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Egg Envelope Cytodifferentiation in the Colonial Ascidian *Botryllus schlosseri* (Tunicata)

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Abstract

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The formation and cytodifferentiation of egg envelopes were studied at the ultrastructural level in blastozooids of *Botryllus schlosseri*. The process was divided into five recognized stages of oogenesis. First, the small young oocytes (stage 1) are contacted by scattered cells (primary follicle cells—PFC) which adhere to the oolemma at several junctional spots. PFC extend all around the growing oocyte, acquire polarity, and form a layer covered externally by a thin basal membrane (stage 2). At stage 3 isolated cells are recognizable between the PFC layer and oocyte. They never form junctions with the oocyte and represent prospective inner follicle cells (IFC) and test cells (TC), the latter being progressively received in superficial depressions in the oocyte. The layer of PFC, which maintains junctions with the oolemma, represents prospective outer follicle cells (OFC). PFC are considered to be the source of the three cellular envelopes because a contribution from mesenchymatous elements was not observed. At the beginning of vitellogenesis (stage 4), the vitelline coat (VC) becomes recognizable as a loose net covering the oocyte and TC. It is crossed by the oocyte microvilli and OFC projections which meet and form numerous small junctional plaques, some of them resembling gap junctions. IFC, VC and TC show marked signs of differentiation with approaching ovulation. OFC differentiate completely before ovulation (stage 5) and are engaged in intense synthesis of proteins which may be transferred and taken by endocytosis into the oocyte for yolk formation. Experiments with injected horseradish peroxidase also revealed that proteins present in the blood may reach the oocyte *via* the intercellular pathway, overcoming OFC and IFC. The possible roles of all the egg envelopes are discussed.

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Introduction

The class Ascidiacea comprises both solitary and colonial filter-feeding species. Different modalities of sexual reproduction are reported for the two groups: solitary species are mostly oviparous and produce a great number of relatively small eggs (about 150 μm in diameter), whereas colonial species are ovoviviparous with a smaller number of heavily yolked eggs (about 250–400 μm in diameter). However, some colonial species are viviparous and produce alecithal eggs even 25 μm in diameter, as in *Hypsistozoa fasmeriana* (Brewin 1956).

In any case, ascidian eggs are very complex, since they typically possess a series of envelopes: a layer of outer (OFC) and inner follicle cells (IFC) and test cells (TC) encased on the oocyte surface; moreover, a fibrous acellular vitelline coat (VC) is located between TC and IFC.

The function of these envelopes has long been debated (Review in Kessel 1983; Cloney 1990*a*). It is still not completely elucidated, but in some cases proposed hypotheses are supported by convincing evidence, e.g., the VC involved in species-specific recognition of gametes (De Santis *et al.* 1980; Monroy & Rosati 1983; Rosati 1985; Honegger 1986; Kawamura *et al.* 1991). Various roles are assigned to IFC that remain to cover the oocytes shed in seawater (Lambert & Lambert 1978; Kawamura *et al.* 1988) and to test cells. The latter have been proposed to participate in synthesis of yolk precursors (Mancuso 1965; Gianguzza & Dolcemascolo 1979), production of granules contributing to the formation (Cavey 1976; Reverberi 1978; Zaniolo & Manni 1990) or chemical characterization of the tunic (Cloney 1990*b*). On the other hand, the role of OFC has received relatively little attention. Zaniolo *et al.* (1987) and Martinucci *et al.* (1988) hypothesized a relation between the development of the OFC and the amount of yolk, on the basis of evidence that colonial species with yolky eggs possess particularly well-developed OFC (Mukai 1977; Zaniolo *et al.* 1987), whereas solitary species with small eggs have flattened or apparently absent OFC (Berrill 1950; Cloney 1990*a*).

Discussion on egg envelopes has occasionally been extended to their origin and differentiation. There is no agreement on the origin of the first single layer of cells surrounding young previtellogenic oocytes (primary follicle cells (PFC)) (Berrill 1941; Tucker 1942; Ermak 1976; Mukai & Watanabe 1976; Sugino *et al.* 1987), nor on the modality of its differentiation. Indeed, some authors maintain that the egg envelopes derive only from PFC (Ermak 1976) while others suggest that they derive from PFC with further contribution from coelomic cells (Mukai & Watanabe 1976).

We have now investigated at an ultrastructural level

the origin, differentiation and possible role of egg envelopes in *Botryllus schlosseri*, a colonial species which possesses a thick layer of OFC. Moreover, in order to verify whether large molecules from blood can pass the follicle envelopes, to reach the oocyte, we have injected horseradish peroxidase (HRP) in some colonies and localized the reaction product in the tissues. Some observations on TC are reported in a previous paper (Zaniolo *et al.* 1989).

The ascidian *B. schlosseri* forms colonies composed of a great number of small zooids (about 2 mm) embedded in a common tunic through which blood vessels run connecting all the zooids and giving the colonies their functional unity.

Three blastogenic generations coexist in each colony: filter-feeding adults, their buds, and the buds of the latter. All zooids of the same generation develop synchronously and their developmental stages are linked temporarily to those of the parental generation. Every 7 days, at 19°C, all adults undergo complete regression, their buds begin to feed and new buds are produced, re-establishing the coexistence of the three blastogenic generations.

The possibility to follow *in vivo* the development of the colonies and to select zooids renders *B. schlosseri* a particularly useful species for experimental studies.

Materials and Methods

For this study, we used colonies of *Botryllus schlosseri* (Pallas, 1776) (Botryllidae, Stolidobranchiata) cultured in the laboratory according to the technique of Sabbadin (1960) and selected at suitable developmental stages.

Transmission electron microscopy

Fragments of mature colonies were dissected and fixed in 1.5% glutaraldehyde buffered with 0.2 M sodium cacodylate plus 1.6% NaCl. After washing in buffer and post-fixation in 1% OsO_4 in 0.2 M cacodylate buffer, specimens were dehydrated and embedded in glycidether 100 (Serva). Thick sections (1 μm) were counterstained with toluidine blue. Thin sections, stained with uranyl acetate and lead citrate, were examined under a Hitachi H-600 electron microscope.

Scanning electron microscopy

For scanning electron microscopy, zooids or isolated eggs were fixed as described for transmission electron microscopy. After dehydration, zooids were dissected in order to eliminate any tissue covering the eggs. Specimens were then critical-point dried, sputter-coated with gold–palladium, and observed under a Cambridge Stereoscan 260.

Experiment with HRP

Some colonies were injected with a small quantity of a 3% HRP solution in filtered seawater. The protein was injected into the blood vessels of the tunic using a microinjection pipette mounted on a micromanipulator. Volumes of liquid were first calibrated by test injections into glycerol fluid and preliminary controls using fluorescein isothyocyanate-dextran, to verify the diffusibility of the injected substances into the vessels and organs.

Thirty min after injection, colonies were fixed in 1.5% glutaraldehyde in 1.6% NaCl buffered at pH 7.4 with 0.2 M sodium cacodylate and cut into small pieces. After washing (30 min total) in the same buffer, the specimens were processed with Graham & Karnowsky's (1966) diaminobenzidine technique for peroxidase, then washed in buffer, post-fixed in 1% OsO_4 buffered in sodium cacodylate, dehydrated in alcohol and embedded in glycidether 100. Ultrathin sections were observed unstained under a Hitachi H-600 electron microscope.

Results

In thick sections of *B. schlosseri* buds, gonads are recognizable in the mantle, on either side of the zooid. They are formed from plurilobated testes and eggs at different stages of development (Fig. 1). Each egg is like a small ovary, being composed of the oocyte with envelopes and its own oviduct.

The gonadal blastema first appears in the young bud when the inner vesicle is closing and segregating from the peribranchial wall of the parent. Our observations confirm previous reports by Berrill (1941), Mukai & Watanabe (1976) and Sabbadin & Zaniolo (1979). Egg and bud develop in co-ordination, so that oogenesis is completed and eggs ovulate when buds initiate filtration. In each blastozooid only one or a few oocytes mature among those initially present in the gonad. A number of oocytes stop development at a previtellogenic stage and, moving through the blood vessels, may reach and mature in buds of successive blastogenic generations (Izzard 1968; Sabbadin & Zaniolo 1979).

Mainly based on the nucleo/cytoplasm ratio, oocyte dimension and yolk formation, the oogenesis of *B. schlosseri* may be subdivided into five stages, to which different stages of the egg envelopes correspond.

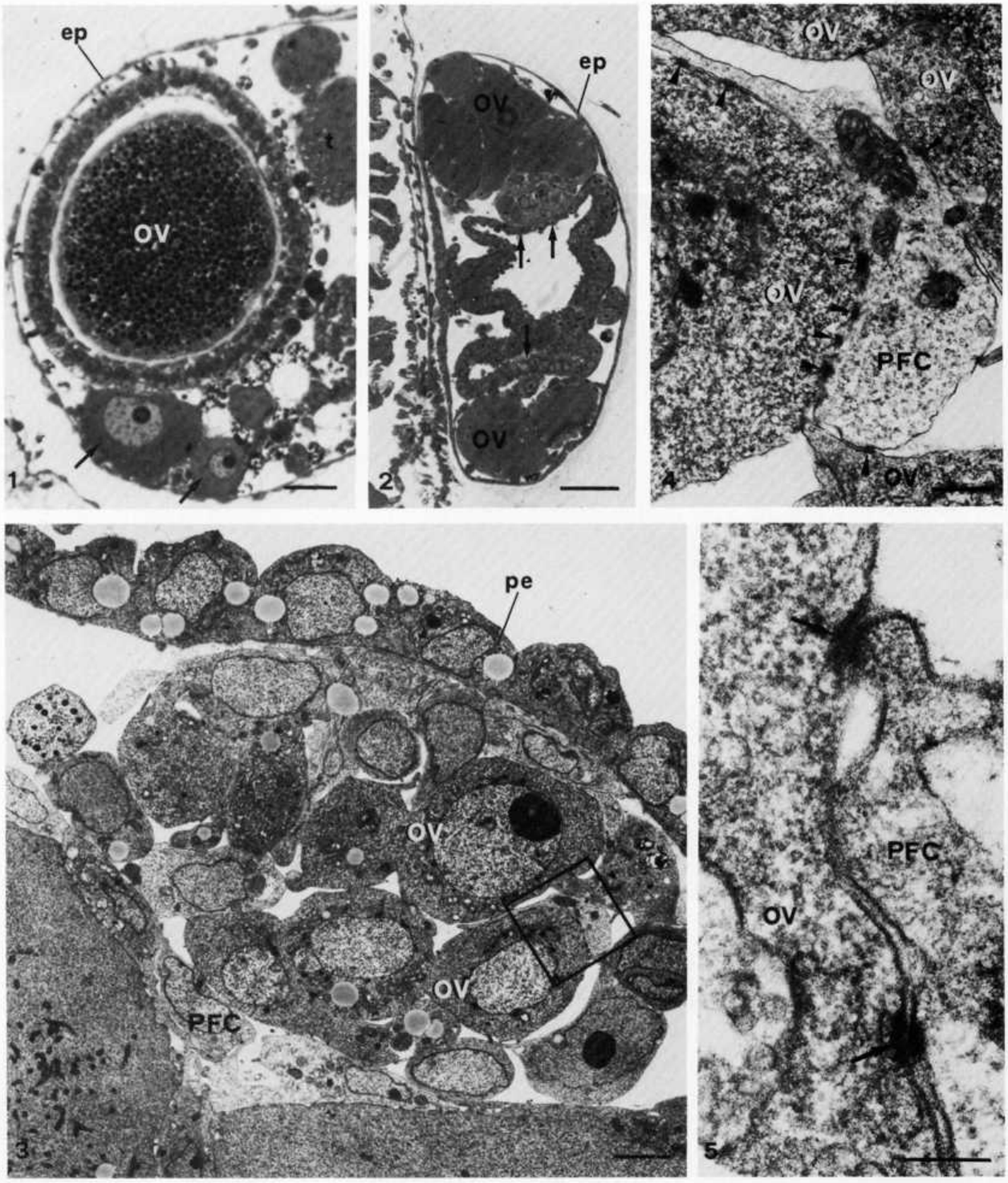
Previtellogenesis. Stage 1. Oocyte about 10 μm in diameter; high nucleo/cytoplasm ratio; cytoplasm with rare organelles; scarce mitochondria; nucleus at early prophase stage. *Stage 2.* Growth of oocyte to diameter about 50 μm; marked nucleus/cytoplasm exchange; several mitochondria in perinuclear areas; nucleus at metaphase I. *Stage 3.* Oocyte diameter about 80 μm; mitochondria scattered in cytoplasm; first appearance of microvillar extensions of oolemma.

Vitellogenesis. Stage 4. Oocyte diameter about 120 μm; initial vitellogenesis with small scattered yolk granules of different density; long oolemmal microvilli. *Stage 5.* Oocyte diameter about 220 μm; late vitellogenesis with most yolk granules about 1.2 μm, homogeneously dense.

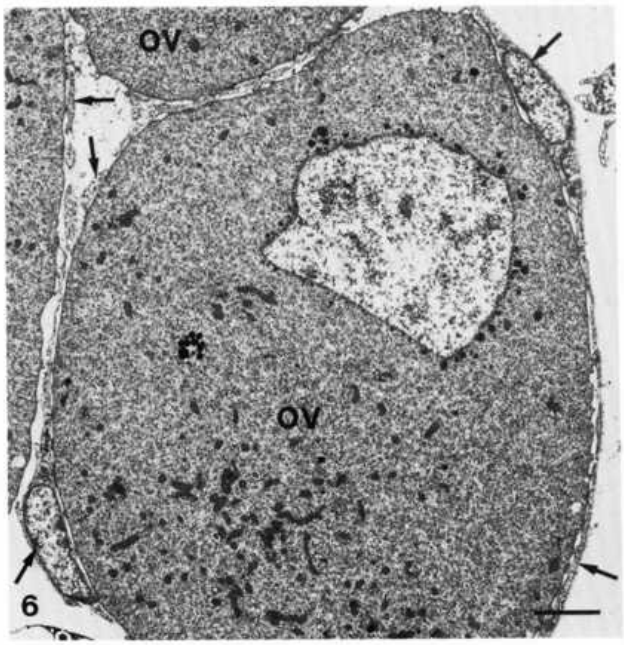
Egg envelope differentiation

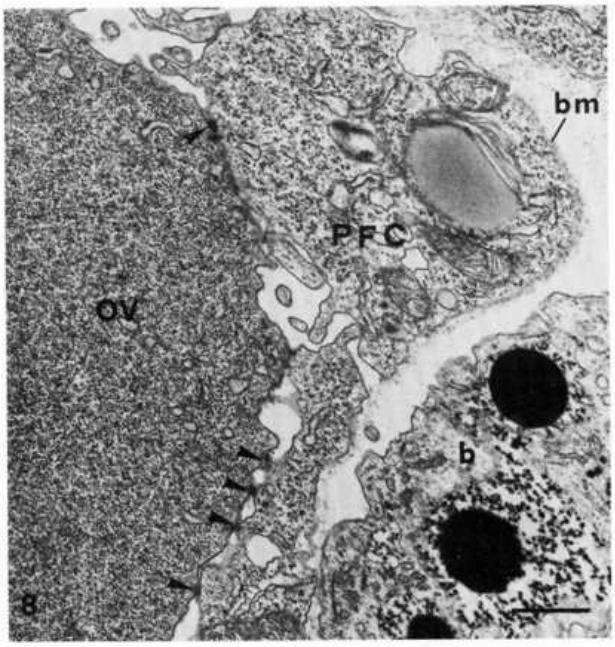
Stage 1. In the mantle of a young bud like that shown in Fig. 2, the gonadal blastema presses against the presumptive external peribranchial wall (Fig. 2) and is bathed by blood flowing in the main sinus of the bud (equatorial sinus; Burighel & Brunetti 1971). Most cells are undifferentiated but small oocytes at stage 1 are clearly recognizable among them (Fig. 3).

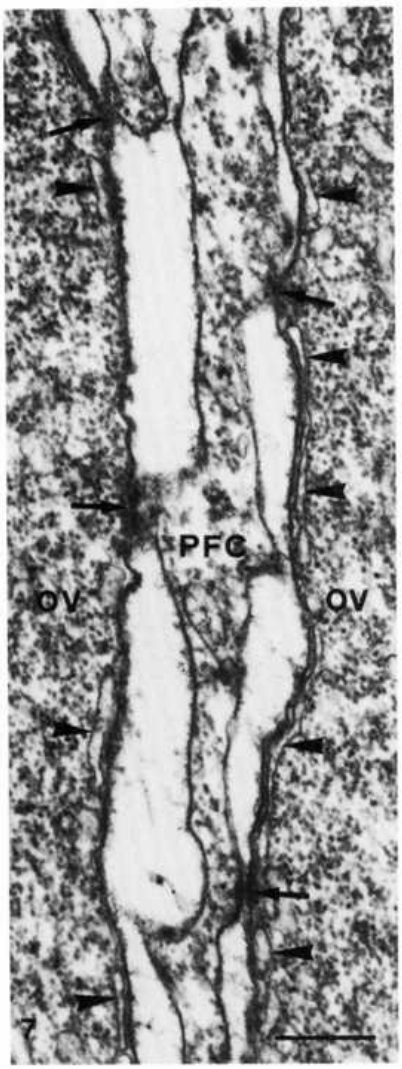
Elongated cells (primary follicle cells, PFC) wrap around the oocytes with cytoplasmic extensions; their cytoplasm is rich in ribosomes while mitochondria and

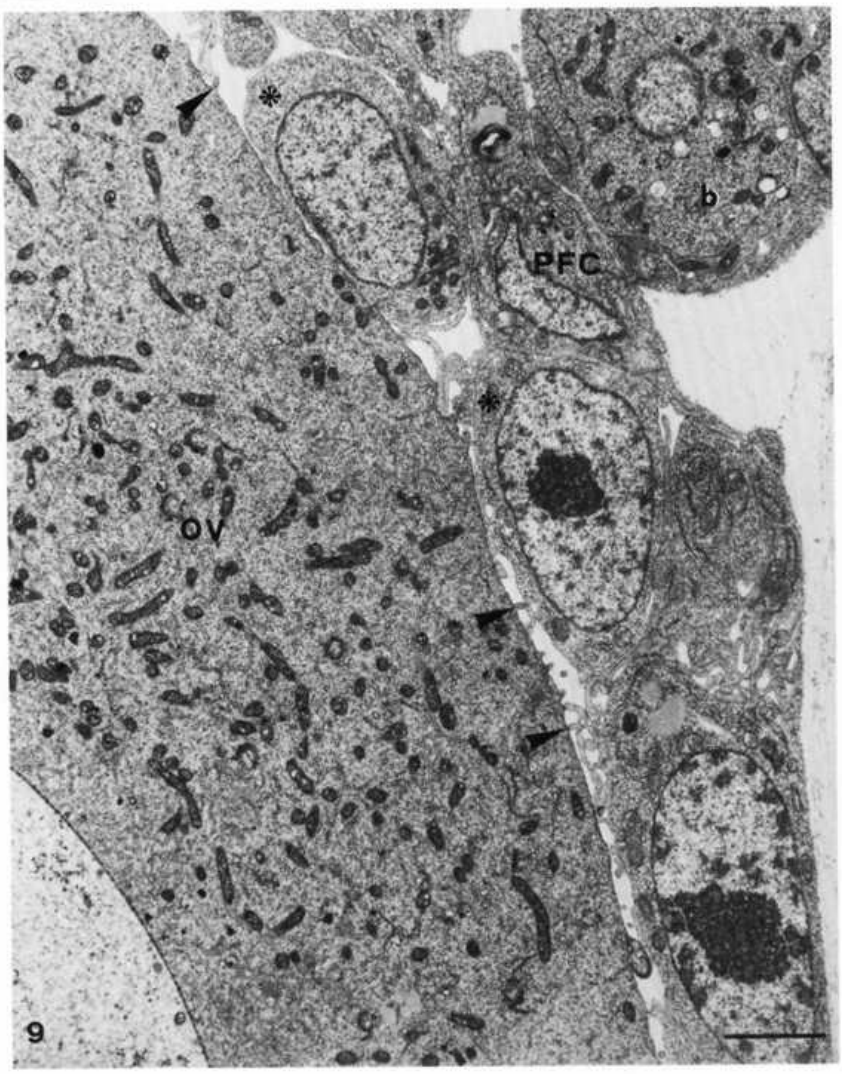


Figs 1–5. Botryllus schlosseri.—Fig. 1. Thick sections of a bud showing a large vitellogenic oocyte (*OV*) with its envelopes (see also Fig. 13). Some young previtellogenic oocytes (*arrows*) and portion of testis (*t*) are also recognizable in the mantle. Toluidine blue. Scale bar 14 μm.—*Fig. 2.* Thick sections of a young bud. Gonadal anlage lies in mantle between prospective peribranchial epithelium (*arrows*) and epidermis (*ep*). Several previtellogenic oocytes (*OV*) produced in preceding blastogenic generations are present on both sides of the bud. Toluidine blue. Scale bar 25 μm.—*Fig. 3.* Electronmicrograph of gonadal anlage in a bud like that of Fig. 2. In undifferentiated blastema, small oocytes (*OV*) are recognizable owing to small dense plaques in perinuclear area. At *bottom* and *left*: portion of two large previtellogenic oocytes (at stage 2) enveloped by PFC (*PFC*). The *rectangle* delimits the area enlarged in Fig. 4. Scale bar 3 μm.—*Fig. 4.* Detail of Fig. 3, showing contact between first PFC (*PFC*) and several young oocytes (*OV*). *Arrowheads* dense junctional spots. Scale bar 0.9 μm.—*Fig. 5.* Detail of area of contact between a primary follicle cell (*PFC*) and a small oocyte (*OV*). Two spot desmosome-like junctions (*arrows*) with dense material on both cytoplasmatic faces are seen. Scale bar 0.2 μm.

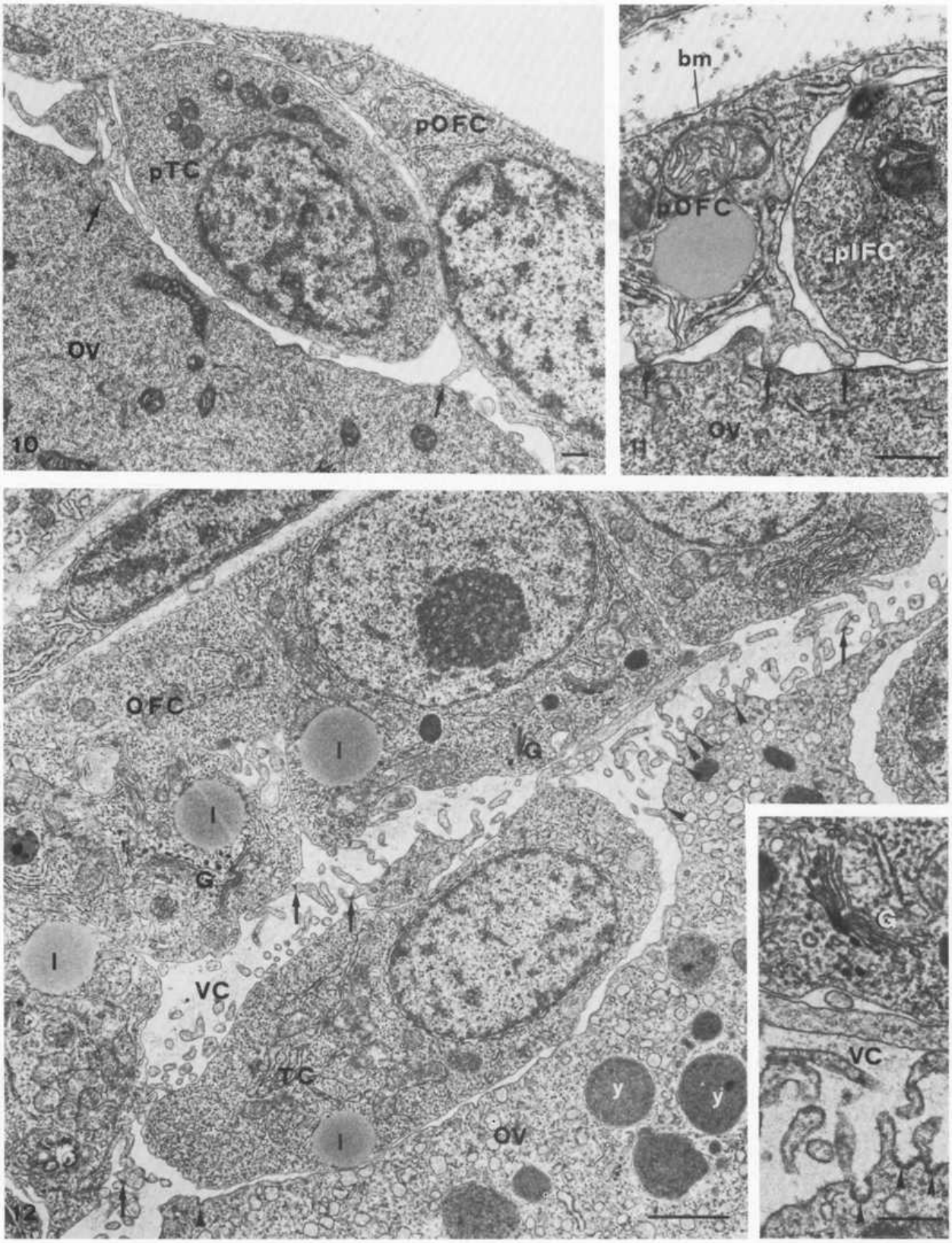








Figs 6–9. Botryllus schlosseri.—Fig. 6. Squamous PFC (*arrows*) form a thin layer around growing previtellogenic oocytes (*OV*), which are rich in ribosomes and have scattered mitochondria. Scale bar 4 μm.—*Fig. 7.* Detail of cytoplasmic extension of a PFC (*PFC*) penetrating between and contacting two previtellogenic oocytes (*OV*), both provided with long flattened suboolemmal cisternae (*arrowheads*). Junctional areas form on both faces of PFC. Scale bar 1 μm.—*Fig. 8.* Around a previtellogenic oocyte (*OV*) PFC (*PFC*) show signs of differentiation, consisting of an increase in the number of free ribosomes and formation of several RER cisternae. *Arrowhead* desmosome-like junctions. Scale bar 3 μm.—*Fig. 9.* In growing oocyte possessing numerous mitochondria distributed throughout cytoplasm (stage 3), isolated cells (*asterisks*) penetrate between oolemma and PFC. Short microvilli (*arrowheads*) merge from the oocyte and no sign of VC is recognizable. Scale bar 3 μm.



Figs 10–12. Botryllus schlosseri.—Figs 10, 11. Oocytes at same stage as in Fig. 9. In Fig. 10, the isolated cell (presumptive TC) (*pTC*) is received in a depression forming on the oocyte surface (*OV*). Junctional areas (*arrows*) exist between extensions of PFC (presumptive OFC)(*pOFC*) and the oocyte but are lacking in the isolated cell. Figs 10, 11: scale bars 0.5 μm.—*Fig. 12.* Oocyte in early vitellogenesis (stage 4). TC (*TC*) is undergoing final location into the oocyte surface while VC (*VC*) appears to be separating TC from OFC (*OFC*). OFC form a thick layer and possess well-developed Golgi and several RER profiles. From the oocyte surface, numerous polymorphous microvilli elongate, contacting OFC extensions with small dense junctional spots (*arrows*). *Inset* a confronting area of OFC and oocyte: note Golgi complex (*G*) formed from several cisternae, network of VC and endocytotic pits (*arrowheads*). Scale bar 2 μm; inset scale bar 0.5 μm.

endoplasmic reticulum (ER) are scarce. PFC contact the oocytes with scattered spot desmosome-like junctions (Figs 4, 5, *arrows*) which are marked by the presence of dense fibrous material on the cytoplasmic side of both PFC and oocyte and by dense material in the 12–16 nm intercellular cleft (Fig. 5). In addition to these young oocytes, other larger ones (commonly at stage 2) are often present, arriving by vascular migration from the zooids of older blastogenic generations (Fig. 2).

Stage 2. Numerous elongated and flattened ER cisternae which closely parallel the oolemma are formed in the oocytes (Fig. 7). PFC arrange to form a continuous thin layer all around the oocyte (Fig. 6). Their cytoplasm contains numerous free ribosomes, some ER cisternae, mitochondria and rare lipid droplets (Fig. 8).

The side of PFC facing the oocyte establishes numerous junctional contacts with the oolemma while the opposite side produces a thin basal membrane. However, when laminar extension of the PFC penetrates between two oocytes, both their surfaces form junctional contacts with the oolemma (Fig. 7).

Stage 3. Only a few of the oocytes initially present reach this stage. Short microvillar projections emerge from the oolemma, while suboolemmal cisternae disappear (Fig. 9).

Isolated roundish cells appear in the space between the PFC layer and oolemma and, increasing in number, form a sort of discontinuous internal layer (Fig. 9). Some of them appear to be partially lodged in shallow depressions in the oocyte surface (Fig. 10) and represent presumptive TC (Zaniolo *et al.* 1989). The presumptive elements of the three cellular envelopes of the oocytes are recognizable for the first time at this stage: OFC, i.e., original PFC forming the external layer; IFC and TC, the internal layer. Junctional contacts are never seen among presumptive TC and IFC and the oolemma. Instead, junctional contacts are maintained or newly established between oolemma and cytoplasmic extensions of the OFC, whose cells differentiate extended RER cisternae (Fig. 11).

Stage 4. At EM, the first small yolk platelets appear, of various size and density. The oolemma projects numerous polymorphous microvilli. Endocytotic pits form at their base, originating coated vesicles which then migrate and fuse to each other and to the yolk granules (Fig. 19). Early in stage 4, the first evidence of the VC appears as a loose network of fibrillar material extending over the plasmalemma and separating TC from IFC (Fig. 12). The VC becomes progressively thicker owing to an increase in fibrillar material (Figs 14–16, 19).

The TC are encased in the oocyte, with their external sides partially covered by long ovular microvilli (Figs 12–14). They have a large nucleus with nucleolus, mitochondria, some RER cisternae, Golgi apparatus and many free ribosomes (Fig. 12).

The IFC are polygonal cells not forming a continuous layer, with a large nucleus and cytoplasm rich in free ribosomes. They are covered by OFC and rest on the VC (Figs 13–15).

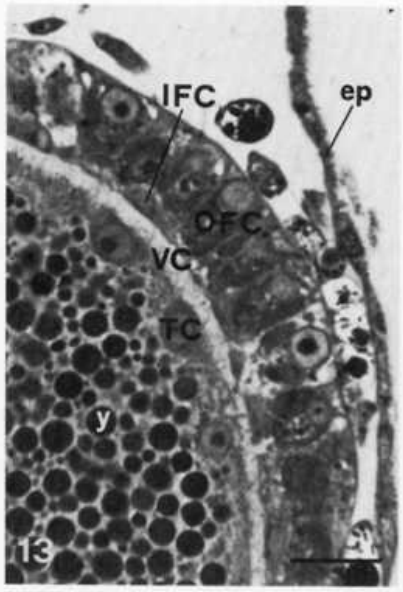
The OFC layer becomes thicker; its cells have a large round nucleus towards the basal lamina, many mitochondria, lipid droplets and long RER profiles. The cytoplasmic area towards the oocyte is usually occupied by a large Golgi apparatus, with numerous flattened cisternae and vesicles (Figs 12, 16). Round dense granules, different in size and provided with a membrane, occur in the Golgi area and seem to originate by fusion of Golgi vesicles (Fig. 20). These cells extend their apical area into the space among the IFC and cross the VC by means of cytoplasmic projections which make junctions with the microvillar extensions of the oocyte (Figs 17, 18). Most junctions are small, with densities on the cytoplasmic sides of membranes (Fig. 17), but the junctional area is sometimes more extended and resembles a gap junction, the intercellular cleft being reduced to 3 nm (Fig. 18).

Stage 5. The oocyte completes vitellogenesis. The TC are deeply encased on the oocyte surface and in their cytoplasm well-developed Golgi together with electron-dense granules increase in number when ovulation approaches (Fig. 21). In the full-grown oocyte, the VC compacts and, especially close to the IFC, is formed of parallel fibres, while towards the oocyte it appears as a loose net. At the same time, the microvilli and OFC extensions shorten and are reduced in number, so that the junctions between the two are progressively lost (Fig. 21).

The IFC grow and establish closer relations with each other, to form a sort of continuous layer. Nevertheless, intervals do remain between them, in which OFC projections penetrate towards the VC.

At this stage, the IFC appear to be involved in synthesis of proteins which fill the RER cisternae as homogeneous, finely granular material. These cisternae often become bigger and, in some cases, large vesicles with rare membrane-bound ribosomes filled with similar material are seen in the proximity of well-developed Golgi. Golgi vesicles seem to fuse with them, contributing to their content (Figs 22, 23).

Figs 13–20. Botryllus schlosseri.—Fig. 13. Thick section detail of a fully grown oocyte with its envelopes: *OFC, IFC, VC, TC.* Scale bar 10 μm. *Figs 14, 15.* Details of fully grown oocytes as seen by SEM. In Fig. 14 OFC (*OFC*) are completely removed and partially detached (Fig. 15) to show their deeper face. IFC rest on VC and contact each other leaving discrete spaces which, *in vivo*, are crossed by basal OFC extensions. TC (*TC*) are covered by VC and partially by oolemmal microvilli (*mv*) seen in the fractured oocyte of Fig. 14. Figures 14, 15: scale bars 5 μm.—*Figs 16–18.* Oocyte at the end of stage 4.—*Fig. 16.* OFC (*OFC*) are tall and differentiated with long flattened parallel cisternae of RER and active Golgi apparatus which produces dense granules and is sited at the cell apex towards the oocyte. VC (*VC*) is crossed by OFC extensions (*arrows*) and oolemmal microvilli (*mv*) forming intercellular dense junctions, shown in detail in Fig. 17 (*arrowheads*). In some cases (Fig. 18) these junctions recall gap junctions for narrow intercellular clefts (*arrow*). Figure 16 scale bar 2 μm; Fig. 17 scale bar 0.2 μm; Fig. 18 scale bar 0.1 μm.—*Figs 19, 20.*—*Fig. 20.* Oocytes at stage 4.—*Fig. 19.* In the oocyte, numerous coated vesicles originating from endocytotic pits (*arrows*) migrate in the cytoplasm and fuse with yolk granules.—*Fig. 20.* Detail of OFC showing well-developed Golgi apparatus formed by a stalk of several parallel cisternae. Numerous dense vesicles (*arrows*) seem to bud from cisternae to contribute to formation of the round granules. Figures 19, 20: scale bars 0.5 μm.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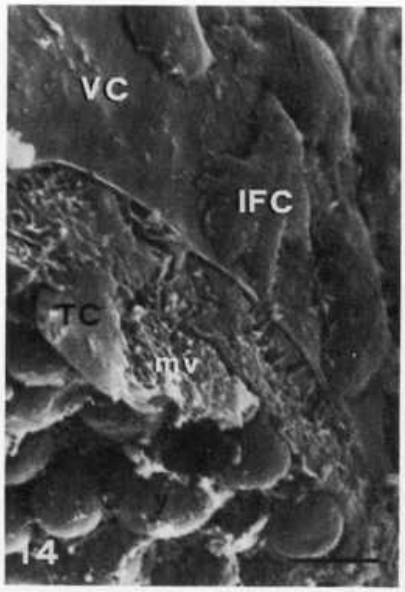
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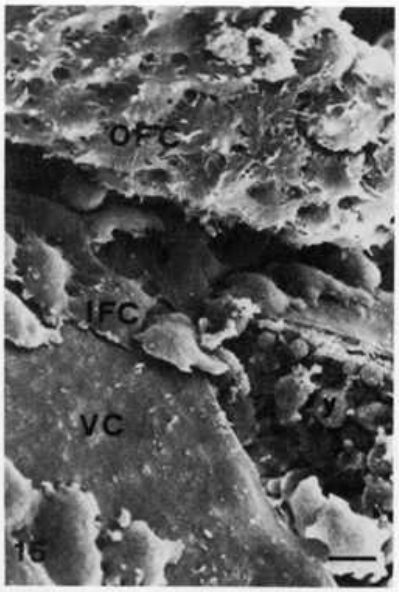
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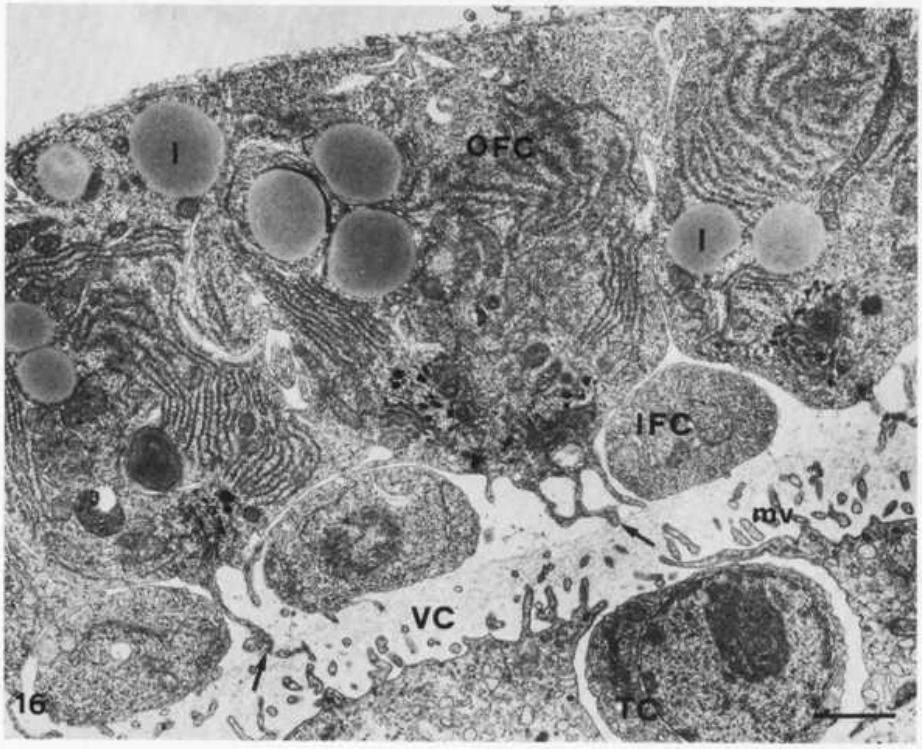
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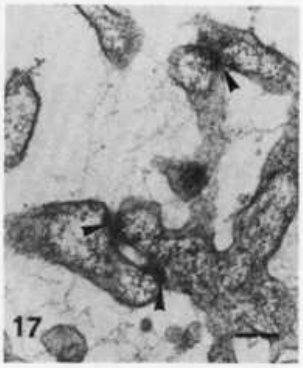
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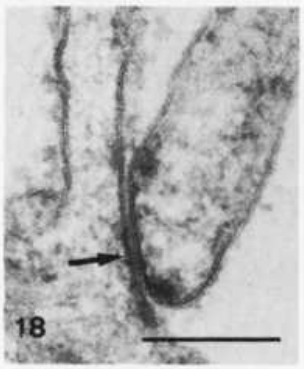
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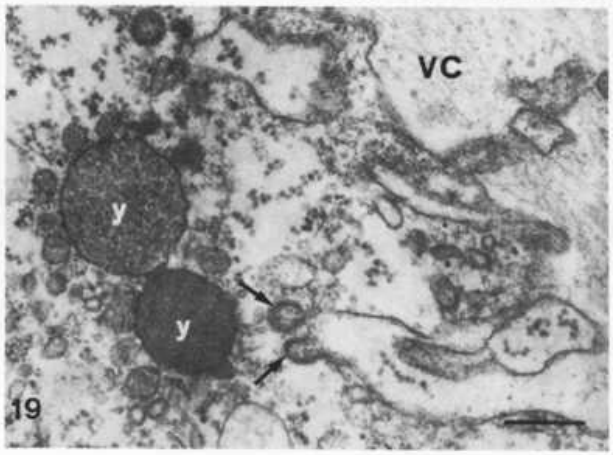
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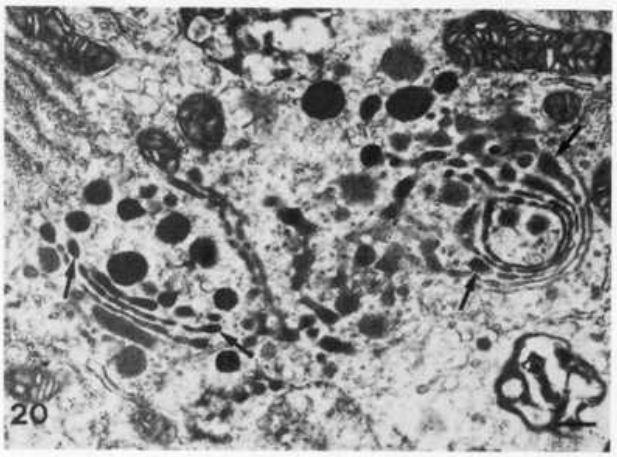
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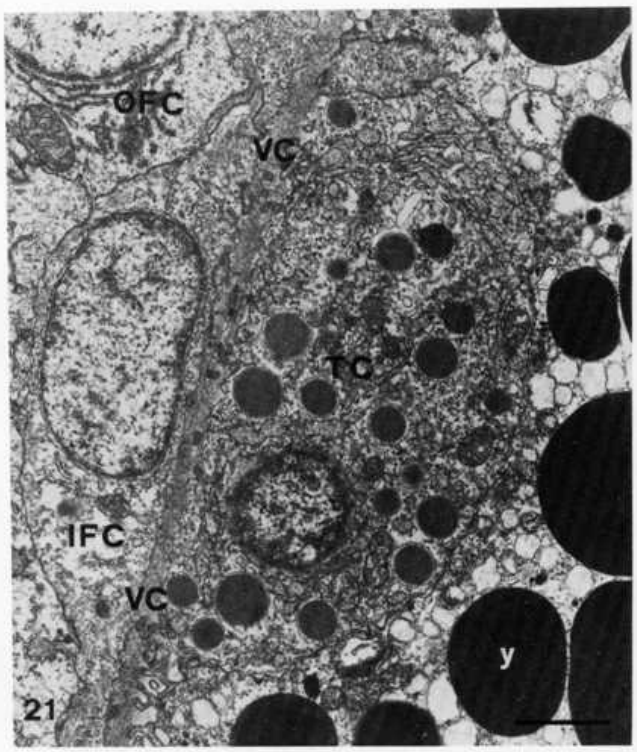

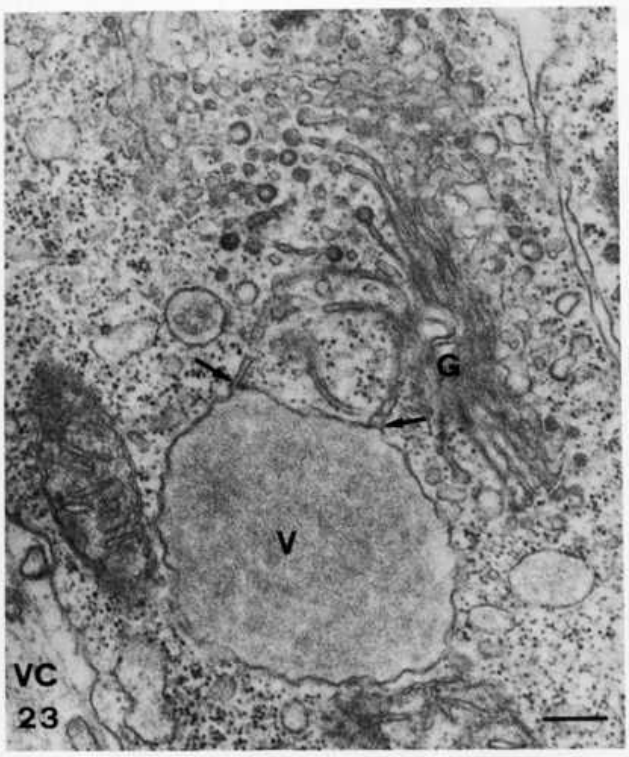

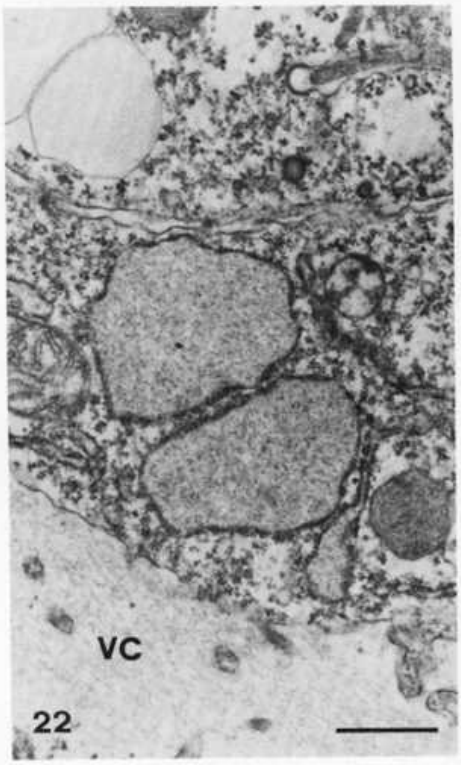

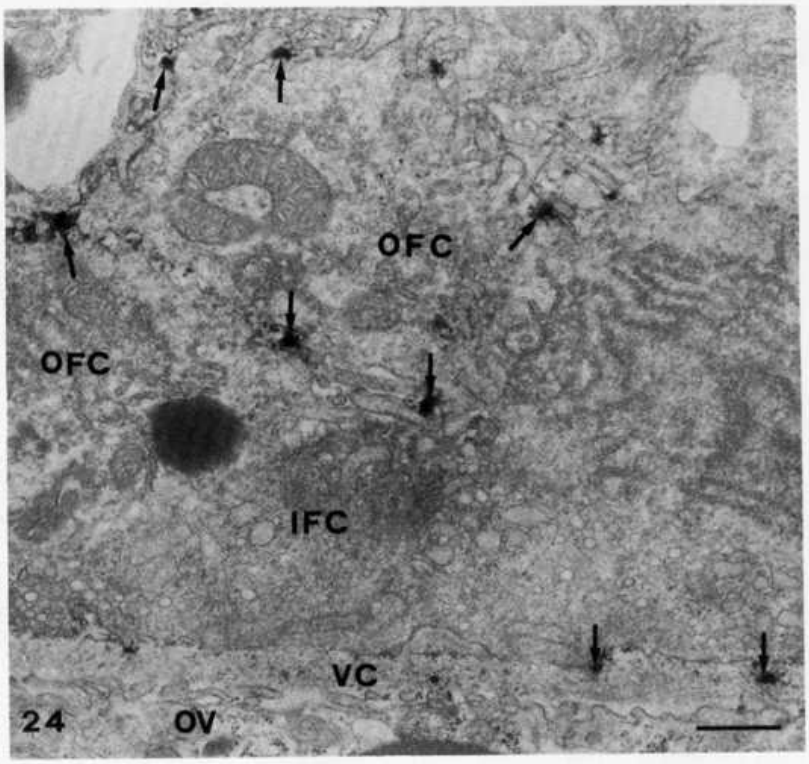

Figs 21–24. Botryllus schlosseri.—Figs 21–23. Oocyte at stage 5.—*Fig. 21.* A TC (*TC*) encased in an oocyte approaching ovulation shows numerous round dense granules. VC (*VC*) is compacted under flat IFC (*IFC*).—*Fig. 22.* RER cisternae of IFC filled with finely granular homogeneous material are seen.—*Fig. 23.* A large smooth vesicle (*V*) with fine content in the Golgi field (*G*), which seems to be receiving contributions from Golgi cisternae (*arrows*). Figure 21 scale bar 2 μm; Figs 22, 23: scale bars 0.5 μm.—*Fig. 24.* Vitellogenic oocyte from a colony injected with HRP. Products of reaction (*arrows*) are recognizable as dense spots in the intercellular spaces between OFC (*OFC*), IFC (*IFC*) and at the VC (*VC*) level. Unstained thin section. Scale bar 1 μm.

The OFC become taller, and the number of long parallel cisternae of RER and the Golgi area further increases. Several dense granules may be seen mainly near the Golgi field or scattered in the cytoplasm. At ovulation, the OFC are discharged in the mantle of the blastozooid, so that the egg remains externally covered by IFC (Zaniolo *et al.* 1987).

A schematic drawing illustrating the main features of cellular envelope differentiation is shown in Fig. 25.

HRP injected colonies

In unstained sections of colonies injected with HRP, reaction product may be seen at EM in the form of dense deposits. In colonies involved in yolk formation such deposits are seen in the blood lacunae and also in the intercellular spaces of OFC and IFC layers and at the IFC–VC border (Fig. 24).

Discussion

Our results provide evidence that, in *Botryllus schlosseri*, egg envelope differentiation is co-ordinated with the developmental phases of the oocyte. In particular, TC, VC and IFC show main differentiation aspects in late oogenesis, whereas OFC undergo chief cytodifferentiation at the beginning of vitellogenesis and are discharged

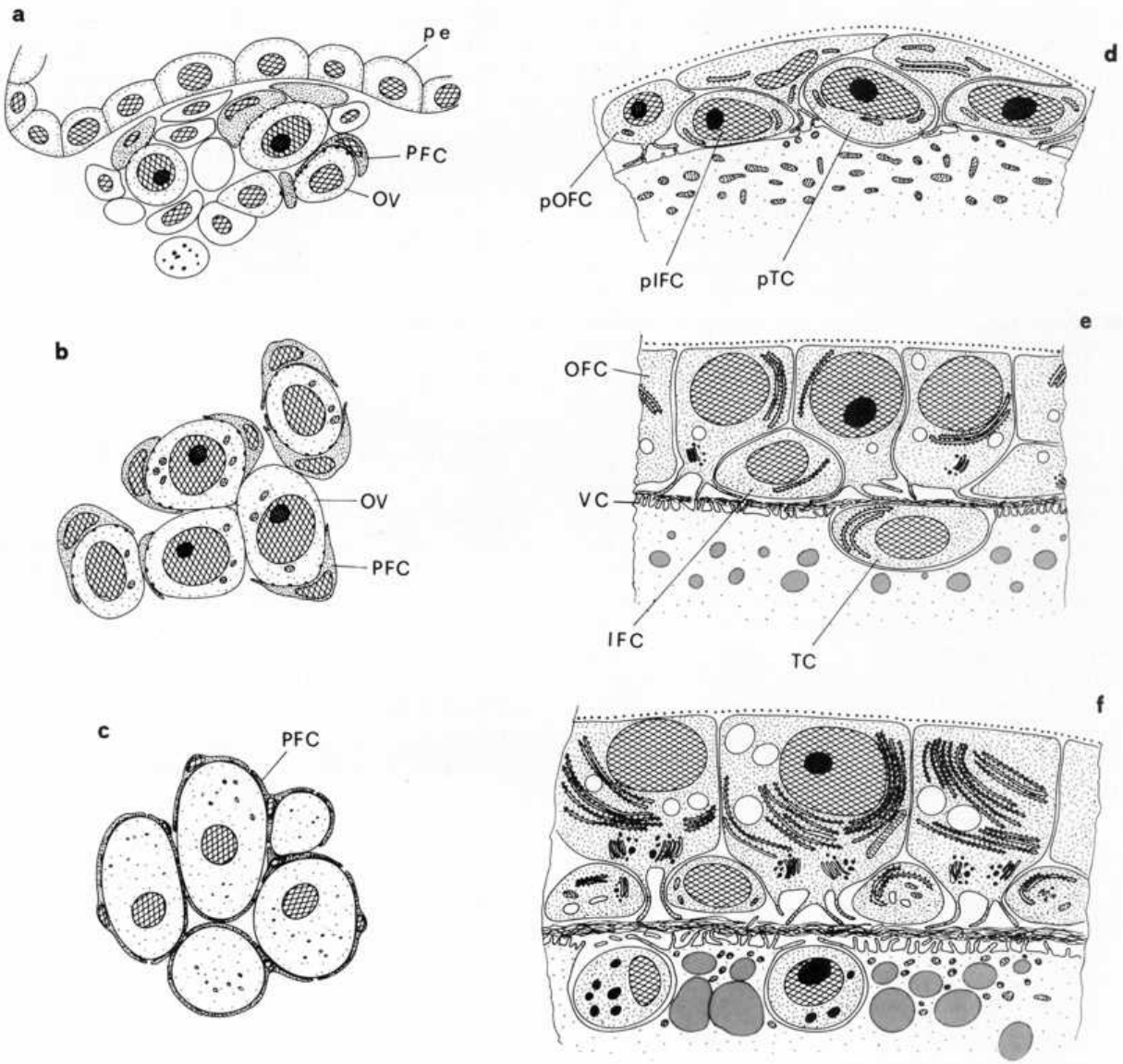

Fig. 25. Schematic drawing illustrating origin and cytodifferentiation of egg envelopes in *Botryllus schlosseri*.—*a*, *b*, *c*. In young oocyte (*OV*) a layer of primary follicle cells (*PFC*) is formed.—*d*, *e*, *f*. PFC give rise to presumptive OFC (*pOFC*), presumptive IFC (*pIFC*) and presumptive TC (*pTC*), the latter being received in the oocyte depression and covered by a VC net (*VC*). OFC form a continuous external layer and establish constant junctional relationships with the oolemma, their cytoplasmic projections penetrating between IFC and crossing the VC. Considering our subdivision of oocyte development, *a* and *b* are referred to stage 1; *c*, *d*, *e*, and *f*, respectively, to stages 2, 3, 4, and 5.

at ovulation. The main role of TC, VC and IFC is therefore probably played in the full-grown egg or after ovulation, unlike OFC whose main activity is shown during vitellogenesis.

The presence of thick OFC was previously suggested to be related to egg size and yolk quantity in colonial species (Zaniolo *et al.* 1987; Martinucci *et al.* 1988). We found evidence that OFC in *B. schlosseri* acquire marked polarity precociously and produce a complex machinery for protein synthesis, as revealed by the enormous quantity of RER, the particularly well-developed Golgi apparatus, and the number of dense granules. The latter do not accumulate in the cells and their contents, secreted into the intercellular spaces, may be taken into the oocyte by pinocytosis to contribute to yolk formation, as suggested by the intense flow of endocytotic vesicles which fuse with yolk granules.

On the other hand, the oocyte can also receive a contribution from blood. The existence of an oocyte–blood pathway which can be traversed by molecules of considerable size is supported by the demonstration that injected HRP rapidly passes the OFC basal membrane and intercellular spaces of the egg envelopes to approach the oocyte. It is therefore probable that, in *B. schlosseri*, both precursors from OFC and blood may participate in yolk formation.

Moreover, exchange may also occur by direct communication between OFC and oocyte. In fact, numerous junctions are formed between the oolemmal microvilli and OFC protrusions, a situation not previously reported for other ascidians. In *B. schlosseri*, intercellular junctions are present throughout the examined phases of oogenesis. Single junctional areas are often restricted, so that it is difficult to establish definitively to what kind of junctions they can be referred on the basis of thin section studies. Most junctions, especially those formed during previtellogenesis, are desmosome-like; they show marked densities on the cytoplasmic faces and may play a role in mechanical adhesion (Fig. 5). Moreover, mainly in oocytes undergoing vitellogenesis, some large junctions are recognizable, with a narrow intercellular cleft and features recalling those of gap junctions found in different tissues of *Botryllus* (Lane *et al.* 1986) (Fig. 18). In other animals, gap junctions represent an important pathway for oocyte–follicle cell exchange (Anderson & Albertini 1976) and this may also be the case for *Botryllus*, in which substances and information may be exchanged between oocyte and OFC and vice versa until the end of vitellogenesis, when retraction of OFC and microvillar protrusions occurs.

To conclude, although other roles for OFC cannot be excluded, e.g., a postovulatory one (Zaniolo *et al.* 1987), all the above-reported features are in agreement with the idea that OFC may contribute to the synthesis and transfer of yolk precursors and other molecules to the oocyte. In a general sense, this hypothesis may clarify the evidence that thick OFC are typical of the large, yolk-rich eggs of ovoviviparous species (Zaniolo *et al.* 1987; Martinucci *et al.* 1988).

Ascidian IFC have been repeatedly investigated and different roles attributed to them (see Kessel 1983), especially on the basis that they present different morphologies in embryos of different species. In *B. schlosseri*, a close relation seems to be established between IFC and VC, mainly at the end of vitellogenesis. This is in agreement with the hypothesis, also suggested by Cotelli *et al.* (1981) for *Ciona*, that IFC participate in the structural characterization of VC where the sites for species-specific recognition are located. With approaching ovulation, the IFC show signs of active differentiation, such as accumulation of fine homogeneous material in large RER vesicles and Golgian granules. The IFC form the vesicles which, after ovulation, help to cement the embryo to the cup-like 'placenta' (Zaniolo *et al.* 1987).

Recently, Sakairi & Shirai (1991) have shown that the IFC of *Halocynthia roretzi* release a factor triggering germinal vesicle breakdown. A similar mechanism is widespread in many animals (Schuetz & Glad 1985; Shirai & Walker 1988) and may be shared by *B. schlosseri*, but at the moment we cannot attribute some of the differentiation aspects of IFC to the production of a factor activating this mechanism.

The role of the TC has also been long debated. Our data show that, with approaching ovulation, their cytoplasm is filled up with electron-dense granules produced by the Golgi apparatus. These granules are present and increase in number during embryogenesis, so that our observations confirm previously reported data (Zaniolo *et al.* 1989) that in *B. schlosseri*, as in other species, the main role of the TC is played during embryogenesis rather than oogenesis. This agrees with results recently obtained by Cloney (1990*b*), comparing a number of species. He gave evidence that TC granules, secreted into the perivitelline space and deposited on the surface of the embryonic tunic, make the surface of the larval tunic hydrophilic and modify it.

Origin of egg envelopes

Our study was directed to the origin and differentiation of egg envelopes in *B. schlosseri*, starting from the young oocytes enveloped by rare cells, which we call PFC.

The first evidence of PFC was found in oocyte stage 1 of the initial gonadal blastema. In agreement with Ermak (1976), our data suggest that PFC represent the stem cells for all egg envelopes. In *Ciona savignyi*, Sugino *et al.* (1987, 1990) identified at the EM two kinds of cells, clear and dark, enveloping the young oocyte. These represent the precursors of the OFC, IFC, and TC, respectively. We were not able to identify two kinds of cells like those seen in *Ciona*, on the basis of morphological features. However, we cannot exclude the fact that the cells which in *B. schlosseri* differentiate into IFC and TC are already determined when they are in the PFC layer. In *B. schlosseri*, PFC give rise to the OFC, which possess a basal membrane and maintain junctional contacts with the oolemma, and to the interposed isolated cells, which differentiate into IFC and TC. Since we never observed features indicating the possibility that external elements may infiltrate the PFC, we think that the isolated cells derive from the PFC by mitosis, as do Tucker (1942), Ermak (1976) and other authors who have studied ascidians from various groups. On the basis of light microscope observations, Mukai & Watanabe (1976) reported that in *Botryllus primigenus* oocytes in early developmen-

tal stages are surrounded by 'primary follicle cells' from which the IFC originate. Later, OFC derive from a second layer formed by migration from neighbouring cells; TC derive from the IFC and move across the growing VC. Our data provide evidence that, in *B. schlosseri*, presumptive TC become recognizable before VC formation and are received into shallow depressions in the oocyte, as in *Halocynthia* (Sugino *et al.* 1987). The TC soon become segregated from the IFC by VC net fibres, which are probably secreted by the oocyte as suggested by Cotelli *et al.* (1981) for *Ciona intestinalis*.

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Abbreviations Used in the Figures

ep	epidermis
G	Golgi apparatus
IFC	inner follicle cells
l	lipid droplet
mv	oolemmal microvilli
OFC	outer follicle cells
OV	oocyte
pe	peribranchial epithelium
PFC	primary follicle cells
pIFC	presumptive inner follicle cells
pOFC	presumptive outer follicle cells
pTC	presumptive test cells
t	testis
TC	test cells
V	vesicle
VC	vitelline coat
y	yolk

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