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Ovulation and embryo-parent relationships in *Botrylloides leachi* (Ascidiacea, Tunicata)

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Summary

The ultrastructural events of ovulation and differentiation of the brood pouch are studied in the colonial ascidian *Botrylloides leachi*. Each zooid matures one or two yolked oocytes enveloped by test cells, vitelline coat, and inner and outer follicle cells. A small vesicular oviduct lies between the outer follicle cells and the brood pouch, a flask-shaped structure differentiated from the wall of the atrial chamber. The oviduct epithelium fuses with the outer follicle and brood pouch cells and breaks, creating a pathway which drives the oocyte to the brood pouch. Embryos develop in isolation in the latter whose epithelium undergoes differentiation: the cell surface increases enormously, forming deeper and deeper baso-lateral foldings, cell interdigitations and apical polymorphic protrusions. Extended tight junctions separate the different domains of the plasmalemma and create a barrier between the pouch lumen and the blood. Large quantities of glycogen, mainly in the form of rosettes, are formed and accumulate progressively in extensive apical regions. Glycogen, presumably released by apocrine secretion, fills the space between the brood pouch epithelium and the inner follicle cells surrounding the embryo. All features are indicative of a transfer of substances from the blood toward the embryo. The significance of the embryo-parent exchange in *B. leachi* and comparative aspects of ovoviviparity and viviparity in ascidians are discussed.

Key words: Ascidians, embryo-parent exchange, glycogen, ovoviviparity, viviparity, ovulation, ultrastructure

Abbreviations: ae: atrial epithelium; bc: blood cells; bm: basement membrane; BP: brood pouch; cl: corpus luteum; E: embryo; ep: epidermis; G: Golgi complex; gl: glycogen; IFC: inner follicle cells; L: BP lumen; m: mitochondrion; N: nucleus; O: oocyte; OD: oviduct; OE: ovular envelope; OFC: outer follicle cells; pl: BP plug; PS: perivitelline space; RER: rough endoplasmic reticulum; st: stigma; t: tunic; TC: test cells; tj: tight junction; VC: vitelline coat; y: yolk globules.

Introduction

In ascidians, sessile filter-feeding tunicates, various reproductive modes can be found: oviparity, ovoviviparity and viviparity. In both ovoviviparity and viviparity, development is intraparental and the embryo depends mainly on yolk or parent, respectively, for nutrition.

In oviparous species, generally solitary forms, many small eggs are produced which are freely spawned in seawater enveloped only by inner follicle cells (IFC). Ovoviviparous species, commonly colonial forms, have larger yolked eggs and the embryos develop in the atrial chamber, either floating in it and held by a network of filaments, as in *Dendrodoa grossularia* (Millar, 1954), or retained in specialized structures called brood pouches (BPs), as in some species belonging to the *Botrylloides* and *Perophora* genera (Mukai, 1977; Mukai et al., 1983). True viviparous species are colonial and produce small alecithal eggs, like the Styelidae *Botrylloides lenis* and *Botrylloides violaceus* (Mukai et al., 1987) and the Holozoinae *Hypsistozoa fasmeriana* (Brewin, 1956).

As in other groups of invertebrates and vertebrates, in ovoviviparous ascidians true viviparity may occur in the same group with variation in yolk content, specialization of maternal structures, and progressive increase in embryo-parent exchange. The most carefully studied group is that of the subfamily Botryllinae (Styelidae), in which Mukai et al. (1987) stressed the different reproductive modes of three species of *Botrylloides*, indicating different adaptive solutions of ovoviviparity and viviparity. In particular, on the basis of histological studies (Berrill, 1947; Mukai et al., 1987), *B. leachi* was suggested to be a species in which the BP epithelium secretes a coagulable substance to pass to the embryo, but detailed supportive observations of the possible embryo-parent nutritional exchange were not further reported. In this respect the only species analysed at the ultrastructural level was *Botryllus schlosseri*, an ovoviviparous species close to *B. leachi*, the BP of which consists of a simple placental cup and which does not show evident signs of embryo-parent exchange (Zaniolo et al., 1987).

In this paper we report on the ultrastructural differentiation of the egg envelopes and BP epithelium, indicating that a close embryo-parent nutritional relationship is established in *B. leachi*.

Materials and Methods

Botrylloides leachi (Savigny) is a colonial ascidian forming a great number of zooids embedded in a common tunic. Three blastogenic generations coexist in the colony: filtering adults, their buds, and the youngest buds produced by the latter; when the adults die, the oldest buds open the siphons and the yongest ones form new buds. The zooids are hermaphrodite and gonads develop on each side of the body close to the atrial wall. Each female gonad, posterior to the testis, has one mature egg and several small previtellogenic oocytes. Fertilization occurs when the ripe egg ovulates in the BP; here, the embryo develops and the larva escapes just before the maternal zooid degenerates.

All specimens used in this study were obtained from the lagoon of Venice and grown in the aquaria at the Stazione Idrobiologica of Chioggia.

Morphological study

Part of the colonies were fixed in 1.5% glutaraldehyde buffered with 0.2 M sodium cacodylate plus 1.6% NaCl. After washing in buffer and postfixation in 1% OsO_4 in 0.2 M cacodylate buffer, specimens were dehydrated, embedded in glycidethere 100 (Serva) and sectioned with an LKB IV Ultratome. Thick sections (1 μm) were stained with toluidine blue. Thin sections were counterstained with uranyl acetate and lead citrate and examined under a Hitachi H-600 electron microscope.

Tests for polysaccharides and glycoproteins for electron microscopy

1. The periodic-acid thiosemicarbazide silver proteinate method of Thiéry (1967) was applied on thin sections. After treatment with 1% periodic-acid for 30 min, sections were incubated with 0.2% thiosemicarbazide in 20% acetic acid in water for periods ranging from 30 min to 72 h. Sections were then incubated for 30 min in the dark with 1% silver proteinate in water. Suitable sections were also prepared for control.

2. Thin sections were treated with 1% tannic acid in distilled water for 30 min and then stained with uranyl acetate and lead citrate according to Afzelius's (1992) method.

Results

Egg and ovulation of Botrylloides leachi

The full-grown oocyte, about 300 μm in diameter, is filled with electron-dense yolk globules and has the typical envelopes: test cells (TC), IFC and outer follicle cells (OFC). Between TC and IFC is the acellular vitelline coat (VC), consisting of a loose network of filamentous material (Figs. 1, 2, 6A).

The TC are roundish and encased in deep indentations of the oocyte. They are rich in rough endoplasmic reticulum (RER) and characterized by a well-developed Golgi apparatus and numerous dense bodies with non-homogeneous content (Figs. 2, 3). The IFC are mainly flat, show several cisterns and vesicles of RER with finely granular content, and lie on the VC (Fig. 2). The IFC are in contact with one another by means of cytoplasmic protrusions, thus forming a loose cellular layer. The OFC are characterized by a great number of elongated and parallel cisterns of RER and by roundish dense granules next to the Golgi apparatus in the cell region facing the oocyte (Fig. 2). The OFC form a continuous layer of cylindrical cells externally covered by a thin basement membrane. They insinuate between the IFC, with protrusions which cross the VC and contact the ovular microvilli (Fig. 2).

A very short oviduct is interposed between and touches the OFC and the atrial epithelium (Figs. 1, 6A). The oviduct is a small vesicular structure with a thin single-layered wall. The atrial epithelium is also commonly flat but, in the area next to the oviduct, it thickens and forms a flask-shaped structure, the BP, communicating with the seawater-filled atrial chamber (Figs. 1, 6A). The BP has a cylindrical epithelium with a thick basement membrane. Its cells have large nuclei with conspicuous nucleoli. The apico-lateral borders of both oviducal and BP cells are joined to one another all round by short, dense, tight junctions (Fig. 4).

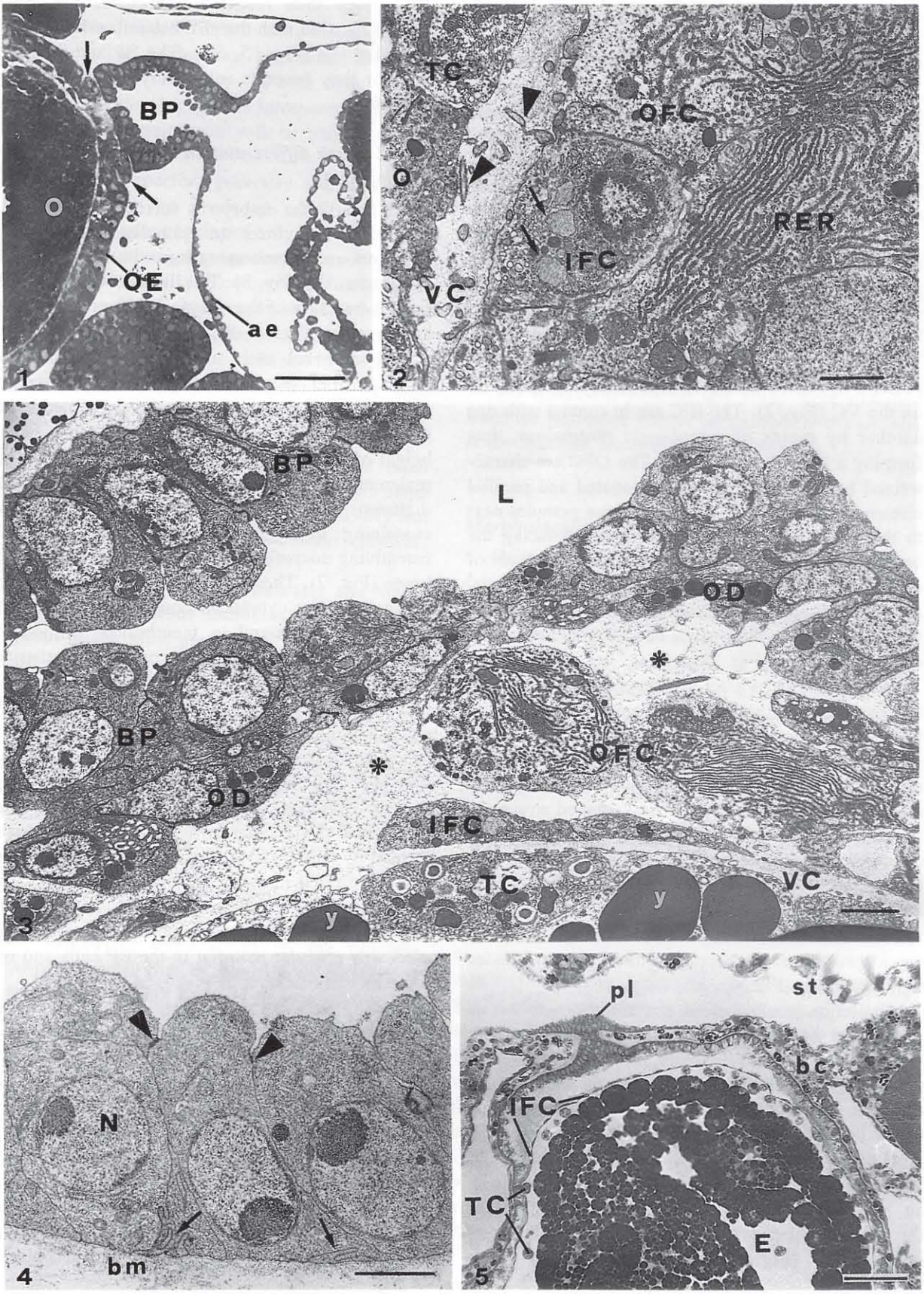
Ovulation occurs when the zooid opens its siphons and begins filtration for the first time: the OFC layer and oviducal epithelium break down at their point of contact (Figs. 3, 6B) and soon, on the opposite side, corresponding fusion and breaking occur between the oviducal and BP epithelia, thus creating a pathway for the oocyte (Figs. 6B, C). Discharging the OFC, which remain in the mantle as an ephemeral *corpus luteum*, the oocyte passes through the short oviduct and enters the BP enveloped by IFC (Fig. 6D). Fertilization occurs in the BP, which then restricts and closes the original communication with the atrial chamber by fusing the epithelial lips (Figs. 5, 6D). The BP and developing embryo thus become completely isolated from the seawater.

Brood pouch differentiation during embryo development

In the BP the embryo is surrounded by the IFC (Fig. 5) which form an epithelial sheet with the basement membrane mingling with the underlying continuous VC (Fig. 8). TC, liberated after ovulation from the surface of the oocyte, are free in the perivitelline space (Figs. 5, 8).

BP epithelial cells are cylindrical during egg cleavage, but they now begin to show irregular apices (Figs. 5, 8). Adjacent cells are joined to each other by tight junctions located at less than half the height of the cell (Figs. 7, 8). The basolateral plasmalemma folds; and contiguous cells become interdigitated, defining restricted cytoplasmic regions containing homogeneous, thin fibrillar material, resembling microfilaments parallel to the basal membrane (Fig. 7). These regions are lacking or poor in organelles. The cytoplasm contains numerous ribosomes free or bound to membranes; smooth and rough cisterns and vesicles are present together with several mitochondria. Dense granules, not bound by membrane and mostly consisting of clusters of a few small subunits, are scattered in the cytoplasm, but often crowd in cytoplasmic areas poor in mitochondria and other organelles. The subunits are larger than the ribosomes and are about 20 nm in diameter, like typical β-glycogen particles, so that the clusters formed by their aggregation (about 80 nm in diameter) correspond in size and form to the α-glycogen rosette granules of other animals (Fig. 7) (Geddes, 1986). Thiéry's test and tannic acid staining also mark this granular material in the BP cells and reinforce its identification as glycogen.

During embryo development, in the medio-apical region the BP cells further develop various indentations and cytoplasmic projections branching into the BP lumen (Figs. 9, 11). The cells are usually connected to each other only at the basolateral area by interdigitations, which are more extended than in the previous stages (Figs. 9, 11, 12). They have a regular intercellular cleft crossed by faint striations. Several mitochondria are found in the cytoplasmic area next to the interdigitations, but they do not establish close spatial relationships with the plasma-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lemma (Fig. 12). Tight junctions are now deeper than in previous stages (Fig. 12 inset). Large areas of cytoplasm are deprived of organelles and filled with glycogen particles (Figs. 9-11). In particular, glycogen progressively accumulates in most of the cytoplasmic extensions penetrating the space between BP and IFC (Fig. 9). Large numbers of glycogen particles are frequently seen to be released from the apical regions and fill the BP lumen, also approaching the IFC (Figs. 9, 13, 14). The IFC (Figs. 11, 14) always form a continuous flat epithelium round the embryo and adhere to the VC. They are rich in endoplasmic reticulum, mainly in the form of round vesicles. Several coated pits are occasionally recognizable on the cell side facing the BP (Fig. 14).

In advanced phases of embryo development, the IFC are absent in some regions around the embryo, presumably owing to rupture and subsequent dislocation (Fig. 16). The embryo secretes the tunic, formed of various layers of fibrous matrix (Figs. 16, 17). The epithelial cells of the BP are extremely polymorphic and interdigitate one another more deeply than those of previous stages (Fig. 15).

In the apical region the cells extend long irregular protrusions, defining labyrinthic spaces (Figs. 15–17). Nuclei are commonly basally located but are sometimes seen in the apical extensions (Fig. 17).

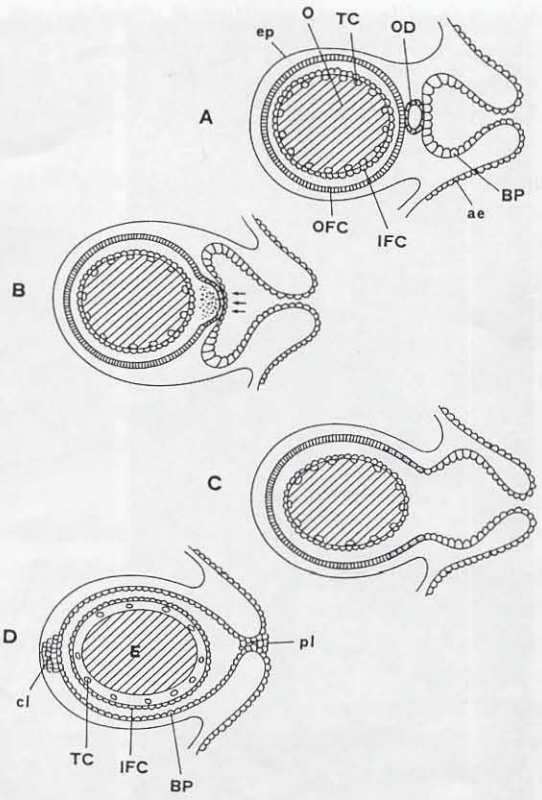
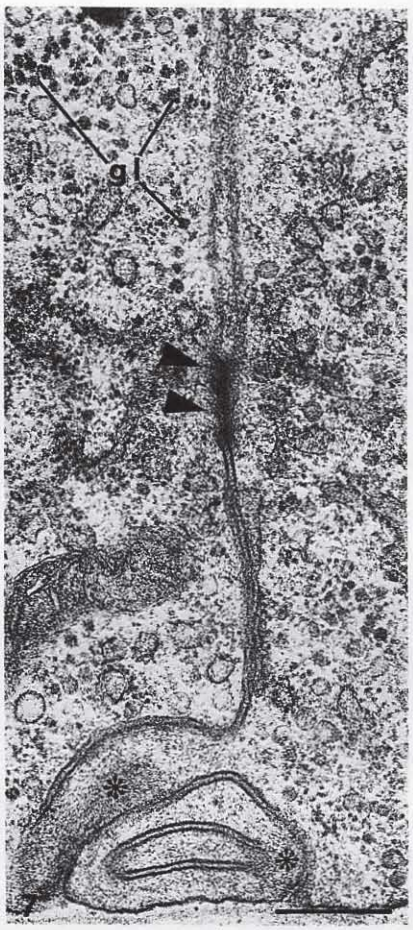


Fig. 6. Schematic drawing of ovulation in *B. leachi*. A: Before ovulation oocyte (O) is in mantle enveloped by OFC, IFC and TC. A small vesicular oviduct (OD) lies between oocyte and BP, a thickened outgrowth of atrial epithelium (ae). B: Oviduct is broken in fusion zone with OFC. On opposite side, oviducal and BP epithelia are still fused (arrows). C: Oocyte, enveloped by IFC, moves towards BP, passing along oviduct. D: After fertilization BP cuts off communication with atrial chamber and forms a plug (pl) so that embryo (E), contained in BP, becomes isolated from seawater. Discharged OFC form a provisional *corpus luteum* (cl).

Golgi fields, numerous mitochondria and long ER cisterns are found in the cytoplasm. Glycogen frequently accumulates in the distal regions of cells, often forming large bubbles protruding into the lumen of the BP towards the embryo (Figs. 15, 16). As embryo development progresses, these regions become poorer and poorer in particles and various cytoplasmic areas appear empty of glycogen (Figs. 16, 17). Also in the BP lumen, glycogen becomes progressively less frequent (Fig. 17); at the same time particles corresponding in size and cytochemical response to glycogen become recognizable in the cells of embryonic tissues, free or packed in small masses (Fig. 17).

Plate I. Fig 1. Thick section of *Botrylloides leachi* showing one full-grown oocyte (O) surrounded by ovular envelopes (OE) and thickened BP, communicating with atrial chamber. Arrows: tangential section of oviduct between BP and oocyte. Scale bar: 35 μm. Toluidine blue. **Fig. 2.** Details of egg envelopes: OFC show numerous long RER cisterns; IFC have several RER vesicles (arrows). Loose net of VC is crossed by ovular microvilli and OFC protrusions (arrowheads). Scale bar: 1.5 μm. **Fig. 3.** At ovulation, due to breaking of oviducal wall (OD) and OFC, oocyte enveloped by IFC and VC penetrates oviduct lumen (asterisks), where some OFC are free. On opposite side, BP lumen (L) is delimited by oviducal and BP epithelia fused together. TC are encased in oocyte depressions (cfr. Fig. 6B). Scale bar: 3.5 μm. **Fig. 4.** At ovulation BP epithelium is composed of cylindrical cells with large nuclei (N) and nucleoli. In basolateral area cells are poorly intergiditated (arrows) and are connected lateroapically by short tight junctions (arrowheads). Scale bar: 2.5 μm. **Fig. 5.** Thick section of an embryo (E) in BP enveloped by IFC, with TC free in perivitelline space. BP wall is completely bathed by blood. Original communication of BP lumen with seawater of atrial chamber is closed by cell plug (pl) (see Fig. 6D). Toluidine blue. Scale bar: 50 μm.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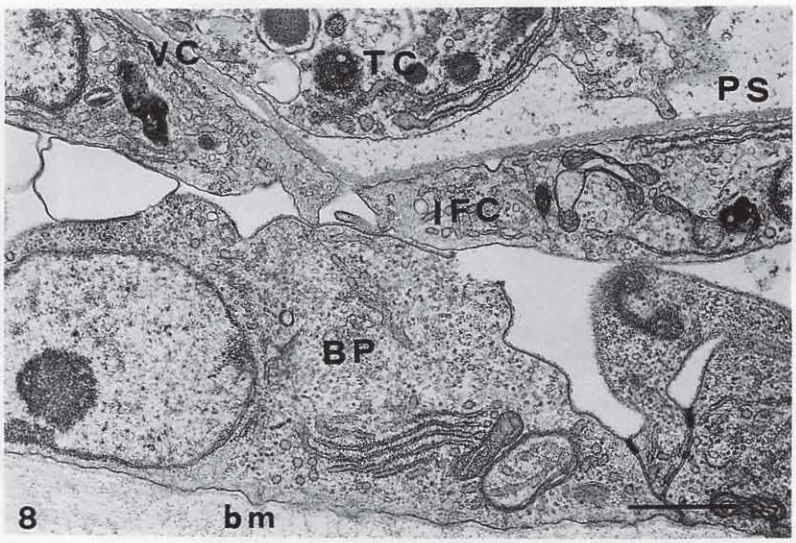
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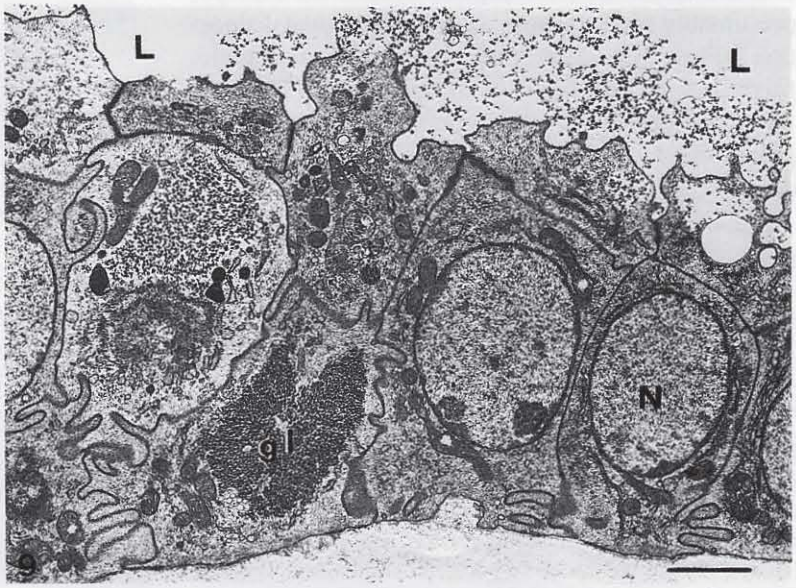
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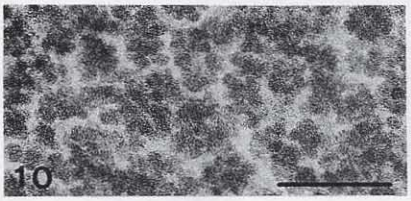
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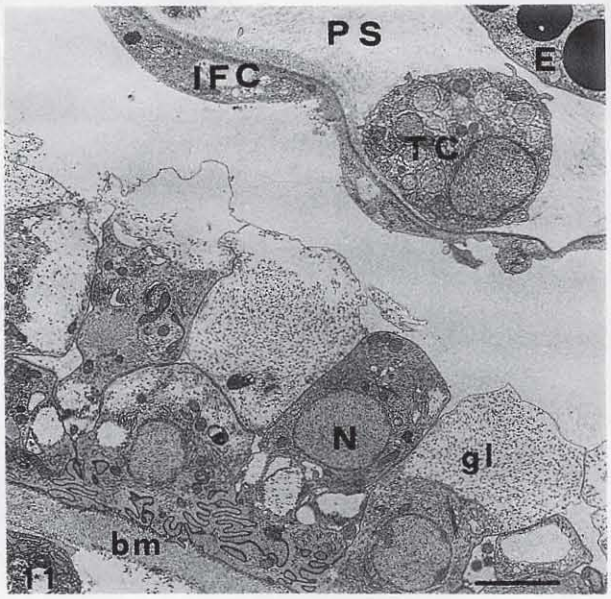
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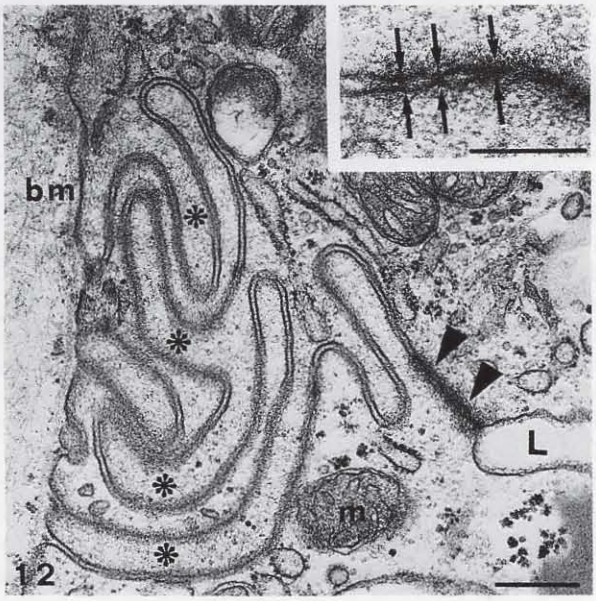
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The main changes which the BP epithelium undergoes during embryo development are summarized in Fig. 18.

Discussion

Egg envelopes and ovulation

In *B. leachi* the number of maturing eggs is reduced to one to two per zooid. The eggs are rich in yolk and have well-developed OFC with signs of intense protein synthesis, probably yolk precursors, as also suggested for other species with yolked eggs and comparable OFC (Martinucci et al., 1988; Sabbadin et al., 1992; Manni et al., 1993, 1994).

Our results agree with those of Mukai et al. (1987) in that the egg enters the BP through the short vesicular oviduct, discharging the OFC. The ultrastructure of this process shows that the oviducal epithelium fuses with OFC and BP epithelium and then breaks so that a temporary canal is opened, the continuous walls of which, provided with tight junctions, separate seawater coming from the atrial chamber from blood circulating in the mantle.

As in other botryllids (Mukai, 1977; Mukai et al., 1987), the BP arises as an outgrowth of the atrial epithelium — a mode of formation commonly found in the subfamily Botryllinae, but unlike that of *Hypsistozoa fasmeriana* (Brewin, 1956) and *Simplegma japonica* (Mukai and Watanabe, 1989) in which the BP is formed of the enlarged distal portion of the oviduct.

In *B. leachi*, after entry of the egg and fertilization, the BP cuts off the original communication with the atrial chamber and becomes more vascularized by circulating blood. The embryo, surrounded by IFC and VC, develops in a space completely isolated from the external environment and the BP wall becomes the compulsory way through which the embryo communicates with its parent and with the outside world.

Brood pouch cytodifferentiation

The BP epithelium, initially poorly differentiated, undergoes progressive changes, all indicating that its cells are involved in the transport, synthesis, storage and release of various substances, in particular sugars. The chief features are: a) extension of the basolateral plasmalemma with the formation of numerous deep cellular interdigitations; b) persistence and reinforcement of tight junctions; c) progressive increase in the apical plasmalemma, with the formation of numerous polymorphic cytoplasmic protrusions; d) synthesis, accumulation and mobilitation of large quantities of glycogen.

Cell interdigitations increase and become extremely extensive during embryo development. The great increase in the basolateral plasmalemma favours surface exchange between adjacent cells and the underlying blood (Berridge and Oschmann, 1972). In *B. leachi* no close association of mitochondria with the plasmalemma indicative of the presence of mitochondrial pumps has been observed: although a number of mitochondria are scattered in the cytoplasm, the BP epithelium lacks this characteristic association, a sign of intense and active transport, as found in arthropod cells (Noirot-Timothée and Noirot, 1980) and ascidian tissues (Burighel et al., 1985). Cytoskeletal elements which are particularly well-developed in the areas of cell interdigitation may be involved in the regulation of cell contractility, as a response to variations in the volume and pressure in both cells and BP lumen.

Apicolateral tight junctions separate domains of apical and basolateral plasmalemma permitting membrane polarization and seal the intercellular cleft, creating a barrier between luminal and basolateral spaces, as commonly occurs in other ascidians and

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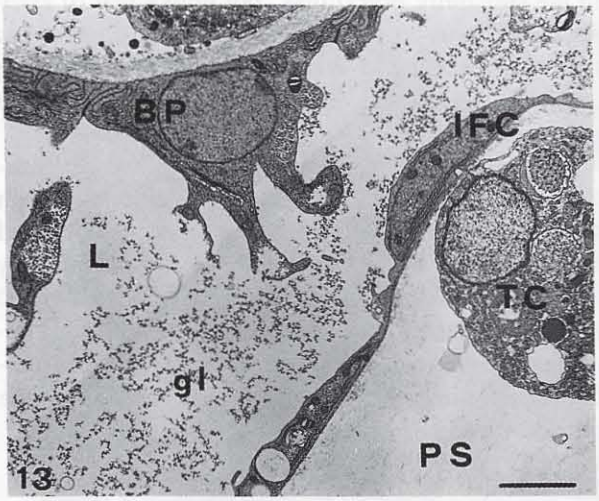
Plate II. Fig. 7. At early stage of embryo development, adjacent cells of BP increase interdigitations in basolateral area, rich in microfilaments (asterisks). Apical portions of cells are well extended over level of the tight junctions (arrowheads). Glycogen particles (gl) are scattered in cytoplasm. Scale bar: 0.5 μm. **Fig. 8.** Sequence of cell layers around embryo. TC in perivitelline space (PS). IFC form a continuous layer resting on VC. BP epithelium has irregular apical profiles and thick basement membrane (bm). Scale bar: 1.5 μm. **Figs. 9-14.** Intermediate stages of embryo development. **Figs. 9, 10. Fig. 9.** Oblique section of BP epithelium. In cytoplasm, areas lacking organelles are filled with dense glycogen particles (gl), shown in detail in Fig. 10. Cell apices are extremely irregular and glycogen particles are also free in BP lumen (L). Scale bar Fig. 9: 2 μm; scale bar Fig. 10: 0.2 μm. **Fig. 11.** Apical regions of BP cells completely filled with glycogen particles (gl). Scale bar: 3.5 μm. **Fig. 12.** Detail of basolateral membrane of BP cells showing very deep interdigitations. An extended tight junction (arrowheads) seals paracellular pathway separating luminal (L) from intercellular lateral spaces. Asterisks: extensions of same cell. Inset: arrows mark several points of membrane apposition in a tight junction. Scale bar: 0.4 μm, inset scale bar: 0.2 μm.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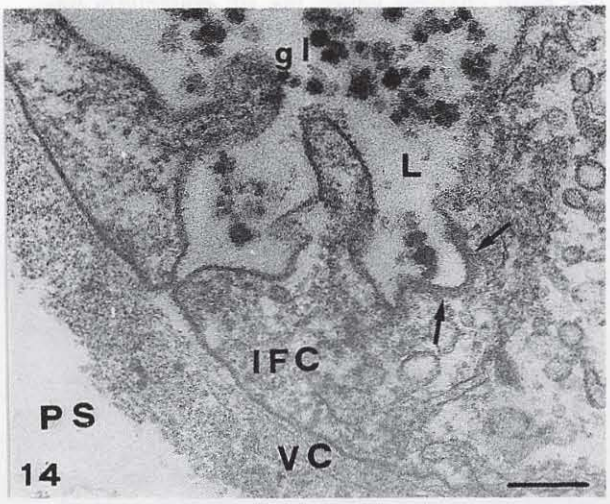
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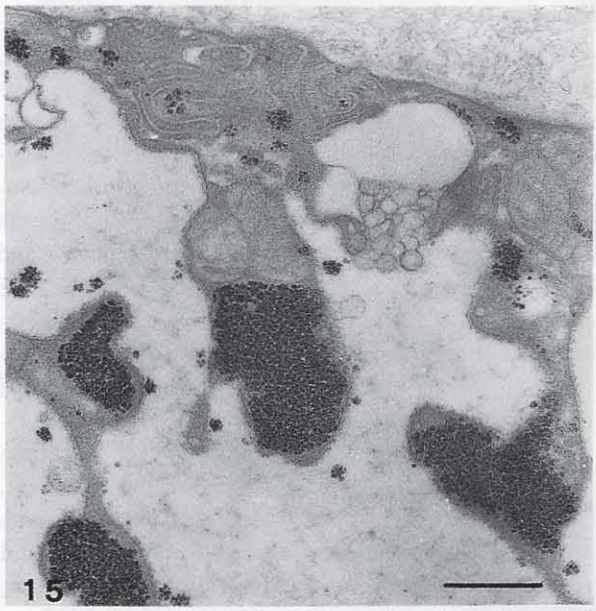
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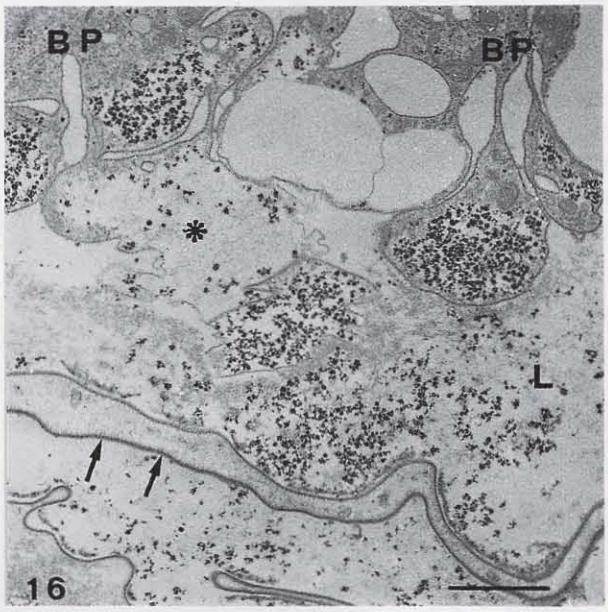
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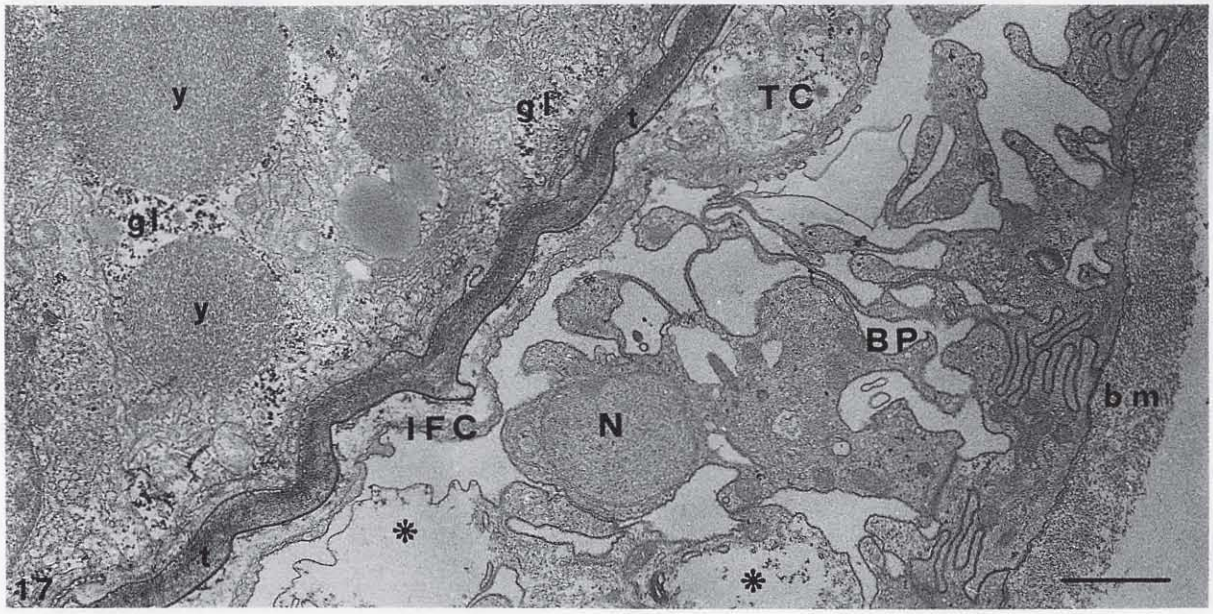
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vertebrates (Lane et al., 1986; Schneerberger and Lynch, 1992). The BP lumen content, completely isolated, can thus be modified by exchanges with blood only by the transcellular pathway of the BP epithelium. Although the cell volume is relatively limited, the apical plasmalemma becomes progressively more and more extensive, giving single cells exceptionally large areas of differentiated membranes for exchange with the lumen of the pouch.

Another important aspect of differentiation of BP cells is the appearance and accumulation of glycogen, also identified by means of cytochemical reactions. In BP epithelium glycogen is not recognized before egg cleavage; later, in the early stages of embryo development, it is found as free particles, which later accumulate progressively in large areas not bound by membranes and lacking organelles. In more advanced stages, glycogen completely fills the tips of the apical protrusions of BP cells and many glycogen rosettes are also found free in the BP lumen. The presence of a increasingly intense gradient of accumulation and mobilitation of glycogen from the BP epithelium toward the embryo suggests that glycogen represents the pathway for the exchange of substances from parent to embryo.

In agreement with Berrill (1947), Mukai et al. (1987) also noted that the BP cells of *B. leachi* are "rich in secretory granules" and that "they clearly secrete a coagulable substance from their plasmodial ends." By means of the Thiéry and tannic acid tests, we demonstrate that the "secretory granules" may correspond to the large accumulation of glycogen particles in the apical areas of cells, and that the free glycogen particles in the lumen may correspond to the "coagulable substance" previously seen with light microscopy. However, it cannot be excluded that the BP epithelium is also engaged in the secretion of morphologically undetectable molecules necessary for embryo development.

As in hepatic parenchymal cells (Hems and Whitton, 1980), through glycogenolysis, glycogen is commonly mobilized from cells as free glucose. But in *B. leachi* very many extracellular glycogen parti-

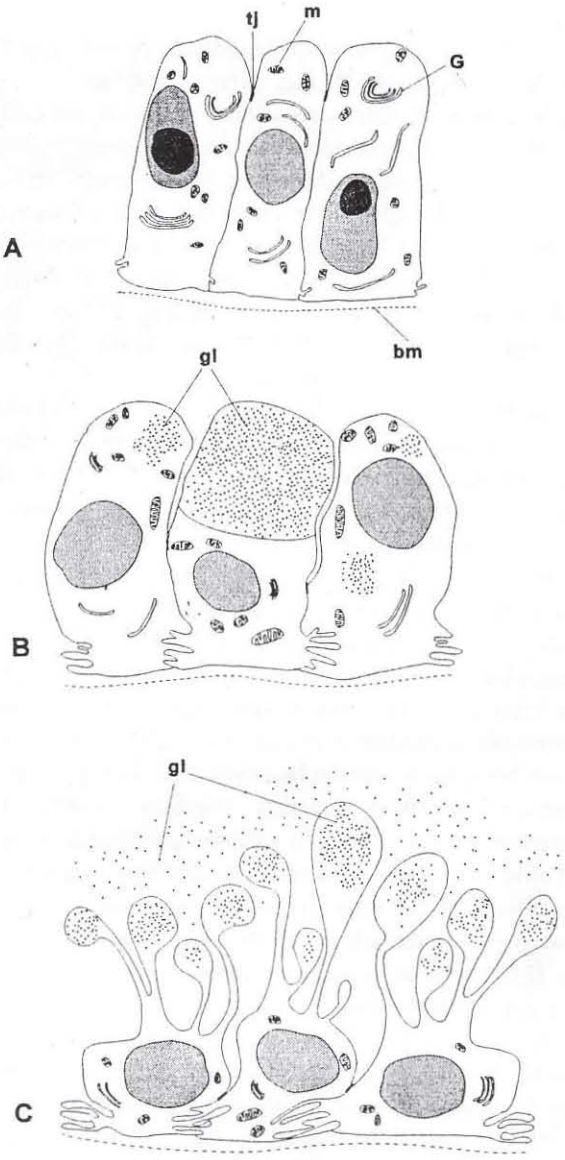

Fig. 18. Schematic drawing of BP cell changes from ovulation (A) to intermediate (B) and advanced (C) stages of embryo development. Note progressive increase in baso-lateral interdigitations, polymorphic apical protrusions, tight junction extensions (tj), glycogen storage (gl) and release from apical regions of BP.

Plate III. Figs. 13, 14. Glycogen particles (gl) are free and fill BP lumen (L), approaching but not passing IFC. In Fig. 14 arrows indicate a coated vesicle. Tannic acid staining. Scale bar Fig. 13: 3.5 μm; scale bar Fig. 14: 0.3 μm. **Fig. 15.** Thiéry's method reveals large quantities of dense glycogen particles in polymorphic apical regions of BP cells. Scale bar: 0.8 μm. **Fig. 16.** A fine silver precipitate covers glycogen particles, filling some apical areas of BP and free in lumen (L) approaching tadpole caudal fin (arrows); other apical areas (asterisk) are empty of glycogen. IFC are lacking in this region. Thiéry's staining method. Scale bar: 2 μm. **Fig. 17.** When larva approaches moment of hatching, large masses of glycogen particles can no longer be recognized (asterisks) in extremely polymorphic BP cells. Larval cells possess glycogen particles (gl) among yolk globules (y). A dense layer of larval tunic (t) is recognizable. Tannic acid staining. Scale bar: 2 μm.

cles are frequently seen in the BP lumen and close to the IFC. Although artifact aspects may not be excluded, these features recall a process of apocrine secretion. Glycogen released by apocrine secretion has previously been described in different tissues such as in the glandular epithelial cells of human postovulatory endometrium (Spornitz, 1993) and in the mantle epithelial cells of *Mytilus edulis* during gametogenesis (Pipe, 1987). According to Pipe, the process represents a particular mechanism for dissemination of great quantities of nutrient reserves.

In *B. leachi* it seems unlikely that extracellular glycogen particles could reach the embryo, either through the intercellular spaces of IFC and VC fibrils or overpassing IFC by a mechanism of polarized uptake and release, although suggestive features of particle endocytosis by IFC have been encountered. Nutrient reserves are probably passed to the embryo in the form of free glucose derived from intracellular glucogenolysis in the BP and/or IFC epithelia or from extracellular digestion in the BP lumen, as degradative enzymes for glycogen breakdown have been seen to be associated with glycogen granules in other animals (Geddes, 1986). In advanced stages glycogen rosettes are found in embryonic cells, but it cannot be established whether they derive from yolk granule metabolism or extraembryonic contribution. In the full-grown embryo, the IFC break at several points and glycogen particles approach the larval tunic.

In conclusion, our observations show that in *B. leachi* the BP is a highly differentiated structure which isolates the embryo from the environment and which is completely bathed by the bloodstream. BP cells undergo progressive changes which strongly increase the areas of exchange with blood and BP lumen, while cell polarization becomes even further marked by the great quantities of glycogen progressively accumulated and released from the apical regions. In *B. leachi*, despite the large amount of yolk of the oocyte, the parent may furnish important nutrients for embryo development. This species therefore represents an interesting intermediate example between ovoviviparity and viviparity in ascidians.

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