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Brood pouch differentiation in *Botrylloides violaceus*, a viviparous ascidian (Tunicata)

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Summary

Botrylloides violaceus, a viviparous colonial ascidian, produces small alecithal eggs which develop into large tadpole larvae after a long period of gestation (about 1 month) in the colonial tunic. Unlike other botryllids, embryo development occurs in a brood pouch which soon becomes detached from the atrial epithelium to reach the colonial tunic and is independent of parent life. This study analyzes the ultrastructural differentiation of the brood pouch—from ovulation to larva release—in order to verify its role in the production, secretion and transport of nutrients for the embryo. Results show that brood pouch cells undergo modifications during embryogenesis, including: (a) increase in baso-lateral foldings of membranes and formation of long basal membranal channels; (b) shifting of tight junctions towards the base of the cells and increase in apical cell region by means of polymorphic, cytoplasmic protrusions; (c) synthesis, storage and secretion towards the embryo of proteinaceous materials and glycogen. All these features indicate that the brood pouch epithelium is involved in nutritional exchanges between the blood flowing into the tunic and the embryo. Aspects of viviparity in ascidians and comparison between the brood pouches of *B. violaceus* and of the ovoviviparous *Botrylloides leachi* are discussed.

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

Introduction

Ascidians, benthic filter-feeding tunicates, include oviparous, ovoviviparous and viviparous species. Commonly, solitary species are oviparous and spawn many small eggs in seawater, while colonial species are ovoviviparous, with a few yolked eggs retained during embryonic development in the atrial chamber, in oviduct, or tunic (Kott, 1985, 1990, 1992; Martinucci et al., 1988; Zaniolo et al., 1994a; Burighel and Cloney, 1997). Some solitary ascidians may be oviparous or ovoviviparous according to the inhabited localities, or show different brooding strategies in the same genus (Lambert et al., 1995).

The viviparous species so far known are the Polycitoridae *Hypsistozoa fasmeriana* and the Styelidae

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This paper is dedicated to Prof. Armando Sabbadin on the occasion of his retirement from the Department of Biology, University of Padova, Italy.

Botrylloides lenis and *Botrylloides violaceus* (Brewin, 1956; Mukai et al., 1987). The former is considered the species with the most advanced form of viviparity because of the specialization of the oviduct for nutrition, exceptionally long gestation (5.5 months) and the development of a larva with numerous buds starting from a tiny alecithal egg (diameter 25 μm). The two species of the genus *Botrylloides* display examples of viviparity unlike that of *Hypsistozoa*; in particular, the atrial epithelium, not the oviduct, is involved in brood pouch (BP) formation. Both these botrylloids produce only a few small alecithal eggs (less than 100 μm in diameter), and the brooding embryos develop into free-swimming larvae in about 3 weeks (*B. lenis*) or more than a month (*B. violaceus*) (Mukai et al., 1987).

In all three viviparous species, intense embryo-parent exchange occurs because, starting from eggs lacking yolks, large larvae are produced after a long period of gestation. However, fine studies of the BP during embryogenesis and its structure and role in production and/or transport of nutrients for the embryo have not yet been reported. In this respect, the only species analyzed at the ultrastructural level are two ovoviviparous Botryllinae with yolked eggs: *Botryllus schlosseri*, in which the simple placental cup does not show specialization for embryo-parent exchange (Zaniolo et al., 1987) and *Botrylloides leachi*, whose BP epithelium synthesizes and releases large amounts of glycogen towards the embryo by means of apocrine secretion (Zaniolo et al., 1994b).

This paper analyzes the ultrastructural modifications of BP epithelium in *B. violaceus*, starting from ovulation to the release of gigantic larvae, in order to verify its possible role in the production, secretion and transport of nutrients from the parental bloodstream to the embryo. Differentiation of the BP of this viviparous species is also compared with that of the closely related ovoviviparous *B. leachi*, and various aspects of viviparity in ascidians are discussed.

Materials and Methods

B. violaceus Oka, 1927 (*sensu* Saito et al., 1981) is a Pacific colonial ascidian recently collected by us in the lagoon of Venice where it is now spreading and may be supplanting the local congeneric species *B. leachi* (Savigny, 1816). The two species differ greatly in the size and morphology of the larva which in *B. violaceus* has a trunk about 1 mm in length with generally 30–32 ampullae and in *B. leachi*, a trunk of about 450 μm and only eight ampullae. Moreover, the oozooid of *B. violaceus* has buds on both sides while that of *B. leachi* only has them on the right.

Colonies of *B. violaceus* were cultured in the laboratory and attached to glass plates according to the technique of Sabbadin (1960). Each colony was formed from a great number of zooids embedded in a common tunic and vascularized by means of a common circulatory system. Three blastogenic generations of zooids may coexist in the colony: filtering adults, their buds, and the youngest buds produced by the latter. When the adults undergo degeneration, the oldest buds open their siphons and the youngest ones form a new generation of buds. The zooids are hermaphroditic, with gonads on each side of the body close to the atrial wall. One or two eggs usually mature, sometimes on the right side only (Saito et al., 1981). Fertilization occurs when ripe eggs ovulate in the BP; in laboratory conditions (19°C) embryogenesis lasts about one month.

For histological purposes, pieces of colonies were fixed in Bouin's solution made with seawater. Paraffin sections 7 μm thick were stained with Delafield's hematoxylin and eosin.

For electron microscopy, selected fragments of colonies were fixed in 1.5% glutaraldehyde in a 0.2 M sodium cacodylate buffer, pH 7.2–7.4, with added 1.5% sodium chloride. Specimens were later post-fixed in 1% osmium tetroxide buffered in 0.2 M cacodylate. Fragments were then dehydrated in alcohol and embedded in glycidether 100 (Serva). Thick sections (1 μm) were stained with toluidine blue. Thin sections, contrasted with uranyl acetate and lead citrate, were examined under a Hitachi H-600 electron microscope.

To reveal polysaccharides and glycoproteins, thin sections were treated according to the periodic-acid thiosemicarbazide silver proteinate method of Thiéry (1967): 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 as controls. Other sections were prepared according to Afzelius's (1992) method: they were treated with 1% tannic acid in distilled water for 30 min and then stained with uranyl acetate and lead citrate.

For scanning electron microscopy, pieces of colony containing developing larvae were fixed as described for electron microscopy. After dehydration, dissection was performed in order to expose the BP lumen and the embryo. Specimens were critical-point dried, sputter-coated with gold-palladium, and observed under a Cambridge Stereoscan 260.

Results

Ultrastructure of ovary, oviduct and brood pouch

The female gonads of *B. violaceus* are located in the mantle close to the atrial wall (Fig. 1) on both sides of the zooid. As in other Botryllinae, one or two eggs, each with its own oviduct, mature per zooid. During oogenesis, the area of the atrial epithelium close to the egg forms an outgrowth, the brood pouch, which receives the egg at ovulation (Mukai et al., 1987).

Our ultrastructural observations on ripe eggs are consistent with the previous reports by Manni et al. (1995). The oocyte (80 μm in diameter) lacks electron-dense yolk granules, but has cytoplasm filled with clear vesicles and peripheral scattered lipid droplets. The nucleus is eccentric, placed towards the oviduct, and possesses a large nucleolus. The oolemma bears numerous microvilli, which decrease in number and extent at ovulation. The oocyte is surrounded by four envelopes (Fig. 2): test cells encased in the oolemma and rich in RER cisterns, Golgi stacks, and granules; inner follicle cells (IFC) and outer follicle cells (OFC), which form two layers with similar cytoplasmic characteristics; and the vitelline coat (VC) which forms a homogeneous fibrillar layer between the IFC and the oolemma, and compacts with approaching ovulation.

The oviduct (Figs. 1,2) is a very small, closed vesicle adhering to and interposed between the oocyte and the BP. It is composed of a single epithelium whose cells, latero-apically sealed by tight junctions, define a small lumen.

The epithelium of the BP is single and thick, and thins out at the periphery into the squamous epithelium of the atrial chamber. The sac-like BP is open towards the atrial chamber and filled with seawater (Figs. 1–3). The BP forms during early oogenesis; as ovulation approaches, BP cells are cylindrical and rich in ribosomes, both free and bound to the endoplasmic reticulum. Basal bundles of microfilaments, some large vesicles of RER containing a homogeneous matrix, an apical Golgi, scattered mitochondria and a large round nucleus are also present. Adjacent cells are sealed by short tight junctions (Figs. 3, 4).

Ovulation and BP segregation

Ovulation takes place when the blastozooid becomes adult, opening its siphons and beginning filtration. At this time, the BP bottom extends deeply into the mantle (Fig. 5); the oviduct fuses with the OFC and BP wall and then breaks to create a canal through which the egg enters the BP, discharging the OFC (Figs. 6, 7). BP cells, particularly rich in bundles of microfilaments, extend to embrace the entering egg (Figs. 8, 9). Cell debris remains for a time between IFC layer and BP epithelium, probably derived from OFC and oviduct cells removed during ovulation (Fig. 8). Shortly after ovulation, the VC begins to elevate below the IFC, forming the perivitelline space into which test cells move, accumulating in the side opposite the BP aperture (Fig. 7). While test cells are free in the growing perivitelline space, the oocyte profile becomes smooth (Figs. 8, 10). Since the BP is in communication with the atrial chamber, the egg is bathed by seawater driving sperm for fertilization, after which the lips of the BP approach each other and fuse, closing communication with the atrial chamber and completely separating the BP lumen, with the embryo, from the external environment. The BP, accompanied by epidermis and amply bathed by the bloodstream, becomes detached from the atrial epithelium and moves towards the tunic, herniating from the body of the parent. The space between BP and epidermis is in communication with tunic vessels and is filled with flowing blood (Fig. 10).

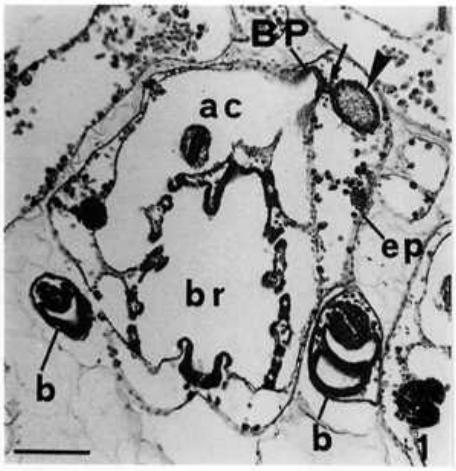
The whole successive development of the embryo is thus independent of the parent and the asexual colonial cycle because it occurs in a lacuna in the tunic. Since blastogenic generation takes place weekly and embryogenesis lasts about one month, the same colonial tunic may contain many embryos at various developmental stages, produced by successive blastogenic generations (see Fig. 21).

Fig. 14 shows the main events occurring during ovulation.

BP differentiation during embryogenesis

Both BP cells and egg envelopes undergo an elaborate series of morphological changes concomitantly with the development of the embryo. Early in embryogenesis (Figs. 10–13), BP cells become cylindrical, with tight junctions located in the apico-lateral position. The nucleus is basally located. The perinuclear cistern has membrane-bound ribosomes and sometimes enlarges, filling with homogeneous material similar to that of some RER cisterns. Rosettes of glycogen, whose presence was confirmed by the cytochemical tests of Thiéry and Afzelius, are widespread (Fig. 13). The IFC constitute a flat cellular layer on the compact VC (Figs. 10, 12). Their cytoplasm contains scattered mitochondria and RER vesicles; their apices, facing the BP, form numerous long microvilli, but evidence of pinocytosis is lacking. The test cells are rich in mitochondria, RER cisterns and granules, and have a well developed Golgi.

During successive embryo development, the BP epithelial cells modify dramatically. The tight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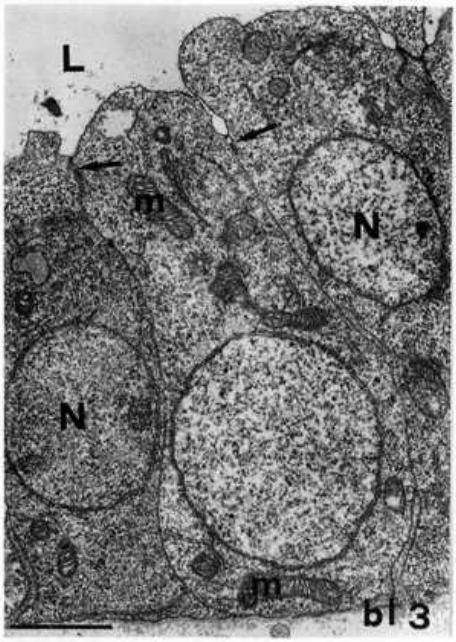
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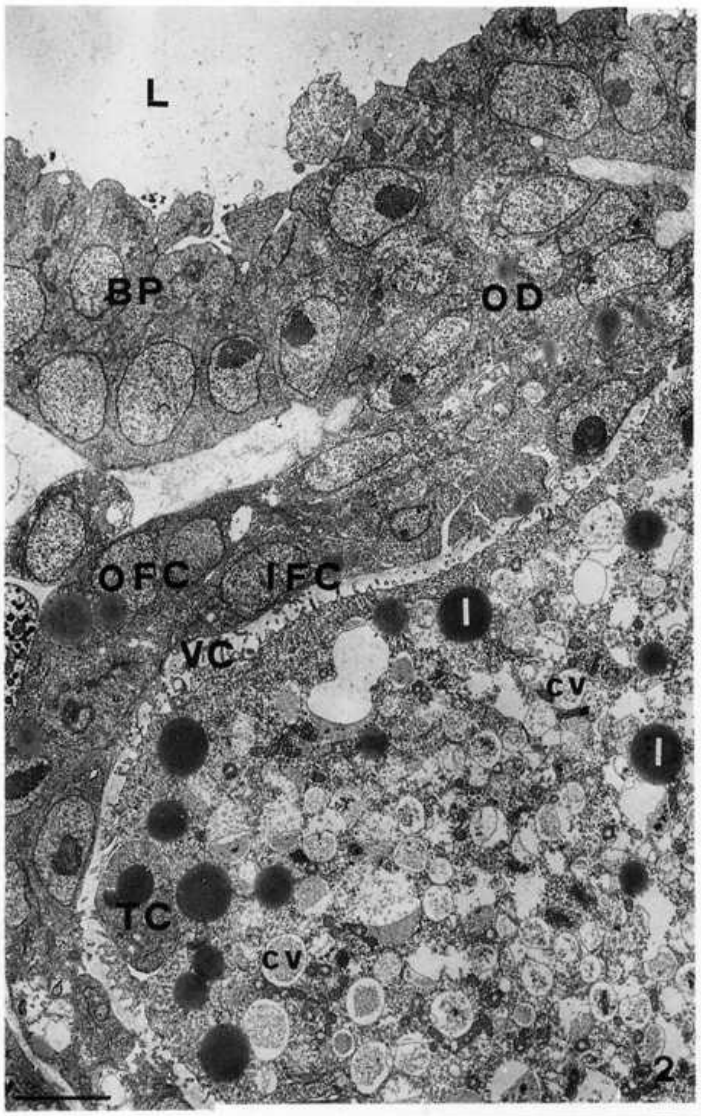
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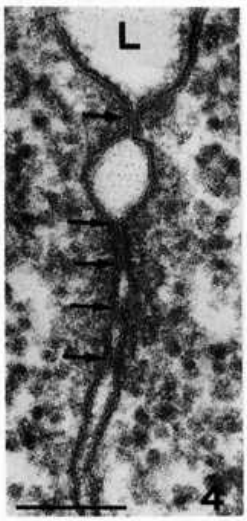
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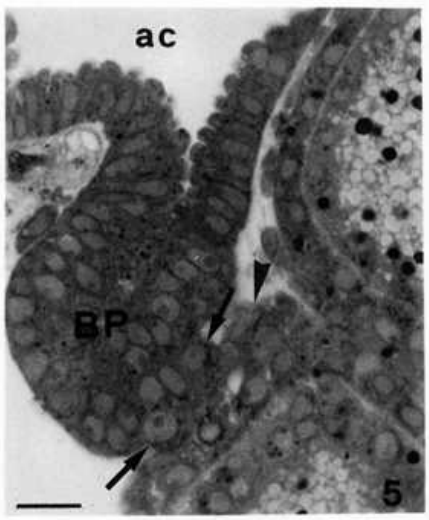
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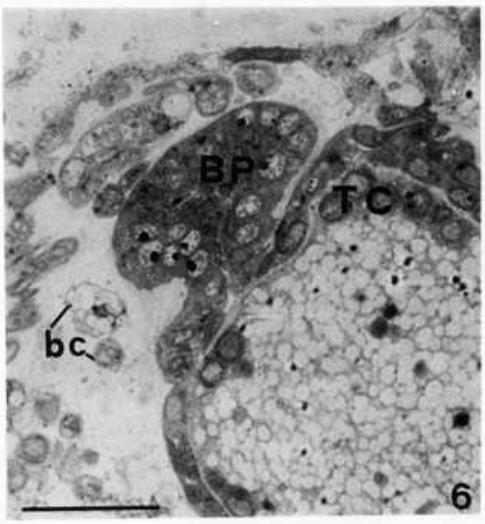
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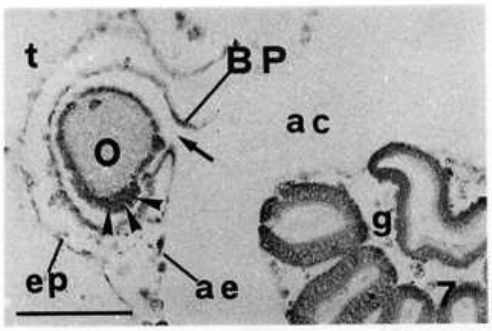
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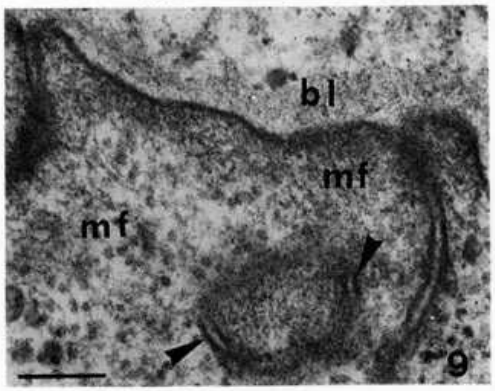
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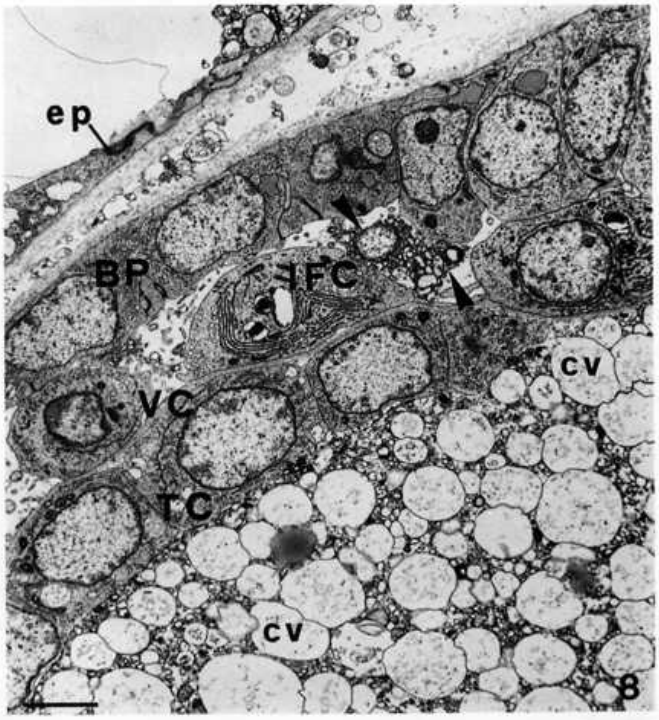
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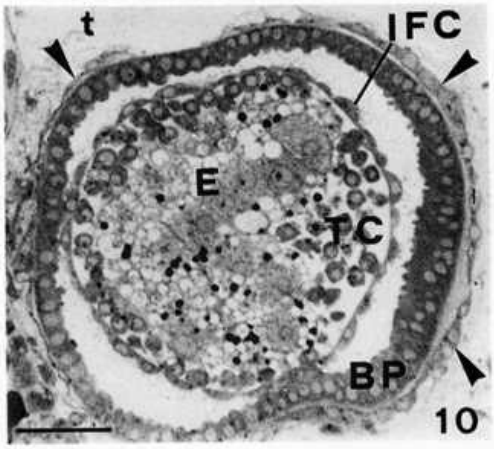
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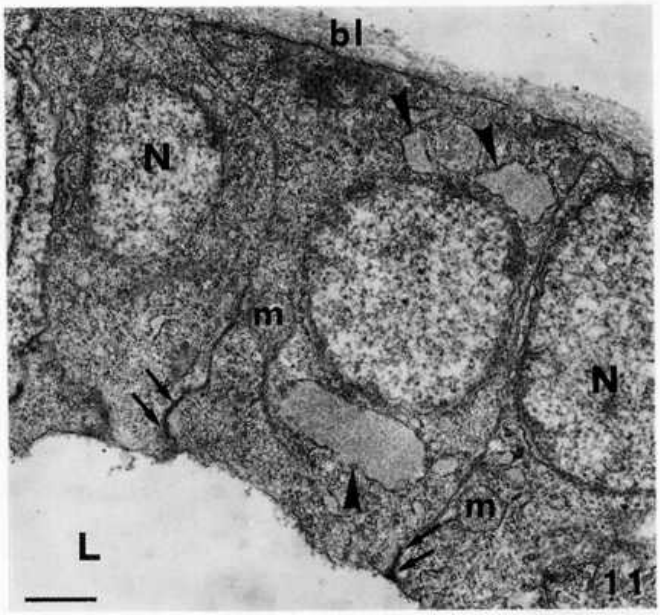
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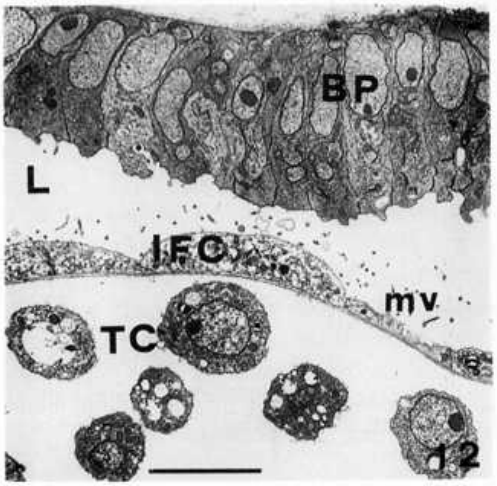
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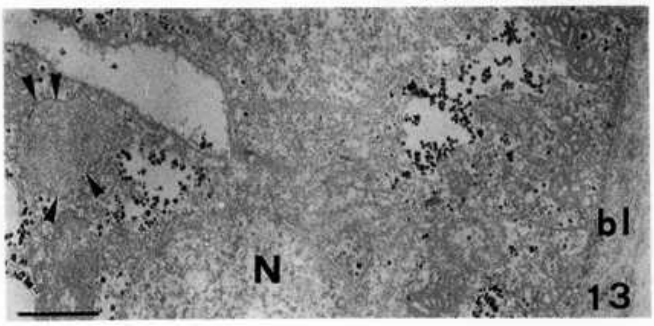
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Figs. 1–6. Fig. 1. Section of part of *Botrylloides violaceus* colony showing an old bud containing an oocyte (arrowhead), connected by its small oviduct (arrow) to brood pouch (BP). ac: atrial chamber; b: young bud; br: branchial sac. ep: epidermis. Hematoxylin-eosin. Scale bar 65 μm. Fig. 2. Growing oocyte has cytoplasm filled with clear vesicles (cv) and some dense lipid droplets (l), and bears short microvilli penetrating vitelline coat (VC). Test cells (TC) are encased in oocyte depressions; outer follicle cells (OFC) and inner follicle cells (IFC) envelope oocyte. Oviduct (OD) is a small vesicular structure interposed between OFC and BP (BP). L: BP lumen. Scale bar 5 μm. Figs. 3,4. Before ovulation, BP cells are cylindrical with large nuclei (N), scattered mitochondria (m) and RER cisterns, resting on basal lamina (bl), sealed apically by tight junctions (arrows) (shown in detail in Fig. 4). Arrows: points of apposition between adjacent junctional membranes. L: BP lumen. Scale bar for Fig. 3: 2 μm. Scale bar for Fig. 4: 0.16 μm. Figs. 5, 6. Thick section of *B. violaceus* showing brood pouch (BP) and egg at onset of (Fig. 5) and during ovulation (Fig. 6). In Fig. 5 oviduct (arrows), here in tangential section, fuses with both outer follicle cells (arrowhead) and brood pouch epithelium. In Fig. 6 oocyte enters BP which penetrates mantle rich in blood cells (bc). Part of another egg is recognizable at top right of Fig. 5. ac: atrial chamber; TC: test cells. Scale bar for Fig. 5: 10 μm. Scale bar for Fig. 6: 20 μm.

Figs. 7–13. Fig. 7. A just ovulated egg is in brood pouch (BP), still communicating (arrow) with atrial chamber (ac). Test cells (arrowheads) accumulate at one pole of oocyte. Toluidine blue. ae: atrial epithelium; ep: epidermis; g: gut; t: tunic. Scale bar: 70 μm. Fig. 8. At end of ovulation, egg lies in restricted lumen of brood pouch (BP). Test cells (TC) are no longer encased in ovular depressions, and inner follicle cells (IFC) are scattered over the thin vitelline coat (VC). BP lumen contains cell fragments (arrowheads). cv: clear vesicles in oocyte; ep: epidermis. Scale bar: 3 μm. Fig. 9. Basal regions of BP cells lie on a basal lamina (bl) and are rich in microfilaments (mf). Arrowheads: foldings of baso-lateral plasmalemma. Scale bar: 0.2 μm. Figs. 10–13: Early embryogenesis. Fig. 10. Thick section of an embryo (E) at gastrula stage in brood pouch (BP), in a colonial vessel bounded by wall (arrowheads). Numerous test cells (TC) are free in perivitelline space under inner follicle cells (IFC). t: tunic. Scale bar: 15 μm. Fig. 11. BP cells are rich in RER vesicles (arrowheads) filled with homogenous granular material, and joined by tight junctions (arrows) towards lumen (L). bl: basal lamina; m: mitochondria; N: nuclei. Scale bar: 1 μm. Fig. 12. Inner follicle cells (IFC) constitute a layer on vitelline coat and extend numerous long microvilli (mv) into lumen (L) of brood pouch (BP). Test cells (TC) lie in perivitelline space and have granules and vacuoles. Scale bar: 7 μm. Fig. 13. Glycogen granules, evidenced with Thièry's cytochemical test, in cytoplasm of BP cells. RER vesicles (arrowheads) are not marked. bl: basal lamina; N: nucleus. Scale bar: 1 μm.

junctions become more extended and shift towards the basal lamina, while the apical region of cells, often containing nuclei, protrudes into the lumen (Fig. 15). Adjacent cells contact each other at baso-lateral sites, interdigitating more and more. At the same time, a complex system of extracellular channels, formed by infoldings of the basal plasmalemma, ramifies and penetrates the cells deeply (Fig. 16). The basal lamina accompanies these basal infoldings. The large nucleus lies in the mid-region and its enlarged envelope contains homogeneous material (Fig. 15). The more apical zone is poor in organelles except for glycogen rosettes and large RER vesicles with homogeneous contents; empty vacuoles may also be seen (Figs. 17, 19). The apical region of cells extends in protrusions which contact each other to define ramified channels continuous with the luminal compartment (Fig. 17). Apical plasmalemmata possess a conspicuous glycocalix, formed of a 50 nm deep layer from which tufts of fibrils emerge and fuse when protrusions of adjacent cells come into contact (Fig. 18). The lumen contains glycogen particles and loose homogeneous granular material, possibly proteinaceous in nature, being secreted by the RER cisterns, some of which may be seen close to the apical plasmalemma (Fig. 19).

In advanced stages, when the tail of the embryo begins to encircle the trunk (Fig. 21), all these BP features become extremely marked. The basal region of cells is very flat, and adjacent cells contact each other by means of very deep interdigitations creating an enormous extension of the baso-lateral plasmalemma (Fig. 22). A thick basal lamina is always present. Most of the cellular body branches and stretches out into the BP lumen (Figs. 20, 22). Long, narrow cell projections float in the lumen, several of which contain nuclei whose envelopes are no longer expanded in the cisterns. Cells contain a well developed Golgi (Fig. 24), mitochondria and numerous tubular cisterns of RER with homogeneous material. Glycogen rosettes are widespread but preferentially accumulate in blebs at the tips of the cytoplasmic branches (Fig. 25 and inset). As in the previous stages, the apical plasmalemma is covered by a thick glycocalix and apical protrusions contact each other. The whole BP lumen contains abundant flocculent, homogeneous material and some glycogen rosettes (Figs. 22, 25).

The IFC now become extremely flat and lengthen to form a delicate sheet around the growing embryo. The space between them and the embryo epidermis contains material, different from the BP lumen, organized in bundles of fibrils 25 nm in diameter, oriented in various directions (Fig. 22). Many fibrils

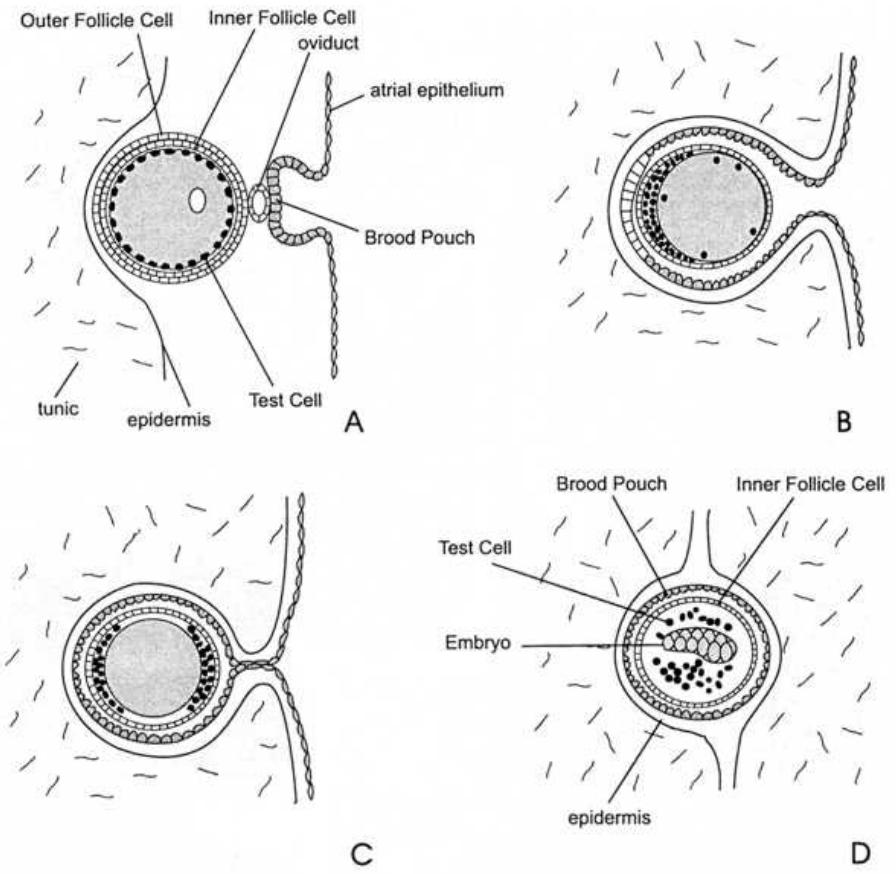


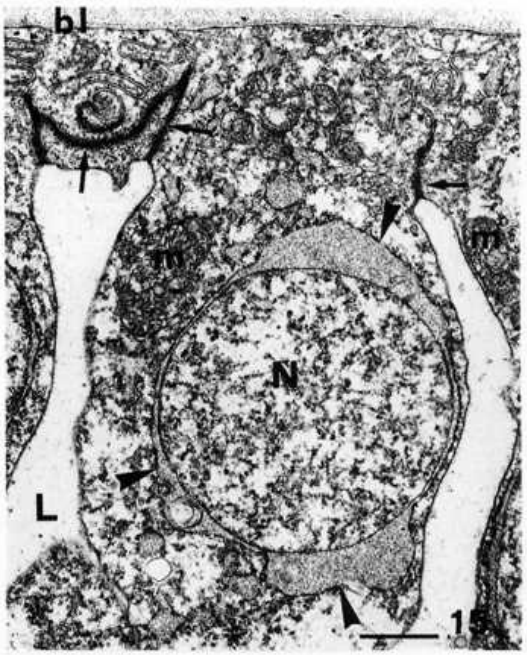
Fig. 14. Sketches of main phases occurring at ovulation and during segregation of fertilized egg in colonial tunic of *B. violaceus*. A: Ripe oocyte in mantle, enveloped by outer follicle cells, inner follicle cells and test cells; a small vesicular oviduct lies between outer follicle cells and BP. B: Egg in BP exposed to seawater coming from atrial chamber; outer follicle cells are discharging, test cells accumulate in perivitelline space at pole opposite to aperture of BP; accompanied by epidermis, BP herniates into tunic. C: Fertilized egg, enveloped by inner follicle cells and with free test cells, segregates into tunic; it is contained in BP which is still connected to atrial epithelium. D: BP with developing embryo is now in tunic, completely separated from zooid. (Vitelline coat is omitted for clarity.)

are attached perpendicularly to the membrane of the IFC. In some points the VC is no longer recognizable (Fig. 26).

The cells of the embryo epidermis have long, very indented apices, and no trace of embryonal or adult tunic secretion is recognizable, although the embryo is well differentiated with the tail encircling the trunk (Figs. 21, 22). Test cells (Figs. 21, 22) are completely embedded in the fibrous matrix and are rich in round granules 1–1.5 μm in diameter. Several of these granules are homogeneously dense; others are heterogeneous and filled with a thread-like matrix, sometimes with a dense cap (Fig. 23). Some large vacuoles are also recognizable, presumably derived from granules which released their contents. Vacuoles may fuse occasionally to form a large, apparently empty vacuole which displaces the nucleus laterally.

When the larva is well formed, and both the outer and inner cuticle and tunic compartments are secreted (Fig. 27), the cytoplasmic protrusions of the BP cells become thinner. The IFC, because of the movements of the larva, break at some points and are displaced. The whole BP lumen is occupied by the flocculent material, but bundles of fibers run parallel to the surface of the larval fin or attach to the membrane of the test cells. The latter are poor in granules and have some empty vacuoles of varying sizes (Fig. 27).

The ripe larva leaves the colony by breaking through the tunic. It is very large, measuring about 3 mm and is equipped with about 28 vascular ampullae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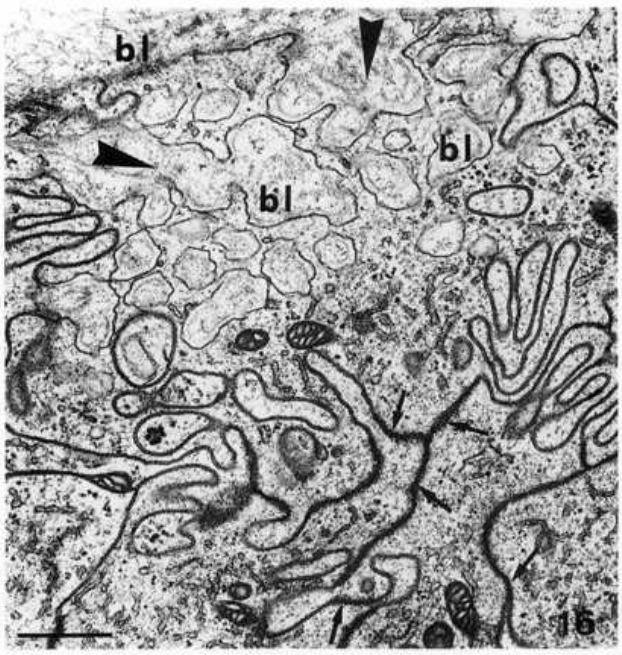
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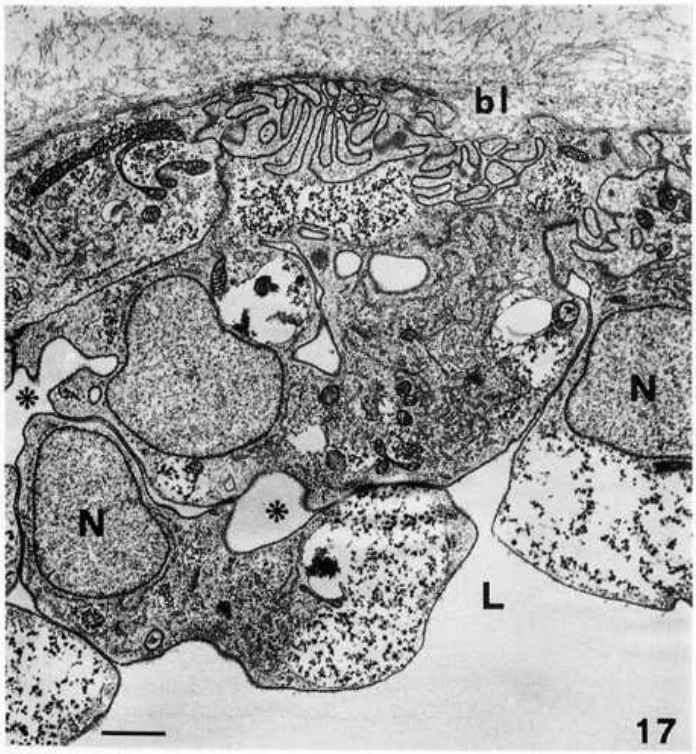
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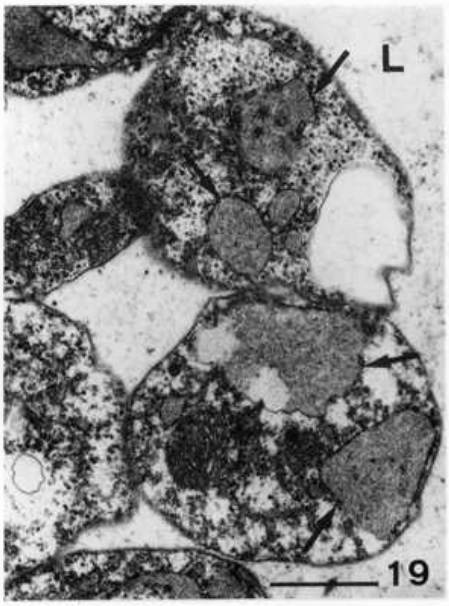
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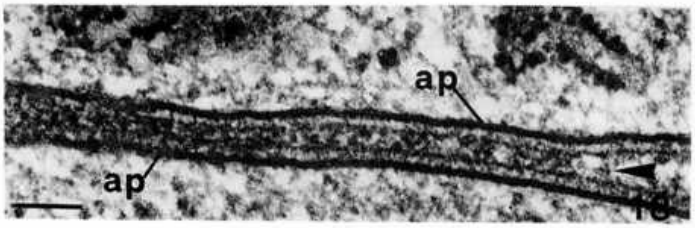
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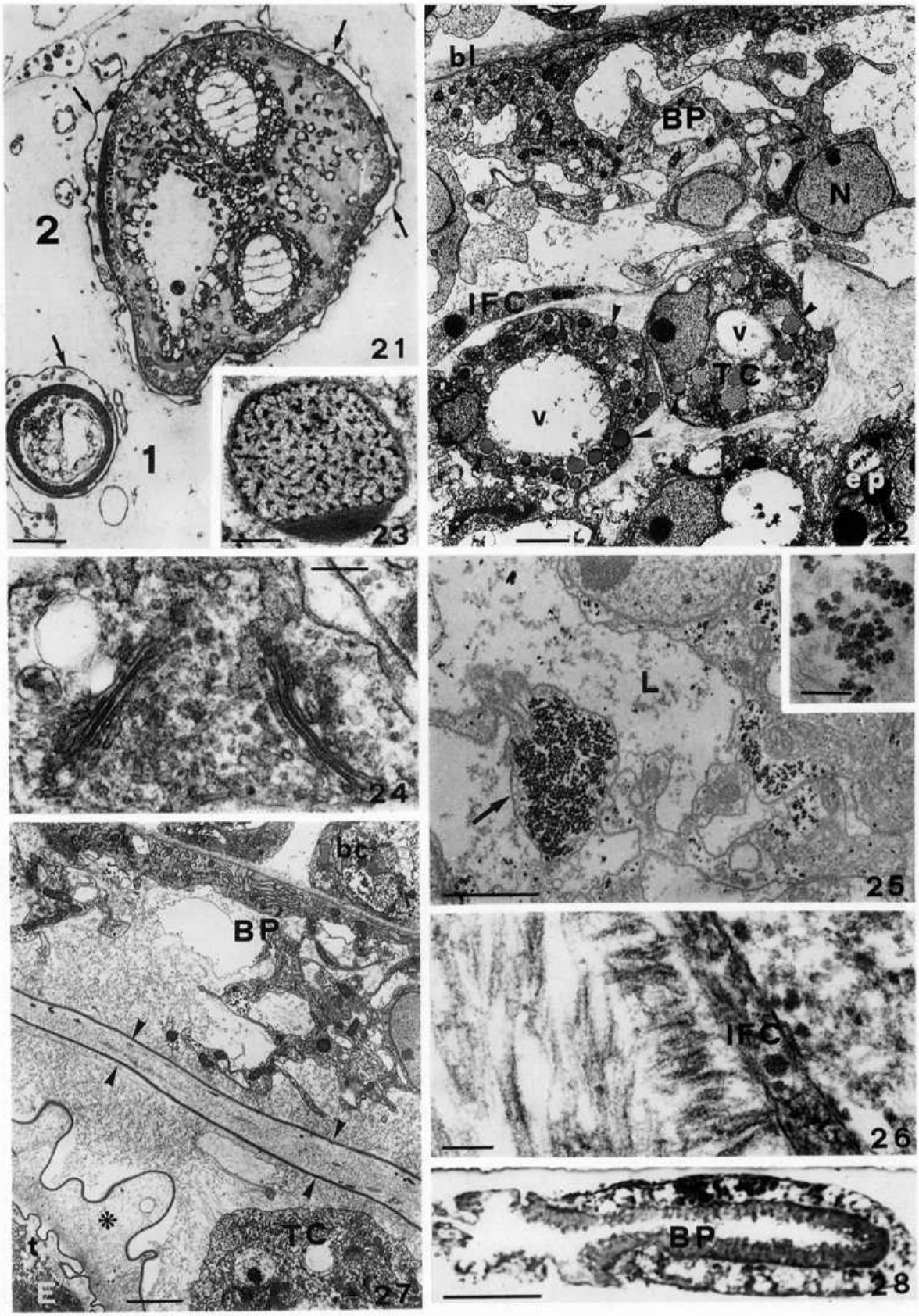
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Figs. 15–19. Mid-embryogenesis. Fig. 15. Medio-apical regions of BP cells protrude in lumen (L); nuclei (N) are large; outer nuclear envelope is rough and enlarged to contain homogeneous granular material (arrowheads); extended dense tight junctions (arrows) are displaced toward basal lamina (bl) under nuclei. m: mitochondria. Scale bar: 1 μm. Fig. 16. Tangential section of BP cells showing large channels (arrowheads) formed by extension of basal plasmalemma, accompanied by basal lamina (bl). Adjacent cells are amply interdigitated. Dense tight junction network (arrow) is visible in lower right corner. Scale bar: 1.5 μm. Figs. 17, 18. Apical regions of BP cells contain ample areas rich in many dense glycogen particles. Adjacent cells are in contact with each other, defining luminal spaces (asterisks in Fig. 17), and intermingle with glycocalix (arrowhead in Fig. 18). Lateral membranes of adjacent cells interdigitate basolaterally. ap: apical plasmalemma; b: basal lamina; L: lumen; N: nuclei. Scale bar for Fig. 17: 2 μm. Scale bar for Fig. 18: 0.15 μm. Fig. 19. Apical areas of BP cells containing RER vesicles (arrows) filled with homogeneous granular material, before discharge into lumen. Note granular material in BP lumen (L). Scale bar: 1.6 μm. Fig. 20. Advanced embryogenesis. Scanning electron microscopy. Embryo removed to reveal apex of polymorphic BP cells protruding into pouch lumen. Scale bar: 10 μm.

Figs. 21–26: Advanced embryogenesis. Fig. 21. Thick section. Colonial tunic showing early (1) and advanced (2) embryos; both are contained in BP bathed by blood. Arrows: vessel epithelium. Scale bar: 50 μm. Fig. 22. Brood pouch (BP) cells interdigitate deeply baso-laterally and have very long apical polymorphic protrusions, some of which contain large nuclei (N). Inner follicle cells (IFC) are squamous. Test cells (TC) contain large vacuoles (v) and numerous round granules (arrowheads), one of which appears in Fig. 23. Embryo epidermis (ep) has an irregular profile. Loose granular material is present in space between BP epithelium and inner follicle cells, while fibrillar material lies between inner follicle cells and embryo epidermis. bl: basal lamina. Scale bar: 3 μm. Fig. 23. Granule of test cell, containing both a thread-like matrix with dense dots and a dense cup. Scale bar: 0.4 μm. Fig. 24. Golgi apparatus in apical region of a BP cell. Scale bar: 0.3 μm. Fig. 25. Thièry method. Glycogen rosettes (shown in detail in inset) fill cytoplasmic protrusions of BP cells (arrow). Granular material stained by Thièry method is also found in lumen (L) of BP. Scale bar: 2 μm; inset scale bar: 0.25 μm. Fig. 26. Inner follicle cells (IFC) separate compartment of BP lumen (right) rich in particulate from embryonal area with many filaments (left). Fibrous material appears to be attached to IFC membrane. Scale bar: 0.2 μm. Fig. 27. Micrograph showing relationship between brood pouch (BP) and larva (E) before hatching. BP cells have thin cytoplasmic protrusions. IFC are displaced. Larva possesses both larval (asterisk) and adult tunic (t); larval fin is formed (arrowheads). Test cell (TC) is poor in granules and possesses vacuoles. bc: blood cells. Scale bar: 3.5 μm. Fig. 28. Residue of brood pouch (BP) in tunic after hatching of larva. Toluidine blue. Scale bar: 60 μm.

and buds. The BP epithelium remains in the tunic (Fig. 28) and undergoes necrotic degeneration: nuclei lose their electrondensity, chromatin disperses, glycogen rosettes disappear, and cytoplasm and organelles undergo vacuolization.

Discussion

Viviparity has evolved several times independently in most animal taxa, commonly accompanied by an increase in the duration of embryo retention in the maternal body, and a corresponding decrease in both egg size and duration of larval life after birth. Embryo retention in the female body often requires the formation of specialized structures with functions for gas exchange and the provision of nutrients.

As a reproductive strategy, tunicates have developed viviparity in thaliaceans (Bone et al., 1985) and various groups of ascidians (Satoh, 1994). In the latter, the Styelidae family (Stolidobranchia) may be regarded as an outstanding example, with different evolutionary steps from oviparity to viviparity. Indeed, in addition to solitary species with external fertilization, the Styelidae include ovoviviparous colonial species in which nutritional parent-embryo exchange is absent, as in *Botryllus schlosseri* (Zaniolo et al., 1987) or present, as in *B. leachi* (Zaniolo et al., 1994b), and also true viviparous species such as *B. lenis* and *B. violaceus* (Mukai et al., 1987). Our data show that viviparity in *B. violaceus* involves precocious segregation of embryos into the common tunic and that the BP epithelium differentiates into a specialized tissue for embryo nutrition.

Ovulation and BP formation

Although the oviduct in *B. violaceus* is a small vesicle, by fusing with OFC and BP it creates the channel driving the egg into the BP. Tight junctions seal adjacent cells of the oviduct and create a barrier preventing seawater from reaching the blood in the mantle. Thus, the ultrastructure and significance of the oviduct in *B. violaceus* is similar to those of the other botryllids, including *Botryllus schlosseri*, although in the latter the oviduct also participates in the formation of the cup-like "placenta" which keeps the embryo in the atrial chamber (Zaniolo et al., 1987).

Ascidians have a variety of incubatory pouches, differing in their modalities of formation and nutritional role for the embryo (for reviews, see Kott, 1985, 1990, 1992; and Zaniolo et al., 1994a). In *B. violaceus*, the BP derives from the atrial epithelium; the presence of a well-developed cytoskeleton in its cells may explain their capacity to expand to receive

the egg and to modify the cells themselves during embryo development. Unlike other botryllids, the BP, filled with the just ovulated egg, soon closes its aperture with the atrial chamber, immediately becomes enclosed in a small sac of the mantle, and is incorporated into the tunic. The microfilaments in the basal region of the BP cells probably take part in all the morphogenetic processes occurring in these phases, i.e., in adapting the shape of the pouch to egg entry, closing the aperture, and retracting the BP into the tunic. Activity may take place in the epidermis and tunic, but it is not so evident as in, for example, *Diplosoma listerianum*, in which embryo segregation into the tunic mainly depends on the epidermis and tunic contraction (Burighel et al., 1987; Martinucci et al., 1988). However, it is noteworthy that, although in both species the embryos are isolated in the colonial tunic, only in *B. violaceus* are they contained in a BP bathed by an intense flow of blood arriving from the circulatory system of the tunic. In the ovoviviparous *Diplosoma listerianum*, the embryo develops in a isolated epidermal sac, with no apparent contribution from the parent.

The possibility of the embryo growing in the tunic, isolated from the parent, is advantageous because it permits gestation to occur independently of the blastogenic cycle of the zooids. In *B. lenis*, embryos may briefly survive their parent by developing in a mantle sac, a residue of the regressed parent. But among botryllids, *B. violaceus* is the only species having embryos segregating into the tunic immediately after fertilization and undergoing development completely independently of the mother zooid. The presence of embryos in the colony (Mukai et al., 1987) does not affect the regular sequence of asexual reproduction.

Brood pouch cytodifferentiation

On the basis of histological observations, Mukai et al. (1987) hypothesized that BP epithelium plays a major role in the transfer of nutrients in *B. violaceus*.

Our data demonstrate that extraembryonic nutrition involves differentiation of BP epithelium for synthesis and transport of nutrients and that a temporal correlation exists between cell modifications and embryo development. The most important changes in BP cells are: (a) enormous increase in the plasmalemma in the basolateral region, with the formation of many deep cell interdigitations and branching infoldings in the basal region; (b) displacement of tight junctions towards the base of the cell and of the nucleus to the apical region; (c) progressive extension of the plasmalemma in the apical region of cells, with the formation of many polymorphic cytoplasmic protrusions; (d) signs of intense synthetic activity for proteins and glycogen, which are released in the pouch lumen. Glycogen especially is accumulated as dense rosettes in the apical protrusions and may be released by apocrine secretion, as suggested for *B. leachi* (Zaniolo et al., 1994b).

The BP of *B. leachi* shows a number of modifications similar to those of *B. violaceus*, thus revealing that the BP supplies nutrients to the embryo in both species. However, in *B. leachi* the time for extraembryonic nutrition is shorter and hypertrophy of BP cells is less marked than in *B. violaceus*. In *B. leachi* the only detectable substance secreted is glycogen, whereas in *B. violaceus*, in addition to glycogen, homogeneous material is accumulated in large cisterns of the RER and is especially secreted in the early phases of embryo development. Thus, both proteins and sugars are required for the lengthy development of a large larva starting from an alecithal egg. The presence of other undetectable substances cannot be excluded. In both species, the BP secretion may be the "coagulable substance" seen in the lumen of the pouch by previous authors (Berrill, 1947; Mukai et al., 1987).

The enormous increase in the plasmalemmal surface improves the capacity of BP epithelium to transport fluids and substances taken up by blood, as occurs in *B. leachi* (Zaniolo et al., 1994b) and also commonly in many transporting epithelia in many animals (Berridge and Oschman, 1972). In this respect the well developed Golgi may have an important role in the traffic of the vesicles. Moreover, by sealing the paracellular pathway and separating the domains of the apical from the basolateral plasmalemma, tight junctions help in maintaining cell polarization and the intracellular flow of materials from blood to embryo. As in *B. leachi*, *B. violaceus* cells are not particularly rich in mitochondria and lack the characteristic association indicative of transport against a gradient (Noirot-Thimothée and Noirot, 1980).

Inner follicle cells and test cells

To reach the embryo, nutrients have to pass through the IFC layer. In the ovarian egg, this envelope is formed of scattered cells (Manni et al., 1995) which, after ovulation, become organized in a thin continuous epithelium.

As a rule, in both external and internal fertilization, the IFC rest on the VC and remain as the boundary separating the developing embryo compartment from the environment. In *B. violaceus*, the latter is represented by the BP lumen, isolated from the blood by tight junctions.

The IFC may mediate nutritional exchanges, especially driving the first phases of development when they are provided with microvilli on their apical surface. In any case, they create a compartment which, in the last phases, contains fibrillar material derived from granules of test cells. The latter are first mainly dense and homogeneous, but later change in feature and number, while vacuoles, possibly remnants of granules which underwent secretion, appear. All this accompanies the appearance and increase in the fibrillar material around the embryo, thus suggesting its derivation from the granules of test cells. Secretion of these cells may contribute to tunic formation and render it hydrophilic (Cloney, 1994; Okada et al., 1996). The tunic is only formed at a more advanced stage of embryo development than in other ascidians (Burighel and Cloney, 1997) and, as the ample caudal fin grows, it breaks and displaces the IFC. The presence of an epidermis not covered by the tunic during the greater part of development, and with irregular apices to create an extensive exchange surface, probably facilitates the absorption of nutrients arriving from the BP.

Because a self/non-self recognition system is well developed in botryllids, the possibility of embryo resorption into the tunic should be investigated. The IFC prevent larva of *B. violaceus*, provided with a number of ampullae, from coming into contact with parental tissues. However, by sealing the lumen, the tight junctions of the BP may also create an effective barrier between embryonal antigens and blood, thus avoiding activation of the parental immune system towards the embryo for fusion or repulsion, as occurs in botryllids when two conspecific individuals come into contact (Hirose et al., 1990; Rinkevich and Weissman, 1992; Sabbadin et al., 1992).

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