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Oogenesis and oocyte envelope differentiation in the viviparous ascidian *Botrylloides violaceus*

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Summary

Oogenesis and oocyte envelope differentiation were examined in the viviparous *Botrylloides violaceus*, a compound ascidian producing small alecithal eggs about 80 μm in diameter. Oogenesis was divided into five stages, defined according to ultrastructural changes in envelopes and oocyte growth. In the gonadal blastema of young buds, each oocyte in the early meiotic prophase is partially enveloped by a few primary follicle cells (stage I) which adhere to the oolemma at several junctional points. In stages II and III, these cells give rise to an external continuous layer (presumptive outer follicle cells) and to underlying scattered elements which later differentiate into test cells and inner follicle cells (stage IV). The direct contribution of mesenchymatous elements was not observed in inner follicle cells and test cells. Observations on the time of appearance and development of organelles, microvilli and endocytotic activity in growing oocytes are reported. Oocytes progressively accumulate "clear" vesicles containing a loose mat and small spherical bodies resembling the previtellogenic bodies observed in other botryllids; they are seen to receive contributions from Golgian vesicles. However, the typical electron-dense yolk globules described in the oocytes of ascidians so far examined were never observed. The meaning of these clear vesicles and the possible role of envelopes in oocyte development are discussed, and the small alecithal eggs of *B. violaceus* are compared with the yolky eggs of other ascidians.

Key words: Ascidians, oogenesis, oocyte envelope differentiation, ultrastructure, viviparity

Introduction

Hermaphroditic ascidians have eggs which vary in size from small (150 μm) in diameter in the solitary oviparous species (Kessel, 1983, for a review), to the relatively large and yolk-rich eggs of the ovoviviparous colonial species, and up to 720 μm in *Ecteinascidia turbinata* (Cloney, 1990a). Moreover,

some colonial species are viviparous and produce tiny alecithal eggs, as small as 25 μm in *Hypsistozoa fasmeriana* (Brewin, 1956).

Various authors have investigated ascidian oogenesis ultrastructurally (Reverberi, 1971, and Kessel, 1983, for reviews), but their studies were devoted to oviparous and ovoviviparous species in which eggs undertake yolk production by autosynthesis and/or auto-heterosynthesis (Kessel, 1966, 1983; Mancuso, 1967; Anderson, 1974; Cotelli, et al., 1982; Manni et al., 1993, 1994a).

The two phylogenetically related genera *Botryllus* and *Botrylloides* (Botryllinae) are of interest because they contain species with different situations of ovoviviparity, viviparity and yolk accumulation (Mukai et al., 1987; Zaniolo et al., 1994a). Most have yolky eggs, but some have eggs poor in or deprived of yolk, such as the 80 μm eggs of *Botrylloides violaceus* (Mukai et al., 1987). In all species both body sides of mature zooids contain a female gonad reduced in size with only a few ripe eggs. For example, *Botrylloides leachi* matures only one or two eggs per zooid; and each one with the envelopes — test cells (TC), vitelline coat (VC), and inner (IFC) and outer (OFC) follicle cells – and oviduct is commonly considered a "small ovary" (Berrill, 1950; Sabbadin, et al. 1992).

Recently, oogenesis and oocyte envelope differentiation have been ultrastructurally analyzed in *Botryllus schlosseri*, a species producing yolky eggs of about 300 μm. Their OFC show some of the features of protein-secreting cells, probably with the role of producing yolk precursors (Manni et al., 1993, 1994a). Various exogenous contributions to yolk have occasionally been claimed in oviparous species possessing small eggs with squamous OFC. According to Kessel and Kemp (1962) and Mancuso (1965), IFC can synthesize or store yolk precursors, and TC have also been considered able to transfer their granules to the oocyte (Kessel and Kemp, 1962; Mancuso, 1965; Reverberi, 1978; Gianguzza and Dolcemascolo, 1979). However, different functions have also been assigned to both IFC (Miller, 1975; Lambert and Lambert, 1978; Cotelli et al., 1981; Martinucci et al., 1988; Bates and Mallet, 1991; Kawamura et al., 1991; Sakairi and Shirai, 1991; Zaniolo et al., 1994a) and TC (Cloney, 1990b).

The viviparous *Botrylloides violaceus* has small eggs with poorly developed IFC and OFC; they ovulate into a brood pouch and are then isolated in the colonial tunic where large larvae develop (Mukai et al., 1987). Hence, this species is an interesting candidate for comparative studies on nutrient storage and the significance of envelopes. In this paper we analyze oogenesis in *B. violaceus* ultrastructurally to compare the development and maturation of its small, alecithal eggs with those of the yolky eggs of oviparous and ovoviviparous species. We also examine the differentiation of the envelopes to verify their role on the basis of hypotheses made for other ascidians.

Materials and Methods

Colonies of *Botrylloides violaceus* (*sensu* Saito et al., 1981) were collected in the lagoon of Venice and cultured in the laboratory attached to glass plates, according to the technique of Sabbadin (1960) for *Botryllus schlosseri*. Embedded in a common tunic and vascularized by a common circulatory system, three blastogenic generations always coexist in the colonies (adult zooids, their buds and the buds of the latter), all developing synchronously. The possibility of following the development of the colonies *in vivo* allows zooids to be selected at suitable developmental stages.

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

For electron microscopy fragments of colonies were first fixed in 1.5% glutaraldehyde in 0.2 M sodium cacodylate, at pH 7.2–7.4 and buffered with 1.5% sodium chloride. Specimens were later post-fixed in 1% osmium tetroxide and 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 according to Reynolds (1963), were examined under a Hitachi H-600 electron microscope.

To reveal polysaccharides and glycoproteins, 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

In *B. violaceus*, the gonads lie on both sides within the thickness of the mantle (between epidermis and atrial epithelium), bathed by circulating blood (Fig. 1). They are composed of a lobulated testis and a very few small ripe eggs (usually one per zooid in cultured colonies). In the region next to each mature oocyte, the atrial wall thickens and forms a sac-like outgrowth, the prospective brood pouch. A small vesicular oviduct is interposed and touches the egg and the brood pouch wall. Oogenesis and maturation of the female gonad are coordinated with bud development: ripe eggs ovulate when the buds become adult and begin filtration.

The gonadal rudiment begins to be recognizable when the young budlets are at the two-vesicle stage in the form of a mass of germ and somatic cells (Fig. 2), close to the prospective wall of the atrial chamber. A number of oocytes soon became recognizable. They may remain in the gonadal area or migrate through the bloodstream and mature in the gonads of buds of successive generations.

Oogenesis is subdivided here into five stages. The three early stages may be compared with those of previtellogenesis, the last two with those of vitellogenesis, following the sequence proposed for *Botryllus schlosseri* (Manni et al., 1993, 1994a).

Stage I

The oocyte is about 10 μm in diameter with a high nucleus/cytoplasm ratio, the nucleus at the early prophase stage, a few organelles in cytoplasm and a few cells partially wrapped around oocyte.

The young oocytes are mainly located in the gonadal rudiment of budlets (Fig. 3) but may also be recognized in the gonads of older buds at later stages. Oocytes are in the early stages of the meiotic prophase (Fig. 5) and are morphologically identifiable according to the characteristics of their nucleus, containing condensed chromatin with synaptonemal complexes and an only slightly prominent nucleolus. The cytoplasm is rich in free ribosomes; mitochondria and Golgi stacks are scarce. Oocytes that have just entered the growth phase (diplotene) (Fig. 4) have homogeneous nucleoplasm and a single, spherical, large nucleolus. The ooplasm is generally devoid of organelles except for free ribosomes and aggregations of dense material not limited by membrane ("nuage") in perinuclear regions. Nuage is sometimes in intimate association with mitochondria.

Oocytes at stage I are partially wrapped by the cytoplasmic extensions of a few somatic cells, the primary follicle cells (PFC) (Figs. 4 and 5). These cells insinuate themselves among the oocytes and have free ribosomes, a few mitochondria, some RER profiles and Golgi apparatus. PFC sometimes touch the oocytes, forming scattered punctate adhering junctions. These are similar to those of successive stages and are characterized by densities on the cytoplasmic side of both cells and by fibrous material in the intercellular 20 nm-wide cleft (Fig. 11).

The gonadal blastema seems to adhere to the epithelium of the prospective atrial wall by means of its PFC, whose cytoplasmic extensions are always interposed between oocytes and the thin basement membrane of the epithelium (Figs. 4 and 6). The latter never shows signs of cell proliferation contributing to the blastema.

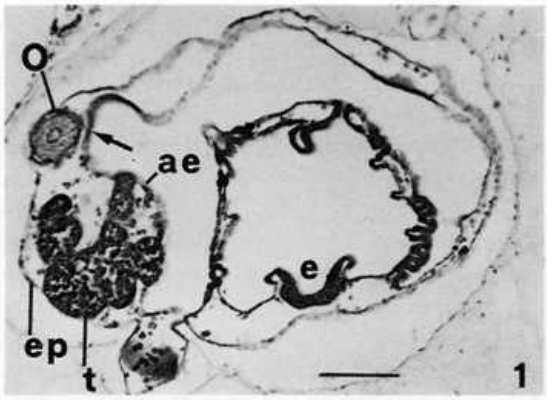
Stage II

The oocyte is about 15 μm in diameter with marked signs of nucleo-cytoplasmic exchange, the nucleus at metaphase I, several mitochondria and oocyte surrounded by PFC.

These oocytes are commonly found in the gonads of both young and mature buds; some have also been seen to be transported by the bloodstream in the colony.

Oocytes contain a voluminous nucleus with nucleolus (Fig. 6), as in the successive stages of oogenesis. The nuclear envelope is usually smooth and may have some vesicles derived from the inner nuclear membrane (Fig. 9). Mitochondria are numerous and preferentially crowded in clouds in the perinuclear regions (Fig. 6). Dense aggregations of nuage are found mainly in perinuclear regions but may also be scattered throughout the ooplasm; the association between nuage and mitochondria is still evident (Fig. 8). Golgi stacks formed of five or six flat cisterns give off numerous small vesicles. Short cisterns of ER with associated ribosomes are also found (Figs. 7 and 10), some in suboolemmal regions. Moreover, scattered smooth vesicles, called here "clear" and containing a loose fibrillar mat, begin to be recognizable; they sometimes also contain small spherical bodies 40–50 nm in diameter.

Oocytes are surrounded by a flat layer of PFC,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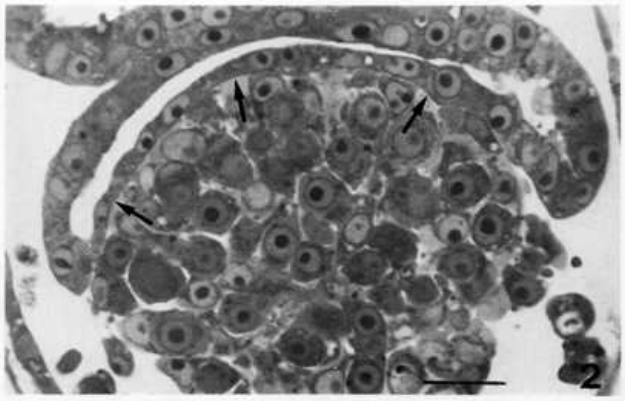
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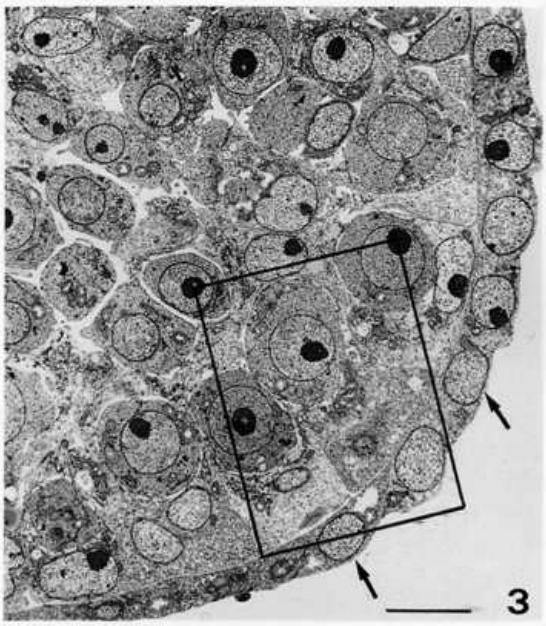
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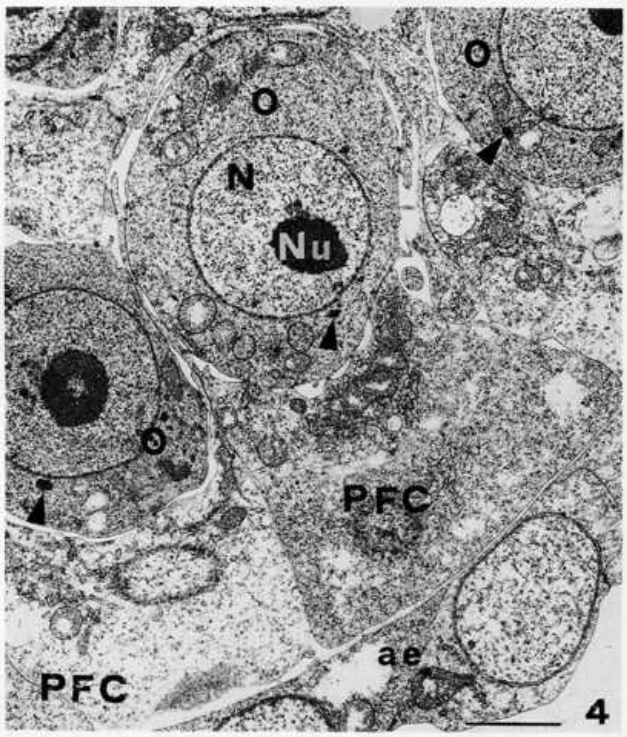
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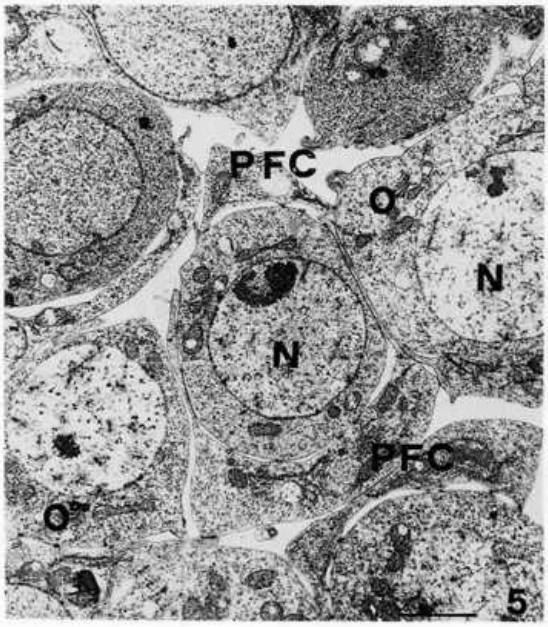
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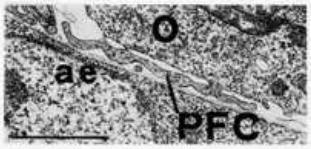
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which lengthen (Fig. 6). The latter have an elliptical nucleus with nucleolus, mitochondria, RER cisterns and active Golgi complexes (Figs. 6 and 7). PFC show polarity, being provided with a thin basement membrane on the external side and forming punctate adhering junctions with the oolemma on the apical side (Fig. 11).

Stage III

The oocyte is about 30 μm in diameter; mitochondria and other organelles are scattered in the cytoplasm. There is a first appearance of microvillar extensions of oolemma and scattered cells between presumptive OFC and oocyte.

Only a few oocytes reach this stage. The cytoplasm undergoes changes. Organelles are abundant (Figs. 12 and 16); small vesicles and short cisterns of RER increase in number and are distributed throughout the cytoplasm, sometimes also lying parallel and close to the oolemma (Fig. 15), which at sites begins to be irregular in outline and forms simple microvillar extensions (Fig. 13). Most of the organelles are located towards the central area of the oocyte, being scarce in the cortical regions. Mitochondria are numerous (Fig. 16); Golgi stacks producing secretory vesicles appear in close association with RER cisterns, and transition vesicles are found among these organelles (Fig. 16). Clear vesicles containing a fibrillar matrix and small spherical bodies become larger and more numerous (inset, Fig. 16).

Two layers of oocyte envelopes begin to be recognizable (Figs. 12–14). The external one (presumptive OFC) is continuous and has a basement membrane. Its cells insinuate themselves among those of the internal layer, which is discontinuous, to touch the oocyte's extensions with punctate adhering junctions. Presumptive OFC are elongated and possess mitochondria, RER profiles and Golgi stacks, often more than one placed close together. The cells of the internal layer (presumptive IFC and TC) are roundish or oval in shape, sometimes placed in shallow depressions in the oolemma; they have only a few organelles and are never seen to form junctions with the oolemma.

Stage IV

The oocyte is about 60 μm in diameter with diffuse clear vesicles throughout the cytoplasm. It has long ovular microvilli, and all oocyte envelopes are recognizable.

Oocytes have numerous clear vesicles, irregular in outline and scarce towards the periphery (Figs. 17 and 18). Dense lipid droplets and mitochondria are scattered among them. The ER is highly developed in the form of small vesicles and short cisterns and also occurs in the cortical region. Golgi stacks are scattered, extended and bud off numerous vesicles.

While vesicles increase in number, microvilli elongate in irregular, branching extensions (Figs. 18 and 19). Some microvilli become very long and can penetrate deep channels at OFC apices (Fig. 19). Moderate pinocytotic activity occurs at the base of microvilli (Fig. 20). Punctate adhering junctions are still recognizable between ovular microvilli and OFC extensions.

All oocyte envelopes become recognizable at this stage (Figs. 17 and 18). TC are roundish, wedged

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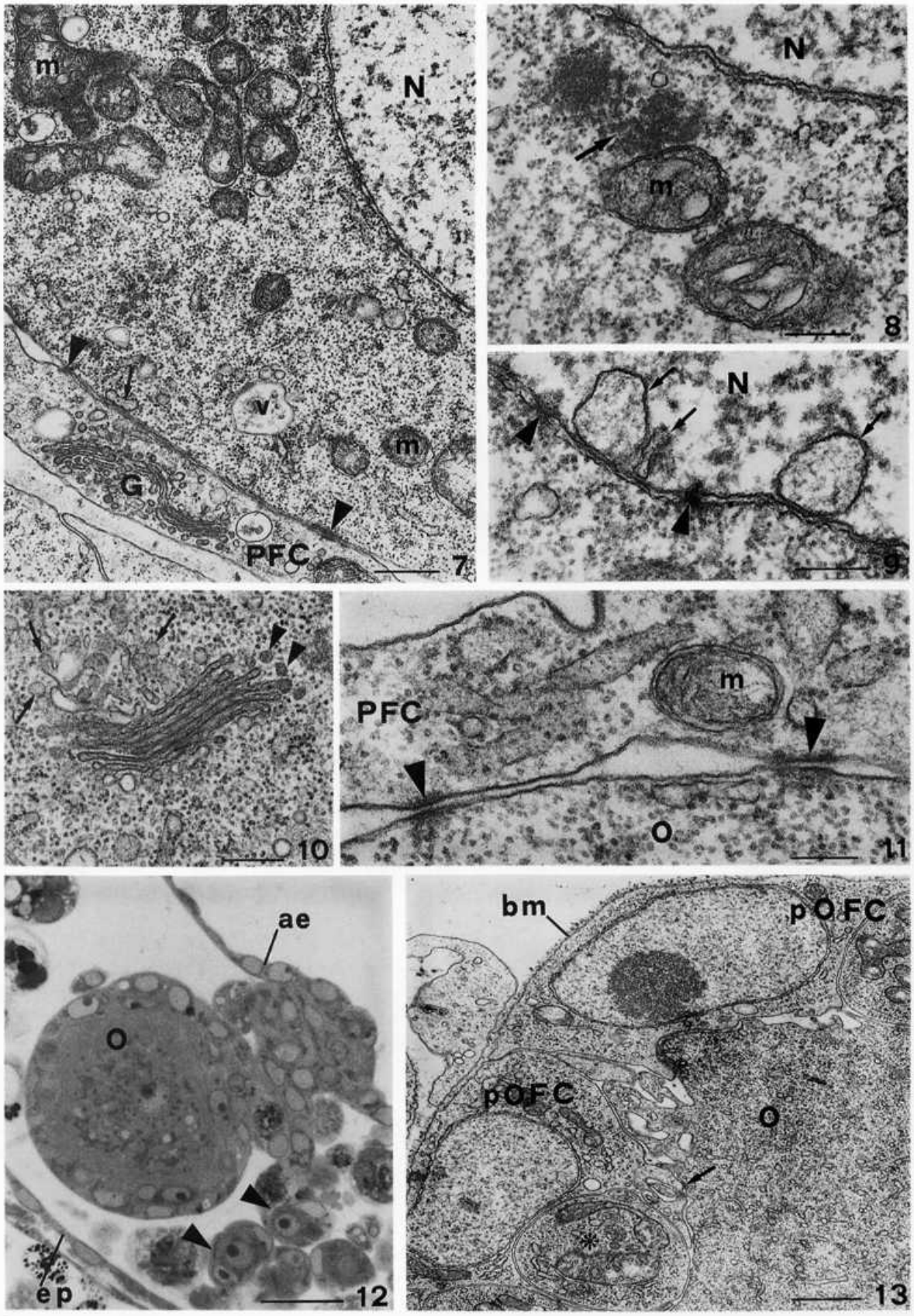
Fig. 1. *Botrylloides violaceus*. Paraffin section of one bud showing an egg (O) and testis (t) in mantle, defined by atrial epithelium (ae) and epidermis (ep). Sac-like outgrowth of brood pouch is also recognizable (arrow). e: endostyle. Hematoxilin-eosin. Scale bar: 50 μm. **Figs. 2–5.** Oocytes at stage I. **Fig. 2.** Thick section of a young bud showing, in mantle, a germinal mass very close to prospective wall of atrial chamber (arrows). Toluidine blue. Scale bar: 40 μm. **Fig. 3.** Electron micrograph of gonadal rudiment in a bud like that of Fig. 2. Undifferentiated blastema is composed of male and female germ cells and somatic cells. Rectangle defines area enlarged in Fig. 4. Arrows: epithelium of prospective wall of atrial chamber of bud. Scale bar: 7 μm. **Fig. 4.** Detail of Fig. 3, showing some oocytes (O) recognizable because of nuclear structural characteristics and dense aggregations of nuage (arrowheads). Oocytes are partially wrapped by extensions of primary follicle cells (PFC) between them and prospective wall of atrial chamber (ae). N: nucleus; Nu: nucleolus. Scale bar: 2.5 μm. **Fig. 5.** Cluster of oocytes (O) at early stages of meiotic prophase, characterized by unhomogeneous nucleoplasm with condensed chromatin. Several primary follicle cells (PFC) with cytoplasmic protrusions adhere to them. N: nuclei. Scale bar: 2.5 μm. **Fig. 6.** Oocytes at stage II (O) have round nuclei (N) with large nucleoli. A number of mitochondria (arrows) crowds next to nucleus. A single layer of flat primary follicle cells (PFC) envelopes oocytes so that they never directly touch atrial epithelium (ae) (see inset); rectangle defines area enlarged in inset. Inset: extension of PFC insinuating between oocyte and atrial epithelium. Scale bar: 7 μm; inset scale bar: 2 μm.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in superficial ovular depressions in cortical regions, and partially covered by ovular microvilli. They have a large nucleus and nucleolus, many free ribosomes, some mitochondria and lipid droplets. The small perivitelline space appearing between the surface of the oocyte and the overlying IFC tends progressively to widen, as the oocytes grow. Within this space, the VC becomes recognizable in the form of a mesh of loose, fibrous material (Figs. 18–20).

OFC and IFC form a double layer with the cells encased in each other. However, OFC insinuate themselves among IFC and, crossing the VC, contact the ovular microvilli. OFC form a continuous layer, while IFC are discontinuous and lie on the VC. Both follicular cell types have a large nucleus and some long RER cisterns, mitochondria, lipid droplets and Golgi stacks (Fig. 18).

Stage V

Oocytes reach their final size (80 μm in diameter); cytoplasm is filled with clear vesicles, and oocyte envelopes complete differentiation.

In mature oocytes (Fig. 21), the nucleus is eccentric and has an irregular profile with indentations and intranuclear annulate lamellae at the periphery (Fig. 23); a nucleolus is always present. Clear vesicles are homogeneously distributed in the cytoplasm (Fig. 21), the size varying from 0.2 μm to 2 μm in diameter. Most of them possess a loose mat and some small spherical bodies. Scattered dense lipid droplets prevail in the peripheral ooplasm; RER vesicles and active Golgi stacks are diffuse (Figs. 22 and 24). Granules of β-glycogen, revealed by cytochemical tests, are found among organelles (Fig. 25).

The space between TC and IFC is more pronounced than in the previous stage and is crossed by ovular microvilli (Fig. 22) which establish punctate adhering and some gap-like junctions with OFC apices (Fig. 26). With approaching ovulation, the oocytes reduce their microvillar extensions, and the VC compacts to form a layer of apparently homogeneous fibrillar material, no longer crossed by microvilli or OFC extensions (Fig. 29).

TC (Fig. 22) are numerous and partially encased in depressions of oocytes; they contact one another and cover extended regions of the oolemma. Their cytoplasm contains well developed RER vesicles of finely granular material and rich Golgi stacks budding dense vesicles (Fig. 28). Some electron-dense granules and lipid droplets may also be observed. IFC are polygonal, and now form a continuous layer (Figs. 22 and 27). They possess a developed RER, whose cisterns often dilate to contain fibrillar material. Round dense lipid droplets and Golgi stacks are common. OFC maintain their epithelial disposition and show cytoplasmic characteristics similar to IFC (Fig. 27).

At ovulation, mature oocytes discharge OFC, pass through the vesicular oviduct and are received in the brood pouch where they are fertilized (Mukai et al., 1987).

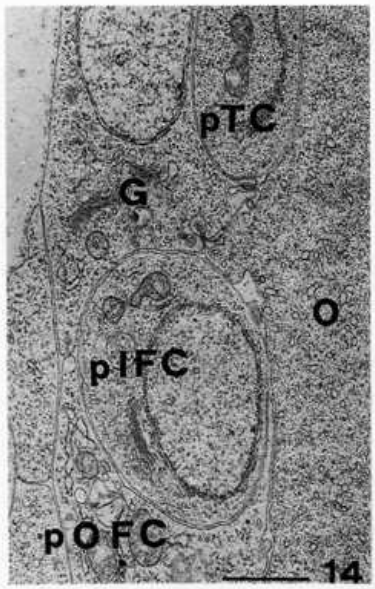
Fig. 30 shows a synopsis of the ultrastructural features of oogenesis.

Discussion

Oocyte origin and differentiation

In *B. violaceus*, as in other botryllids (Mukai, 1977; Sabbadin and Zaniolo, 1979; Manni et al., 1993, 1994a), the gonadal blastema is first recognizable in young buds in the form of a mass of

Figs. 7–11. Oocytes at stage II. **Fig. 7.** Several mitochondria (m), numerous free ribosomes, short RER cisterns and scattered smooth vesicles (v) containing a loose mat and small spherical bodies are in cytoplasm. Note Golgi complex (G) and punctate adhering junction (arrowheads) with oolemma in primary follicle cells (PFC). N: nucleus; arrows: short RER cisterns in suboolemmal region. Scale bar: 600 nm. **Fig. 8.** Clump of dense nuage (arrow) associated with mitochodrion (m). N: nucleus. Scale bar: 300 nm. **Fig. 9.** Intranuclear vesicles (arrows) emanating from inner nuclear membrane. N: nucleus. Arrowheads: nuclear pores. Scale bar: 300 nm. **Fig. 10.** Active Golgi complex in oocyte with several flattened cisterns blebbing vesicles (arrowheads) at periphery. Note Golgian vesicles (arrows) associated with smooth endoplasmic reticulum in maturing face. Scale bar: 300 nm. **Fig. 11.** Intercellular junctions (large arrowheads) between primary follicle cells (PFC) and oocyte (O) have cytoplasmic densities close to membrane and fibrous material in intercellular cleft. m: mitochondrion. Scale bar: 200 nm. **Figs. 12, 13.** Oocytes at stage III. **Fig. 12.** Detail of gonadal area in differentiating bud, showing one oocyte (O) at stage III and a cluster of oocytes (arrowheads) at stage I. ae: atrial epithelium; ep: epidermis. Thick section. Toluidine blue. Scale bar: 15 μm. **Fig. 13.** Oocyte (O) forms irregular microvillar extensions and junctional spots (arrow) between them and presumptive outer follicle cells (pOFC). Junctional areas are lacking in roundish cells (asterisk) interposed between presumptive OFC and oocyte. bm: basement membrane. Scale bar: 1.5 μm.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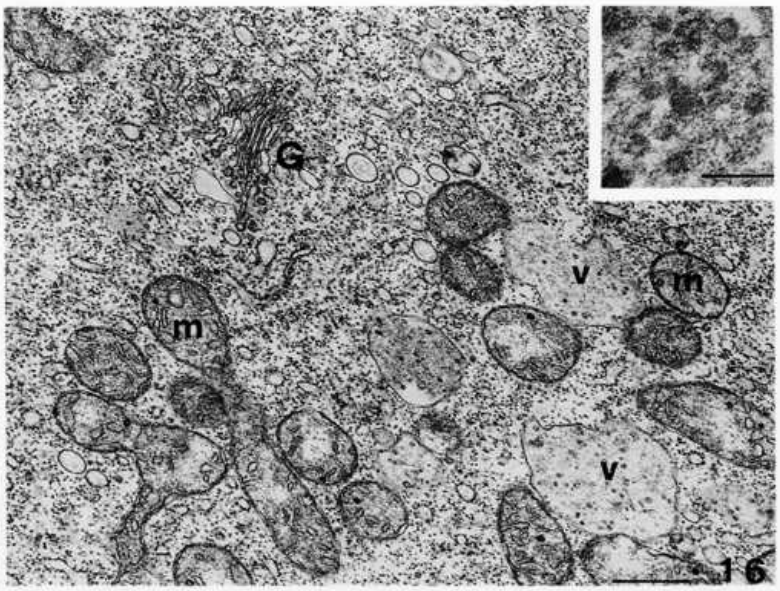
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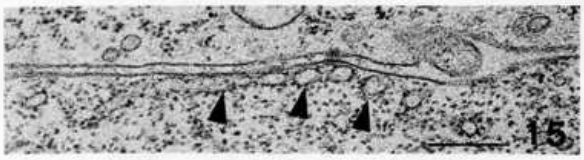
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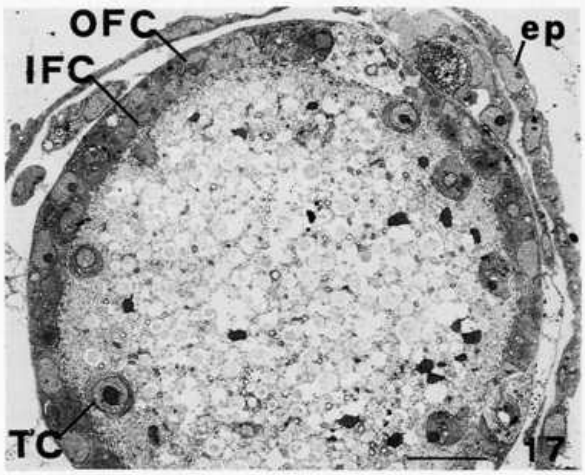
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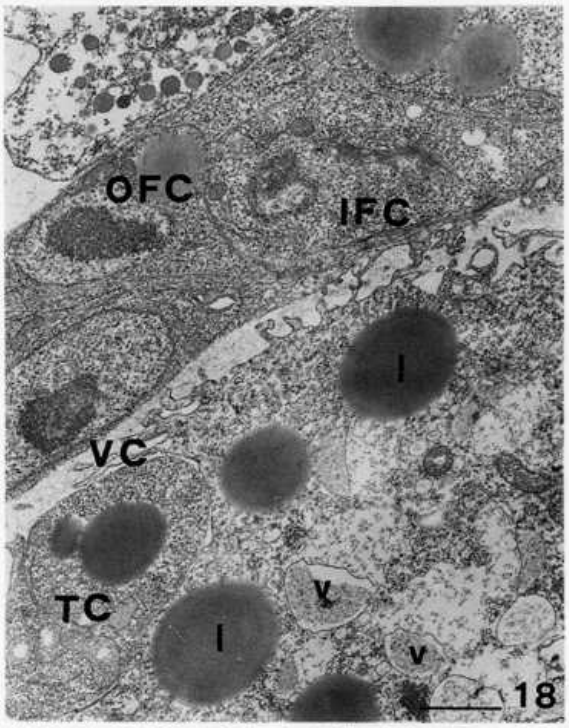
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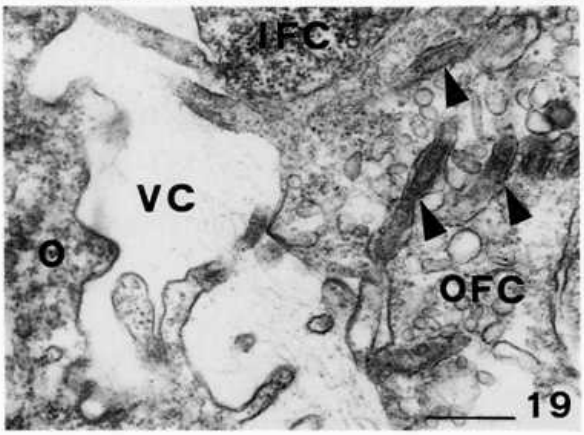
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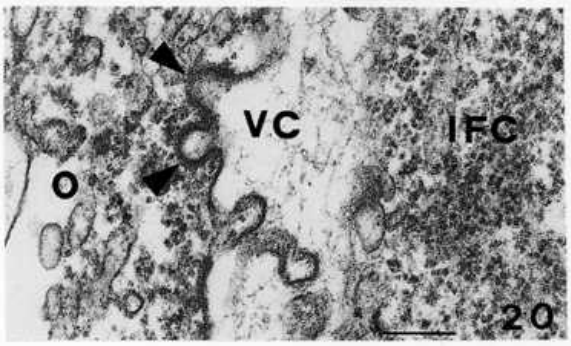
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germ and somatic cells. As in other ascidians, young oocytes are characterized by small size, a high nucleus-cytoplasm ratio, and the presence of PFC, which adhere to the oolemma by means of punctate adhering junctions (Sugino et al., 1990; Manni et al., 1994a).

The origin of the gonadal blastema in botryllids (Berrill, 1950; Mukai, 1977; Sabbadin and Zaniolo, 1979; Manni, 1993, 1994a) is still an open problem. In *B. violaceus*, the blastema adheres to the wall of the prospective atrial chamber, but the oocytes never directly touch this epithelium due to the interposition of cytoplasmic extensions of PFC. We never found any unequivocal evidence that gonadal blastema can proliferate from the prospective atrial wall, as claimed by Berrill (1941) for *Botryllus schlosseri*. Rather our findings are in agreement with reports that the germ cells reach the gonadal blastema by migration through the bloodstream (Izzard, 1968; Mukai and Watanabe, 1976; Sabbadin and Zaniolo, 1979; Manni et al., 1994a) and that they can be transferred through several generations before they mature (Sabbadin and Zaniolo, 1979).

The early developmental stages of *B. violaceus* oocytes are similar to those of other ascidians (Kessel, 1966; Mancuso, 1967; Sugino et al., 1990; Okada and Yamamoto, 1993; Manni et al., 1994a), although some peculiarities were noted. In *B. violaceus* oocytes are poor in nuage, the electron-dense material originating from the nucleus considered to be a specific germ-line character (Eddy, 1975; Wallace and Selman, 1990). Moreover, they lack annulate lamellae, the porous membrane system commonly found in the germ cells of solitary ascidians (Kessel, 1985, 1989, 1992), nor do they display any association between porous cisterns and vesicles, as observed in *Botryllus schlosseri* (Manni et al., 1994b). It cannot be excluded that the paucity of nuage and the absence of annulate lamellae or other porous membrane associations in *B. violaceus* are peculiar to these oocytes which, unlike most ascidians, do not accumulate dense yolk globules.

In the successive stages of differentiation, *B. violaceus* shows striking qualitative and quantitative differences with other ascidians, the main one being the complete absence of the typical electron-dense yolk globules. *B. violaceus* produces clear membrane-bound vesicles which begin to form in the previtellogenic phase, concomitantly with the increased number of organelles and the appearance of microvilli. They probably originate from specialized zones in endoplasmic regions and grow with the contribution of Golgian vesicles. Clear vesicles resemble the membrane-bound previtellogenic bodies of *Botryllus schlosseri* from which dense yolk granules are formed with the contribution of exogenous pinocytosed material (Manni et al., 1994a). In *Botryllus schlosseri* the previtellogenic bodies progressively disappear, while the yolk granules concomitantly increase in number and grow to fill the cytoplasm of ripe oocyte completely. In *B. violaceus* pinocytosis is less well represented than in *Botryllus schlosseri* and the process of maturation of previtellogenic bodies in dense yolk granules does not take place, so that the mature oocyte fills with clear vesicles. This suggests that in *B. violaceus* the lack of yolk synthesis is an adaptation to the special reproductive requirements of this viviparous ascidian. Clear vesicles, from which some contents may be extracted during tissue processing, are the way to furnish the first nutrients to the embryo, in addition to glycogen granules and lipid droplets.

In total, the number of eggs produced and the amount of nutrient reserves accumulating in the

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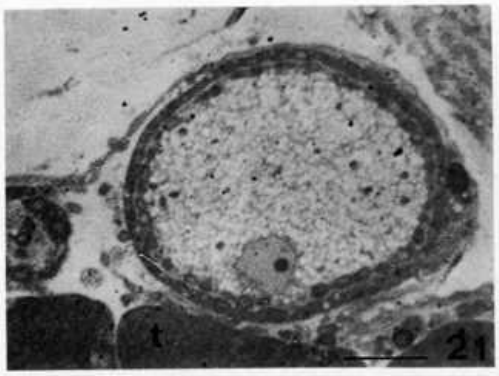
Figs. 14–16. Oocytes at stage III. **Fig. 14**. A presumptive test cell (pTC) amply touches oocyte surface, while presumptive inner follicle cells (pIFC) are partially enveloped by presumptive outer follicle cells (pOFC). G: Golgi complexes; O: oocyte. Scale bar: 1.5 μm. **Fig. 15**. Arrowheads indicate short cisterns of RER parallel and close to oolemma. A profile of PFC on top. Scale bar: 350 nm. **Fig. 16.** Clear vesicles (v) containing fibrillar matrix and very small spherical bodies (enlarged in inset) scattered among mitochondria (m) and short RER cisterns. G: Golgi complex. Scale bar: 600 nm; inset scale bar: 200 nm. **Figs. 17-20**. Oocytes at stage IV. **Fig. 17**. Low-magnification electron micrograph of oocyte surrounded by outer follicle cells (OFC), inner follicle cells (IFC) and test cells (TC). Ooplasm filled with many clear vesicles. ep: epidermis. Scale bar: 10 μm. **Fig. 18**. Vitelline coat (VC) separates outer follicle cells (OFC) and inner follicle cells (IFC) from test cells (TC) located in oocyte depressions. IFC and OFC form a double-layered epithelium and are similar in thickness. Numerous ovular microvilli cross VC, contacting OFC extensions. l: lipid droplets; v: vesicles. Scale bar: 2 μm. **Fig. 19**. Long ovular microvilli (arrowheads) deeply penetrate outer follicle cells (OFC). IFC: inner follicle cells; O: oocyte; VC: vitelline coat. Scale bar: 500 nm. **Fig. 20**. A facing area of inner follicle cell (IFC) and oocyte (O): note loose mat of vitelline coat (VC) and endocytotic pits (arrowheads). Scale bar: 250 nm.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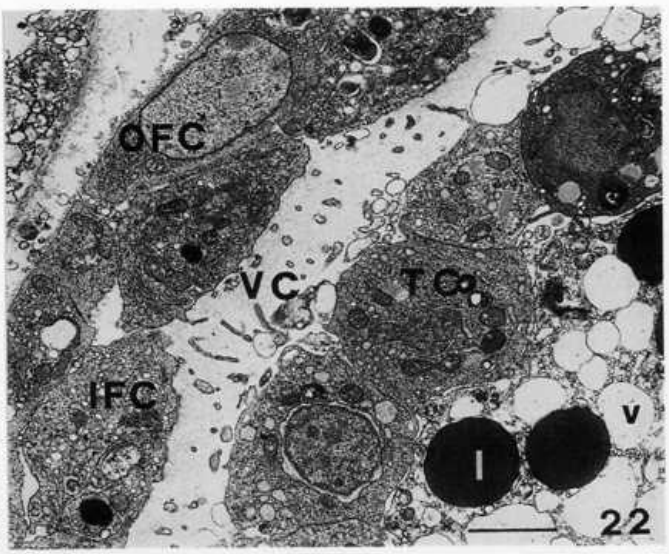
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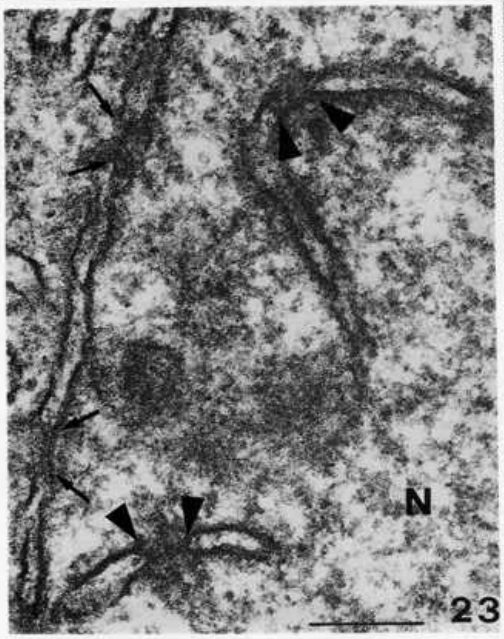
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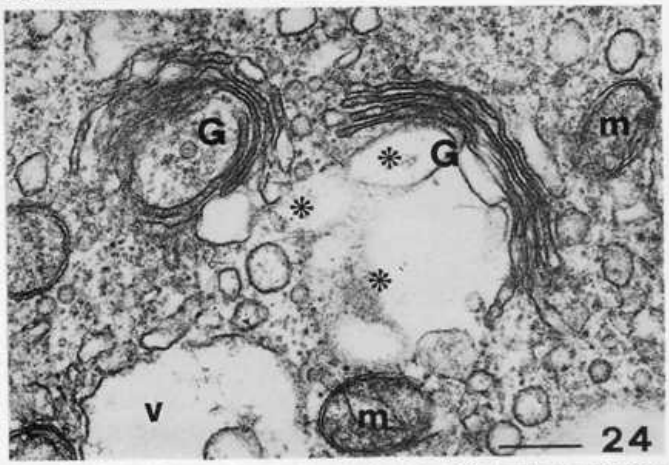
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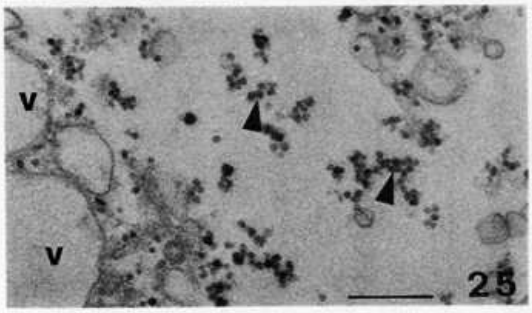
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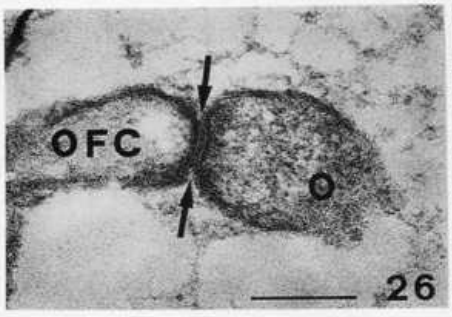
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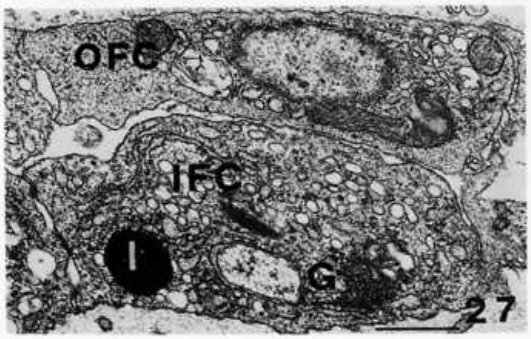
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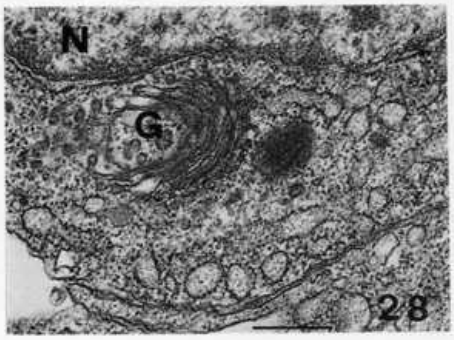
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small oocytes of *B. violaceus* are extremely low when compared with those of oviparous and ovoviviparous ascidians. This means that, in *B. violaceus*, the investment of parental energy for reproduction is very low during oogenesis and occurs after fertilization, when embryos develop through exchange with the parent (Mukai et al., 1987).

Oocyte envelope origin and differentiation

Our data suggest that all the cellular envelopes of *B. violaceus* derive from the PFC, probably originating from free cells of the hemocoel, as Mukai and Watanabe (1976) suggested for *Botryllus primigenus*. The pattern of cytodifferentiation of oocyte envelopes in *B. violaceus* is similar to that described for *Botryllus schlosseri* (Manni et al., 1993): PFC may be stem cells which are transformed in the external layer of OFC and the internal scattered cells, source of IFC and TC. These isolated elements are morphologically similar to the PFC from which they are presumed to derive, although we were not able to detect mitotic divisions of PFC, as Tucker (1942) and Ermak (1976) did. However, since we never saw mesenchymal cells infiltrating the PFC, we are not inclined to think that in *B. violaceus* the IFC and TC originate from hemoblasts which gain access to oocytes through this epithelium, as suggested for other ascidians (Knaben, 1936; Kalk, 1963; Gianguzza and Dolcemascolo, 1978).

The ripe eggs of *B. violaceus* have differentiated IFC and OFC similar in thickness and aspect. Some authors (Mukai, 1977; Zaniolo et al., 1987; Martinucci et al., 1988; Sabbadin et al., 1992; Manni et al., 1993, 1994a) correlate the development of OFC with egg size and yolk quantity: small eggs with little yolk have flat OFC, and large eggs rich in yolk have columnar OFC. In the oviparous *Ciona intestinalis*, OFC are so squamous that their role in oogenesis has never been considered, while in a colonial species such as *Botryllus schlosseri* the OFC are rich in RER cisterns and Golgi stacks and produce dense granules which may be secreted, contributing to yolk (Manni et al., 1993, 1994a). The small eggs of *B. violaceus* without yolk globules have cubic and not particularly differentiated OFC, lacking marked signs of protein-secreting cells. OFC show a close relationship with the oocyte as evidenced both by the presence of deep interdigitations between ovular microvilli and apices of OFC and by gap-like junctions, which may allow exchange of ions and small molecules (Anderson and Albertini, 1976). A similar relation between OFC and ovular microvilli has only been reported for *Botryllus schlosseri* (Manni et al., 1993, 1994a).

The significance of IFC has long been debated. They have been reported to participate in the structural characterization of VC (Cotelli et al., 1981), to release a factor triggering germinal vesicle breakdown (Sakairi and Shirai, 1991), to produce sperm attractants (Miller, 1975) or to facilitate sperm behavior (Villa and Patricolo, 1993) and sperm interaction with VC (Kawamura et al., 1991). IFC remaining to cover ovulated eggs may serve as flotation devices (Lambert and Lambert, 1978), produce adhesive material anchoring eggs to benthic substrates (Bates and Mallet, 1991), or contribute to the formation of placental structures (Mukai et al., 1987; Zaniolo et al., 1987, 1994a, 1994b). In *B. violaceus*, as seen also in *Botryllus schlosseri* (Manni et al., 1993), IFC differentiate in the last stages of oogenesis, becoming rich in RER vesicles

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Figs. 21–29. Oocyte at stage V. Fig. 21. Thick section of a fully grown oocyte with its envelopes. Cytoplasm is filled with clear vesicles and scattered dark lipid droplets; nucleus with nucleolus is eccentric. t: testis. Scale bar: 25 μm. Fig. 22. Electron micrograph showing envelopes (OFC, IFC, VC and TC) and oocyte with clear vesicles (v) and lipid droplets (l). Note envelopes forming continuous layers around oocyte. Several ovular microvilli cross VC. Scale bar: 2μm. Fig. 23. Intranuclear annulate lamellae with features (arrowheads) resembling nuclear pores near nuclear envelopes. N: nucleus. Arrows: nuclear pores. Scale bar: 200 nm. Fig. 24. Two active Golgi stacks (G) with several cisterns blebbing vesicles at periphery, associated with numerous condensing vacuoles (asterisk) in maturing face. Vacuoles lie close to clear vesicles (v) of similar content. m: mitochondria. Scale bar: 300 nm. Fig. 25. β-glycogen granules (arrowheads) scattered among clear vesicles (v). Afzelius staining method. Scale bar: 300 nm. Fig. 26. Intercellular gap-like junction (arrows) between extension of outer follicle cells (OFC) and oocyte (O). Intercellular junctional cleft reduced to about 4 nm. Scale bar: 150 nm. Fig. 27. Electron micrograph showing outer (OFC) and inner (IFC) follicle cells forming a two-layer epithelium. RER cisterns and vesicles are developed in both envelopes. G: Golgi complex; l: lipid droplet. Scale bar: 900 nm. Fig. 28. Detail of test cell with well-developed Golgi complex (G) and RER vesicles. N: nucleus. Scale bar: 500 nm. Fig. 29. Detail of oocyte (O) approaching ovulation. Vitelline coat (VC) becomes more compact and ovular microvilli are absent. IFC: inner follicle cell; TC: test cell. Scale bar: 800 nm.

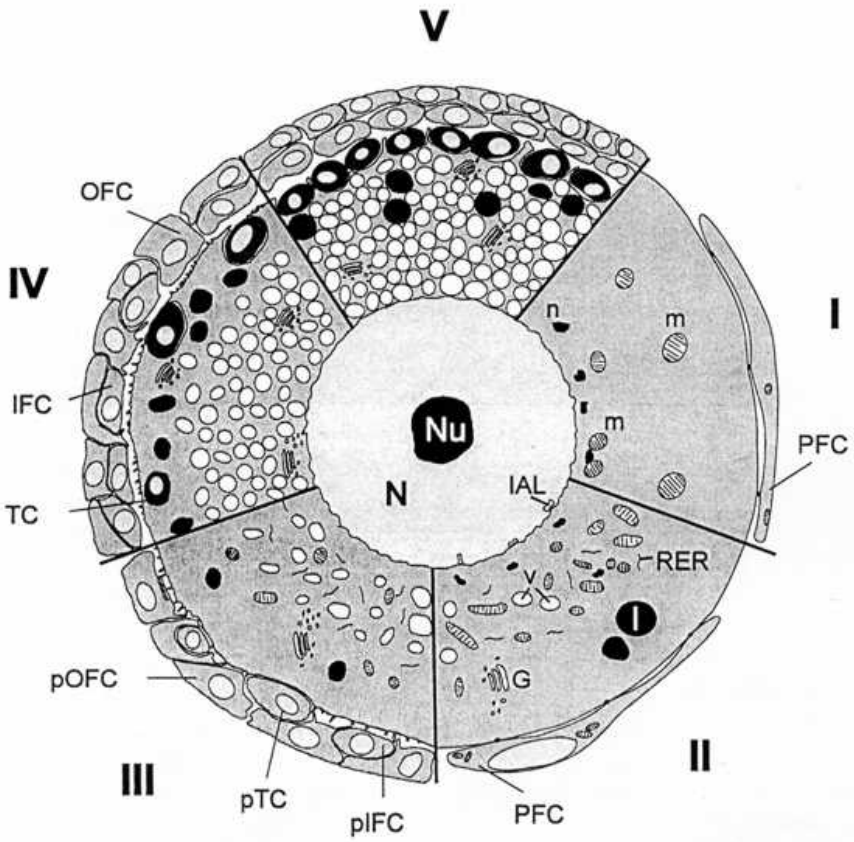

Fig. 30. Sketch illustrating stages I-V of oogenesis. Oocyte at stage I shows nuage (n) in perinuclear ooplasm, sometimes associated with mitochondria (m). Stage II: Golgi complex (G), RER cisterns (RER), some clear vesicles (v) and lipid droplets (l) also appear. Primary follicle cells (PFC), adhering to oolemma by means of junctional points, envelope oocyte at stages I and II. Stage III: a number of clear vesicles, appearance of short ovular microvilli and presence of scattered cells between presumptive outer follicle cells (pOFC) and oocyte. Stage IV: all ovular envelopes are recognizable; clear vesicles are scattered throughout cytoplasm and microvilli are elongated. Stage V: cytoplasm becomes filled with clear vesicles and oocyte envelopes are completely differentiated. Oolemma becomes smooth with approaching ovulation. VC omitted for clarity. IAL: intranuclear annulate lamellae; N: nucleus; Nu: nucleolus; IFC: inner follicle cells; OFC: outer follicle cells; pIFC: presumptive inner follicle cells; pTC: presumptive test cells; TC: test cells.

containing fibrillar material. Thus they may play their role either at ovulation or during embryogenesis, when they constitute the epithelium interposed between embryo and parent (Mukai et al., 1987, and unpublished data).

The main function of VC in species-specific recognition of gametes is well established (De Santis et al., 1980; Monroy and Rosati, 1983; Rosati, 1985; Honegger, 1986; Kawamura et al., 1991). During oogenesis, VC fibers, probably secreted by the oocyte (Cotelli et al., 1981), separate oocyte and TC from IFC. With approaching ovulation, they compact into an apparently homogeneous layer which does not show several separate zones, as occurs in other species (Honegger, 1986).

In comparison with other ascidians, TC seem to be relatively very numerous in *B. violaceus* because they crowd closely all around the oocyte. Probably the total number of TC is independent of oocyte size but correlated to larva size, although this hypothesis requires further analysis. TC show signs of differentiation in late oogenesis when dense granules are produced by their Golgi apparatus. As we never observed the secretion of these granules, we do not think that they are transferred and used for yolk synthesis, as proposed for other ascidians (Kessel and Kemp, 1962; Mancuso, 1965; Reverberi, 1978; Gianguzza and Dolcemascolo, 1979). In *B. violaceus*, the great number of granules which are kept after ovulation (unpublished data) suggest that their role is played in embryonal or larval phases, contributing to the chemical characterization of the surface of the larval tunic, as proposed for other species (Cloney, 1990b, 1994).

In conclusion, *B. violaceus* eggs are remarkable, differing from those produced by oviparous and ovoviviparous species in size, absence of electron-dense yolk globules, and relative extension of IFC and OFC. However, when compared with the squamous OFC of solitary species, the presence of cuboid OFC, despite the absence of yolk, and their morphological relationships with the oocyte may be seen as the remains of the OFC of the large yolky eggs of ancestors, according to the hypothesis that viviparous species may be regarded as evolutionary derivatives of ovoviviparous species (Mukai et al., 1987).

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