Ovulation and placentation in *Botryllus schlosseri* (Ascidiacea): an ultrastructural study

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The mode of ovulation and placentation was studied by light and electron microscopy in the ovoviviparous ascidian *Botryllus schlosseri* using colonies from the laboratory. The full-grown oocyte is surrounded by the outer and inner follicle cell layers, the acellular vitelline coat (chorion), and the test cells, and it is furnished with its own vesicular oviduct which is interposed between the egg and the atrial epithelium. In contrast to most ascidians, the outer follicle is thick and has an ultrastructure consistent with intense protein synthesis. At ovulation the outer follicle shows signs of involution where it contacts the oviduct. When the oviducal wall breaks and the egg moves through the oviduct, the outer follicle cells are discharged in the mantle to form a sort of corpus luteum. The egg remains hanging in the atrial chamber by means of a cuplike "placenta." The placental tissues are all of maternal origin, being derived from both the atrial and oviducal epithelia together with some of the inner follicle cells. These latter anchor to the oviducal epithelium by means of junctional spots and a filamentous cementing secretion. Our results suggest that the main role of the "placenta" is to attach the embryo to the parent, thus exposing it to the flow of seawater.

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Le mode d'ovulation et de formation du « placenta » chez l'ascidie ovovipare *Botryllus schlosseri* a été étudié par examen aux microscopes électronique et photonique de colonies de laboratoire. L'ovocyte à maturité est entouré par les couches externe et interne de cellules folliculaires, le revêtement vitellin acellulaire (chorion) et les cellules de la thèque et il possède son propre oviducte vésiculaire qui est coincé entre l'œuf et l'épithélium de l'atrium. Contrairement à ce qui prévaut chez la plupart des ascidies, le follicule externe est épais et son ultrastructure possède les propriétés nécessaires à une synthèse importante de protéines. Au moment de l'ovulation, le follicule externe semble s'involuer à l'endroit où il est en contact avec l'oviducte. Lorsque la paroi de l'oviducte se rompt et que l'œuf se déplace dans l'oviducte, les cellules du follicule externe se répandent dans le manteau pour former une sorte de *corpus luteum*. L'œuf demeure suspendu dans l'atrium grâce à un « placenta » en forme de coupe. Les tissus placentaires sont tous d'origine maternelle et proviennent à la fois de l'épithélium de l'atrium et de l'épithélium de l'oviducte et contiennent aussi quelques cellules de la couche folliculaire interne. Ces cellules s'accollent à l'épithélium de l'oviducte par des points de jonction et par l'intermédiaire d'une sécrétion filamenteuse fixative. Nos résultats indiquent que le rôle principale du « placenta » est de maintenir l'embryon attaché au parent de façon à ce qu'il soit exposé au courant d'eau de mer.

[Traduit par la revue]

Introduction

Ascidians are hermaphroditic sessile animals in which both external and internal fertilization are encountered. During oogenesis their eggs are enveloped by distinct layers: the follicle cells, which in several species (e.g., botryllids) are differentiated into the outer follicle cells (OFC) and the inner follicle cells (IFC), the acellular vitelline coat (chorion), and the test cells. A great number of papers have discussed these envelopes (see Kessel 1983 for review), but most of the questions about their origin and possible function remain open, though recently Monroy and collaborators showed that the vitelline coat is involved in the specific recognition of spermatozoa (see Monroy et al. 1984 for review).

During ovulation the eggs shed the OFC. The spawned eggs remain covered externally by the IFC; in the species with external fertilization the IFC are highly vacuolated, thus making the eggs more buoyant, whereas in the species with internal fertilization they are extremely flattened (Berrill 1950).

Internal fertilization occurs commonly in the colonial forms where the egg develops into a larva which escapes by swimming from the colony. In many cases, the embryos are retained within the parent by means of specialized structures, frequently called "incubatory pouches," "brood pouches," or "placentae." In the botryllids, the origin, morphology, and function of the "placenta" have been repeatedly debated. Recently, they were reconsidered by Mukai (1977) using the light microscope (LM) in a comparative study of the reproduction of four botryllid species.

We have studied ovulation and placentation in *Botryllus schlosseri* with the LM and the electron microscope (EM). This study has shown that the OFC are characterized by very well developed rough endoplasmic reticulum (RER) and during ovulation they form an ephemeral corpus luteum, whereas some of the IFC participate in the formation of the placenta by establishing a specialized relationship with the oviducal epithelium.

Aspects of this study have appeared in abstract form (Zaniolo et al. 1986).

Materials and methods

Botryllus schlosseri (family Botryllidae, order Stolidobranchiata) is a colonial ascidian composed of a great number of small zooids embedded in a common tunic. All specimens used in this study were from our laboratory where colonies adhering to glass are routinely cultured with Sabbadin's (1960) technique.

In each colony three blastogenic generations coexist: the filtering adults, their buds, and the buds of the latter. A fine regulatory mechanism allows all zooids of the same generation to develop synchronously. Weekly (at 18°C), all adults regress and are absorbed; their buds become new adults and then begin filtering.

Zooids have hermaphroditic gonads on either side (Figs. 1, 2), but self-fertilization is prevented since the testes mature later than the eggs (Sabbadin 1971). The mature eggs are ovulated into the peribranchial chamber just when the zooids initiate filtering activity. Light acts as a stimulus for ovulation, as has been reported for *Botryllus primigenus* (Mukai and Watanabe 1978). The transparency of the colonies allows the study of ovulation *in vivo* under the stereomicroscope, making the selection of appropriate developmental stages possible (Fig. 1).

Selected fragments of colonies, cut with a small blade, 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, the specimens were dehydrated and embedded in Epon. Thick sections (1 μm) were stained with toluidine blue. Thin sections, stained with uranyl acetate and lead citrate, were examined with a Hitachi H-600 electron microscope.

Results

Organization of the female gonad

In each zooid of *Botryllus schlosseri* only a few oocytes reach maturity (Sabbadin and Zaniolo 1979). A single egg is, in fact, a one-egg ovary, since it is surrounded by four layers (the outer and inner follicles, the vitelline coat, and the test cells) and is furnished with its own oviduct (Figs. 3, 4). The oviduct, before ovulation, is a vesicle interposed between the OFC and the atrial epithelium.

The growth of the oocyte is synchronized with the development of the zooid, so the egg reaches maturity and is ovulated just when the zooid begins filtration. Figures 4 and 5 show details of fully grown oocytes. The eggs lie in the blood lacuna, surrounded by a large number of different hemocytes (Fig. 4). The outer follicle is thick and consists of a single layer of cells characterized by distended RER and round nuclei with finely dispersed chromatin. The RER is formed by a great number of flattened parallel cisternae which have a homogeneous content (Figs. 5, 9, 11). Round, strongly electron-dense granules are generally associated with the Golgi field, which is situated towards the IFC. The OFC follow the profile of the IFC (Fig. 5), but sometimes protrusions of the OFC cross the IFC contacting the vitelline coat.

The IFC are thinner than the outer ones and their surface is mammillary in shape (Fig. 5). They are rich in endoplasmic reticulum, mainly composed of smooth vesicles of different sizes or short cisternae. Ribosomes are abundant, free or attached to the membranes of the endoplasmic reticulum. The Golgi apparatus shows many anastomosed cisternae and numerous small dense vesicles. Round, membrane-limited granules of different sizes and densities are present, some with a homogeneous fine granular content and others with areas of different densities (Figs. 6, 7). They seem to be formed by coalescence of numerous small vesicles. The IFC are interconnected by junctional complexes.

The vitelline coat is an acellular, moderately dense layer. Before ovulation it is a thick felt of apparently disorganized fibrillar material. It is attached to the basal surfaces of the IFC and is penetrated by numerous microvilli extending from the oocyte surface (Fig. 5).

The test cells are scattered and encased by deep indentations of the oocyte (Figs. 4, 5). They are easily recognized by the presence of several round dense granules and by their cytoplasm being less dense than the yolk platelets. The test cells are rich in RER, largely in the form of vesicles and short cisternae, and possess a well-developed Golgi apparatus (Fig. 5).

Ovulation

A schematic drawing of the principal steps of ovulation and placentation is shown in Fig. 8. During oogenesis the oviducal vesicle is formed by a single layer of cuboid cells whose basal membranes interlace with the basal membranes of the OFC and the peribranchial epithelium. A narrow space remains between the cell membrane of the contiguous epithelia (Fig. 9).

As ovulation approaches, the oviduct enlarges (Fig. 10) and its cells become more and more flattened; the cell interdigitations, earlier well developed (Fig. 9), become progressively less conspicuous. Several oviducal cells show in their apical zones scarce cytoplasm and cytoplasmic blebs into the cavity; their luminal surfaces are covered by a marked fuzzy coat (Fig. 9).

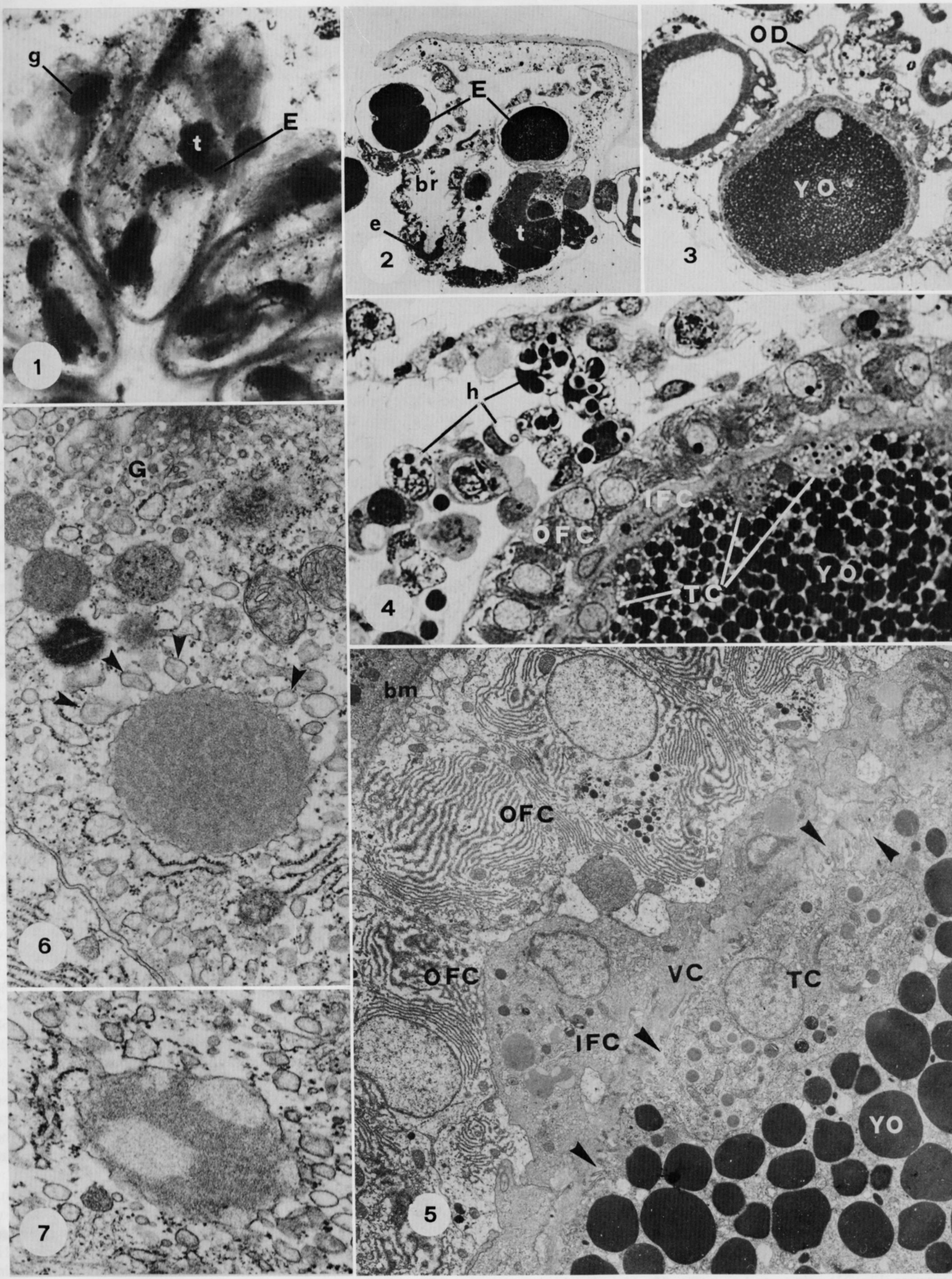
Figures 10 and 11 show eggs at the moment of ovulation. The oviducal epithelium has become flattened with cells now contacting one another by means of their narrow zones (Fig. 11). The connections of the oviducal epithelium with the OFC and atrial epithelium become so intimate that the space between the basal membranes is reduced to about 10 nm. Those OFC that contact the oviduct display autophagic bodies and light vacuoles of various sizes, some extending almost the entire height of the cell (Fig. 11). The vitelline coat appears as a 0.3 μm thick layer of filamentous material over the oocyte surface (Fig. 11). The test cells remain encased within indentations of the oocyte surface, and no perivitelline space is recognizable before fertilization.

During ovulation the oviducal epithelium and the outer follicle break so that the egg penetrates the oviduct after shedding the OFC. These latter cluster in the blood lacuna to form a postovulatory follicle called the corpus luteum by Arnback-Christie-Linde (1923) because of the similarity in formation with that of vertebrates (Figs. 12, 13). The corpus luteum keeps the connection with the oviducal epithelium and occludes the oviducal break through which the egg has passed (Figs. 12, 14). A number of granulocytes and phagocytes with large heterophagic vacuoles crowd around the corpus luteum, the cells of which initially maintain their earlier characteristics (Fig. 13). Then, luteal cells undergo involution and are engulfed by phagocytes so that by the day after ovulation only a few cell residues are present.

Placentation

The egg moves through the oviduct and enters the atrial chamber where it remains hanging on the atrial wall by means of a cup that surrounds almost one-third of it (Figs. 8, 12). The cup has a double wall: the external wall is a prolongation of the atrial epithelium, and the internal wall derives from the oviducal epithelium. The interspace is bathed with blood (Figs. 15, 16).

FIG. 1. Detail of a living colony of *B. schlosseri*. An embryo (E) and the testis (*t*) are recognizable because of the transparency of the adult. *g*, hermaphroditic gonad of a bud. ×25. FIG. 2. Thick transverse section of a zooid showing two embryos (E) in the atrial chamber. *br*, branchial chamber; *e*, endostyle; *t*, testis. ×70. FIG. 3. Thick section of a one-egg ovary in the mantle. Because of the plane of this section, the oviduct (OD) does not show the actual connection with its egg. YO, yolk. ×170. FIG. 4. The three cellular envelopes are recognizable in this thick section of an ovarian egg: OFC, IFC, and test cells (TC). Numerous hemocytes (*h*) adjoin the egg. YO, yolk. ×1150. FIG. 5. Detail of an ovarian egg showing the ultrastructure of envelopes. The OFC are rich in RER and have dense granules in the Golgi field, situated towards the IFC. The latter have mammillary surfaces and are basally in contact with the vitelline coat (VC) which is penetrated by microvilli of the ovum (arrowheads). One test cell (TC), exhibiting round granules, is encased by the egg. *bm*, basement membrane of the OFC; YO, yolk. ×4000. FIGS. 6 and 7. Details of IFC before ovulation. In Fig. 6, a large granule with homogeneous content seems to be formed by contribution of vesicles (arrowheads). The Golgi (G) is rich in cisternae and small vesicles. In Fig. 7, the granule resembles that of Fig. 6 but has areas of lower density. Fig. 6, ×23 000; Fig. 7, ×30 000.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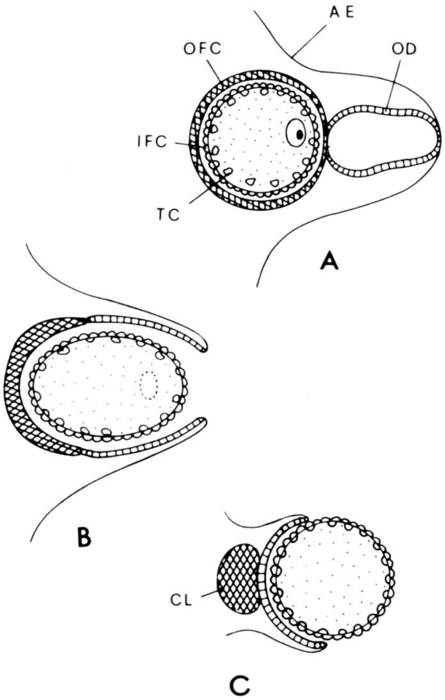


FIG. 8. Schematic drawing of the process of ovulation and placentation in *B. schlosseri*. (A) Before ovulation, the oocyte lying in the mantle is covered by its outer (OFC) and inner (IFC) follicle cells and test cells (TC), and is furnished with its own vesicular oviduct (OD). The latter contacts closely, on opposite sides, the OFC and the atrial epithelium (AE). (B) The oviducal vesicle at ovulation. The OFC slide on the egg and crowd in the mantle to form the corpus luteum. The oocyte, now covered externally by the IFC, moves through the oviduct. (C) The egg is hanging on the atrial wall. The "placenta" is formed by a double-walled cup, the inner one being derived from the oviducal epithelium and the outer one from the atrial epithelium. In the placenta, the IFC adhere to the oviducal epithelium. The corpus luteum (CL) will be resorbed before gastrulation.

The cup and the contiguous IFC enveloping the egg constitute the "placenta."

Shortly after ovulation, a relatively large space remains between the IFC and the oviducal epithelium (Fig. 18), except at the edge of the cup where the two epithelia contact each other forming junctional spots (Figs. 15–17). The IFC rest on the underlying vitelline coat and are connected by extended intermediate junctions, which show extracellular spaces (4 to 7 nm) crossed by dense striations (3–4 nm wide). Filaments associate with the cytoplasmic sides of the junctional membranes (Fig. 19). The IFC possess granules and vacuoles of various sizes and of prevailingly filamentous contents (Figs. 14, 16, 20, 21). The space between the IFC and the oviducal epithelium becomes progressively reduced and crossed by cytoplasmic protrusions (Figs. 21, 22). At first a thin layer of filamentous material covers the surface of the cell and condenses at some points, especially at the level of the cicatrix of the oviduct close to the corpus luteum (Fig. 14). Then, the space reduces and is filled with filamentous material (Figs. 21, 22), closely resembling the content of the large vacuoles in the IFC; the IFC, which are not part of the placenta and are bathed by seawater, also have similar vacuoles and cytoplasmic protrusions (Fig. 20). Most of these vacuoles appear to be derived through transformation of the granules present before ovulation, as can be seen by comparing Figs. 6, 7, 14, 20, 21, and 23.

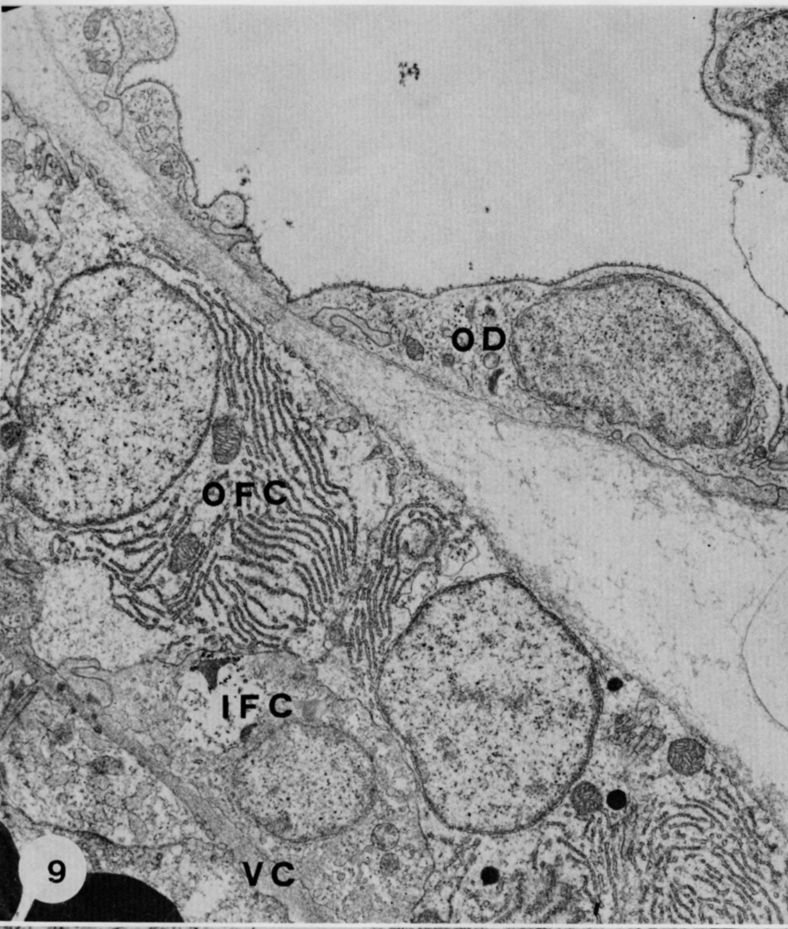
Contiguous vacuoles are frequently interconnected by short tubules, about 80 nm in diameter, whose walls appear dense because their cytoplasmic sides are reinforced by a filamentous felt (Fig. 23). Many vacuoles also show a similar, though more dispersed, felt adhering to their walls, continuous with the remaining cytoskeleton (Fig. 25). Occasionally, a tubule with a thickened wall seems to connect a large vacuole with the external space, creating a potential path for the extrusion of the vacuole contents (Fig. 24).

During early embryonic development, the vitelline coat always remains attached to the IFC. It lies close to the embryo, and only in some zones does it detach from the embryo to form a

FIG. 9. Detail of a one-egg ovary before ovulation. The oviducal vesicle (OD), which is interposed between the atrial epithelium (AE) and the OFC, is bounded by filamentous material continuous with the basement membranes. The oviducal cells are interdigitated and their apical cytoplasm is scarce. Cellular blebs, probably artifactual, are evident in the oviducal lumen. VC, vitelline coat. ×6000. FIG. 10. Thick section of a one-egg ovary at ovulation. The oviduct (OD) is enlarged and its flat epithelium is apparently fused (arrowheads) with the atrial epithelium (AE) and the OFC. ×700. FIG. 11. Detail of a one-egg ovary at ovulation. The flat oviducal epithelium (OD) is closely apposed to the OFC. The latter show vacuolization and autophagic bodies (arrowheads). The apical cell membranes in the oviduct are covered by a thick, dense, fuzzy coat. The compact vitelline coat (VC) appears in the lower left corner. ×16 000.

FIG. 12. Thick section showing an egg just after ovulation, enveloped by the IFC and hanging in the atrial cavity by means of the placenta, the edges of which are indicated by arrows. The OFC discharged during ovulation form in the mantle a sort of corpus luteum (CL). The vitelline space is very narrow. The zone indicated by a bracket corresponds to that of Fig. 14. Arrowheads indicate stigmata. ×270. FIG. 13. Electron micrograph showing a portion of the corpus luteum (CL) of Fig. 12. Many granulocytes and phagocytes (arrowheads) are close to the corpus luteum. Luteal cells show the typical features of the OFC in addition to conspicuous vacuolization. ×2500. FIG. 14. Electron micrograph of a zone corresponding to that indicated in Fig. 12. The gap of the oviduct (OD) through which the egg passed is now occluded by the corpus luteum (CL) and by filamentous material (arrowheads) resembling the content of granules in the IFC. VC, vitelline coat. ×6000. FIG. 15. Thick section through the edge of the placenta showing the relation between the placental cup and the embryo. Some hemocytes (*h*) are in the interspace of the cup. AE, atrial epithelium; OD, oviducal epithelium; TC, test cells; YO, yolk. ×500. FIGS. 16 and 17. Detail of the edge of the placental cup showing its connections with the IFC. Junctional spots (arrowheads) are visible in Fig. 17 which is an enlargement of the area indicated in Fig. 16. *gc*, granulocyte; VC, vitelline coat. Fig. 16, ×4000; Fig. 17, × 20 000.

FIG. 18. Shortly after ovulation, in the zones far from the cup edge, there is a large space between the IFC and the oviducal epithelium (OD), which bears scattered fuzzy material (arrowheads). VC, vitelline coat. × 12 000. FIG. 19. Large junctional area between two IFC. The intercellular space, crossed by dense striations, is reduced to 4 nm near the vitelline coat (VC). × 100 000. FIG. 20. The IFC exposed to seawater also possess numerous vesicles and vacuoles of filamentous content like the IFC adhering to the oviduct (see Figs. 14 and 23). × 20 000. FIGS. 21 and 22. The composition of the placental tissues is evident: the atrial epithelium (AE), the oviducal epithelium (OD), and the IFC. The embryo is in segmentation (Fig. 21) or in gastrulation (Fig. 22). Note that the space between OD and IFC (stars) is progressively filled with filamentous material and decreases in size (see also Fig. 18). *bs*, blood space; VC, vitelline coat; YO, yolk. Fig. 21, × 12 500; Fig. 22, × 10 000.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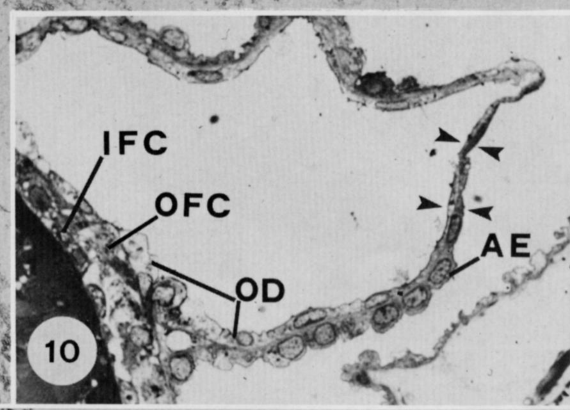
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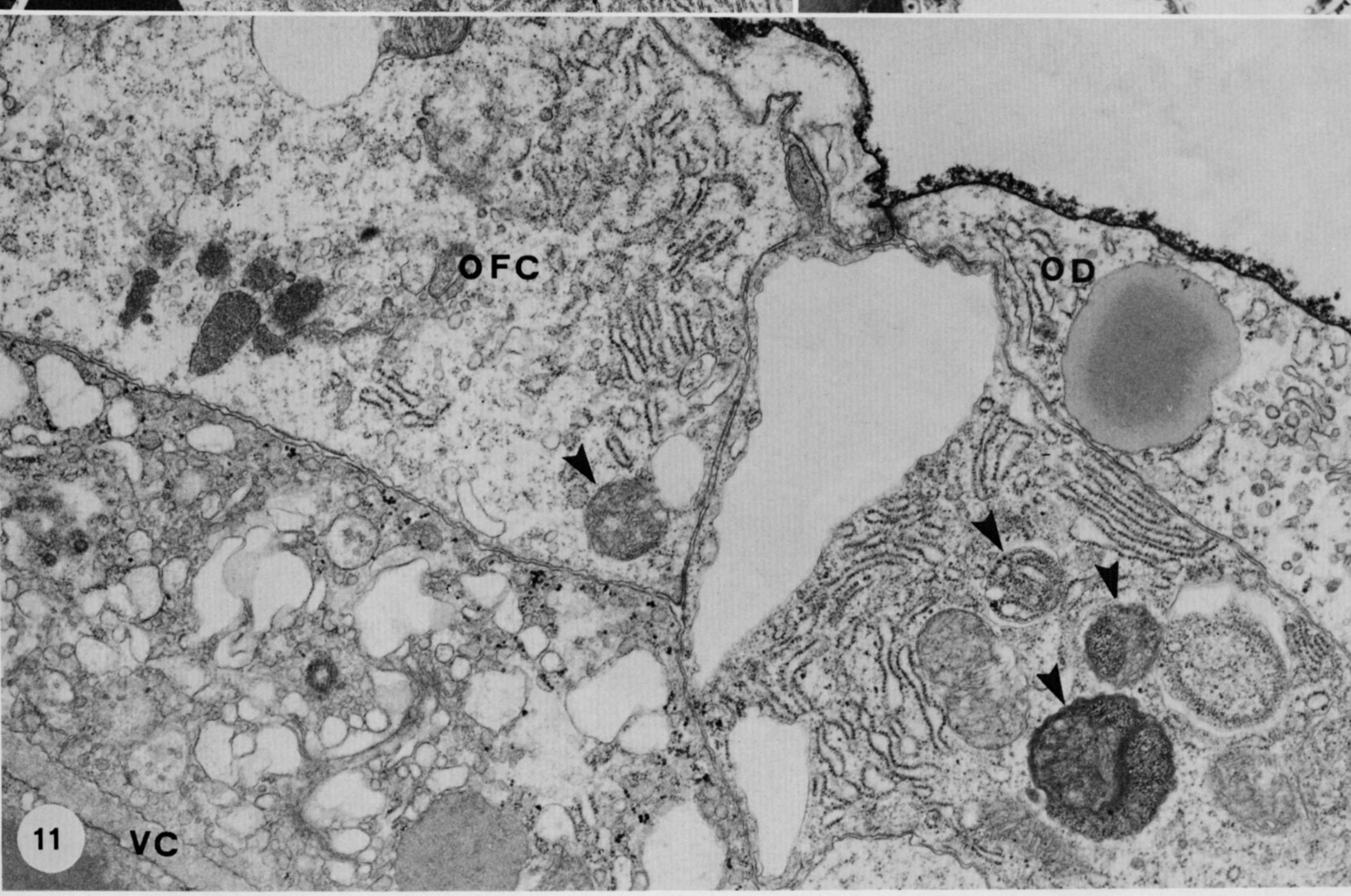
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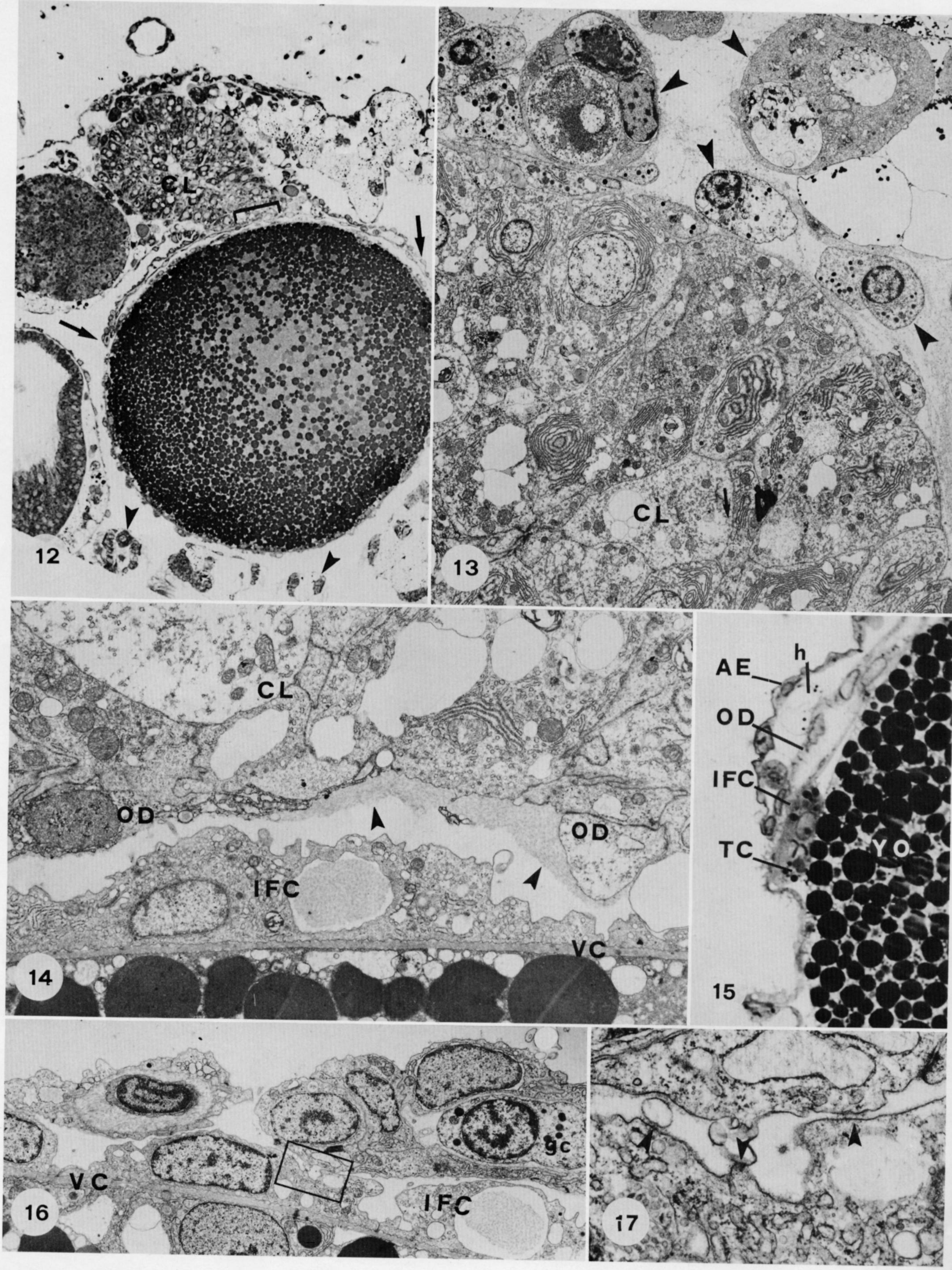
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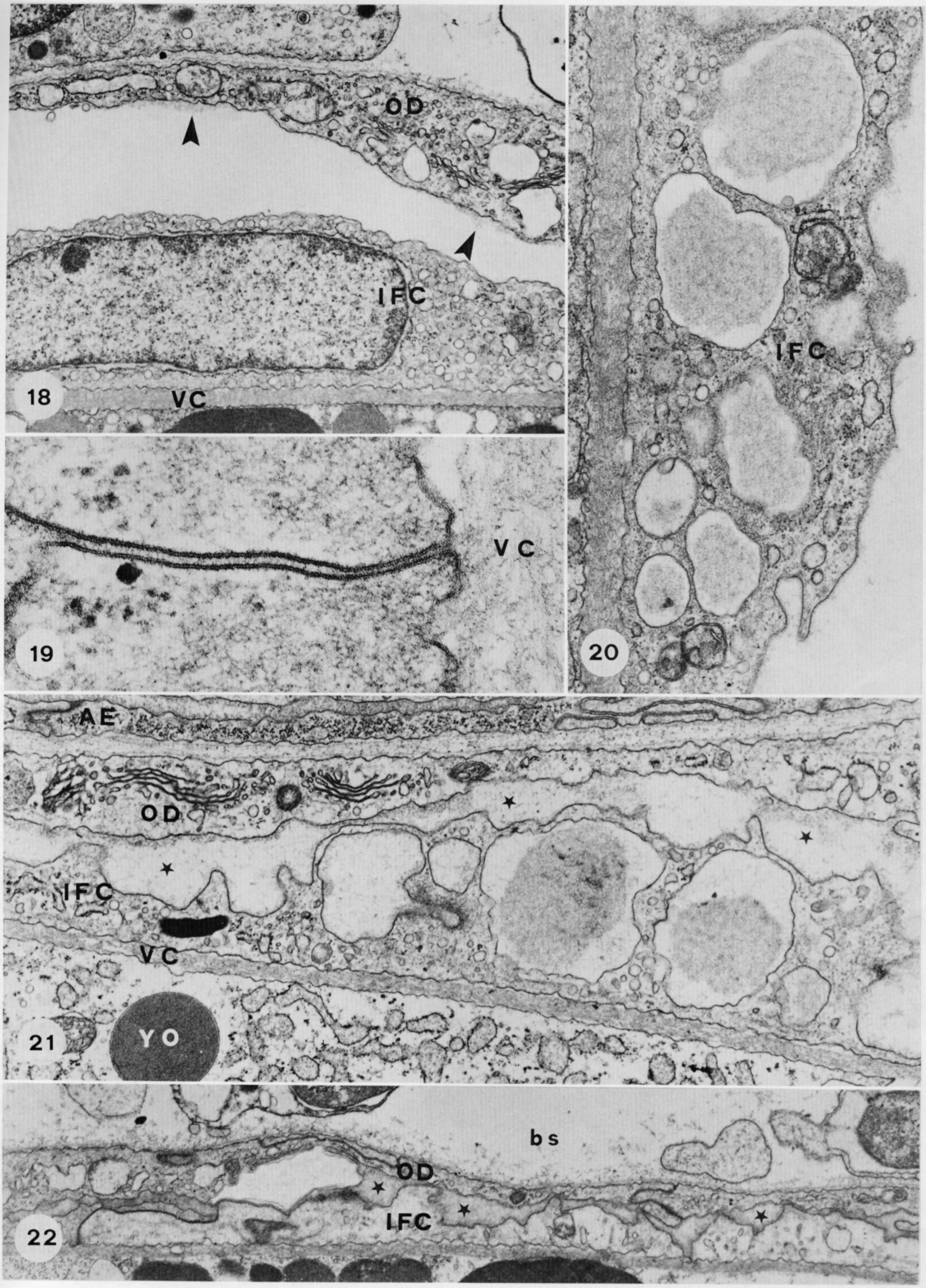
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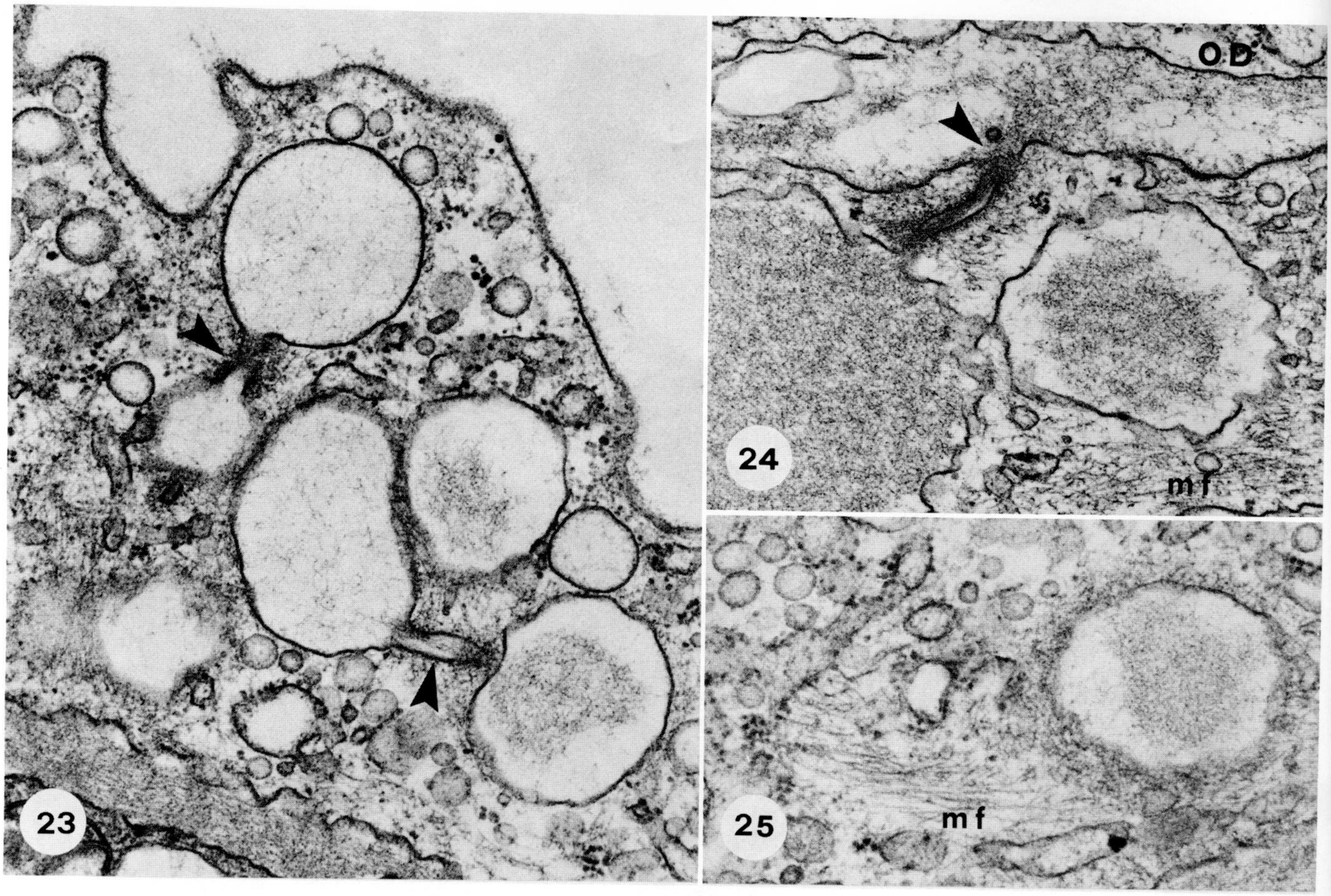

FIG. 23. Detail of an IFC in the placenta. Vacuoles with filamentous content are connected to one another by tubular channels (arrowheads) whose walls are reinforced by a felt of cytoplasmic filaments continuous with the rich cytoskeleton. Filaments also adhere to the vacuolar walls. × 40 000. FIG. 24. Detail of a placenta. The content of a vacuole of an IFC is apparently extruded through a narrow tubular channel (arrowhead) the walls of which are covered on the cytoplasmic side with dense filaments. Numerous microfilaments (*mf*) are in the cell. OD, oviducal epithelium. × 35 000. FIG. 25. Detail of IFC after ovulation. A great number of smooth vesicles and microfilaments (*mf*) occur in the cytoplasm. Many filaments are in bundles parallel to the basal wall (*mf*); others form a couche around the vesicles. × 46 000.

perivitelline space in which free test cells can be found occasionally (Fig. 15).

Discussion

In ascidians with internal fertilization, embryos establish various relationships with the parent. In ovoviviparous species, embryos develop using their own yolk nutrition, while in viviparous species, they depend on the parent for nutrients. In the latter species, the eggs can be extremely small and alecithal with a prolonged gestation period, as long as 5 months in *Hypsistozoa fasmeriana* (Brewin 1956).

In the ovoviviparous *Botryllus schlosseri*, few eggs mature; they are quite rich in yolk and about 300 μm in diameter. The embryos are held in the atrial chamber by a placenta formed by maternal tissues. We have not seen signs of intense parent–embryo exchange of nutrients, so the embryos may develop using mainly their own yolk. This is consistent with the possibility of obtaining in some cases a complete development of young embryos removed from the zooids (Milkman 1967). As reported for salps (Bone et al. 1985), the main role of the placenta seems to be the attachment of the embryo to the atrial wall, thus permitting the irrigation of the embryo by the continuous flow of seawater created by the branchial stigmata. However, it cannot be completely excluded that some parent–embryo exchange can occur.

Outer follicle cells

The OFC of *B. schlosseri* show signs of intense protein synthesis in contrast to cells from ascidians with external fertilization where the outer follicle is not recognizable or is reduced to a very thin layer (Cotelli et al. 1981; Kessel 1983). This seems to be the cause of confusing nomenclature of the egg envelopes. Indeed, some authors (e.g., Berrill 1950; Mansueto and Villa 1983; Satoh et al. 1982) mention "outer" and "inner" follicle layers in the spawned eggs, referring to the true inner follicle layer and the test cells, respectively. Other authors introduce ambiguous terms, e.g., "chorion cells" of Ursprung and Schabtach (1968) and Markert et al. (1968), to indicate the IFC.

The true role of the OFC of *B. schlosseri* has not been elucidated despite the evidence of their involvement in protein synthesis. These cells are presumably engaged in processing material to be passed to the egg. However, they might also be involved in the production and (or) regulation of hormones. In this context it is noteworthy that the OFC, discharged during ovulation, form a postovulatory follicle recalling a sort of corpus luteum (Arnback-Christie-Linde 1923). The mode of degradation of the OFC by wandering phagocytes recalls that encountered during tissue regression in the adults at the end of the life cycle (Burighel and Schiavinato 1984). The possibility that the corpus luteum of *B. schlosseri*, as in vertebrates, could have some physiological influence on the tissues, facilitating the early attachment of the egg to the placenta, cannot be excluded. In favor of this view the luteal cells appear to be active during the period of egg attachment. Consistent with this view, a conspicuous corpus luteum was described in ovoviviparous species of the genera *Botryllus* and *Botrylloides* (Arnback-Christie-Linde 1923; Mukai 1977) but not in oviparous species, such as *Ciona intestinalis* (Pérès 1954; Cotelli et al. 1981).

Oviduct

Mukai (1977) referred to the oviduct as a "follicle stalk," but in agreement with Arnback-Christie-Linde (1923), we prefer to call it "oviduct" in consideration of its roles in conducting the egg and in forming the placenta. The wall of the oviduct undergoes transformations during maturation and implantation of the egg. It is possible that at ovulation the apical cytoplasmic modifications occur concomitantly with entrance of liquid into the lumen, and swelling of the oviduct. This could facilitate the rupture of the oviduct and the movement of the egg towards the atrial chamber.

Pizon (1893) and Arnback-Christie-Linde (1923) gave an erroneous interpretation about the tissues forming the placental cup. In agreement with Mukai (1977), we have observed that the internal wall of the cup consists of oviducal epithelium. The edge of the cup is responsible for the early retention of the egg, since here the follicle cells and oviducal epithelium stick to one another by means of scattered junctional spots. Moreover, the final anchoring of the egg into the cup is assured by means of the fibrillar cementing material secreted by the follicle cells.

Inner follicle cells

The spawned eggs of ascidians are surrounded externally by a cell layer derived from the IFC to which multiple functions have been attributed (see De Santis et al. 1980; Jeffery 1980). It is supposed that these cells function as a nonspecific filter regulating the passage of the spermatozoa towards the underlying vitelline coat which bears species-specific receptors on its outer surface (De Santis et al. 1980). The large content of microfilaments and microtubules observed in *B. schlosseri* might be responsible for a contractile mechanism regulating the passage of the spermatozoa between contiguous cells, as claimed for *Ciona* by De Santis et al. (1980). In addition, the developed cytoskeleton together with the extended junctions would be necessary to maintain the continuity of the follicle layer against the mechanical stresses caused by the embryo movements and the external medium. Moreover, during differentiation, the IFC constantly maintain a close relationship with the vitelline coat, probably influencing the assembly of its outer surface, as suggested for *Ciona* (Cotelli et al. 1981). In the mature eggs of most ascidians there is a wide perivitelline space, where the test cells reside (Berrill 1950). In *B. schlosseri* no similar space exists and the test cells remain encased in oocyte indentations; only during embryonic development does a perivitelline space become evident and contain extruded test cells.

The IFC represent a barrier between the external medium and the developing embryo. A protective role could require a continuous secretion of a covering substance; perhaps the granules seen in *B. schlosseri* serve such a function. A characteristic of the IFC are the large vacuoles interconnected by thick-walled tubules and having a filamentous content similar to the material filling the space between the IFC and the oviducal epithelium. There is no doubt that the vacuole content is extruded even though we have never observed aspects of typical secretion. The IFC secretion could serve to cement the embryo to the cuplike placenta. Indentations and microvilli might increase the surface area for the attachment of the extracellular material and also for some parent–embryo exchange.

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