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The Follicle Cells of Styela Plicata (Ascidiacea, Tunicata): A Sem Study

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ABSTRACT—The morphological aspect of the follicle cells of *Styela plicata* eggs is described by means of scanning electron microscope investigations. The follicular layer is made of spaced, cylindrical box-like cells which are arranged hexagonally. They adhere to the egg through a complex network of membrane extensions making an overall thin layer on the vitelline coat. The walls of the follicle cells are plentifully provided with microvilli, filopodia and lamellipodia, which allow a connection among the cells. At their apical end lies a large vacuole containing a granule, probably involved in secretion. At insemination the majority of spermatozoa is distributed on the apical membrane of the follicle cells. The membrane often breaks after sperm-egg impact and the granule is therefore displaced. By means of the present investigations it is once again suggested a role played by follicle cells in ascidian eggs at fertilization.

INTRODUCTION

The ovular envelopes of Ascidians (follicle cells, vitelline coat and test cells) have been widely studied in several species and their functions are almost understood (for references see Cloney, 1990; Burighel and Cloney, 1997)

For the follicle cells, which constitute the outer envelope, many functions have been proposed, according to the species considered ,e. g. RNA and protein synthesis (Jeffery, 1980); flotation (Lambert and Lambert, 1978); secretion of adhesive material, a sticky mucus coat that attaches the egg to the substrate (Young *et al*, 1988; Bates and Mallett, 1991); secretion of a factor that triggers germinal vesicle breakdown (Sakairi and Shirai, 1991).

In *Ciona intestinalis*, a self-sterile species, it has been found that gamete self-incompatibility is established during oogenesis and is controlled by follicle cells overlying the vitelline coat (De Santis and Pinto, 1991; Pinto *et al.*, 1995); the cells prevent self fertilization by releasing self-sterility factors, of a protein nature, that bind to the vitelline coat (Marino *et al.*, 1999). At fertilization the follicle cells influence sperm behaviour through the production of chemotactic substances such as sperm-attractans (Miller, 1975;1982), and through phagocytosis and the entrapment of spermatozoa (Villa and Patricolo, 1993). These cells play also a role in sperm activation and contribute to sperm-egg interaction by facilitating sperm aggregation on the vitelline coat *of C. intestinalis*, and the consequent sperm penetration (Kawamura *et al*, 1988). Their presence, indeed, is necessary for fertilization in *Halocynthia roretzi* (Ishikawa *et al.*, 1978; Fuke, 1983); in *Ascidia nigra* their removal decreases the fertilization rate; in *Ascidiella aspersa* (Villa and Patricolo, 1992) they seem to induce a sort of “capacitation” of the spermatozoa of their own species. The follicle cells are also involved in sperm mitochondrion translocation (Villa and Patricolo, 1992; 1993), in sperm guiding into the interfollicular clefts (Villa and Patricolo, 1993; Fukumoto and Numakunai, 1995), and in creating an interspecific block to fertilization (for references see Patricolo and Villa, 1995).

In this paper the authors extend their observations to *Styela plicata* eggs. The peculiar morphology of the follicle cells in this ascidian species is highlighted by SEM studies; the results support the previously suggested role played by these cells at fertilization, i.e that of facilitating sperm penetration.

MATERIALS AND METHODS

Biological material

Adult specimens of *Styela plicata* were collected in the Gulf of Palermo. Female and male gametes were removed from the gonoducts of dissected animals. Since this species is self-sterile hermaphrodite, insemination was accomplished by mixing the gametes of two or more individuals. Before insemination, dry sperm was diluted to a final concentration of approximately 0.1% v/v. Some lots of eggs were defollicled by a gentle shaking. Ten minutes after insemination the eggs were washed twice in Millipore filtered sea water (MFSW) at pH 7.8, at about 22°C. Part of the living eggs was examined at light microscope (LM), part was fixed for scanning electron microscopy (SEM).

Scanning Electron Microscopy

Inseminated eggs were fixed at room temperature with 2.5% gluteraldehyde in 0.1 M cacodylate buffer in MFSW at pH 7.4 for 30

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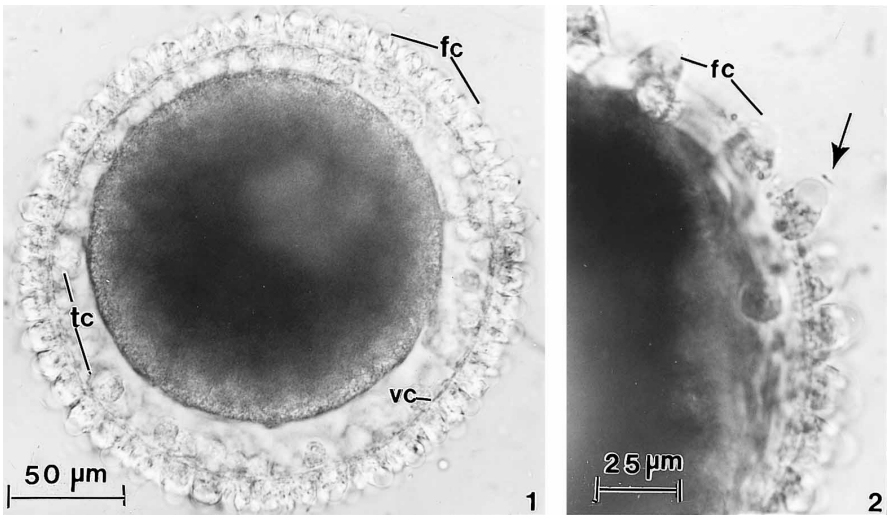
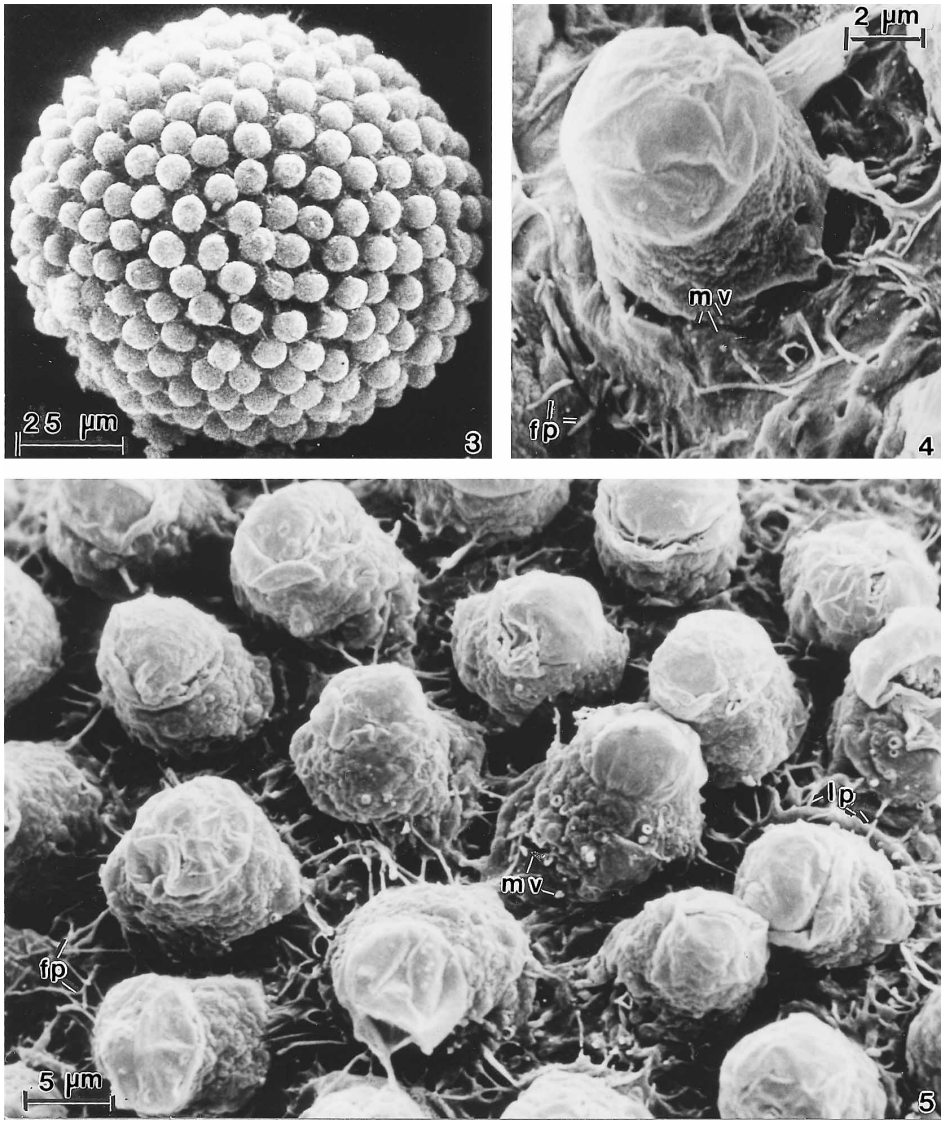


Figs. 1, 2. Light micrographs of *Styela plicata* egg. Arrow points to the spermatozoon placed on the great vacuole. **fc** = follicle cell; **tc** = test cell; **vc** = vitelline coat.



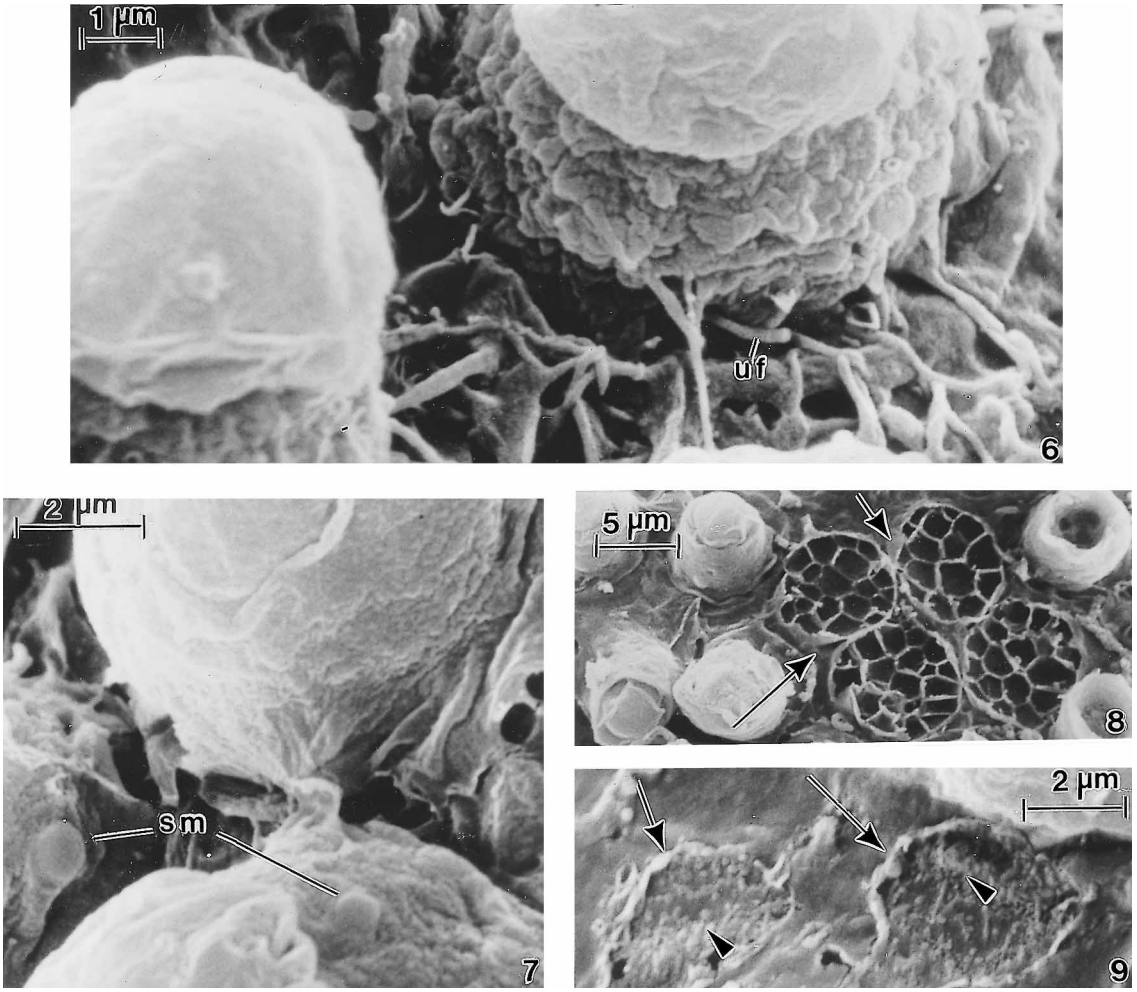
Figs. 3–5. SEM micrographs of *S. plicata* eggs: general view (Fig. 3); details of **fc** (Fig. 4) and of the **fc** layer (Fig. 5). **fp**=filopodia; **lp**= lamellipodia; **mv**=microvilli.

min. They were then rinsed twice in the same buffer and post fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for 1 hr. After dehydration the samples were critical point dried, sputter coated with gold and observed with a Cambridge Stereoscan 200 scanning electron microscope.

RESULTS

S. plicata eggs are about 230 µm in diameter and are surrounded by large follicle cells that at LM appear as highly vacuolated and columnar in shape, with a height of about 18 µm (Figs. 1,2). At the apical end of each cell lies a very large and clear vacuole, half of which extends inward among the smaller vacuoles that fill the rest of the cell. Spermatozoa, placed on or pointing towards the protruding vacuole, are frequently observed in inseminated eggs (Fig. 2, arrow). First cleavage occurs at approximately 60 min. The removal of the follicle cells delays this event of 30 min; moreover a decrease of 50% of fertilization rate is also obtained.

The follicle cells, as observed at SEM, are arranged in a single layer of largely spaced and hexagonally arranged box-like cells (Fig. 3). The plasma membrane shows a variety of surface specializations in different regions. The lateral portions of the cell, i.e. the walls of the elongated box, are folded and/or wrinkled, while the apical region, i.e. the top of the box, appears to be thinner and generally less folded (Fig. 4). The cell surface, in fact, shows a peculiar structure, more developed in the lateral regions, consisting of a complex of membrane extensions, i.e. microvilli, filopodia and lamellipodia. Filopodia, which are very prominent compared to the other extensions, from the basal region of the plasma membrane irradiate in all directions towards the adjacent cells, thus creating a complex network which undoubtedly aids the follicle cells to anchor to one another (Fig. 5). Occasionally unattached filopodia are also visible stemming from the basal region of the follicle cells (Fig. 6). Microvilli of various length and shape are also scattered on the follicle cell surface; round formations likely made of secretory material, perhaps buds of some product to be discharged, can be observed among the microvilli (Fig. 7). The basal surface of the follicle cells also appears to be provided with lamellipodia of various shape. In regions where the network of membrane protrusions is less developed, the basal region of the follicle cells is evidenced

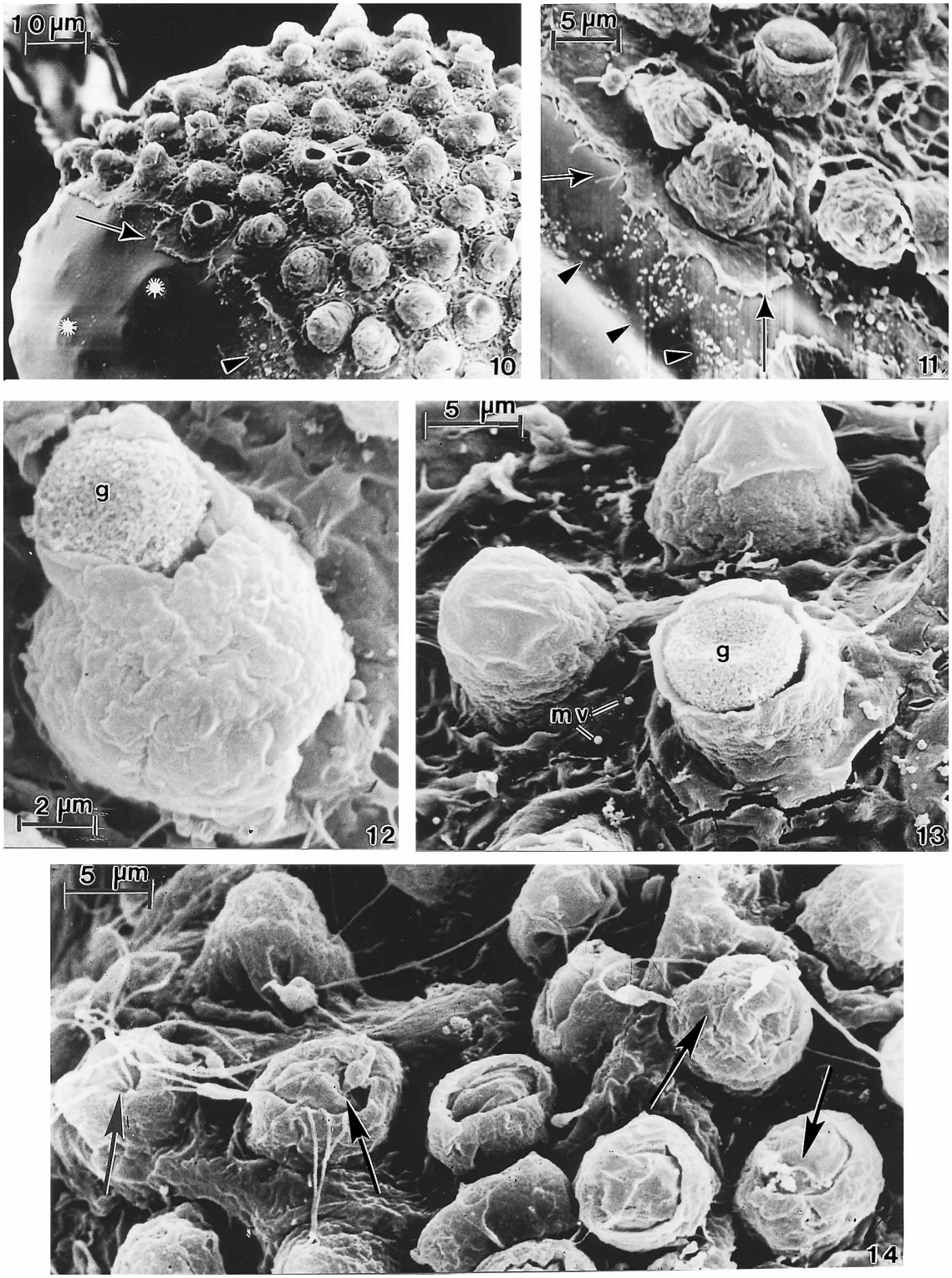


Figs. 6 – 9. SEM micrographs of *S. plicata* eggs: details of **fc**s (Figs. 6, 7) and of their impressions (Figs. 8, 9). Arrows indicate the basal region of detached **fc**s (Fig. 8) and remnants of their cell boundaries (Fig. 9); arrowheads indicate **vc** microvilli.
mv=microvilli; **sm**=secreted material (?); **uf**=unattached filopodia.

and the interdigitations among cells can be observed.

The whole of the membrane extensions forms a thin yet compact layer, through which the follicle cells are tightly attached to one another and to the vitelline coat (Figs. 10, 11, arrows).

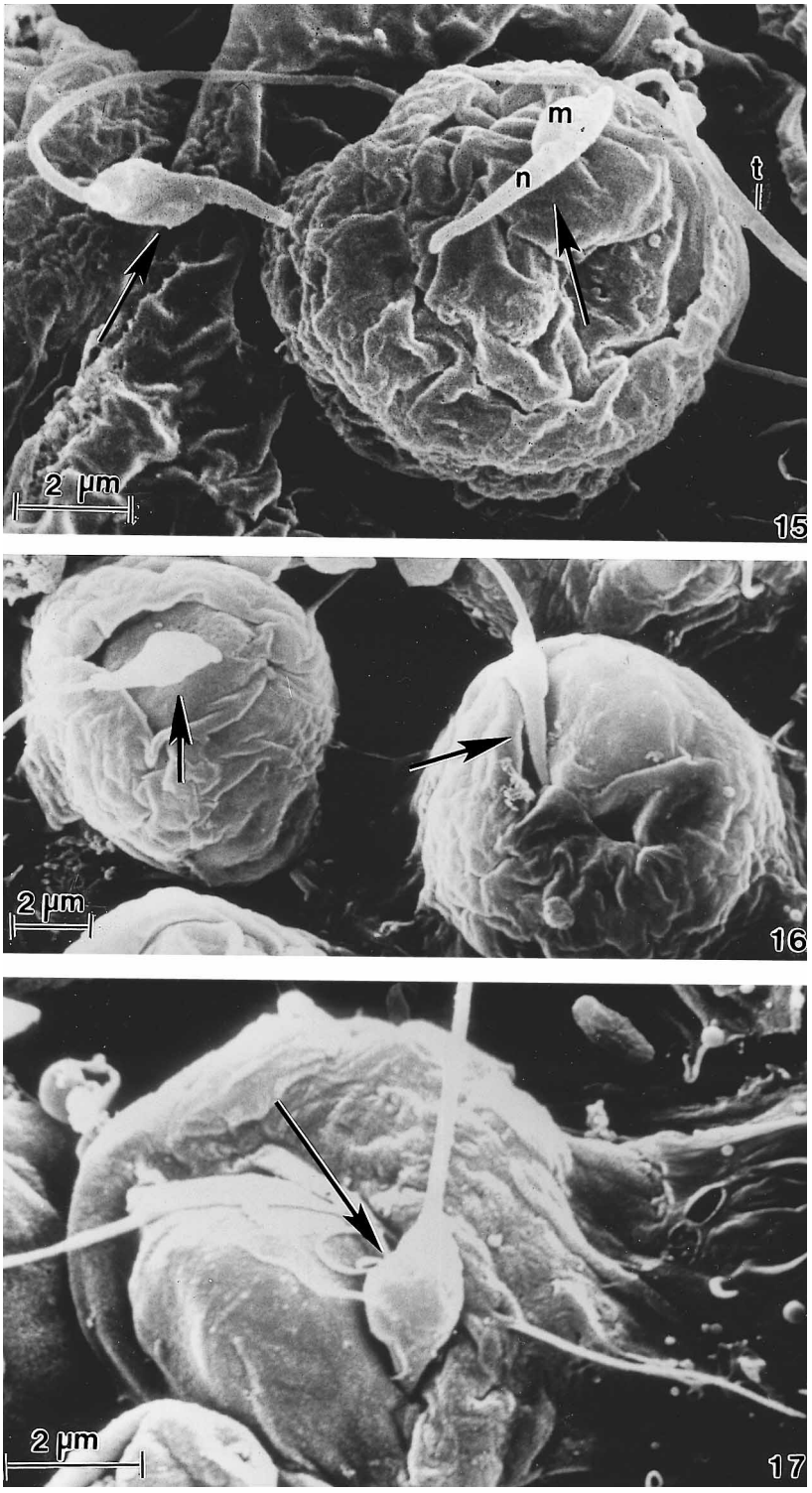
The follicle cells often fit inside niches created by projections of the vitelline coat, as visible at Transmission electron microscope (work in progress). The cohesion between the



Figs. 10–14. SEM micrographs of *S. plicata* eggs. Figs. 10 and 11 show a part of uncovered **vc**; arrowheads indicate **vc** microvilli; arrows point to the basal layer of the **fc**s; asterisks indicate **tc**s below. Figs. 12 and 13 show the **fc** apical granule (**g**) after rupture of the membraneous cover. Fig. 14: preferential sperm location on the **fc**s after insemination (arrows).

projections of the plasma membrane of the follicle cells and the vitelline coat is clearly shown in SEM micrographs of specimens damaged by the technique; in this case only the impression of the cell base is still observed adherent to the vitelline coat, the rest of the cell being lost (Fig. 8, arrows). In other specimens showing just remnants of the cell boundaries upon loss of the follicle cell base (Fig. 9, arrows), the naked vitelline coat appears to be largely microvillated (Figs. 9–11, arrowheads).

Uncovering of the apical region, likely due to mechanical impact with the spermatozoon head, evidences a large sponge-like granule (Figs. 12, 13). Inseminated eggs show a



Figs. 15–17. SEM micrographs of inseminated *S. plicata* eggs showing the position of spermatozoa (arrows) on the apical cover of the **fc**s. Fig. 15 is a magnification of the upper right side of Fig. 14. **m**=mitochondrion; **n**=nucleus; **t**=tail.

peculiar sperm distribution, with interfollicular spaces often empty (Fig. 14) and spermatozoa preferably scattered on the thin cover of the follicle cell (Figs. 14–17, arrows). When uncovered, follicle cells show spematozoa inside; moreover, sperm mitochondrion translocation cannot be observed at this stage.

DISCUSSION

The follicle cell layer of *Styela plicata* is made of cylindrical and highly vacuolated box-like cells. Tucker (1942) described them as "foam cells" for their extreme vacuolization. This feature represents the final stage of development since in the ovarian egg follicle cells make an inner and an outer layer, whose structure is morphologically different. In eggs discharged from the ovary the thin outer follicle epithelium is absent and thus the outer layer of the envelopes consists only of the inner follicle cells.

In *S. plicata* eggs, differently from other species, the follicle cells are largely spaced. SEM observations of the cell basal regions highlight numerous membrane projections which build a highly dynamic and protective layer on the external part of the vitelline coat. This carpet-like membranous system is a newly found feature which could be considered as an egg device to balance the large gaps between the follicle cells. In this way in fact spermatozoa are offered a safe anchorage within the network of membrane projections. This suggestion is supported by the absence, in the follicle cells of *S. plicata*, of the alveolar structure which aids the crossing of spermatozoa, discovered in *Ascidia malaca* and *A. aspersa* (Villa and Patricolo, 1993). In the latter the authors have hypothesized that the alveolar structure, made of membrane protrusions arranged in a honey-comb fashion, could act as a "railway" in guiding the sperm across the interfollicular clefts towards the vitelline coat. A similar surface ornamentation of the follicle cells was also later described in *H. roretzi* by Fukumoto and Numakunai (1995).

In inseminated eggs of *S. plicata* the interfollicular spaces are almost devoid of spermatozoa, which instead are preferably scattered on the follicle cell covers. In other ascidian species, such as *A. malaca* and *A. aspersa* (Villa, 1977; Villa and Patricolo, 1992; 1993; Patricolo and Villa, 1995) sperm behaviour seems quite different, as spermatozoa mainly crowd the narrow interfollicular clefts and show mitochondrion translocation. In *S. plicata,* spermatozoa in contact with the egg do not present mitochondrion translocation; moreover, it seems that on impact with the follicle cell layer the spermatozoon head could cause the rupture of the follicle cell apical membrane owing to weakness of the latter.

The follicle cells, described at LM as having a "clear vacuole" (Tucker, 1942) involved in flotation, in the present observations possesses a large, sponge-like granule, well visible after cell uncovering. The granule endows the cell with other functions; for instance, the frequent localization of spermatozoa on the follicle cell tops suggests for the latter a primarily attractant function. Moreover the displacement of the granule after sperm-egg impact could produce a shorter passage-way for sperm penetration through the vitelline coat. This likely passageway function of the follicle cells at fertilization must be added to the already described ones, even if at present it is not satisfactorily demonstrable; this hypothesis could be only supported by our observations on the decrease in fertilization rate of defollicled eggs.

It is widely known that the follicle cells represent the earlier barrier encountered by spermatozoa after insemination and that, besides the role they have in common with all species, they also play species-specific functions. One of these is the early discrimination of heterospecific spermatozoa; it has been observed, in fact, that in ascidian interspecific fertilization, e.g. *A. malaca* and *A. aspersa* (Villa and Patricolo, 1992; Patricolo and Villa, 1992; 1995) the follicle cells constitute a hybridization barrier, acting as the primary recognition site. Foreign spermatozoa initially interacting with the follicle cell layer remain on its surface, leaving the interfollicular clefts empty; however, other functions are performed, such as mucus secretion, sperm attraction, entrapment and phagocytosis and, to a lesser extent, sperm mitochondrion translocation. Therefore, even if the species-specific block is ensured by the vitelline coat (Rosati and De Santis, 1978; Honegger, 1986; Lambert, 1986) the follicle cells undoubtedly act as the first obstacle to heterospecific sperm penetration.

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