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Sexual reproduction in the compound ascidian *Diplosoma listerianum* (Tunicata). II. Sperm penetration through ovary wall and evidence of internal fertilization

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Abstract. *Diplosoma listerianum* differs from most ascidians in that, at ovulation, eggs are emitted at the bottom of the ovary and segregated into the tunic, so that fertilization occurs far from seawater. A fertilization canal, a hollow extension of the ovary, conducts sperm towards the egg. In the present paper, ultrastructural evidence is reported on the morphological relationship between the ovary, egg envelopes and oocyte and on the mechanism by which sperm-egg interaction is established. In the ovary, the very complex sperm, equipped with a spiral "dense groove", undergo metamorphosis as the first step in a sperm reaction and then pass through the ovary epithelium insinuating themselves between the intercellular junctions which appear to be mouldable, although able to maintain the egg-ovary barrier. Sperm then reach the vitelline coat, where a further step in the sperm reaction occurs. Before the egg abandons the ovary, the sperm head is incorporated into the oocyte by a process recalling phagocytosis, with the formation of an engulfing pocket. Sperm-egg contact and incorporation in *D. listerianum* occur in a way, never previously reported for other ascidians, in which fusion of plasma membranes takes place immediately after sperm-egg contact. Unlike other cytoplasmic components, the dense groove persists until the sperm enters the egg. It gives a corkscrew-like configuration to the sperm head and allows close adhesion to cell membranes, facilitating sperm movement. Expulsion of numerous cortical granules and features of a cortical reaction were observed in the egg penetrated by the sperm. The mode of internal fertilization of this species in comparison with that of other tunicates and phylogenetic aspects are discussed. Ripe colonies of *D. listerianum* collected in the Lagoon of Venice, Italy in 1986 and 1991 and colonies reared on glass in aquaria were used for our investigations.

Introduction

In the last two decades a number of papers have been published on aspects of sexual reproduction in ascidians, especially as regards gamete morphology and sperm-egg interactions (Tuzet et al. 1972, Woollacott 1977, Rosati and De Santis 1978, Cloney and Abbott 1980, Fukumoto 1981, 1990a, Villa 1981, Monroy and Rosati 1983, Burighel et al. 1985, Rosati 1985, Honegger 1986, De Santis and Pinto 1988, Koch and Lambert 1990, Jamieson 1991). In fact, ascidians represent very suitable material for studying the fertilization mechanism, owing to the fact that their eggs have a complex envelope that can be experimentally isolated, that fertilization occurs in seawater, and that "simple" sperm undergo a striking sperm reaction. However, in these respects ascidians are not such a homogeneous group as superficial examination of the literature would suggest, because most research has been directed towards a few species with external fertilization, which are widespread and easy to observe (Koch and Lambert 1990, for review). But several studies on other species have revealed very different situations as regards the structure of gametes and mode of reproduction (Jamieson 1991, for review).

Most solitary ascidians are free-spawning with external fertilization, whereas most compound ascidians retain the embryos in the atrial chamber, which is filled with seawater (Berrill 1950). However, although in both cases sperm are emitted and meet the egg in the same environment, i.e., seawater, the complexity of the sperm seems to vary if species with either external or internal fertilization are compared. This assumption is valid even when comparison is extended to species from all classes of tunicates. In particular, very complicated sperm are found in colonial forms with internal fertilization occurring in the zooids, far from seawater, both in ascidians and in other tunicates, the thaliaceans (Burighel et al. 1985, Holland 1988, 1989, 1990).

The compound ascidian *Diplosoma listerianum* produces large eggs which at ovulation are segregated into the tunic where the embryos develop and, by rupturing the tunic, escape from the colony as free-swimming larvae. Several studies have begun to shed light on the sexual reproductive mode of this species (Burighel et al. 1985, 1987, Brunetti et al. 1988, Martinucci et al. 1988, Ryland

and Bishop 1990, Bishop and Ryland 1991, Burighel and Martinucci 1994). In particular, we now know that: (1) oocytes grow on the wall of the hollow ovary and are segregated into the tunic enveloped by the epidermis, after having discharged two follicle layers (outer and inner), which remain in the mantle; (2) the ovary can extend to form a fertilization canal opening into the cloacal chamber; (3) metamorphosed sperm differing from those of the sperm duct are found in this canal; (4) this ovarian canal is the presumptive site where zooids can store exogenous viable sperm for long periods.

These findings reveal the existence of an intrazooidal pathway by means of which the sperm approaches the egg; however, they do not give information about the mechanism of gamete interaction, which would be interesting, especially as the lumen of the ovary, where sperm are found, is separated from the egg envelope by its epithelium which possesses intercellular tight junctions (Burighel and Martinucci 1994).

With the aim of investigating this mechanism, we studied mature zooids of *Diplosoma listerianum* using light and electron microscopy. In the present paper we report ultrastructural evidence that sperm pass through the ovary epithelium and that sperm-egg interaction occurs in a different way from that commonly reported for other ascidians.

Material and methods

Diplosoma listerianum (Milne-Edwards, 1841) (Aplousobranchia, Didemnidae) forms colonies with numerous small zooids. In each zooid a short peduncle separates the thorax from the abdomen, where the viscera and gonads are sited. The peduncle is crossed by the oesophagus, terminal intestine, sperm duct and fertilization canal, an extension of the ovary ending in the common cloacal cavity (Burighel and Martinucci 1994).

We used ripe colonies collected in the Lagoon of Venice and colonies reared on glass in aquaria. The transparency and thinness of the tunic allowed us to follow the phases of ovulation and egg segregation *in vivo* and to select zooids at opportune stages.

Specimens were fixed in 1.5% glutaraldehyde buffered with 0.2 *M* sodium cacodylate plus 1.6% NaCl and post-fixed in 1% OsO_4. They were embedded in Epon after orientation and cut serially. Thick sections (1 μm) were stained with toluidine blue. Thin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H 600 electron microscope.

Results

Ovary-egg relationship

The eggs of *Diplosoma listerianum* grew on the ovary wall one at a time, so that in an ovary one full-grown egg and one or two less developed oocytes could be present at the same time (Fig. 1 A, B). The full-grown egg had a complex vestment formed of a thick layer of outer follicle cells (OFC) rich in rough endoplasmic reticulum (RER), a thin, discontinuous layer of inner follicle cells (IFC), an acellular fibrous vitelline coat (VC) and test cells (TC) encased in superficial depressions of the oocyte. The latter was more than 300 μm in diameter and filled with round yolk platelets (Fig. 1 A) (see also Martinucci et al. 1988).

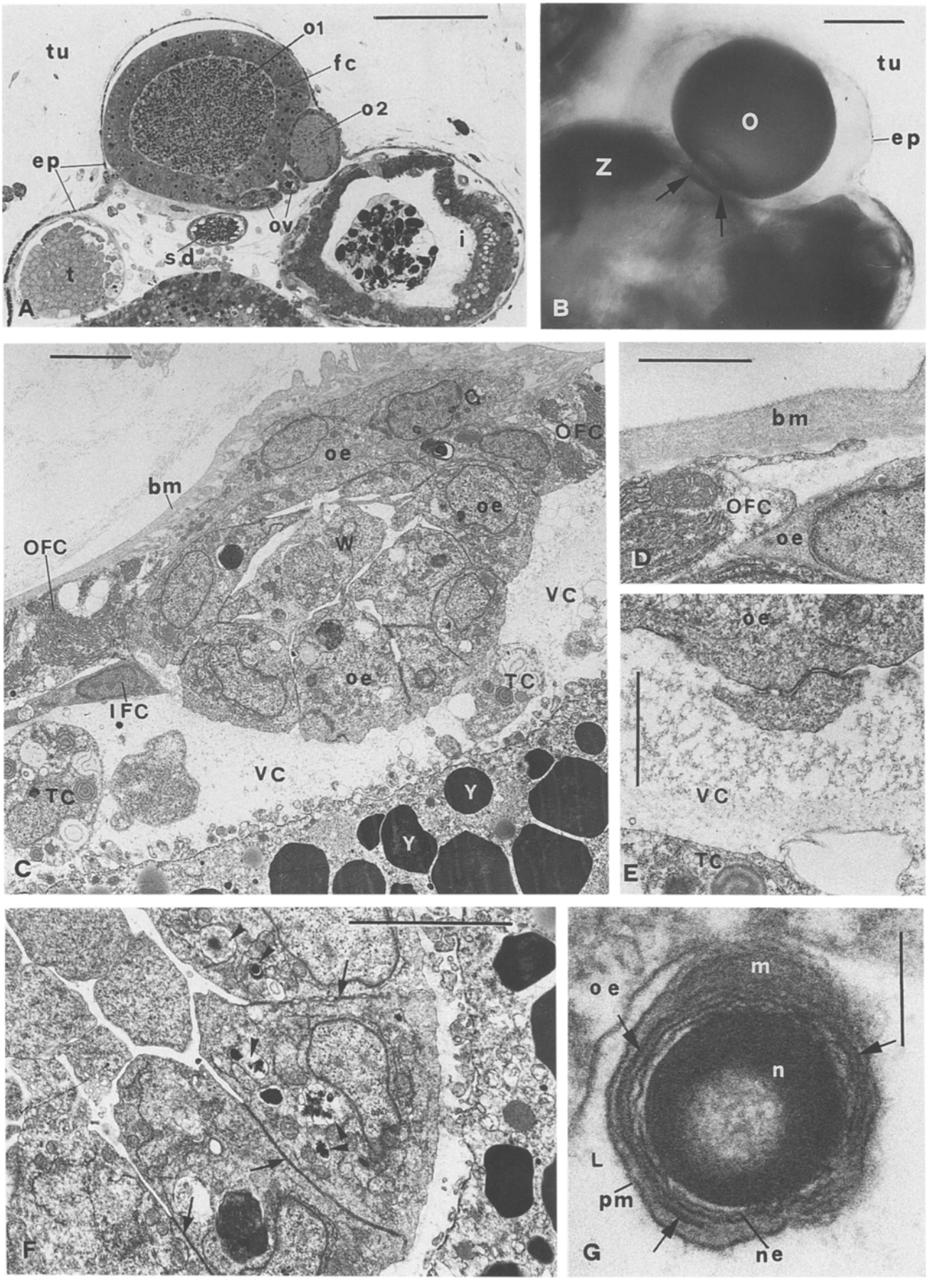
During growth, the egg herniated almost completely out of the ovary wall, but maintained contact with it in such a way that the region of the ovary close to the egg became incorporated in the OFC layer. The basal membranes of the OFC and ovary epithelium were in contact with each other (Fig. 1 C). They were very thick and composed of several layers of fibrillar material (Fig. 1 D). Where the ovary faced the oocyte (Fig. 1 C), its basal membrane was very thin, loose and difficult to recognize, because it appeared to be intermingled with the fibres of the VC.

The ovary wall and growing egg were always linked until ovulation when the oocyte was enveloped by the epidermis and segregated into the colonial tunic. The discharged OFC and IFC remained in the mantle of the zooid, attached to the deep region of the ovary (Burighel et al. 1987, Martinucci et al. 1988).

Ovary epithelium

In some cases the ovary epithelium approached the oocyte until contact was made at certain points, but direct communication between ovary lumen and perivitelline space was never found (Fig. 1 C, F, see Fig 3A). The ovary epithelium was formed of a monolayer of cells joined to each other by very extended intercellular junctions that ran along almost all their lateral borders from the apex (Fig. 1 C, F). These junctions showed several points of apposition between the two half-leaflets of adjacent membranes and a reduced intercellular space. Moreover, the junctional area was reinforced on the cytoplasmic sides by dense fibrous mats close to the membranes (Fig. 2 C–E). These junctions resembled the tight junctions described in other ascidians and in the fertilization canal of *Diplosoma listerianum* (Burighel and Martinucci 1994).

Fig. 1. *Diplosoma listerianum.* (A) Thick section through mature zooid showing vitellogenic oocyte (o1) with envelope of follicle cells (fc) and previtellogenic oocyte (o2). Sperm duct (sd) is next to ovary (ov). ep: epidermis; i: intestine; t:testis; tu: tunic. (B) Detail of colony mounted in toto showing narrow collar (arrows) which connects segregating egg (O) to parental zooid (Z). (C) Ovary crosses both outer (OFC) and inner (IFC) follicle cells and contacts vitelline coat (VC); oocyte (at bottom) contains yolk granules (Y). Ovary wall (oe) is covered only externally by thick basal membrane (bm) and not on the side facing oocyte. Wandering cell (W) is in the lumen of the ovary. TC:test cells. (D) Thick bm covers externally both OFCs and oe at their point of contact. (E) Only fibrous VC separates oe from test cells and oocyte (not shown). (F) Extended junctions (arrows) connect ovary epithelial cells, some of which contain heterophagic vacuoles with sperm remnants (arrowheads). (G) Cross section through posterior region of one sperm head contacting oe. Only remnants of mitochondrion (m) with its alar expansions (arrows) are seen in thin cytoplasm. L: ovary lumen; n: nucleus; ne: nuclear envelope; pm: plasma membrane. Scale bars in (A) = 50 μm; in (B) = 100 μm; in (C), (F) = 5 μm; in (D), (E) = 2 μm; in (G) = 0.2 μm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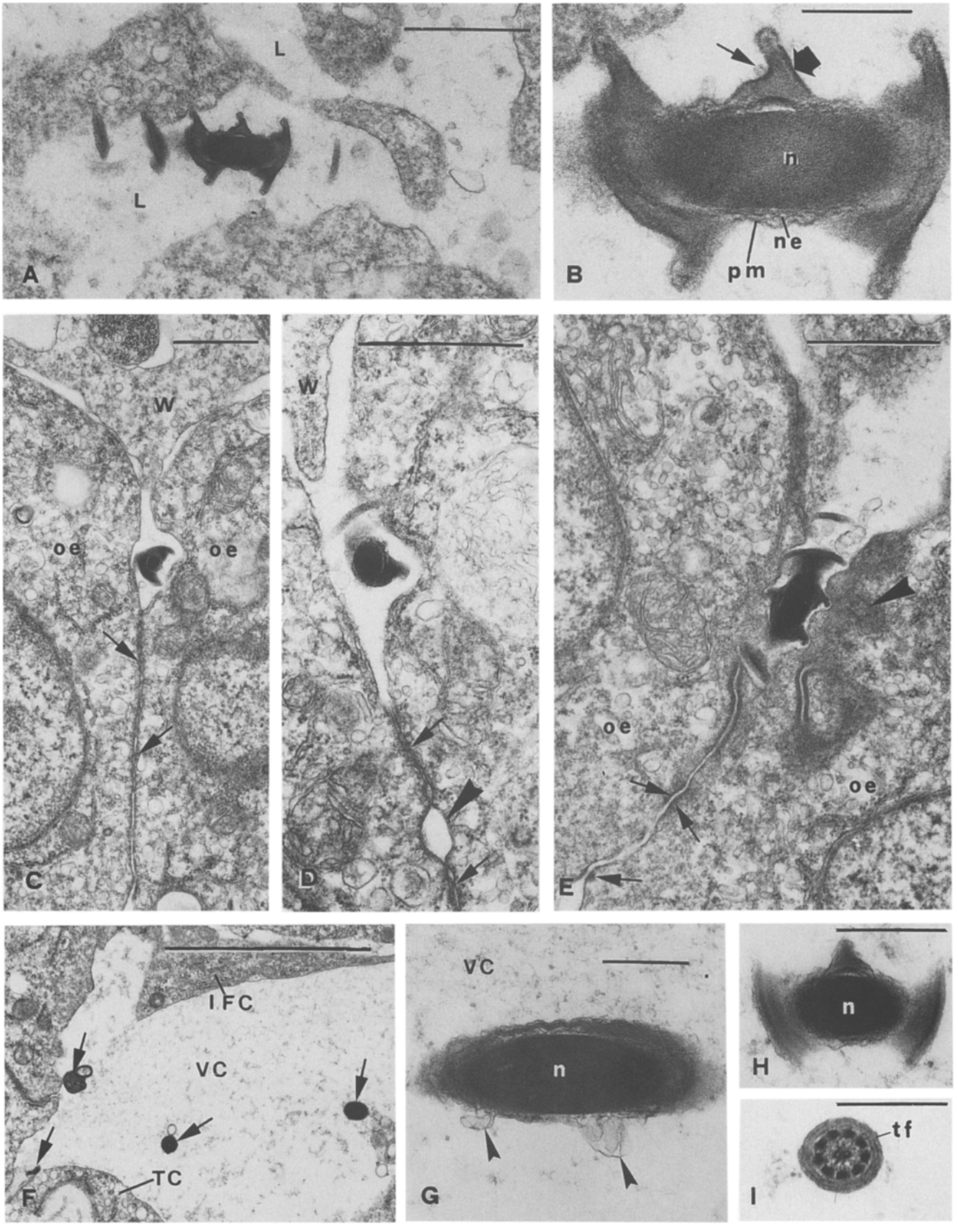
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Wandering cells and spermatozoa

The lumen of the ovary often contained wandering cells which did not show any junctional relationship with the surrounding epithelium. They were found also deep in the ovary, next to the full-grown oocyte (Fig. 1 C). These wandering cells and the cells of the ovary wall showed sperm or remnants of sperm at different phases of degeneration in the intracellular vacuoles (Fig. 1 F).

Occasionally, several sperm were encountered free in the lumen of the ovary, although wandering cells were also present (Figs. 1G, 2A, C). These sperm appeared to be modified with respect to those found in the sperm duct and looked like the metamorphosed sperm of the fertilization canal (Burighel and Martinucci 1994), although they revealed even more variations. In the basal region of the head, the spiral groove was missing, and neither the mitochondrion, nor endoplasmic tubules with typical disposition were recognizable. Only a thin cytoplasmic area persisted around the nucleus, with some remnants of the mitochondrion and its alar expansions close to the nuclear envelope; a glycocalyx of short dense filaments emerged from the plasmalemma and contacted the ovary cell membranes (Fig. 1 G).

The anterior portion of the sperm head revealed a dense coiled structure spiralling round the straight nucleus, protruding outside and giving the former a corkscrew-like aspect (Fig. 2 A). Under large magnification (Fig. 2 B), the coiled structure revealed its correspondence with the dense groove of normal and metamorphosed sperm. It was connected to the nuclear envelope by dense material and externally showed two paired ridges emerging at the bottom of the plasmalemmal invagination (Fig. 2 B).

Sperm passing through ovary epithelium

Before segregation into the tunic, a sort of dimple became recognizable in the ovulating egg (Fig. 1 B). Sperm in close relation with the ovary wall were found in zooids fixed at this stage. Some of them were present near the tight-junctional limits between contiguous cells (Fig. 2 C). Different sections of the same tissue (Fig. 2 C, D) revealed that the cells were not joined by junctions extending all along their lateral borders but that scattered, sac-like intercellular spaces occurred among the junctional areas (Fig. 2 D). Corresponding features were also found in zones lacking evidence of sperm (Fig. 1 C). Together with the tight junctions and sac-like intercellular spaces, other extended junctions were identified, characterized by a dense mat positioned in the cytoplasm close to the facing parallel membranes and a regularly 25-nm wide intercellular space crossed by fibrillar material (Figs. 2E, 3B). These junctions recall the adhering junctions previously described in other tissues of *Diplosoma listerianum* (Lane et al. 1986). Some sperm were seen to infiltrate their coiled head deeply into the intercellular spaces of the ovary wall and to corkscrew between adjacent cells by the hooks of their dense groove (Fig. 2 E). In some cases, the cytoplasmic areas next to the infiltrated sperm showed a diffuse dense mat like that of adhering junctions (Fig. 2 E).

Sperm were also identified in the perivitelline space among the filamentous VC (Fig. 2 F). Sections of the anterior head with spiral dense groove (Fig. 2 H) and of the posterior head with remnants of cytoplasm (Fig. 2 G) were frequently cut, but occasionally also sections of the proximal region of the tail flagellum were encountered (Fig. 2 I). Sperm found next to the oocyte, in contact with the VC, always showed cytoplasmic remnants attached to the plasmalemma, especially in the form of swollen and rearranged membranes (Fig. 2 F, G).

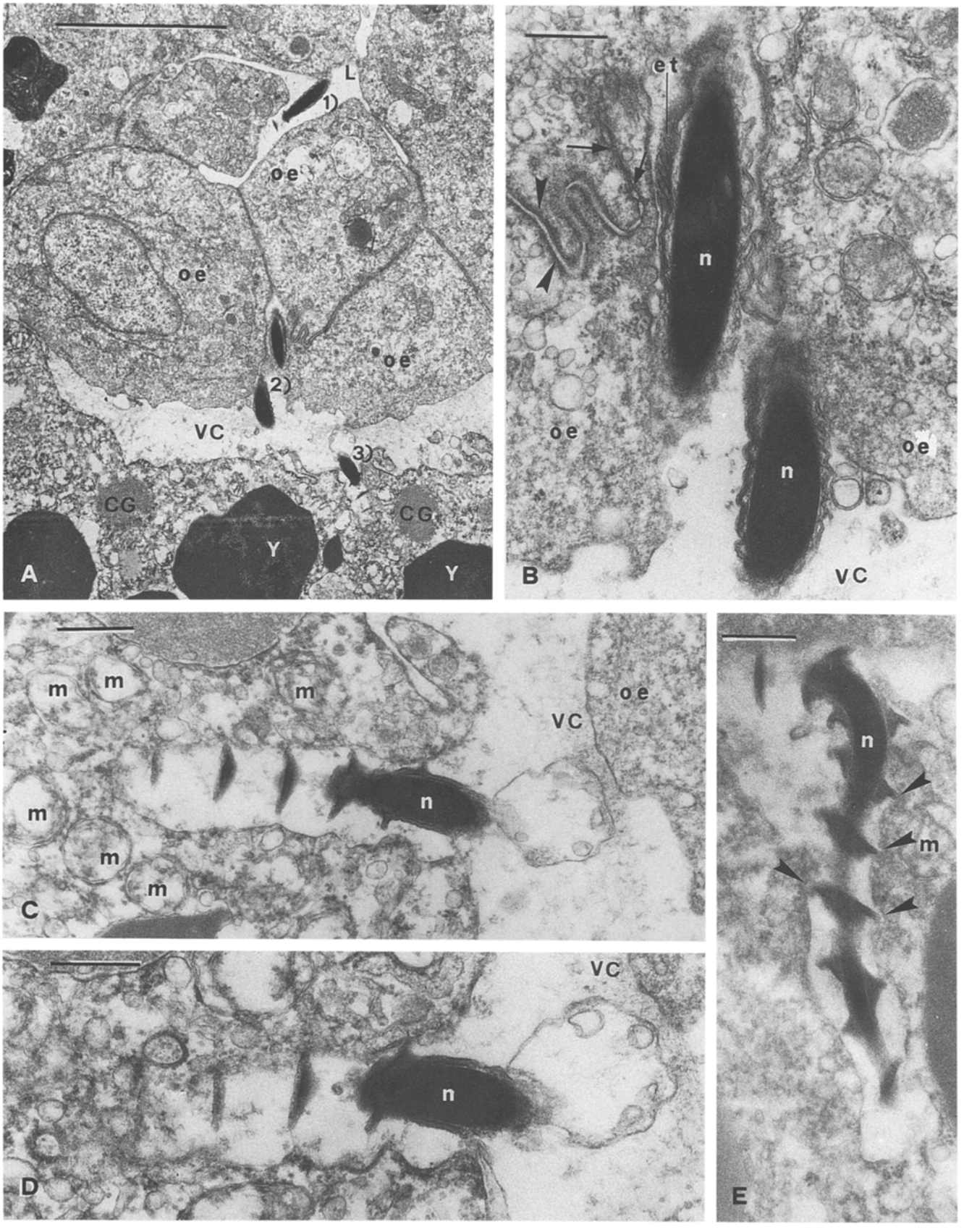
Fig. 2. *Diplosoma listerianum.* (A), (B) Oblique section of a corkscrew-like sperm head contained in the lumen (L) at bottom of ovary. Detail in (B) shows spiral structure (dense groove) around straight nucleus (n). Dense groove (large arrow) includes plasmalemmal invagination, possessing two ridges protruding outside (arrow), connected by dense material to nuclear envelope (ne). pm: plasma membrane. (C), (D) Two sections of same ovary epithelium (oe) showing one sperm infiltrating between cells. Note that sac-like spaces (arrowhead) are between intercellular junctions (arrows). W: wandering cell. (E) Spiral groove of sperm head is penetrating the intercellular space of ovary epithelium and is received in depressions of plasma membranes. At level of intercellular junctions plasmalemma is flanked by dense material arranged in spots (arrows) and parallel laminae (adhering junctions). Fibrous dense material is also in cytoplasm next to junctional areas (arrowhead). (F)–(I) Sections of sperm (arrows) in vitelline coat (VC). Some of them are eliminating cell debris (arrowheads). IFC: inner follicle cells; TC: test cells. In (H) and (I), sections of anterior head and proximal region of tail flagellum (tf) are shown. Scale bars in (A), (C)–(E) = 1 μm; in (B) = 0.2 μm; in (F) = 5 μm; in (G)–(I) = 0.5 μm

Sperm-oocyte interaction

Fig. 3 A is a highly representative picture of fertilization in *Diplosoma listerianum*. It shows one metamorphosed sperm free in the ovary lumen, two sperm (or two sections of the same sperm) passing through the ovarian epithelium, and one sperm being engulfed by the egg after having crossed the VC. The spermatozoa partially trapped in ovarian epithelium (Fig. 3 B) are pressed in the intercellular space next to junctions like those described above. They possess a nuclear envelope and plasma membrane, and also endoplasmic tubules, reduced to a few remnants on one side of the nucleus (Fig. 3 B).

Fig. 3 C and D illustrate two sections of the same sperm-egg contact. The sperm head is engulfed in a deep depression of the egg bounded by the oolemma. The borders of the protruding dense groove of this sperm are in close contact with the oolemma and a number of small round mitochondria are in the cytoplasm near the engulfing pocket. The swollen cytoplasm, containing some empty vesicles, is emitted from the sperm and appears to be bound to VC fibrils close to the ovary epithelium (Fig. 3 C, D).

As visible also in another spermatozoon (Fig. 3 E) in the process of being engulfed by the egg with the formation of a long pocket bounded by the oolemma, the dense groove persisted. It coiled at least eight to nine times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around the anterior portion of the sperm nucleus and established contact with the oolemma in the pocket.

Egg reaction

Before ovulation, the egg was studded with numerous test cells which were encased, often very deeply, in its superficial depressions (Fig. 4A). The test cells stood out from the dense yolk platelets due to their roundish shape and especially due to the presence of round granules of various densities; a number of these granules displayed a thread-like net of interconnected lamellae that were sometimes concentrically arranged in the homogeneous matrix (Fig. 4A, B). After segregation of the egg into the tunic, test cells are always found in the perivitelline space (Burighel et al. 1987). But also in the egg with evidence of sperm penetration, test cells were seen free in the perivitelline space, trapped in the loose net of the VC (Fig. 4C). Their granules were variable in content: several resembled those described above, and other larger ones had dispersed contents which seemed to be secreted outside the cells (Fig. 4C).

Moreover, in the same egg the cortical layer was modified. Before ovulation, full-grown oocytes have rather smooth oolemma with a reduced number of microvilli (Fig. 4A). Several round granules of different sizes (Fig. 4A, B) were found in the cortical layer, intermingled with the yolk platelets and characterized by their finely granular, homogeneous content. In eggs penetrated by sperm, the cortical region showed spectacular activity. A great number of cytoplasmic protrusions, having round granules with homogeneous content, stood out from the edge of the egg. Other granules, often larger but with similar content, were still in the cortical cytoplasm (Fig. 4C, D). Moreover, cytoplasmic protrusions containing empty vesicles and cytoplasmic debris extended out of the egg. In addition to the cytoplasmic protrusions, numerous coated pits and vesicles were present: a homogeneous granular material, looking like that of the cortical granules, adhered to the lumenal face of the membranes of both the vesicles and pits (Fig. 4D).

The process of sperm penetration through ovary wall and sperm-egg contact is illustrated in the schematic drawing of Fig. 5.

Fig. 3. *Diplosoma listerianum*. (A) Sperm are in ovary lumen (L) 1) passing through ovary epithelium (oe), 2), and penetrating into the egg, 3) after having crossed vitelline coat (VC). CG: cortical granules; Y: yolk granules. (B) Detail shows sperm heads with remnants of endoplasmic tubules (et). Ovary cells possess intercellular junctions that in (B) resemble tight junctions (arrows) and adhering junctions (arrowheads). Note, (A) on the left, ovary epithelium very close to oolemma. n: nucleus. (C), (D) Two contiguous sections of sperm head, which having crossed vitelline coat, penetrates into engulfing pocket of the egg. A number of mitochondria (m) are in the proximity of this pocket. Sperm remnants (swollen mitochondrion?) are discharged and their membrane is bound to vitelline coat which adheres to ovary epithelium. (E) Longitudinal section of another sperm penetrating egg. Note that border of spiral groove makes close contact with oolemma of engulfing pocket (arrowheads). Scale bars in (A) = 5 μm; in (B)–(E) = 0.5 μm

Discussion

Many observations have been published on sperm-egg interactions in ascidians with external fertilization but, as far as we know, nothing on species such as *Diplosoma listerianum*, whose sperm-egg contact takes place far from seawater. Previous reports on *D. listerianum* demonstrated the presence of metamorphosed sperm in the hollow ovary (Martinucci et al. 1988, Burighel and Martinucci 1994) and its capacity to store viable exogenous sperm (Bishop and Ryland 1991). The present results now add information on the major steps involved in sperm-egg interaction and suggest that *D. listerianum* is a very interesting species for further comparative study. Sperm-egg contact occurs in *D. listerianum* before the egg abandons the ovary. This behaviour is different from that commonly seen in ascidians, where the sperm fertilizes the egg after ovulation, i.e., after the egg, enveloped by IFC, has been emitted through the oviduct. At first, basing our research on the current state of knowledge, we looked for morphological signs of sperm-egg interaction in early segregated eggs, by searching for fertilizing sperm in the VC and perivitelline space. We thought that fertilization could occur, as in other colonial ascidians (Mukai 1977, Zaniolo et al. 1987), after discharge of OFC, and this hypothesis also fitted the report of Brunetti et al. (1988): the first egg cleavage happens up to 2 d after the egg is laid in the tunic. Attempts at finding sperm in segregated eggs were unsuccessful. Instead, evidence of sperm-egg contact was found before segregation of the egg, i.e., before discharge of OFC and IFC. Present observations show that OFC and IFC do not constitute any sort of barrier for sperm, because the two envelopes are invaded by the ovary epithelium, which insinuates itself between them and contacts the VC. This means that the actual barriers for sperm reaching the egg are the ovary epithelium itself and the VC fibrils.

In the distal ovary, i.e., the fertilization canal, sperm undergo morphological changes in comparison with sperm located in the sperm duct (Burighel and Martinucci 1994). This metamorphosis presumably corresponds to the sperm reaction by which the mitochondrion is discarded in other ascidians (Lambert and Epel 1979, Lambert 1982). At first the reaction consists of the release of the dense groove which is unhooked from the base of the sperm head and coils around its anterior half. This movement presses the mitochondrion and endoplasmic tubules posteriorly toward the base of the head. At the bottom of the ovary, we found sperm still further modified in that neither mitochondrion nor endoplasmic tubules had the aspect or disposition of those seen in the fertilization canal. Moreover, only rare sections of tail flagella were observed, which may indicate that most of the tail had been lost before. In the ovary lumen, sperm were free or contained in intracellular vacuoles of wandering cells. At the moment, we do not have sufficient evidence to state whether or not sperm reach the bottom of the ovary free and actively, or if they are transported *via* wandering cells and then liberated. The finding in these cells of vacuoles containing sperm at different phases of degeneration renders the latter hypothesis less probable but does not exclude it completely.

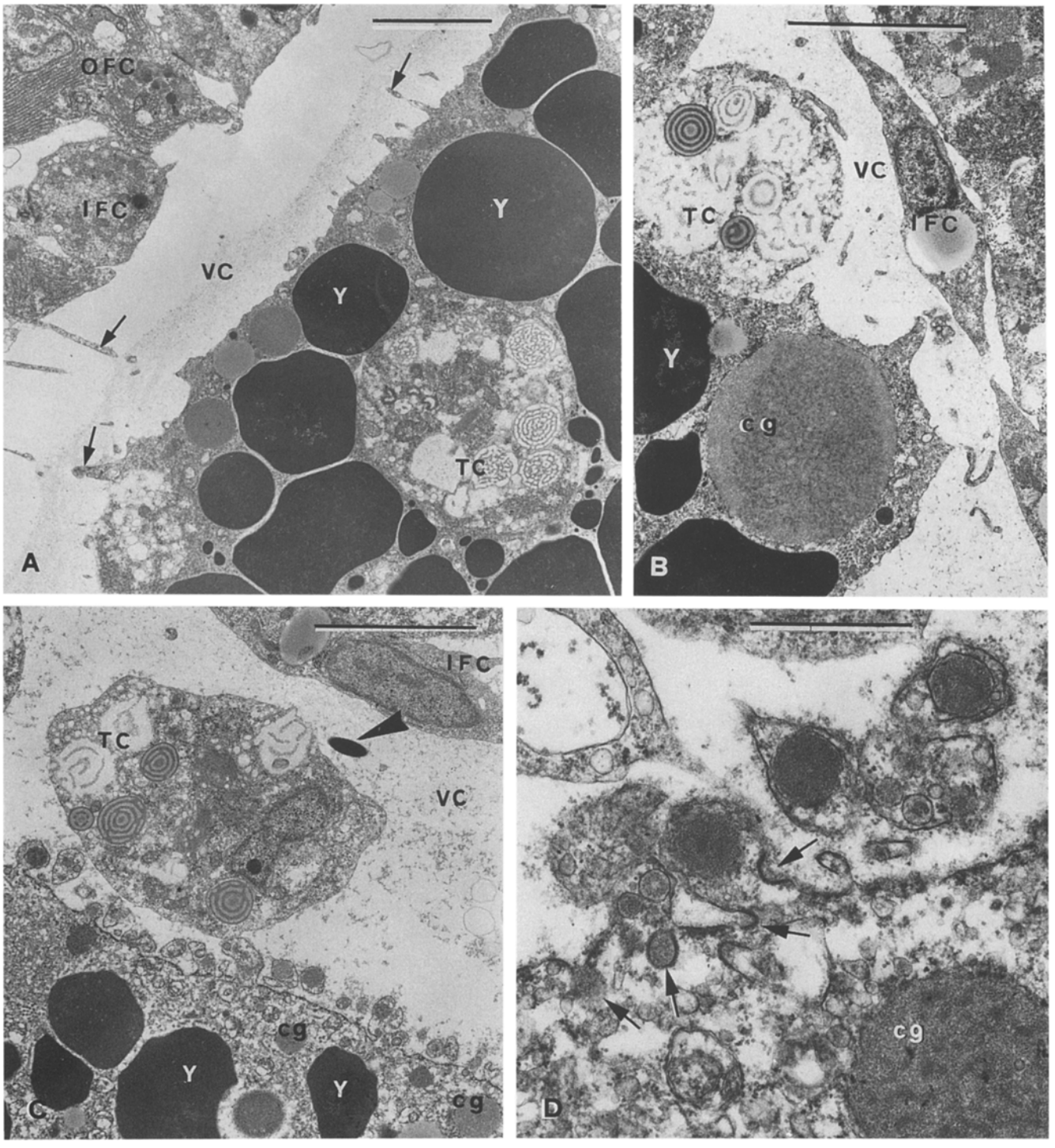

Fig. 4. *Diplosoma listerianum.* (A), (B) Full-grown oocytes before meeting with sperm. (A) Test cells (TC) are encased in oocyte showing granules different in size and density often containing concentric lamellae [see also (B) and (C)]. Only a few protrusions of oocyte (arrows) penetrate vitelline coat (VC). Outer (OFC) and inner (IFC) follicle cells are seen. Y: yolk granules. (B) One test cell protrudes toward vitelline coat and large cortical granule (cg) with coarse and fine granular content is next the oolemma. (C), (D) Presumptive cortical reaction in egg met by sperm. Numerous cytoplasmic protrusions extend from egg, often containing round granules with content looking like that of cortical granules. Note frequent aspects of endocytotic pits and vesicles (arrows). In (C), test cells are in perivitelline space and have secreting granules with dispersed content. One sperm (arrowhead) is kept by vitelline coat fibrils. Scale bars in (A)–(C) = 5 μm; in (D) = 1 μm

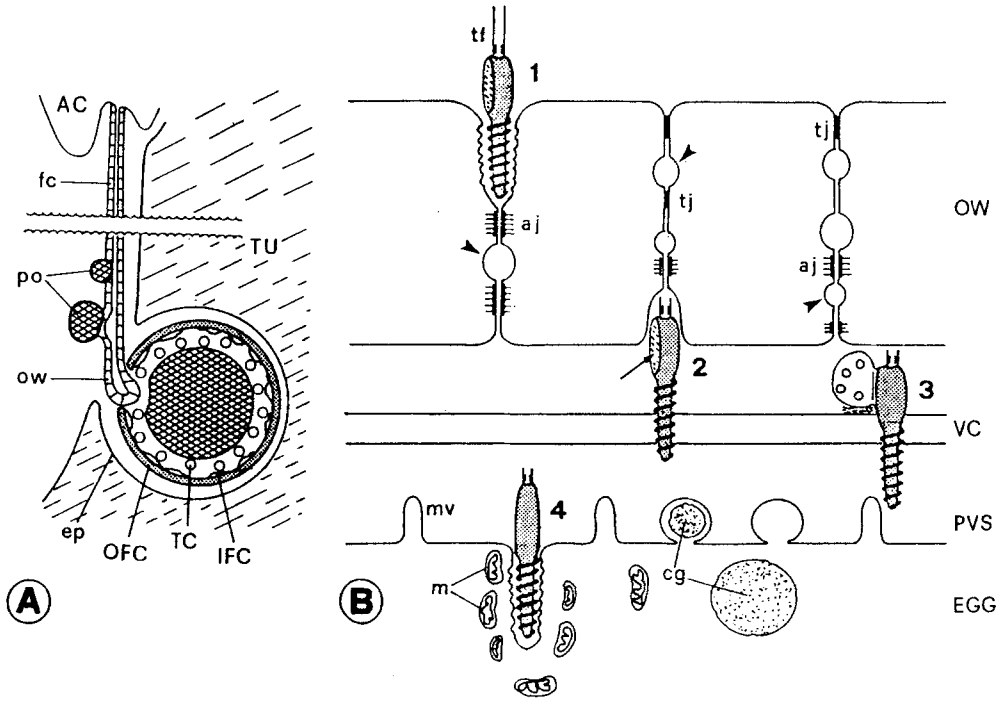

Fig. 5. Schematic drawing of (A) female gonad and (B) internal fertilization in *Diplosoma listerianum*. (A) Relationships between full grown egg protruding into tunic (TU) and ovary wall (ow) with its fertilization canal (fc) open in atrial chamber (AC). Note that ovary wall approaches egg going beyond outer (OFC) and inner (IFC) follicle cells. Test cells (TC) are located in perivitelline space. Sperm and amoebocytes in ovary lumen and vitelline coat are omitted. ep: epidermis; po: previtellogenic oocyte. (B) Several steps of sperm penetration through ovary wall (OW) and vitelline coat (VC) and sperm-egg contact. (1) Corkscrew sperm head insinuates itself between intercellular spaces, overcoming tight junctions (tj), adhering junctions (aj) and sac-like zones (arrowheads); (2) sperm, still equipped with remnants of mitochondrion and endoplasmic tubules (arrow), leaves ovary wall and meets VC; (3) sperm crosses VC and discharges remnants of cell organelles entering perivitelline space (PVS); (4) sperm penetrates egg, beginning at tip of corkscrew head. Oolemma of engulfing pocket outlines spirals of head. Microvilli (mv) and cortical granules (cg) shown at periphery of egg. m: mitochondria; tf: sperm tail flagellum

Sperm crossing ovary epithelium and VC barriers

Our data give evidence that sperm cross the ovary epithelium by means of an original mechanism. First, they present their corkscrew-like heads at the point where contiguous cells are bound by junctions and begin to infiltrate, winding into the intercellular spaces. Probably the coiled head strongly help sperm during penetration, but junctions also appear very mouldable, so as to create intercellular sac-like compartments and facilitate sperm progress. The mechanism recalls that suggested for the testis of vertebrates (Dym and Fawcett 1970, Russell and Peterson 1985), in which the modulation of tight junctions serves to create intercellular compartments so that the blood-testis barrier is maintained while germ cells move into the epithelium towards the lumen. In *Diplosoma listerianum*, a comparable mechanism with intercellular compartments at times communicating with each other or at times sealed may maintain the barrier between the ovary lumen and the perivitelline space, while the ovary epithelium is crossed by sperm actively moving towards the VC.

So, in *Diplosoma listerianum* the pathway of sperm towards the egg appears to be long, complicated and strongly selective. The first step in selection must lie in approaching and entering the narrow opening of the fertilization canal, under attraction of chemical messages that, as in other ascidians (Reverberi 1971, Miller 1982), the egg could release. The path followed by metamorphosed sperm along the ovary lumen, controlled by the wandering phagocytes, is also strongly selective. Selection may also occur during the passage through the ovary epithelium, so that only a limited number of sperm are expected to reach the VC.

In other ascidians, selection of sperm may be made by the IFC, which are considered capable of blocking heterologous sperm (Patricolo and Villa 1992), but it has also been demonstrated that contact with the VC is an obligatory, determinative step in fertilization (Rosati and De Santis 1978, Monroy and Rosati 1983, Honegger 1986, De Santis and Pinto 1988, Fukumoto 1990b, Koch and Lambert 1990). Sperm which pass through the clefts between the IFC find sites for species-specific recognition and binding on the VC (Rosati 1985). Sperm may then cross the VC by means of a mechanism which involves anchorage of the mitochondrion to the VC fibrils and its later translocation along the tail to drive the sperm head into the egg (Lambert and Lambert 1983, Lambert and Koch 1988).

In *Diplosoma listerianum* as well, the VC may play an important role. At its level a further step towards sperm modification occurs, as revealed by the swelling cytoplasmic organelles, and this may correspond to the final phase of the ascidian sperm reaction. In *D. listerianum*,

selection of heterologous sperm possibly occurs during previous stages, before they reach the VC. So the VC, like that of other ascidians (Rosati and De Santis 1978, Fuke 1983, Kawamura et al. 1987), may be able to recognize homologous sperm and impede self-fertilization, which is reduced in this species (Ryland and Bishop 1990). The possibility that at the VC level an acrosomal reaction occurs has been repeatedly discussed (Fukumoto 1990b, for review), but in *D. listerianum* we did not find signs of such a reaction which, considering the very small size of the acrosome, if present at all, (Burighel et al. 1985) must be very reduced. In other ascidian sperm, evidence was found for proteases or glycosidases necessary for fertilization (Woollacott 1977, Hoshi et al. 1981, 1985, Godknecht and Honegger 1991). The sperm of *D. listerianum* retains the ridges of the dense groove until it penetrates the egg. Although these ridges recall the ridge-like surface ornamentations of *Ciona intestinalis* sperm where proteases may be localized (Woollacott 1977), their actual significance remains to be investigated.

Sperm-egg penetration

In solitary species of ascidians the contact between reacted sperm and oolemma has sometimes been reported and it has been seen to occur by fusion of the plasma membranes of the sperm with oolemma, after the sperm acrosomal reaction. In particular, the sperm membranes of the post acrosomal region and the apical processes appear involved in gamete fusion in *Phallusia mammillata* (Honegger 1986) and *in Ciona intestinalis* (Fukumoto 1988, 1990a), respectively. In *Diplosoma listerianum*, the meeting of the two gametes occurs differently, because the sperm initially penetrate a deep cortical invagination of the oolemma. Penetration seems to be an active process for both gametes, as indicated by the close contact between the border of the spiral groove and the oolemma and by the number of mitochondria present in the neighbouring ooplasm. Both when crossing the ovary epithelium and when entering the oocyte, sperm give signs of active penetration. Its presumptive tool is the dense groove which, unlike all the other cytoplasmic constituents, is maintained until the sperm head enters the egg. We have no information on the eventual fate of the dense groove or on the complete penetration of the sperm nucleus into the ooplasm. An interval of time may be required for the steps following sperm entry and this would explain the delay of beginning cleavage after egg segregation observed by Brunetti et al. (1988) and, to a lesser extent, by Ryland and Bishop (1990) in *D. listerianum*.

Cortical reaction

The existence in ascidian eggs of cortical granules and/or a cortical reaction comparable to those of other invertebrates and vertebrates (Longo 1987) has been always denied or questioned. In *Ciona intestinalis*, which lacks cortical granules, Rosati et al. (1977) reported that immediately after fertilization small electron-dense granules appear beneath the oolemma and some of them are extruded into the perivitelline space. Occasionally, also the test cells themselves were considered to be analogous to cortical granules (Guraya 1982).

In *Diplosoma listerianum*, we observed that the test cells are extruded into the perivitelline space before the egg becomes isolated in the tunic. Because in the unfertilized egg they are deeply encased in the oocyte, their sorting must occur with modification of the cortical layer of the egg. In our opinion, this takes place immediately before or contemporaneously with sperm entry into the egg. Indeed, our data, although limited in number, show unequivocally that, when the sperm enters the egg, the test cells are free in the perivitelline space and that the cortical layer displays marked signs of extrusion and endocytosis. This strongly indicates that a noticeable cortical reaction takes place in *D. listerianum* with increase of egg surface area, rearrangement of excess membrane in folds and microvilli and successive removal of membranes by endocytosis as occurs in seaurchins (Fisher and Rebhun 1983). The homogeneous granules scattered in full-grown oocytes and in eggs with penetrating sperm may correspond to the cortical granules of other animals (Longo 1987). Their secretion, together with that of test cell granules, may participate in changing VC characteristics, so as to avoid polyspermy. The capacity of the fertilized egg to release rapidly a factor-blocking polyspermy was demonstrated by Lambert (1986) in a solitary ascidian. Nevertheless, in one case, by means of serial sections of *D. listerianum* we were able to see that the same egg was penetrated by two sperm.

Phylogenetic considerations based on sperm morphology

Comparing different marine groups whose fertilization is external in seawater, Franzen (1956, 1970) introduced the concept of "primitive sperm". This type of sperm has organelles assembled in the antero-posterior sequence: a cap-like acrosome surmounting the ovoid nucleus, a midpiece composed of few rounded mitochondria, two centrioles, and the tail with the typical 9+2 axoneme (Baccetti and Afzelius 1976, Jamieson 1991). Franzen (1956, 1970) claimed that, rather than reflecting phyologenetic relationships, sperm morphology was due mainly to fertilization biology, especially to the mode of sperm transmission. However, Jamieson (1987), considering that the common ground plan characteristics of primitive sperm might be genuinely plesiomorphic for many metazoan groups, termed this "plesiosperm".

The subphylum Tunicata includes the classes Appendicularia, Ascidiacea (suborders Aplouso-, Phlebo- and Stolidobranchia) and Thaliacea (orders Pyrosomatida, Doliolida and Salpida). Tunicata usually possess sperm of a derived type with respect to the primitive one (Jamieson 1991). The sperm of the appendicularian *Oikopleura dioica* (Flood and Afzelius 1978, Holland et al. 1988) is nearest to plesiosperm and appears as the least-derived of all tunicate sperm, in both form and function (Holland 1991). In contrast, the sperm of all other tuni-

cates show: acrosome reduced or absent, nucleus elongated with the mitochondrion lateral to it, absence of mid-piece, presence of distal centriole only, and tail with 9+2 axoneme (Jamieson 1991, for review). Among Ascidiacea and Thaliacea, the least-derived sperm occur in solitary ascidians with external fertilization (Jamieson 1991), while more a complex type is found in colonial forms with internal fertilization such as thaliaceans (Holland 1988, 1990) and aplousobranch ascidians (Burighel et al. 1985, Franzen 1992).

Because coloniality appears several times in the evolutionary branches of Tunicata, as indicated by different modes of asexual reproduction, it is reasonable that sperm morphology varies as an adaptation to differently evolved reproductive solutions. In our opinion, similarities in sperm morphology between didemnid ascidians (Burighel et al. 1985) and thaliaceans (Holland 1988, 1990) first depend on adaptation of these species to "true" internal fertilization. This is indirectly revealed by the fact that the aplousobranch *Clavelina lepadiformis*, closely related to didemnids but with fertilization in seawater, has "simple" sperm which conforms to the general pattern of the solitary ascidians (Burighel and Martinucci 1991, Franzen 1992).

At the moment, information on the mechanism of internal fertilization in both pyrosomids and salps is too scanty to permit comparison with *Diplosoma listerianum*. The three taxa have sperm with spiralled head, an adaptive solution that presumably improves sperm mobility along narrow oviducts and/or epithelial barriers. It would also be of interest to know if the mechanism of sperm-egg approach and egg penetration in these thaliaceans corresponds with that which we observed in *D. listerianum*.

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