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Sexual reproduction in ascidians:
comparative aspects of oviduct, ovulation and spawning

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ABSTRACT - In the evolution of ascidians different reproductive strategies operated. One of the most important aspects was the passage from external to internal fertilization and then from oviparity to ovoviviparity and viviparity with consequent morpho-functional modifications to organs and gametes. This paper presents a short review on the ovary and oviduct, the plasticity of which had played a critic role in permitting the evolutive changes of sexual reproduction.

INTRODUCTION

Ascidians are hermaphroditic sessile marine animals which include both solitary and colonial forms and which show a variety of adaptive solutions in sexual reproduction. Oviparous, ovoviviparous and viviparous species are present, and various morphologies of female and male gonads and gametes, kinds of fertilization and development of accessory structures for embryo retention have developed during the evolution of these animals (for reviews, see Berrill, 1975; Kessel, 1983; Cloney, 1990; Sabbadin *et al.*, 1992).

The aim of this paper is to focus attention on the morphological and functional adaptations of the oviduct, the consequent variability in mechanism of ovulation and spawning, and the involvement of the oviduct in the formation of structures for embryo brooding.

ENTEROGONIDS AND PLEUROGONIDS

On the basis of the position of the gonads, ascidians are divided into Enterogona (suborders Aplousobranchia and Phlebobranchia) and Pleurogona (suborder Stolidobranchia), two natural groups which are assumed to be evolved independently (Kott, 1985, 1990, 1992; Wada *et al.*, 1992). In Enterogona, the gonads lie inside or behind the intestinal loop; in Pleurogona, they are found far from the gut, inside the mantle.

In the Enterogona the gonads are sited far from the atrial cavity and siphon. A single long sperm duct and an oviduct open near the anus, at the base of the atrial siphon. Conversely, in Pleurogonids the gonads tend to become bilateral and to subdivide into several units, better contained within the thickness of the mantle. The subdivision of the gonad and its nearness to the atrial siphon lead the single gonadal units to open independently into the atrial cavity. The process of subdivision reaches the extreme case in the Pleurogonid compound forms with small zooids, such as the Botryllinae, where the reduced size of zooids, paralleled by increased egg size, results in reduction in size of the ovaries, which contain only a few functional ova. For example, every zooid of *Botryllus schlosseri*, starting from a small gonadic blastema, matures very few oocytes and each one,

with its envelopes and its own oviduct, may be considered as a "small ovary" (Berrill, 1950; Sabbadin and Zaniolo, 1979; Sabbadin *et al.*, 1992).
With a few exceptions, all Aplousobranchia are colonial, while Phlebobranchia and Stolidobranchia include both solitary and colonial forms.

OVARY AND EGG ORGANIZATION

The solitary oviparous *Ciona intestinalis* (Aplousobranchia), is considered a species with primitive characteristics (Kott, 1990). Its ovary is a pear-shaped body lying in the intestinal loop and
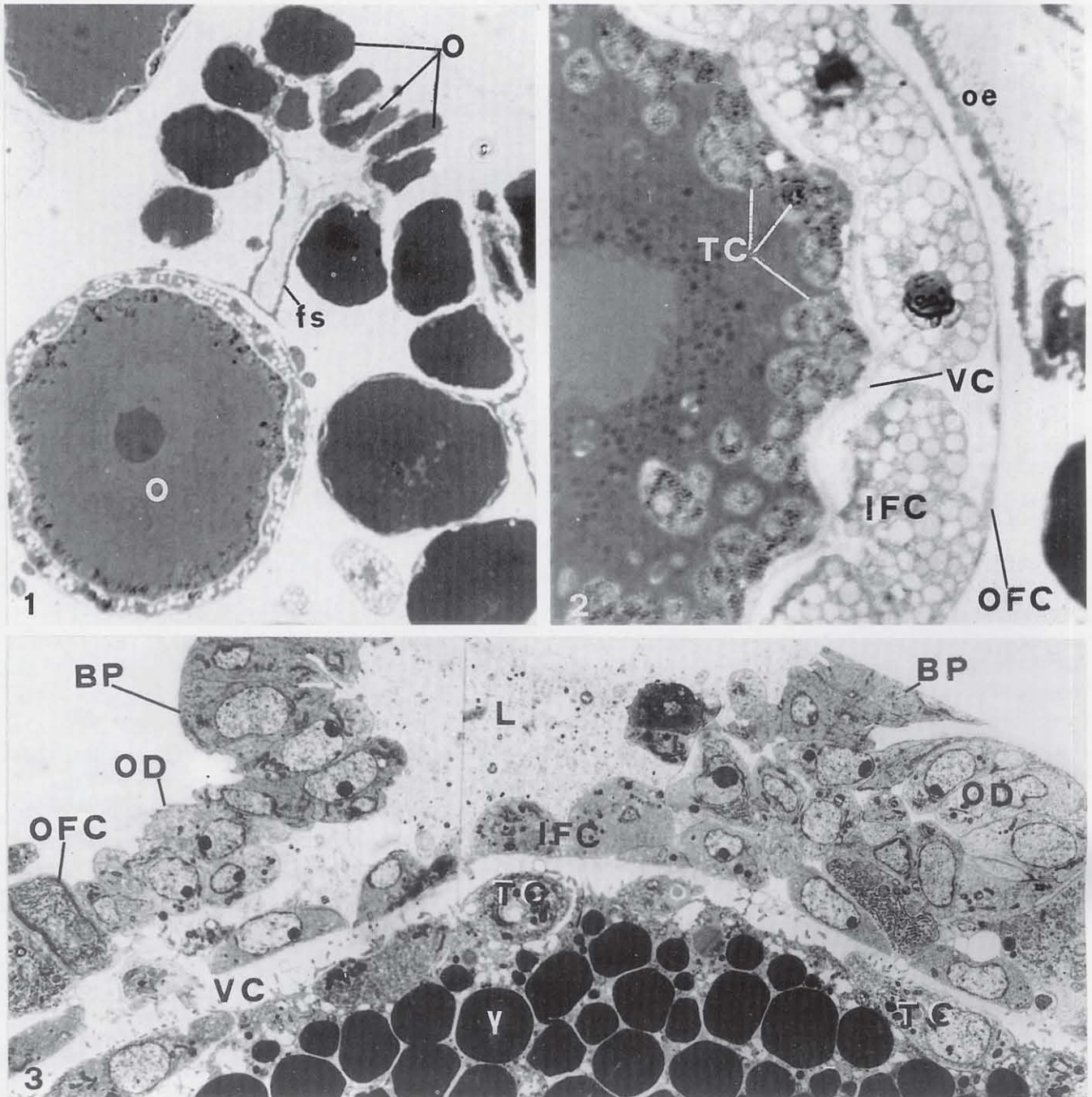
Figs. 1, 2. Ovary of *Ciona intestinalis*. Thick sections. Fig. 1. A cluster of oocytes (**O**) at different developmental stages protrude from germinal epithelium. Fig. 2. Detail of a vitellogenetic egg and its envelopes. Note thin outer follicle cells (**OFC**) with respect to large vacuolated inner follicle cells (**IFC**). **fs**: follicle stalk; **oe**: ciliated ovary epithelium; **TC**: test cells; **VC**: vitelline coat. Toluidine blue. Fig. 1: x 300; Fig.2: x 1200.

Fig. 3. Botrylloides leachi electronmicrograph. At ovulation small oviduct (**OD**) (cfr. Fig. 9) opens after fusion with outer follicle cells (**OFC**) and brood pouch epithelium (**BP**), so that egg, enveloped by test cells (**TC**) and inner follicle cells (**IFC**), penetrates original oviducal lumen (**L**). **Y**: yolk; **OD**: vitelline coat. x 6000.

anteriorly continuous with the oviduct, running forward to the dorsal side of the intestine. The ovary possesses a branched ovarian cavity, defined by a folded epithelium bearing clusters of oocytes at various stages of oogenesis (Fig. 1). In the central areas of the ovary the epithelium is ciliated, like the wall of the oviduct. During oogenesis, oocytes enlarge and protrude from the germinal epithelium into the ovarian stroma (the mesenchymal tissue comprised between the germinal epithelium and the external wall of the ovary), but always remain connected to the germinal epithelium by narrow tubular structures called follicle stalks (Millar, 1953, Sugino *et al.,* 1987, 1990). The ripe egg possesses a differentiated complex of ovular envelope that, in *Ciona*, as in general in other solitary ascidians, is composed of test cells encased in a superficial depression of the oocyte, the acellular vitelline coat, a layer of high and vacuolated inner follicle cells, and a layer of squamous outer follicle cells connecting the egg to the follicle stalk (Figs. 1, 2) (see Kessel, 1983).

The situation of the female gonad seen in *Ciona intestinalis* can be generalized to many ascidians (Tucker, 1942; Kessel and Kemp, 1962; Ermak, 1976; Mukai 1977a; Mukai *et al.,* 1983; Sugino *et al.,* 1987, 1990; Kott, 1990) but, as mentioned above, the variety in reproductive strategies has necessarily involved changes in the gonoducts. So, in some species of the compound styelids (Stolidobranchia) the oviduct may be non-ciliated (e.g. in *Symplegma reptans* and *Symplegma japonica*) and its aperture in the atrial cavity may be simple (*Symplegma reptans*), complexly lobated (*Metandrocarpa uedai*), or surrounded by a "flaring pavilion" as in *Distomus variolosus* (Newberry, 1968; Mukai and Watanabe, 1989). In *Polyandrocarpa misakiensis* (Mukai and Watanabe, 1989) the oviduct is so reduced that the ovarian cavity itself acts as an oviduct. Short oviducts opening near the atrial opening (as in Plurellidae - Phlebobranchia) have a possible advantage in ensuring rapid release of ova in sea-water (Kott, 1992).

Most of the solitary ascidians produce a great number of relatively small eggs like those of *Ciona intestinalis*, but colonial species produce large yolked eggs with flat inner follicle cells and highly developed outer follicle cells (Zaniolo *et al.,* 1987; Martinucci *et al.,* 1988; Manni *et al.,* 1993, 1994). However, despite the differences in the female gametes, ovulation, i.e. the process of expelling the mature oocytes from the rupturing ovarian follicle into the oviduct, follows a common pattern in most ascidians as regards ovular envelopes: the oocyte is shed and passes through the follicle stalk, accompanied by the inner follicle cells, while the outer follicle cells remains *in situ* (Figs. 3, 5, 6, 8, 9). An exception to this pattern, as in the Aplousobranch Didemnidae (Fig. 10) (see below), may be seen as an extreme specialization. In *Halocynthia roretzi*, Sakairi and Shirai (1991) have shown that a signal, probably produced by follicle cells, triggers the germinal vesicle breakdown of the oocyte, vitelline coat elevation, test cells scattering and, shortly thereafter, ovulation.

Among Enterogona, matured eggs are ovulated one by one and masses of eggs are stored in the large oviduct before they are spawned. At the time of spawning all gametes in the gonoduct are released, probably as a result of tissue contraction (Woollacott, 1974). In contrast, most stolidobranchs have no such storage gonoducts and the eggs are released directly from the gonad (Svane and Young, 1989; Sakairi and Shirai, 1991). Many ascidians spawn shortly after exposure to light (Mukai and Watanabe, 1978; Svane and Young, 1989).

OVIDUCT, OVOVIVIPARITY AND BROODING

In many ascidians, as in Polyzoinae and some species of *Molgula* (all Stolidobranchia), the gonoducts open far from the atrial aperture and the oviducts are sometimes turned ventrally away from the atrial aperture. These adaptations may reflect a tendency toward ovoviviparity and viviparity, ensuring retention of eggs in the atrial cavity. In *Pyura littoralis* (Stolidobranchia) and no other known species the female duct is subdivided into numerous short openings, and this probably favours retention of eggs in the atrial chamber (Kott, 1985).

Fertilization occurs externally in oviparous species, whereas ovoviviparous and viviparous ascidians have internal fertilization which occurs, according to the species, in different zones of the parent, such as specialized brood pouch, proximal end or top of the oviduct, or atrial chamber. Internal fertilization is almost universal in colonial species. Its adaption required changes both on female and male gametes and, once it has evolved, it appears
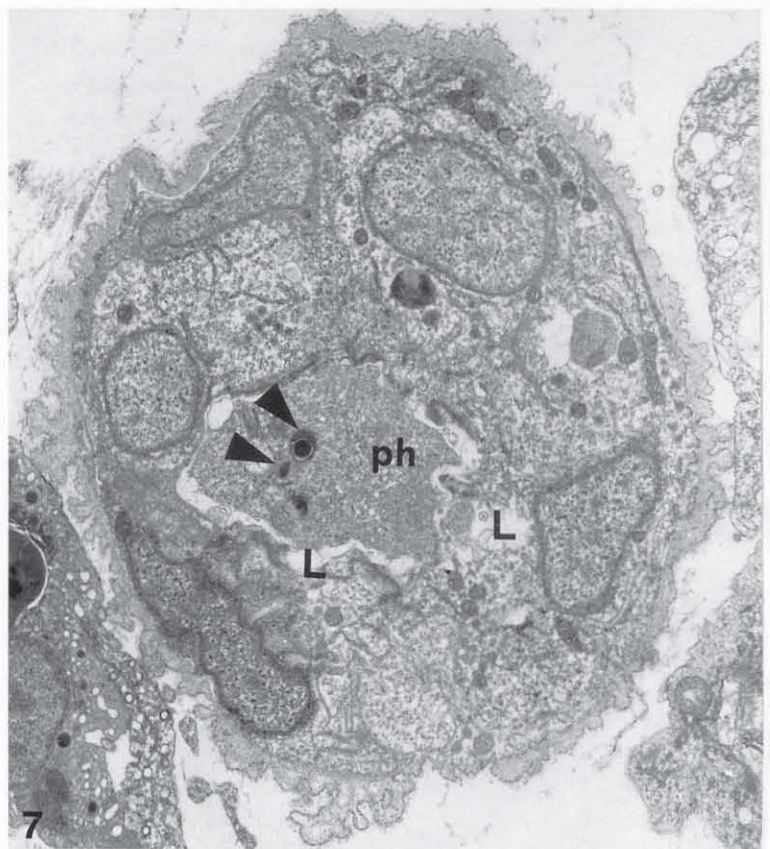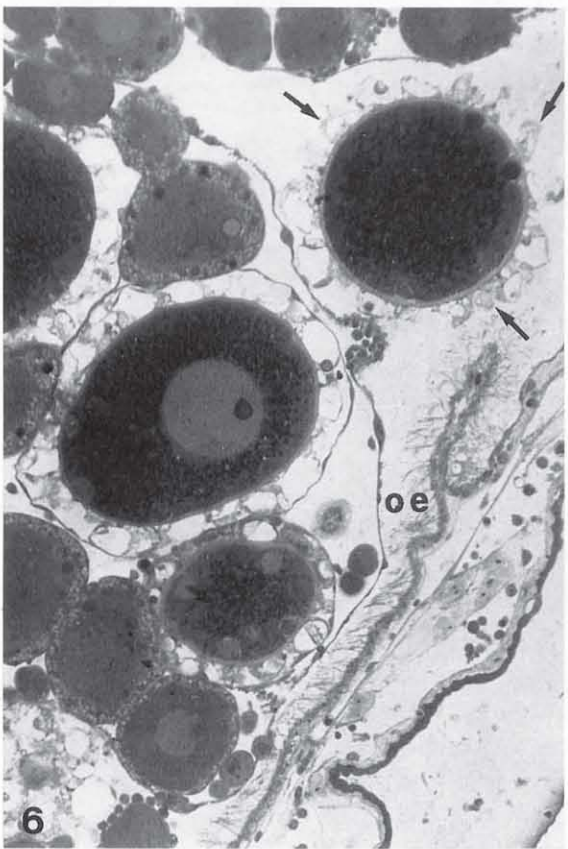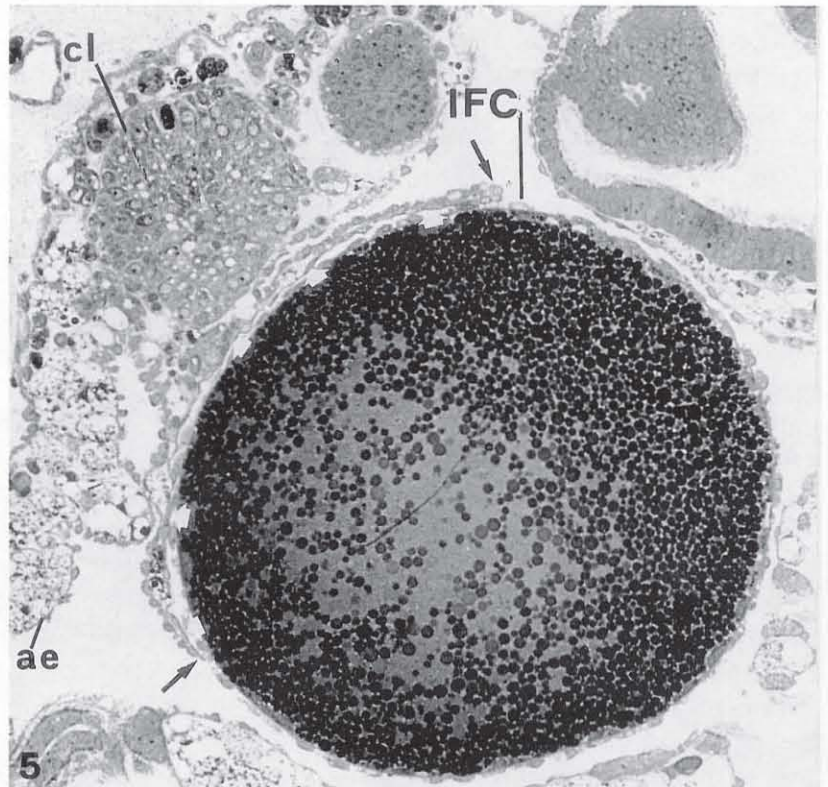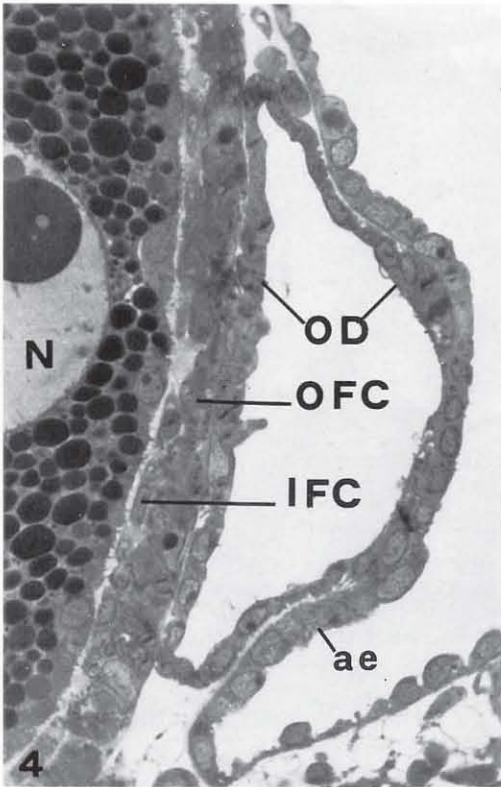
never to be lost (Kott, 1992). In most ascidians, sperm are "simple" and tailed with oval nucleus flanked by a single mitochondrion, although some species which have developed internal fertilization have also evolved more specialized sperm (Burighel et al., 1985; Jamieson, 1991).

Moreover, a considerable morphological modification of the oviduct is reached in the compound Botryllinae species (Stolidobranchia) in which the ovarian epithelium is absent and, as mentioned above, isolated large ova mature in the mantle. These eggs are attached individually to the atrial epithelium by way of an oviduct reduced to a small vesicle, which is not continuous with the outer follicle cells and does not communicate with the atrial chamber (Figs. 4, 8, 9) (Mukai, 1977b; Zaniolo et al., 1987). This oviduct, also called follicle stalk, is homologous to the follicle stalk of other Styelid ascidians (Mukai and Watanabe, 1976). At ovulation, the egg enters the brood pouch, an outgrowth of the atrial epithelium, through the short oviduct. The ultrastructure of this process, studied in Botrylloides leachi (Zaniolo et al., 1994), shows that the oviductal epithelium fuses with outer follicle cells and brood pouch epithelium at opposite sides and then breaks, opening a temporary canal (Fig. 3). Oviducal cells and outer follicle cells are discharged into the mantle and the egg is fertilized in the brood pouch (Fig. 9). So the function of this small vesicle seems to regulate the contemporaneous opening of brood pouch epithelia and outer follicle cells and exposes the egg to sea-water, separating sea-water coming from the atrial chamber from blood circulating in the mantle.

In Botryllus schlosseri the vesicular oviduct plays an additional role (Mukai, 1977b; Zaniolo et al., 1987) because, not only as in Botrylloides leachi drives the egg into the atrial chamber, but also because it takes part in the formation of a placental cup for embryo retention (Figs. 4, 5, 8). In

this species, the placental cup has a double wall: an external one derived from the atrial epithelium, and an internal one derived from the oviducal epithelium (Zaniolo et al., 1987). The main role of this "placenta" is to attach the embryo to the parent, thus exposing it to the sea-water flow in the atrial chamber (Fig. 5).

In several ascidians, the oviduct is specialized for incubation of embryos as, for example, in Placentelidae (Aplousobranchia), in which a pouch forms at the distal end of the oviduct where the eggs are fertilized and brooded. Moreover, in other ascidians many embryos in a marked developmental sequence, from zygotes to larvae, are found in the oviduct (see Ryland and Bishop 1993). Brewin (1956) has carefully described the development of the oviducal brood pouch in the viviparous Hypsistozoa fasmeriana (Aplousobranchia). In this species, a week or more before the egg leaves the ovary, the oviduct develops distally a short hairpin bend which projects outwards, forming a sac-like brood pouch. The lining cells of the brood pouch modify, assuring the passage of nutrients for the embryo, which retains its connexion with the parent zooid for approximately 5 months.

An interesting functional modification of the oviduct occurs in the family Didemnidae (Aplousobranchia) in which embryos are incubated free in the tunic. The particular mechanism of ovulation has been analyzed in detail in Diplosoma listerianum (Burighel et al.,1987; Martinucci et al., 1988). In this species the growing egg, herniating from the ovary wall, presses on the overlying epidermis and causes it to evert into a sac which receives the egg (Fig. 10). At ovulation, outer and inner follicle cells are discharged and remain in situ, but fertilization occurs before the egg leaves the ovary and segregates inside the tunic, surrounded by epidermis. Thus, the egg does not pass along the oviduct, is never exposed to sea-water and a true internal fertilization takes

■ Fig. 4. Thick section of a mature oocyte (left) and its vesicular oviduct (**OD**) in Botryllus schlosseri (cfr. Fig. 8). Oviduct possesses flat epithelium contacting both developed outer follicle cells (**OFC**) and atrial epithelium (**ae**). **IFC**: inner follicle cells; **N**: nucleus. Toluidine blue. x 950.

Fig. 5. Thick section showing egg of Botryllus schlosseri just after ovulation (cfr. Fig. 8), enveloped by inner follicle cells (**IFC**) and retained in atrial chamber by means of placental cup (edges indicated by arrows). Outer follicle cells discharged during ovulation form corpus luteum (**cl**) in mantle. White arrowheads: internal wall of placental cup derived from oviducal epithelium. **ae**: atrial epithelium. Toluidine blue. x 300.

Fig. 6. Thick section of ovary of Molgula socialis. Oocytes at various stages of development in ovarian stroma. Note just ovulated egg externally covered by inner follicle cells (arrows) in ovary lumen. **oe**: ciliated ovary epithelium. Toluidine blue. x 250.

Fig. 7. Cross-section of fertilization canal of Diplosoma listerianum. Note in lumen (**L**) a phagocyte (**ph**) containing sperm in regression (arrowheads). x 7000.
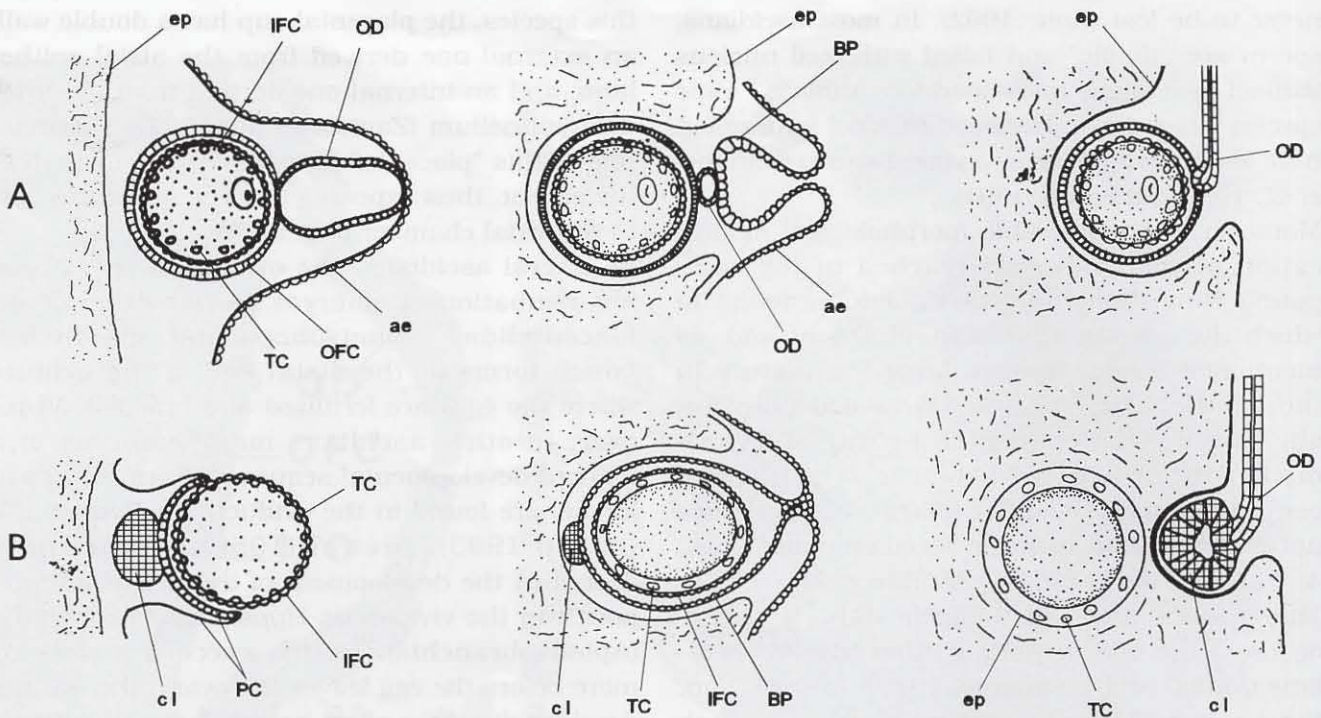
Figs. 8-10. Ovaries of *Botryllus schlosseri* (Fig. 8), *Botrylloides leachi* (Fig. 9) and *Diplosoma listerianum* (Fig. 10) just before (A) and after (B) ovulation. In all cases outer follicle cells (**OFC**) are discharged to form a sort of *corpus luteum* (**cl**). In *Botryllus schlosseri* and *Botrylloides leachi* egg passes through oviduct (**OD**) which, in the former, gives part of placental cup (**PC**). In *Botrylloides leachi*, egg is eventually contained in brood pouch (**BP**). In *Diplosoma listerianum*, egg is not shed through oviduct but, after discharge of outer and inner follicle cells, is segregated in tunic surrounded by epidermis. Fertilization occurs by sperm from oviduct. **ae**: atrial epithelium; **ep**: epidermis; **IFC**: inner follicle cells; **TC**: test cells.

place. This means that the extension of the ovary communicating with the atrial cavity has lost the function of driving eggs externally and acquires the significance of a fertilization canal (Figs. 7, 10). A controlled environment where sperm reaction occurs is created in the lumen of the ovary. Sperm, with modified features with respect to those present in the male duct, are stored in the lumen of the ovary and must fertilize eggs at the bottom of the ovary after crossing the ovary wall (Burighel and Martinucci, 1994a, b). Storage of exogenous sperm, which remain viable for a long time in the ovary before undergoing regression (Fig. 7), has been proved (Bishop and Ryland, 1991; Ryland and Bishop, 1993; Burighel and Martinucci, 1994a,b).

CONCLUSIONS

The presumptive evolution from oviparity to ovoviviparity and viviparity in ascidians is accompanied by reduction in number and increase in size of eggs per zooid, passage from external to internal fertilization, important changes in sperm morphology, and also pecu-

liar oviduct specializations. The shortening of the oviduct, which moves away from the cloacal siphon and opens at the base of the atrial chamber, favoured the keeping and subsequent brooding of embryos. But the oviduct also underwent modifications, partly or completely forming sacs for embryo brooding and, in some cases, differentiating its epithelium for the placental function of prolonged nourishment of the embryos. Moreover, in didemnids the oviduct lost its original function of driving eggs outside, assuming its new function as fertilization canal for storage and/or driving of sperm to egg before its segregation. All the above data shown that the plasticity of the ovary and oviduct have played an important and successful role in sexual reproductive strategies in ascidians.

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