An ultrastructural study of spermatogenesis and the mature spermatozoon of the ascidian *Pyura stolonifera*

R. Biseswar* and T. Maslamoney
Department of Zoology, University of Durban-Westville, Private Bag X54001, Durban, 4000 Republic of South Africa

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In *Pyura stolonifera*, there is an orderly arrangement of the male germ cells within the testicular follicles. Cells in early stages are located at the outer surface of the follicle while those in later stages of differentiation, including mature spermatozoa, lie nearer to the lumen. Developing spermatocytes are connected by cytoplasmic bridges which persist up to the late spermatid stage. The cytoplasmic bridge is reinforced on each side by an osmiophilic layer. Microtubules appear in the cytoplasm around the outside of the nuclear membrane in the mid-spermatid stage. The mature spermatozoon consists of an elongate, spindle-shaped head and a long tail. The head, which is about 9 μm in length, is slightly curved and tapers anteriorly. It comprises an elongate, electron-dense nucleus, a single large mitochondrion and a thin layer of cytoplasm. There is no middle piece. The mitochondrion is disposed lateral to the nucleus. Granules (presumably glycogen) occur in the cytoplasm between the nucleus and the mitochondrion. Neither an acrosome nor any other structure corresponding to it was observed in sections of the apical region. A 'fuzzy' material, however, surrounds the apex. The tail is about 40 μm in length and 0,2 μm in diameter. The axoneme has the 9 + 2 arrangement of microtubules.

In *Pyura stolonifera* is daar 'n reëlmatige rangskikking van manlike kiemselle in die testisfollikels. Selle in die vroeë fase is op die buite oppervlak van die follikels teenwoordig terwyl hierdie selle in later fases van differensiasie, insluitend ryp spermatozoa, nader aan die lumen gelee is. Ontwikkelende spermatosiete is deur sitoplasmiese brûe verbind tot by die laat spermatiedfase. Die sitoplasmiese brûe is versterk aan elke kant deur 'n osmiofiliese laag. Mikrotubules verskyn in die sitoplasma aan die buitekant van die kernmembraan gedurende die mid-spermatied stadium. Die volwasse spermatozoon bestaan uit 'n spoelvormige kop en 'n lang stert. Die kop, wat 9 μm lank is, is gebuig en na voor gespits. Dit bestaan uit 'n verlengde, elektrondigte kern, een groot mitochondrion en 'n dun laag sitoplasma. Daar is geen middelstuk nie. Die mitochondrion is lateraal ten opsigte van die kern, en korrels kom voor wat vermoedelik glikogeen is en in die sitoplasma tussen die kern en die mitochondrion voorkom. Geen akrosoom of enige ander struktuur wat daarmee ooreenstem is waargeneem in seksies van die voorpunt van die sperm. Ongedifferensieerde materiaal omsingel egter die voorpunt. Die stert is ongeveer 40 μm lank en 0,2 μm breed. Die aksoneem het 'n 9 + 2 rangskikking van mikrotubules.

* To whom correspondence should be addressed

Although several investigators have reported on the process of spermatogenesis and the morphology of the mature sperm in various species of ascidians (Schabtach & Ursprung 1965; Franzén 1976; Woollacott 1977; Kubo, Ishikawa & Numakunai 1978; Cloney & Abbott 1980; Cotelli, De Santis, Rosati & Monroy 1980; Fukumoto 1981; 1983; 1985; 1986; 1988; Villa 1981; Villa & Tripepi 1982; 1983; Jamieson 1991), no such studies have been undertaken on *Pyura stolonifera*. *Pyura stolonifera* is a dominant species in the infratidal fringe of the rocky shores of southern Africa extending to a depth of about 10 metres.

It is evident from the literature that some of the ultrastructural components of the mature sperm of ascidians need further investigation. One of the controversial issues that still exists is whether an acrosome or its equivalent structure is present. Furthermore, a better understanding of the reproductive biology of *P. stolonifera* is essential as this species is exploited as bait by fishermen.

In this paper, sperm differentiation and the ultrastructure of the mature spermatozoon of *P. stolonifera* are described. The results are discussed and compared with other species of ascidians.

Materials and Methods

Specimens were collected intertidally from the rocky shores of Isipingo Beach (29°05'S / 20°56'E), on the Natal coast during February, May and June 1987. In the laboratory, the specimens were removed from the tests and dissected to expose the gonads.

For transmission electron microscopy, small pieces of the gonads were fixed in 3% phosphate-buffered glutaraldehyde at 4°C and left overnight. After washing in phosphate buffer (pH 7,2), small pieces of tissue were post-fixed in 1,0% osmium tetroxide for one hour, dehydrated and embedded in Spurr's resin. Thin sections were cut on a glass knife, stained with uranyl acetate and lead citrate and examined with a Philips TEM 301.

For scanning electron microscopy, sperm were obtained by rupturing pieces of gonoducts and gonads. After gentle centrifugation of the mixture (sea-water and sperm), the sperm were fixed in cold 3% glutaraldehyde in sea-water for 1 hour. Sperm smears were prepared on glass coverslips, air-dried and dehydrated in a graded series of ethanol. The coverslips were then coated with gold and examined with a Philips SEM 300.

Results

Gonads

Pyura stolonifera has two large gonads, each of which is divided into a variable number of polycarps. Each polycarp

contains the testis and the ovary. The testis consists of numerous spherical follicles containing male germ cells in various stages of development (Figures 1 & 2).

Spermatogenesis

The male germ cells are arranged circumferentially in the lumen of the testicular follicles with cells in early stages of development being located close to the wall of the follicle. As these cells develop, divide and mature they move towards the inner region. Thus there is a developmental sequence that proceeds roughly from the outer edge of the follicle to the inner cells where the completed, mature spermatozoa are found (Figures 1 & 2). No morphological differences between the spermatozoa in the lumen of the follicles and in the gonoducts were observed.

Spermatocytes

The earliest germ cells seen were the primary spermatocytes, located close to the wall of the testicular follicles. They are spherical to somewhat oval cells with large nuclei (Figure 3). A Golgi apparatus and several vesicles are present in the cytoplasm. The vesicles tend to be confined in a specific region of the cytoplasm.

The secondary spermatocytes are spherical cells occurring in many clusters (Figures 4 & 5). Several cells within a cluster are connected by means of cytoplasmic bridges (Figures 5 & 6). Each secondary spermatocyte has a large nucleus which contains irregular patches of chromatin and synaptonemal complexes. The diameter of each cell ranges from 2,5 to 3,5 μm and that of the nucleus from 1,8 to 2,5 μm. A few mitochondria and several vesicles are present in the cytoplasm (Figure 5). A pair of centrioles, the proximal and distal centrioles, are arranged orthogonally to one another (Figure 7). The flagellum differentiates precociously from the distal centriole and thus marks the longitudinal axis of the spermatocyte.

Intercellular bridges (Figures 5 & 6) between the spermatocytes persist until the late spermatid stage. The cytoplasmic bridge is reinforced on each side by an osmiophilic layer (Figure 6). The exact number of cells within a cluster could not be ascertained from the study of thin sections or even from the scanning electron micrographs.

Young spermatids

The young spermatids (Figure 8) are much smaller than the preceding spermatocytes. Their diameters range from 0,6 to 1,4 μm. The nuclear material begins to condense in concentric bands. The single elongate mitochondrion, which results from the fusion of several randomly dispersed spermatocyte mitochondria, becomes more or less adjacently aligned to the nucleus. Groups of young spermatids still remain connected by cytoplasmic bridges.

Intermediate spermatids

The intermediate spermatids are elongated cells (Figure 9). In cross-section, microtubules appear in the cytoplasm around the outside of the nuclear membrane. Cytoplasmic bridges continue to connect groups of these cells (Figure 9)

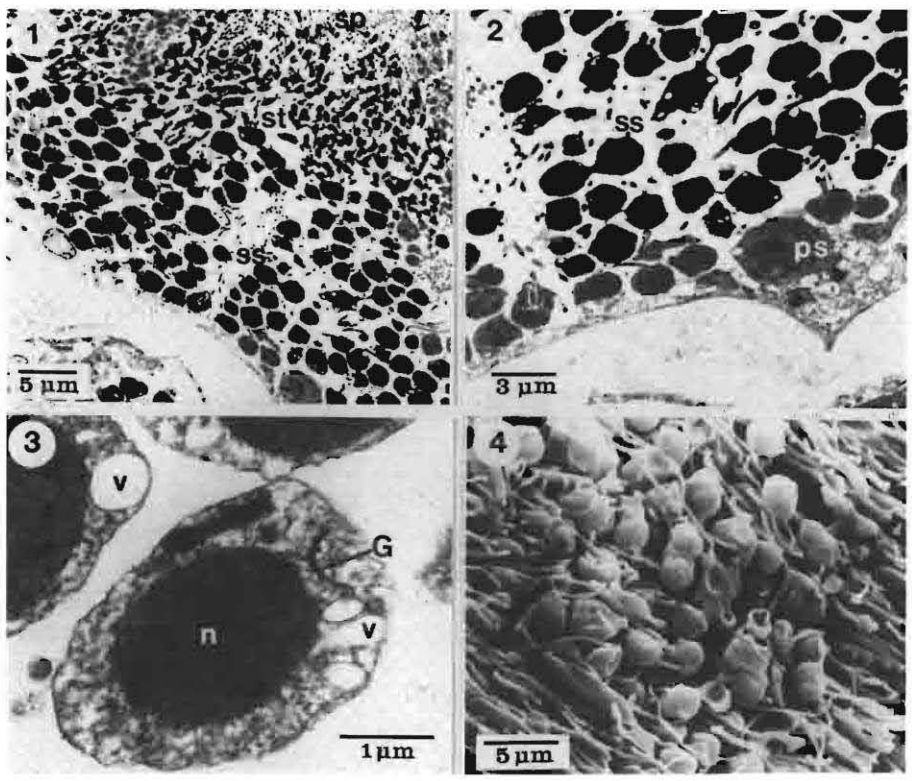
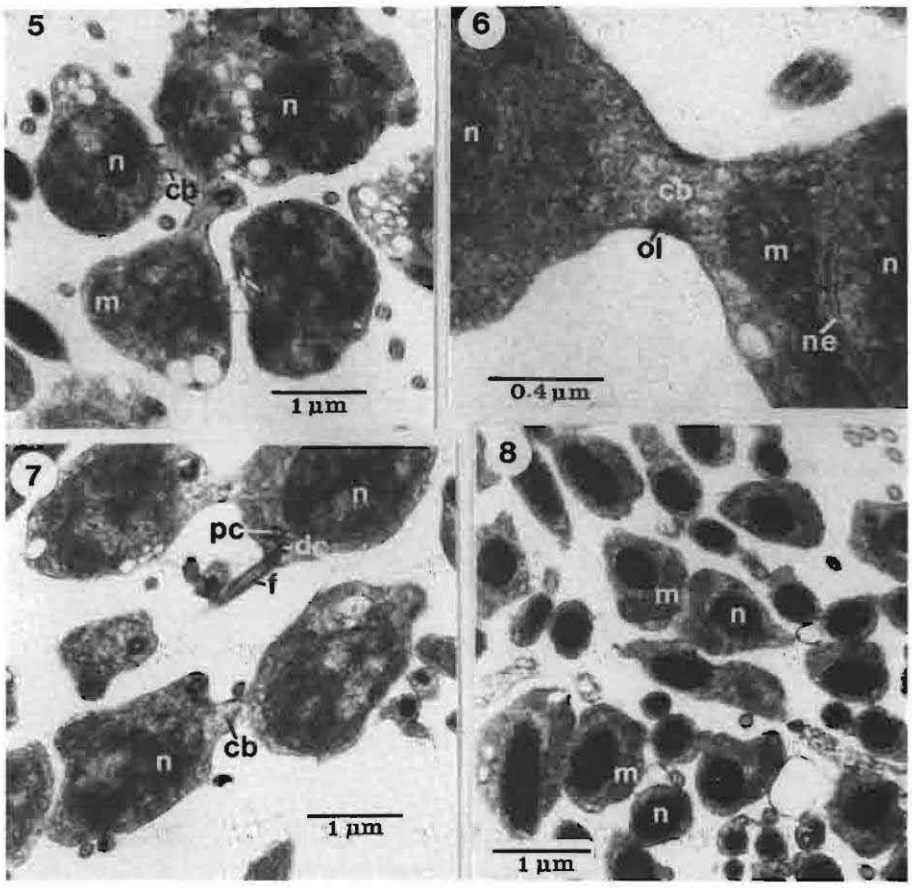
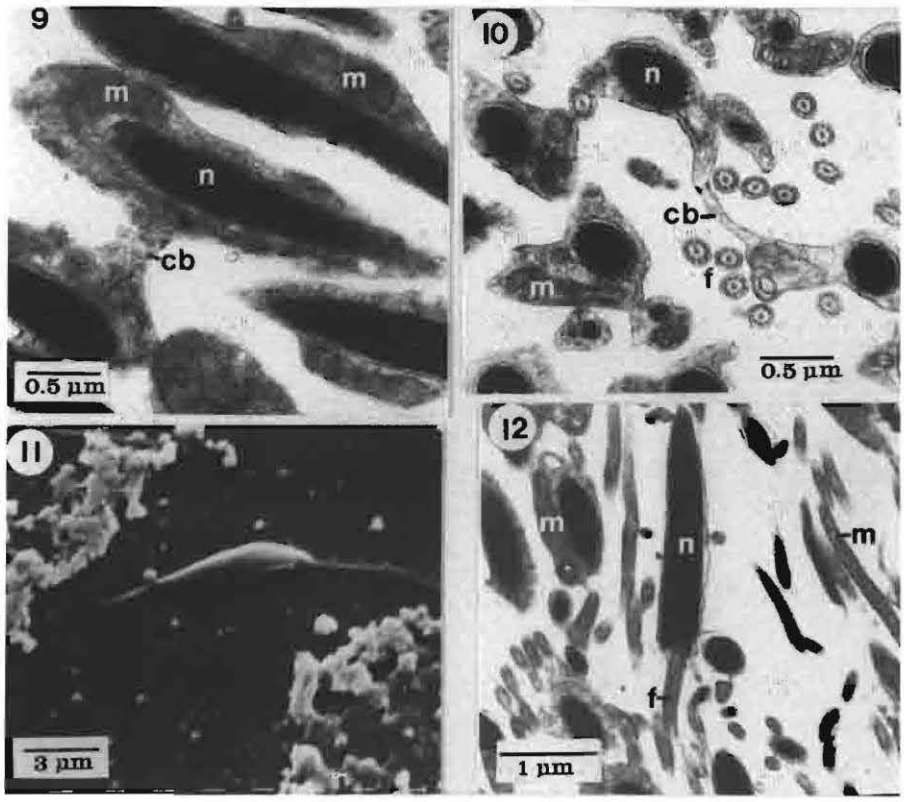


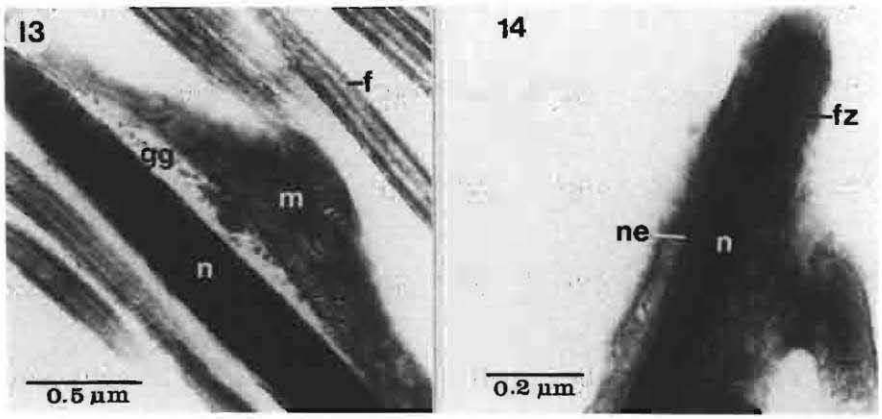
Figure 1–4. **1.** Section through testicular follicle showing germ cells in various stages of development. **2.** Section through testicular follicle showing primary and secondary spermatocytes. **3.** Section through primary spermatocytes. **4.** Scanning electron micrographs of secondary spermatocytes. Abbreviations: (G) Golgi apparatus; (n) nucleus; (ps) primary spermatocyte; (Sp) spermatozoa; (ss) secondary spermatocyte; (st) spermatid.



Figures 5–8. **5.** Section through a cluster of secondary spermatocytes. **6.** Section through a cytoplasmic bridge showing osmiophilic layers. **7.** Section through secondary spermatocytes showing precocious development of a flagellum. **8.** Section through young spermatids showing partially condensed nuclear material. Abbreviations: (cb) cytoplasmic bridge; (dc) distal centriole; (f) flagellum; (m) mitochondrion; (n) nucleus; (ne) nuclear membrane; (ol) osmiophilic layer; (pc) proximal centriole.



Figures 9–12. **9.** Section through intermediate spermatids. **10.** Section through late spermatids showing elongate cytoplasmic bridges. **11.** Scanning electron micrograph of a mature spermatozoon. **12.** Section through mature spermatozoa. Abbreviations: (cb) cytoplasmic bridge; (f) flagellum; (m) mitochondrion; (n) nucleus.



Figures 13–14. **13.** Longitudinal section through a mature spermatozoon. **14.** Longitudinal section through the apical region of a mature spermatozoon. Abbreviations: (f) flagellum; (fz) fuzzy material; (gg) glycogen granules; (m) mitochondrion; (n) nucleus; (ne) nuclear material.

and the osmiophilic layers still persist. The concentric bands of the nuclear material remain unchanged from the previous stage.

Late spermatids

Cytoplasmic bridges are still present in the late spermatids but they are now much narrower and considerably longer (Figure 10). This is probably the phase when the individual spermatids begin to separate from the cluster. Nuclear shaping, initiated in the preceding stage, continues. The nucleus now contains electron-dense material and nuclear condensation appears to be complete.

Mature spermatozoa

Each spermatozoon consists of an elongate, spindle-shaped head and a long tail. The head, which is about 9 µm in length, is slightly curved and tapers anteriorly (Figure 11). The diameter of the head at the extreme anterior end is about 0,1 µm and in the widest portion it measures about 1,3 µm. The head comprises an elongate, electron-dense nucleus, a single, large mitochondrion and a thin layer of cytoplasm (Figures 12 & 13). Granules (presumably glycogen granules) occur in the cytoplasm between the nucleus and the mitochondrion (Figure 13). The mitochondrion is elongate-oval and laterally disposed to the nucleus.

Neither an acrosome nor any other structure corresponding to it was observed in sections of the apical region. A 'fuzzy' material, however, is found around the apex (Figure 14). The axoneme has the 9 + 2 arrangement of microtubules.

Discussion

In *P. stolonifera*, there is an orderly pattern of differentiation of the germ cells, in that, the cells in progressively more advanced stages of development are found towards the centre of the follicles. A similar arrangement of the germ cells has also been reported in *Ciona intestinalis* (Cotelli *et al.* 1980) and *Perophora formosana* (Fukumoto 1981). In several other ascidians, on the other hand, the differentiating germ cells have been reported to be randomly distributed within the testicular follicles (Kubo *et al.* 1978; Villa 1981; Villa & Tripepi 1982).

The presence of intercellular bridges is a characteristic feature of several species of ascidians. It has been stated that the events of differentiation within a cluster appear to be synchronized and that the protoplasmic continuity is the basis for this synchrony. In *P. stolonifera* these intercellular bridges connect groups of spermatocytes and persist until the later spermatid stage. According to Lambert (1982), remnants of these cytoplasmic bridges are present even in spawned, mature spermatozoa of styelid ascidians. The complete absence of these interconnecting structures, however, has been reported in *Molgula impura* and *Styela plicata* (Villa 1981) and in *Ascidia malaca* (Villa & Tripepi 1983).

The mature spermatozoa of *P. stolonifera* show morphological features which are characteristic of ascidian spermatozoa in general. According to Jamieson (1991), the ascidian sperm comprises an elongated nucleus, no acrosome or only a very simple acrosome, a flagellum with the 9 + 2 arrangement of microtubules and a single large mitochondrion located laterally to the nucleus.

In *P. stolonifera*, a Golgi apparatus and numerous vesicles were observed in the spermatocytes and even in the young and intermediate spermatids. The fate of these vesicles, however, could not be traced in later stages of development. As in *Corella parallelogramma* (Franzén 1976); *Ciona intestinalis* (Woollacott 1977); *Halocynthia roretzi* (Kubo *et al.* 1978); Molgula impura and *Styela plicata* (Villa 1981); *Microcosmus sabatieri* (Villa & Tripepi 1982); *Ascidia malaca*, *Ascidiella aspersa* and *Phallusia mamillata* (Villa & Tripepi 1983), no acrosome or acrosomal-like structure was observed. Villa & Tripepi (1982; 1983), however, have reported the presence of vesicles in an apical protrusion in spermatids of *Microcosmus sabatieri* and *Phallusia mamillata*. According to the latter authors, these vesicles were no longer visible at the end of sperm-shaping.

On the other hand, several workers have mentioned the presence of an acrosome or its equivalent structure in ascidians. Furthermore, it is interesting to note that a structure initially identified as an acrosome in *Ascidia nigra* by Schabtach & Ursprung (1965) was later shown by Cloney & Abbott (1980) to correspond with the details of the nuclear envelope of the spermatozoon of *Ascidia callosa*. According to Schabtach & Ursprung (1965: p. 359), it is not known

'.... whether the very electron dense line at the tip of the sperm is merely an artefact of preparation or whether it reflects close apposition of two boundaries that are seen to enclose the acrosomal vesicle further back'. The electron micrographs of Cloney & Abbott (1980), Cotelli *et al.* (1980) and Fukumoto (1981; 1983; 1986), however, clearly show membrane-enclosed vesicles at the anterior tip of the sperm. Some workers who have observed anteriorly located vesicles, do not consider these to represent an acrosome *per se;* rather, they speak of a putative acrosome (Cloney & Abbott 1980; Cotelli *et al.* 1980) or an acrosome-like structure (Fukumoto 1983). According to Fukumoto (1983), the acrosome in ascidians is estimated to occupy only 0,1 – 0,2% of the volume of the head of the sperm compared with 2,5 – 16% of the volume of the echinoderm sperm head. The small size of these acrosomal vesicles shows that they are not a major storage place for lysins (Fukumoto 1986). This is further substantiated by the fact that the spermatozoon of *Ciona intestinalis* passes through the chorion without releasing any acrosomal substance and without any detectable changes occurring in the plasmalemma of the apical region (Fukumoto 1986).

The paper by Fukumoto (1988) describes the fine structure of the apex of the sperm and its morphological changes during the process of fertilization in *Ciona intestinalis*. According to this author, the acrosome in *C. intestinalis* is a flattened vesicle filled with moderately electron-dense material in its central region. Fukumoto also mentions the presence of an apical substance at the anterior tip of the sperm head. The acrosome remains intact after binding to the chorion and there is no observable alteration in the plasmalemma in the apical region (Fukumoto 1988). In contrast, spermatozoa that have entered the perivitelline space lack an acrosome. Instead of an acrosome, apical processes, considered to be equivalent to acrosomal processes in other marine invertebrates, are observed at the apex of the sperm. Gamete fusion seems to occur between the egg membrane and some of these apical processes (Fukumoto 1988). The possibility of the occurrence of tiny apical vesicles has not been ruled out in *Clavelina oblonga* sperm by Holland (1989).

As in *Ciona intestinalis*, a fuzzy material also surrounds the external surface of the plasmalemma at the tip of the mature spermatozoon of *P. stolonifera*. The binding of the spermatozoa to the chorion seems to be established by this fuzzy material (Fukumoto 1988).

According to Franzén (1956), the primitive metazoan sperm is characterized by a short, rounded or oval head, a mid-piece containing four mitochondrial spheres and a tail consisting of a long filament. This primitive sperm is considered to have an acrosome with a shallow cap-shape. Such a sperm is found in many invertebrate groups (Franzén 1956). Lambert (1982) states that the ascidian sperm are simplified by lacking a mid-piece and proximal centriole and by having the single mitochondrion located lateral to the nucleus. Villa & Tripepi (1982) are of the view that the small size of the acrosome, and the absence of this structure in some species, has resulted from a simplification, which led to the disappearance of the acrosome during evolution of the ascidians. The assumption that the most primitive acrosome consists of many small Golgi vesicles, suggests that a primitive character has been retained in this group of animals (Villa 1981). Fukumoto (1988), on the other hand, states that the spermatozoa of ascidians may be highly modified to effect penetration through the thick and tough chorion so as to achieve successful fusion with the female gamete.

According to Lambert (1982), there is a close correlation between sperm size and structure on the one hand, and mode of reproduction on the other. The length of the head of the spermatozoa of ascidians ranges from about 3 to 14 μm. The only exception being that of *Perophora formosana* which measures 90 μm in length (Fukumoto 1981). The internally fertilizing forms such as the aplousobranchs and compound phlebobranchs are considered to have large spermatozoa with relatively larger head : tail ratios (Lambert 1982). The large size of the spermatozoa of *Perophora formosana* indicates that the eggs of this species are internally fertilized. In *P. stolonifera*, where fertilization is external, the sperm are small, thus adding further support to Lambert's hypothesis.

During spermatogenesis in *P. stolonifera*, the spherical spermatocyte nucleus is transformed into an elongate structure in the mature sperm. This nuclear elongation becomes evident in the spermatid stage. Fawcett, Anderson & Phillips (1971) and Villa (1981), consider nuclear shaping to be effected by a genetically controlled pattern of aggregation of chromatin while others suggest that microtubules are responsible. Microtubules, surrounding the elongating spermatid nucleus, have been described in several animal groups, including some ascidians (Franzén 1976). In *P. stolonifera*, also, microtubules were clearly evident in the mid-spermatid stage which coincided with the period of nuclear elongation. Fawcett *et al.* (1971) are of the view that the microtubules are essential for the redistribution of cytoplasm that takes place during spermatid elongation but that they are not directly involved in the shaping of the nucleus.

Fukumoto (1983) and Jamieson (1991) mention the presence of longitudinal tube-like structures in the matrix of spermatid mitochondria of *Pyura haustor* and *P. vittata*, respectively. Intramitochondrial tubules, however, were not observed in spermatids of *P. stolonifera* by the present authors. It is deduced that in *Pyura*, at least, they contribute to mitochondrial elongation (Jamieson 1991).

The findings so far tend to suggest that, although an acrosome appears to have been demonstrated in some ascidians, evidence for its presence in others is equivocal. Further studies based on biochemical and morphological changes occurring at the apex of the spermatozoa during the process of fertilization will probably shed more light on the problem.

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