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Spermatology of the genus *Lepidodasys* Remane, 1926 (Gastrotricha, Macrodasyida): towards a revision of the family Lepidodasyidae Remane, 1927

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Abstract

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The spermatozoa of *Lepidodasys unicarenatus* and *Lepidodasys* sp. are filiform and composed of a cork-screw shaped acrosome, a helical nucleus surrounding a mitochondrial axis, and a $9 \times 2 + 2$ flagellum as in the basic structural model of the macrodasyidan sperm. The genus *Lepidodasys* has a debated phylogenetic position and has been linked in turn to the family Lepidodasyidae and the family Thaumastodermatidae. The sperm features of the two *Lepidodasys* species examined are distinct from those typical of the two families: the absence of the periaxonemal cylinder, a character shared only with Turbanellidae among Macrodasyida, could be considered as a symplesiomorphy, suggesting a basal position of the genus along the Macrodasyida clade. Moreover, a comparison of the spermatogenic process of *Lepidodasys* sp. with those of *Acanthodasys aculeatus* (Thaumastodermatidae) and *Cephalodasys maximus* (Lepidodasyidae) has revealed that the process of acrosome formation and nuclear morphology during spermatogenesis are peculiar in *Lepidodasys* sp. and differences are evident especially in the late stages of spermatogenesis. Penetrated spermatozoa were observed in the oocytes at all maturation stages in *L. unicarenatus.*

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Introduction

Gastrotricha Macrodasyida includes 240 protandric or simultaneous hermaphroditic species distributed in six families and 30 genera (Kisielewski 1987, 1991; Ruppert 1988; Evans and Hummon 1991; Hummon *et al.* 1993). The evolutionary relationships among families are little known and uncertainty also persists within group alliances because species descriptions are often incomplete and there is a general lack of knowledge about the microscopic anatomy of taxa perceived to be 'primitive', or at least possessing plesiomorphic features. These problems are also highlighted by two recent phylogenetic reconstructions of Gastrotricha based on morphological traits (Hochberg and Litvaitis 2000, 2001) as well as one based on the 18S rRNA gene sequences (Todaro *et al.* 2003). These studies confirm previous doubts about the monophyly of some families of Macrodasyida, such as Lepidodasyidae and Planodasyidae (Travis 1983; Ruppert 1991).

The family Lepidodasyidae is especially problematic because it contains seven morphologically and biologically disparate genera with no known synapomorphies. Ironically, species of the type genus *Lepidodasys* Remane, 1926 share more potentially homologous characters with the species of Thaumastodermatidae than with the species of Lepidodasyidae, including: Y cells with myofilaments, absence of circular muscles in the lateral body regions, and structural complexity of the cuticle (Rieger and Rieger 1977; Ruppert 1978). Still, species of *Lepidodasys* are exceptional among Macrodasyida because of two unique characters: a non-striated pharyngeal myo-epitelium (all other species of Gastrotricha possess cross-striated myoepithelia), and the absence of pharyngeal pores (apomorphic to Macrodasyida) (Ruppert 1978).

Recently, comparative studies of the gastrotrich spermatozoa have provided useful information on taxonomic and phylogenetic relationships within the phylum (Balsamo 1992; Balsamo *et al.* 1999). In this study, we compare the spermatozoa of two species of *Lepidodasys*, *L. unicarenatus* Balsamo, Fregni & Tongiorgi, 1994 and *Lepidodasys* sp. (cf Todaro *et al.* 1995), with those of other macrodasyidans to gain insight on the phylogenetic position of the genus. We also examine the development of the spermatogenesis of *Lepidodasys* sp. compared to that of *Cephalodasys maximus* (Lepidodasyidae; Fischer 1994) and *A. aculeatus* (Thaumastodermatidae; Guidi *et al.* 2003), because the latter species are representative of the two families to which *Lepidodasys* has been linked. Finally, because specimens of *L. unicarenatus* also show a number of oocytes at different maturation stages, each containing several spermatozoa within the ooplasm, the fine morphology of the penetrated spermatozoa has been surveyed and compared with that of the testicular spermatozoa.

Materials and methods

Adult specimens of *Lepidodasis unicarenatus* were collected on 8 June 2002 from Porthu de la Rena, Castelsardo, Sardinia (Italy) at 13 m water depth (Balsamo and Todaro, unpublished data), while those of *Lepidodasys* sp. were collected on 25 September 1994 from the shallow sublittoral of Panama City beach, Florida, US (cf. Todaro *et al.* 1995). In laboratory, living gastrotrich were extracted by means of the narcotization and decantation technique (Higgins and Thiel 1988); thereafter, the Italian specimens were fixed in sucrose paraformaldehyde glutaraldehyde (SPAFG) (Ermak and Eakin 1976) while the American ones were fixed in 2% glutaraldehyde. All the animals were then washed in 0.1 M phosphate buffer (PBS) at pH 7.2 and post fixed in 2% osmium tetroxide solution for one hour at room temperature. All fixatives were in PBS with 10% sucrose. After a rinsing in PBS, the specimens were dehydrated in a graded acetone series, stained *en bloc* in uranyl acetate in 70% acetone, and embedded in araldite. Ultra thin sections were cut with an LKB Ultratome 2088 V, contrasted with lead citrate, and observed under a Philips 300 and a Zeiss 902 transmission electron microscope. Additional specimens of both species were whole-mounted on slides and observed using Nomarski differential interference contrast (DIC) optics under a Leitz Dialux 20 microscope. These specimens were photographed with a Nikon 995 Coolpix digital camera, and measured with an ocular micrometer; location of the testes along the body was reported in percentage units (U) from anterior to posterior.

Results

Mature spermatozoon of Lepidodasis unicarenatus

The mature spermatozoon of *L. unicarenatus* is a filiform cell, 83.8 μm long, composed of an elongated acrosome, a nucleus surrounding a single mitochondrion and a tail (Fig. 1G,H). The acrosome, 19.6 μm long, is clearly distinct in three regions. The apical one is 13.4 μm long and 0.08 μm in diameter, cork screw-shaped, and shows alternating dark and light thin streaks (Fig. 1A,B). The middle region, 2.6 μm long and 0.5 μm wide, is weakly twisted and full of moderately electron-dense material (Fig. 1B,C). The basal one, 3.6 μm long and 0.35 μm in diameter, is weakly twisted and contains a more electron-dense material; numerous, tiny electron-dense vesicles close to one another are located between the acrosomal membrane and the inner material (Fig. 1C,D). The spring-shaped nucleus, 12.2 μm in length and 0.35 μm in diameter, is composed of fully condensed chromatin organized into well-spaced coils and surrounding a single, long and twisted mitochondrion (Fig. 1E, F). A simple clasp-like structure (*sensu* Teuchert 1976) connects the nucleus to the 52 μm long flagellum. The $9 \times 2 + 2$ axoneme is devoid of any accessory structure. The axoneme doublets run parallel to each other for the entire length of the axoneme (Fig. 1F).

Mature spermatozoon of Lepidodasys *sp.*

The mature spermatozoon of *Lepidodasys* sp. is filiform and composed of an elongated acrosome, a nucleus surrounding a single mitochondrion, and a tail (Fig. 2E). The acrosome is clearly distinct in two regions. The apical one is 0.15 μm in diameter, cork screw-shaped, made up of at least 10 coils, and full of a very electron-dense material, whereas the basal one, is 5 μm long and 0.38 in diameter, slightly twisted and composed of a high number of disks (89 counted in one specimen) stacked on top of each other. Each disk is moderately electron-dense and is separated from each adjacent one by an electron-dark septum, which is obliquely arranged with respect to the main cell axis. The disks show a rectangular or trapezoidal shape in longitudinal section generating acrosome twisting (Fig. 2A,B). The nucleus (10 μm in length, 0.53 μm in diameter) is made of fully condensed chromatin organized into 30 coils (Fig. 2C). The inner nuclear cavity includes a single, thin and straight mitochondrion (Fig. 2B). The nucleus-flagellum connection is a simple clasp-like structure. The basal, short, tract of the $9 \times 2 + 2$ axoneme shows a parallel arrangement of the external microtubules, which then coil around the central doublets giving the tail an obliquely striated appearance in longitudinal sections (Fig. 2D). The axoneme is devoid of accessory structures.

Spermatogenesis in Lepidodasys *sp.*

Two very elongated, lateral testes lie close to the anterior region of the gut. They appear slightly asymmetrical, the left posterior (U43.5) being smaller than the anterior one (U41) (Fig. 3A). Each testis is entirely wrapped by a basal lamina and consists of a thin band of germ cells maturing in a caudocephalic direction so that spermatogonia, spermatocytes,

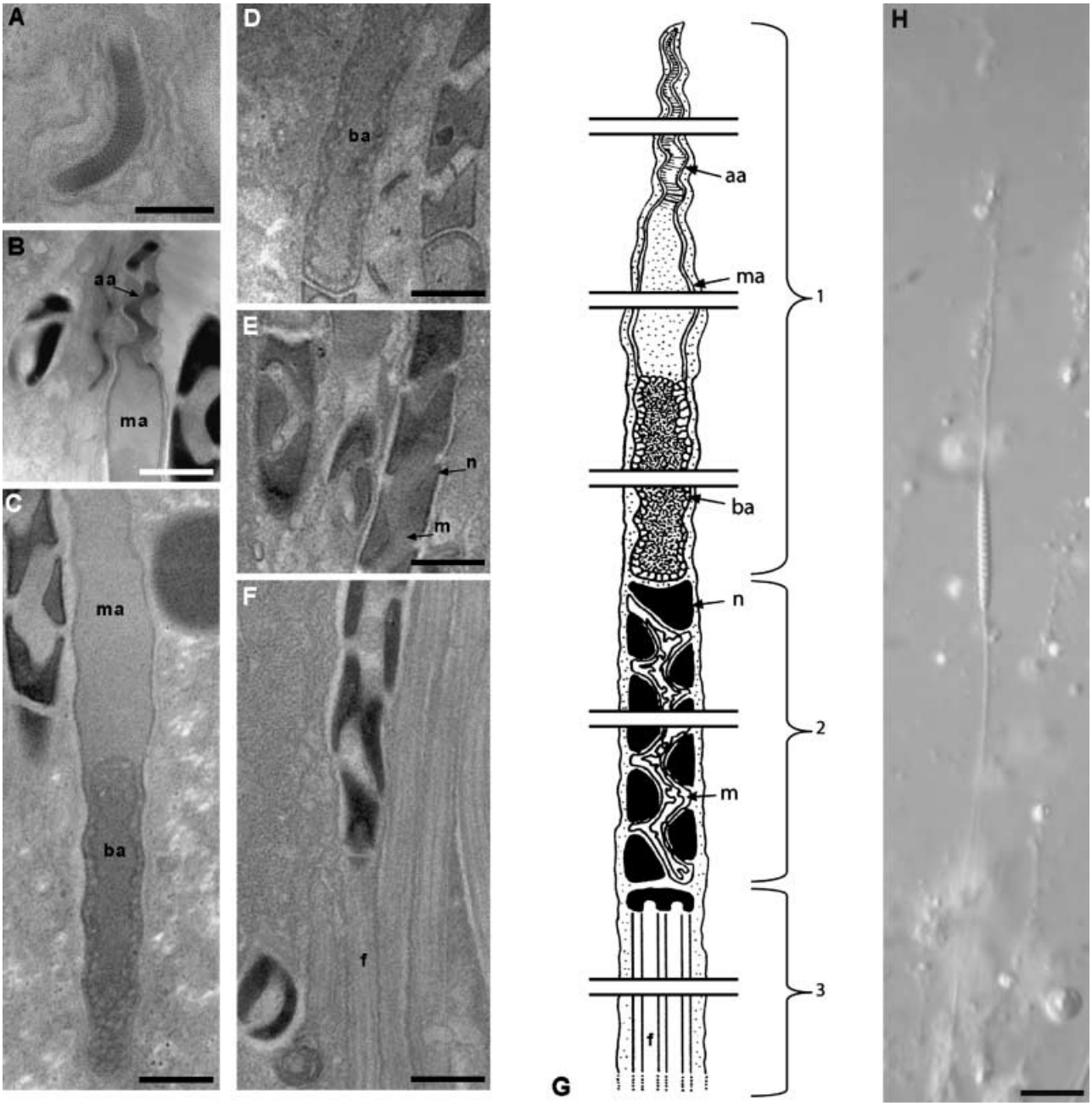


Fig. 1—Mature spermatozoa of *Lepidodasys unicarenatus*. —**A**. High magnification of the acrosomal apical portion. —**B**. Apical and middle portion of the acrosome. —**C**. Middle and basal portion of the acrosome. —**D**. Basal portion of the acrosome and the beginning of the nucleus. —**E**. The nucleus surrounding the mitochondrion. —**F**. Nucleus and proximal portion of the flagellum. —**G**. Schematic drawing of the mature spermatozoon: 1. The acrosome; 2. the helical nucleus and the mitocondrion 3. the flagellum. —**H**. A single spermatozoon as seen by differential interference contrast optics. Scale bars: A = 0.25 μm; B, C, D, E, F = 0.5 μm; H = 5 μm. ma – middle region of the acrosome, f – flagellum, ba – basal region of the acrosome, aa – apical region of the acrosome, n – nucleus, m – mitochondrion.

and spermatids are linearly arranged (Fig. 3B). At the caudal end of each testis, two types of spermatogonia are recognizable, some showing signs of degeneration (Fig. 4A) and the others showing a conventional morphology (Fig. 4B). The first cells are characterized by a strongly condensed chromatin and a crowding of cytoplasmic organules, two markers of the apoptotic processes (Fig. 4A). The conventional spermatogonia, about 2.5 × 4 μm in diameter, are irregular in shape,

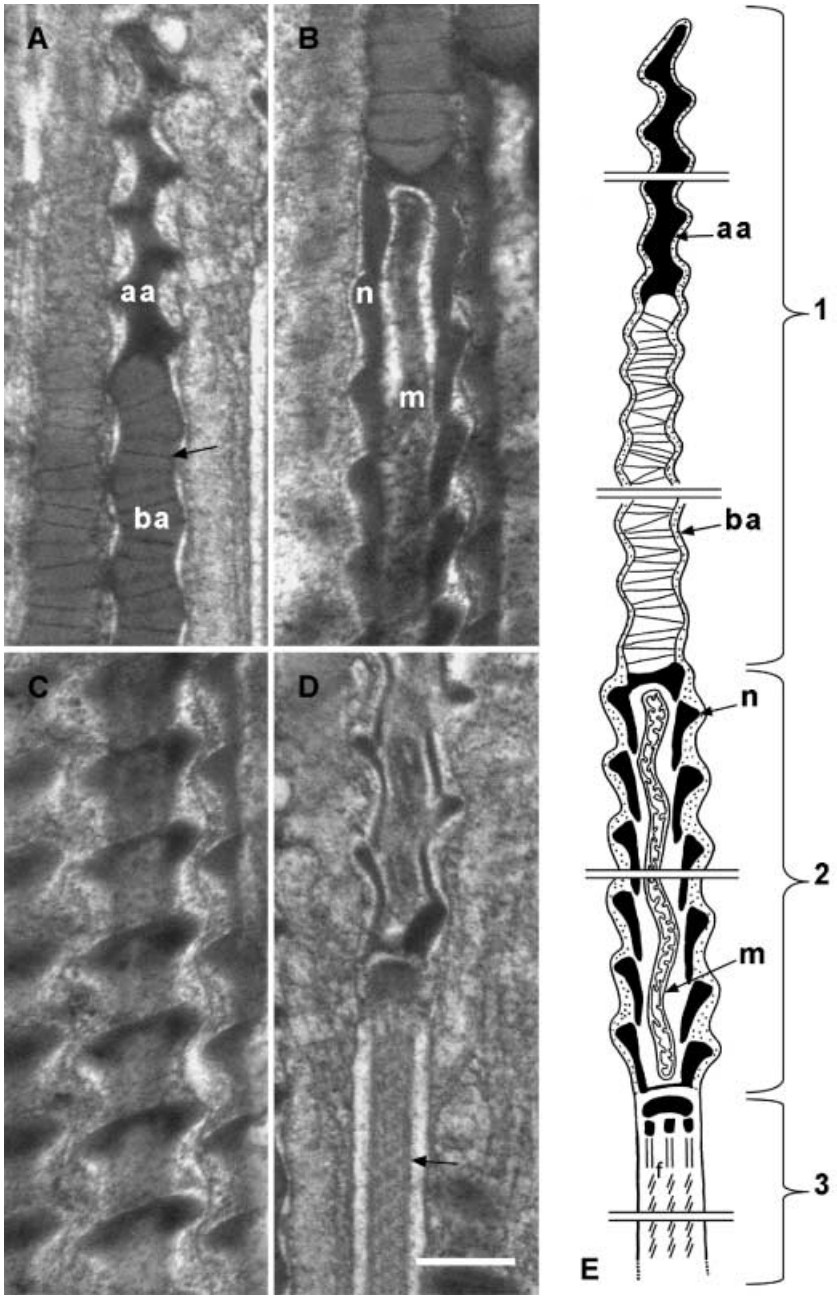
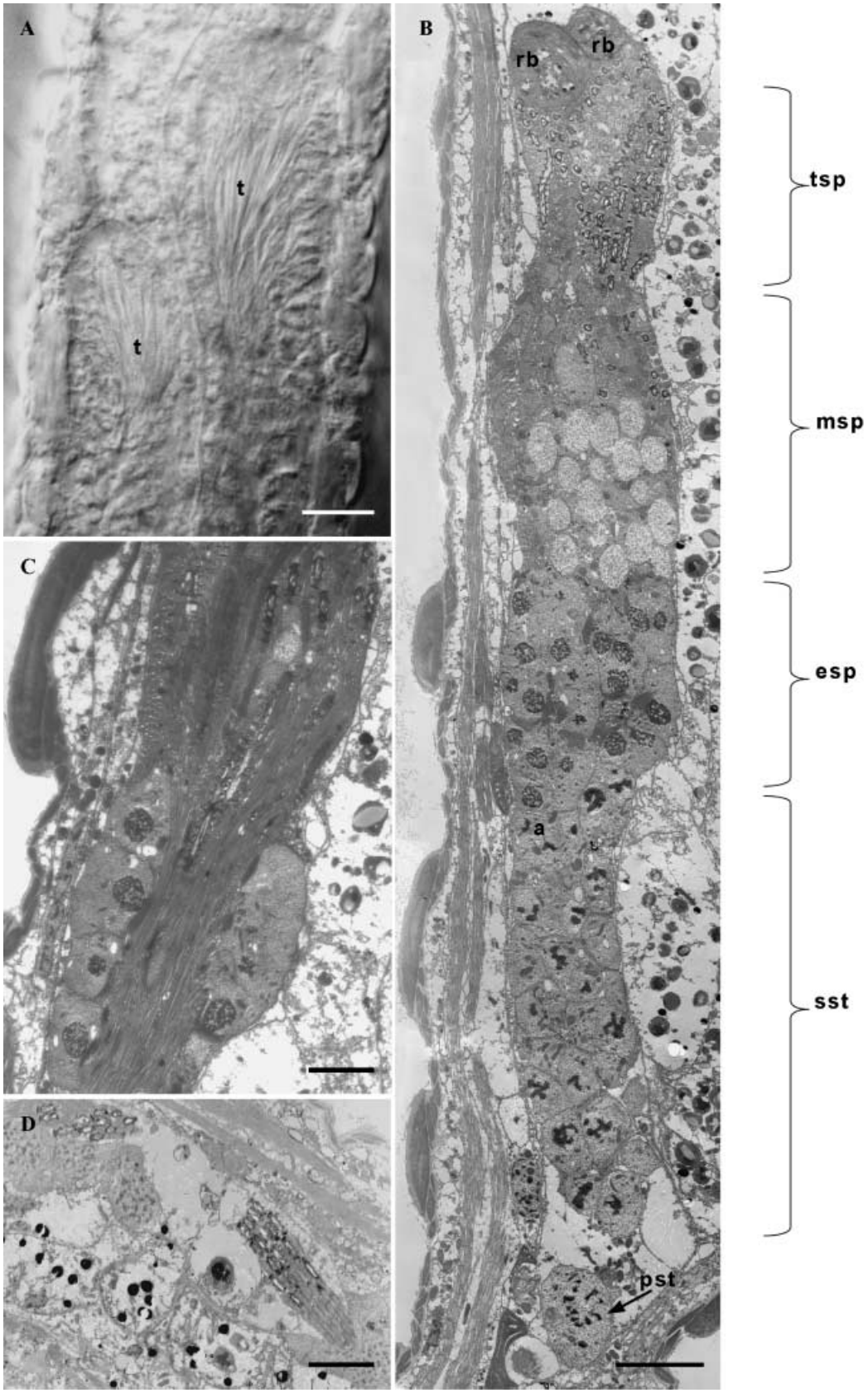


Fig. 2—Mature spermatozoa of *Lepidodasys* sp. —**A**. Apical and basal regions of the acrosome. The acrosomal base is formed by a pile of disks (arrow) stacked on top of each other. —**B**. Acrosomal base and apical portion of the nucleus inside which the mitochondrion is located. —**C**. Tangentially sectioned nucleus showing the fully compacted chromatin organized into coils. —**D**. Basal portion of the nucleus and proximal portion of the flagellum (arrow). The axoneme, with a $9 \times 2 + 2$ microtubular array, shows an obliquely striated appearance because the external doublets coil around the central microtubules. —**E**. Schematic drawing of the mature spermatozoon: 1. The acrosome; 2. the nucleus surrounding the mitochondrion; 3. the flagellum. Scale bars: A, B, C, D, E, F = 0.5 μm.

with an eccentric, round nucleus and dense chromatin patches. Many thin, elongated, mitochondria, some lysosome-like bodies and many Golgi cisternae filled with electron-lucent material are visible in the cytoplasm (Fig. 4B). Primary leptotenic spermatocytes, about 5.2×3.1 μm, are connected by cytoplasmic bridges (Fig. 4C). Their nucleus is eccentric and ovoid, about 2.8×2 μm in size. A large number of round vesicles are grouped at the cell pole opposite to the nuclear one

Fig. 3—Testes. —**A**. Mid portion of the body of *Lepidodasys* sp. showing the two asymmetrical testes. —**B**. Longitudinal section of *L.* sp. testis formed by a band of germ cells developing in a caudo-cephalic direction. —**C**. Group of spermatids sliding ventrally towards the caudal region of the testis. —**D**. Mature spermatozoa located between the youngest germinal cells and the basal lamina. Scale bars: A, C = 2 μm; B = 4 μm; D = 6 μm. t – testis, rb – residual body, tsp – terminal spermatids, msp – middle spermatids, esp – early spermatids, pst – primary spermatocyte, sst – secondary spermatocytes.



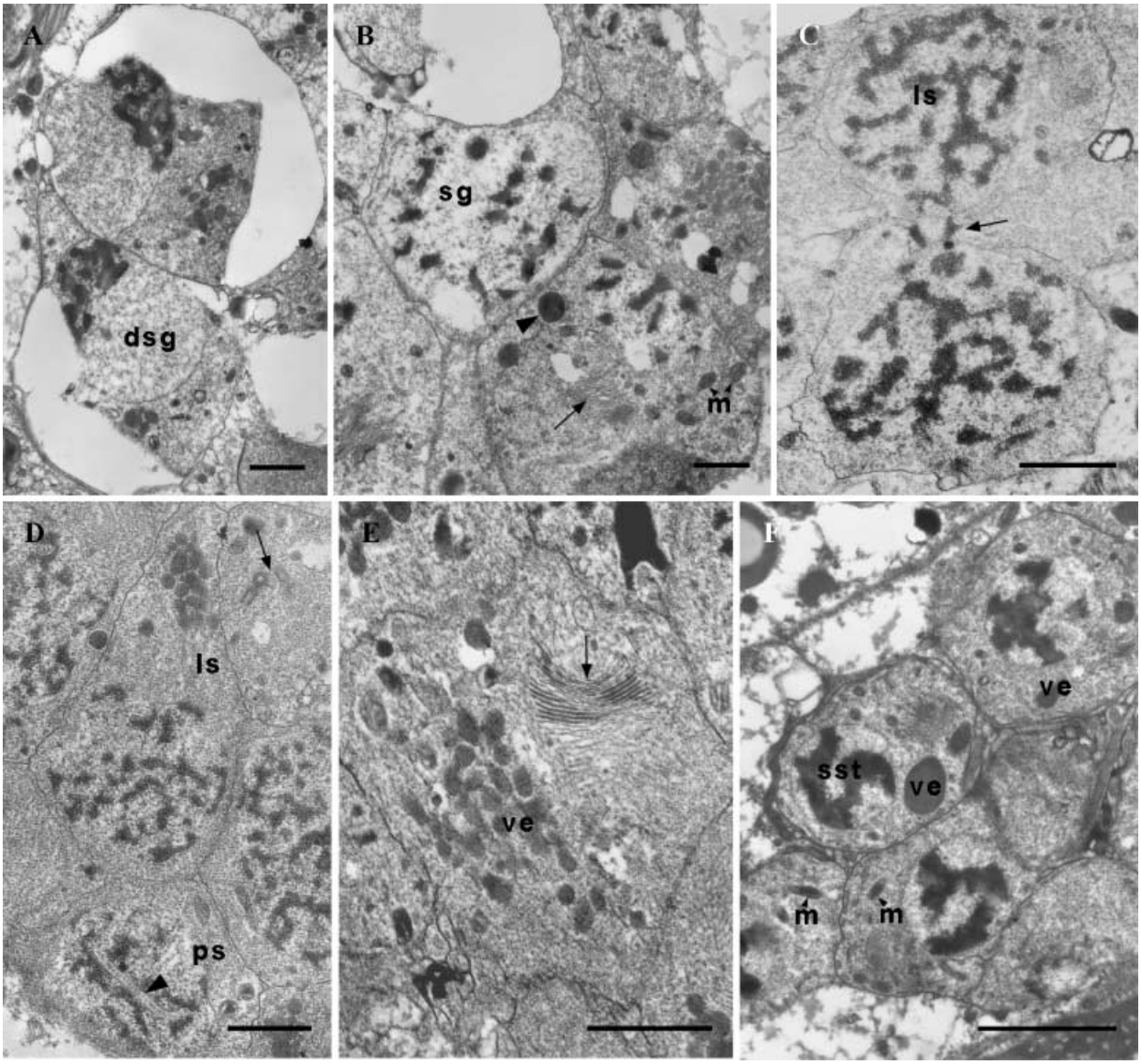


Fig. 4—Spermatogenesis of *Lepidodasys* sp. —**A**. Spermatogonia showing signs of apoptotis. —**B**. Spermatogonia with normal morphology. In the cytoplasm mitochondria, Golgi apparatus (arrow) and lysosome like bodies (arrow-head) are visible —**C**. Primary leptotenic spermatocytes connected by a cytoplasmic bridge (arrow). —**D**. Primary spermatocytes in leptotene and in pachytene. In the cytoplasm of the leptotenic spermatocytes duplicated diplosomes are visible (arrow); in the nucleus of pachytenic ones synaptonemal complexes are present (arrow-head). —**E**. Detail of a leptotenic spermatocyte showing a group of vesicles, located close to a Golgi apparatus (arrow). —**F**. Secondary spermatocyte. In the cytoplasm, large ovoidal vesicles are present. Scale bars: A, B, C, D, E, F = 1 μm. dsg – spermatogonia with degenerating signs, sg – spermatogonia with normal morphology, ls – leptotenic spermatocyte, ps – pachytenic spermatocytes, ve – vesicles.

(4D), close to numerous Golgi cisternae, the contents of which appear with the same electron-density as that of the vesicles (Fig. 4E). Duplicated diplosomes have also been observed in several cases (Fig. 4D). In primary pachytenic spermatocytes, synaptonemal complexes appear and become more visible as the chromatin gradually condenses at their sides (Fig. 4D). Secondary spermatocytes have an irregular shape, about 3.15×2.05 μm, and an eccentric, round nucleus, about 1.2 μm in diameter, with electron-dense chromatin patches. In the examined specimens, most of them appeared in interphase, some were binucleated, and only one was in anaphase (Figs 3B and 4F). Many thin elongated mitochondria and several ovoid vesicles, derived from the fusion of the smaller ones at the previous stage, are visible in the cytoplasm (Fig. 4F). Three successive spermatid stages can be distinguished. The early spermatids already show developing

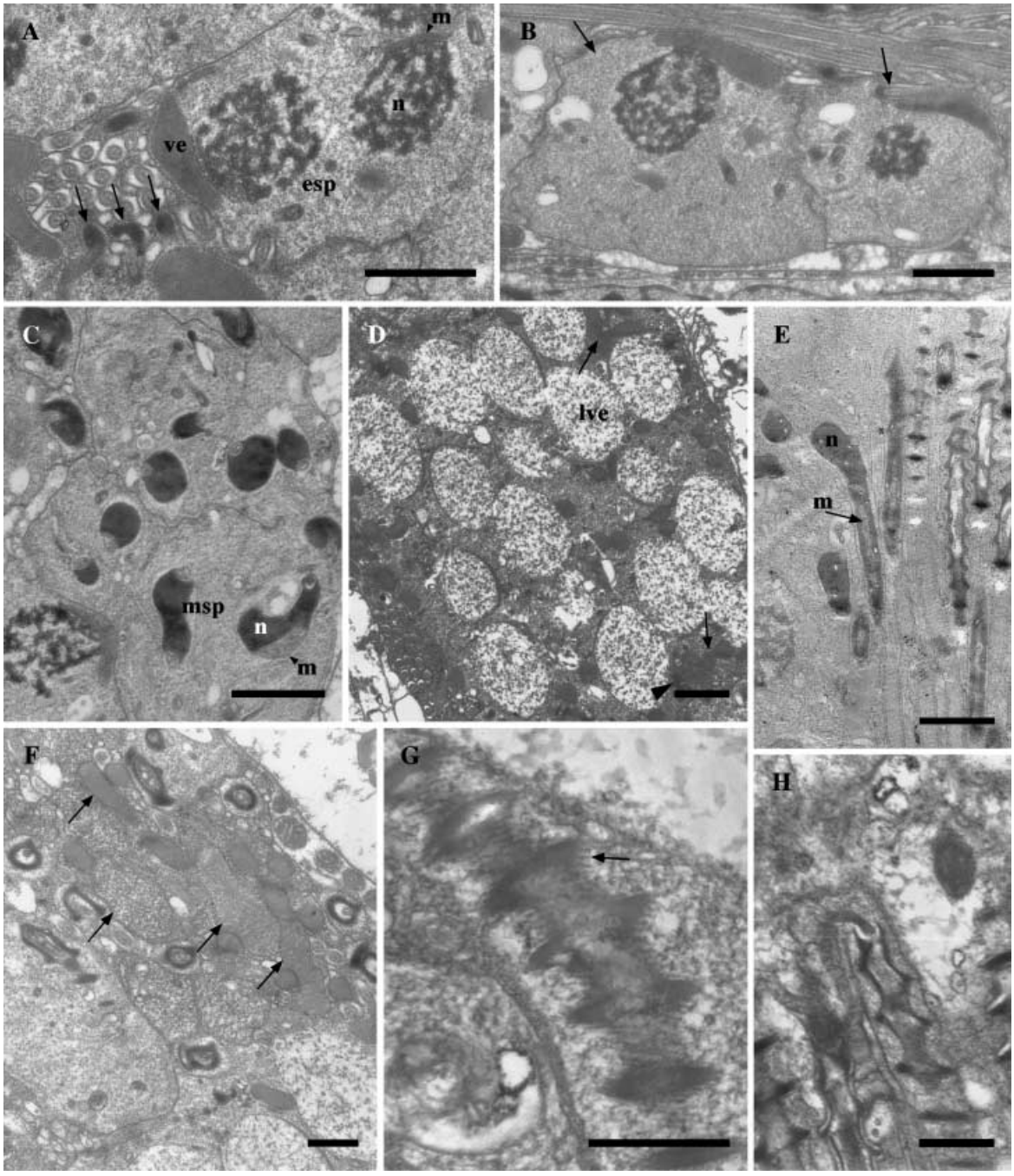


Fig. 5—Spermiogenesis of *Lepidodasys* sp. —**A**. Early spermatids. The nucleus is filled with a condensed chromatin network; an elongated mitochondrion is close to the nuclear envelope on the cytoplasm side. Large cytoplasmatic vesicles, which represent the pro-twisted acrosomal region, are visible. Some of them have a more electron-dense extremity (arrows). —**B**. Two early spermatids: the one on the right side shows the axoneme (arrows). —**C**. Mid spermatids: the elongated convoluted nuclei, with highly compacted chromatin, are located in the cellular body. —**D**. Cytoplasmic region of mid spermatids with large vesicles full of granular and fibrillar material. Other vesicles (arrows), with the same appearance as the basal tract of the acrosome of the mature spermatozoon are visible among the large ones. These vesicles will fuse with the twisted apical acrosomal region, as may be seen in one case (arrowhead). —**E**. Terminal spermatids: the mitochondrion is inside the completely condensed nucleus. —**F**. Terminal spermatids with many vesicles derived from the condensation of the ones visible in Fig. 5(D). Four kinds of vesicles, in intermediate stages of condensation, are visible (arrows). —**G**. Magnification of a terminal spermatid nucleus surrounded by a manchette of microtubules (arrow). —**H**. Terminal, U-shaped, spermatid with nucleus and axoneme in a parallel arrangement. Scale bars: A, B, C, D, F, H = 1 μm; E = 2 μm; G = 0.5 μm. lve – large vesicles.

acrosomes and flagella (Figs 3B and 5B). The nucleus is convoluted, round in transversal sections (about 1 μm in diameter), and characterized by a condensed chromatin network. Some large, irregular vesicles are present in the cytoplasm (Figs 3B and 5A,B). These vesicles, arising from the fusion of the smaller ones of secondary spermatocytes, show a homogeneous content of moderate electron-density, in which a denser region can sometimes be recognized (Fig. 5A). These vesicles represent the 'pro-twisted acrosomal region', as they will give rise to the twisted, apical region of the acrosome. A long mitochondrion formed by the merging of many smaller ones sticks to the nuclear envelope surrounding it progressively; then the 'pro-twisted acrosomal region' starts adhering to the mitochondrion (Fig. 5A). Mid spermatids are large cells with a nuclear and a cytoplasmic pole. They have an elongated, convoluted nucleus with highly condensed chromatin (Fig. 5C) and many cytoplasmic vesicles with different morphology: most of them are large and full of a granular and fibrillar content (Figs 3B and 5D), whereas others, in a more limited number, look like the basal region of the acrosome of the mature spermatozoon. The twisted apical region of the acrosome is already formed (Fig. 5D). The long mitochondrion, coiled around the nucleus, progressively sinks into its chromatin (Fig. 5C,E), while the axoneme lengthens further on. Terminal spermatids have a completely condensed nucleus, surrounding the mitochondrion (Fig. 5E,F), and a high number of cytoplasmic vesicles probably derived from the condensation of the large ones visible in the previous stages. These gradually decrease in width through a condensation process developing in four morphologically distinct stages to form the smallest vesicles, 0.5 μm wide, which look like that of the acrosomal base (Fig. 5F). These vesicles finally fuse with each other, lengthening the acrosomal base. At the same time the nucleus lengthening is completed and it appears surrounded by a manchette of microtubules (Fig. 5G): it first turns medially and then posteriorly, so giving the spermatid a clear U shape (Figs 3C and 5H). The axoneme lengthening is completed, and the centriole localizes in a hollow at the posterior end of the nucleus (Fig. 5H). Cytoplasmic debris are removed from terminal spermatids by extruding droplets, which gather in a large residual body at the most anterior part of each testis (Fig. 3B). At this point, spermatids start sliding ventro-laterally along each testis moving to its posterior end (Fig. 3C) to reach a location just ventral to the primary spermatocytes. Here sperm maturation ends with the rotation of the flagellum, which at the end lines up with the nucleus-acrosome. Mature spermatozoa migrate into the narrow space defined by the youngest germinal cells at the caudal region of each testis and its basal lamina: this space actually acts as a sperm duct (Fig. 3D).

Penetrated spermatozoa of L. unicarenatus

Several spermatozoa were observed between the basal lamina of the ovary and the oocytes membrane (Fig. 6A,D) and also in the ooplasm of all oocytes, at any maturing stage (Fig. 6B,C,E). The spermatozoa still external to the oocytes showed the same structure as the testicular ones, with a complete, elongated acrosome, a nucleus and a tail (Fig. 6A); on the contrary, those penetrated into the ooplasm showed only the nucleus and the basal acrosomal region (Fig. 6B,C,E). Moreover, several spermatozoa were observed in the secretory cells forming the wall of an accessory organ, which extends for the entire length of the ovary, of unclear function (Fig. 6A,B,C,D,E).

Discussion

Spermatozoon ultrastructure

Both the morphology and ultrastructure of the spermatozoa of *Lepidodasis unicarenatus* and *Lepidodasys* sp. agree with the basic structural model of the Macrodasyida sperm (Ferraguti and Balsamo 1994–1995; Balsamo *et al.* 2002): a filiform sperm with cork-screw shaped acrosome, helical nucleus surrounding a mitochondrial axis, and a $9 \times 2 + 2$ flagellum, and thus clearly places the genus among the Gastrotricha Macrodasyida. However, a comparison with the other species of Lepidodasyidae (*Mesodasys laticaudatus*, *Cephalodasys maximus*) and Thaumastodermatidae (*Acanthodasys aculeatus*, *Diplodasys ankeli*, *Pseudostomella etrusca*, *Tetranchyroderma papii*, *T.* sp. 1, *T.* sp. 2) studied so far in detail shows several different, ultrastructural details. The spermatozoa of both *Lepidodasys* species have a simple acrosome like *M. laticaudatus*, but unlike *C. maximus* and all the Thaumastodermatidae, which are provided with a hollow striated acrosomal tube. The absence of a striated cylinder surrounding the axoneme in the spermatozoa of *Lepidodasys* species is a character shared only by Turbanellidae among the Macrodasyida; even if it does not prove a close relationship of the two taxa because it can be considered a possible simplesiomorphy, however, it may indicate their basal position along the Macrodasyida clade.

Spermatogenesis in Lepidodasys *sp.*

The structure of the testes and spermatogenesis development in Macrodasyida is well documented from three species: *Lepidodasys* sp. (the present study), *C. maximus*, Lepidodasyidae, and *A. aculeatus*, Thaumastodermatidae (Fischer 1994; Guidi *et al.* 2003). Among these species, only *Lepidodasys* sp. is characterized by strip-shaped testes lacking both an internal lumen and a true sperm duct, and showing germinal cells arranged into a band. This condition contrasts with the more typical club-shaped and hollow testes of the other species of Macrodasyida. Moreover, no mitotic spermatogonial divisions have been seen in *Lepidodasys* sp. contrary to *A. aculeatus*, in which the observation of duplicated diplosomes in the spermatogonia proved that mitoses occur in the male germ cells of Gastrotricha, a phylum known for its euthely. Because

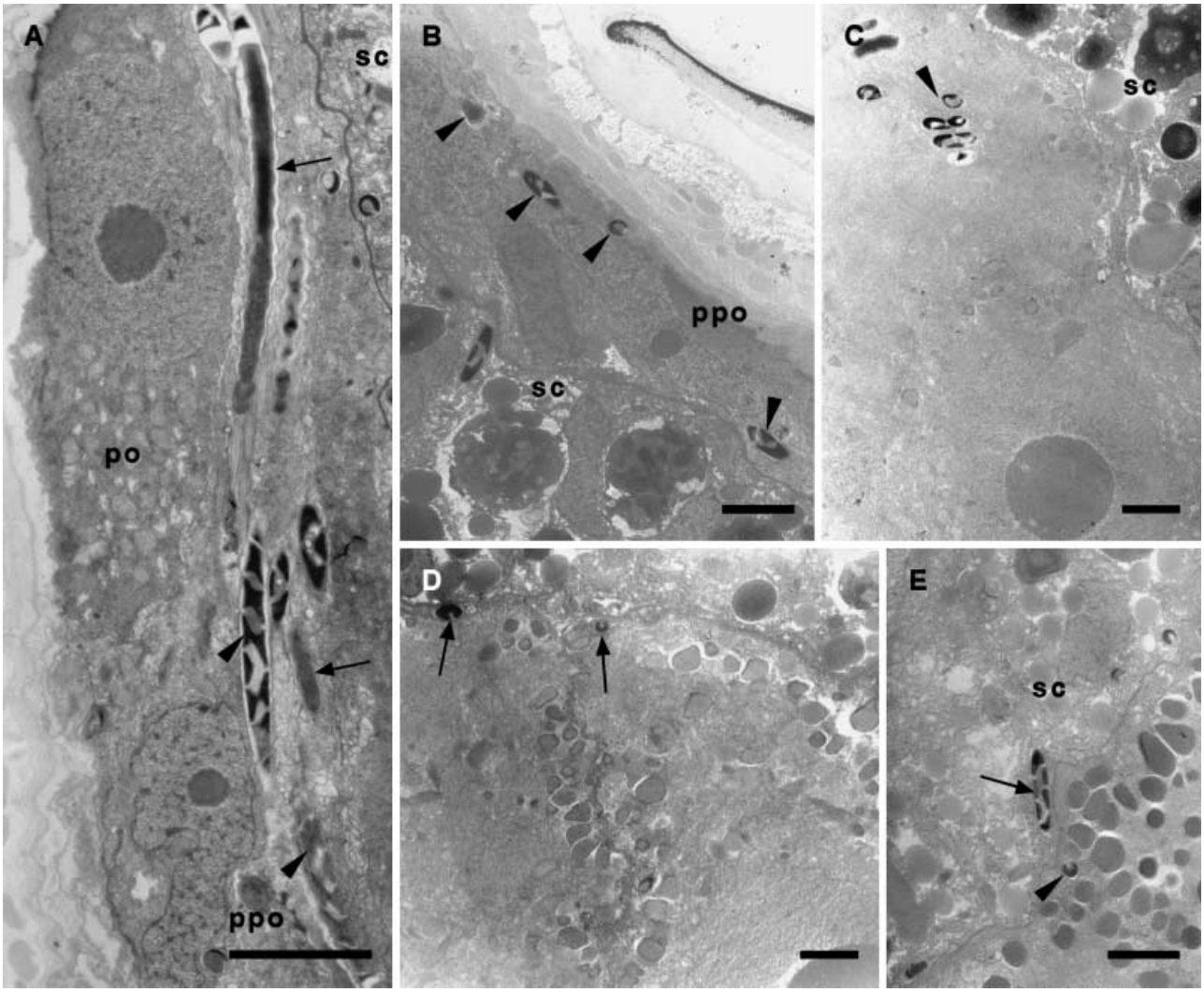


Fig. 6—Penetrated spermatozoa of *Lepidodasys unicarenatus*. —**A**. Some spermatozoa with acrosome (arrows), nucleus (arrowheads) and tail are visible externally to the cytoplasmic membranes of a previtellogenic and a pre-previtellogenic oocyte. —**B**. Pre-previtellogenic oocyte with several spermatozoa (arrowheads) into the cytoplasm. —**C**. Oocyte in early vitellogenesis: on the top a group of spermatozoa (arrowhead) is visibile. —**D**. Two spermatozoa (arrows) located between the basal lamina of the ovary and the cytoplasmic membrane of the oocyte in middle vitellogenesis are visible. —**E**. A spermatozoon (arrowhead) is present in the cytoplasm of an oocyte in late vitellogenesis – another sperm in the cytoplasm of a secretory cell is visible (arrow). Scale bars: A, B, C, D, E = 2 μm. po – previtellogenic oocytes, ppo – pre-previtellogenic oocytes, sc – secretory cell.

apoptosis has been recognized as an important mechanism for the removal of unwanted cells (Hetts 1998; Shinoda *et al.* 2000), the presence of numerous spermatogonia showing apoptotic markers both in the cytoplasm and in the nucleus suggests that an apoptotic process plays this role in the testis of *Lepidodasys* sp. Detailed observations of the spermatogenesis in this species imply a two-step process in sperm formation: first the fusion of mitochondria into a single element, then the penetration of the mitochondrial element into the nucleus. This process is similar to that observed for *C. maximus* (Fischer 1994), whereas in *A. aculeatus*, the two phases follow each other in an opposite sequence. Both the acrosome formation and the nucleus morphology during spermatogenesis appear to be peculiar in *Lepidodasys* sp. the distal and the proximal regions of the acrosome derive from the fusion of many vesicles, not from a single one as in the other macrodasyidans, and the whole process completes later. Moreover, the nucleus keeps a convoluted shape throughout nearly the whole spermatogenesis length, and becomes straight and parallel to the axoneme only at the final spermatid stage, contrary to the other macrodasyid species in which the spermatids show a U-shaped morphology just from the beginning of the spermatogenesis.

The removal of the residual cytoplasm is also carried out in different ways: in *Lepidodasys* by the expulsion of a residual body, whereas in *A. aculeatus* by lysosome intracellular digestion, and consequent reabsorbing (Guidi *et al*. 2003).

Penetrated spermatozoa of L. unicarenatus

The observation of penetrated spermatozoa not only between the ovary and its basal lamina, but even in the oocytes at any development stage, is new for Gastrotricha; in fact, Fischer (1996) reported sperm in *Dactylopodola baltica* only in post-vitellogenetic oocytes. The spread presence of penetrated spermatozoa in all the oocytes implies that fertilization may occur in oocytes at any development stage, and not only in the mature ones, as generally believed for Macrodasyida: that is also suggested by the presence of a sexual accessory organ close to the large, fully grown oocyte and storing allosperm (Ruppert 1991).

Conclusions

To summarize, a number of morphological features of the testis structure and of the spermatozoa appear to be exclusive of *Lepidodasys* species with respect to all the other Macrodasyida species studied so far. A comparison of the spermatogenesis in *Lepidodasys* and in other Macrodasyida shows that the general process occurs in a similar way, but differences are evident especially in the late stages of the process. Thus, from a spermatological point of view, the genus *Lepidodasys* clearly fits within the Gastrotricha Macrodasyida, but stands out among all the other members of the order. It appears to belong to a separate, basal clade, according to the results of recent molecular analyses based on 18S rRNA gene which suggest various locations of the genus in the phylogenetic tree of Gastrotricha, but in any case on a separate clade (Todaro *et al*. 2003). On account of these observations, the study of spermatozoa of additional species of Lepidodasyidae family as well as of potential out-group species will be of great importance in order to make the Macrodasyida basic sperm plan clearer and to understand the in-group relationships of the phylum.

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