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ORIGINAL ARTICLE

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Ultrastructural observations of the spermatogenesis of the hermaphroditic solitary coral *Balanophyllia europaea* (Anthozoa, Scleractinia)

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Abstract Spermatogenesis ultrastructure was studied in a simultaneous hermaphrodite population of the solitary coral *Balanophyllia europaea*. In this species, spermatogenesis takes place in spermatocysts located within gametogenetic mesenteries surrounded by a bilayered boundary. Spermatogonia and spermatocytes are large flagellate cells, densely packed at the outermost edges of the spermatocyst. Spermatids and sperm are loosely distributed near the centre of the spermatocyst. The cytoplasm of spermatogonia and primary spermatocytes often contains short lengths of free axonemes, probably derived from the reabsorption of a primitive flagellum. Maturing spermatids either contain long intracytoplasmic axonemes, that may be stages of the tail synthesis, or have a flagellum. The morphological features of the sperm of this hermaphroditic scleractinian, very similar to those observed in the sperm of gonochoric taxa, support the hypothesis that the hermaphroditism of this population is an adaptive condition.

A. Introduction

In the Cnidaria, extensive ultrastructural studies on gametogenesis have been conducted in Hydrozoa (Stagni and Lucchi 1970; Littlefield et al. 1991; Thomas and Edwards 1991; Franzén 1996). Information on the origin and differentiation of germ cells in Scyphozoa and Anthozoa is still very limited (Fautin and Mariscal 1991; Lesh-Laurie and Suchy 1991).

The origin of male germ cells in Anthozoa is controversial. Some authors postulate that they derive from gastrodermal cells of the mesentery (Miller 1983; Fautin and Mariscal 1991). Others suggest they could originate from undifferentiated cells as, for example, the interstitial cells located in the gastrodermis (Hinsch and Moore 1992). Spermatogonia migrate in groups or individually from the gastrodermis to the mesenteric mesoglea. Here they regroup in structures called spermatocysts in which spermatogenesis takes place (Larkman 1984; Fautin and Mariscal 1991). Larkman (1980), Schmidt and Hoeltken (1980) and Hinsch and Moore (1992) studied the early phases of Anthozoa spermatogenesis. Clark and Dewel (1974), Lyke and Robson (1975) and Schmidt and Zissler (1979) carried out most of the available observations on spermiogenesis in Anthozoa. Recently, much attention has been given to the ultrastructural features of mature sperm in Scleractinia (Harrison 1988; Steiner 1991, 1993; Steiner and Cortés 1996). However, no ultrastructural data exist on spermatogenesis in this taxon of Anthozoa except for a study by Schmidt and Zissler (1979) on *Cladocora caespitosa* (Linné, 1767).

According to Schumacher and Zibrowius (1985), *Balanophyllia europaea* (Risso, 1826) is a zooxanthellate non-constructional scleractinian coral. It belongs to the Dendrophylliidae family and has a known distribution that includes the rocky coast of the Mediterranean Sea and the Atlantic coast of Spain (Zibrowius 1980, 1983; Aleem and Aleem 1992). The sampled population of *B. europaea* was from the Calafuria area (northern Tyrrhenian Sea, Italy). Goffredo and Telò (1998) discovered that this population is a simultaneous hermaphrodite and brooder.

The study of the male gametogenesis of *B. europaea*, besides providing further information on the origin and maturation of sperm in Anthozoa, may give a contribution to the question concerning the environmental adaptation of the sexuality in these organisms (see Bacci 1975; Rossi 1975; Glynn et al. 1991; Fautin 1992). The present ultrastructural investigation is part of an extensive research project that includes the study of the reproductive biology and population dynamics of *B. europaea* in the northern Tyrrhenian Sea.

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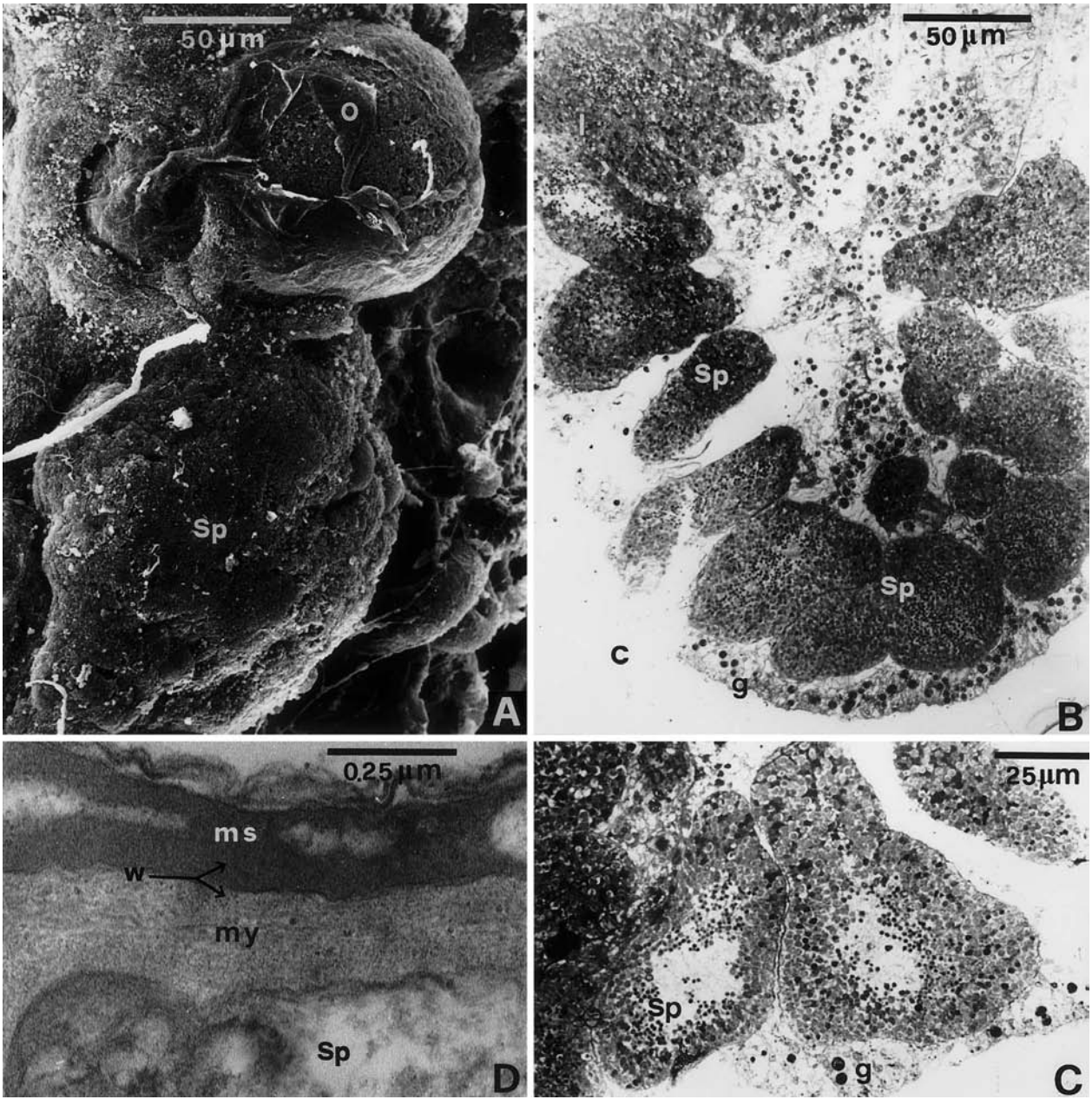


Fig. 1A–D *Balanophyllia europaea* gametogenetic mesenteries. **A** Mesentery showing an oocyte adjacent to spermatocysts. **B** Mesentery with several spermatocysts. **C** Spermatocysts with immature cells densely packed at the periphery and mature cells loosely distributed near the centre. **D** The bilayered structure of the spermatocyst wall. The mesoglea makes up the outer layer and the myofibrils the inner layer. *C* Coelenteron, *g* gastrodermis, *ms* mesoglea, *my* myofibrils, *O* oocyte, *Sp* spermatocyst, *w* spermatocyst wall

B. Materials and methods

In February and March 1998, ten large adult polyps of *B. europaea* were collected by SCUBA divers at 6.5 m depth in the Calafuria area (Leghorn, northern Tyrrhenian Sea, Italy; 43°28.4'N, 10°20'E). The maximum diameter of the oral disc was 16 mm (SD=4). Samples were collected during the period of maximum fertility of the population.

For transmission electron microscopy samples were fixed in 2.5% glutaraldehyde in phosphate buffer 0.2 M (pH 7.2) for 2 h at 4°C. Specimens were washed in the same buffer. Gametogenetic mesenteries were extracted after EDTA decalcification (60 h). They were then rinsed in phosphate buffer 3 times (15 min), and postfixed in 1% OsO_4 in the same buffer for 1 h at 4°C. Mesenteries were processed through graded acetone series and propylene oxide and embedded in an Epon-Araldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and were observed with a Philips EM 410 electron microscope. Semithin sections, 0.5–1.0 µm thick, were stained with toluidine blue for light microscopy observation.

For scanning electron microscopy gametogenetic mesenteries were extracted, then fixed in 2.5% glutaraldehyde in cacodylate buffer 0.2 M (pH 7.2) for 1 h at 4°C and postfixed in 2% OsO_4 in cacodylate buffer at 4°C. They were processed through a graded acetone series, critical-point dried, fixed on stubs with double-sided adhesive tape and vacuum coated with gold, 3 min at 30 mA, before being viewed with a Jeol JSM 5200 scanning electron microscope.

C. Results

I. Spermatocyst structure

Male germ cells of *B. europaea* group together in structures called spermatocysts (Fig. 1). These are located in the gametogenetic mesenteries from the body wall to the inner margin of the mesentery. Spermatocysts are found in progressively diminishing numbers from the oral to the aboral region. Spermatocysts have a spherical or oval shape, depending on their proximity to other spermatocysts or oocytes. Their maximum diameter varies from 40 to 150 µm (Fig. 1A–C). Growth of spermatocysts is asynchronous, thus, in the same polyp, spermatocysts at different stages of differentiation are present.

A wall surrounds the spermatocysts. This wall is irregular in thickness and has a bilayered structure: the outer layer is made up of electron-dense mesoglea while the inner one is made up of myofibrils. The maximum width of each layer is approximately 0.3 µm (Fig. 1D). Within the spermatocyst, immature sperm cells are densely packed at the periphery and mature sperm cells are loosely arranged near the centre. The more advanced spermatocysts appear hollow because of the decrease in cell volume, a characteristic of the later spermatogenetic stages (Fig. 1C).

II. Spermatogonia and spermatocytes

Densely packed flagellate cells are present at the outer margin of the spermatocyst. Each cell contains a large, spherical nucleus with a mean diameter of 5 µm (SD=0.9, *n*=103). The nucleus is completely euchromatic and contains a single nucleolus that is normally found adjacent to the nuclear membrane (Fig. 2A,C). These cells have a high nucleus-cytoplasm ratio and are identified as spermatogonia. In spermatogonia cytoplasm are found small mitochondria, a Golgi complex, few lipid vesicles, free ribosomes, primary lysosomes and a pair of centrioles lying perpendicular to each another (Fig. 2A,C,D). Short lengths of free axonemes are also present (Fig. 2B). Intercellular bridges are not observed.

When spermatogonia enter meiosis, they do not undergo substantial morphological or dimensional modifications. Primary spermatocytes were identified by the presence in the nucleus of a typical structure associated with meiosis I prophase, the synaptonemal complexes (Fig. 2E). The nucleus of primary spermatocytes is round in shape with an average diameter of 5 µm (SD=1.0, *n*=97); it does not contain a nucleolus (Fig. 2E). The number of recognised secondary spermatocytes is low compared to other spermatogenetic stages. Secondary spermatocytes are smaller than primary ones and were almost observed during their second meiotic division (Fig. 2F).

III. Spermatids and sperm

Early spermatids originate at the end of the second meiotic division. Their nucleus is spherical with a mean diameter of 2.5 µm (SD=0.4, *n*=107). It contains a few irregularly distributed clumps of chromatin and a nucleolus (Fig. 3A,B). The scant cytoplasm is segregated at one of the poles of the cell (Fig. 3A). Three or four mitochondria per cell, with a mean diameter of 0.5 µm (SD=0.1, *n*=196), were often observed adjacent to the nuclear membrane (Fig. 3A,C,E,G). In the cytoplasm of early spermatids, lipid vesicles, a conspicuous Golgi complex and multivesicular bodies are also evident (Fig. 3A,D,E). The latter are bound by a simple membrane and contain numerous vesicles of various forms and sizes (Fig. 3E). A membrane-bound lamella filled with electron-dense material is present in the cytoplasm of early spermatids (Fig. 3A,C,G). It derives from the fusion of Golgi complex cisterns (Fig. 3D). The lamella has few annular-like, slightly electron-dense formations where the original cisterns have fused (Fig. 3A,D). It is initially sealed lengthways, appearing ring-shaped when cross-sectioned; later, it opens up and takes on a sheet-like appearance (Fig. 3A,C,G).

Early spermatids often have a flagellum. In some cases the centriolar structures of the flagellum are not in contact with the nuclear membrane. In other cases, they press the nuclear fossa, a shallow indentation at the base of the nucleus (Fig. 3B). The proximal centriole stands at a slight angle to the distal centriole. Both are connected to each other by an intercentriolar strand (Fig. 3B). The distal centriole has a pericentriolar apparatus consisting of nine arms arranged in a radial pattern. These arms arise from the triplets of the centriole (Fig. 3G,H). The cytoplasm of early spermatids that do not have a flagellum often contains long lengths of free axonemes and several disassembled doublet microtubules (Fig. 3C,F).

In *B. europaea* spermiogenesis (Fig. 4), the maturation of cytoplasm and nucleus occurs asynchronously; therefore, we will describe these two processes separately. Nuclear maturation of the spermatid begins with chromatin condensation in scattered areas (Fig. 4A). Chromatin condensation proceeds between the anterior and posterior nuclear poles where two plates of electron-dense material become evident. As condensation continues, a cylindrical mass of chromatin, 1 µm in diameter, takes shape. It is surrounded by residual nucleoplasm in which uncondensed chromatinic filaments are present (Fig. 4B). Continuing with the condensing process, the chromatin mass takes on a cone-like shape (Fig. 4C,E,H). At the nuclear apex, a roughly conical, slightly electron-dense area known as the anterior process, is observed (Fig. 4C,H). It is made up of granular matter and is in continuity with the chromatin. The residual nucleoplasm is gradually eliminated during spermiogenesis.

During spermatid cytoplasm maturation, mitochondria decrease in number and grow in size due to the fusion of their membranes (Fig. 4D). Multivesicular bodies and a long sheet-like membrane-bound lamella are pres-

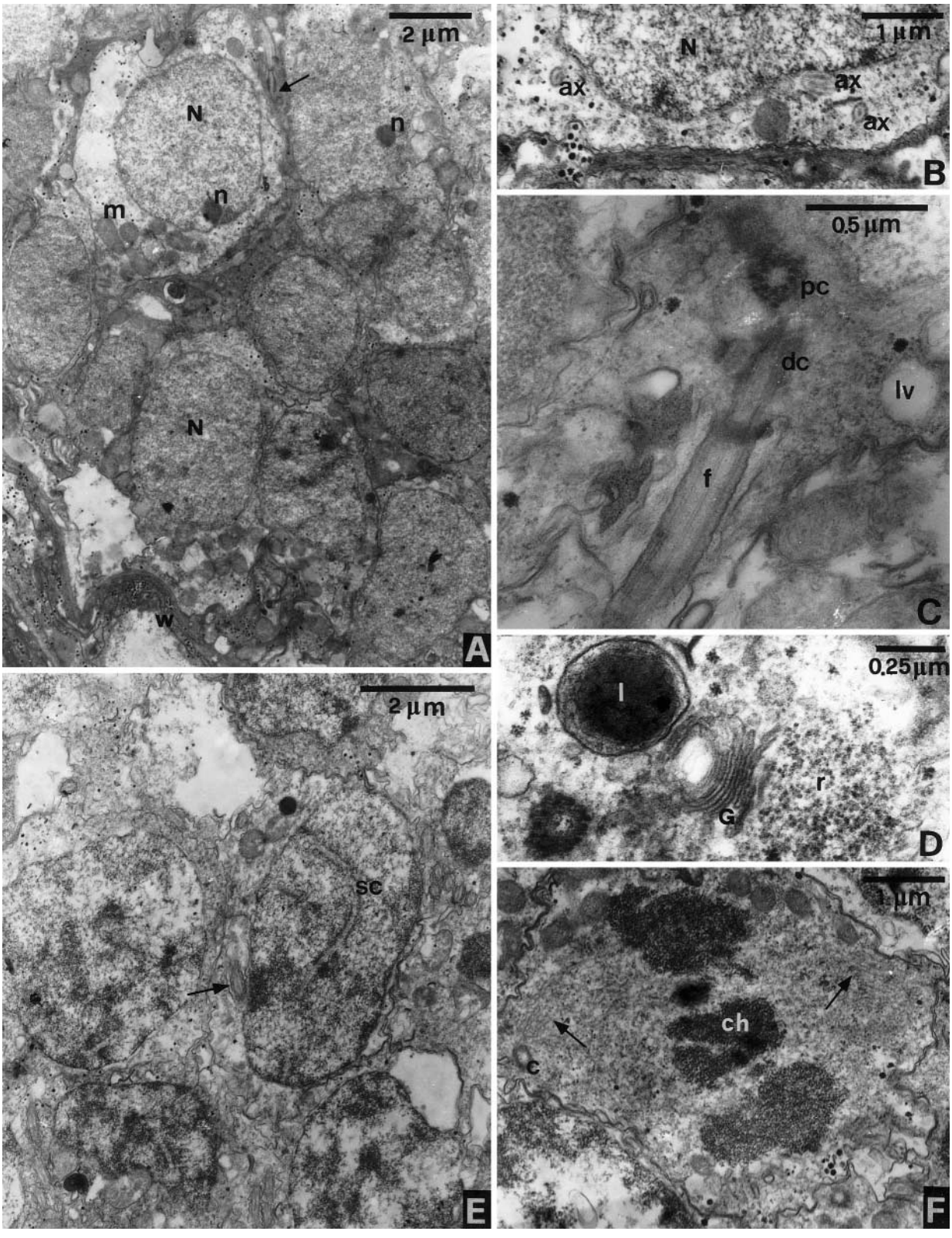
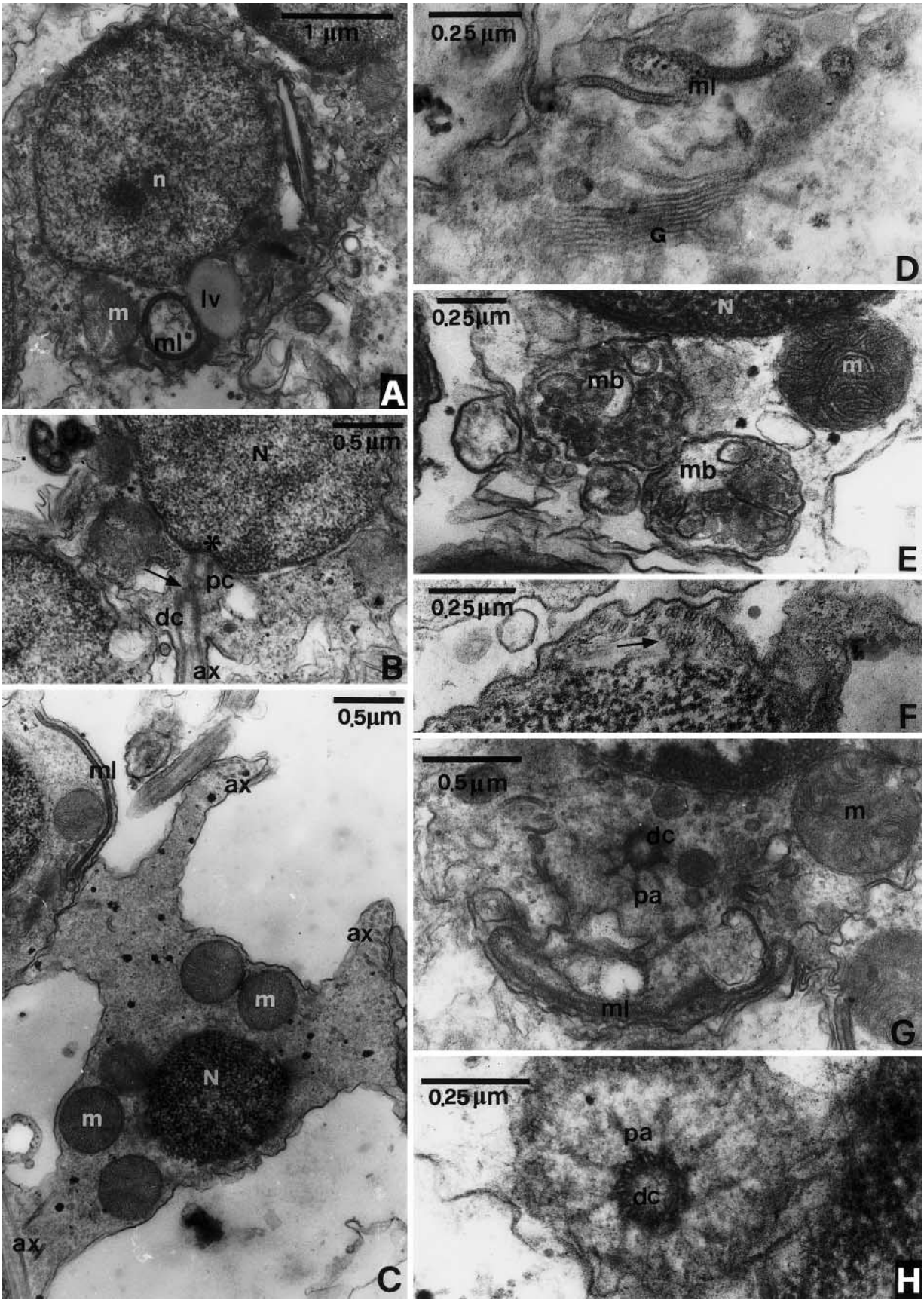


Fig. 2A–F *B. europaea* early spermatogenesis. **A** Densely packed spermatogonia at the spermatocyst periphery. Note the large nuclei containing finely scattered chromatin and a nucleolus, and in the cytoplasm, the flagellum (*arrow*). **B–D** Cytoplasm features of spermatogonia. **B** Sectioned free axonemes found in the cytoplasm. **C** Flagellum with centrioles lying at right angles to each other. **D** Free ribosomes and primary lysosome are close to Golgi complex. **E** Primary spermatocytes in meiosis I prophase. Synaptonemal complexes are evident. Flagella often appear in channels running through the cytoplasm (*arrow*). **F** Secondary spermatocyte in meiosis II metaphase. Chromosomes, microtubules of the meiotic spindle (*arrows*) and one centriole may be observed. *ax* Axoneme, *c* centriole, *ch* chromosomes, *dc* distal centriole, *f* flagellum, *G* Golgi complex, *l* primary lysosome, *lv* lipid vesicle, *m* mitochondrion, *N* nucleus, *n* nucleolus, *pc* proximal centriole, *r* ribosomes, *sc* synaptonemal complex, *w* spermatocyst wall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ent (Fig. 4C,E). During spermiogenesis, this sheet-like membrane-bound lamella moves from the distal cell area to the head region and is found first laterally and then apically to the nucleus (Figs. 3G, 4C,E). The spermatid discards residual cytoplasm and organelles through long processes that eventually separate from the cell. In advanced stages, even the sheet-like membrane-bound lamella is partially eliminated (Fig. 4E). A few small lamella fragments, next to the mitochondria, remain in late spermatid and sperm. Long lengths of free axonemes may be observed in the cytoplasm of spermatids that do not have a flagellum (Fig. 4A,E,F).

In late spermatids and sperm, two or one mitochondria per cell are commonly found with a mean diameter of 0.9 µm (SD=0.1, *n*=191) and with a conspicuous lipid vesicle nearby (Fig. 4H). In the nuclear fossa, the proximal centriole is connected to the nucleus by two fascias of thin strands ending up against an electron-dense plate. The two centrioles eventually line up in a coaxial position and are joined by a marked ligament (Fig. 4C,G). Pericentriolar apparatus arms extend from the distal centriole to the plasma membrane (Fig. 4C).

D. Discussion

I. Sexuality

The solitary coral *B. europaea* living in the Calafuria area is a simultaneous hermaphrodite (Goffredo and Telò 1998). In every sexually mature individual of this population, spermatogenesis takes place mainly in the oral region and ovogenesis mainly in the aboral region (S. Goffredo and T. Telò unpublished data).

II. Spermatocyst wall structure

The spermatocysts of *B. europaea* have a bilayered wall. The inner layer is made up of myofibrils and the outer layer consists of a highly electron-dense mesoglea. In the literature regarding Anthozoa spermatogenesis, ultrastructural descriptions of the spermatocyst wall are rare. In sea anemones, the wall has been described as a trilayered structure made up of fibrils (Dewel and Clark 1972). The bilayered structure of the spermatocyst wall of *B. europaea* can be explained following the model proposed by Larkman (1984) for the sea anemone *Actinia fragacea* Stephenson, 1935. Therefore, one can hypothesise that *B. europaea* spermatogonia, moving from the gastrodermis towards the mesoglea, push against the myofibril layer of epitheliomuscular cells that lies at the base of the gastrodermis. Thus, when entry into the mesentery is complete, the spermatocyst has the appearance of a roughly spherical group of cells bound by a bilayered wall made up internally of myofibrils and externally of mesoglea.

III. Spermatogenesis

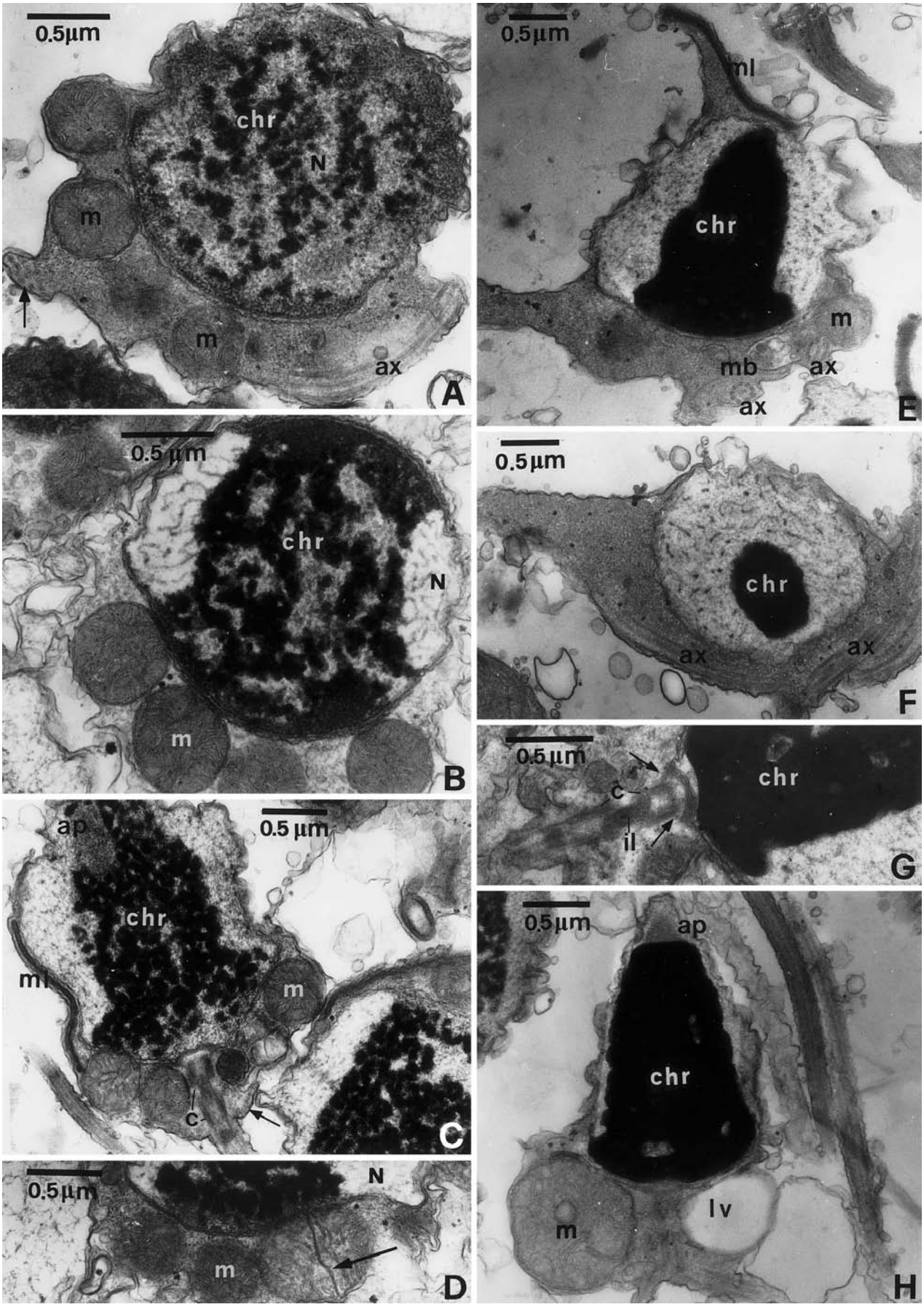
The number of secondary spermatocytes identified in our specimens is low compared to the other spermatogenetic stages. This is probably due to the quick succession of the two meiotic divisions. The ephemeral existence of secondary spermatocytes has already been noted in many Metazoa (Nath 1965; Adiyodi and Adiyodi 1983).

Cytoplasmic continuity has not been observed among *B. europaea* early spermatids. In contrast, intercellular bridges have been observed in spermatids of other Anthozoa (see Dewel and Clark 1972; Clark and Dewel 1974; Larkman 1980). Via cytoplasmic continuity, spermatogenetic cells of common origin may attain a synchronisation of their differentiating process (Fawcett l971). Thus, it is probable that the absence in *B. europaea* of this synchronisation factor causes the maturation phase-difference among adjacent spermatids observed in our preparations.

The main nuclear maturation stages in *B. europaea* spermatids are substantially the same as those described in other Anthozoa (see Dewel and Clark 1972; Clark and Dewel 1974; Lyke and Robson 1975; Larkman 1980;

◀ **Fig. 3A–H** *B. europaea* early spermatids. **A** The large round nucleus contains a centrally located nucleolus and a few clumps of finely granular chromatin. The cytoplasmic organelles (mitochondria, membrane-bound lamella and lipid vesicle) are located in the posterior region of the cell. **B** Flagellum. The distal centriole is at a slight angle to the proximal centriole that presses the nuclear fossa (*asterisk*). An intercentriolar strand (*arrow*) connects the centrioles. **C** Axonemes within the peripheral cytoplasm. **D–H** Cytoplasm features of early spermatids. **D** Membrane-bound lamella originating from Golgi complex. **E** Multivesicular bodies and a mitochondrion. **F** Disassembled doublet microtubules (*arrow*) lateral to the nucleus. **G** Membrane-bound lamella and pericentriolar complex of distal centriole. **H** Cross-section of distal centriole with its pericentriolar complex. Pericentriolar complex arms emanate from the triplets of the centriole. *ax* Axoneme, *dc* distal centriole, *G* Golgi complex, *lv* lipid vesicle, *m* mitochondrion, *mb* multivesicular body, *ml* membrane-bound lamella, *N* nucleus, *n* nucleolus, *pa* pericentriolar complex arm, *pc* proximal centriole

Fig. 4A–H *B. europaea* spermiogenesis. **A** Spermatid. Beginning ▶ of chromatin condensation. In the posterior region of the cell an axoneme, many disassembled doublet microtubules (*arrow*) and four mitochondria are present. **B** Spermatid. Chromatin is condensing between the anterior and posterior pole of the nucleus. **C** Spermatid chromatin has formed a thicker central mass still surrounded by uncondensed nucleoplasm. The anterior process is already visible. Note the following features in the cytoplasm: the membrane-bound lamella lateral to nucleus, the tail centrioles in tandem position and one pericentriolar apparatus arm (*arrow*). **D** Spermatid posterior region. Aggregation and fusion of mitochondria are evident (*arrow*). **E** Late spermatid with completely condensed chromatin shedding residual cytoplasm as cellular protrusions. **F** Late spermatid showing long lengths of free axonemes within the cytoplasm. **G** Sperm posterior region. The centrioles are connected by the intercentriolar ligament. Two arrays of thin strands (*arrows*) are between the proximal centriole and the nuclear fossa. **H** Sperm. Note the bullet-shaped anterior process at the apex of the nucleus. In the posterior region of the cell, a single large mitochondrion and a lipid vesicle are visible. *ap* Anterior process, *ax* axoneme, *c* centriole, *chr* chromatin, *il* intercentriolar ligament, *lv* lipid vesicle, *m* mitochondrion, *mb* multivesicular body, *ml* membrane-bound lamella, *N* nucleus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Schmidt and Hoeltken 1980; Hinsch and Moore 1992). Cytoplasmic microtubules, which play a role in nuclear shaping of many Metazoa (Baccetti 1970; Adiyodi and Adiyodi 1983), were not observed. For this reason, one can assume that nuclear shaping in *B. europaea* is determined by chromatin aggregation and condensation, as already suggested by Lyke and Robson (1975) for other anthozoans. When chromatin condensation is complete, a small bullet-shaped area of slightly electron-dense material is evident at the anterior tip of the nucleus in *B. europaea* sperm. This is the so-called "Spitzenkorper" or anterior process. The anterior process has been found in the sperm of gonochoric scleractinians (Schmidt and Zissler 1979; Harrison 1985; Steiner 1991, 1993; Steiner and Cortés 1996) where, in Steiner's (1991) opinion, it serves a possible acrosomal function. This is the first time that the anterior process is described in the sperm of a hermaphroditic scleractinian.

In early spermatid cytoplasm of *B. europaea* a membrane-bound lamella filled with electron-dense material is always present. It originates from the Golgi complex, initially assuming a ring-like shape; later it opens and assumes a sheet-like shape. During spermiogenesis, this membrane-bound lamella shifts forwards reaching the spermatid anterior pole where it disappears. Among Anthozoa, a single sheet-like membrane-bound lamella has been observed in mature sperm of some scleractinians (Harrison 1985; Steiner 1991, 1993; Steiner and Cortés 1996). According to Steiner (1993), this sheet-like lamella structurally resembles the Golgi-derived membrane-bound vesicles that vary in size, number and location and are usually found in many anthozoan sperm. The exact function of these vesicles has not been ascertained, but they are interpreted as being proacrosomal vesicles by Hinsch (l974), Miller (1983) and Steiner (1993).

The sperm cytoplasm of *B. europaea* has a single large lipid vesicle, which probably originates from the fusion of the smaller vesicles found in early spermatogenetic stages. Since sperm cytoplasm appears to lack glycogen, this vesicle may serve as an energy store, as suggested for sea anemones (see Dewel and Clark 1972). A single large lipid vesicle is a distinguishing trait of the sperm of the gonochoric scleractinians studied by Harrison (1985), Steiner (1991, 1993) and Steiner and Cortés (1996). This is the first time that this kind of vesicle has been found in the sperm of a hermaphroditic scleractinian.

The spermatogonia and primary spermatocytes of *B. europaea* have a flagellum with centrioles lying at right angles to each other as found in other anthozoans (Dewel and Clark 1972; Schmidt and Hoeltken 1980; Larkman 1984). According to Schmidt and Hoeltken (1980), these findings might be evidence that male germ cells arise from flagellate gastrodermal cells. In more advanced spermatogenetic stages, the centrioles become coaxial and enter into contact with the nucleus. This is a usual occurrence during maturation of flagellate sperm (Sotelo and Trujillo-Cenoz 1958; Baccetti 1970).

Lengths of intracytoplasmic free axonemes were observed during whole spermatogenesis of *B. europaea*. An explanation for their presence in the earlier spermatogenetic stages is that they originate from the absorption of the primitive flagellum as described for some Algae, Fungi and Protozoa, in epithelial cells of bird oviduct and during the early phases of spermatogenesis in Diptera (Bloodgood 1974; Boisvieux-Ulrich et al. 1980; Quagio-Grassiotto and Lello 1996). Absorption of the flagellum by early spermatogenetic cells could be a necessary condition for the centrioles to be engaged in the organisation of mitotic and meiotic spindles. In the later spermatogenetic stages one can assume that the intracytoplasmic axonemes are involved in the tail synthesis as observed in some natural and experimental systems (Sorokin 1962; Schuster 1963; Yasuzumi et al. 1970; Lemullois et al. 1988; Cifrian et al. 1992). When the tail synthesis is completed, these axonemes are not found.

IV. Sperm structure and sexuality environmental adaptation

The sperm of the hermaphroditic scleractinian *B. europaea* has a conical shaped head, an anterior process at the tip of the nucleus, a single large lipid vesicle and a large mitochondrion; these features are described as typical of the gonochoric scleractinian sperm (Harrison 1985; Steiner 1991, 1993; Steiner and Cortés 1996). Therefore, our findings do not support the assumption of a correlation between sperm morphology and sexual condition.

B. europaea belongs to the Dendrophylliidae. According to Harrison (1985), 94% of the dendrophylliid corals studied is gonochoric. There are roughly 50 recorded *Balanophyllia* species (Cairns 1977). Among these, the sexual condition is known only for *B. pruvoti* Lacaze-Duthiers, 1897 from Lions Gulf near Marseilles (France), *B. elegans* Verril, 1864 from Monterey Bay (California) and *B. europaea* from Calafuria (Italy). The first two are gonochoric while the third is a simultaneous hermaphrodite (Lacaze-Duthiers 1897; Fadlallah and Pearse 1982; Beauchamp 1993; Goffredo and Telò 1998). One could tentatively hypothesise that in Dendrophylliidae, gonochorism is a primitive condition, while the simultaneous hermaphroditism of *B. europaea* is a derived trait with adaptive significance. The mean population density of *B. europaea* in the Calafuria area [9 corals/m^2 (SD=17); Goffredo and Telò 1998] is significantly low compared to that of *B. elegans* from Monterey Bay [563 corals/m^2 (SD=393); Fadlallah 1983] which is the only known datum concerning a gonochoric dendrophylliid. The low density of *B. europaea* reduces breeding opportunities among individuals of the same population. Under these conditions, simultaneous hermaphroditism becomes adaptive because, although energetically more expensive for the individual, it maximises the rate of fertilisation (Ghiselin 1969). That the morphological features of *B. europaea* sperm reflect the typical struc-

ture of gonochoric scleractinian sperm (see Harrison 1985; Steiner 1991, 1993; Steiner and Cortés 1996) seems to support this hypothesis.

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