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Spermatogenesis in the carnivorous sponge *Lycopodina hypogea* (Porifera, Demospongiae)

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Running title: Spermatogenesis in sponges

Abstract

The ultrastructural features of spermatogenesis of the carnivorous sponge *Lycopodina hypogea* displays unusual features in sponges. The male cells derive from large archaeocyte-like cells from the sponge body, the prospermatogonia, the ultrastructure of which is described. During its first divisions in spermatogonia, then in spermatids, the prospermatogonium is already surrounded by a thin follicle made by a single cell, at the origin of the spermatic cyst in which evolve the sperm cells. The spermatid nucleus progressively elongates and its granular chromatin condenses into twisted fibers. Spermatozoa, tightly packed inside the spermatic cyst, are long and narrow monoflagellated cells. They are unusual for sponges, i.e. dart-shaped, with an anterior filament, an electron-dense, rod-like outgrowth terminating in an acrosome, an opaque cone-shaped vesicle separated from the tip of the nuclear filament by a basal plate. The proximal region of the long flagellum is located within a cytoplasmic tunnel until about one third of the cell length. In the course of spermiogenesis, a secondary envelope made by several cells extraordinarily intertwined by their pseudopodia formed around the first unicellular envelope. Species-specific symbiotic bacteria are usually present between the sperm cells. During maturation, the sperm cyst, which may be termed spermatophore, migrates from the sponge body to the sponge filaments and acquires two bundles of special forceps spicules, included in the cyst envelope. The mature spermatophores are released from the filaments. These peculiarities of spermatogenesis in Porifera are discussed in relationship with the special characters of the carnivorous sponges, Cladorhizidae, i.e. absence of aquiferous system and choanocytes.

Keywords: Spermatogenesis, Carnivorous sponge, *Lycopodina*, Porifera, Acrosome, Spermatophore

Introduction

Sponges have no organized gonads or tissue that can be identified as reproductive (Simpson, 1984; Boury-Esnault & Jamieson, 1999; Ereskovsky, 2010). Their spermatogenesis occurs through transdifferentiation of somatic cells into germ cells inside temporary sperm cysts distributed in non-specific regions of the animal body. These germ cells, spermatogonia, very generally derives from choanocytes, the flagellate collar cells characteristic of sponges and responsible of their filtering activity, with most often whole choanocyte chambers transforming directly into spermatic cysts, which could be considered as fragmented gonads (reviews in Boury-Esnault & Jamieson, 1999; Ereskovsky, 2010). Some observations, however, suggest that in some cases archaeocytes may be also directly at the origin of the germinal male line (Hoppe & Reichert, 1987; Ilan, 1995). After

maturation, the spermatozooids are released free in the surrounding water, sometimes in spectacular, synchronous clouds, through the oscules of exhalant canals of the sponge (Reiswig, 1976). In viviparous sponges, they are then caught for fertilization by another sponge, generally through transport to the oocyte by a modified choanocyte (the so-called “carrier cell”).

As shown by Riesgo *et al.*, (2007a), this general scheme of reproduction relying on the specific canal system of Porifera, is necessarily strongly modified in carnivorous sponges, in which there is no more aquiferous system. The spermatogenesis, and more generally sexual reproduction, is poorly documented in carnivorous sponges, due to their scarceness and to the difficulty of investigations on deep-sea animals. Some observations have been made on specimens collected from the deep-sea, mainly in the genus *Chondrocladia*, which although still having choanocytes, has shown reproductive characters unusual in sponges (Kübler & Barthel, 1999; Lee *et al.*, 2012). But most of the knowledge relies on two species of *Lycopodina*, *L. occidentalis* (Lambe, 1893) and *L. hypogea* (Vacelet & Boury-Esnault, 1996). The first one is of relative easy access in the North Pacific, at 100-120m depth, and its gametogenesis and embryogenesis have been thoroughly described by Riesgo *et al.*, (2007a). The second, *L. hypogea*, with a bathyal distribution in the Mediterranean and some NW European coasts, presents two very interesting characters. Firstly, it is able to colonize cold water caves in the SCUBA zone. These caves are rather uncommon because their temperature must remain nearly the same as in the deep Mediterranean, around 13 °C, which in the littoral zone could occur only in rare topographic conditions allowing trapping of winter water masses. One of these, the 3 PP cave at 20 m depth near Marseille, where the sponge was first discovered (Vacelet & Boury-Esnault, 1995), offers an abundant population of *L. hypogea* with an easy SCUBA access. Secondly, these cave specimens prove to be very easy to maintain for years in laboratory conditions, with the full reproductive cycle available, an opportunity which is not so common in sponges and especially in deep-sea sponges.

Preliminary observations in *L. hypogea* (Vacelet & Boury-Esnault, 1996; Vacelet, 1996; Vacelet, 2007) have shown that spermatogenesis occurs through the development in the main body of spermatocysts very unusual in Porifera, which migrate from the ovoid body part to outer filaments normally used for prey trapping, where they become free and able to fertilize another sponge. These cysts are surrounded by two envelopes, the inner one unicellular and the outer one made by intertwined cells. The cysts possess two tufts of forceps spicules which were supposed to be flotation devices or possibly used for capture by another sponge (Vacelet, 2007). These processes were confirmed and more completely described by Riesgo *et al.*, (2007a) in *L. occidentalis*.

Here we present a more complete study of spermatogenesis in *L. hypogea*, based on confocal microscopy, SEM and mostly TEM pictures, one of which only was published in Vacelet (2007). These pictures remained unpublished, partly because the phenomenon appeared rather similar to what is described in *L. occidentalis* by Riesgo *et al.*, (2007a). However, it appears that there are some differences in the two species, and also that these observations bring some complement.

MATERIALS AND METHODS

The specimens of *Lycopodina hypogea* (Vacelet & Boury-Esnault, 1996) (class Demospongiae, order Poecilosclerida, family Cladorhizidae) were collected by scuba diving in 3PP cave, 1.2 km south-west of La Ciotat on the French Mediterranean coast (43° 09' 47 N - 05° 36' 01 E), 18-23 m deep, where they live at 13.0-14.5 °C all year round (Vacelet *et al.*, 1994). They were fixed *in situ* or after raising in the laboratory, where they were kept in small tanks (500 ml) in a dark room at a temperature of 12° to 14 °C. Once a week, the sponges were fed live small crustaceans such as mysids or *Artemia* nauplii of brine shrimps. The water of the tanks was gently changed every two months. The sponges survived for years and successfully reproduced under these conditions.

For light microscopy, sponges were fixed for 30 min with 4% paraformaldehyde in filtered seawater and then permeabilized for 20 min at room temperature with 0,2 % Triton X-100 in TS buffer (150 mM NaCl, 25 mM Tris, pH 7.5). Fixed and permeabilized sponges were labelled with DAPI as described previously (Le Goff *et al.*, 2015). DIC and DAPI images were obtained using a Zeiss LSM880 Fast Airyscan confocal microscope at the DBS-Optique MRI facilities (Montpellier, France).

For scanning (SEM) and transmission electron microscopy (TEM), the specimens were fixed in 2.5% glutaraldehyde in a buffer composed of 0.4 M sodium cacodylate / sea water (1/1) for approximately 20 hours, followed by postfixation in 2% osmium tetroxide in sea water for 2 hours, and embedded in Araldite after desilicification in HF 5%. Semithin sections were stained with toluidine blue. For TEM, thin sections, contrasted with uranyl acetate and lead citrate, were observed under a Hitachi Hu 600 or a Zeiss EM 912 electron microscope. For SEM, the specimens were fixed in a mixture of osmium tetroxide and mercury chloride (6/1) (Johnston & Hildemann, 1982). They were fractured in liquid nitrogen, then critical-point-dried, sputter coated with gold-palladium, and observed under a Hitachi S 570 electron microscope.

RESULTS

The structure, skeleton and cytology of *L. hypogea* were described by Vacelet & Boury-Esnault (1996). It is a minute sponge, with a maximum size of 20 mm, composed of a small fixation base, a cylindrical peduncle, and an ovoid body, 1.9-6.5mm/0.7-1.2 mm, bearing when fasting 30 to 60 slender filaments up to 10 mm long and 50-80 µm in diameter (Fig. 1A). At least seven cellular types have been described.

Spermatogenesis has been observed frequently and all year round, contrary to oogenesis and embryogenesis, more rarely documented. However, the determinism of the occurrence of spermatogenesis has not been clearly determined, even in aquarium conditions. Mature or sub-mature spermatocytes with their cellular envelopes, which we will refer to in the text as “spermatophores”, are often observed as white swellings along the filaments (Fig. 1A,B). Various stages of spermatogenesis are simultaneously present in the same individual. Development is nearly synchronous within a sperm cyst (Fig. 1C,D).

The first stages of spermatogenesis occur inside the ovoid body part of the sponge. Solitary cells with very few or no pseudopodia, up to 33 µm in size, thus larger than the somatic cells observed in *L. hypogea* which generally do not exceed 15 µm, are interpreted as the prospermatogonia (Fig. 2A-C). Their nucleus, generally with an irregular outline, 6 -14 µm in diameter, has a large nucleolus, 2.8-3.3 µm in diameter, always located at the center of the nucleus. The cytoplasm contains numerous free ribosomes, rough endoplasmic reticulum (RER) typically arranged in long cisternae that may extend for several micrometers, and mitochondria, oval and elongated, 0.8 x 1.5 to 0.8 x 6 µm in section, with a dense matrix (Fig. 2A-C), that are usually clustered into groups rather than randomly scattered through the cytoplasm. Rare intracellular bacteria and phagosomes may be present. These prospermatogonia are often already partially surrounded by an amoeboid cell, sometimes with dark inclusions (Fig. 2A-C) 0.6-0.9 µm in diameter, which appears to be the first stage of the follicular cell that will constitute the unicellular, inner layer of the follicle of the spermatocyst.

The prospermatogonium divides mitotically, giving rise to spermatogonia inside a spermatocyst. The younger cysts observed on fracture in SEM (Fig. 3A), 30-36 µm in diameter, are completely surrounded by a unicellular follicle deriving from the follicular cell already observed near the prospermatogonium. These cysts contain closely packed young spermatogonia, 10-14 µm long and 4-6 µm wide, which are more or less conical on section and appear radially arranged. The nucleus, about 3 µm in diameter, is located at the outer, large pole. Their distal pole appears to end in a poorly differentiated central zone of the cyst. At this stage, the number of cells in a cyst could be approximated on fracture to 30.

In the earliest stage observed on section in TEM (Fig. 3B), probably a little more advanced than in SEM, the spermatogonia appear as polygonal to trapezoid cells, more or less closely packed together in the cyst, with smaller cell sections in the center of the cyst. They appear less closely appressed than in the early stage observed in SEM and do not completely fill in the cyst. The cells are 3.3-5.5 μm in maximum size on section, with a nucleus 2.4-3.2 μm in diameter, often with denser irregular zones, but always anucleolated (Fig. 3B). In a given cyst, they appear to be heterogeneous by the density of the cytoplasm, most having a dense cytoplasm, but some cells, apparently similar, being clearer. The cytoplasm contains clear vesicles, a few mitochondria and some dense inclusions 0.1-0.5 μm in diameter. A well-developed Golgi apparatus is present near the nucleus. They appear devoid of flagellum.

At this stage, the follicle surrounding the cyst is composed of a single cell, the follicle cell, with a nucleolated nucleus 3.5 μm in diameter and a cytoplasm which may be thinner than 0.1-0.2 μm by place, with few inclusions. However, some outer cells with pseudopodia extending over the follicle cell probably represent an early stage of the external, multicellular layer of the cyst follicle (Fig. 3B). During the whole development of the cyst, symbiotic bacteria are absent or very rare inside the cyst.

In a later stage (Fig. 3C,D), the cells are more numerous in the cyst and a flagellum is present (Figs 2D, 3C-E). There is still a distinction between clear and dense cells (Fig. 3F). The nuclei of primary spermatocytes contain synaptonemal complexes (Fig. 4C). In a presumed later stage, the cells appear more uniform in density, still with a large nucleus and with some dense cytoplasmic inclusions, 0.2 to 0.3 μm in diameter, which are most often surrounding the nucleus. The cytoplasm also contains oval mitochondria, 0.2 μm in diameter, with defined laminar cristae, a Golgi apparatus, lipid droplets and several small electron-clear vesicles. The flagella appear more numerous on the sections (Figs 3C,D, 4A,B).

The first meiotic division is asynchronous, primary spermatocytes and secondary spermatocytes occur at the same time within each cyst. In a further stage the secondary spermatocytes take a more irregular shape (Fig. 3F), with the same cytological characters and with numerous cytoplasmic bridges. These secondary spermatocytes are smaller cells, around 2.5-3.3 μm with a nucleus 1.5-1.6 μm in diameter (Fig. 4D), with the chromatin forming electron-dense areas. The flagella are more numerous on sections and their insertion in the cell is often visible. The follicle cell, with a nucleolated nucleus 4.3 μm in diameter surrounded by Golgi vesicles, has more numerous dense inclusions, 0.3-0.6 μm in diameter in its thin cytoplasm.

The secondary spermatocytes then evolving in spermatids are very irregular in shape (Fig. 5). They are frequently joined by cytoplasmic bridges (Fig. 4D). The cytoplasm of spermatids contains clear vacuoles and numerous dense, round inclusions, 0.2-0.7 μm in diameter, often surrounded by a clear zone, probably lipid in nature, which become more numerous and fill in the cytoplasm, often making bumps at the surface of the cell (Fig. 6A,E). Glycogen particles are numerous. The nucleus, first with a slightly elongate shape, progressively elongates up to about 3.6 μm for 0.75 μm in diameter (Fig. 6B-D) during the full compaction of its granular chromatin. Aggregation in the nucleus is uneven and leaves clear areas in which the chromatin condenses into twisted fibers (Fig. 6C). A group of chromatin fibers separates into a bundle that begins to form the anterior (distal) filament (Fig. 6B,C). A cytoplasmic invagination forms a pit in the plasma membrane around the basal part of the flagellum. The basal part of the axoneme is associated with a basal body (Fig. 6E, F). As the compaction and elongation of the nucleus occur, the spermatids take an elongated, conical shape, 6.2 – 7 μm in length, with an expanded posterior end bearing the flagellum. The volume of the cytoplasm started to decrease by shedding off the residual cytoplasm at the basal side of the cell into the lumen of the spermatid cyst. This process ends with the transformation of the spermatids in spermatozoa.

Intercellular bridges are absent between the late spermatids and spermatozoa. Spermatozoa, tightly packed inside the cyst, are long and narrow monoflagellated cells, averaging 6.8 μm in

length and 0.9 μm in width (Fig. 7A), schematic view in Fig. 8. The nucleus is elongate and oval (3.7 μm / 0.84 μm), with electron dense chromatin, denser in the central part (Fig. 7A). An anterior filament, an electron-dense, thin and long rod-like outgrowth (3.1 μm / 0.1 μm), is fully extended. At its tip, the acrosome (Fig. 7A,B), an opaque cone-shaped vesicle, 0.3 μm in high and 0.25 μm in diameter, appears separated from the tip of the nucleus by a basal plate. The proximal region of the flagellum is located within a cytoplasmic tunnel until about a third of the cell length (up to 2.3 μm depth), then becomes free (Fig. 7C,D). Mitochondria are located in the cytoplasm near the flagellum (Fig. 7D). Lipid spheres are concentrated in the proximal part of the spermatozoon and form oval protrusions on the surface (Fig. 7A,D). Glycogen particles are numerous. In the cyst, the flagella are most often disposed in bundles, indicating a precise orientation of the sperm cells within the cyst. Some symbiotic bacteria are usually present between the sperm cells (Fig. 7C).

At this stage two groups of sclerocytes, the cells forming forceps spicules in the cyst, develop inside large invaginations of the follicular cell at opposite poles of the cysts. At the stage of development observed in TEM (Fig. 9A,B,E,F), these invaginations open in the space between the two layers of the cyst envelope, and remain encircled by the outer envelope. Two invaginations are present by cyst, each containing seven to ten sclerocytes. These sclerocytes are elongate (10.3 – 12 x 1.6 – 1.8 μm in sections), with an irregular outline and an oval nucleus 1.5 μm in diameter. Each sclerocyte includes two sections of spicule, 0.25 – 0.35 μm in diameter, with a polygonal axial canal 0.07-0.1 μm visible on desilicified sections. These sections of spicule are most certainly from the two branches of the hairpin-like forceps specific of the mature spermatophores. The origin of these sclerocytes is unknown, but they probably come from the outer envelope of the cyst.

During this development of the spermatozoa in the body of the sponge, the envelope of the cyst shows profound changes. The inner layer, composed of a single follicular cell already present in the first stages of the formation of the cyst (Fig. 9B-D) becomes thicker, with an increasing number of dense spherules, 0.3-0.7 μm in diameter. A secondary envelope made by several cells forms around this first envelope. This outer envelope, up to 5 to 6 μm thick, is always separated from the inner layer by a clear space, 0.9 to 1.2 μm thick, containing collagen fibrils, a variable number of rod-like bacteria similar to the symbiotic ones, and pseudopodia mostly grown from the follicular cell and some from cells of the outer layer (Fig. 9C-D). The cells of this outer envelope are extraordinarily intertwined by their pseudopodia. The cytoplasm is poor in inclusions. The nucleus, 1.5-2 μm in diameter, is devoid of nucleolus and usually irregular in shape, often with a deep indentation on the inwards face (Fig. 9A,D). This layer of intertwined cells is outwardly lined by an extremely thin pinacocyte layer. There are numerous connections between the inner follicular cell and the outer pluricellular layer by means of pseudopodia apparently mostly arising from the follicular cell. In a few cases, the same outer envelope has been observed surrounding two or even three cysts, each with its own follicular cell (Fig. 9A).

The mature cyst, which could be named “spermatophore”, migrates from the sponge ovoid body part to the surface of the filaments, according to an unknown mechanism. On the filaments they determine oval swellings, 35-52 μm in diameter which could be very numerous in some specimens, sometimes about 50 spermatophores per filament (Figs 1, 10A-C). The slight increase in size from the first stages of the spermatocyst to the mature spermatophore is probably mostly due to the formation and progressive thickening of the outer layer. The forceps spicules are not visible in SEM on these spermatophores still attached to the filaments. It is likely that they are still included in the cyst envelope, the diameter of which is slightly greater than the length of the forceps (25-34 μm according to Vacelet & Boury-Esnault, 1996).

Fully mature spermatophores then become free in the water (Fig. 10D), where they can be observed by chance only, even in laboratory conditions. In sections under optical microscope (Fig. 10A,C), they are 35-50 μm in diameter, sometimes up to 60 μm when two cysts occur in the same envelope, slightly oval, with the two follicular envelopes still conspicuous. Two opposite tufts of diverging forceps spicules are present on each side (Fig. 10D). It is presumed that these tufts of

forceps, spicules which are found in the sponge only during spermatogenesis, help the fertilization of another sponge by allowing the capture of spermatophore by the velcro-like cover of anisochelae of its filaments.

Discussion

The present observations on spermatogenesis in *Lycopodina hypogea* confirm and precise the first descriptions already obtained in this species (Vacelet & Boury-Esnault, 1996; Vacelet, 1996; Boury-Esnault & Jamieson, 1999; Vacelet, 2007) and in *L. occidentalis* (Riesgo *et al.*, 2007a). In the absence of choanocytes, of filtering activity and of canal system in carnivorous sponges, the processes of spermatogenesis and of fertilization necessarily widely differ from the general scheme in Porifera. The origin of the male germ cells, the formation of special spermatophores allowing their liberation, as a sperm packet, and the structure of the mature spermatozoa are the most remarkable differences.

Origin of the sperm line

The origin of the sperm cells in the carnivorous sponges already studied is a first unusual character in sponge spermatogenesis. The spermatozoa inside a sperm cyst derive from a single large cell of the archaeocyte type, the prospermatogonium, while in non-cladorhizid Demospongiae and Homoscleromorpha spermatogenesis usually begins with the transformation of whole choanocyte chambers into spermatocysts, with choanocytes individually transforming into spermatogonia. In Hexactinellida, in which choanocytes are anucleate (Boury-Esnault & Vacelet, 1994), sperm cells derive from archaeocytes forming clusters before starting spermatogenesis (Boury-Esnault *et al.*, 1999). The prospermatogonia that we observed in *L. hypogea*, probably also present in *L. occidentalis* but not yet observed, are unusual for sponges. They are very large compared to the somatic cells, twice the size of the larger one, archaeocytes, when all up to now described sponge spermatogonia are nearly of the size of choanocytes (Efremova *et al.*, 1987; Gaino *et al.*, 1986; Barthel & Detmer, 1990; Boury-Esnault & Jamieson, 1999). These observations of prospermatogonia in *L. hypogea*, show that they have cytoplasmic characteristics similar to those of spermatogonia of unicellular origin known in some other metazoans, for example Anthozoa (Larkman, 1984). In particular, their clear cytoplasm includes numerous large mitochondria not found in somatic cells, a large number of ribosomes, and unusually long RER cisternae.

Spermatogonia and spermatocytes

The further development of male germ cells occurs according to a common metazoan scheme: mitotic divisions of the prospermatogonium, then mitotic division of spermatogonia leading to an increase in their number and a decrease in their size. Spermatogonia later pass into the stage of primary spermatocytes and secondary spermatocytes. Interestingly, the flagellum appears at a stage during the transition from the spermatogonia to primary spermatocytes. This is in agreement with most Metazoans, in which the flagellum appears only at an advanced stage of spermatogenesis (Reunov & Hodgson, 1994), but differs from most sponges, in which the choanocyte flagellum is retained in the spermatogonia and during spermatocyte division. This has been shown, for example, in the demosponges *Aplysilla rosea* (Tuzet *et al.*, 1970), *Halichondria panicea* (Barthel & Dermer, 1990), *Crambe crambe* (Tripepi *et al.*, 1994; Riesgo & Maldonado, 2009), *Iophon proximum* (Vasconcellos *et al.*, 2019), some freshwater sponges (Sukhodolskaya & Papkovskaya, 1985; Paulus 1989), and also in some homoscleromorphs (Gaino *et al.*, 1986; Riesgo *et al.*, 2007b). However, in some other demosponges the spermatogonia seem to lose their flagellum of choanocyte origin during the first division and to produce a new one at a late spermatid stage: *Suberites massa* (Diaz & Connes, 1980), *Iophon piceum* (Efremova *et al.*, 1987), *Spongia officinalis* (Gaino *et al.*, 1984), *Hippospongia lachne*, *Spongia barbara*, *S. graminea* (Kaye & Reisinger, 1991), *Tedania ignis* (Vasconcellos *et al.*, 2019).

Primary spermatocytes differ from spermatogonia by a lighter-density of the cytoplasm, a change in the nuclear/cytoplasmic index and the development of synaptonemal complexes in the nucleus. The first meiotic division leads to the formation of secondary spermatocytes, with a reduction in size. These stages of spermatogenesis are in agreement with the general scheme for Demospongiae in particular (Boury-Esnault & Jamieson, 1999) and for metazoans in general.

Spermiogenesis and structure of mature spermatozoa

More drastic transformations occur during spermiogenesis – spermatozoa formation, and result in mature spermatozoa somewhat unusual in Porifera, possibly related to fertilization processes in the absence of aquiferous system and carrier cell.

One of the remarkable transformations is a substantial elongation of the spermatids, with the nucleus stretching accordingly, and condensation of its chromatin. Similar modifications were described in another carnivorous sponge, *L. occidentalis* and also in the Poecilosclerida *Crambe crambe* (Riesgo *et al.*, 2007a; Riesgo & Maldonado, 2009). As in many animals, chromatin compaction in the nucleus is uneven. However, an interesting feature of this process in *L. hypogea* is that in the first stages of compaction, the chromatin becomes fibrillar, twisted and elongated along the long axis of the nucleus. The twisting of the chromatin fibres during condensation may lock in torsion, as has been shown to occur in mesogastropods, some chitons, and in anthozoans (Walker, 1969; Dewel & Clark, 1972; Buckland-Nicks & Chia, 1981; Buckland-Nicks *et al.*, 1990).

These transformations result in a mature elongated, uniflagellate spermatozoon with an unusual character for Porifera, i.e. a dart-shaped nucleus with a long nuclear filament that terminates in a tiny acrosome vesicle. Sponge elongated spermatozoa were described only in Demospongiae, mostly in the order Poecilosclerida: in *Crellomima imparidens*, *Hymedesmia irregularis* (Ereskovsky, 2005), *Crambe crambe* (Riesgo & Maldonado, 2009), *Tedania ignis*, *Iophon piceum* and *I. proximum* (Vasconcellos *et al.*, 2019), and in one species of the order Suberitida – *Halichondria panicea* (Barthel & Detmer, 1990), and in the other studied carnivorous sponge, *Lycopodina occidentalis* (Riesgo *et al.*, 2007a). The elongated shape of spermatozoa and the presence of an elongated nucleus unequivocally categorize this spermatozoon as “modified” or as “derived” (Reunov, 2005).

Electron microscopic studies have shown the presence of an acrosome in diverse sponges, a feature earlier thought to be associated with more evolutionary advanced animals. In particular, acrosomes were revealed in the spermatozoa of all the homoscleromorph species studied (Baccetti *et al.*, 1986; Boury-Esnault & Jamiesson, 1999; Ereskovsky, 2005; Riesgo *et al.*, 2007b) and in one species of Calcarea, *Sycon calcaravis* (Nakamura *et al.*, 1998). However, among Demospongiae, the most species-rich sponge taxon, acrosome bearing spermatozoa were found only in some species from the order Poecilosclerida: *Crellomima imparidens*, *Hymedesmia irregularis*, and *Crambe crambe* (Ereskovsky, 2010; Riesgo & Maldonado, 2009). We confirm in this work the presence of an acrosome in *L. hypogea*, but its origin remains poorly known. We do not know if in this species the acrosome derives from the Golgi apparatus, as for example in the case of Homoscleromorpha (Riesgo *et al.*, 2007b), or if the Golgi cisternae disappear but with persistence of dense proacrosomal vesicles near the nucleus, possibly representing a rudimentary acrosome, as in *Suberites massa*, *Spongia officinalis*, *Lycopodina occidentalis*, *Petrosia ficiformis*, and *Geodia phlegraei* (Diaz & Connes, 1980; Gaino *et al.*, 1984; Riesgo *et al.*, 2007a; Maldonado & Riesgo, 2009; Koutsouveli *et al.*, 2020). A possible formation of the acrosome at the tip of the sperm, completely independent of the Golgi apparatus, would be very unusual. In *L. hypogea*, a remarkable transformation during spermiogenesis is that a bundle of nuclear chromatin projects forward into an anterior filament, an elongate structure containing nuclear extension with an acrosome at its apical part. Such an anterior filament with acrosome has never been described in sponges.

Our investigation on spermiogenesis in *L. hypogea* revealed amazing parallelisms in the structure of the spermatids and spermatozoa with some chitons (Mollusca). In particular, a dart-shaped nucleus with a long nuclear filament that terminates in a tiny acrosome was described only

in some polyplacophoran mollusks (Buckland-Nicks, 2006, 2008; Buckland-Nicks *et al.*, 1990). Such shape of spermatozoa in Polyplacophora may be correlated with peculiarities in the mechanism of fertilization (Buckland-Nicks, 2006, 2008). In chitons, this structure may help to the penetration through the vitelline layer and the oocyte membrane, in which the acrosome digests a minute hole through which the nuclear filament is inserted. A similar role in fertilization of this structure could be supposed in carnivorous sponges. There the sperm cell, directly trapped through a spermatophore in the absence of aquiferous system and carrying cells, has to go through the living tissue up to the oocyte and may need special structure. But this remains highly hypothetic in the absence of available data on the fine mechanisms of fertilization. It could be noted that insemination with a spermatophore often results, both in aquatic and terrestrial invertebrates, in a highly elongated sperm shape (Brown, 1983; Eekhaut & Jangoux, 1991).

Similar to other invertebrate sperms, the flagellum of *L. hypogea* spermatozoon arises from the distal centriole. A long portion of the flagellum is included in a deep flagellar tunnel, which also includes a long portion of the proximal axoneme of elongated spermatozoon. Long cytoplasmic tunnel with axoneme is also known in some spermatozoa of demosponges: *Lubomirskia baikalensis* (Efremova & Papkovskaya, 1980), *Ephydatia fluviatilis*, *Spongilla lacustris* (Sukhodolskaya & Papkovskaya, 1985; Paulus & Wiesenfels, 1986; Paulus, 1989), and *Crambe crambe* (Riesgo & Maldonado, 2009). This structure is similar in general organization to that described in some phoronids (Zimmer, 1991; Reunov & Klepal, 2004), scorpions (Jespersen & Hartwick, 1973), Tetragnathid Spiders (Michalik *et al.*, 2005), or in *Nemertoderma* (Xenacoelomorpha) (Tyler & Rieger, 1975).

Spermatophore

Contrary to Calcarea, in which the poorly known spermatogenesis seems to have a diffuse character without sperm cysts, in Hexactinellida, Demospongiae and Homoscleromorpha spermatogenesis proceeds through formation of sperm cysts, temporary spherical structures bounded by flattened somatic cells (for review see Reiswig, 1983; Boury-Esnault & Jamieson, 1999; Ereskovsky, 2018). Development of male gametes within a cyst may be synchronous (Hexactinellida, Demospongiae) or asynchronous (Homoscleromorpha). These spermatocysts, surrounded by follicle cells deriving from endopinacocytes or archaeocytes, are temporary structures inside the sponge body, and the spermatozoa are released as free cells in the exhalant water current.

In cladorhizids the known spermatocysts are quite different, and most probably related to facilitation of fertilization in the absence of filtering system and in deep-sea conditions, with a possible relationship with the special characters of the spermatozoa, as already discussed higher. They are more permanent structures with a rather complex cell envelope and specific spicules, transmitting a sperm packet to another sponge for fertilization. They correspond to the definition of “spermatophores”, a well-known structure in some vertebrates and in many invertebrates (Mann, 1984), but unknown in Porifera with the exception of the carnivorous Cladorhizidae (Fig. 11). This term was already used by Lee *et al.*, (2012) for the sperm cysts of the cladorhizid *Chondrocladia lyra* and we think that it is well appropriate. The wall of these spermatophores consists of two layers: a primary unicellular layer made of a very thin follicular cell and a secondary one, much thicker and made of several cells with numerous long outgrowths forming a complex interdigitated structure. In addition to its cellular envelopes, the spermatophore of the two studied species of *Lycopodina* includes special spicules (forceps), absent in non-reproducing specimens, which are formed by sclerocytes that are included in the cyst in a late stage. Such spermatophores, well observed in the present work and in Riesgo *et al.*, (2007a) seems to be also present in other carnivorous sponges. They have most likely a role in fertilization in such sponges which are not filtering large amount of water and furthermore live in low density populations in deep-sea environments. As already suggested by Riesgo *et al.*, (2007a) the complex cellular envelope likely has a role in the cyst migration toward the end of the filaments where they are released. When free

in the water, the special spicule complement of the spermatophore may add to its flotation ability and likely contributes to its capture and attachment to another sponge to fertilize, through the Velcro-like cover of chelae, normally designed for prey capture. In other animals where spermatophores are well documented, such as insects (Mann, 1994), they may have an additional nutritional role for the female, including possibilities of specific stimulants of oogenesis. We have no element for such a hypothesis, but this could be interesting to investigate in the future.

Our research shows for the first time the presence of some symbiotic bacteria inside the spermatophores, which is likely transmitted as a whole to the sponge to fertilize. We do not know the role of these microbes in the cysts, and we never saw the penetration of bacteria into male cells, nor the phagocytosis of these bacteria by follicle cells. Given the particularities of fertilization in *Lycopodina* by capturing the spermatophore and its further penetration into the mesohyl, we can hypothesize that this may be a new unique mechanism of male vertical transmission of symbionts through generation. Different ways of vertical transmission of the microbiome is well known for the female reproductive elements, by eggs, embryos and larvae (Ereskovsky & Boury-Esnault, 2002; Ereskovsky *et al.*, 2005). Participation of the male gametes in this mechanism was described only once in *Chondrilla australiensis* (Usher *et al.*, 2005), a case in which the presence of cyanobacteria has been shown inside of free spermatozoa cytoplasm using fluorescent microscopy.

It is interesting to note that such a complex structure, the spermatophore, widely involved in indirect insemination of numerous groups of Metazoa and previously unknown in Porifera, first appeared in the evolution in Porifera, but in a highly specialized group, the Cladorhizidae (Fig. 11).

Spermatogenesis in other Cladorhizidae

In addition to the descriptions of spermatogenesis in *L. occidentalis* and in *L. hypogea*, more fragmentary observations have been made in several deep-sea carnivorous sponges, generally during their description as new species. For instance, sperm cysts have been observed in *Chondrocladia gigantea*, *Cladorhiza methanophila*, *Cladorhiza pteron*, *Abyssocladia koltuni*, *Chondrocladia lyra*, *Cladorhiza kensmithi*, *Abyssocladia oxyasters*, *Abyssocladia microstrongylata*, and a few others (Kübler & Barthel, 1999; Vacelet & Boury-Esnault, 2002; Reiswig & Lee, 2007; Ereskovsky & Willenz, 2007; Lee *et al.*, 2012; Lundsten *et al.*, 2014; Ekins *et al.*, 2020; Vacelet, 2020). All these observations, although generally made on rare and poorly preserved specimens collected by chance from deep-sea environments, indicate that the scheme described in the two *Lycopodina* species may be general in Cladorhizids. In the most detailed observations, in *C. lyra*, the sperm cells are clearly enclosed in spermatophores located at maturity on the special spheres on which normally occurs prey capture (Lee *et al.*, 2012). However, we do not know if in *Chondrocladia*, in which at least some species have conserved an aquiferous system, the sperm cells originate from choanocytes or from archaeocytes. In all cases, including our present study, it is difficult to make a distinction between the mature spermatophores ready to be released from the filaments or spheres normally designed for prey-capture, and those captured for fertilization from another individual.

Conclusions

Although *L. hypogea* is a very propitious material for reproduction study due to its easy access in some field locations and to the possibility of maintaining for years reproducing populations in laboratory condition, several points of its reproductive biology would need complementary studies. Both in the special cave in rather stable temperature condition, around 13°-14°C where the sponge is common, and in laboratory at the same temperature conditions, it seems that sexual reproduction occurs all year round, as also observed in the same cave in the normally deep-sea hexactinellid *Oopsacas minuta* (Boury-Esnault *et al.*, 1999). However, oogenesis and embryogenesis, frequently observed in the natural environment for *L. occidentalis* by Riesgo *et al.*, (2007a), and occurring sometime in our laboratory condition, are more rarely observed than spermatogenesis. Spermatogenesis occurs frequently, but very irregularly, some individuals having

a great number of spermatophores when others have few or none, with a determinism which remains unknown. Feeding on a large prey, which means in deep reorganization of the sponge including a complete regression of the filaments, could interfere with the formation and maturation of the spermatophores and especially with their migration and release along the filaments. There is also the possibility that oogenesis is induced by spermatophore reception, as envisaged by Lee *et al.*, (2012) for *Chondrocladia lyra*. But more precise and specifically dedicated studies are needed for these points.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

Figure legends

Figure 1. *Lycopodina hypogea*. A - *In situ, in vivo* with numerous sperm cysts at the filaments. B – DIC image of filaments with sperm cysts; C – Semi-thin section of sponge body with sperm cysts at different stages of development; D – SEM photo of the body section during spermatogenesis.

fi - filaments, sc – sperm cysts.

Scale bars: A – 5mm; B – 1mm; C – 100µm; D – 50µm.

Figure 2. Spermatogonia. A,B, Prospermatogonia. C, Primary spermatogonia. D, Nuclear area of primary spermatogonia with intranuclear electron dense granules (arrowheads). E, F, Secondary spermatogonia.

er - cisternae of rough endoplasmic reticulum, f – flagellum, fo – follicle during formation; G - Golgi complex; mt – mitochondria, n – nucleus, nu – nucleolus, ph – phagosomes.

Scale bars: A, B – 5µm; C – 3µm; D – 2 µm.

Figure 3. Sperm cysts at early stages of spermatogenesis.

A, B, Sperm cyst with primary spermatogonia. C, Sperm cyst with primary spermatocytes. D - Sperm cysts with a more advanced stages of primary spermatocytes. Formation of second outer layer of sperm cyst is visible – future spermatophore wall. E - Primary spermatocyte with flagellum; F, Sperm cysts with-primary spermatocytes.

bb – basal body of sperm axoneme, f – flagellum, fo – follicle of sperm cyst, l - lipid inclusions, n – nucleus, sw – cells of future spermatophore wall.

Scale bars: A, B, F – 15µm; C, D – 5µm; E – 1µm.

Figure 4. Spermatogenesis: primary spermatocytes (A-C) and secondary spermatocytes (D).

b - symbiotic bacteria, br - cytoplasmic bridges, f – flagellum, fo – follicle; G - Golgi complex; l - lipid inclusions, n – nucleus; sy - synaptonemal complexes

Scale bars: A – 5µm; B, D – 2 µm, C – 3µm.

Figure 5. Spermiogenesis: sperm cysts with secondary spermatocytes and spermatids.

fo – follicle; s2 - secondary spermatocytes, st – spermatids, sw – cells of spermatophore wall.

Scale bars: A-C – 10 µm.

Figure 6. Spermiogenesis: spermatids. A, Spermatids in the sperm cyst; B, Nuclear chromatin aggregation and formation of the anterior filament and acrosome; C, Nuclear chromatin condensed into fibres, which become twisted; D, Developed spermatid with compact chromatin and axoneme tunnel; E, F, Fragments of spermatids showing the basal body of the axoneme and axoneme tunnel.

a – acrosome, af- anterior filament of a nucleus, ax – axoneme, b – symbiotic bacteria, bb – basal body of sperm axoneme, bp –basal plate of acrosome, fo – follicle; gl – glycogen rosettes, l - lipid spheres, mt – mitochondria, n – nucleus; st - spermatids.

Scale bars: A – 2.5µm; B-D – 1µm; E – 2µm; F– 0.5µm.

Figure 7. Spermatozoa. A, A longitudinal section of a spermatozoon inside of sperm cyst; B, Acrosome and anterior filament of a nucleus. Inset - acrosome; C, A longitudinal section of a central part of spermatozoon ; D, Cross-section of a middle part of spermatozoon.

a – acrosome, af- anterior filament of a nucleus, ax – axoneme of sperm, b – symbiotic bacteria, bb – basal body of sperm axoneme, bp –basal plate of acrosome, gl – glycogen rosettes, l - lipid spheres, mt – mitochondria, n – nucleus.

Scale bars: A – 1µm; B – 0.2µm; C – 0.5µm; D – 0.2 µm.

Figure 8. Scheme of *Lycopodina hypogea* spermatozoon.

a – acrosome, af- anterior filament of a nucleus, ax – axoneme of sperm, bb – basal body of sperm axoneme, gl – glycogen rosettes, l - lipid spheres, mt – mitochondria, n – nucleus.

Figure 9. Last stages of spermatogenesis and spermatophore wall formation and structure. A, Sperm cysts covered by spermatophore wall during their migration at the surface of filament. B, Formed spermatophore bearing two bundles of spicules going into the water. C, D, Spermatophore wall structure. E, F, Longitudinal and cross sections of the bundles of sclerocytes, bearing spicules.

b – symbiotic bacteria, ex – exopinacocyte, n – nucleus, s – spicule, sb – bundle of sclerocytes, bearing spicules, sc - sperm cysts, scl – sclerocyte, sp – spermatozoon, sw - spermatophore wall.

Scale bars: A, B – 10µm; C, D, E – 3µm; F – 1.5µm;

Figure 10. Spermatophores of *Lycopodina hypogea*. A, Confocal microscopy image of mature spermatophores in the filament, with DAPI labeling. B –SEM image of the mature spermatophores at the surface of filaments; C – Semi-thin section of mature spermatophores; D - A spermatophore, floating in the water.

fi - filaments, sb – bundle of spicules, sf – spermatophore.

Scale bars: A, B, C – 50µm; D – 30µm.

Figure 11. Scheme of phylogenetic distribution of spermatophores in animals.