Departamento de Biología Celular
Universidad de Barcelona

Sexual reproduction in demosponges: ecological and evolutive implications

Reproducción sexual en demosponjas: implicaciones ecológicas y evolutivas

Ana Riesgo Gil

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Chapter 7:

▦ Introduction

In many cases among invertebrates and vertebrates, the totality of produced sperm is never released during spawning (e.g., Jørgensen and Lützen 1997; Quintana et al. 2004; Kalachev and Reunov 2005). Phagocytosis of unspawned sperm and/or the resorption of the whole testis are the two events that immediately take place in the empty testes of most animals. Phagocytic activity is carried out by somatic cells. Depending on the animal group, these cells have been identified as Sertoli cells (Buckland-Nicks and Chia 1986; Jørgensen and Lützen 1997) or coelomocytes, amoebocytes, and macrophages (Pacey and Bentley 1992; Kalachev and Reunov 2005). In most cases these cells phagocytose not only unspawned sperm but also spermatocytes or spermatozoa that are identified as aberrant or abnormal during the course of spermatogenesis, both in invertebrates (Buckland-Nicks and Chia 1986; O'Donovan and Abraham 1987; Jørgensen and Lützen 1997) and vertebrates (Griswold 1995, 1998; Nakanishi and Shiratsuchi 2004).

Sperm-phagocytic cells are found both in invertebrates and vertebrates. Among the invertebrates, such cells occur in plathelmints (O'Donovan and Abraham 1987), marine gastropods (Buckland-Nicks and Chia 1986), echinoderms (Chia and Buckland-Nicks 1987; Reunov et al. 2004; Kalachev and Reunov 2005), ascidians (Jørgensen and Lützen 1997), and cephalochordates (Holland and Holland 1989). However, they have not always been called Sertoli cells, but "accessory cells", "auxiliary cells", "nutritive phagocytes" or "wall cells". In order to simplify terminology, Buckland-Nicks and Chia (1986) proposed to use the term Sertoli cells to those cells with an analogous

functioning to them. The relationship between the germinal cells and the Sertoli cells in testis development as well as in spermatogenesis is obligatory in many animals (Griswold 1995, 1998). They are usually located in the walls of the testis and seminiferous tubules, but in some cases they can detach and migrate into the lumen (Jørgensen and Lützen 1997; Ramofafia et al. 2003). When in the walls of the testis, Sertoli cells provide crucial factors that facilitate the successful progression of germ cells into spermatozoa. This facilitation may be in the form of either physical support, creating a blood-testis barrier, or biochemical stimulation by supply of growth factors and/or nutrients (Buckland-Nicks and Chia 1986; Griswold 1998). In addition, Sertoli cells appear to phagocytose sperm cells to control germ cell numbers in testis (Buckland-Nicks and Chia 1986; Griswold 1995; Jørgensen and Lützen 1997).

Coelomocytes, amoebocytes, and macrophages, which are cells usually involved in elimination of unwanted cellular and non-cellular material from the coelom of invertebrates (Dhainaut and Porchet-Henneré 1989), can additionally play the same function as Sertoli cells in bivalves (Vaschenko et al. 1997) and polychaetes (Pacey and Bentley 1992).

Sponges are gonad-lacking organisms (Bergquist 1978), with no definite line of germ cells (Fell 1983; Simpson 1984; Boury-Esnault and Jamieson 1999). Depending on the species, 2 somatic cell types have been postulated as the origin of spermatogonia, archaeocytes -amoeboid totipotent cells- and, more frequently, choanocytes -flagellated collar cells involved in particle capture and sponge feeding- (Reiswig 1983; Boury-Esnault and Jamieson 1999). Spermatogenesis takes place in spermatic cysts (spermatocysts) located within the internal tissue of sponges -i.e. choanosome or mesohyl depending on species- (Reiswig 1983; Simpson 1984). Spermatic cysts are generally, but not always, surrounded by follicle cells apparently derived from functional adult cells, that in most cases are pinacocytes (pseudo-epithelial cells) or archaeocytes (Boury-Esnault and Jamieson 1999). Sperm release has been observed in very few demosponges, being a population synchronous event in most gonochoristic, oviparous species (Reiswig 1970, 1976; Lévi and Lévi 1976; Hoppe and Reichert 1987; Ritson-Williams et al. 2004). The fate of unspawned sperm has rarely been approached so far. In empty spermatic cysts of *Halichondria panicea*, phagocytic vacuoles appeared within the follicle cells after the spawning events (Barthel and Detmer 1990). Such vacuoles were interpreted as phagosomes, which would indicate that follicle cells

derived from archaeocytes. Diaz and Connes (1980) suggested that follicle cells of the spermatocysts of *Aplysilla rosea* can eliminate abnormal sperm by phagocytosis, although the issue was not further investigated. In the current study, we investigate the fate of unspawned sperm by examining spermatic cysts of pre- and post-spawning individuals of two gonochoristic demosponge species, *Raspaciona aculeata* and *Petrosia ficiformis*.

Material and methods

For a long-term monitoring of the spermatogenesis of *Raspaciona aculeata* and *Petrosia ficiformis* we sampled and tagged several individuals (see below) from a sublittoral rocky community in Blanes (northeastern Mediterranean coast of Spain). Five individuals of *R. aculeata* were tagged and collected monthly from January 2004 to November 2005. During the first year, we assessed the duration of the spermatogenic cycle, which ended in November. Hence, the second year we increased number of sampled individuals (N=13) from September to November, and we stopped the sampling in November. Unfortunately, we missed the last stages of spermatogenesis, since the cycle was unexpectedly delayed this year. Likewise, five individuals of *P. ficiformis* were tagged and collected monthly, but in this case the monitoring was undergone from October 2003 to December 2006. Number of sampled individuals increased to 19 in November 2005 and 25 in November-December 2006.

We collected small tissue pieces (approx. 0.7 x 0.5 x 0.3 cm of *Raspaciona* and 1 x 1 x 0.5 cm of *Petrosia*) from each sponge using scuba and surgical scissors at each sampling time. Tissue samples were divided into two pieces, one for light microscopy and the other for electron microscopy.

Tissue samples for light microscopy were maintained in ambient seawater for transportation to the laboratory and fixed within 2 h after collection in 4% formaldehyde in seawater for 24 h. Then, samples were desilicified with 5% hydrofluoric acid for 5 h, rinsed in distilled water, dehydrated through a graded ethanol series, cleared in toluene, and embedded in paraffin to cut them into 5 μm-thick sections with an Autocut Reichert-Jung microtome 2040. After deparaffining with xylene, sections were stained

with Hematoxylin-PAS, and studied through a Zeiss Axioplan II compound microscopy.

Samples for electron microscopy were always fixed in 2.5% glutaraldehyde in 0.2 M Milloning's phosphate buffer (MPB) and 1.4 M sodium chloride, and stored until the light microscopy study of the individuals recommended post-fixation. Samples were then rinsed with MPB for 40 min, post-fixed in 2% osmium tetroxide in MPB, dehydrated in a graded acetone series, and embedded in Spurr's resin. Ultrathin sections obtained with an Ultracut Reichert-Jung ultramicrotome were mounted on gold grids and stained with 2% uranyl acetate for 30 min, then with lead citrate for 10 min. Observations were conducted with a JEOL 1010 transmission electron microscope (TEM) operating at 80 kV and provided with a Gatan module for acquisition of digital images.

Results

Motile phagocytic cells in *Raspaciona aculeata*

The dynamic of spermatogenesis, which extended through October in 2004 and from October to November in 2005, is reported in Chapter 1. Spermatic cysts containing spermatocytes II measured approximately 200 μm in their largest diameter (Fig. 1A) and were enveloped by a single layer of follicle cells. Follicle cells were flat, measuring approximately 15-20 μm in largest diameter (Fig. 1B). They showed usually a nucleolate nucleus and many phagosomes within the cytoplasm, as well as a large Golgi apparatus with lamellae oriented parallel to the external nuclear membrane (Fig. 1B-C). There were small spaces between follicle cells in few occasions, which could be either fixation artefacts or true spaces. Occasionally, some follicle cells left the envelope and entered the cyst (Fig. 1C). Once inside the cyst, we referred to these cells as "motile phagocytic cells (MPCs)". Apart from spermatocytes and MPCs, free bacteria were also observed in the lumen of the cysts (Fig. 1D). Large MPCs (up to 15 μm in diameter) were found in the lumen of the cysts intermingled with spermatocytes II, which at this stage measured 2 μm in diameter (Fig. 1A, D; 2A-B). MPCs were amoeboid, with a round nucleolate nucleus measuring approximately 3-4 μm (Fig. 1C-D; 2A). Their cytoplasm showed, as that of follicle cells (Fig. 1B), multiple phagosomes in different

digestion stages (Fig. 1C-D), many electron-clear vesicles (Fig. 1C-D; 2A-B), dictyosomes (Fig. 1C), and mitochondria (Fig. 2A). These cells were observed: a) engulfing bacteria and presumably exocytosed excess of the spermatocytes' cytoplasm (Fig. 2B), b) approaching spermatocytes and developing pseudopodia to start engulfing them (Fig. 1D); and c) showing already engulfed spermatocytes, which located in membrane-bound vesicles (Fig. 2A).

For unknown reasons, occasionally, large cells (approximately 15 μm in their largest diameter) were observed in the mesohyl of the sponge close to spermatocysts phagocytosing secondary spermatocytes (Fig. 2C-D). Surprisingly, they were engulfing secondary spermatocytes within the mesohyl (Fig. 2D), although they were very close to a spermatic cyst. Those large cells were somewhat different from motile phagocytic cells. They showed few phagosomes within their cytoplasm, and, looked like regular archaeocytes of the sponge. They possessed a large nucleus (approx. 3.5 μm), apparently anucleolate, with finely condensed chromatin (Fig. 2D). Golgi apparatus and some vesicles containing lipid droplets appeared in the cytoplasm (Fig. 2D). Therefore, they were interpreted as archaeocytes.

Motile phagocytic cells in *Petrosia ficiformis*

We examined ultrastructurally spermatic cysts (Fig. 3A) after the spawning event occurred in November 2006. At this stage, cysts were lax, lined by a discontinuous layer of cells (Fig. 3A), and virtually empty of sperm. Small round cells (approx. 5 μm) were found inside the spermatic cysts, which contained some unspawned spermatozoa (Fig. 3A), and in the mesohyl of the sponge (Fig. 3D). These cells located around unspawned spermatozoa, sometimes containing engulfed sperm (Fig. 3B-D; 4A). As they displayed a similar phagocytic activity as in *Raspaciona aculeata*, we referred them as "motile phagocytic cells (MPCs)". MPCs were amoeboid to round cells, showing a nucleolate nucleus (Fig. 3B-D; 4A). They often emitted multiple pseudopodia, presumably to complete engulfment of unspawned spermatozoans within the cysts (Fig. 3B-D). Numerous phagosomes in different stages of digestion were observed within their cytoplasm (Fig. 3D; 4A). Many large vacuoles
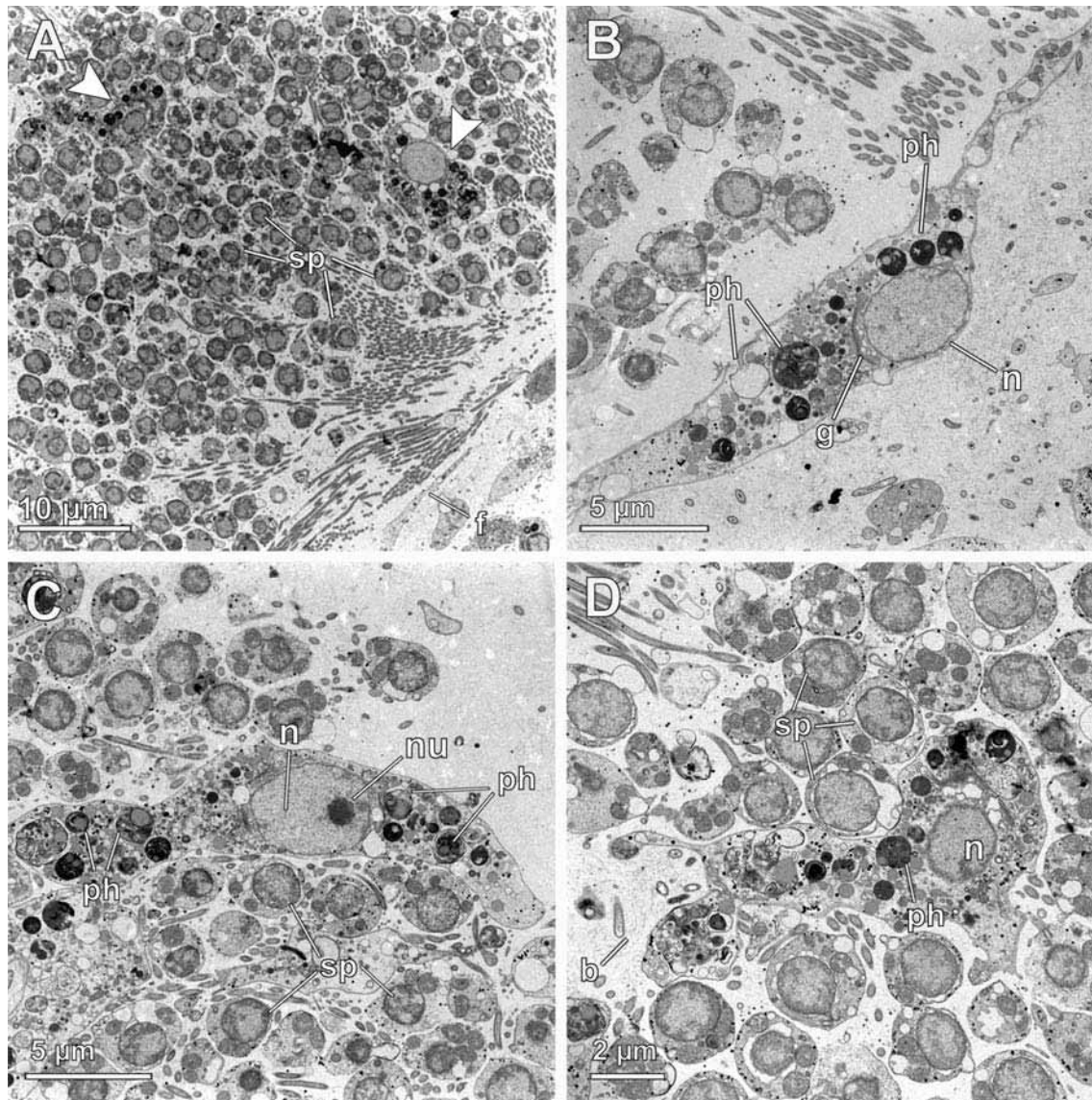
Figure 1. Spermatic cysts and motile phagocytic cells in *Raspaciona aculeata*. **(A)** Spermatic cyst containing secondary spermatocytes (sp) and motile phagocytic cells (arrow heads). Note the follicle cell partly enclosing the cyst (f). **(B)** Follicle cell lining the spermatic cyst showing an oval nucleus (n), a well-developed Golgi apparatus (g), and multiple phagosomes (ph) within the cytoplasm. **(C)** Follicle cell detaching from the follicle and entering the spermatic cyst that contained secondary spermatocytes (sp). Note the nucleolate (nu) nucleus (n) of the cell, and the many phagosomes within the cytoplasm (ph). **(D)** Motile phagocytic cell intermingled with secondary spermatocytes (sp) inside the spermatic cyst. Note the nucleus (n) and the multiple phagosomes (ph) of the cytoplasm. Free bacteria (b) can be detected in the lumen of the cyst.
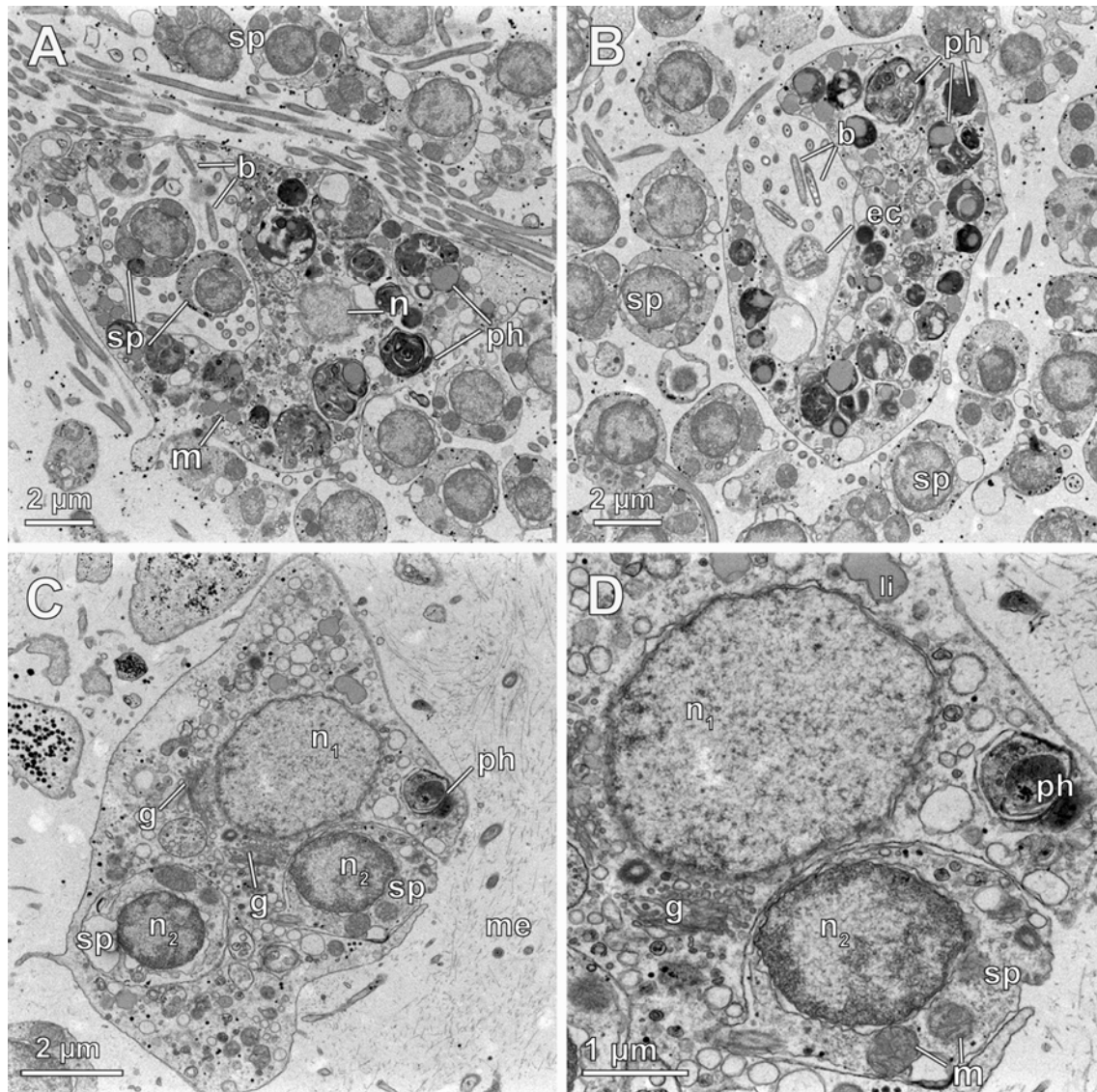
Figure 2. Motile phagocytic cells within the spermatic cysts and within the mesohyl in *R. aculeata*. **(A)** Motile phagocytic cell inside a spermatic cyst phagocytosing secondary spermatocytes (sp) and bacteria (b). The nucleus appeared to be cross-sectioned (n). Note the numerous phagosomes (ph) and the mitochondria (m) within the cytoplasm. **(B)** Motile phagocytic cell inside a spermatic cyst engulfing free bacteria (b) and presumable excess of cytoplasm (ec) jettisoned by secondary spermatocytes. **(C)** Phagocytic cell in the mesohyl of the sponge (me) engulfing two secondary spermatocytes (sp). In the cytoplasm of the phagocytic cell can be observed the nucleus (n_1), several Golgi apparatus (g), and phagosomes (ph). Note the different appearance of the secondary spermatocyte's nucleus (n_2). **(D)** Magnification of C, showing the phagocytic cell's nucleus (n_1), the Golgi apparatus (g), the phagosome (ph), and lipid droplets (li). Note the nucleus (n_2) and the two mitochondria (m) of the secondary spermatocyte (sp).
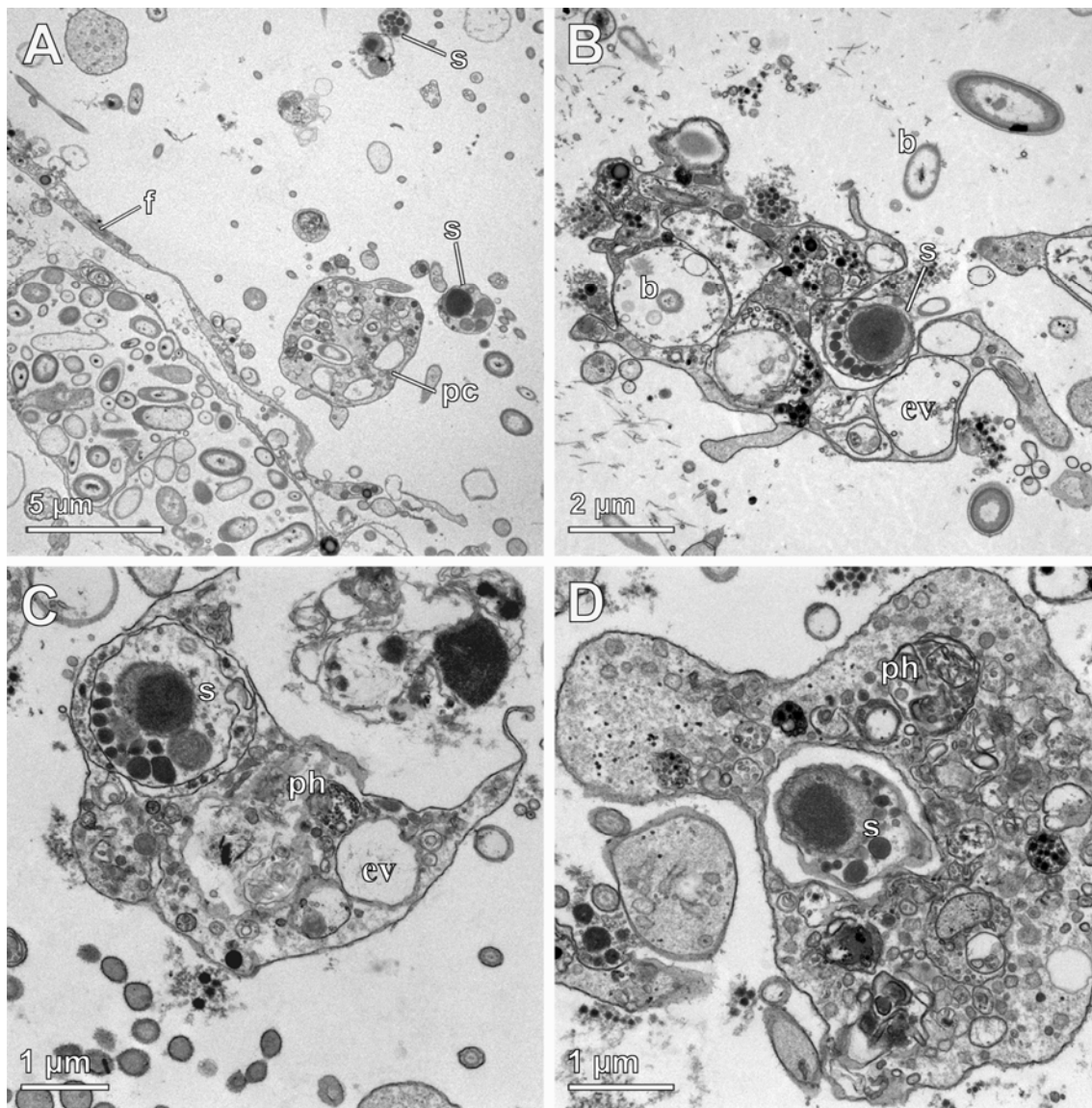
Figure 3. Motile phagocytic cells within the spermatic cysts of *Petrosia ficiformis*. **(A)** View of a virtually empty spermatic cyst lined by a single cellular follicle (f), containing few mature spermatozoans (s), and a motile phagocytic cell (pc). **(B)** Motile phagocytic cell inside an almost disassembled spermatic cyst engulfing a mature spermatozoan (s). Note the occurrence of free bacteria (b) in the lumen of the cyst, and in a phagocytic vacuole of the cell, and the electron-clear vacuole (ev). **(C)** Motile phagocytic cell showing an already engulfed spermatozoan (s). Note the heterogeneous appearance of the pseudo-digested content of a phagocytic vacuole (ph), and occurrence of electron-clear vacuoles (ev). **(D)** Motile phagocytic cell with a phagocytosed spermatozoan (s) in a large vacuole and many phagosomes (ph).
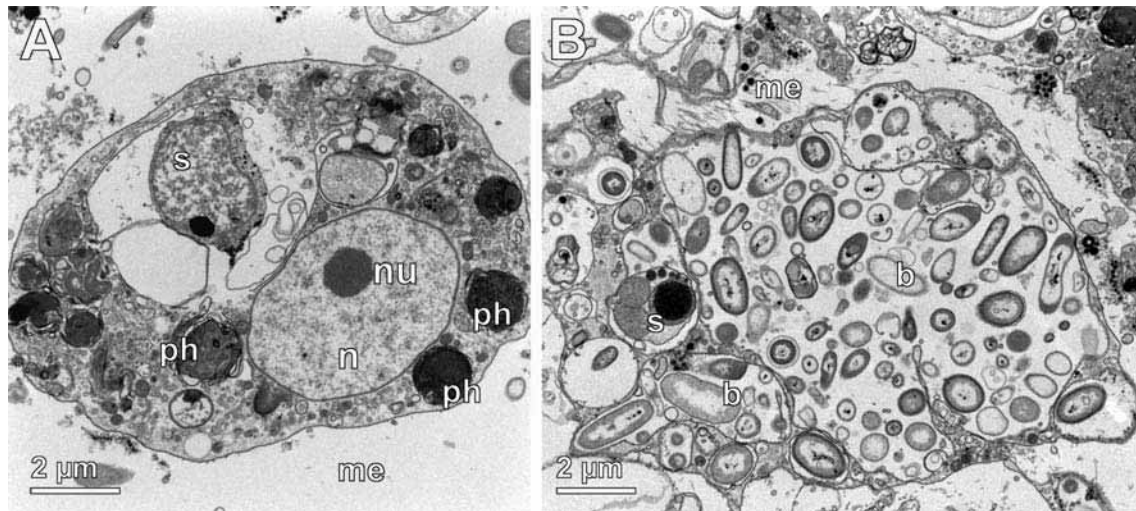
Figure 4. Motile phagocytic cells and bacteriocytes in the mesohyl of *Petrosia ficiformis*. **(A)** Phagocytic cell in the mesohyl (me), during the digestion of a sperm cell (s). Note the nucleolate (nu) nucleus (n) of the cell and the many phagosomes (ph) within the cytoplasm. **(B)** Bacteriocyte in the vicinity of an empty spermatic cyst showing large vacuoles charged with bacteria (b) and a mature spermatozoan (s).

also occurred in their cytoplasm, some of them containing phagocytosed bacteria (Fig. 3B), as well as large electron-clear vacuoles (Fig. 3B-C).

In few cases, we found large bacteriocytes in the mesohyl containing engulfed unspawned sperm (Fig. 4B). They located close to empty spermatic cysts. Phagocytosed sperm was in large vesicles that also contained bacteria (Fig. 4B).

Discussion

Gamete resorption is a widespread process in marine invertebrates. Oocyte resorption has been documented in cnidarians (Szmant-Froelich et al. 1980; Kruger and Schleyer 1998; Neves and Pires 2002), nemertines (Bierne 1983), acanthocephalans (Crompton 1983), molluscs (Jong-Brink et al. 1983; Dorange and Le Pennec 1989; Fabioux et al. 2005), polychaetes (Olive et al. 1981), tardigrades (Bertolani 1983), and brachiopods (Chuang 1983). However, phagocytosis of unfertilized oocytes has only been observed in sipunculans (Rice 1983) and crustaceans (Adiyodi and Subramoniam 1983), while in the rest of the cases, degeneration of oocytes was the rule. In contrast, phagocytosis of sperm (unspawned or abnormal) in marine invertebrates is more

frequent than simply sperm degeneration (Buckland-Nicks and Chia 1986; O'Donovan and Abraham 1987; Chia and Buckland-Nicks 1987; Holland and Holland 1989; Jørgensen and Lützen 1997; Reunov et al. 2004). Phagocytosis of sperm is usually carried out by somatic cells which are usually accessory cells located in the testis wall (generally called Sertoli cells) (Buckland-Nicks and Chia 1986), or in the lumen of the testis (Pacey and Bentley 1992; Jørgensen and Lützen 1997; Vaschenko et al. 1997; Ramofafia et al. 2003; Kalachev and Reunov 2005).

In sponges, the fate of waste gametes (i.e., unfertilized or unspawned) has been investigated only for oocytes. Degeneration and resorption of unfertilized oocytes has been reported in *Grantia compressa* (Duboscq and Tuzet 1937), *Halisarca dujardini* (Lévi 1956), *Sycon raphanus* (Colussi 1958), *Hymeniacidon sanguinea* (Sarà 1961), *Petrobiona massiliana* (Vacelet 1964), *Haliclona ecbasis* (Fell 1969), *Spongilla lacustris* (Gilbert 1974), *Verongia cavernicola* and *Verongia aerophoba* (Gallissian and Vacelet 1976), *Suberites massa* (Diaz 1979) and *Halichondria okadai* (Tanaka-Ichihara and Watanabe 1990). In *Haliclona permollis* oocytes were found to be further numerous than embryos, suggesting an important oosorption after fertilization (Elvin 1976). In *Haliclona loosanooffi* (Fell 1976a) and *Corticium candelabrum* (Riesgo et al. 2007) production of new oocytes was documented simultaneously to maturation and fertilization of mature oocytes, being these new oocytes presumably resorbed or used as food for the latter ones.

In contrast to oocytes, the issue of sperm resorption during spermatogenesis and after spawning has never been thoroughly investigated in sponges to date. Diaz and Connes (1980), in their remarkable ultrastructural study of the spermatogenesis of *Aplysilla rosea*, suggested that follicle cells could remove sperm waste (unspawned sperm) by phagocytosis. Likely, Barthel and Detmer (1990) noticed that the follicle cells of the empty spermatic cysts of *Halichondria panicea* contained phagocytic vacuoles, and interpreted them as phagosomes that would support their archaeocytic origin. Nevertheless, those phagosomes could well be the result of sperm phagocytosis.

Phagocytosis of unspawned sperm (Fig. 5A) has been noted in *Chondrilla nucula* (Maldonado and Riesgo, unpublished). After a massive sperm spawning in a Bahamian population, samples of male individuals were fixed and examined by TEM, revealing phagocytic activity of archaeocyte-like motile cells over unspawned sperm located within the mesohyl (Fig. 5B). The fact that aberrant sperm has been found in

Chondrilla australiensis prior and after spawning (Usher et al. 2004), led us to think that the phagocytic cells of the genus *Chondrilla* could be involved in elimination of both unspawned and aberrant sperm cells.
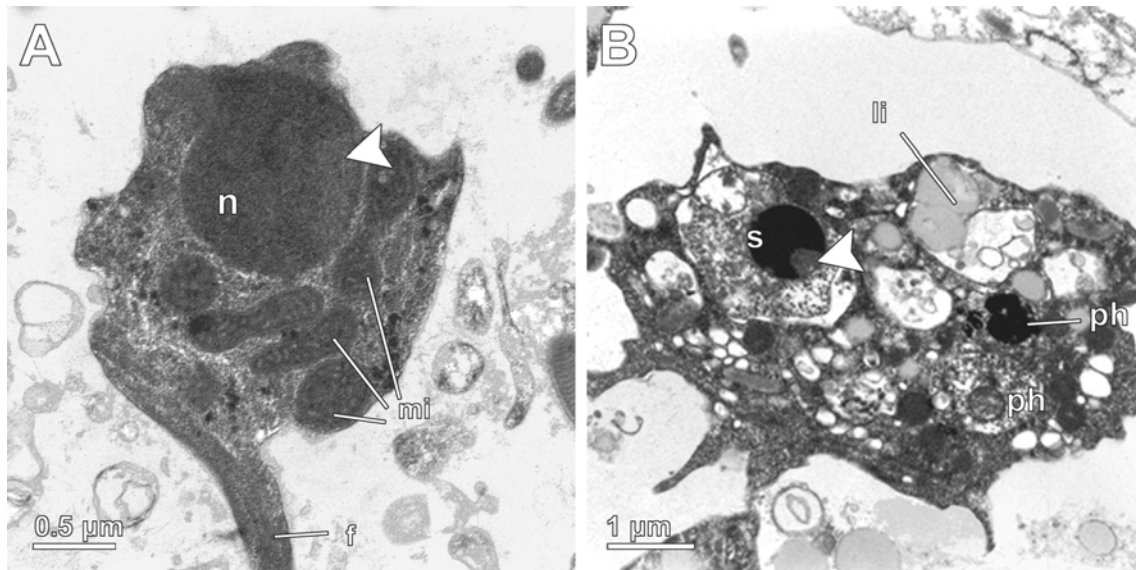

Figure 5. Phagocytosis of sperm in *Chondrilla nucula*. **(A)** Unspawned mature spermatozoon of *Chondrilla nucula* found within the mesohyl of the male. Note the clearer region (arrowhead) of the nucleus (nu), the multiple mitochondria (mi), and the single flagellum (f). **(B)** Archaeocyte-like cell showing phagocytosed sperm (s) with a clear region in the nucleus (arrowhead), and phagosomes (ph) in different digestion stages.

Motile phagocytic cells found in the cysts of *Raspaciona aculeata* appeared to be follicle cells that detach and penetrate the lumen of the cyst. In addition, archaeocyte-like cells also contained engulfed spermatocytes, but they occurred outside the cysts, in the mesohyl of the sponge. Therefore, although follicle cells can migrate into the cyst to operate somehow spermatocyte removal in *R. aculeata*, we can not discard that archaeocytes can also enter the cysts and participate in this task. In *Petrosia ficiformis*, the objective of the MPCs was fairly distinct. Motile phagocytic cells that occurred in spermatic cysts and the mesohyl removed completely mature sperm that had not been spawned. In this particular case, phagocytic cells were more similar to sponge archaeocytes than to follicle cells of the cyst. Archaeocytes are typical constituents of demosponge mesohyl, being amoeboid motile cells with a nucleolate nucleus and evident phagocytic activity (Simpson 1984). These cells have also been pointed out as

the most probable origin of oocytes and follicle cells of both oocytes and spermatocysts in many demosponges (Simpson 1984). Since they are motile cells their occurrence in the lumen of spermatic cysts is not an unthinkable option. Moreover, the contact between follicle cells of the spermatic cysts of *P. ficiformis* and many other demosponges occasionally, is not very tight. Thus, penetration of archaeocytes to the lumen of cysts can be facilitated.

The present study shows that sponge MPCs are involved in both removal of unspawned sperm (as observed in *Petrosia ficiformis*) and elimination of presumably aberrant sperm during spermatogenesis (probably the case in *Raspaciona aculeata*). Although we assume that the spermatocytes phagocytosed within the cysts of *R. aculeata* were abnormal, other hypotheses, in which sperm abnormality does not occur, can be taken into account to explain these observations.

1. The gametogenic cycle of *Raspaciona aculeata* was investigated in Chapter 1, finding that the sponge is oviparous. It was observed that spermatogenesis caused a mesohyl disruption in male individuals, a phenomenon previously reported for other sponges (Tanaka-Ichihara and Watanabe 1990; Tsurumi and Reiswig 1997; Ereskovsky 2000). This mesohyl disruption implies a loss of nearly all choanocyte chambers, which is the main structure involved in capture of bacteria and particulate material for feeding (Simpson 1984). The presumable starvation suffered by males while producing their sperm may lead to situations of sperm self-predation, in order to regain critical nutritive reserves to palliate starvation. Although the direct costs of sperm production are poorly understood in invertebrates and vertebrates (Wedell et al. 2002), it has been observed that sometimes sperm production reduces life-span, as in *Caenorhabditis elegans* (Van Voorhies 1992), or produces a great loss of body-mass, as detected in the adder *Vipera berus* (Olsson et al. 1997). Thus, a mechanism aimed to recapture some reserves and palliate starvation should not be unlikely.

2. Griswold (1995, 1998) proposed an alternative explanation. He considered that the essential role of Sertoli cells during spermatogenesis was to prevent proliferation of sperm cells beyond an unsatisfactory threshold by phagocytosing them. In *Hydra* testis, apoptotic sperm precursors are removed by the epithelial cells that line the testis (Kuznetsov et al. 2001).

Although it has been interpreted as a "quality control" developed by such cells, the authors did not discard Griswold's alternative explanation for their findings. Apoptosis of sperm cells and subsequent removal from the testis could be directed to maintain or achieve the precise homeostasis for each sperm cell and/or contribute to maintain a critical cell number ratio between differentiating spermatogonia and epithelial cells. A similar explanation may not be ruled out in the case of *Raspaciona aculeata*.

The occurrence of active phagocytic cells in spermatogenesis of sponges has further implications. Sponges are considered as primitive organisms with very simple characteristics and simple behaviour. Regarding their gametogenesis, it is widely accepted that sponge gametes possess "primitive" features (Reunov 2005), such as spherical or conical shape with a flagellum running from it, few large mitochondria within the sperm head, and the absence of an acrosome (Afzelius 1972; Baccetti and Afzelius 1976; Baccetti 1984, 1986). However, many relatively recent discoveries regarding sponge reproduction and development made this "simplicist" vision swayed. Proacrosomal vesicles, similar to those reported in cnidarians (Franzén 1996), have been found in few sponges (Diaz and Connes 1980; Gaino et al. 1984), and a true acrosome has been ultrastructurally described in sperm of different demosponge species (Tripepi et al. 1984; Baccetti et al. 1986; Riesgo et al. 2007; Riesgo and Maldonado, in press). Extremely modified sperm morphologies are also found in demosponges (Tripepi et al. 1984; Barthel and Detmer 1990; Riesgo and Maldonado, in press). Regarding embryogenesis, many authors refer the sponge larva as a blastula (e.g., Simpson 1984; Rupert and Barnes 1996; Ereskovsky 1999). Although the occurrence of gastrulation in sponges is still a matter of discussion (Ereskovsky and Dondua 2006), such process appears to be unequivocally confirmed by some authors (Leys 2004; Maldonado 2004). All these examples appear to support the vision that in sponges both simple and complex features coexist. The occurrence of MPCs involved in sperm elimination prior or after completion of spermatogenesis comes to the still incomplete list of complex features displayed by sponges that are widely known in higher invertebrates. Furthermore, it shows that processes aimed to regulate tissue functioning in higher metazoans have their equivalent in sponges. Although MPCs lack of the complexity displayed by Sertoli cells, their functionality can be considered analogous.

Consequently, we tentatively suggest referring to MPCs that enter spermatic cysts in sponges as Sertoli-like cells.