

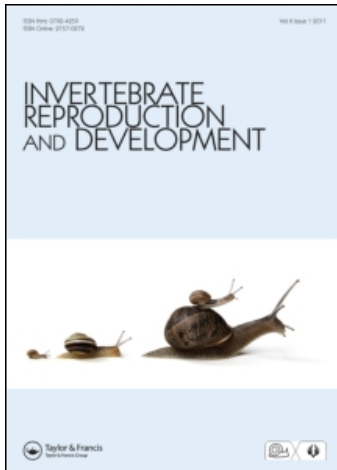
This article was downloaded by: [Riesgo, Ana]

On: 4 March 2011

Access details: Access Details: [subscription number 934405814]

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## Invertebrate Reproduction & Development

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t926968007>

### Ultrastructure of the gametogenesis of the common Mediterranean starfish, *Echinaster (Echinaster) sepositus*

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First published on: 04 March 2011

**To cite this Article** Riesgo, Ana , Villamor, Adriana and Becerro, Mikel(2011) 'Ultrastructure of the gametogenesis of the common Mediterranean starfish, *Echinaster (Echinaster) sepositus*', Invertebrate Reproduction & Development,, First published on: 04 March 2011 (iFirst)

**To link to this Article:** DOI: 10.1080/07924259.2011.558182

**URL:** <http://dx.doi.org/10.1080/07924259.2011.558182>

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## Ultrastructure of the gametogenesis of the common Mediterranean starfish, *Echinaster (Echinaster) sepositus*

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(Received 2 July 2010; final version received 21 January 2011)

*Echinaster (Echinaster) sepositus* is one of the most abundant sea stars in western Mediterranean rocky bottoms, yet its reproductive biology remains virtually unknown. Here we report the ultrastructure of its gametogenesis over 2 consecutive years. It is a gonochoric species with an annual reproductive cycle spawning gametes in late summer and early autumn. Each arm of every individual contained two gonads (dark red in females and yellow in males). In both sexes, the gonad was a single, large sac composed of several smaller sacs. The gonad wall consisted of two multilayered sacs, outer and inner, separated by the genital haemal sinus. The histology of the gonad wall was consistent with that found in other asteroids. Oogenesis was continuous during the year, but eggs were spawned only in late summer. Oocytes were in close relationship with follicular cells that are suggested to transfer nutrients to the oocytes. Spermatogenesis was restricted to 5–6 months in spring-summer. It occurred in columns with an axial interstitial cell supporting each column, and producing processes towards the lumen that remained connected to spermatogenic cells by intercellular junctions. Developing sperm cells were found along the length of the column, while spermatozoa were found free in the testis lumen. Spermatogenesis followed the pattern described for echinoderms, to give rise to an acrosome-bearing, round-shaped spermatozoon. The histology and cytology of the reproductive process in *E. sepositus* followed the general pattern found for asteroids.

**Keywords:** oogenesis; spermatogenesis; Asteroidea; gamete; Echinasteridae

### Introduction

Understanding the reproductive patterns of a given species is an essential step to understand most of its biological traits, such as its life cycle, distribution, population dynamics, ecology, and ultimately its evolution. Reproduction of sea stars, like that of other echinoderms, has long fascinated biologists. Even though asteroids can reproduce either by asexual or sexual means (Chia and Walker 1991), asexual reproduction is restricted to few genera and species (Ottesen and Lucas 1982; Chia and Walker 1991; Mladenov 1996; Knott et al. 2003), and therefore the sexual reproduction of starfish has attracted most attention. Sexual reproduction in asteroids ranges from gonochorism, through labile hermaphroditism, to functional hermaphroditism (Cognetti and Delavault 1962; Mercier and Hamel 2009).

With regard to the reproductive biology of the class Asteroidea, the order Forcipulatida is amongst the best studied taxa, perhaps because it includes common shallow-water species worldwide, many of them

considered keystone predators. Investigations include the ultrastructure and physiology of gametogenesis, exogenous and endogenous factors affecting reproduction, and the cytology and physiology of gamete development (Chia and Walker 1991). The genus *Asterias* has attracted most attention (e.g., Walker 1974, 1980; Schoenmakers et al. 1981; Beijinink et al. 1984b), providing important information about the progression of gametogenesis in starfish. In comparison, the sexual reproduction of the remaining asteroid orders has been addressed less frequently (e.g., Lawson-Kerr and Anderson 1978; Tyler et al. 1984; Alves et al. 2002; Byrne et al. 2003). Presumably, that is the reason why features observed in forcipulatids are often considered a common pattern for asteroids.

The asteroid family investigated here is the family Echinasteridae, which belongs to the order Spinulosida, whose most distinct characteristic is the small number or complete absence of pedicellariae (Hyman 1955). The family Echinasteridae contains four genera, *Henricia*, *Echinaster*, *Poraniopsis*, and *Stegnaster*, with widely distributed species, including

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deep-sea animals. The reproductive biology has only been studied in *Henricia* and *Echinaster*. In the genus *Henricia* the reproduction has been investigated in *H. sanguinolenta*, *H. abyssicola*, and *H. lisa* (Chia and Walker 1991; Benitez-Villalobos et al. 2007; Mercier and Hamel 2008), species that are gonochoric and brood their young (Chia and Walker 1991; Pearse et al. 1991; Benitez-Villalobos et al. 2007; Mercier and Hamel 2008).

The genus *Echinaster* comprises two subgenera (*Echinaster* and *Othilia*). Species presenting glass tubercles on the aboral surface of arms and disc are included in the subgenus *Othilia*. Those without such tubercles are included in the subgenus *Echinaster* (Clark and Downey 1992). The genus *Echinaster* contains 38 species, but the sexual cycle has only been described in two of them: *Echinaster (Othilia) echinophorus* and *Echinaster (Echinaster) sepositus*, using light microscopy. Both *Echinaster* species are gonochoric, and neither of them is apparently a brooding species (Delavault 1960a; Cognetti and Delavault 1962; Ferguson 1974). *Echinaster (Echinaster) sepositus* (RETZIUS, 1873) is one of the most common asteroid species on the rocky bottoms of the northwestern coast of Spain and is also common elsewhere in the Mediterranean (Tortonese 1954; Hayward and Ryland 1990; Southward and Campbell 2006). It is a bright orange-red starfish that can measure up to 20 cm along the largest arm, and is traditionally considered carnivorous (Vasserot 1961; Ferguson 1969). It inhabits both rocky and sandy bottoms to 250 m deep. Its reproductive cycle has only been described in moderate detail in a population of the Naples Gulf by Delavault, but it is known to vary along the Mediterranean coasts (Delavault 1960a, 1960b). Although *E. sepositus* is a gonochoric species, hermaphrodite individuals are reported (Delavault 1960a). Ultrastructural studies on the gametogenesis of *Echinaster* have not yet been performed, although the ultrastructure of the gonad wall of the ovary follicle of *E. sepositus* is briefly described (Tangapregassom and Delavault 1967). There is one study of light microscopy of the sperm of *E. sepositus* (Field 1895), but there are no details of the spermatogenesis. Here, we describe the ultrastructure of the gonad wall and the oogenesis and spermatogenesis of *Echinaster (Echinaster) sepositus* from the Northwestern coast of Spain.

## Material and methods

### Sampling

In order to assess the reproductive dynamics of *Echinaster (Echinaster) sepositus*, we collected five individuals in the sublittoral rocky communities of Blanes (North-eastern Mediterranean coast of Spain, 41° 11' 18"N, 2° 45' 2"W). Sample collection was

performed monthly from July 2007 to December 2008 by scuba divers, and consisted on the removal of the arm III of each individual (the madreporite is located between arms I and II counting counter-clockwise). The whole arms were transported in seawater to the laboratory (1 h) where the gonads were immediately removed from the animal.

### Transmission electron microscopy

One of the two gonads was prefixed in 2.5% glutaraldehyde in 0.1M PBS for at least 2 h. After primary fixation with glutaraldehyde, samples were rinsed with MPB for 40 min, post-fixed in 2% osmium tetroxide in PBS, dehydrated in a graded acetone series and embedded in Spurr's resin. Both semithin and ultrathin sections were obtained with an Ultracut Reichert-Jung ultramicrotome. Semithin sections were 1–2 µm in thickness and stained with methylene blue. Ultrathin sections (64 nm) were mounted on gold grids and stained with 2% uranyl acetate for 30 min, followed by lead citrate for 10 min. Observations were conducted with a JEOL 1010 transmission electron microscope operating at 80 kV and fitted with a Gatan module for acquisition of digital images.

## Results

### General morphology of the gonads

In *Echinaster (Echinaster) sepositus*, each arm contained two gonads (10 reproductive units in total), attached proximally near the disc on the inner face of the lateral wall of the ray and projecting freely into the coelomic cavity along the entire length of the arm. Although the gonads were present during the entire year, they were smaller after spawning in August and September. Each gonad was a single large sac composed of several smaller sacs. Mature ovaries were dark red-colored, while mature testes were light yellow. The rachis formed the axis of the gonad, suspending it by few projections from this axial piece to the coelom.

### Histology of the gonads

The gonad wall composed of several layers arranged in two structures, the outer sac (OC) and the inner sac (IS), separated by the genital haemal sinus (GHS) (Figures 1A and 4B). The dimensions and appearance of these layers varied depending on the gametogenic stage of the gonad and the part of the gonad depicted in Figures 1 and 4B.

In order of arrangement, the outer sac was comprised of a single stratum of visceral peritoneal cells (visceral peritoneum, VP) lying onto a layer of fibrous content (connective tissue layer, CTL) containing myoepithelial cells, a basement membrane, and a

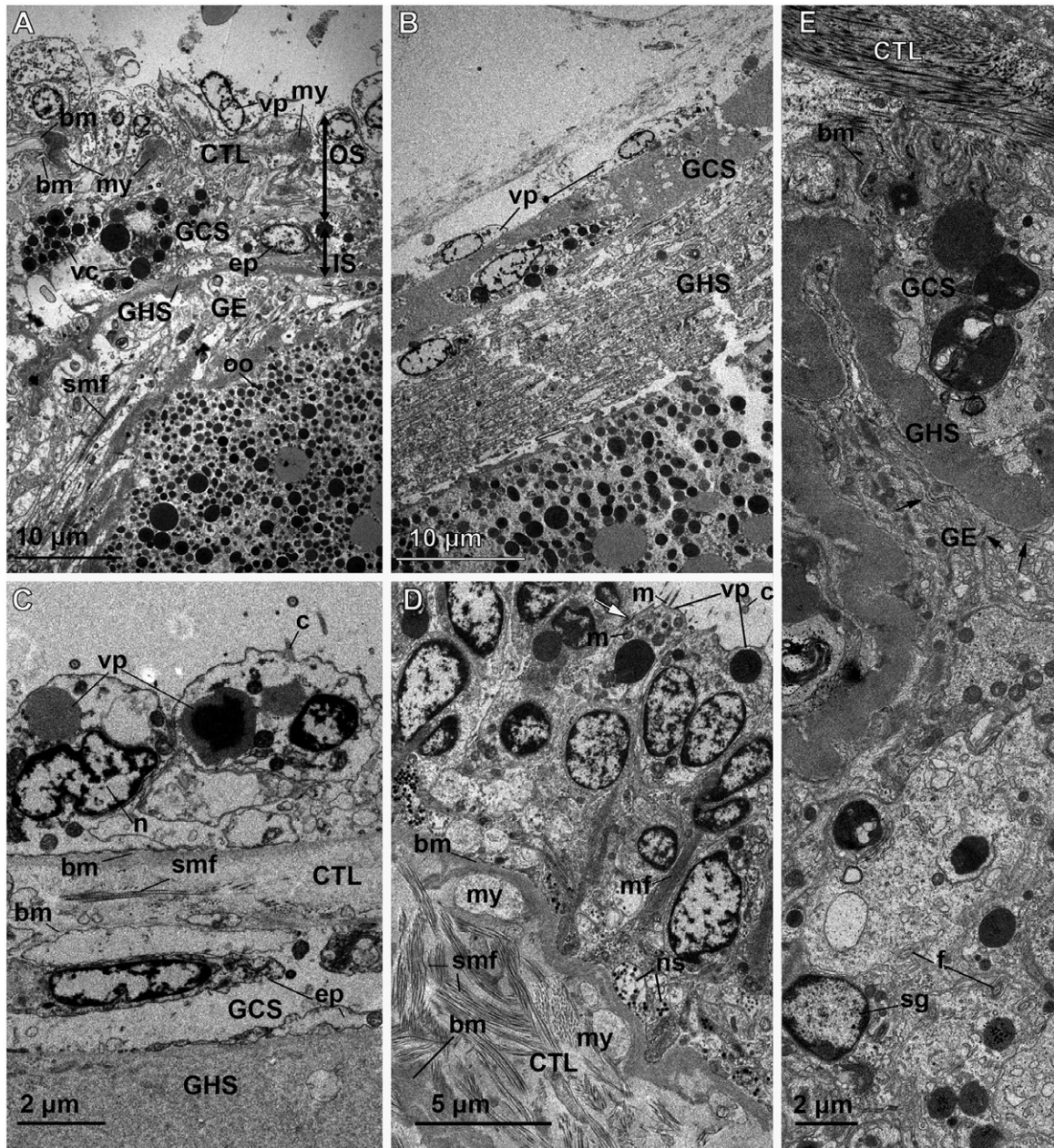


Figure 1. Morphology of the gonadal wall in *Echinaster (Echinaster) sepositus*. (A) Wall of the female gonad in October showing the layers of which the outer (OS) and the inner sac (IS) are comprised. The first layer was the visceral peritoneum (vp) made up of round cells, a basement membrane (bm), the connective tissue layer (CTL) made of myoepithelial cells (my), another basement membrane (bm) and a layer of epithelial cells (ep) that was part of the genital coelomic sinus (GCS). The GCS also contained "vesicular cells" (vc). Below the GCS, it was the genital haemal sinus (GHS), which in this case was thin and filled with flocculent material. The germinal epithelium (GE) lied below the GHS, and consists of growing oocytes (oo) and also striated muscle fibers (smf). (B) Female gonad wall in August, before spawning the oocytes (oo). The only layers that were obvious at this time are the visceral peritoneum (vp), the genital coelomic sinus (GCS) filled with flocculent material and cells, and the genital haemal sinus (GHS), which appeared vesiculated. (C) Male gonad wall in February. The wall was comprised by ciliated cells (c) forming the visceral peritoneum (vp), a basement membrane underneath (bm), the connective tissue layer (CTL) with striated muscle fibers (smf), another basement membrane (bm), the genital haemal sinus (GCS) with epithelial cells (ep), and the genital haemal sinus (GHS) filled with flocculent material. (D) Male gonad wall in August. The ciliated epithelial cells (vp) that build the visceral peritoneum increased in size and became columnar, containing bundles of filaments (mf) oriented along the long axis. They showed a single cilium (c) per cell, abundant microvilli (mi), and several mitochondria. In the base of the epithelial cells, neurons containing neurosecretory granules (ns) were found. Both the basement membranes (bm) and the connective tissue layer (CTL) became thicker, the last one showing many more striated muscle fibers (smf) than in previous months. (E) Male gonad wall in September. The connective tissue layer (CTL) was still a thick band comprised of myoepithelial cells but the basement membranes (bm) became thinner. The GCS was filled with large cells while the GHS was filled with flocculent material. The germinal epithelium (GE) was made up of spermatogonia and interstitial cells (not shown), which were connected by intercellular junctions (arrows). The flagella (f) of the spermatogonia were packed among the large cells of the germinal epithelium.

layer of epithelial cells (EP) (Figures 1A–E and 4B). Peritoneal cells were usually small and round (5–7  $\mu\text{m}$  in diameter) in both sexes and formed a cuboidal epithelium (Figure 1A–C). However, in the middle of the reproductive cycle, and before the gonad was mature, the visceral peritoneal cell layer changed to a pseudostratified columnar epithelium (Figure 1D) of cells measuring approximately 10  $\mu\text{m}$  of maximum length. Before spawning, when the gonad was mature, peritoneal cells appeared as flat cells with virtually no contact between them (Figure 1B). Peritoneal cells were epithelial, flagellated-collar cells with an ovoid conspicuous nucleus, several mitochondria, and inclusions (Figure 1A–D). Their collar was comprised of numerous microvilli (Figure 1D). In testes, when they appeared as columnar cells, their cytoplasm showed a longitudinal reinforcement of filaments (Figure 1D). Peritoneal cells were joined distally by zonulae adherentes (Figure 1D). Neurons with neurosecretory granules were found at the base of peritoneal cells (Figure 1D).

The CTL was limited by basement membranes on either side. The CTL consisted of fibroblasts, myoepithelial cells, a matrix of collagen, and striated muscle fibers running in different directions (Figure 1A–D). In males, the CTL was thickest in August, before spawning (Figure 1C–E). Internal to the CTL, the epithelial cells lined the genital coelomic sinus (GCS) (Figures 1A, C and 4B).

The inner sac of the gonad was usually very thin and difficult to interpret. It comprised the haemal sinus and associated tissues. The first layer was the epithelium that underlain the GCS (Figures 1A–C, E and 4B), which sometimes contained myoepithelial cells (not shown). The haemal sinus (GHS) was composed of the haemal sinus space filled with haemal fluid and with a few cells (Figure 1A–C and E). The GHS width changed depending on the part of the gonad examined (Figure 4B). However, besides this variation, in males the GHS was thicker and completely filled with flocculent material during the proliferative phase of the spermatogenesis (Figure 4B) appearing as a narrower layer during the aspermatogenic phase (i.e., spawning and spermatogonia proliferation) (Figure 1E). In the ovaries, the GHS acquired a vacuolated appearance prior to gamete release (Figure 1B). Changes in the GHS during the rest of the cycle were difficult to discern because oogenesis was a continuous process. The germinal epithelium of the gonad was located below the haemal sinus (Figures 1A, B, E, and 4B).

### Oogenesis

Oogenesis was a continuous process in *Echinaster* (*Echinaster*) *sepositus*, with only one spawning event

in August–September. There was no rest period in *E. sepositus* and the female gonads of *E. sepositus* always contained oocytes in different degrees of maturation. However, at the end of the annual oogenic cycle (July–September), when the ovaries were full of fully-grown oocytes, immature oocytes were scarcer than in previous months (Figure 2A). The total amount of time required to complete the oocyte growth was difficult to calculate, since different cohorts of oocytes coexisted in the ovaries.

A cellular follicle enveloped each oocyte (Figures 2A, C–D and 3A, B), and was comprised by flat cells containing different types of inclusions (Figure 2B). Their nuclei were oblong with chromatin masses attached to the inner nuclear membrane (Figure 3A and B). Their cytoplasm was filled with granular material, Golgi complexes, and mitochondria (Figures 2C and 3A–C). Follicular cells emitted numerous pinocytotic vesicles towards the oocyte (Figure 2C–D) and also processes that contacted the oolemma (Figure 3A–C).

Oogonia were usually located near the gonadal wall within the germinal epithelium, intermingled with follicular and other non-germinal cells (Figures 2A and 3B). Their cell body was spherical to columnar about 10  $\mu\text{m}$  in their longest dimension. Their nuclei were ovoid and measured approximately 5  $\mu\text{m}$  in longest diameter. Nuclei stained densely, showing chromatin masses attached to the inner nuclear membrane and a large nucleolus of about 3  $\mu\text{m}$  in longest dimension (Figure 3B). In contrast, their cytoplasm appeared as very electron-lucent, with very few organelles: a Golgi apparatus and several inclusions (Figure 3B).

The youngest primary oocyte found in the germinal epithelium reached 30  $\mu\text{m}$  and contained a few proteid yolk granules and few scattered lipid droplets (Figure 2B). The nucleolated nucleus (nucleus: 3–4  $\mu\text{m}$ ; nucleolus: 1  $\mu\text{m}$  in diameter) contained round chromatin masses in the center and also in the periphery (not shown). The first yolk granules appeared scattered throughout the entire ooplasm at this stage (Figure 2B), although later in the development the periphery of the oocyte appeared devoid of them (Figure 2C). Proteid yolk was formed within vesicles of 0.5–2  $\mu\text{m}$ , and it first appeared as flocculent material, before acquiring higher electron-density (Figure 2C–D). Fully-grown yolk granules and lipid droplets were 2 and 5  $\mu\text{m}$  in diameter, respectively (Figure 2C). Lipid droplet formation started after yolk granule formation (Figure 2B and C). In the ooplasm, later in the growth phase, bundles of filaments were found, their length varying between 2 and 5  $\mu\text{m}$  (Figure 2C and D). As the oocyte reached *ca.* 60–90  $\mu\text{m}$  in diameter, yolk granules were distributed to the periphery of the ooplasm, filling it entirely (Figure 2D). Immature oocytes extended numerous

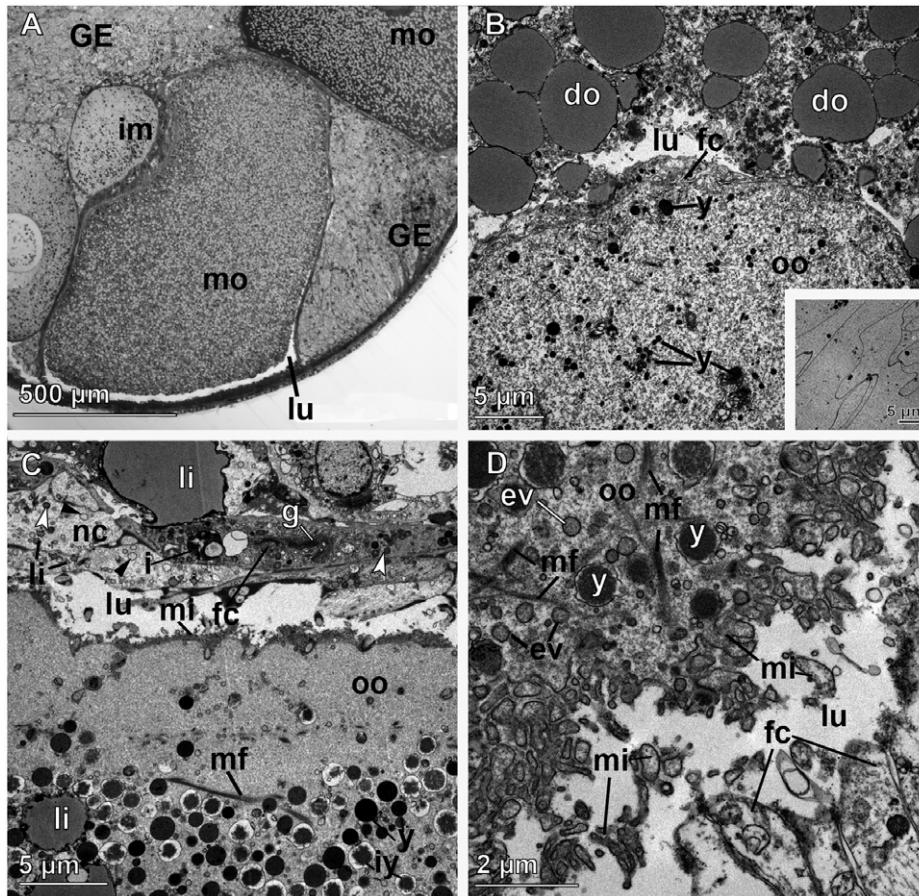


Figure 2. Early stages of oogenesis in *Echinaster (Echinaster) sepositus*. (A) Ovary collected in September, prior to oocyte release. Mature oocytes (mo) shared the space with immature oocytes (im), follicular cells, and germ cells (oogonia) (GE). (B) Degerating oocyte (do) in close relation to a normal young oocyte (oo) undergoing vitellogenesis. The yolk granules (y) of the young oocyte started to be formed throughout the entire cytoplasm. Note the thin layer of follicular cells (fc) enveloping the young oocyte. (B, inset) Convolutd nuclear membrane of a degenerating oocyte. (C and D) Early oocyte (oo) surrounded by a layer of follicular cells (fc) and other non-germinal cells (nc). Follicular cells showed Golgi complexes (g), few mitochondria (white arrowheads) and inclusions (i) in their cytoplasm. The non-germinal cells (nc) contained lipid droplets (li), mitochondria (white arrowheads) and rough endoplasmic reticulum (black arrowheads). The plasmalemma emitted numerous microvilli (mi) towards the lumen (lu) of the ovary. The cytoplasm of the oocyte showed yolk platelets both mature (y) and immature (iy) and numerous endocytotic vesicles (ev). It also contained lipid droplets (li) and bundles of fibres (mf). Abbreviations: lu, lumen.

microvilli from the oolemma (Figure 2C and D) invading the ovarian lumen. Non-germinal cells besides follicular cells were seen in small numbers around small oocytes (Figure 2C). They possessed an electron-lucent cytoplasm, with abundant granular endoplasmic reticulum, several mitochondria, and lipid droplets (Figures 2C and 3A). Degerating (atretic) oocytes were present during the entire year (Figure 2B) showing a convoluted arrangement of the nucleus membrane (inset of Figure 2B).

Mature oocytes were round, bright, red cells that reached 1 mm diameter (Figure 2A) and were fully packed with yolk granules, lipid droplets, and bundles of microfilaments (Figure 3A–D). Rough endoplasmic and large Golgi complexes (comprised of multiple cisternae and vesicles) became relatively abundant in

advanced oocytes (Figure 3D and E). A few mitochondria were found in small clusters (Figure 3E). The nuclear pores also increased in number during the last growth phase (Figure 3F).

In late stage oocytes, follicular cells extended processes to the oocyte (Figure 3A–C), contacting the oocyte in several points (Figure 3A and B). Such extensions were apparently involved in the transference of different materials to the oocyte, which occurred presumably by endocytosis because numerous endocytotic vesicles were observed (Figure 3C).

### Spermatogenesis

The germinal layer in each testis was composed of interstitial somatic cells and spermatogenic cells in

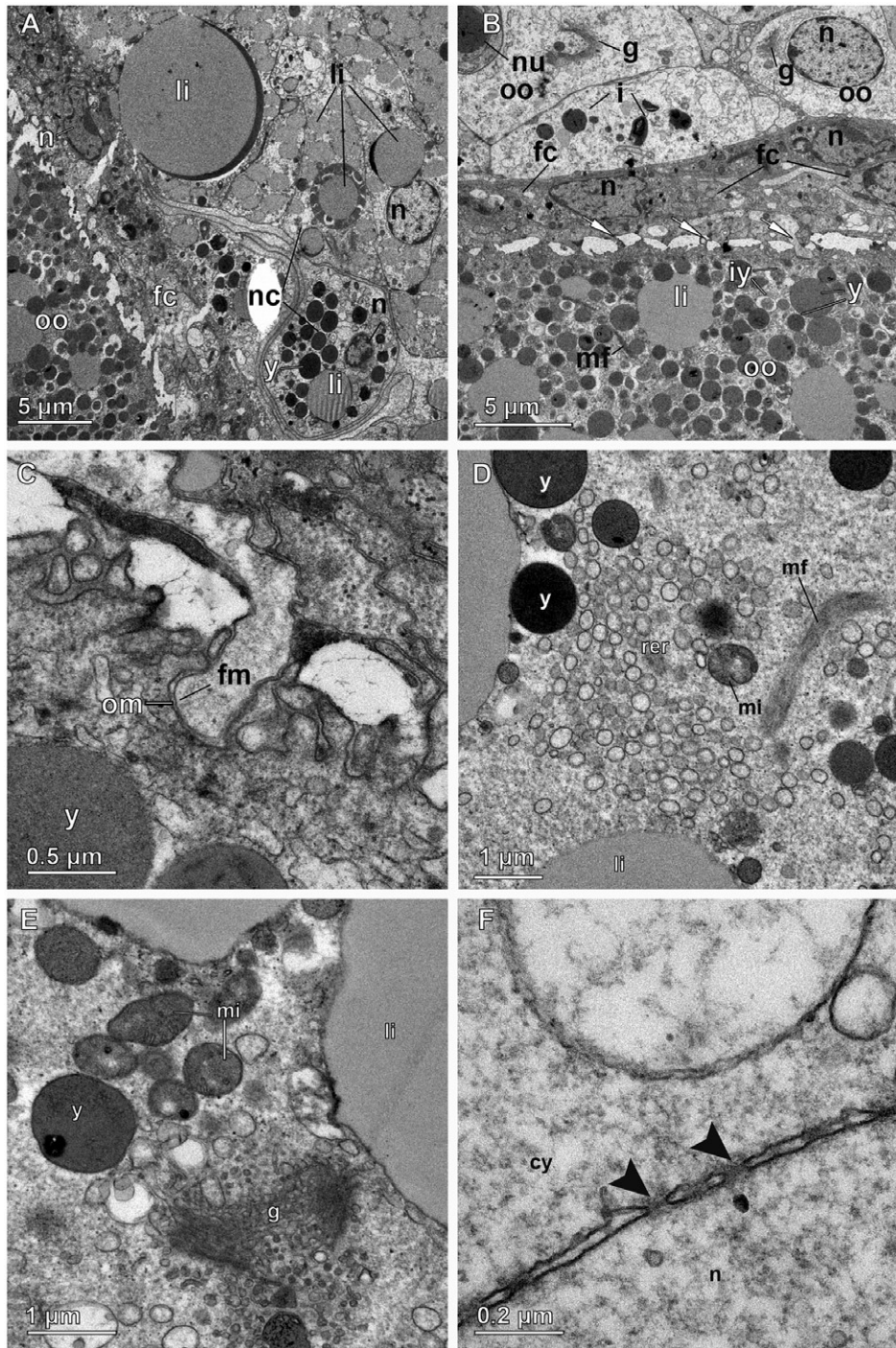


Figure 3. Maturation of oocytes in *Echinaster (Echinaster) sepositus*. (A) Developing oocyte (oo) surrounded by follicular cells (fc) and a thick layer of non-germinal cells (nc) containing lipid droplets (li) and yolk inclusions (y). (B) Higher magnification of the oolemma of a mature oocyte (oo) showing the connections (arrows) between it and follicular cells (fc). Above the follicular cells and within the germinal epithelium (GE), oogonia with a nucleolated nucleus (nu) containing inclusions (i) and Golgi apparatus (g) can be observed. The cytoplasm of the developing oocyte contained immature (iy) and mature yolk (y), lipid droplets (li), and bundles of microfibers (mf). (C) Close-up of the processes emitted by the follicular cells towards the oocyte. Note the close apposition of the follicular cell membrane (fm) and the oolemma (om). The cytoplasm of follicular cells appears to be filled with a granular material. (D and E) Cytoplasm of a mature oocyte containing rough endoplasmic reticulum (rer), mitochondria (mi), bundles of filaments (mf), yolk platelets (y), Golgi complexes (g), and lipid droplets (li). (F) Nuclear pores (black arrowheads) of the nucleus (n) of a mature oocyte. Abbreviations: cy, oocyte cytoplasm; n, nucleus; y, yolk.

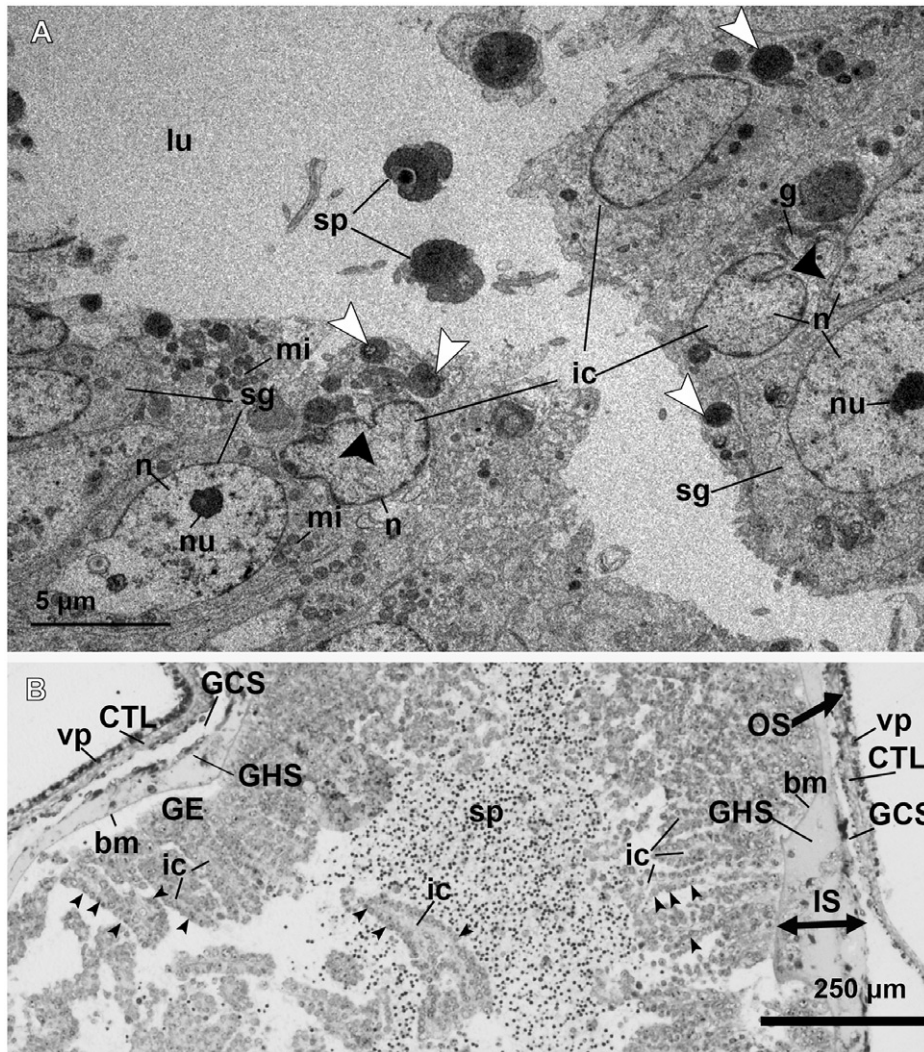


Figure 4. Male gonads of *Echinaster (Echinaster) sepositus*. (A) Germinal epithelium of the testis showing spermatogonia (sg) and interstitial cells (ic) prior to form the columns. Spermatogonia possessed a round nucleolated (nu) nucleus (n) and a few mitochondria (mi), whereas interstitial cells showed irregular anucleated nucleus (n) with depressions (black arrowheads), numerous mitochondria (mi), inclusions (white arrowheads), and Golgi apparatuses (g). Note the presence of residual spermatozoans (sp) in the lumen of the testis. (B) Developing testis with the typical arrangement of spermatogenic cells (black arrowheads) in columns. Note the interstitial cell forming the axis (ic) of the column and the mature spermatozoans (sp) filling the lumen of the testis.

Abbreviations: vp, visceral peritoneum; CTL, connective tissue layer; GCS, genital coelomic sinus; GHS, genital haemal sinus; GE, germinal epithelium; bm, basement membrane.

different stages of development arranged in columns (Figure 4A and B). Immediately after spawning, only interstitial cells, amitotic spermatogonia, and residual spermatozoa were found in the lumen of the testis (Figure 4A). The events related with the fate of residual sperm will be described later.

The beginning of the spermatogenic phase was characterized by the increase in the number of mitotic spermatogonia (Figure 5A and B), which appeared in the gonad intermingled with interstitial cells (Figures 4A and 5A). These were elongated cells with an irregular anucleated nucleus showing the nuclear depression typical of interstitial cells, Golgi apparatus, and several inclusions (Figure 4A). Spermatogonia

were flagellated columnar cells up to 10  $\mu\text{m}$  in maximum diameter (Figures 4A and 5A), which at this stage were not arranged in distinguishable columns (Figure 5A and B). Their cytoplasm contained small mitochondria clustered in two groups of 10–15 and located below and underneath the nucleus (Figures 4A and 5A). Nuage was observed among the mitochondrial cluster located distally and consisted of several granules, some of them bigger and more electron-dense (Figure 5C). The cytoplasm also contained a well-developed Golgi apparatus (Figures 4A and 5A, B). The round nucleus of the mitotic spermatogonium was 5  $\mu\text{m}$  in diameter and contained a centric nucleolus of 1  $\mu\text{m}$  in diameter



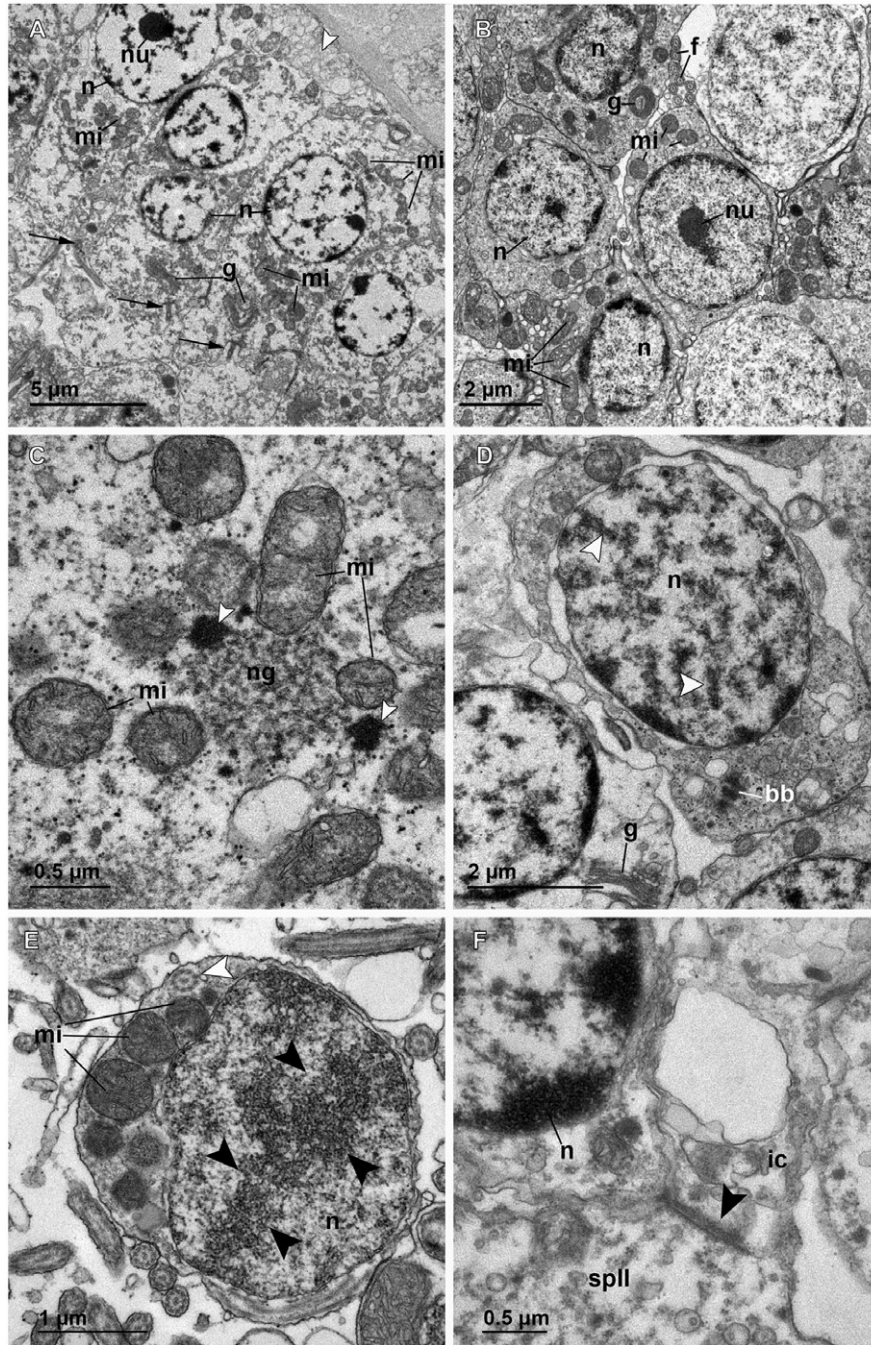


Figure 5. Spermatocytogenesis of *Echinaster (Echinaster) sepositus*. (A and B) Germinal epithelium showing spermatogonia, each containing a nucleolated (nu) nucleus (n), Golgi apparatus (g), two groups of mitochondria located over and below the nucleus (mi) and a flagellum (arrows). In Figure 5A, note the interstitial cell processes (white arrowheads). (C) Cytoplasm of a mitotic spermatogonium showing the nuage (ng) within a mitochondrial cluster (mi). Note the two highly electron-dense granules (white arrowheads) intermingled with the more diffusely granulated material. (D) Primary spermatocytes showing the synaptonemal complexes (white arrowheads) within the nucleus (n), the Golgi apparatus (g), and the basal body (bb). (E) Secondary spermatocytes showing the chromatids aligned along the equatorial plate (black arrowheads) within the nucleus (n). Note the larger size of the mitochondria (mi) at this stage, and the flagellum insertion (white arrowhead). (F) Intercellular junction (black arrowhead) connecting a secondary spermatocyte (spII) and an interstitial cell (ic). Note the nucleus (n) of one of the secondary spermatocytes adjacent to them.

(Figure 5A and B). The flagellum of each spermatogonium extended towards the lumen of the testis (Figure 5A). Spermatogonia were connected by intercellular junctions (not shown).

After the massive proliferation of spermatogonia, each spermatogenic column consisted of an axial interstitial cell that gave rise to a long process that extended towards the testis lumen. The gonad wall was

lined by spermatogenic cells (Figure 4B). Spermatogenesis took place along the column, with developed stages located more distally than the early stages. Primary spermatocytes were very similar to spermatogonia, except that they showed synaptonemal complexes typical of prophase I (Figure 5D). At this stage, sister cells were not connected by intercellular bridges.

Secondary spermatocytes were round cells measuring 3  $\mu\text{m}$  in diameter, with larger and fewer mitochondria than those in spermatogonia and primary spermatocyte. Mitochondria were located in one pole of the cell (Figure 5E). Within the nucleus, chromatids started to align along the equatorial plate (Figure 5E). Secondary spermatocytes were attached to interstitial cells by intercellular junctions (Figure 5F).

The nuclei of spermatids underwent drastic changes, decreasing in size as the chromatin condensed (Figure 6A–C). Spermatids were also round cells, 2–2.5  $\mu\text{m}$  in maximum diameter, with 5–6, 1- $\mu\text{m}$ -long mitochondria surrounding the flagellar insertion (Figure 6B). The flagellum arose from the distal centriole (not shown), but the proximal centriole was also evident. The acrosome, which was formed from the Golgi apparatus, was a round vesicle partially surrounded by the nucleus (Figure 6A and B). At this stage, it appeared as a single vesicle with a homogeneous content (Figure 6A and B), which acquired more electron-density as spermiogenesis progressed (Figure 6A and B). Spermatids were interconnected by cytoplasmic bridges (Figure 6B), through which nuclear material was sometimes observed (Figure 6B).

Mature spermatozoa detached from the columns and were free in the testis lumen (Figure 4B). They were 2–3  $\mu\text{m}$  in diameter, with 2–3 mitochondria (Figure 6C and D) and glycogen (not shown). The flagellum arose from the distal centriole and was supported by an electron-dense ring that occurred at the flagellar base (Figure 6D). Nuclear chromatin was condensed and the nucleus was ovoid in shape, only interrupted by the acrosomal fossa (Figure 6C and D). The acrosome was a round membrane-bound vesicle with a highly electron-dense homogeneous material surrounded by fine granular, moderately electron-dense periacrosomal material (Figure 6C–F). A slightly less electron-dense, round substructure was seen on the posterior end of the acrosome (Figure 6C).

After the spawning, phagocytes entered the testis lumen and phagocytosed sperm relicts (Figure 6E and F). These cells had an electron-lucent nucleus of 5  $\mu\text{m}$  in maximum diameter and several inclusions within vesicles (Figure 6E and F).

## Discussion

We found that *Echinaster (Echinaster) sepositus* is a gonochoric species with an annual reproductive cycle and spawning occurring from August to September. In contrast to what Cognetti and Delavault (1962) found in the Naples Gulf, we did not observe hermaphroditic individuals. However, these authors saw only 4% hermaphrodites in 162 individuals, so the absence of hermaphroditic individuals in our samples may be due to smaller sample size ( $n = 90$ ).

The ovaries of *E. sepositus* contained oocytes in different stages of maturation, while the testes contained only spermatogenic cells from February–March to September–October, as previously reported by Cognetti and Delavault (1962). However, individuals collected in Banyuls were mature in March–April (Cognetti and Delavault 1962). Variations in the timing of gonad maturation between different populations are also reported for other sea stars (e.g., *Marthasterias glacialis* (Cognetti and Delavault 1962)), and may result from the complex interplay between endogenous and exogenous signals to which populations are subjected (Mercier and Hamel 2009).

## Histology of the gonads

Echinoderm gonads are sac-like organs, with large lumina and located in the perivisceral coelom or the genital (or periaemal) coelom. They are intimately associated with major aboral or oral haemal or sometimes periaemal channels that interconnect all gonads and also lie in contact with all major systems within an individual. Gonads usually open to the exterior by a single or multiple gonopores (Walker 1982; Chia and Koss 1994). The gonad wall in *E. sepositus* shows the typical arrangement found in asteroids, echinoids, and ophiuroids, comprised of two sacs (outer and inner), each composed of several layers (Walker 1982; Chia and Walker 1991). Our findings are complementary to the preliminary description of the gonad wall of *E. sepositus* made by Tangapregasson and Delavault (1967), although they did not report the variation in shape and size of each layer during the reproductive cycle. Columnar peritoneal cells of *E. sepositus* contain thick bundles of filaments in their cytoplasm. This has never been reported before in the peritoneal cells of echinoderm gonads. Such filaments might be involved in reinforcing the cell while it acquires a more elongated shape. Cells filled with neurosecretory granules are common near the base of peritoneal cells in *E. sepositus*. Although their function and significance is not known, they might serve an endocrine function in oocyte development, as suggested for other asteroids (Ferrand 1983).

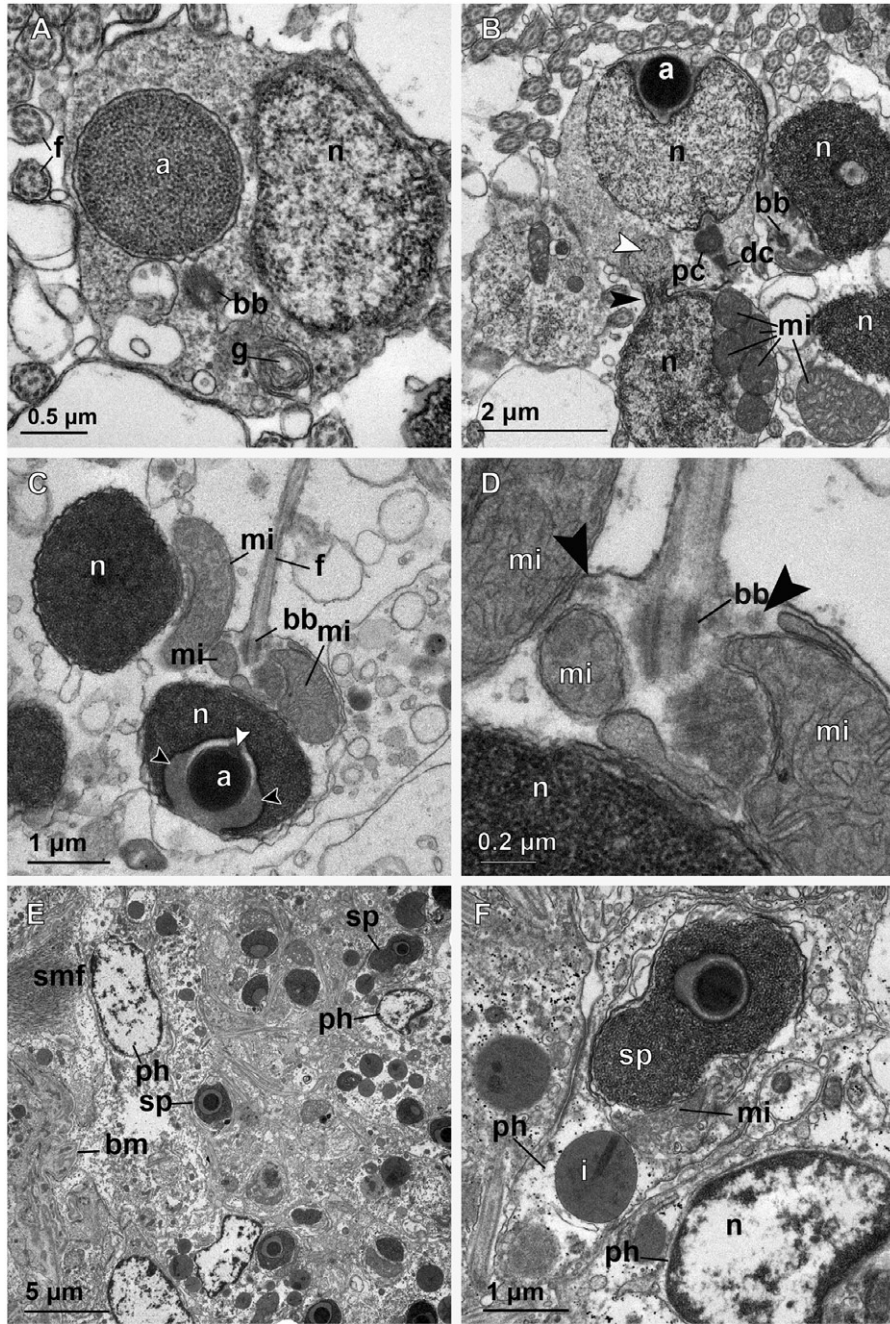


Figure 6. Spermiogenesis and phagocytosis of sperm after spawning in *Echinaster* (*Echinaster*) *sepositus*. (A) Spermatid showing the partially condensed acrosome (a) and nucleus (n). Note the Golgi apparatus (g) within the cytoplasm and the basal body (bb). (B) Sister spermatids connected by a cellular bridge (black arrowhead). Both spermatids showed a partially condensed nucleus (n), larger and fewer mitochondria (mi) than in previous stages. Note the conformation of the flagellum insertion, comprised of a proximal centriole (pc) and a distal centriole (dc) from which the flagellum arose (not shown). Nuclear material (white arrowhead) passed through the cellular bridge from one spermatid to the other. (C) Spermatozoon showing the nucleus (n) containing highly condensed chromatin and the fully formed acrosome (a). The cytoplasm contained three mitochondria (mi) and the basal body (bb) from which the flagellum (f) arose. Note the acrosomal fossa surrounded by the nucleus (black arrowheads) and the round substructure of the acrosome (white arrowhead). (D) Detail of the flagellum insertion of the spermatozoon of Figure 6C showing the basal body (bb) and an electron-dense ring (black arrowheads). (E) Lumen of testis containing phagocytotic somatic cells (ph) and remaining spermatozoans (sp) after spawning. (F) Detail of a spermatozoon (sp) phagocytosed by a phagocytotic somatic cell (ph) containing inclusions (i) within its cytoplasm. Note the nucleus (n) of an adjacent phagocytotic somatic cell and the partially digested mitochondria (mi) within the phagocytosed spermatozoon. Abbreviations: bb, basal body; bm, basal membrane; f, flagellum; mi, mitochondria; smf, striated muscle fibers.

The predictable changes in the content of the GHS seen during oogenesis and spermatogenesis suggest the function of this layer as temporary intragonadal storage of nutrients (Walker 1980). Since spermatogenesis in *E. sepositus* is an annual process, such changes are easily detectable. Nutrient accumulation begins during the proliferative phase of the cycle, and they are apparently used to depletion. The strong relationship of the interstitial axial cells located in the middle of spermatogenic columns with the GHS also suggests that such cells are somehow involved in the distribution of nutrients to spermatogenic cells. Follicular cells surrounding oocytes might be involved in the same function in females. However, little evidence of nutrient transference from interstitial cells to spermatogenic cells has been shown so far. The evidence of follicular cells transferring nutrients to oocytes is discussed below.

### Oogenesis

Oogenesis in *Echinaster (Echinaster) sepositus* is a continuous and asynchronous process, as also found in the population of the Naples Gulf and in the other echinasterids *Henricia abyssicola* and *H. lisa* (Delavault 1960a; Benitez-Villalobos et al. 2007; Mercier and Hamel 2008). The oocytes in *E. sepositus* are not attached to the ovarian wall as seen in the forcipulid sea stars (Schoenmakers et al. 1981; Beijinink et al. 1984b), but within the lumen of the ovaries, filling the entire space. Because of this, the germinal layer is not apparent in *E. sepositus*, similar to that found in other asteroids (Ottesen and Lucas 1982; Benitez-Villalobos et al. 2007; Mercier and Hamel 2008) and crinoids (Byrne 1989). As typical for echinoderm oogenesis, the oocytes of *E. sepositus* form numerous microvilli along the oolemma during early stages (Delavault and Tangapregassom 1967). The follicle, formed by somatic cells, provides structural support for developing oocytes, and may be involved in the maintenance of the microenvironment required for development. The multiple contact points between the follicular cells and the oocytes may suggest that they play a role in the nutrition of oocytes, as suggested for other asteroids (Chia 1970; Beijinink et al. 1984a).

In *Echinaster (Echinaster) sepositus*, yolk granules appear before lipid droplet synthesis, in contrast to that found for another echinasterid, *Henricia sanguinolenta* (Chia 1970). The homogeneous, electron-dense appearance of yolk granules in *E. sepositus* is typical from echinoderm oogenesis, as well as its mechanism of formation (Chia 1970; Beijinink et al. 1984a; Byrne 1989; Chia and Walker 1991). Cortical granules were not observed in the eggs of *E. sepositus*, even though they are common in the periphery of the oocyte in

other species (Holland 1980; Byrne 1989). Degenerating oocytes were present during most of the year, as previously reported for *E. sepositus* (Delavault 1960a). Abortive oocytes are common in asteroids, echinoids, crinoids, and ophiuroids, and it has been suggested that they contribute nutrients to other successful primary oocytes (Mortesen 1936; Walker 1982). We were not able to resolve the formation of the vitelline membrane, which apparently occurs very late in oogenesis in other asteroids (Holland 1980).

Spawned eggs of *Echinaster (Echinaster) sepositus* were large, red, round cells measuring about 1 mm. Other echinasterids have similarly sized eggs, e.g., *E. echinophorus*, 800  $\mu\text{m}$ , and *Henricia sanguinolenta* and *H. lisa*, both 1.2 mm (Chia 1970; Chia et al. 1993; Mercier and Hamel 2008).

### Spermatogenesis and sperm phagocytosis

Spermatogenesis in *Echinaster (Echinaster) sepositus* occurs in spermatogenic columns (first denominated as “leisteinfirmigen erhabungen” in *Asterias rubens* by Ludwig (1877) and later “colonnettes” in *E. sepositus* by Delavault (1960a, 1960b)). This columnar arrangement is typical from asteroids and ophiuroids (Chia and Walker 1991). Spermatogenic columns consist of a central interstitial cell surrounded by spermatogenic cells that develop along the axis towards the lumen of the testis (Delavault 1960a, 1960b; Walker 1980; Chia and Walker 1991). Therefore, spermatids are usually found near the tips of the columns, while spermatogonia and spermatocytes are found at the base of the column. Mature spermatozoa, however, are located freely in the lumen of the testis.

In some ways, the relationship between the axial interstitial cells and the spermatogenic cells is analogous to that involving Sertoli cells in mammalian testes. Such similarity noted before by Walker (1980) in *Asterias rubens*. Sertoli cells (or Sertoli-like cells) have already been found in many different phyla other than chordates (Hinsch 1980; Buckland-Nicks and Chia 1986; Guraya 1995; Jørgensen and Lützen, 1997; Erkan and Sousa 2002; Reunov et al. 2004; Riesgo and Maldonado 2008), displaying one or some of the functions usually attributed to mammalian Sertoli cells: skeletal and nutritive support, production of cleansing elements with phagocytic properties, aid in recycling nutrients and residual spermatozoa, and production of synthetic elements for control of spermatogenesis. In *E. sepositus*, interstitial cells are important for scaffolding and supporting the columnar structures of the testes, and for removing the unspawned, residual spermatozoa. Support and phagocytic functions of gonadal interstitial cells have been reported previously in a variety of echinoderms

(e.g., Hinsch and Dehn 1979; Bickell et al. 1980; Buckland-Nicks et al. 1984; Walker 1980; Chia and Walker 1991; Reunov et al. 2004). The typical junctional complexes between Sertoli cells and spermatogenic cells have not been reported in echinoderms, but intercellular junctions between interstitial cells and spermatocytes have been described in the brittle-star *Amphipholis squamata* (Buckland-Nicks et al. 1984) and in this study. However, the morphology of interstitial cells is quite different to that of Sertoli cells (Buckland-Nicks et al. 1984) and Chia and Buckland-Nicks (1987) demonstrated that they do not function as a true permeability barrier.

Spermatogenesis of *Echinaster (Echinaster) sepositus* follows the same pattern described for other asteroids (reviewed in Chia and Walker 1991), also being very similar across all echinoderms (Longo and Anderson 1969; Bickell et al. 1980; Buckland-Nicks et al. 1984; Hendler 1991; Holland 1991; Pearse and Cameron 1991; Smiley et al. 1991). After proliferation of flagellated spermatogonia by mitosis, spermatogenic cells arrange in columns. Although the onset of spermatocytogenesis does not require any chemical stimulus, it is still unclear how meiosis is initiated simultaneously in cells of different columns of the testes of *E. sepositus*. Interestingly, spermatogonia and spermatocytes of asteroids are not interconnected by cytoplasmic bridges (Chia and Walker 1991). In asteroid female gonads, a substance called 1-methyladenine is involved in triggering the onset of meiosis in the ovarian lumen (Kanatani 1973). This substance has been found to cause the release of both types of gametes after a variable latent period in *Echinaster (Echinaster) modestus* (Turner 1976), but whether it could function in terminating meiotic arrest is not known.

The morphology of the spermatozoon of *Echinaster (Echinaster) sepositus* resembles the generalized “primitive” echinoderm spermatozoon, composed of a spherical head with the acrosome positioned at the anterior end of the nucleus in a depression, while the mitochondrial middle piece of *E. sepositus* takes the form of an annular band at the posterior end of the nucleus (Chia et al. 1975). The measurements of the sperm head and middlepiece are consistent with those given by Field (1895). The flagellum insertion consists of two centrioles, the proximal and the distal centriole, from which the flagellum arises, as also seen in other asteroids (e.g., Dehn and Hinsch 1981). It also presents an electron-dense ring near the base of the flagellum but we were unable to detect the pericentriolar processes typical for other echinoderm spermatozoa (Dehn and Hinsch 1981; Buckland-Nicks et al. 1984; Chia and Walker 1991). The acrosome does not possess any striking modification. It is a homogeneous, electron-dense, round structure surrounded by a membrane that bears a posterior round substructure slightly

less electron-dense. The acrosome lies within a subacrosomal, membrane-bound vesicle containing a matrix of fine granular material.

In conclusion, the gonadal wall morphology and the gametogenic process in *Echinaster (Echinaster) sepositus* follow the general pattern for asteroids, showing very few modifications or peculiarities. This study sheds some light in the reproductive cycle of this very important Mediterranean asteroid species and at the same time contributes to the general knowledge on the ultrastructure of the reproductive cycle and gametogenesis of the understudied order Spinulosida.

### Acknowledgements

We are indebted to Virginia Jiménez, Oriol Sacristán, Joao Gil, Fernando Riesgo, Sergio Taboada, Carmen Gutiérrez, and Anna Hervás for help with field sampling and sample processing. We also thank Almudena García and Nùria Cortadellas for help with TEM samples. Comments, suggestions, and corrections of two anonymous reviewers were very helpful to improve the quality of the manuscript. This study was funded by the Spanish Ministry of Science and Education (MPA-STAR, grant 200730I005 and MARMOL, CMT2007-66635).

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