Estudi de la biologia reproductiva de la cabra de mar, *Maja brachydactyla*: aparell reproductor i qualitat de les postes en captivitat

Carles Garcia Simeó

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Resultats

1. L'aparell reproductor

Article 1

Títol: Internal anatomy and ultrastructure of the male reproductive system of the spider crab, *Maja brachydactyla* (Decapoda: Brachyura)

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Informe de la contribució del doctorand
La hipòtesi de treball, la metodologia de mostreig i dissecció, i les tècniques a utilitzar varen estar seleccionades per la Dra. Rotllant i el Dr. Ribes. El doctorand va realitzar les disseccions, els mostrejos dels teixits i el processat de les mostres per microscòpia òptica amb el suport de la Dra. Rotllant. Les mostres per microscòpia electrònica es van prendre amb el suport del Dr. E. Ribes, i foren processades pel doctorand i pels Serveis Cientificotècnics de la Universitat de Barcelona. Les imatges de microscòpia òptica i electrònica foren realitzades pel doctorand amb el suport del Dr. Ribes. La descripció i interpretació de les imatges i la redacció del manuscrit foren realitzades pel doctorant amb la col·laboració dels coautors.

Dra. Guiomar Rotllant Estelrich

Resum

La morfologia i funció de l'aparell reproductor masculí de la cabra de mar, *Maja brachydactyla*, és descrita mitjançant microscòpia òptica i electrònica. Els mascles adults foren capturats a la Ria d'A Coruña (Galícia), i transportats a l'IRTA per a la seua dissecció. L'aparell reproductor masculí fou extret i els fragments de les diferents regions foren processades seguint els protocols estàndards de microscòpia òptica per a tincions d'hematoxilina-eosina i de Mallory; i de microscòpia electrònica de rastreig i transmissió. L'aparell reproductor de la cabra de mar segueix el patró observat en altres braquiürs, i està format per un parell de testicles, conductes deferents i conductes ejaculadors. El testicle és de tipus tubular, i està format per un únic tub seminífer altament enrotllat. El tub seminífer està dividit longitudinalment per una capa epitelial interna que separa les zones germinal, de transformació i evacuació. La zona germinal es troba en un pol de la secció transversal del tub seminífer, i conté espermatogonis. La zona de maduració ocupa la zona central del tub seminífer i és la zona on té lloc l'espermatogènesi. La zona d'evacuació recull i transporta els espermatozoides fins el conducte deferent. El conducte deferent (CD) és un tub enrotllat dividit en 3 parts en base a característiques anatòmiques i funcionals. El CD anterior (CDA) és un tub llis en la porció proximal i amb alguns diverticles aïllats en la porció distal. La formació de l'espermatòfor es produeix al llarg del CDA. El CD mitjà (CDM) està enrotllat helicoïdalment i presenta els diverticles a un pol del CD. Els espermatòfors, juntament amb secrecions pròpies del CDM s'emmagatzemen en els diverticles i la llum del CDM. El CD posterior (CDP) és un tub curt que presenta una glàndula accessòria de gran mida formada per varis diverticles altament ramificats on es produeixen i emmagatzemen grans quantitats de fluids seminals. La paret del CD està formada per una capa externa de teixit connectiu, una capa intermèdia de fibres musculars i una capa epitelial interna. L'epiteli és monoestratificat al CDA i CDM, i presenta activitat secretora d'exocitosi. L'epiteli del CDA produeix dues substàncies (anomenades substàncies I i II) involucrades en la formació de la paret de l'espermatòfor. La substància I divideix la massa d'espermatozoides provinent del testicle en grups, i la substància II envolta i separa els diferents grups d'espermatozoides per formar els espermatòfors. L'epiteli del CDM també secreta materials per exocitosi, els quals s'acumulen entre els espermatòfors emmagatzemats als diverticles d'aquesta regió. La ultraestructura de les cèl·lules epitelials del CDA i CDM és similar. Les cèl·lules epitelials presenten nuclis basals i voluminosos, normalment lobulats, amb la cromatina condensada en la perifèria i varis nuclèols. El citoplasma conté vesícules de reticle endoplasmàtic i nombrosos complexos de Golgi. La regió apical de la cèl·lula està coberta

per microvilli, mentre que la membrana plasmàtica de la zona basal presenta nombroses invaginacions. La paret del CDP presenta una capa muscular molt desenvolupada, amb feixos de musculatura orientada en vàries direccions i un epiteli pseudoestratificat sense activitat secretora. El citoplasma de les cèl·lules epitelials conté uns pocs mitocondris i alguns complexos de Golgi poc desenvolupats. La zona apical presenta llargs microvilli ramificats, i la zona basal presenta nombroses invaginacions associades a projeccions de làmina basal. La paret dels diverticles de glàndula accessòria del CDP presenta una estructura semblant a aquella observada al CDA i CDM, amb una capa simple de feixos de musculatura i un epiteli amb activitat secretora. Tanmateix, el mode de secreció de l'epiteli de la glàndula accessòria sembla ser apocrina. El nucli de les cèl·lules epitelials és lobulat, amb la cromatina condensada en la perifèria. El citoplasma conté grans quantitats de reticle endoplasmàtic organitzat en vesícules aplanades. Unes vesícules endosòmiques que contenen mitocondris, membranes i material granular s'agrupen fer formar uns grànuls que finalment són abocats a la llum del diverticle. El conducte ejaculador (CE) presenta una paret similar al CDP, amb una capa de musculatura molt desenvolupada i un epiteli pseudoestratificat i no secretor. La funció del CE és l'extrusió dels espermatòfors i fluids seminals cap els gonòporus. La glàndula andrògena s'ha identificat com un teixit adherit al CE, format per una massa de cèl·lules irregulars amb nuclis centrals arrodonits i signes de vacuolització sota la membrana plasmàtica.

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Internal anatomy and ultrastructure of the male reproductive system of the spider crab *Maja brachydactyla* (Decapoda: Brachyura)

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ABSTRACT

The morphology and function of the male reproductive system in the spider crab *Maja brachydactyla*, an important commercial species, is described using light and electron microscopy. The reproductive system follows the pattern found among brachyuran with several peculiarities. The testis, known as tubular testis, consists of a single, highly coiled seminiferous tubule divided all along by an inner epithelium into germinal, transformation, and evacuation zones, each playing a different role during spermatogenesis. The vas deferens (VD) presents diverticula increasing in number and size towards the median VD, where spermatophores are stored. The inner monostratified epithelium exocytoses the materials involved in the spermatophore wall formation (named substance I and II) and spermatophore storage in the anterior and median VD, respectively. A large accessory gland is attached to the posterior VD, and its secretions are released as granules in apocrine secretion, and stored in the lumen of the diverticula as seminal fluids. A striated musculature may contribute to the formation and movement of spermatophores and seminal fluids along the VD. The ejaculatory duct (ED) shows a multilayered musculature and a nonsecretory pseudostratified epithelium, and extrudes the reproductive products towards the gonopores. A tissue attached to the ED is identified as the androgenic gland.

1. Introduction

The morphology and histology of the male reproductive system of brachyurans have been extensively studied. The reproductive system, located in the cephalothorax, lies on the hepatopancreas and extends longitudinally at both sides of the median body plane. The male reproductive system consists of paired testes, vasa deferentia, and ejaculatory ducts (Krol et al., 1992). The testes are tubular organs in the anterior region of the body that extend along the sides of the stomach (Fasten, 1915; Mouchet, 1931) and are commonly united by a medial commissure between the posterior end of the stomach and the anterior region of the heart (Fasten, 1915, 1917; Mouchet, 1931; Hoestlandt, 1948; Estampador, 1949; George, 1963; Ryan, 1967; Uma and Subramoniam, 1984; Suganthi and Anilkumar, 1999; Garcia and Silva, 2006; Castilho et al., 2008). The testes of brachyurans have been classified into 2 morphological types: lobular and tubular (Nagao and Munehara, 2003). Lobular testes are composed of numerous seminiferous lobules, acini or cysts connected by a seminiferous duct as a central axis, whereas tubular testes consist of a single, highly convoluted testicular tubule (Minagawa et al., 1994; Nagao and Munehara, 2003). The wall of seminiferous lobules and tubules is generally composed of 2 layers: an outer connective tissue layer and an inner flat epithelium (Krol et al., 1992). Spermatogonial cells are usually concentrated in a band along the periphery of the lobule or tubule, while developing sperm cells accompanied by accessory cells fill the central region (Cronin, 1947; Hoestlandt, 1948; George, 1963; Minagawa et al., 1994). The morphology and function of the accessory cells vary depending on the stage of spermatogenesis (Krol et al., 1992).

A short, small duct known as vas efferens, which connects the testis to the vas deferens and presents typhlosolar-like expansions, has been described in *Callinectes sapidus* (Cronin, 1947) and *Portunus pelagicus* (George, 1963). However, testes are in general continued by the vas deferens (VD), a pair of elongated and convoluted tubules which extend longitudinally in the posterior region of the body (Ryan, 1967; McLaughlin, 1983). According to morphological and functional criteria, the VD is usually divided into 3 regions: anterior (AVD), median (MVD), and posterior vas deferens (PVD) (Adiyodi and Anilkumar, 1988). Functionally, the AVD is considered the site of spermatophore formation, whereas the MVD and PVD store spermatophores and seminal fluids (Krol et al., 1992). The wall of the VD is composed of an outer layer of connective tissue, an intermediate muscular layer, and an inner secretory epithelium (Fasten, 1917). Despite the great amount of information regarding

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the VD, only few preliminary data exist about the ultrastructure of epithelial cells and their role in spermatophore and seminal fluid production. Epithelial cells studied in *Libinia emarginata* and *Libinia dubia* (Hinsch and Walker, 1974), *Ocypode ceratophthalmus* (Sudha Devi and Adiyodi, 1995) and *Chionoecetes opilio* (Benhalima and Moriyasu, 2000) contained organelles associated with protein synthesis and secretion, such as highly developed rough endoplasmic reticulum and Golgi complex.

The ejaculatory duct (ED) is a smooth, narrow duct, extending ventro-posteriorly between the musculature of the coxa of the fifth walking leg towards the gonopores (Krol et al., 1992). The wall of the ED presents a thick muscular layer composed of several layers of striated fibers and an inner columnar epithelium. The role of the ED is the extrusion of seminal products towards the gonopores (Cronin, 1947).

The spider crab *Maja brachydactyla* is an important commercial fishery species distributed in the Northeast Atlantic (Le Foll, 1993). In Spain, it has an important socio-economic value, specially in the NW coast (González-Gurriarán et al., 1993; Freire et al., 2002). The reproductive cycle has been intensely studied in natural populations of the Galician coast (NW Spain) (González-Gurriarán et al., 1993, 1995, 1998). In addition, information regarding the morphology and ultrastructure of the ovaries and the seminal receptacle, as well as seasonal changes in the maturity of gonads, is already available (Brosnan, 1981; Diesel, 1991; González-Gurriarán et al., 1993, 1995, 1998; Rotllant et al., 2007). However, despite the important role of males on maintaining the balance of fished decapod populations, as recently proved (Hankin et al., 1997; Rondeau and Sainte-Marie, 2001; Carver et al., 2005; Sato et al., 2006), very little is known about the morphology and physiology of the male reproductive system of *M. brachydactyla*. A few scattered studies have dealt with different aspects of the male sexuality of *M. brachydactyla*, such as the gross morphology of the VD and spermatophore formation (Mouchet, 1931), spermatogenesis (Meusy, 1972), and gonopod morphology (Neumann, 1996, 1998). The aim of this study is the detailed description of the morphology and ultrastructure of the internal male reproductive system of the spider crab *M. brachydactyla*.

2. Materials and methods

Ten adult males of the spider crab *M. brachydactyla* Balss, 1922 were captured in Galicia, NW Spain, by artisanal coastal fishery using gillnets from November 2005 to May 2007 and were transported in dry and high humidity conditions to IRTA facilities (Tarragona, NE Spain). Once in the laboratory, carapace length (CL) and weight (*W*) were measured, being in average CL = 149.68 ± 18.43 mm and *W* = 1118.0 ± 436.4 g (mean ± SD). Spider crabs were then placed on ice during 10 min and dissected. The whole reproductive system was measured, extracted, and processed for light and electron microscopy. The different regions of the reproductive system were measured using a digital caliper, before extraction (testes and vas deferens) and after extraction (accessory gland and ejaculatory duct). The length of the seminiferous tubule was measured after the extraction of a whole testis. Then, the layer of connective tissue that covers the testis was removed, the seminiferous tubule was uncoiled manually into a unique strand, and its total length was measured using a ruler.

For light microscopy (LM) the testis, vas deferens, accessory gland, and ejaculatory duct were fixed in Bouin's solution between 24 and 48 h depending on tissue size. Tissues were rinsed and stored in 70% ethanol until processing. Dehydration was carried out in a tissue processor (Histolab, Myr) applying an increasing alcohol series of 70% (3 h), 96% (1 h), and twice in 100% (1 h each), followed by a wash of absolute ethanol:xylene (1:1) and twice in xylene (1 h each). Finally, tissues were immersed in two consecutive paraffin baths (3 and 11 h) and embedded in paraffin. Sections of 3 μm were cut on a Leica RM 2155 rotary microtome and stained with Harris's hematoxilin–eosin (H–E) and Mallory's stain. Sections were observed under an Olympus BX61 light microscope using bright field optics, and photographs were taken with an Olympus DP70 camera connected to the microscope.

Samples of the testis, vas deferens, accessory gland, and ejaculatory duct for transmission electron microscopy (TEM) and for scanning electron microscopy (SEM) were extracted, dissected, and fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 24 h at 4 °C. Samples were rinsed in cacodylate buffer (3 times for 10 min and 3 times for 30 min) and postfixed in 1% osmium tetraoxide in cacodylate buffer (twice for 1 h and 30 min, respectively) at 4 °C. Then, the samples were rinsed in cacodylate buffer twice for 5 min and once for 30 min. The TEM samples were dehydrated applying an increasing acetone series (30% for 15 min, 50% for 15 min, 70% for 15 min, 70% overnight incubation at 4 °C, 90% for 60 min, 75 min for 95% and twice 100% for 30 min) and embedded in Spurr's resin. Ultrathin sections were made in a Leica UCT ultramicrotome and counterstained with uranyl acetate and lead citrate. Observations were made on a Jeol EM-1010 transmission electron microscope at 80 kV. Progressive dehydration of SEM samples was made in an increasing ethanol series (30% for 15 min, 50% for 15 min, 70% for 15 min, 70% overnight incubation, 90% for 60 min, 95% for 75 min and twice 100% for 30 min). Later, samples were dried at their critical point with CO_2 and then sputter-coated with gold-palladium. Observations were made on a Hitachi S-2300 scanning electron microscope at 10-15 kV.

Measurements of the testis, vas deferens, and ejaculatory duct as well as cellular measurements (n = 10–30) of the muscular and epithelial layers were made using an image analyzing system (AnalySIS, SIS). Results are shown as mean ± SD.

3. Results

3.1. Gross morphology

The male reproductive system of *M. brachydactyla* is composed of paired tubular testes, paired vasa deferentia, and paired ejaculatory ducts located in the cephalothoracic cavity (Fig. 1A). The reproductive system runs parallel to the median plane of the body, lying dorsal to the hepatopancreas lobes. A layer of connective tissue encloses and attaches the reproductive system to different components of the body cavity.

The testes are located in the anterior half of the body extending longitudinally from the anterior end of the branchial chamber, close to the insertion of the epipodite of the first maxilliped, to the base of the eyestalk (Fig. 1A). Then, the testes run laterally along the stomach, passing around the dorsal end of the mandibular tendons and meeting in the posterior end of the stomach. From this point, the testes run close to each other parallel to the median body plane until they reach the anterior region of the heart, where the vas deferens begins. No commissural connection in the distal region of the testes has been observed. Testes are white tubular organs, 77.08 ± 4.30 mm long in adult crabs, composed of a single, highly coiled seminiferous tubule, which is circular in section and has a diameter of 0.66 ± 0.29 mm (Figs. 1B and 2A and B). The length of the uncoiled seminiferous tubule reaches up to 3 meters when the connective tissue that covers the reproductive system is removed.

The vasa deferentia (VD) are complex coiled tubes in the posterior half of the body within the branchial cavities and over the intestine (Fig. 1A). The length of the coiled vas deferens is 56.48 ± 1.15 mm in adult crabs. The VD has been divided into 3

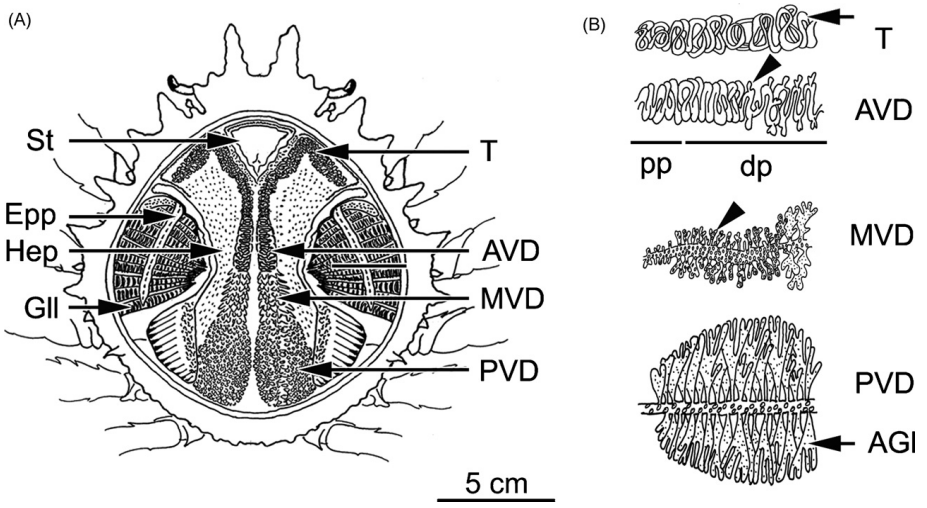

Fig. 1. *Maja brachydactyla*. Internal male anatomy. A. Diagrammatic representation. The reproductive system lies on the hepatopancreas and is composed of testis and vas deferens divided into anterior, median, and posterior vas deferens. The ejaculatory duct, not represented, is located in the coxa of the fifth walking leg. B. Diagrammatic representation of the testis and vas deferens. The testis is composed of a single, highly coiled seminiferous tubule (arrow). The anterior vas deferens is a folded duct divided into a smooth, proximal portion and a distal portion showing small, isolated diverticula (arrowhead). The median vas deferens is a twisted tube with numerous diverticula (arrowhead) located at one side of the duct. The narrow, short, posterior vas deferens presents an associated large accessory gland composed of a few, highly ramified diverticula. AGl, accessory gland; AVD, anterior vas deferens; dp, distal portion of the AVD; Epp, epipodite; Gll, gills; Hep, hepatopancreas; MVD, median vas deferens; pp, proximal portion of the AVD; PVD, posterior vas deferens; St, stomach; T, testis.

distinct regions, based on morphological and functional criteria: anterior (AVD), median (MVD), and posterior (PVD) vas deferens (Fig. 1A and B). The AVD and MVD appear as white opaque, coiled masses beneath the heart. The AVD, which is connected to the testis, has been divided into 2 portions: the proximal portion, a smooth narrow duct, and the distal portion, which presents a few short, isolated diverticula (see arrowhead in AVD diagram of Fig. 1B). The diameter of the AVD increases from the proximal portion (0.55 ± 0.42 mm) to the distal portion (2.57 ± 0.24 mm). The MVD is a twisted tube with an average diameter of 2.67 ± 0.56 mm. Numerous ramified diverticula are located only in one half of the duct, increasing in size towards the PVD. The PVD is a short (approximately 2.5 mm long) and narrow (diameter of 1.12 ± 0.32 mm) tubule in the posterior end of the body and is covered by an accessory gland. The accessory gland is a white or pink, large globular mass (diameter of 26.94 ± 2.74 mm) composed of 7 or 8 highly ramified diverticula attached to the dorsal side of the PVD.

The ejaculatory duct (ED) passes through the openings of the endophragmal system and extends between the muscles of the coxa of the fifth walking leg. The ED is a long (57.97 ± 5.71 mm), narrow, smooth duct, circular in section and with a constant diameter (1.12 ± 0.08 mm). The terminal portion of the ED slightly increases in diameter and finishes as a terminal ampoule that surrounds the internal opening of the gonopore.

3.2. Histology

3.2.1. Testis

The wall of the seminiferous tubule presents a layer of connective tissue, a muscular layer of scattered striated fibers, and a single layer of epithelial cells (Fig. 2C). The connective tissue layer surrounds the muscular layer and is composed of an outer layer (lamina adventitia) and an inner layer (lamina propria). The inner epithelial layer divides the seminiferous tubule into 3 zones: the germinal zone (GZ), at one pole of the tubule; the transformation zone (TZ), filling the central region; and the evacuation zone (EZ) or collecting tube, in the opposite pole to the GZ (Fig. 2D and E).

The GZ contains two cellular types: spermatogonia and accessory cells. Both cellular types lie directly on the connective tissue layer due to the absence of the inner epithelial layer of the tubule wall (Fig. 2F). Accessory cells are small cells with oval nuclei located between the spermatogonia. The outline of the cell is not well defined, and cytoplasm fills the spaces left by the spermatogonia. The size of the GZ increases, concomitantly to the decrease of the TZ, as spermatogenesis progresses. The GZ appears as a thin, convex layer in the periphery of the seminiferous tubule when spermatocytes and spermatids fill the TZ (Fig. 2E and F). However, the GZ is more prominent, occupying larger areas of the seminiferous tubule, at the end of spermiogenesis when spermatozoa fill the TZ (Fig. 2D).

The TZ presents germ cells in different stages of spermatogenesis and spermiogenesis accompanied by accessory cells (Fig. 2D and E). Although cells at all stages can be seen along the testis, cells belonging to the same transversal section are usually in the same stage of development or in two successive stages, such as spermatocytes and spermatids or spermatids and spermatozoa (Fig. 2F). Differentiation of germ cells also involves changes in the accessory cells and the inner epithelial layer. At the beginning of spermatogenesis, the accessory cells are still undifferentiated – appearing as small, oval cells similar to those found in the GZ – and are located among the spermatocytes in the periphery of the seminiferous tubule. When the spermatocytes mature into spermatids, the accessory cells appear as radial extensions from the wall of the seminiferous duct towards the central region of the TZ, with the cytoplasm forming several projections that surround adjacent spermatids (Fig. 2E and F). Epithelial cells remain as a thin layer until germ cells mature to the spermatozoa stage. Then, the epithelium appears as a monostratified layer of columnar cells with basal, rounded highly basophilic nuclei. The cytoplasm contains lightly electron-dense

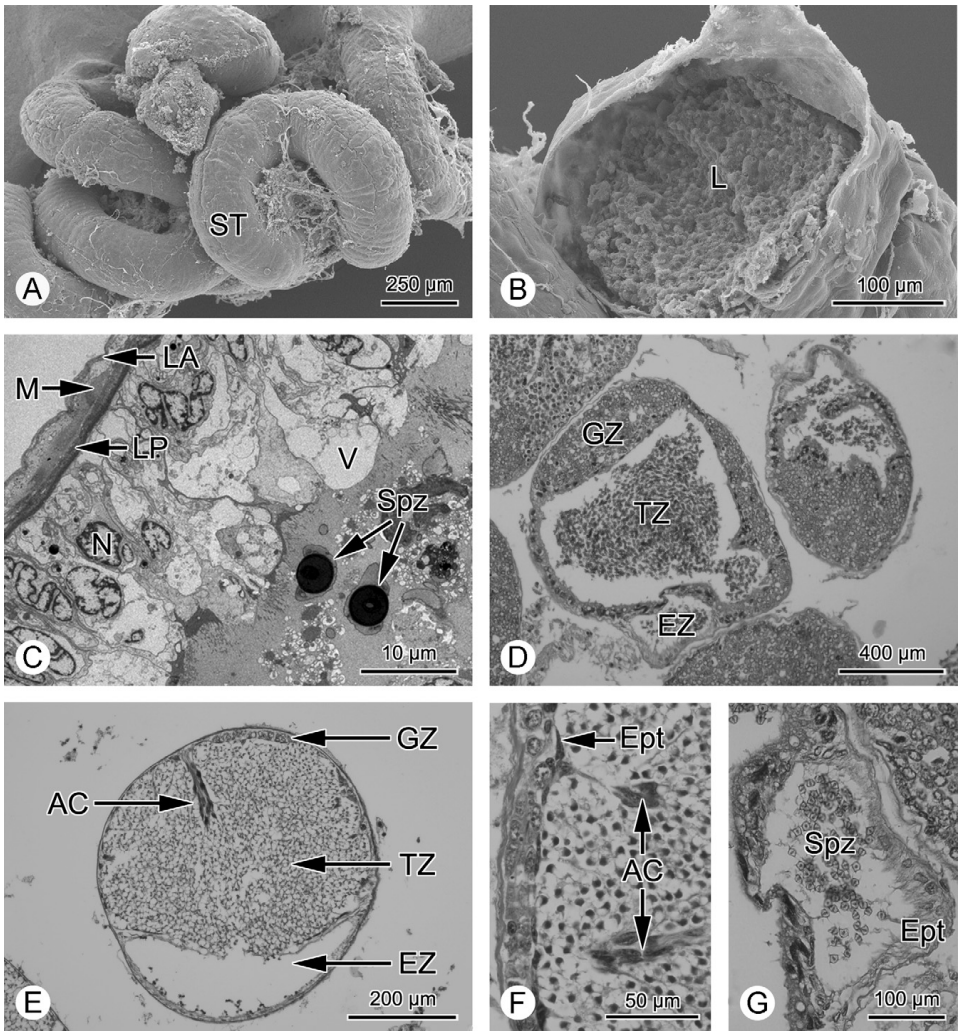

Fig. 2. *Maja brachydactyla*. Seminiferous tubule. A and B. General view of the testis. SEM. Each testis consists of a single, highly coiled seminiferous tubule that contains developing gametic cells in the lumen. C. Ultrastructure of the wall. TEM. The musculature is composed of a single layer of striated muscular fibers. The inner epithelium is columnar with basal nuclei when spermatids transform to spermatozoa, and the cytoplasm contains an enlarged vacuole in the apical region. D. Transversal section. LM, H–E. The seminiferous tubule is divided in germinal, transformation, and evacuation zones. The germinal zone is well developed. The transformation zone in the central area of the seminiferous tubule contains spermatozoa, which will be released to the evacuation zone. E. Transversal section. LM, Mallory's staining. The germinal zone appears as a thin band opposite to the evacuation zone. The accessory cells increase in size towards the center of the seminiferous tubule when the spermatids fill the transformation zone. F. Detail of the germinal and transformation zones. LM, H–E. The transformation zone, containing spermatids, presents enlarged accessory cells. G. Detail of the evacuation zone. LM, H–E. The evacuation zone only contains spermatozoa and is lined by columnar epithelium with basal nuclei. AC, accessory cells; Ept, epithelium; EZ, evacuation zone; GZ, germinal zone; L, lumen; LA, lamina adventitia; LP, lamina propria; M, muscular layer; N, nuclei; Spz, spermatozoa; ST, seminiferous tubule; TZ, transformation zone; V, vacuole.

material and a prominent vacuole associated to the apical region (Fig. 2C). However, no secretory activity has been observed.

The EZ is located in the opposite pole to the GZ and contains only the mature spermatozoa originated in the TZ (Fig. 2D and E). The outer edge of the EZ presents a monostratified epithelium whose cells vary in height from flattened to columnar. The cells contain basal nuclei that are flattened and highly basophilic and a cytoplasm that shows vacuoles of transparent material. Spermatozoa are surrounded by seminal fluids and generally fill the central region of the lumen of the EZ (Fig. 2G). The EZ collects the spermatozoa pro-

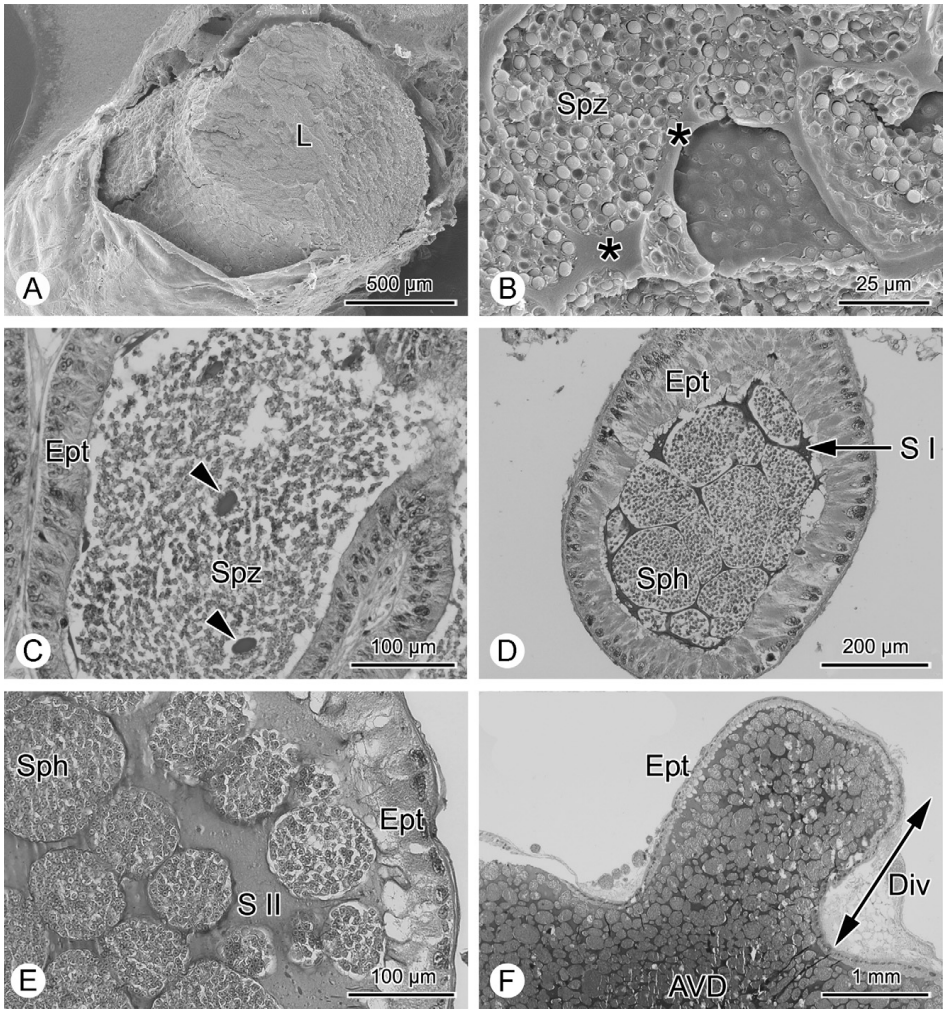

Fig. 3. *Maja brachydactyla*. Anterior vas deferens (AVD). A and B. SEM. Spermatozoa are divided by epithelial secretions (asterisk) in the lumen. C. Transversal section of the proximal portion close to the testis. LM, H–E. The homogeneous mass of spermatozoa is not yet divided by substance I (arrowheads). D. Transversal section of the proximal portion. LM, H–E. The columnar epithelium lines the wall and secretes substance I, which divides the sperm mass into spermatophores. E. Transversal section of the distal portion. LM, H–E. The epithelium decreases in height in the distal portion and secretes substance II, which surrounds the spermatophores. F. Longitudinal section of the distal portion. LM, H–E. Diverticula containing spermatophores are lined by a columnar epithelium. AVD, anterior vas deferens; Div, diverticula; Ept, epithelium; L, lumen; Sph, spermatophore; Spz, spermatozoa; S I, substance I; S II, substance II.

duced along the testis and brings them to the vas deferens, where they are packaged and stored in spermatophores.

3.2.2. Vas deferens

3.2.2.1. Anterior vas deferens (AVD). Spermatozoa from the testis enter the AVD as a homogeneous mass, which is divided in spermatophores by two consecutive epithelial secretions (Fig. 3A–C). The first substance, named substance I, is secreted by the epithelium of the proximal portion of the AVD (Fig. 3C and D). Substance I has a homogeneous appearance, stains red in hematoxilin–eosin and Mallory's stain, and divides the sperm mass into small oval groups of irregular size (Fig. 3D). The second substance, substance II, is secreted by the distal portion of the AVD, stains blue in hematoxilin–eosin and Mallory's stain, and surrounds each single sperm mass, which becomes a spermatophore (Fig. 3E). Freshly formed spermatophores are found along the distal portion, including the diverticula (Fig. 3F).

The wall of the AVD is composed of a layer of connective tissue, an intermediate muscular layer, and an internal epithelium (Fig. 4A). The connective tissue layer consists of an outer and an

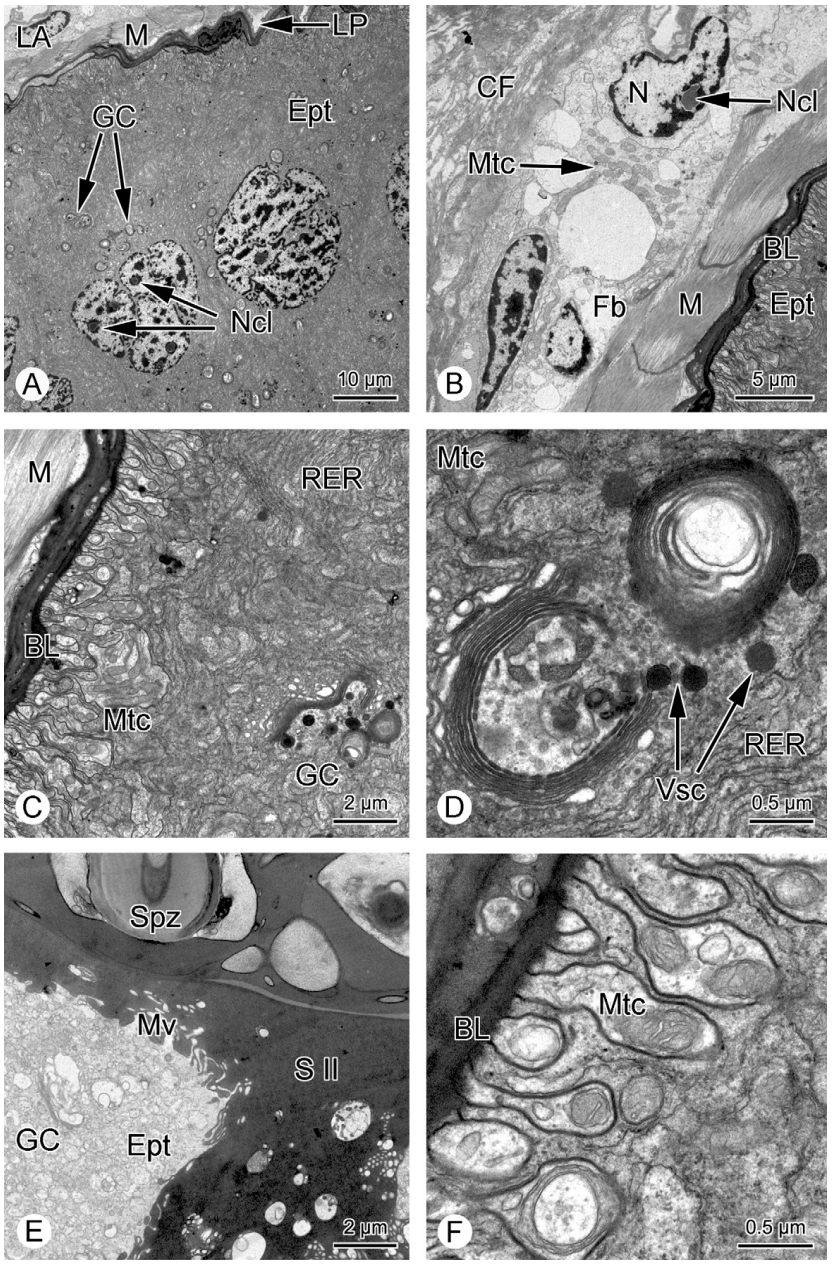

Fig. 4. *Maja brachydactyla.* Anterior vas deferens (AVD). TEM. A. Ultrastructure of the wall. The wall is composed of an outer layer of connective tissue (lamina adventitia), a muscular layer, an inner layer of connective tissue (lamina propria), and an innermost epithelial layer. Epithelial cells present lobed nuclei with several nucleoli and a well-developed Golgi complex. B. Detail of the wall. Fibroblast-like cells, which produce collagen fibers, present oval nuclei and numerous mitochondria in the cytoplasm. The musculature is composed of a single layer of striated muscular fibers. Epithelial cells rest on an electron-dense basal lamina. C. Cytoplasm of the epithelium. The cytoplasm contains large amounts of rough endoplasmic reticulum, a developed Golgi complex, and numerous mitochondria. D. Golgi complex of the epithelium. The Golgi complex

inner layer called lamina adventitia and lamina propria, respectively. The lamina adventitia is composed of a single layer of fibroblast-like cells, which produce collagen fibers. Their nuclei are oval or spindled-shaped with few nucleoli (Fig. 4B). The cytoplasm contains lightly electron-dense material and numerous mitochondria. The lamina propria is intercalated between the musculature and the epithelium, which lies on an electron-dense basal lamina (Fig. 4B). The musculature is composed of a single layer of scattered striated muscular fibers oriented obliquely to the axis of the duct (Fig. 4A and B). The epithelium is composed of a single layer of secretory columnar cells (96.97 ± 12.06 μm) in the proximal portion that decrease in size towards the distal portion (68.31 ± 8.26 μm) (Fig. 3D and E). Epithelial cells of the diverticula are also columnar (80.26 ± 13.70 μm) and present the same characteristics than the epithelial cells of the AVD (Fig. 3F). Their nuclei are prominent and located in the basal region of the cell (Fig. 3D and E). The chromatin is condensed in the periphery of the nucleus, and several nucleoli are dispersed throughout the nucleus (Fig. 4A). The cytoplasm is filled with large amounts of rough endoplasmic reticulum (RER), which is organized in flattened and rounded cisterns (Fig. 4C) that surround the numerous mitochondria (Fig. 4D). The Golgi complex is also well developed and dispersed throughout the cell (Fig. 4A). The units of the Golgi complex produce vesicles of highly electron-dense material (Fig. 4C and D). The vesicles are released to the lumen by the apical region of the cell, which is brushed with microvilli (Fig. 4E). The basal region of the plasma membrane presents large invaginations that are continuous with the basal lamina (Fig. 4C). Mitochondria, granules, and vesicles of translucent material fill the invaginations (Fig. 4F).

3.2.2.2. Median vas deferens (MVD). The MVD stores large amounts of spermatophores embedded in a heterogeneous matrix (Fig. 5A–C). This matrix is composed of the epithelial secretions of the MVD and the remains of the secretions from the AVD. The secretion of the MVD consists of translucent drops that merge in the lumen, acquiring spherical and laminar shapes and forming a complex net in which the spermatophores are embedded (Fig. 5C).

The wall of the MVD presents a connective tissue layer, a single layer of scattered striated muscular fibers, and a monostratified secretory epithelium lying on a basal lamina (Fig. 5D and E). The epithelial cells are cubic or flattened, with a height of 23.68 ± 9.21 μm (Fig. 5C). The epithelial cells in the diverticula also show the same characteristics and have a height of 19.35 ± 8.63 μm. The nuclei are basal and voluminous, filling the width of the cell in both cubic and flattened cells (Fig. 5D). Epithelial cells observed under TEM present irregular lobed nuclei showing rounded or flattened morphology (Fig. 5D). The chromatin appears highly condensed in the periphery of the nucleus as well as forming large granules. Several nucleoli, associated to the peripheral condensed chromatin, are also present in the nucleus. The cytoplasm is mainly filled with flattened and rounded cisterns of RER. The units of the Golgi complex are distributed throughout the cell, in both apical and basal regions, and appear generally in clusters, showing circular and longitudinal morphologies (Fig. 5D). Vesicles produced by the Golgi complex contain highly electron-dense material and are released to the lumen in the apical region of the cell, which is brushed with short microvilli (Fig. 5D and F). The basal region presents invaginations of plasma membrane that contain several mitochondria, also found among the cisterns of the RER (Fig. 5E).

3.2.2.3. Spermatophore. The spermatophores of *M. brachydactyla* are stored in the MVD, are oval – nearly spherical – and have an average major and minor axis of 134.57 ± 38.08 μm and 109.48 ± 36.68 μm, respectively (Fig. 6A). Moreover, the spermatophores show a great variation in size, the major axis ranging from 54.92 to 247.35 μm. The spermatophore consists of a sperm mass embedded in a matrix, whose main component is substance I, and surrounded by a thin acellular wall composed of substances I and II, secreted only in the AVD (Fig. 6 B–D). The thin wall allows the forms of the spermatozoa within to be seen from the outside of the spermatophore (Fig. 6B).

3.2.2.4. Posterior vas deferens (PVD). The PVD is composed of a short duct and an enlarged accessory gland attached to it (Figs. 1B and 7A). The lumen of the PVD presents few spermatophores embedded in an eosinophilic matrix, similar to the secretion produced in the accessory gland (Fig. 7B).

The PVD itself presents a highly developed muscular layer (thickness of 225.67 ± 98.64 μm), intercalated between layers of connective tissue, and a nonsecretory, pseudostratified epithelium (Fig. 7B). The musculature is composed of several layers of striated muscular fibers oriented in multiple directions. The innermost layer of musculature is oriented parallel to the longitudinal axis of the PVD (Fig. 7C). Muscular fibers present great amounts of glycogen-like granules (Fig. 7D). The pseudostratified epithelium is organized in folds of highly prismatic cells (152.90 ± 28.99 μm) with rounded nuclei and chromatin condensed in the periphery (Fig. 7B and E). The cytoplasm appears as a thin layer between greatly folded plasma membranes and contains few mitochondria and a poorly developed Golgi complex, which produces vesicles of lightly electron-dense material (Fig. 7F). The basal region of the cell presents numerous invaginations, containing mitochondria, associated to projections of the thick basal lamina (Fig. 7G). The apical region is brushed with large ramified microvilli (Fig. 7H).

3.2.2.5. Accessory gland (AGl). The accessory gland (AGl) is composed of 7 or 8 highly ramified, enlarged diverticula connected to the dorsal region of the PVD (Figs. 1B, 7A and 8A). The AGl produces and stores large amounts of seminal fluids secreted by the epithelium that lines the diverticula. The epithelial secretions appear in SEM as well-defined drop-shaped granules (Fig. 8B). The epithelial cells use apocrine secretion to release the granules. Thus, the columnar epithelium becomes flattened during the release at the apical region of the cytoplasm (Fig. 8C–E). In LM and TEM observations, the granules appear embedded in a gelatinous and heterogeneous matrix, which stains red in hematoxilin–eosin and blue in Mallory's stain (Fig. 8C, D and F).

The wall of the diverticula is formed by a connective tissue layer, a musculature composed of a single layer of striated, scattered muscular fibers, and a monostratified secretory epithelium (Fig. 8G and H). Both columnar (21.06 ± 4.05 μm) and flattened (9.29 ± 1.92 μm) epithelial cells present voluminous, lobed nuclei (Fig. 8H). In flattened secretory cells, nuclei are centrally or apically placed (Fig. 8H). The chromatin is highly condensed in a thin layer under the inner nuclear membrane, and a few granules of condensed chromatin appear in the nucleoplasm. The cytoplasm is filled with large amounts of endoplasmic reticulum composed of flattened cisterns (Fig. 8H and I). Mitochondria as well as endosomal vesicles, present in both basal and apical regions, are numerous. Endosomal vesicles contain membrane complexes, degenerated

produces vesicles of highly electron-dense material and is surrounded by cisterns of rough endoplasmic reticulum and mitochondria. E. Apical region of the epithelium. The Golgi complex is also present in the apical region of epithelial cells brushed with short microvilli. The lumen contains spermatozoa already packaged in spermatophores surrounded by seminal secretions. F. Basal region of the epithelium. Epithelial cells lie on an electron-dense basal lamina. Numerous invaginations of plasma membrane contain several mitochondria. BL, basal lamina; CF, collagen fibers; Ept, epithelium; Fb, fibroblast-like cell; GC, Golgi complex; LA, lamina adventitia; LP, lamina propria; M, musculature; Mtc, mitochondria; Mv, microvilli; N, nucleus; Ncl, nucleolus; RER, rough endoplasmic reticulum; Spz, spermatozoa; S II, substance II; Vsc, vesicles.

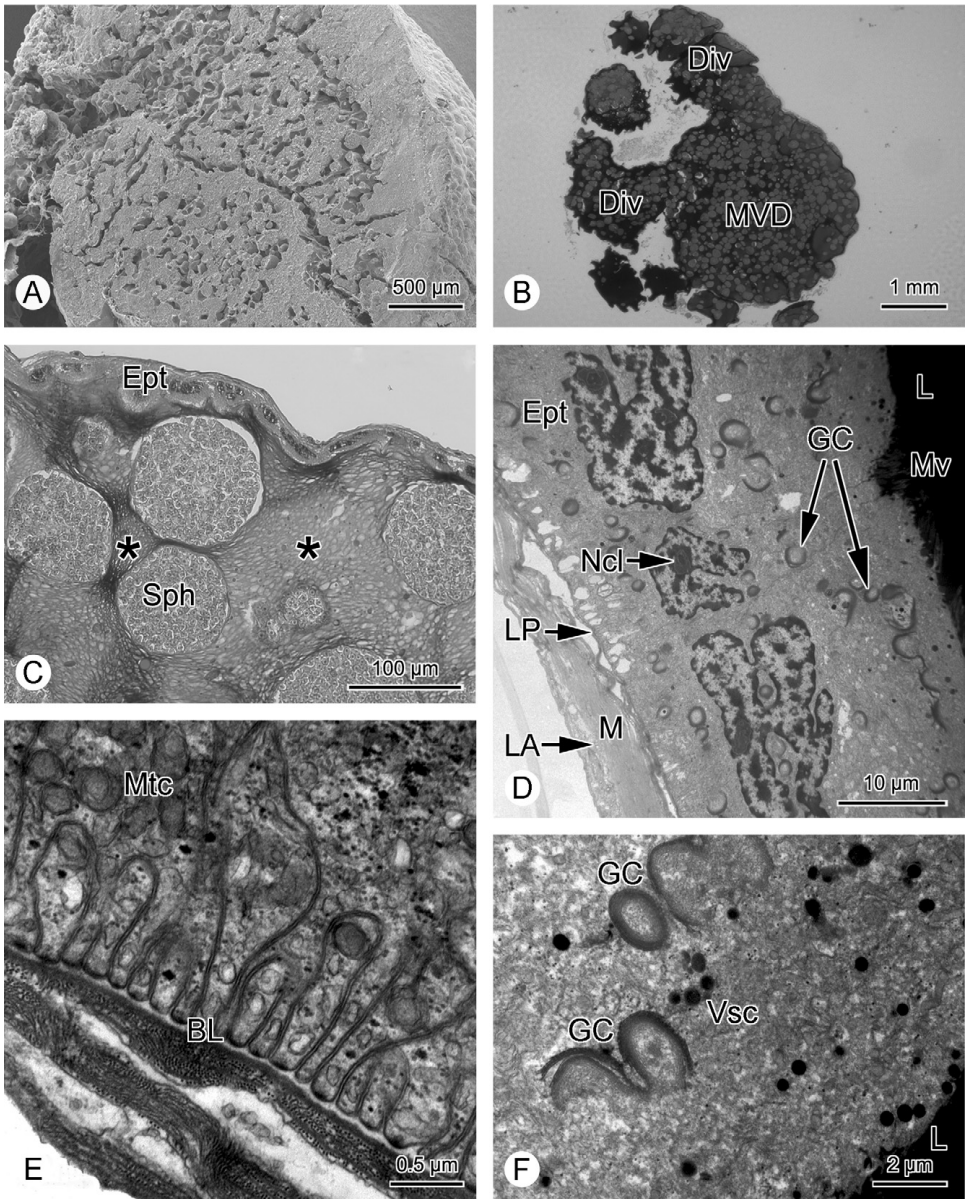

Fig. 5. *Maja brachydactyla*. Median vas deferens (MVD). A. General view. SEM. B. Transversal section. LM, H–E. The MVD stores spermatophores surrounded by seminal fluids. Note that diverticula are located in one pole of the MVD. C. Transversal section. LM, H–E. The spermatophores are embedded in a heterogeneous matrix (asterisk). The MVD is lined by a flattened epithelium. D. Ultrastructure of the wall. TEM. The musculature is composed of a single layer of striated muscular fibers located between the lamina adventitia and the lamina propria. The epithelial cells present lobed nuclei with several nucleoli and contain units of the Golgi complex spread throughout the cytoplasm. The apical region is brushed with microvilli. E. Basal region of the epithelium. TEM. Epithelial cells rest on a basal lamina. Numerous invaginations of the basal plasma membrane contain mitochondria. F. Apical region of the epithelium. TEM. The Golgi complex produces vesicles of highly electron-dense material, which are released to the lumen. BL, basal lamina; Div, diverticula; Ept, epithelium; GC, Golgi complex; L, lumen; LA, lamina adventitia; LP, lamina propria; M, musculature; Mtc, mitochondria; Mv, microvilli; MVD, median vas deferens; Ncl, nucleolus; Sph, spermatophore; Vsc, vesicles.

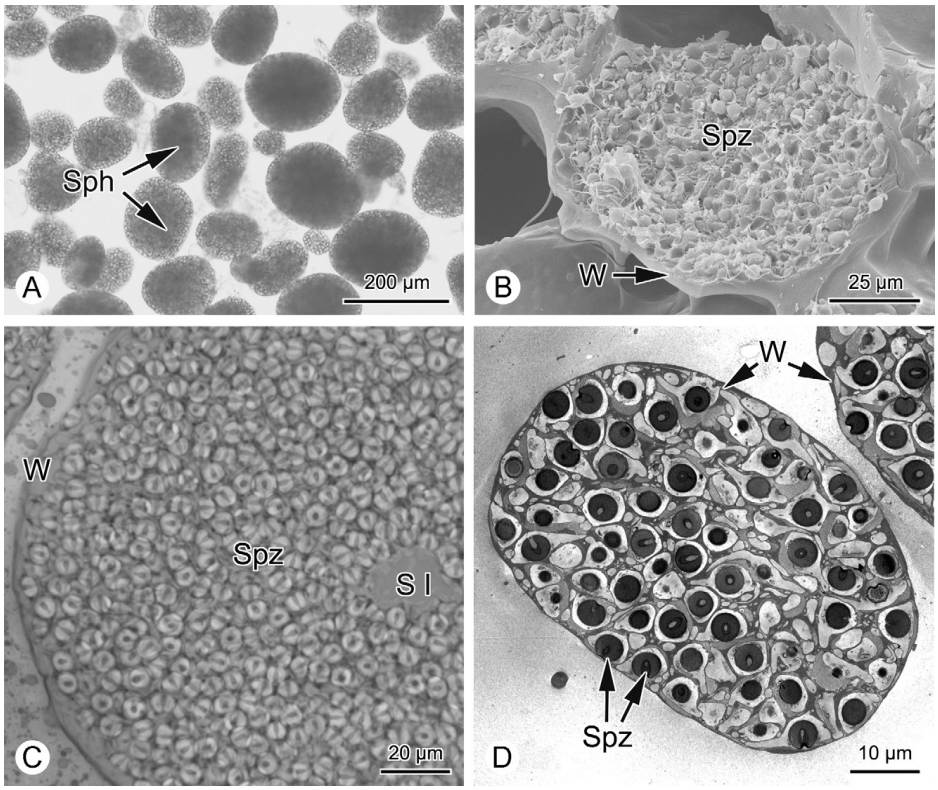

Fig. 6. *Maja brachydactyla*. Spermatophore. A. Fresh smear. LM. Spermatophores are oval or spherical. B. Fractured section. SEM. A thin acellular wall surrounds the spermatozoa. C. Transversal section. LM, H–E. The spermatophore wall is a thin layer composed of both substances I and II. Substance I is also located between the spermatozoa. D. Ultrastructure. TEM. Spermatozoa are surrounded by a thin spermatophore wall. S I, substance I; Sph, spermatophore; Spz, spermatozoa; W, spermatophore wall.

mitochondria, and granular material, all of which is involved in the formation of the secreted material (Fig. 8I). As a result, a voluminous granule is formed in the apical region of the cell, resulting in an apical bulge (Fig. 8H), which is finally secreted to the lumen of the diverticula (Fig. 8F). The basal region does not show membrane invaginations (Fig. 8G), but the apical region presents microvilli (Fig. 8H).

3.2.3. Ejaculatory duct

The ejaculatory duct (ED) is a narrow, smooth duct (Fig. 9A and B), and its lumen is usually closed by an epithelium (Fig. 9B). During copulation or artificially, during dissection, eosinophilic materials similar to that secreted in the accessory gland of the PVD as well as spermatophores from the MVD are seen along the ED (Fig. 9A).

The wall of the ED is mainly formed by a highly developed muscular layer and a nonsecretory, pseudostratified epithelium (Fig. 9A and B). The musculature is composed of multiple layers of striated muscular fibers oriented in different directions, resulting in a thickness of 190.13 ± 47.27 μm (Fig. 9B and D). The epithelium is composed of highly prismatic cells (height of 137.14 ± 24.32 μm) lying on an electron-dense basal lamina (Fig. 9C). Nuclei, ovoid or flattened, are grouped in the basal half of the cell. The chromatin appears condensed in the periphery of the nucleus as well as in granules associated to the nuclear membrane in a central position (Fig. 9C). The cytoplasm contains a small amount of organelles, such as mitochondria and a poorly developed Golgi complex. Short projections of highly electron-dense basal lamina are intercalated within the invaginations of the plasma membrane in the basal region of the cell (Fig. 9C). The apical region of the cell presents numerous cytoplasmic projections towards the lumen of the ED (Fig. 9E).

Tissue identified as the androgenic gland (AG) is attached to the ED through a thin layer of connective tissue (Fig. 9B). The suspected AG is triangular and composed of cellular masses surrounded by connective tissue fibers. The cells of the AG are irregular, slightly polygonal, and have an approximate diameter of 28.09 ± 1.32 μm. Rounded nuclei are centrally located and few cells are binucleate. The chromatin is condensed in the periphery and presents 1 or 2 nucleoli. The cytoplasm presents some signs of vacuolization, especially below the plasma membrane (Fig. 9F).

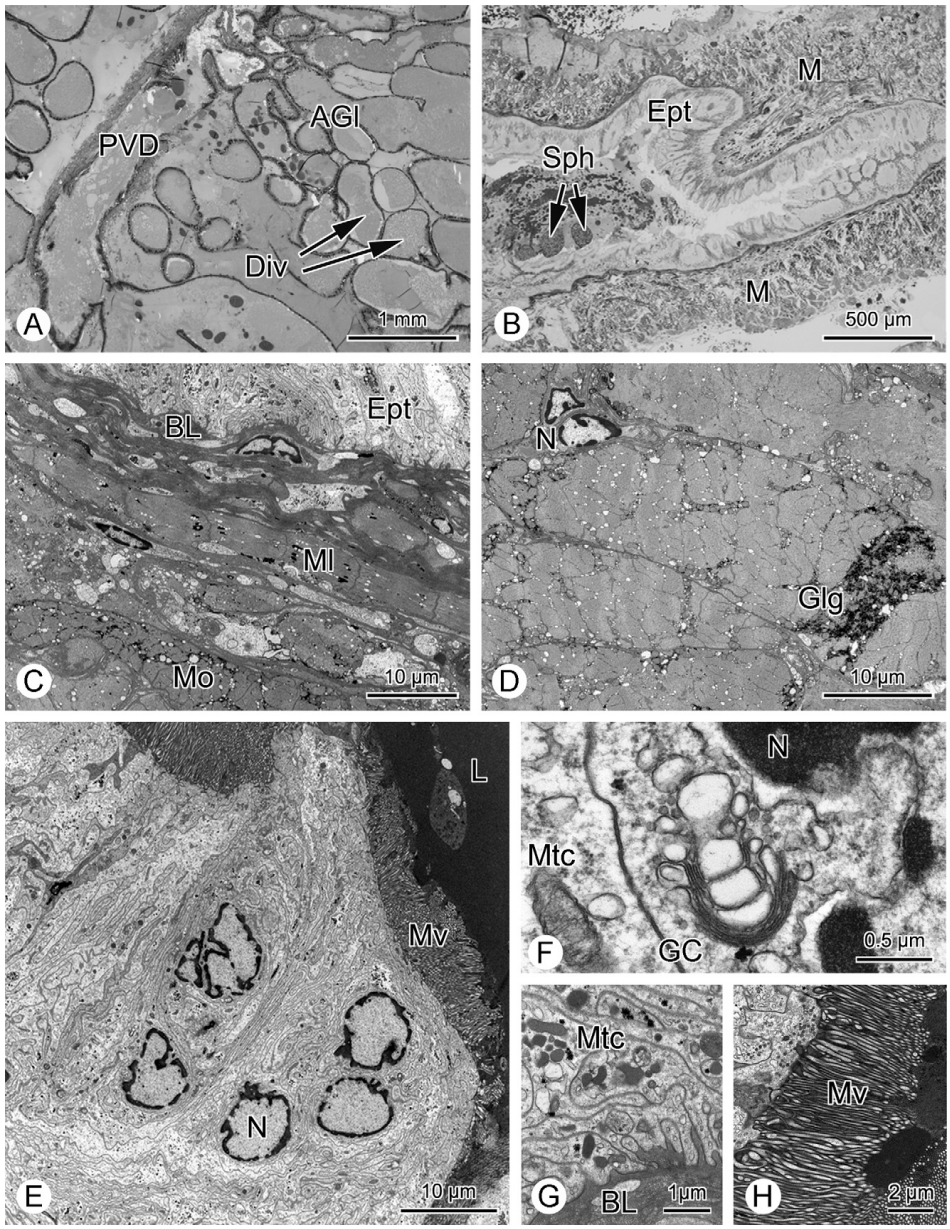

Fig. 7. *Maja brachydactyla*. Posterior vas deferens (PVD). A. General view. LM, H–E. The PVD is formed by a short duct and an associated accessory gland, which is composed of a few highly ramified diverticula. B. Longitudinal section. LM, Mallory's staining. The PVD presents a pseudostratified epithelium and a thick multilayered musculature. The lumen contains few spermatophores. C. Ultrastructure of the wall. TEM. The fibers of the musculature are oriented obliquely and parallel to the longitudinal axis of the PVD. Epithelial cells rest on the basal lamina. D. Ultrastructure of the muscular fiber. TEM. The striated muscular fibers present many glycogen-like granules. E. Ultrastructure of the epithelium. TEM. Epithelial cells present rounded nuclei and greatly folded plasma membranes. The apical region is brushed with long, ramified microvilli. F. Cytoplasm of the epithelium. TEM. The cytoplasm contains few mitochondria and a poorly developed Golgi complex, which produces vesicles of lightly electron-dense material. G. Basal region of the epithelium. TEM. Finger-like projections of basal lamina are inserted between the numerous invaginations of the plasma membrane, which contain mitochondria.

4. Discussion

4.1. Gross morphology

The male reproductive system of *M. brachydactyla* follows the same pattern as other decapoda, showing paired testes, paired vasa deferentia, and ejaculatory ducts (Krol et al., 1992). The reproductive system is symmetric to the median body plane and lies between the hepatopancreas and the hypodermis. Testes, similarly to other Majoidea species (Fasten, 1915, 1917; Sapelkin and Fedoseev, 1981), are located in the anterior region of the body. They extend anterolaterally from the medio-dorsal region of the body cavity along both sides of the stomach, following the outline of the carapace, until the branchial chamber. In this study, we have not observed the commissure indicated by Mouchet (1931). Testes of *M. brachydactyla* are composed of a single, highly coiled seminiferous tubule; consequently, they have been classified as tubular testes according to the categories (lobular and tubular) established by Nagao and Munehara (2003). Tubular testes have been described only in a few species of Brachyura, such as *Menippe mercenaria* (Binford, 1913), *Eriocheir sinensis* (Hoestlandt, 1948), *Chionoecetes opilio* (Kon and Honma, 1970; Sapelkin and Fedoseev, 1981), *Pachygrapsus crassipes* (Chiba and Honma, 1972) and *Chionoecetes bairdi* (Sapelkin and Fedoseev, 1981); however, they seem to be distributed among different groups of Brachyura (Majoidea, Grapsoidea, Xanthoidea). In contrast to the tubular morphology, lobular testes are composed of numerous lobes (also called acini or cysts) connected to a seminiferous duct. Lobular testes, unlike tubular testes, have been widely described among brachyurans (Cronin, 1947; George, 1963; Ryan, 1967; Gupta and Chatterjee, 1976; Hinsch, 1988a; Minagawa et al., 1994; Suganthi and Anilkumar, 1999; Balasubramaniam and Suseelan, 2000; Moriyasu et al., 2002; Nagao and Munehara, 2003; Garcia and Silva, 2006; Cuartas and Petriella, 2007; Castilho et al., 2008). A third testicular morphology, the multiple tubular testes described in *Potamon koolooense* (Joshi and Khanna, 1982), could be considered as lobular testes. Tubular testes seem to be present only in brachyurans since the non-brachyuran Pleocyemata groups studied to date present lobular testes: Achelata (Matthews, 1951, 1954; Burton, 1995), Anomura (Matthews, 1956; Greenwood, 1972; Subramonian, 1981; Manjón-Cabeza and Garcia Raso, 2000; Sokolowicz et al., 2007), Astacidea (Dudenhausen and Talbot, 1983; Haley, 1984; López Greco et al., 2007; Noro et al., 2008), Caridea (Kim et al., 2006), and Dendrobranchiata only present multiple lobular testes (Heldt, 1938; Subrahmanyam, 1965; Malek and Bawab, 1974; Motoh, 1979; Huq, 1981; Chen and Cui, 1986; Champion, 1987; Chow et al., 1991). However, further studies are needed to confirm the exclusiveness of tubular testes in brachyurans, establish phylogenetic relations between groups with tubular testes, and consider the physiological and functional benefits of tubular testes.

The vas efferens, described in *Callinectes sapidus* (Cronin, 1947) and *Portunus sanguinolentus* (George, 1963), is absent in *M. brachydactyla*, where testes are directly connected to the vas deferens (VD). The VD is located in the posterior half of the body over the gut between the branchial chambers. It has been divided according to functional and morphological criteria in anterior (AVD), median (MVD), and posterior (PVD) regions, which is in agreement with previous descriptions of the VD (Mouchet, 1931; Ryan, 1967). Accordingly, the role of the AVD and MVD is spermatophore formation and storage, respectively. The PVD presents a secretory accessory gland (AGl), which produces and stores large amounts of seminal fluids. The present study describes two new features not previously reported by Mouchet (1931). The first one is the division of the AVD into two portions: the proximal, smooth portion and the distal portion, which presents a few isolated diverticula. Each portion plays a different role in the formation of spermatophores. Thus, spermatophore formation begins in the proximal portion and finishes in the distal portion, which also seems to store the spermatophores. The second feature is the characteristic twisting of the MVD and the polar location of the diverticula. The increase of diameter observed from the proximal AVD to the MVD was also reported by Mouchet (1931). The AGl, also called terminal ampoule (Mouchet, 1931), is composed of 7 or 8 highly ramified diverticula connected to the dorsal region of the PVD. The presence of diverticula in the VD of Brachyura has been widely described (Fasten, 1915; Mouchet, 1931; Cronin, 1947; Hoestlandt, 1948; George, 1963; Ryan, 1967; Johnson, 1980; Sapelkin and Fedoseev, 1981; Garcia and Silva, 2006; Castilho et al., 2008), particularly in spider crabs, which present numerous ramified diverticula in the posterior regions of the VD (Mouchet, 1931). Diverticula play an important role increasing the secretion, absorption, and storage of spermatophores and seminal fluids (Adiyodi and Anilkumar, 1988; Diesel, 1991). In *M. brachydactyla* we have considered the diverticula of the PVD as a well-organized secretory gland, since the accessory gland is morphologically and functionally independent from other regions of the VD, similarly to the coral-shaped gland of *Ocypode ceratophthalmus* (Sudha Devi and Adiyodi, 1995). More attention is needed when describing the terminal portion of the vas deferens among Brachyura to avoid confusions, i.e. with the androgenic gland (Sarojini, 1961), and prevent nomenclature misunderstandings, such as the use of terms as rosette (Sapelkin and Fedoseev, 1981) to refer to the PVD of *C. opilio* (Beninger et al., 1988), which could be confused with the rosette glands, located at the base of the first pleopod (Diesel, 1989; Beninger and Larocque, 1998; Brandis et al., 1999). The terminal portion of the reproductive system is the ejaculatory duct, which is a smooth duct extending between the musculature of the fifth walking leg, as already described in Brachyura (Krol et al., 1992).

4.2. Testis

The transversal section of the seminiferous tubule of *M. brachydactyla* is circular and divided by a flattened epithelium into 3 zones called germinal, transformation, and evacuation zone, following the nomenclature given by Hoestlandt (1948). Each zone shows a different content and plays a specific role during spermatogenesis. The germinal zone (GZ) is a thin band in one pole of the seminiferous duct and contains accessory cells and spermatogonia. After maturation mitosis, the spermatogonia become spermatocytes that will mature in the transformation zone (TZ). The TZ fills the central region and contains all stages of developing gametic cells (spermatocytes to spermatozoa) along the seminiferous tubule. The TZ is the zone where spermatogenesis takes place. In a given transversal section of the TZ, sperm cells appear at the same stage or 2 successive stages. However, it has not been possible to establish the pattern of the spermatogenetic wave as in *E. sinensis* (Hoestlandt, 1948), since the length of each stage in the seminiferous tubule seems to be highly variable. The evacuation zone (EZ) is the collecting tubule that contains and carries only mature spermatozoa through the seminiferous duct towards the VD.

The species *M. mercenaria* (Binford, 1913) and *E. sinensis* (Hoestlandt, 1948) also present a seminiferous tubule divided into 3 zones, whereas the snow crabs *C. opilio* and *C. bairdi* (Sapelkin

H. Apical region of the epithelium. TEM. Long, ramified microvilli brush the apical region of the epithelial cells. AGl, accessory gland; BL, basal lamina; Div, diverticula; Ept, epithelium; GC, Golgi complex; Glg, glycogen-like granule; L, lumen; M, musculature; Ml, longitudinal musculature; Mo, oblique musculature; Mtc, mitochondria; Mv, microvilli; N, nucleus; PVD, posterior vas deferens; Sph, spermatophores.

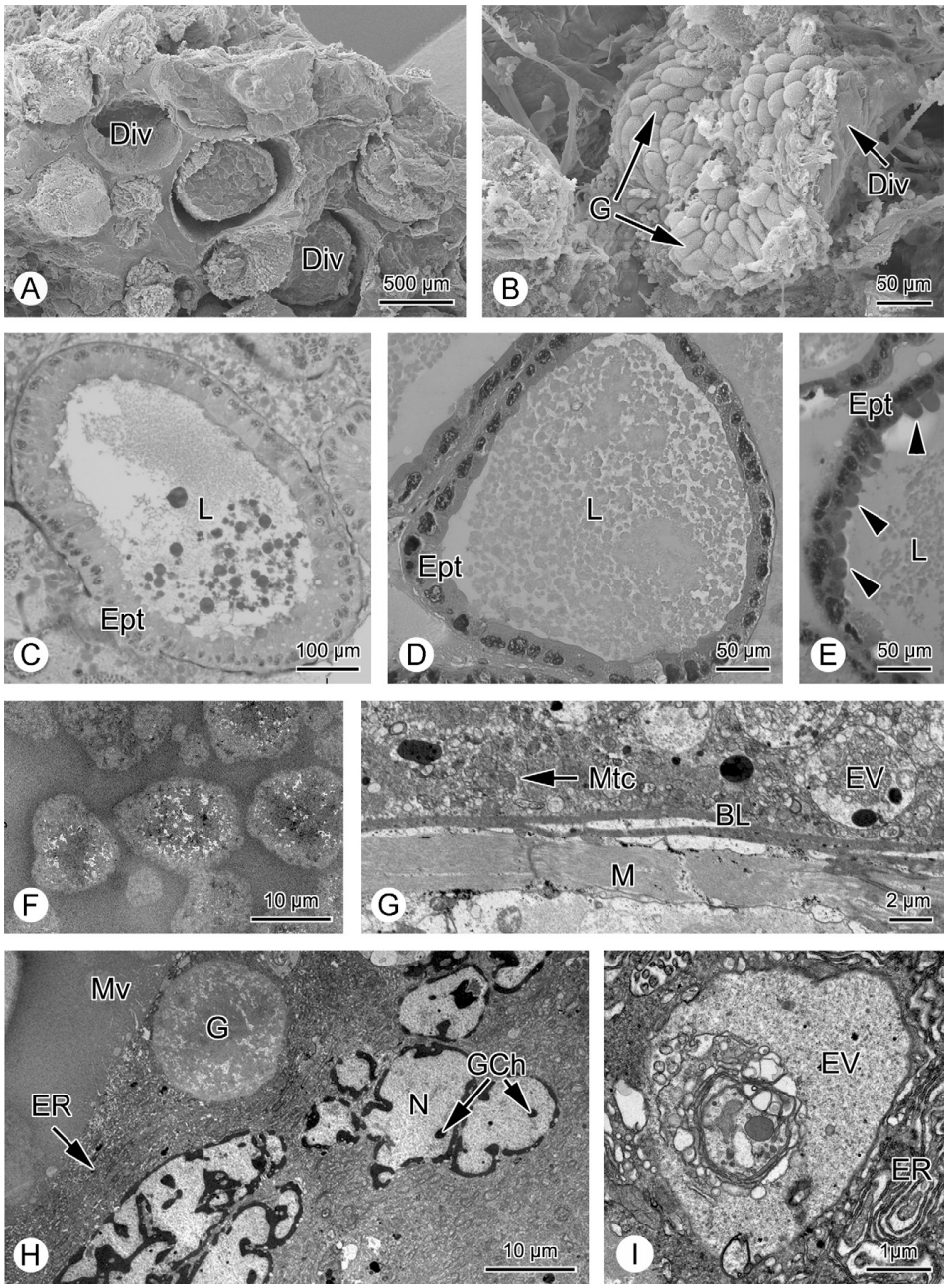

Fig. 8. *Maja brachydactyla*. Accessory gland (AGl) of the posterior vas deferens (PVD). A and B. General view. SEM. The AGl is composed of several diverticula that contain granules secreted by epithelial cells. C–E. Transversal section of the diverticula. LM, C: Mallory's staining, D, E: H–E. The diverticula are lined by a monostratified epithelium with voluminous nuclei. The size of the epithelium decreases due to the release of the apical region in an apocrine secretion (arrowheads). The lumen contains a heterogeneous

and Fedoseev, 1981) only present 2 unequal parts: a larger part containing developing gametic cells and a smaller part, called the seminiferous duct, containing only spermatozoa. Despite the variation in the number of zones, the arrangement of the gametic cells in the seminiferous tubule is similar in all species in which it has been described: a polar organization with the GZ at one pole of the seminiferous duct, the TZ in the center, and the EZ opposite to the GZ. Furthermore, the structure of the seminiferous tubule is morphologically and functionally similar to that of the lobular and multiple lobular testes among decapoda; that is, the spermatogonia are located in the periphery of the lobule or acini, while developing gametic cells fill the central region (Ryan, 1967; Hinsch, 1988a; Chow et al., 1991; Minagawa et al., 1994; Moriyasu et al., 2002).

The wall of the seminiferous tubule of *M. brachydactyla* is composed of 3 layers: a connective tissue layer, composed of an outer lamina adventitia and an inner lamina propria; a muscular layer; and an inner flat epithelium. The musculature has been described only in *E. sinensis* (Hoestlandt, 1948) and may play an important role in the transport of the spermatozoa from the TZ to the EZ and, once here, towards the VD. The inner epithelium divides the lumen of the seminiferous tubule, unlike *E. sinensis*, where thin layers of connective fibers divide the lumen (Hoestlandt, 1948). The flattened epithelium of the TZ becomes a monostratified epithelium of columnar cells when the spermatids undergo the transformation into spermatozoa. Then, the epithelial cells present basal nuclei and an enlarged apical vacuole of lightly electron-dense material, which may be released to the TZ. Similar findings have been observed in *M. mercenaria* (Binford, 1913), suggesting that secretions may push mature spermatozoa towards the EZ. However, the development of a columnar epithelium was attributed to accessory nuclei intercalated between the gametic cells in *C. sapidus* (Cronin, 1947). The accessory cells of *M. brachydactyla* also increase in size during the final stages of spermatogenesis, becoming prominent when the spermatid stage is reached. The development of accessory cells has been also described in *E. sinensis* (Hoestlandt, 1948). Interestingly, prominent accessory cells present a radial disposition with a large cytoplasmic prolongation towards the central region of the seminiferous tubule. Furthermore, apical cytoplasmic extensions surround adjacent spermatids, suggesting a supportive and nutritive role for the accessory cells. In addition, secretory activity has been suggested in *E. sinensis* (Hoestlandt, 1948); although, it has not been observed in this study. The EZ of *M. brachydactyla* contains only spermatozoa produced in the TZ along the seminiferous tubule. A columnar epithelium lines the outer edge of the EZ, as also described in *M. mercenaria* (Binford, 1913), *E. sinensis* (Hoestlandt, 1948), *C. opilio* and *C. bairdi* (Sapelkin and Fedoseev, 1981). The EZ may be structurally and functionally comparable to the collecting tubule found in lobular testes, since the collecting tubule is also lined by a cuboidal or columnar epithelium with possible secretory activity (Krol et al., 1992).

4.3. Vas deferens

The wall of the VD of *M. brachydactyla* is composed of 3 layers: a layer of connective tissue, which consists of a lamina adventitia and a lamina propria; a muscular layer; and an inner epithelium. The musculature presents regional modifications associated to the role of each segment along the VD. Thus, secretory regions such as the AVD, the MVD and the AGl of the PVD present a single layer of scattered, striated muscular fibers obliquely oriented, whereas the PVD shows several layers of striated muscle fibers oriented in different directions. Striated muscular fibers have been also described in the VD of *Penaeus setiferus* (Ro et al., 1990), *Homarus americanus* (Kooda-Cisco and Talbot, 1986) and *Cherax albidus* (Talbot and Beach, 1989). However, in brachyurans only Fasten (1917) and Cronin (1947) described a striated and circular muscular layer. In the AVD and MVD, the musculature may be related to spermatophore formation and movement, since muscular contractions may separate the sperm mass (Ryan, 1967), contribute to the movement of the spermatophore towards posterior regions, and mold the spermatophore by rotation (Mouchet, 1931). In the AGl, the musculature may play an important role discharging the seminal fluids towards the PVD. Thus, the contraction of the musculature in the coral-shaped accessory gland of *O. ceratophthalmus* (Sudha Devi and Adiyodi, 1995) pushes the contents of the lumen towards the posterior MVD. In addition, the obliquely orientation of the muscular fibers in *M. brachydactyla* suggests that the musculature could be arranged helicoidally over the epithelium, improving the release of the secretion towards the base of the diverticula. The highly developed musculature of the PVD may allow the lumen to dilate, facilitating the mixing of the spermatophores from the MVD and the seminal fluids from the AGl, as well as the movement towards the ED.

The epithelium of the VD in *M. brachydactyla* can be grouped in 3 classes according to functional and ultrastructural criteria. The first class is the epithelium of the AVD and MVD, composed of a monostratified layer of secretory cells with exocytosis activity. The second class is the epithelium of the AGl of the PVD, which consists of a monostratified layer of cells with apocrine secretion. Finally, the epithelium of the PVD consists of a pseudostratified layer of nonsecretory cells, similar to the epithelial cells found in the ED. The size of the epithelium decreases from the proximal portion of the AVD, with columnar cells, to the MVD, which presents flattened cells. The decrease of the epithelium has been also observed in *C. sapidus* (Cronin, 1947) and *Chaceon fenneri* (Hinsch, 1988a), although the general pattern among brachyurans is a constant height along the VD. Sapelkin and Fedoseev (1981) pointed out that the secretory activity, the amount of sexual products accumulated in the lumen, and the location of the cells may affect the height of the cells. In *M. brachydactyla*, the decrease in height of the epithelium along the AVD and MVD may be related to a decrease in secretory activity, since the main role of the MVD is the storage of spermatophores.

The epithelial cells of the AVD and MVD present basal and lobed nuclei containing several nucleoli. The cytoplasm is filled with a highly developed rough endoplasmic reticulum and presents numerous mitochondria. The Golgi complex is well developed and produces vesicles containing highly electron-dense material. The vesicles are exocytosed to the lumen of the VD in the apical region, which is brushed with microvilli. In the epithelium, the plasma membrane of the basal region presents large numbers of interdigitations indicating a high degree of ionic exchange. The presence of associated mitochondria to the interdigitations may grant the energetic supply necessary for the ionic exchange. Similar structures have been also observed in the Majoidea *Libinia emarginata* and *Libinia dubia* (Hinsch and Walker, 1974) as well as several decapoda species (Kooda-Cisco and Talbot, 1986; Hinsch and McKnight, 1988; Talbot and Beach, 1989; Ro et al., 1990; Subramonian, 1995). In

mixture of epithelial secretions. F. Lumen of the diverticula. TEM. Granules, surrounded by a heterogeneous matrix, fill the lumen of the diverticula. G. Ultrastructure of the wall. TEM. The musculature is composed of a single layer of striated muscular fibers. The epithelium shows mitochondria and endosomal vesicles. Note the absence of membrane invaginations in the basal region of the epithelial cell. H. Ultrastructure of the epithelium. Flattened epithelial cells present highly condensed chromatin in the periphery of the nucleus, and the cytoplasm shows a granule in the apical region, which is brushed with microvilli. I. Endosomal vesicle. TEM. Endosomal vesicles present a heterogeneous content, such as membrane complexes, mitochondria, and granular material. Note the cisterns of endoplasmic reticulum surrounding the endosomal vesicle. BL, basal lamina; Div, diverticula; Ept, epithelium; ER, endoplasmic reticulum; EV, endosomal vesicle; G, granule; GCh, granules of condensed chromatin; L, lumen; M, musculature; Mtc, mitochondria; Mv, microvilli; N, nucleus.

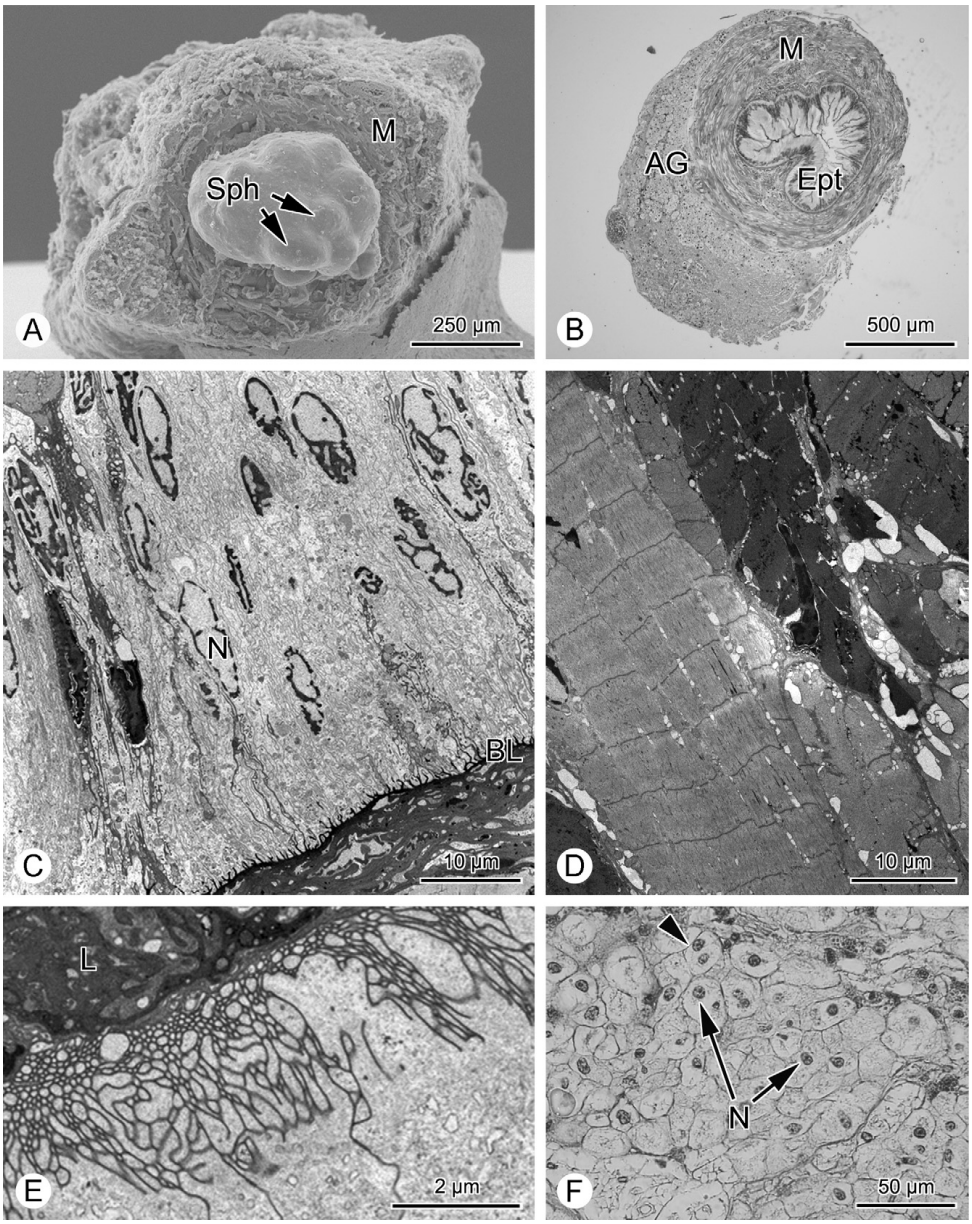

Fig. 9. *Maja brachydactyla*. Ejaculatory duct (ED). A. General view. SEM. The ED presents a thick musculature layer. A few spermatophores surrounded by seminal fluids pass through the lumen of the ED during copulation. B. Transversal section. LM, H–E. The musculature layer surrounds the inner pseudostratified epithelium. Tissue identified as the androgenic gland is attached to the musculature of the ED. C. Ultrastructure of the epithelium. TEM. Epithelial cells present oval nuclei with chromatin condensed peripherally and lie on a thick basal lamina. Note the highly electron-dense projections of basal lamina towards the epithelium in the basal region of the cells. D. Ultrastructure of musculature. TEM. Striated muscular fibers are oriented in different directions. E. Apical region of the epithelium. TEM. Cytoplasmic projections towards the lumen are present in the apical region of the cell. F. Androgenic gland. LM, H–E. Cells are polygonal or irregular, with a central nucleus. Few cells are binucleate (arrowhead). The cytoplasm presents some signs of vacuolization. AG, androgenic gland; BL, basal lamina; Ept, epithelium; L, lumen; M, musculature; N, nucleus; Sph, spermatophores.

addition, the epithelial cells of the MVD in *L. emarginata* and *L. dubia* absorb the secretion products of the AVD, as indicated by the numerous micropinocytotic vesicles at the cell surface (Hinsch and Walker, 1974). However, we have not reported any absorption activity in *M. brachydactyla*, since micropinocytotic vesicles were not observed and the secretions of the AVD remain embedded between the secretions of the MVD.

The epithelial layer of the PVD presents a cytoplasm with a poorly developed Golgi complex and a few mitochondria. The absence of secretory activity is in agreement with its role mixing spermatophores and seminal fluids of the PVD in *M. brachydactyla*. Furthermore, the posterior region of the VD seems to present a great variability of functions among brachyurans. Thus, the epithelium of the PVD in *L. emarginata* and *L. dubia* presents secretory activity and an ultrastructure similar to that in the AVD and MVD (Hinsch and Walker, 1974), whereas the PVD in *C. opilio* presents a phagocytic role of spermatophores and spermatozoa in the distal portion and a storage function of seminal fluids in the proximal region (Benhalima and Moriyasu, 2000).

The epithelial cells of the AGl present lobed nuclei with condensed chromatin and several nucleoli. The cytoplasm contains large amounts of endoplasmic reticulum, which may be involved in the production of the granule. Thus, the material produced by the endoplasmic reticulum seems to accumulate in the cytoplasm forming the granule. In addition, the numerous endosomal vesicles indicate an important degree of autophagy, which may also play an important role in the formation of the granule by degrading cytoplasmic components that will form the granule. Finally, the granule is released to the lumen of the diverticula by apocrine secretion. Similar findings have been described in the coral-shaped accessory gland of the crab *O. ceratophthalmus* (Sudha Devi and Adiyodi, 1995). The materials produced by the epithelial cells of *O. ceratophthalmus* are partially discharged by a merocrine and apocrine secretion. These materials are proteinaceous in nature, since the cytoplasm contains free ribosomes, polyribosomes, a developed RER, and numerous cisterns of the Golgi complex. However it is difficult to establish the nature of the secretion in *M. brachydactyla* due to the heterogeneous source of the materials.

4.4. Ejaculatory duct

The ED transfers the spermatophore masses from the VD to the gonopore during copulation, as in other brachyurans (Cronin, 1947). The wall of the ED is composed of 3 layers: a connective tissue layer, composed of a lamina adventitia and a lamina propria; a muscular layer; and an internal epithelium. The musculature is composed of several layers of striated muscular fibers. The thickness of the muscular layer remains constant, contrary to *Ranina ranina* (Minagawa et al., 1994), whose musculature increases, and *C. opilio* and *C. bairdi* (Sapelkin and Fedoseev, 1981), whose musculature decreases. A multilayered musculature has been also observed in *C. opilio,* in which the muscular fibers are oriented obliquely to the longitudinal axis of the duct (Sapelkin and Fedoseev, 1981). In contrast *M. brachydactyla* outer fibers are circularly oriented, while the inner fibers are longitudinally oriented. The musculature may play an important role in the extrusion by allowing the dilatation of the lumen, normally closed by the epithelium, and subsequently, the movement of spermatophores embedded in seminal fluids to the gonopore.

The inner layer of the ED of *M. brachydactyla* is composed of a highly prismatic, pseudostratified epithelium lying on a thick basal lamina similar to that found in the PVD. The spider crabs *C. opilio* and *C. bairdi* (Sapelkin and Fedoseev, 1981) as well as *Ucides cordatus* (Castilho et al., 2008) also present a pseudostratified epithelium, whereas the portunid crabs *C. sapidus* (Cronin, 1947) and *P. sanguinolentus* (Ryan, 1967) and the Majoidea *L. emarginata* and *L. dubia* (Hinsch and Walker, 1974) present cuboidal or low columnar cells. In *R. ranina* the ED lacks an epithelial layer (Minagawa et al., 1994). Epithelial cells present oval basal nuclei with chromatin condensed peripherally in a thin layer. The cytoplasm is poor in organelles, containing a poorly developed Golgi complex and small mitochondria. No endoplasmic reticulum has been reported. Thus, no secretory activity has been observed in contrast to observations made in *L. emarginata* and *L. dubia* (Hinsch and Walker, 1974), in which the epithelial cells of the ED present a developed RER and Golgi complex, indicating secretory activity. The lumen of the ED is full with epithelial cells, although the remainder of the secretions from the accessory gland and a few spermatophores can eventually be seen. Similar findings have been described in *E. sinensis* (Hoestlandt, 1948), *P. sanguinolentus* (Ryan, 1967), *Scylla serrata* (Uma and Subramoniam, 1984), *Goniopsis cruentata* (Garcia and Silva, 2006), and *U. cordatus* (Castilho et al., 2008), whose ED contains material similar to that secreted in the PVD and the spermatophores.

The ED presents a type of tissue, identified as the androgenic gland (AG), attached to the musculature by connective tissue. The AG is responsible for the secretion of the androgenic gland hormone, which maintains and develops the male sexual characters (Adiyodi and Adiyodi, 1997; Sagi and Khalaila, 2001). The AG appears as a triangular organ in a cross section of the ED and is composed of a cellular mass surrounded by connective tissue. The cells are irregular or polygonal with a central nucleus and vacuoles in the cytoplasm, and only a few cells appear binucleate. Similar descriptions of the AG have been previously reported in *M. brachydactyla* (Charniaux-Cotton, 1958; Charniaux-Cotton et al., 1966). Meusy (1972) described the ultrastructure of AG cells, reporting the presence of a well-developed RER and numerous organelles similar to lysosomes. In addition, he found signs of cellular degeneration, indicating a holocrine mode of secretion.

4.5. Spermatophore formation and structure

The histological observations have revealed the process of spermatophore formation and the role of the epithelium of the VD in *M. brachydactyla*. Thus, the secretions of the AVD are involved in the spermatophore formation: substance I, secreted by the epithelium of the proximal portion, divides the sperm mass into small clumps; and substance II, secreted by the epithelium of the distal portion, consolidates the small clumps resulting into the spermatophore. This pattern has been also observed in several brachyurans (Cronin, 1947; Ryan, 1967; Uma and Subramoniam, 1984; Adiyodi and Anilkumar, 1988), in which 2 substances of different nature secreted in the AVD are responsible for the spermatophore formation.

The spermatophore of *M. brachydactyla* presents the typical brachyuran morphology (Mann, 1984; Adiyodi and Anilkumar, 1988; Hinsch, 1991; Subramonian, 1991). Spermatophores are oval, nearly spherical (134 μm × 109 μm), with a great variation in diameter. The size of the spermatophores is similar to those found in *E. sinensis* (Hoestlandt, 1948), *C. opilio* and *C. bairdi* (Sapelkin and Fedoseev, 1981). The spermatophores of *M. brachydactyla* have an intermediate size, since they are larger than those described in *Inachus phalangium* (Diesel, 1989; Rorandelli et al., 2008), *Carcinus maenas* (Spalding, 1942), and *U. cordatus* (Castilho et al., 2008) but smaller than those in *C. sapidus* (Johnson, 1980) and *P. sanguinolentus* (Ryan, 1967). The spermatophore consists of a sperm mass surrounded by a thin acellular layer, the spermatophore wall. The spermatozoa are embedded in a heterogeneous matrix, presenting a range of electron densities. The main substance filling the spaces between the spermatozoa is also the main component of the spermatophore wall and corresponds to substance I of the AVD. Thus, the spermatophore wall is composed of a single layer, similar to that in *L. emarginata* and *L. dubia* (Hinsch and

Walker, 1974) and *Ovalipes ocellatus* (Hinsch, 1986). In contrast, a spermatophore wall composed of two layers has been reported in several species of Brachyura (Spalding, 1942; Cronin, 1947; Uma and Subramoniam, 1984; Hinsch, 1988b; El-Sherief, 1991; Chiba et al., 1992; Minagawa et al., 1994; Anilkumar et al., 1999). In *M. brachydactyla* the outer surface of the spermatophore is smooth, similarly to other species (Spalding, 1942; Hinsch and Walker, 1974; Hinsch, 1986, 1988b; Garcia and Silva, 2006). In contrast, a convoluted surface has been reported in *Portunus pelagicus* (El-Sherief, 1991) and *Metopograpsus messor* (Anilkumar et al., 1999). The snow crab *C. opilio* (Moriyasu and Benhalima, 1998) presents two spermatophore wall morphologies, smooth and convoluted, produced by males in different developmental stages of sexual maturity.

4.6. New considerations of the role of the striated musculature

Throughout the reproductive system of *M. brachydactyla*, the musculature is composed of striated muscular fibers. The role of the musculature in relation to the different processes that take place in the reproductive system has been described. Thus, the musculature of the testis is involved in the movement of the spermatozoa towards the VD (Hoestlandt, 1948). The musculature of the VD participates in the formation, modeling, and transport of the spermatophores in the anterior and median regions (Mouchet, 1931; Ryan, 1967) and in the discharge of seminal fluids from accessory glands in the posterior region (Sudha Devi and Adiyodi, 1995). Finally, the musculature of the ED plays an important role during copulation, taking part in the extrusion of the spermatophores and seminal fluids through the first long, tubular gonopod towards the seminal receptacle of the female. In addition, the musculature may also play an important role in the reproductive strategies of brachyuran males, especially in the control of the transfer of sperm to the female (sperm allocation). The ability to allocate sperm, as occurs in *C. opilio* (Rondeau and Sainte-Marie, 2001) and *Paralithodes brevipes* (Sato et al., 2006), involves some controls of the reproductive system. Physiologically this means changes in muscular frequency, intensity, and time contractions, for which a striated musculature may be more appropriate than a smooth musculature. Thus, the presence of striated musculature in the male reproductive system of *M. brachydactyla* suggests that the male could apply some controls over the quantity of ejaculate transferred (sperm allocation) to the females. However, the mechanism to control the release of the spermatophores is still unknown in decapod crustaceans, and future research needs to be done to correlate the musculature of the reproductive system with sperm transfer strategies.

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References

Adiyodi, K.G., Anilkumar, R.G., 1988. Accessory sex glands. In: Adiyodi, K.G., Adiyodi, R.G. (Eds.), Reproductive Biology of Invertebrates, vol. 3. John Wiley and Sons, Kerala, pp. 261–318.

Adiyodi, K.G., Adiyodi, R.G., 1997. Sexual differentiation and behaviour. In: Adiyodi, K.G., Adiyodi, R.G. (Eds.), Reproductive Biology of Invertebrates, vol. 5. John Wiley and Sons, Kerala, pp. 325–343.

Anilkumar, G., Sudha, K., Subramonian, T., 1999. Spermatophore transfer and sperm structure in the brachyuran crab *Metopograpsus messor* (Decapoda: Grapsidae). J. Crustacean Biol. 19, 361–370.

Balasubramaniam, C.P., Suseelan, C., 2000. Male reproductive system and spermatogenesis in the deep water crab *Charybdis smithii* McLeay (Brachyura: Portunidae). Indian J. Fish. 47, 275–282.

Benhalima, K., Moriyasu, M., 2000. Structure and function of the posterior vas deferens of the snow crab, *Chionoecetes opilio* (Brachyura, Majidae). Invertebr. Reprod. Dev. 37, 11–23.

Beninger, P.G., Larocque, R., 1998. Gonopod tegumental glands: a new accessory sex gland in the Brachyura. Mar. Biol. 132, 435–444.

Beninger, P.G., Elner, R.W., Foyle, T.P., Odense, P.H., 1988. Functional anatomy of the male reproductive system and the female spermatheca in the snow crab *Chionoecetes opilio* (*O. Fabricius*) (Decapoda: Majidae) and a hypotheses for fertilization. J. Crustacean Biol. 8, 322–332.

Binford, R., 1913. The germ-cells and the process of fertilization in the crab, *Menippe mercenaria*. J. Morphol. 24, 147–201.

Brandis, D., Storch, V., Türkay, M., 1999. Morphology and function of the copulatory system in freshwater of the genus *Potamon*. J. Morphol. 239, 157–166.

Brosnan, D.M., 1981. Studies on the biology, ecology and fishery of the spider crab *Maia squinado* Herbst (1768) of the west coast or Ireland. Master Thesis. University College, Galway, 133 p.

Burton, T.E., 1995. The spermatid pathway and associated reproductive structures of the squat lobster *Thenus orientalis* (Lund, 1793). Invertebr. Reprod. Dev. 28, 53–61.

Carver, A.M., Wolcott, T.G., Wolcott, D.L., Hines, A.H., 2005. Unnatural selection: effects of a male-focused size-selective fishery on reproductive potential of a blue crab population. J. Exp. Mar. Biol. Ecol. 319, 29–41.

Castilho, G.G., Ostrensky, A., Pie, M.R., Boeger, W.A., 2008. Morphology and histology of the male reproductive system of the mangrove land crab *Ucides cordatus* (L.) (Crustacea, Brachyura, Ocypodidae). Acta Zool. 89, 157–161.

Champion, H.F.B., 1987. The functional anatomy of the male reproductive system in *Penaeus indicus*. S. Afr. J. Zool. 22, 297–307.

Charniaux-Cotton, H., 1958. La glande androgène de quelques Crustacés Décapodes et particulièrement de *Lysmata seticaudata*, espèce à hermaphrodisme protérandrique fonctionnel. C.R. Acad. Sci. Paris 246, 2814–2817.

Charniaux-Cotton, H., Zerbib, C., Meusy, J.J., 1966. Monographie de la glande androgéne des crustacés supérieurs. Crustaceana 10, 113–136.

Chen, Q., Cui, W., 1986. On structure and development of the male reproductive system of the Chinese prawn *Penaeus orientalis*. Acta Zool. Sin. 32, 255–259.

Chiba, A., Honma, Y., 1972. Studies on the maturity of the gonad in some marine invertebrates—IV: seasonal changes in the testes of the lined shored crab. Nippon Suisan Gakkai Shi 38, 317–329.

Chiba, A., Kon, T., Honma, Y., 1992. Ultrastructure of the spermatozoa and spermatophores of the zuwai crab, *Chionoecetes opilio* (Majidae, Brachyura). Acta Zool. 73, 103–108.

Chow, S., Dougherty, W.J., Sandifer, P.A., 1991. Unusual testicular lobe system in the white shrimps *Penaeus setiferus* (Linnaeus, 1761) and *P. vannamei* Boone, 1931 (Decapoda, Penaidae): a new character for Dendrobranchiata? Crustaceana 60, 304–318.

Cronin, L.E., 1947. Anatomy and histology of the male reproductive system of *Callinectes sapidus*. J. Morphol. 81, 209–240.

Cuartas, E.I., Petriella, A.M., 2007. Formación inicial de los espermatofóros en el testículo del cangrejo *Uca uruguayensis* (Brachyura: Ocypodidae). Rev. Biol. Trop. 55, 9–14.

Diesel, R., 1989. Structure and function of the reproductive system of the symbiotic spider crab *Inachus phalangium* (Decapoda: Majidae): observations on sperm transfer, sperm storage, and spawning. J. Crustacean Biol. 9, 266–277.

Diesel, R., 1991. Sperm competition and the evolution of mating behaviour in Brachyura, with special reference to spider crabs (Decapoda, Majidae). In: Bauer, R.T., Martin, J.W. (Eds.), Crustacean Sexual Biology. Columbia University Press, New York, pp. 145–163.

Dudenhausen, E.E., Talbot, P., 1983. An ultrastructural comparison of soft and hardened spermatophores from the crayfish *Pacifastacus leniusculus* Dana. Can. J. Zool. 61, 182–194.

El-Sherief, S.S., 1991. Fine structure of the sperm and spermatophores of *Portunus pelagicus* (L.) (Decapoda, Brachyura). Crustaceana 61, 271–279.

Estampador, E.P., 1949. *Scylla* (Crustacea: Portunidae) II. Comparative studies on spermatogenesis and oogenesis. Philip. J. Sci. 78, 95–109.

Fasten, N., 1915. The male reproductive organs of some common crabs of Puget Sound. Puget Sound Mar. Sta. Pub. 1, 35–41.

Fasten, N., 1917. Male reproductive organs of decapoda, with special reference to Puget Sound forms. Puget Sound Mar. Sta. Pub. 1, 285–307.

Freire, J., Bernárdez, C., Corgos, A., Fernández, L., González-Gurriarán, E., Sampedro, M.P., Verísimo, P., 2002. Management strategies for sustainable invertebrate fisheries in coastal ecosystems of Galicia (NW Spain). Aquat. Ecol. 36, 41–50.

Garcia, T.M., Silva, J.R.F., 2006. Testis and vas deferens morphology of the red-clawed mangrove tree crab (*Goniopsis cruentata*) (Latreille, 1803). Braz. Arch. Biol. Technol. 49, 339–345.

George, M.J., 1963. The anatomy of the crab *Neptunus sanguinolentus* Herbst. Part IV: reproductive system and embryological studies. J. Madras Univ. B33, 289–304.

González-Gurriarán, E., Fernández, L., Freire, J., Muiño, R., 1998. Mating and role of seminal receptacles in the reproductive biology of the spider crab *Maja squinado* (Decapoda: Majidae). J. Exp. Mar. Biol. Ecol. 220, 269–285.

González-Gurriarán, E., Fernández, L., Freire, J., Muiño, R., Parapar, J., 1993. Reproduction of the spider crab *Maja squinado* (Brachyura: Majidae) in the Southern

Galician coast (NW Spain). International Council for the Exploration of the Sea. Shellfish Committee, Copenhaguen, CM 1993/K: 19, 15 p.
González-Gurriarán, E., Freire, J., Parapar, J., Sampedro, M.P., Urcera, M., 1995. Growth at moult and moulting seasonality of the spider crab, *Maja squinado* (Herbst) (Decapoda: Majidae) in experimental conditions: implications for juvenile life history. J. Exp. Mar. Biol. Ecol. 189, 183–203.
Greenwood, J.G., 1972. The male reproductive system and spermatophore formation in *Pagurus novaezealandiae* (Dana) (Anomura: Paguridea). J. Nat. Hist. 6, 561–574.
Gupta, R.S., Chatterjee, N.B., 1976. Anatomical observations of the internal male reproductive organs of *Scylla serrata* (Forskal). Ind. J. Physiol. Allied Sci. 30, 34–42.
Haley, S.R., 1984. Spermatogenesis and spermatophore production in the Hawaiian red lobster *Enoplometopus occidentalis* (Crustacea, Nephropidae). J. Morphol. 180, 181–193.
Hankin, D.G., Butler, T.H., Wild, P.W., Xue, Q.L., 1997. Does intense fishing on males impair mating success of female Dungeness crabs? Can. J. Fish. Aquat. Sci. 54, 655–669.
Heldt, J.H., 1938. La reproduction chez les crustacés décapodes de la famille des Pénéides. Ann. Ins. Oceanogr. 18, 31–206.
Hinsch, G.W., 1986. A comparison of sperm morphologies, transfer and sperm mass storage between two species of crab, *Ovalipes ocellatus* and *Libinia emarginata*. Int. J. Invertebr. Reprod. Dev. 10, 79–87.
Hinsch, G.W., 1988a. Morphology of the reproductive tract and seasonality of reproduction in the golden crab *Geryon fenneri* from the eastern gulf of Mexico. J. Crustacean Biol. 8, 254–261.
Hinsch, G.W., 1988b. Ultrastructure of the sperm and spermatophores of the golden crab *Geryon fenneri* and a closely related species, the red crab *G. quinquedens*, from the eastern gulf of Mexico. J. Crustacean Biol. 8, 340–345.
Hinsch, G.W., 1991. Structure and chemical content of the spermatophores and seminal fluid of reptantian decapods. In: Bauer, R.T., Martin, J.W. (Eds.), Crustacean Sexual Biology. Columbia University Press, New York, pp. 290–307.
Hinsch, G.W., Walker, M.H., 1974. The vas deferens of the spider crab, *Libinia emarginata*. J. Morphol. 143, 1–20.
Hinsch, G.W., McKnight, C.E., 1988. The vas deferens of the Spanish lobster, *Scyllarides chacei*. Int. J. Invertebr. Reprod. Dev. 13, 267–280.
Hoestlandt, H., 1948. Recherches sur la biologie de l'*Eriocheir sinensis* en France. Ann. Ins. Oceanogr. 24, 1–116.
Huq, A., 1981. Reproductive system of six species of *Penaeus Fabricius* (Decapoda: Penaeidae). Bangladesh J. Zool. 8, 81–88.
Johnson, P.T., 1980. The reproductive system. In: Histology of the Blue Crab, *Callinectes sapidus*. A Model for Decapoda. Praeger, New York, pp. 327–347.
Joshi, P.C., Khanna, S.S., 1982. Structural and seasonal changes in the testes of a freshwater crab, *Potamon koolooense* (Rathburn). Proc. Ind. Acad. Sci. 91, 439–450.
Kim, D.H., Choi, J.H., Kim, J.N., Cha, H.K., Oh, T.Y., Kim, D., Han, C.H., 2006. Gonad and androgenic gland development in relation to sexual morphology in *Pandalopsis japonica* Balss, 1924 (Decapoda, Pandalidae). Crustaceana 79, 541–554.
Kon, T., Honma, Y., 1970. Studies on the maturity of the gonad in some marine invertebrates—IV: seasonal changes in the testes of the Tanner crab. Nippon Suisan Gakkai Shi 36, 1028–1031.
Kooda-Cisco, M.J., Talbot, P., 1986. Ultrastructure and role of the lobster vas deferens in spermatophore formation: the proximal segment. J. Morphol. 188, 91–103.
Krol, R.M., Hawkins, W.E., Overstreet, R.M., 1992. Reproductive components. In: Harrison, F.W., Humes, A.G. (Eds.), Microscopic Anatomy of Invertebrates, vol. 10. Wiley-Liss, Inc, New York, pp. 295–343.
Le Foll, D. 1993. Biologie et exploitation de l'araignée de mer *Maja squinado* Herbst en Manche Ouest. Université de Bretagne Occidentale. IFREMER. Report nr RI DRV 93–030, 524 p.
López Greco, L.S., Vázquez, F., Rodríguez, E.M., 2007. Morphology if the male reproductive system and spermatophore formation in the freshwater "red claw" crayfish *Cherax quadricarinatus* (Von Martens, 1898) (Decapoda, Parastacidae). Acta Zool. 88, 223–229.
Malek, S.R.A., Bawab, F.M., 1974. The formation of the spermatophore in *Penaeus kerathurus* (Forskal, 1775) (Decapoda, Peneidae) I. The initial formation of the sperm mass. Crustaceana 26, 273–285.
Manjón-Cabeza, M.E., Garcia Raso, J.E., 2000. Morphological reproductive aspects of male of *Diogenes pugilator* (Roux, 1829) (Crustacea, Decapoda, Anomura) from southern Spain. Sarsia 80, 195–202.
Mann, T., 1984. Spermatophores: Development, Structure, Biochemical Attributes and Role in the Transfer of Spermatozoa. Springer-Verlang, Berlin, 217 p.
Matthews, D.C., 1951. The origin, development and nature of the spermatophoric mass of the spiny lobster, *Panulirus penicillatus* (Oliver). Pac. Sci. 5, 359–371.
Matthews, D.C., 1954. A comparative study of the spermatophores of three scyllarid lobsters (*Parribacus anctarticus*, *Scyllarides squammosus*, and *Scyllarides martensii*). Q. J. Microsc. Sci. 95, 205–215.
Matthews, D.C., 1956. The origin of the spermatophoric mass of the sand crab, *Hippa pacifica*. Q. J. Microsc. Sci. 97, 257–268.
McLaughlin, P.A., 1983. Internal anatomy. In: Mantel, L.H. (Ed.), The Biology of Crustacea, vol. 5. Academic Press, New York, pp. 1–52.
Meusy, M.J.J., 1972. La gamétogenèse et la fraction protéique de l'hémolymphe spécifique du sexe femelle chez quelques Crustacés supérieurs: étude descriptive et rôle des glandes androgènes. PhD Thesis. Universite Paris VI, Paris, 165 p.
Minagawa, M., Chiu, J.-R., Kudo, M., Takashima, F., 1994. Male reproductive biology of the red frog crab, *Ranina ranina*, off Hachijojima, Izu Islands, Japan. Mar. Biol. 118, 393–401.
Moriyasu, M., Benhalima, K., 1998. Snow crabs, *Chionoecetes opilio* (*O. Fabricius*, 1788) (Crustacea: Majidae) have two types of spermatophore: hypotheses on the mechanism of fertilization and population reproductive dynamics in the southern Gulf of St. Lawrence. Can. J. Nat. Hist. 32, 1651–1665.
Moriyasu, M., Benhalima, K., Duggan, D., Lawton, P., Robichaud, D., 2002. Reproductive biology of the male Jonah crab, *Cancer borealis* Stimpson, 1859 (Decapoda, Cancridae) on the Scotian shelf, Northwestern Atlantic. Crustaceana 75, 891–913.
Motoh, H., 1979. Preliminary study of the internal genital organ of the giant tiger prawn, Penaeus monodon. Q. Res. Rep. Aquacult. Dept. 3, 15–18.
Mouchet, S., 1931. Spermatophores des Crustacés Décapodes Anomures et Brachyoures et castration parasitaire chez quelques Pagures. Ann. Sta. Océanogr. Salammbô VI, 1–203.
Nagao, J., Munehara, H., 2003. Annual cycle of testicular maturation in the helmet crab *Telmessus cheiragonus*. Fish. Sci. 69, 1200–1208.
Neumann, V., 1996. Comparative gonopod morphology of the European spider crabs of the genus Maja Lamarck 1801. Senckenb. Biol. 75, 143–157.
Neumann, V., 1998. A review of the *Maja squinado* (Crustacea: Decapoda: Brachyura) species-complex with a key to the eastern Atlantic and Mediterranean species of genus. J. Nat. Hist. 32, 1667–1684.
Noro, C., López-Greco, L.S., Buckup, L., 2008. Gonad morphology and type of sexuality in *Parastacus defossus* Faxon 1898, a burrowing, intersexed crayfish from southern Brazil (Decapoda: Parastacidae). Acta Zool. 89, 59–67.
Ro, S., Talbot, P., Leung-Trujillo, J., Lawrence, A.L., 1990. Structure and function of the vas deferens on the shrimp *Penaeus setiferus*: segments 1–3. J. Crustacean Biol. 10, 455–468.
Rondeau, A., Sainte-Marie, B., 2001. Variable mate-guarding time and sperm allocation by male snow crabs (*Chionoecetes opilio*) in response to sexual competition, and their impact on the mating success of females. Biol. Bull. 201, 204–217.
Rorandelli, R., Paoli, F., Cannicci, S., Mercati, D., Giusti, F., 2008. Characteristics and fate of the spermatozoa of *Inachus phalangium* (Decapoda, Majidae): description of novel sperm structures and evidence for an additional mechanism of sperm competition in Brachyura. J. Morphol. 269, 259–271.
Rotllant, G., González-Gurriarán, E., Fernández, L., Benhalima, K., Ribes, E., 2007. Ovarian maturation of the multi-spawning spider crab *Maja brachydactyla* (Decapoda: Majidae) with special reference to yolk formation. Mar. Biol. 152, 383–394.
Ryan, E.P., 1967. Structure and function of the reproductive system of the crab *Portunus sanguinolentus* (Hersbt) (Brachyura: Portunidae) I. The male system. Proc. Sym. Crustacea. 2, 506–521.
Sagi, A., Khalaila, I., 2001. The crustacean androgen: a hormone in an isopod androgenic activity in decapods. Am. Zool. 41, 477–484.
Sapelkin, A.A., Fedoseev, V.Y., 1981. Structure of male reproductive system of Tanner crabs. Sov. J. Mar. Biol. 7, 37–43.
Sarojini, S., 1961. The androgenic organ in some Indian Crustacea I. J. Zool. Soc. India 13, 189–193.
Sato, T., Ashidate, M., Jinbo, T., Goshima, S., 2006. Variation of sperm allocation with male size and recovery rate of sperm numbers in spiny king crab *Paralithodes brevipes*. Mar. Ecol. Prog. Ser. 312, 189–199.
Sokolowicz, C.C., López-Greco, L.S., Goncalves, R., Bond-Buckup, G., 2007. The gonads of *Aegla platensis* Schmitt (Decapoda, Anomura, Aeglidae): a macroscopic and histological perspective. Acta Zool. 88, 71–79.
Spalding, J.F., 1942. The nature and formation of the spermatophore and sperm plug in *Carcinus maenas*. Q. J. Microsc. Sci. 83, 399–423.
Subrahmanyam, T.B., 1965. On the reproductive cycle of *Penaeus indicus* (Milne Edwards). J. Mar. Biol. Ass. India 7, 284–290.
Subramonian, T., 1981. Protandric hermaphroditism in a mole crab, *Emerita asiatica* (Decapoda: Anomura). Biol. Bull. 160, 161–174.
Subramonian, T., 1991. Spermatophores and sperm transfer in marine crustaceans. Adv. Mar. Biol. 29, 129–214.
Subramonian, T., 1995. Light and electron microscopic studies on the seminal secretions and the vas deferens of the penaoidean shrimp, *Syciona ingentis*. J. Biosci. 20, 691–706.
Sudha Devi, A.R., Adiyodi, R.G., 1995. The coral-shaped accessory gland of the ghost crab, *Ocypode ceratophthalmus*. J. Anim. Morphol. Physiol. 42, 69–73.
Suganthi, A.S., Anilkumar, G., 1999. Moult related fluctuation in ecdysteroid titre and spermatogenesis in the crab, *Metopograpsus messor* (Brachyura: Decapoda). Zool. Stud. 38, 314–321.
Talbot, P., Beach, D., 1989. Role of the vas deferens in the formation of the spermatophore of the crayfish (*Cherax*). J. Crustacean Biol. 9, 9–24.
Uma, K., Subramoniam, T., 1984. A comparative study on the spermatophore in *Scylla serrata* (Forskal) (Decapoda: Brachyura) and *Clinabarius longitarsus* (De Haan) (Decapoda: Anomura). J. Mar. Biol. Ass. India 26, 103–108.

Article 2

Títol: Spermatogenesis of the spider crab *Maja brachydactyla* (Decapoda: Brachyura)

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Informe de la contribució del doctorand

La hipòtesi de treball i la metodologia a seguir varen estar realitzades per la Dra. G. Rotllant, el Dr. M. Chiva i el Dr. E. Ribes. El doctorand va realitzar les disseccions i els mostrejos dels teixits amb la col·laboració del Dra. G. Rotllant i la Dra. K. Kurtz. Les mostres foren processades pels Serveis Cientificotècnics de la Universitat de Barcelona. Les imatges de microscòpia electrònica foren realitzades pel doctorand amb el suport del Dr. Ribes. La descripció i interpretació de les imatges, i la redacció del manuscrit foren realitzades pel doctorant amb la col·laboració dels coautors.

Dra. Guiomar Rotllant Estelrich

Resum

Aquest estudi descriu l'espermatogènesi, amb especial èmfasi en l'espermiogènesi, de la cabra de mar mitjançant microscòpia electrònica. Les primeres fases de l'espermatogènesi de *M. brachydactyla* segueixen el mateix patró descrit en altres braquiürs. Els espermatòcits són cèl·lules arrodonides amb un gran nucli en posició central. En els espermatòcits en proleptotè, el citoplasma conté material granular, mitocondris, i vesícules irregulars i aïllades de reticle endoplasmàtic. Els espermatòcits en leptotè presenten cromosomes individualitzats al nucli, mentre que el citoplasma conté un sistema concèntric de membranes i un prominent *nuage*. A l'estadi paquitè, els cromosomes dels espermatòcits estan aparellats i formen complexes sinaptonemals. El sistema concèntric de membranes i el *nuage* encara estan presents al citoplasma. Els cromosomes dels espermatòcits en estadi diplotè estan aparellats i condensats, i els complexes sinaptonemals encara estan presents. Al citoplasma, es formen uns sistemes de membranes concèntrics que contenen mitocondris. A més a més, apareixen les làmines anellades, estructures formades per capes paral·leles de membranes que apareixen associades en alguns casos al nucli. El *nuage* encara és present. Els espermatòcits secundaris només es pogueren detectar en profase II. El nucli conté cromosomes condensats i el citoplasma conté el *nuage* i cisternes irregulars de reticle endoplasmàtic. L'espermiogènesi s'ha dividit en tres fases, d'acord amb el canvis de la condensació de la cromatina, i el desenvolupament i diferenciació de la vesícula proacrosòmica. Les espermàtides primerenques són cèl·lules esfèriques, amb el nucli localitzat en el que serà el pol nuclear de la cèl·lula, mentre que en el pol oposat, el pol acrosòmic, es desenvoluparà l'acrosoma. El nucli és esfèric, amb cromatina granular. Al citoplasma hi ha alguns mitocondris i sistemes de membranes concèntrics de reticle endoplasmàtic. Les espermàtides intermèdies es diferencien per presentar un nucli amb la cromatina poc condensada. Al citoplasma es produeix el desenvolupament i diferenciació del sistema de membranes que dóna lloc al reticle endoplasmàtic i a un sistema de membranes semblant al complex de Golgi, el qual produeix dos tipus de secrecions de diferent electrodensitat. Les vesícules amb material poc electrodens es fusionen al citoplasma, donant lloc a la vesícula proacrosòmica en el pol acrosòmic. Les vesícules més electrodenses formen un gránul esfèric, què després es fusiona amb la vesícula proacrosòmica. A mesura que la vesícula proacrosòmica creix en mida, desplaça el nucli al pol nuclear i el reticle endoplasmàtic i el complex de Golgi es redueixen, quedant com sistema de membranes a la zona equatorial de la cèl·lula. L'espermàtida madura és una cèl·lula altament polaritzada amb el nucli i vesícula proacrosòmica ocupant pràcticament tot el volum cel·lular. Durant la fase d'esper-

màtida madura es produeix la diferenciació de la vesícula proacrosòmica i un profund canvi de la morfologia del nucli. A sobre del gránul de la part apical de la vesícula proacrosòmica apareix una fina capa molt electrodensa que constituirà l'opercle. A la base de la vesícula proacrosòmica, es produeix una invaginació, que és l'origen del perforatori, associada a una fina capa de material granular. El nucli comença la seua reorganització, estenent-se lateralment i envoltant la vesícula acrosòmica, amb el que arrossega el sistema d'estructures (restes de reticle endoplasmàtic i complex de Golgi) i orgànuls (mitocondris i microtúbuls) anomenat complex SO (de l'anglès *structures* i *organelles*) cap a la zona apical de la cèl·lula. La diferenciació de la vesícula proacrosòmica continua amb l'agregació dels materials en dues fases: primer els materials més interns i posteriorment els més externs. La darrera modificació és el desenvolupament del braços radials nuclear sota la regió apical de la cèl·lula, associat al complex SO.

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Spermatogenesis of the Spider Crab *Maja brachydactyla* (Decapoda: Brachyura)

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ABSTRACT This study describes spermatogenesis in a majid crab (*Maja brachydactyla*) using electron microscopy and reports the origin of the different organelles present in the spermatozoa. Spermatogenesis in *M. brachydactyla* follows the general pattern observed in other brachyuran species but with several peculiarities. Annulate lamellae have been reported in brachyuran spermatogenesis during the diplotene stage of first spermatocytes, the early and mid-spermatids. Unlike previous observations, a Golgi complex has been found in mid-spermatids and is involved in the development of the acrosome. The Golgi complex produces two types of vesicles: light vesicles and electron-dense vesicles. The light vesicles merge into the cytoplasm, giving rise to the proacrosomal vesicle. The electron-dense vesicles are implicated in the formation of an electron-dense granule, which later merges with the proacrosomal vesicle. In the late spermatid, the endoplasmic reticulum and the Golgi complex degenerate and form the structures–organelles complex found in the spermatozoa. At the end of spermatogenesis, the materials in the proacrosomal vesicle aggregate in a two-step process, forming the characteristic concentric three-layered structure of the spermatozoon acrosome. The newly formed spermatozoa from testis show the typical brachyuran morphology. J. Morphol. 271:394–406, 2010.

KEY WORDS: sperm morphology; gametogenesis; germ cells; ultrastructure; Majidae

INTRODUCTION

The brachyuran spermatozoon is characterized by a globular shape, the absence of a flagellum, and the presence of a variable number of radial arms (Felgenhauer and Abele, 1991). Numerous morphological and taxonomic studies have provided a clear description of the ultrastructure of the sperm cell (Jamieson and Tudge, 2000). The brachyuran spermatozoon is composed of a cup-shaped nucleus with lateral arms and decondensed chromatin, a thin cytoplasmic layer, and a complex globular acrosome, which is centrally penetrated by the perforatorium. In addition, the role of several spermatozoon components has been proposed, whereas others, such as the different acrosome layers, still remain unclear. Thus, the decondensed chromatin seems to provide the necessary malleability for the acrosome reaction (Krol et al., 1992; Kurtz et al., 2008), the lateral arms may participate in the attachment to the egg, and the perforatorium may play a key role during egg penetration (Brown, 1966; Hinsch, 1971; Goudeau, 1982; Medina, 1992; Medina and Rodríguez, 1992a).

Despite all the information available on the spermatozoal morphology and ultrastructure, little is known about spermatogenesis in brachyurans. The first descriptions of spermatogenesis were done using light microscopy (LM) in *Menippe mercenaria* (Binford, 1913), *Cancer magister* (Fasten, 1918), *Cancer* sp. (Fasten, 1924), *Lophopanopeus bellus* (Fasten, 1926), *Sartoriana spinigera* (Nath, 1932, as *Paratelphusa*), *Eriocheir sinensis* (Hoestlandt, 1948), and *Scylla* sp. (Estampador, 1949). However, these works revealed only the general pattern of spermatogenesis. Later, studies using transmission electron microscopy (TEM) were not conclusive and mainly described the last phases of spermatogenesis (spermiogenesis) in a few brachyuran species: *Eriocheir japonicus* (Yasuzumi, 1960), *Carcinus maenas* (Pochon-Masson, 1962, 1968), *Cancer* sp. (Langreth, 1969), *Pinnixa* sp. (Reger, 1970), *E. sinensis* (Du et al., 1988), *Uca tangeri* (Medina and Rodríguez, 1992b), *Portunus trituberculatus* (Li, 1995), *Scylla serrata* (Wang et al., 1997b), and *Sinopotamon yangtsekiense*

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(Wang et al., 1999). Although in some animal species a transverse section of the testis contains most stages of spermatogenesis (Beninger and Pennec, 1991; Patiño and Redding, 2000; Sasso-Cerri et al., 2004; Cledón et al., 2005; Thongkukiatkul et al., 2008), in brachyurans, cells belonging to the same transverse section of the testis are usually in the same stage of development (Krol et al., 1992). Therefore, obtaining the complete sequence of stages throughout spermatogenesis is a difficult task that would explain why so little information is available in brachyurans. Recent studies have revealed new features of the brachyuran spermiogenesis, such as the maturation of the spermatids in the vas deferens and seminal receptacles of the snow crab *Chionoecetes opilio* (Sainte-Marie and Sainte-Marie, 1999a,b) and the loss of a glycocalyx in the spermatozoa of *Inachus phalangium* in the seminal receptacle of the females (Rorandelli et al., 2008).

The spider crab *Maja brachydactyla* is an important commercial species in the Atlantic Ocean (Freire et al., 2002) that has been often synonymized with *Maja squinado*. Recently, its taxonomic status has been clarified (Neumann, 1998; Sotelo et al., 2008), recognizing the Mediterranean *M. squinado* and the Atlantic *M. brachydactyla* as different species. Only a few studies focused on the morphology of the reproductive system (Mouchet, 1931; Neumann, 1996 as *M. squinado*; Simeó et al., 2009), spermatogenesis (Meusy, 1972 as *M. squinado*), and the spermatozoal ultrastructure (Tudge and Justine, 1994 as *M. squinado*; Simeó et al., in press). In this study, we give a detailed description of spermatogenesis in the spider crab, *M. brachydactyla*, using TEM.

MATERIAL AND METHODS

Twenty-four adult males of *Maja brachydactyla* Balss, 1922 were captured in Galicia, Northwest Spain, by artisanal coastal fishery using gillnets between November 2006 and July 2007. The specimens were transported in dry and high humidity conditions to Institut de Recerca i Tecnologia Agroalimentàries facilities (Tarragona, NE Spain). Before dissection, carapace length and weight (W) were measured, being in average carapace length = 155.55 ± 6.89 mm and W = 1,147.5 ± 218.4 g (mean ± SD). Then, spider crabs were anesthetized on ice for at least 10 min until they did not respond to external stimuli; heart was dissected causing the death of the animal, and pieces of testis were extracted and processed for LM and TEM. The experimental procedure conforms to the current animal protection regulations (86/609/CEE, RD 1201/2005, and D 214/1997).

For LM, the right testis of three animals was extracted and divided into three parts (distal, median, and proximal to the vas deferens). The parts were fixed in Bouin's solution for 24-48 h and then rinsed and stored in 70% ethanol until processing. Samples were dehydrated through a graded series of alcohol and embedded in paraffin. Slides of 3 μm were cut on a Leica RM 2155 rotary microtome and stained with Harris's hematoxylin-eosin dye. Sections were photographed using an Olympus DP70 camera connected to an Olympus BX61 light microscope.

For TEM, small pieces of testis belonging to the 24 males were extracted and fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (0.1 mol L^{-1}, pH 7.4) for 24 h at 4°C. Samples were rinsed in cacodylate buffer several times, postfixed in 1% osmium tetroxide at 4°C, dehydrated in graded series of acetone, and embedded in Spurr's resin. Semithin sections, used for LM, were stained using toluidine blue and observed with an Olympus BX61 light microscope. Ultrathin sections were made in a Leica UCT ultramicrotome and counterstained with uranyl acetate and lead citrate. Observations were made with a Jeol EM-1010 transmission electron microscope at 80 kV.

Sagittal sections of the germ cells were selected and measured using an image analyzing system (AnalySIS, SIS; n = 15, except for early spermatid, in which only four sections were properly oriented). The measurements of mid- and late spermatids were made using the longest axis of the cell, because these stages present an oval shape. For late spermatids, which show irregular nuclear shapes, nuclear measurement refers to thickness of the nucleus in the sagittal section.

RESULTS

Spermatogenesis in the Seminiferous Tubule

Testes of *M. brachydactyla* consist of a single seminiferous tubule, which is divided in transverse section by epithelial cells into three zones: germinal, transformation, and evacuation zones (EZ) (Fig. 1). Each zone contains different stages of germ cells accompanied by accessory cells and plays a different role in spermatogenesis. The germinal zone is located at one side in a transverse section of the seminiferous tubule and contains spermatogonia. The transformation zone (TZ) fills the central region of the seminiferous tubule and contains the different stages of spermatogenesis, from spermatocytes to spermatozoa. As cells belonging to the same transverse section are usually in the same stage of development or in two successive stages, independent of their distance to the vas deferens, we had to dissect a large number of animals to follow the spermatogenesis along the seminiferous tubule. The EZ is diametrically opposed to the germinal zone and only contains mature spermatozoa originating from the TZ. The EZ collects and transports spermatozoa produced along the testis toward the vas deferens, where they are packed and stored in spermatophores. Hence, we have focused our observations on the TZ because it is the place where spermatogenesis takes place.

Spermatocyte Stages

Primary spermatocytes are spherical cells with a spherical nucleus located in the central region of the cell (Figs. 2 and 3A–D). Spermatocytes are the largest germ cells measured in this study (Table 1), and their size and nuclei remain constant during meiotic divisions.

Preleptotene spermatocytes show nuclei with small clumps of heterochromatin (Fig. 2A). The cytoplasm appears homogeneous, with granular material and few organelles. The mitochondria are distributed throughout the cytoplasm, containing

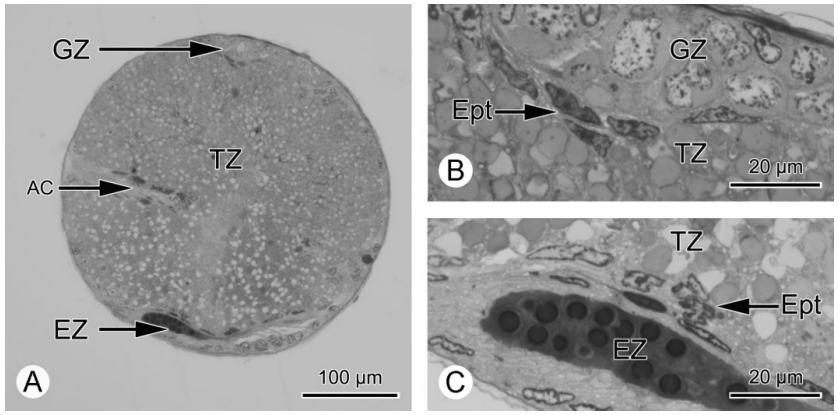

Fig. 1. *Maja brachydactyla*. Light microscopy of a seminiferous tubule. **A**, transverse section of the seminiferous tubule divided into three zones: germinal, transformation, and evacuation zones. In this section, the transformation zone contains spermatids and an enlarged accessory cell. **B**, epithelial layer separating the germinal and transformation zones. **C**, epithelium between the transformation and evacuation zones. AC, accessory cell; Ept, epithelium; EZ, evacuation zone; GZ, germinal zone; TZ, transformation zone.

slightly electron-dense material and few, poorly developed cristae. The endoplasmic reticulum (ER) is composed of isolated, irregular cisternae containing material of low electron density.

Leptotene spermatocytes contain individualized chromosomes condensed into strands (see arrowheads in Fig. 2B,C) and a single nucleolus in the nucleus (Fig. 2B). The cytoplasm exhibits a concentric membrane system, which is composed of flattened concentric cisternae, and an oval-shaped, slightly prominent, electron-dense nucleolus-like body or nuage (Fig. 2C).

In the pachytene stage, spermatocytes show paired chromosomes with synaptonemal complexes (arrowheads and inset in Fig. 2D). In the cytoplasm, the concentric membrane system shows lateral dilatations associated with the peripheral cisternae (asterisks in Fig. 2E). The nuage is still present (Fig. 2D), although it is larger and less electron dense than in the leptotene stage.

In the diplotene stage, chromosomes are paired and condensed (Fig. 3A), and the synaptonemal complexes are still present (Fig. 3D). Elongated membrane cisternae merge in the cytoplasm, forming concentric complexes that contain mitochondria (Fig. 3C). In addition, the cytoplasm contains annulate lamellae, which are composed of several parallel membrane layers that eventually become associated with the nucleus (Fig. 3B,D). The nuage increases in size, appearing as a prominent electron-dense body in the cytoplasm (Fig. 3B).

Secondary spermatocytes in prophase II have condensed chromosomes (Fig. 3E) and a light electron-dense nucleoplasm. The cytoplasm is less electron dense than in the previous stage and contains the nuage and several irregular ER cisternae with light electron-dense material.

For the following secondary spermatocyte stages, we did not obtain sections for TEM; therefore, in the next section, the description of spermatogenesis continues with spermatid maturation.

The accessory cells seem closely related to spermatocytes, showing a spindle-shaped nucleus located at the center of the cell (Fig. 2B). Heterochromatin is condensed mainly in the periphery of the nucleus, and the nucleoplasm is moderately electron dense. A nucleolus, centrally placed, is also present. The cytoplasm contains granular material and is more electron dense when it is found between spermatocytes (arrowhead in Fig. 3A).

Spermiogenesis

Spermiogenesis shows three stages, early, mid, and late spermatids, according to changes in chromatin condensation, and the development and differentiation of the proacrosomal vesicle. Because of morphological changes of the nucleus during the last stage (Table 1), spermatids decrease in size during spermiogenesis.

Early spermatids are slightly polarized, spherical cells with the nucleus located at one pole of the cell (here referred as nuclear pole) and the cytoplasm at the opposite pole (acrosomal pole, Fig. 4A), where the proacrosomal vesicle will arise. The nucleus is spherical and contains granular chromatin, which still appears as condensed clumps distributed throughout the nucleoplasm and is also associated with the nuclear enve-

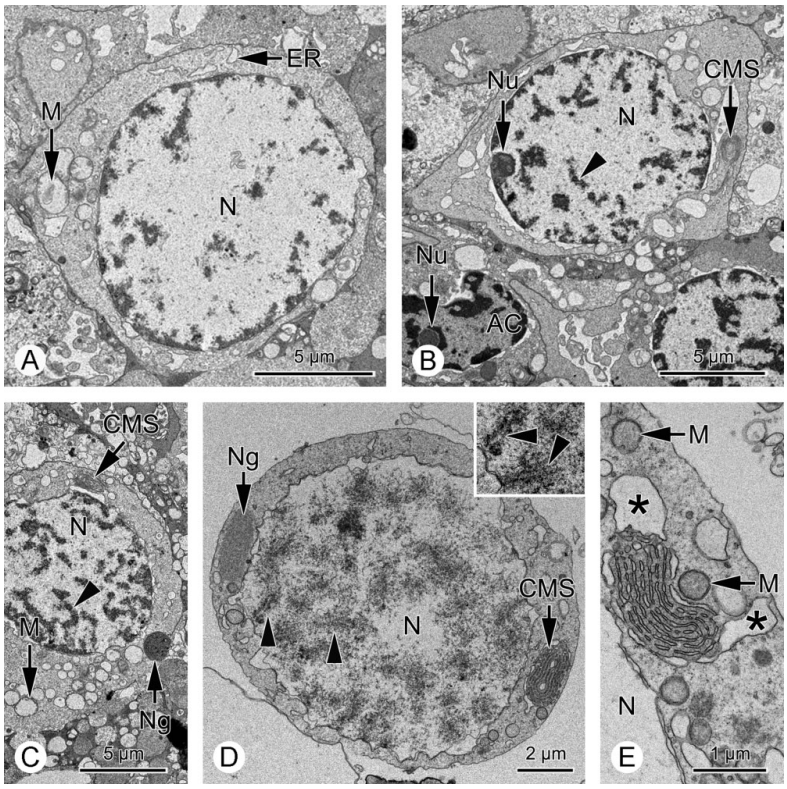

Fig. 2. *Maja brachydactyla*. Transmission electron micrographs of primary spermatocytes. **A**, preleptotene stage. Spermatocytes showing small clumps of heterochromatin in the nucleus. The cytoplasm contains few organelles, such as mitochondria with poorly developed cristae and irregular cisternae, and vesicles of endoplasmic reticulum. **B**, leptotene stage of the spermatocyte and adjacent cells. The nucleus has a single nucleolus and individualized chromosomes (arrow, also in C). A concentric membrane system appears in the cytoplasm. An accessory cell shows a spindle-shaped nucleus with a prominent nucleolus. **C**, leptotene stage of the spermatocyte showing the concentric membrane system, mitochondria with poorly developed cristae, and a nuage in the cytoplasm. **D**, pachytene stage of the spermatocyte showing paired chromosomes and synaptonemal complexes (arrowheads). Inset shows synaptonemal complexes at higher magnification (arrowheads). In the cytoplasm, the concentric membrane system and the nuage are more prominent. **E**, pachytene stage, detail of the concentric membrane system showing lateral dilatations in the periphery (asterisks), with a small electron-dense mitochondrion. AC, accessory cell; CMS, concentric membrane system; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; Ng, nuage; Nu, nucleolus.

lope (Fig. 4A). The nuclear envelope shows nuclear pores, particularly in the region facing the acrosomal pole (arrowhead and inset in Fig. 4B). In the cytoplasm, several small mitochondria with degenerate cristae show electron-dense contents (Fig. 4B). Few concentric membranous arrangements such as those observed in the diplotene stage are still present (Fig. 4A). In addition, flattened cisternae extend longitudinally resembling a poorly developed ER (Fig. 4B).

Mid-spermatids are spherical to oval cells characterized by chromatin decondensation, growth and differentiation of the ER and Golgi complex, and development of the proacrosomal vesicle. The first change observed in mid-spermatids is the decondensation of chromatin (Fig. 4C). Thus, the nucleus contains homogeneous chromatin with few small, condensed clumps (arrowhead in Fig. 4D,E). In the cytoplasm, membrane layers are arranged longitudinally, whereas the annulate lamellae seem associated with the nuclear envelope (Fig. 4D). Later, the membrane layers continue their development and differentiate into the ER and a membranous system resembling a Golgi complex (Fig. 4E,F). The ER is composed of highly packed longitudinal cisternae oriented parallel to the nuclear envelope, whereas the Golgi complex, consists of a few semicircular cisternae, which produces two types of vesicles containing either light or electron-dense materials. Light electron-dense vesicles merge in the

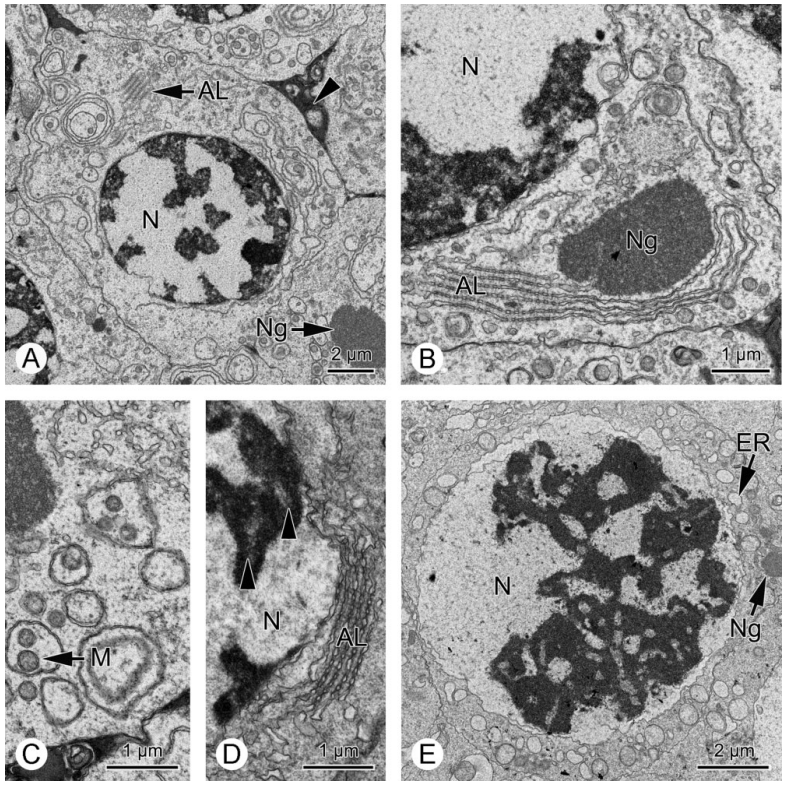

Fig. 3. *Maja brachydactyla*. Transmission electron micrographs of spermatocytes. **A**, primary spermatocyte during diplotene of the spermatocyte showing condensed and paired chromosomes in the nucleus. In the cytoplasm, the nuage is more prominent, several membrane systems are organized in concentric systems, and the annulate lamellae are present. The cytoplasm of the accessory cells (arrowhead) appears as a highly electron-dense material. **B**, detail of the cytoplasm showing the annulate lamellae and the nuage. **C**, detail of the circular arrangement of the cytoplasmic membranes containing mitochondria. **D**, detail of the annulate lamellae closely associated with the nucleus, which still presents synaptonemal complexes (arrowheads). **E**, secondary spermatocyte showing tightly condensed chromatin in the nucleus. The cytoplasm contains several vesicles of endoplasmic reticulum, and the nuage is still present. AL, annulate lamellae; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; Ng, nuage.

acrosomal pole to give rise to the proacrosomal vesicle, which is filled with homogeneous granular material (Fig. 4F).

As spermiogenesis progresses, the proacrosomal vesicle grows in parallel to the ER and Golgi complex, occupying a large region of the mid-spermatid (Fig. 5A). The ER and Golgi complex fill the cytoplasm, which is reduced to a band between the nucleus and the proacrosomal vesicle. In addition, small mitochondria containing electron-dense material are intercalated within the cisternae of the ER and Golgi complex (Fig. 5B,C). The first sign of differentiation in the proacrosomal vesicle is the presence of a single electron-dense granule. This electron-dense granule is a spherical vesicle delimitated by a membrane (white arrows in Fig. 5C) and contains electron-dense material, which seems to originate

TABLE 1. Characteristics of the germ cells during M. brachydactyla spermatogenesis

		Spermatid			
	Spermatocyte	Early	Mid	Late	Spermatozoa
Cellular diameter, *C* (µm)	12.81 ± 1.44	8.50 ± 1.82	8.08 ± 2.58	6.94 ± 0.71	4.32 ± 0.48
Nuclear diameter, *N* (µm)	9.17 ± 1.44	6.54 ± 1.66	5.47 ± 2.28	0.99 ± 0.51	n.d.
Ratio *N*/*C*	0.72	0.77	0.68	0.14	n.d.

Data are shown as mean ± SD.
n.d., no data

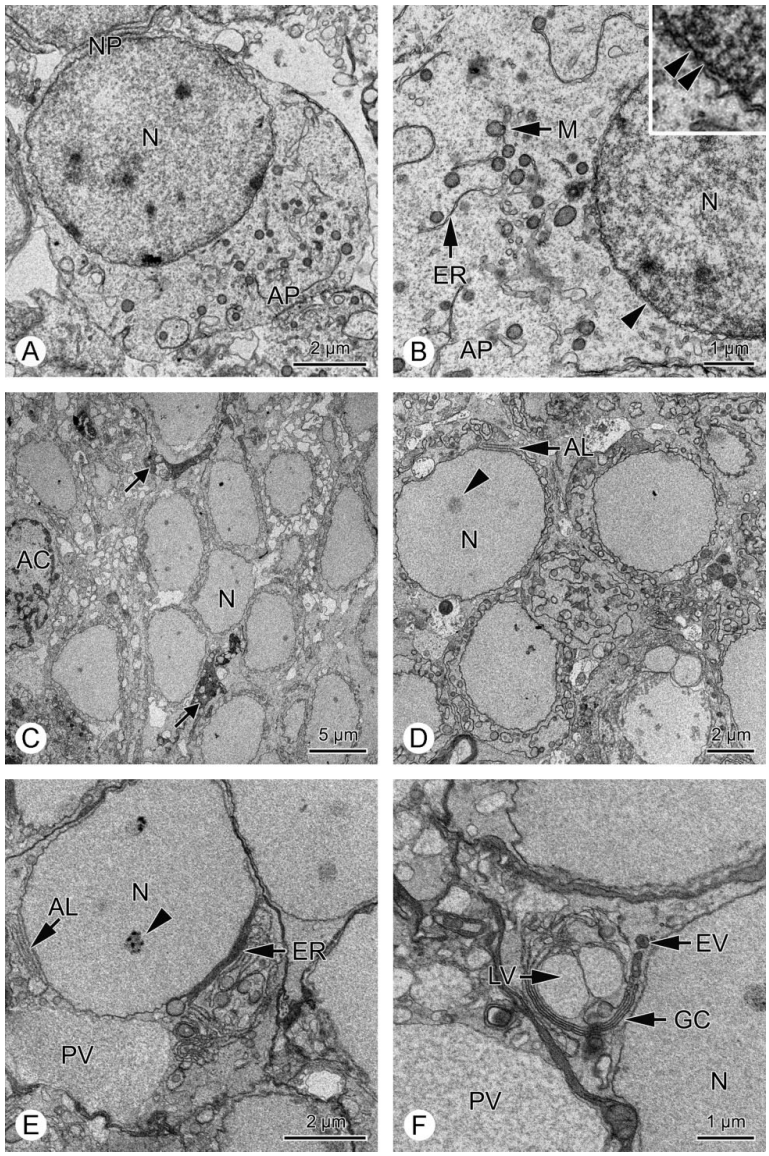

Fig. 4. *Maja brachydactyla*. Transmission electron micrographs of spermatids. **A**, early spermatid showing the nucleus, located in the nuclear pole, with few clumps of condensed chromatin. Note the circular arrangement of the cytoplasmic membrane in the left region of the acrosome pole. **B**, detail of the acrosomal pole of an early spermatid showing small mitochondria and longitudinal cisternae of the endoplasmic reticulum. Nuclear envelope pores (arrowhead) are oriented toward the acrosomal pole. Inset shows nuclear pores (arrowheads) at higher magnification. **C**, seminiferous tubule showing some mid-spermatids with decondensed chromatin and an accessory cell with oval nucleus. The cytoplasm of the accessory cells extends between spermatids, with areas of high electron-density (arrows). **D**, mid-spermatids showing the nuclei with small clumps of condensed chromatin (arrowhead, also in E). In the cytoplasm, the annulate lamellae are associated with the nuclear envelope. **E**, mid-spermatid showing annulate lamellae (left) and the endoplasmic reticulum (right) associated with the nucleus. The proacrosomal vesicle is formed in the acrosomal pole and contains granular homogeneous material. **F**, detail of the Golgi complex of a mid-spermatid producing vesicles of light and medium electron-dense materials. AC, accessory cell; AL, annulate lamellae; AP, acrosomal pole; ER, endoplasmic reticulum; EV, vesicles of electron-dense material; GC, Golgi complex; LV, vesicles of light electron-dense material; M, mitochondria; N, nucleus; NP, nuclear pole; PV, proacrosomal vesicle.

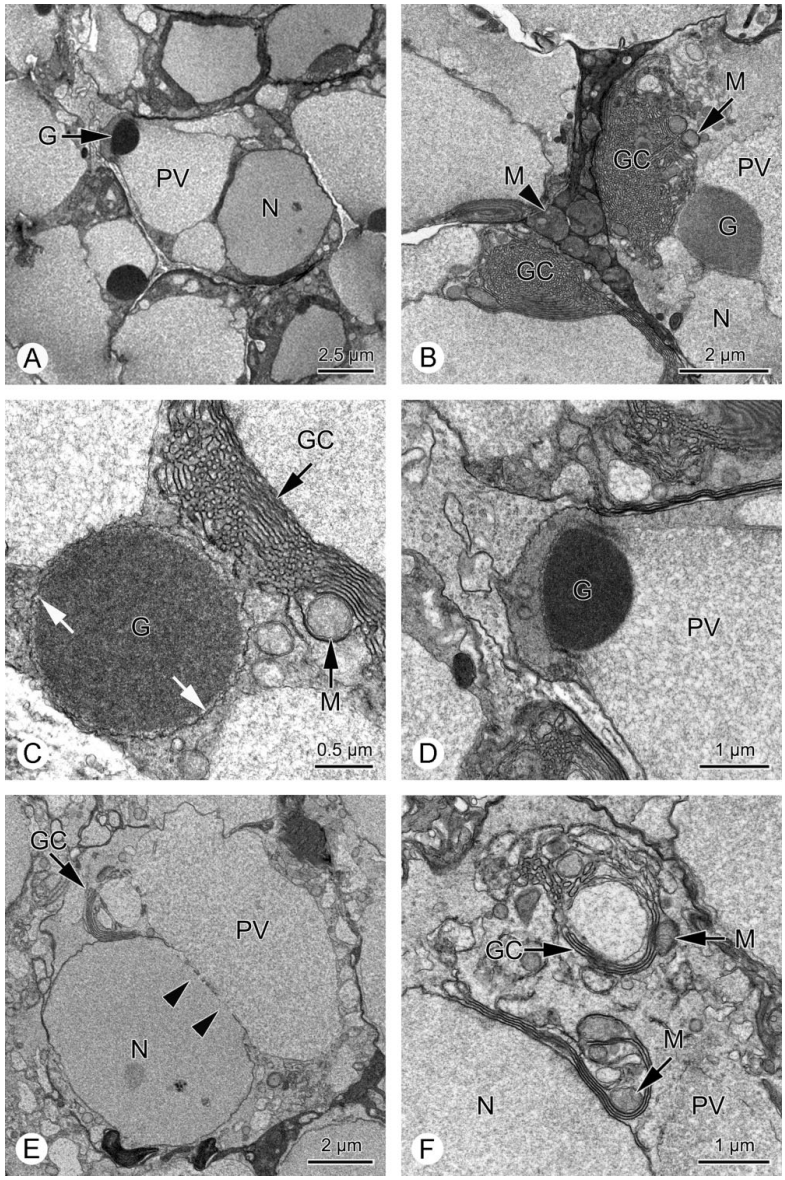

Fig. 5. *Maja brachydactyla*. Transmission electron micrographs of mid-spermatids. **A**, polarized spermatid containing the electron-dense granule in the apical region of the proacrosomal vesicle. **B**, detail of the cytoplasm with tightly packed cisternae of the Golgi complex and adjacent mitochondria. The cytoplasm of the accessory cells, containing mitochondria (arrowhead), is intercalated between the spermatids. **C**, detail of the electron-dense granule surrounded by a thin membrane (white arrows). **D**, detail of the apical granule already merged into the proacrosomal vesicle. **E**, general view of a spermatid at the end of the mid-spermatid stage showing the discontinuous nuclear envelope (arrowheads) in the equatorial region and the degeneration of the Golgi complex. **F**, detail of a degenerating Golgi complex with adjacent mitochondria. G, granule; GC, Golgi complex; M, mitochondria; N, nucleus; PV, proacrosomal vesicle.

in the cytoplasm by the fusion of the electron-dense Golgi vesicles. Later, the membranes of the granule and the proacrosomal vesicle merge, and the granule appears in the apical region of the spermatid (Fig. 5D). Once the proacrosomal vesicle achieves its maximum size, the nuclear envelope breaks in the equatorial

region of the cell, and the ER and Golgi complex degenerate (Fig. 5F).

During the mid-spermatid stage, the accessory cells show features similar to those in the spermatocyte stage. In addition, the cytoplasm has a vacuolized appearance with regions of different electron densities (arrows in Fig. 4C). Some mitochondria in the accessory cells seem associated with the regions of the spermatids where the ER and Golgi complex appear (mitochondria pointed out with arrowhead in Fig. 5B).

Late spermatids demonstrate several important changes in the nuclear morphology and the internal organization of the proacrosomal vesicle. Late spermatids are highly polarized cells, showing a reduced, half-moon shaped nucleus at the nuclear pole and a voluminous proacrosomal vesicle (Fig. 6A,B). The nuclear envelope merges with the plasma membrane, giving rise to a thick, electron-dense membrane (Fig. 6B,C). The cytoplasm is now highly reduced to the margins of the nucleus in the equatorial region and is filled with degenerate mitochondria and a membrane system derived from the degenerated ER and Golgi complex (Fig. 6C). A highly electron-dense band that will give rise to the operculum appears over the apical granule of the proacrosomal vesicle (Fig. 6B). In the base of the proacrosomal vesicle, a thin layer of granular material (arrowhead in Fig. 6B) covers an invagination of cytoplasm, which is the origin of the perforatorium (Fig. 6D). Later, the invagination extends anteriorly while it is surrounded by the posterior extension of the electron-dense apical granule (Fig. 6E). The maturation of the spermatids continues with the lateral extension of the nucleus, appearing as a horseshoe shape in longitudinal section that surrounds the proacrosomal vesicle. In the apical region, the operculum extends laterally covering the perforatorium and, partially, the proacrosomal vesicle (Fig. 6E). The contents of the proacrosomal vesicle aggregate, first into clumps distributed throughout the vesicle and then forming a layer around the perforatorium (Fig. 6F). A homogeneous layer of light electron-dense granular material still remains in the outer region of the proacrosomal vesicle (Fig. 6G). The last modifications of the late spermatid are the development of the nuclear lateral arms in the subapical region of the cell (arrowhead in Fig. 6G) and the condensation of the outer layer of the proacrosomal vesicle.

At the end of the spermiogenesis, the accessory cells present a degenerated aspect. In the nucleus, heretochromatin and nucleoplasm are highly electron dense (Fig. 6A). The cytoplasm, which surrounds late spermatids, also increases in electron density, showing numerous degenerate mitochondria (asterisk in Fig. 6C) and highly electron-dense spherical bodies, which are probably endosomal vesicles with degradative activity (Fig. 6E).

The newly formed spermatozoa are transferred from the TZ to the EZ of the seminiferous tubule (Fig. 7A) and then moved toward the vas deferens, where they are packed in spermatophores. The spermatozoon is the smallest of the germ cell lineage (Table 1). It is composed of a globular acrosome, a thin layer of cytoplasm, and a cup-shaped nucleus with several lateral arms (Fig. 7B). The acrosome presents three layers of different electron density and is encircled in the subapical region by the structures-organelles complex (SO-complex) which consists of membrane layers, degenerate mitochondria, and microtubules.

DISCUSSION

We present a complete sequence of stages throughout spermatogenesis of the spider crab, *M. brachydactyla*. Because the germ cells within a cross section of the seminiferous tubule were all in the same stage or two successive stages, the differentiation of the germ cells had to be followed along the testis. Our work complements previous ultrastructural studies in brachyurans that were focused on spermiogenesis (Yasuzumi, 1960; Pochon-Masson, 1968; Langreth, 1969; Reger, 1970; Du et al., 1988; Medina and Rodríguez, 1992b; Li, 1995; Wang et al., 1997b; Wang et al., 1999). All previous studies were performed with the higher groups within Eubrachyura, but majids appear in the basal positions within this group, and therefore the results presented here carry potential phylogenetic significance (see Jamieson and Tudge, 2000 for phylogenetic discussion). Contrary to former studies (Pochon-Masson, 1983), our morphological observations suggest that the acrosome in *M. brachydactyla* is mainly derived from the Golgi-like complex.

Spermatocytes

During the early phases of spermatogenesis in *M. brachydactyla*, the nucleus of primary spermatocytes contains typical meiotic figures such as the synaptonemal complexes in the pachytene stage. In addition, the cytoplasm contains few mitochondria, a developing ER and other membrane arrangements, and a nuage (Du et al., 1988; Li, 1995; Wang et al., 1997b). Similar findings were also described in the cytoplasm of other decapod crustaceans, such as *Pagurus bernhardus* and *Nephrops norvegicus* (Chevaillier, 1970). In both species, as in *M. brachydactyla*, a low number of mitochondria with poorly developed cristae were present throughout the cytoplasm, the ER was continuous with the nuclear envelope, and the Golgi complex was absent. On the contrary, the cytoplasm of *Procambarus paeninsulanus* (Hinsch, 1993) shows aggregated mitochondria and an ER that breaks up into small tubular aggregates. The

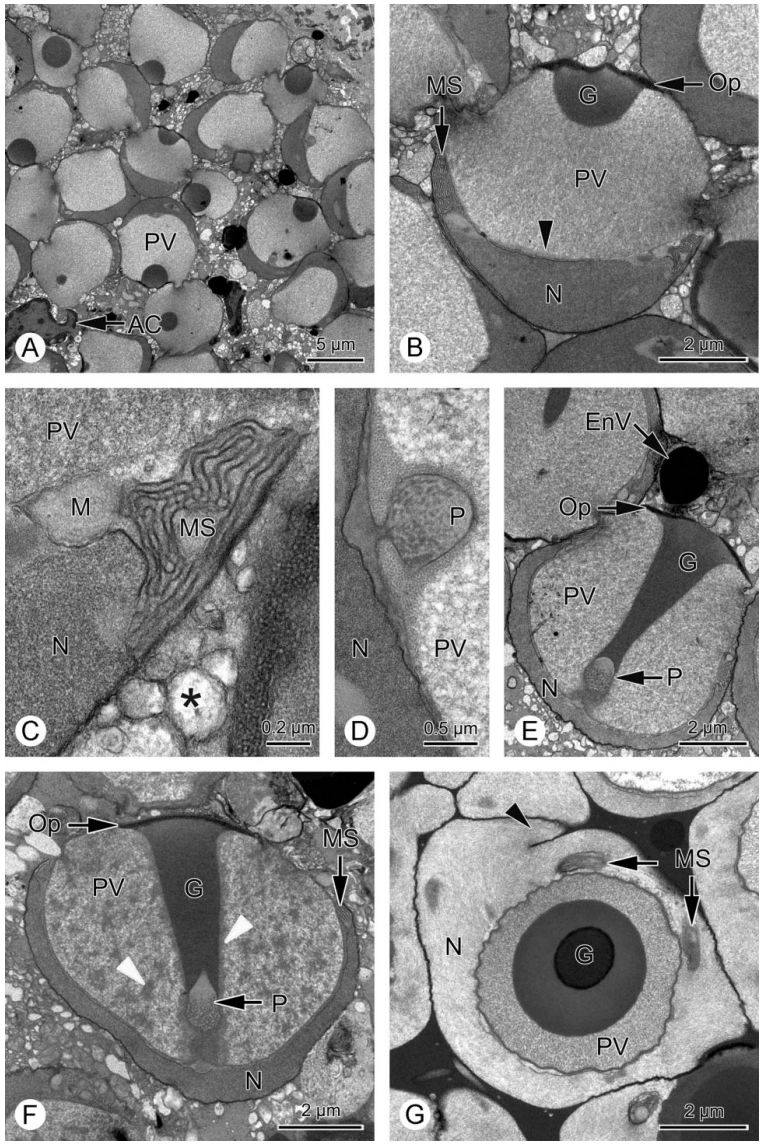

Fig. 6. *Maja brachydactyla*. Transmission electron micrographs of late spermatids. **A**, several late spermatids accompanied by an accessory cell. The nucleus of the accessory cell is electron dense, and its cytoplasm surrounds the spermatids. **B**, spermatid showing the half-moon-shaped nucleus, the reduced cytoplasm, and the proacrosomal vesicle. The apical region of the proacrosomal vesicle already presents the operculum, while a band of fine granular material (arrowhead) appears in the base. **C**, detail of the membrane system and degenerate mitochondria, similar to the mitochondria (asterisk) that belong to the accessory cell. **D**, detail of the basal region in the proacrosomal vesicle. The perforatorium is developed in association to the band of fine granular material. **E**, spermatid with the nucleus surrounding the proacrosomal vesicle. The operculum extends laterally, and the granule surrounds the perforatorium following the central axis of the spermatid. An electron-dense spherical body in the cytoplasm of the accessory cell seems to be an endosomal vesicle with degradative activity. **F**, spermatid showing the aggregation (white arrowheads) of the proacrosomal vesicle materials. **G**, spermatid with the nucleus already showing lateral arms (arrowhead) and the uncondensed, outer layer of the proacrosomal vesicle. AC, accessory cell; EnV, endosomal vesicle of the accessory cell; G, granule; M, mitochondria; MS, membrane system; N, nucleus; P, perforatorium; PV, proacrosomal vesicle; Op, operculum.

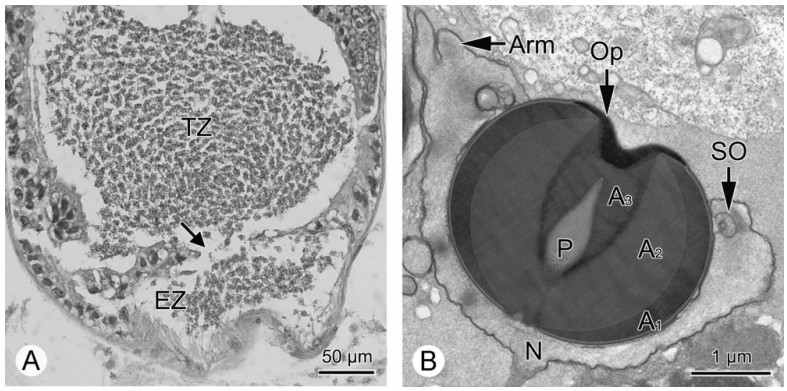

Fig. 7. *Maja brachydactyla*. **A,** light microscopy micrograph (hematoxylin-eosin stain) of the seminiferous tubule. Transverse section showing the newly formed spermatozoa moving from the transformation to the evacuation zone through a discontinuity in the wall that separates both zones (arrow). **B**, transmission electron micrograph of a spermatozoon showing the typical brachyuran structure is composed of a cup-shaped nucleus and a globular, three-layered acrosome centrally crossed by the perforatorium and apically covered by the operculum. A1, external acrosomal layer; A2, intermediate acrosomal layer; A3, inner acrosomal layer; Arm, lateral arm of the nucleus; EZ, evacuation zone; N, nucleus; Op, operculum; P, perforatorium; SO, structures-organelles complex; TZ, transformation zone.

cytoplasm of the spermatocytes in *M. brachydactyla* demonstrates three peculiarities: the nuage, the concentric membrane system, and the annulate lamellae. The nuage appears as an electron-dense body during most primary spermatocyte stages, being especially prominent in the diplotene stage. The concentric membrane system appears in the leptotene and pachytene stages and shows lateral dilatations in pachytene. The annulate lamellae appear during diplotene, and to our knowledge this is the first report of annulate lamellae in the spermatocytes of a crab. Annulate lamellae are described as a network of parallel intracytoplasmic membranes observed in dividing cells, both somatic and germ cells (Kessel, 1992). Their origin and function is still unclear, although recent immunolocalization studies have indicated that the annulate lamellae may act as a reservoir of nuclear envelope and nuclear pore complex proteins (Imreh and Hallberg, 2000).

Spermiogenesis

The basic changes occurring during spermiogenesis were established using LM. These events were summarized as follows: 1) cellular polarization caused by the marginalization of the nucleus, along with the development of the proacrosomal vesicle; 2) formation of a ring by the membranous system; 3) development of the operculum and perforatorium in the acrosomal vesicle; 4) nuclear surrounding of the acrosome; and 5) development of the radial arms (Binford, 1913; Fasten, 1918, 1926; Nath, 1932). Later studies using TEM (Yasuzumi, 1960; Pochon-Masson, 1962, 1968; Langreth, 1969; Reger, 1970; Du et al., 1988; Medina and Rodríguez, 1992b; Li, 1995; Wang et al., 1997b; Wang et al., 1999), including the present work, support these findings.

The first change occurring during spermiogenesis in *M. brachydactyla* is the decondensation of chromatin in the mid-spermatid. The decondensation leads to a nucleus with slightly condensed, fibrillar chromatin, which is highly characteristic of the brachyuran spermatozoon. Similar results have been described at the beginning of spermiogenesis in *Cancer* sp. (Langreth, 1969) and *Pinnixia* sp. (Reger, 1970), where chromatin condensed in clumps appeared in the periphery of the nucleus. However, the nucleus of *U. tangeri* (Medina and Rodríguez, 1992b) presented a granular homogeneous appearance. The molecular basis of chromatin decondensation has largely been investigated. The first studies concluded that the chromatin in decapod spermatozoa was not associated with proteins, because neither histones nor protamines were detected (Vaughn and Locy, 1969; Vaughn and Hinsch, 1972; Vaughn and Thomson, 1972). Recently, a low histone to DNA ratio and a high level of acetylation of these proteins were reported in *Cancer* sp. (Kurtz et al., 2008) and *M. brachydactyla* (Kurtz et al., 2009), which could explain the decondensed chromatin in these species.

Other changes in the nucleus include breakage of the nuclear envelope and a dramatic modification of its morphology. During spermiogenesis in *M. brachydactyla*, the nuclear envelope disinte-

grates near the basal region of the acrosome, similar to *C. maenas* (Pochon-Masson, 1968), *Cancer* sp. (Langreth, 1969), *Pinnixa* sp. (Reger, 1970), and *U. tangeri* (Medina and Rodríguez, 1992b). As a result, the chromatin is in contact with the cytoplasm, giving rise to the so-called nucleo-cytoplasm complex. In addition, the nuclear envelope in *M. brachydactyla* also gives rise to a pentalaminar system when it fuses with the plasma membrane, as has been observed in several brachyurans (Brown, 1966; Langreth, 1969; Reger, 1970; Medina and Rodríguez, 1992b). The nucleus is also subjected to deep morphological changes during spermiogenesis, going from spherical in early and mid-spermatids to half-moon and, finally, horseshoe shaped in late spermatids. During this process, the nucleus extends anteriorly, surrounding the acrosome and developing the nuclear lateral arms, as described for *C. maenas* and *U. tangeri* (Pochon-Masson, 1968; Medina and Rodríguez, 1992b). Nothing is known about the mechanism of the morphological modification of the nucleus and the development of the lateral arms. The lateral arms are usually associated with the membrane and mitochondrial complex of the spermatozoa, and they are sustained by microtubules in some species, such as *C. maenas* (Pochon-Masson, 1965), *Libinia emarginata* (Hinsch, 1969), and *Mithrax* sp. (Hinsch, 1973).

Throughout spermiogenesis in *M. brachydactyla*, the cytoplasm becomes highly reduced until it is finally limited to a thin band between the nucleus and the acrosome. The cytoplasmic reduction is due to the development of the acrosome and, probably, to the release of cytoplasmic regions, which is especially intense at the end of spermiogenesis. In *E. japonicus* (Yasuzumi, 1960), large regions of the cytoplasm become isolated and slough off. As reported for *C. maenas* (Pochon-Masson, 1968) and *Cancer* sp. (Langreth, 1969), the accessory or nurse cells play a key role in phagocytosing and degrading the spermatid residual cytoplasm. The accessory cells could also play a similar role in *M. brachydactyla*, as suggested by the presence of electron-dense spherical bodies (probably endosomal vesicles with degradative activity) in their cytoplasm at the end of spermiogenesis.

The different organelles are also modified during spermiogenesis. As described for *U. tangeri* (Medina and Rodríguez, 1992b), the mitochondria in *M. brachydactyla* are scarce and with degenerate cristae. During spermiogenesis, mitochondria undergo a process of aggregation and number reduction by means of fusion or cristae degeneration (Wang et al., 1997a), which in some cases leads to a loss of their oxidative function (Pearson and Walker, 1975). At the end of the spermiogenesis in *M. brachydactyla*, the mitochondria are integrated in the SO-complex of the spermatozoa, as shown for several species (Pochon-Masson, 1962; Langreth, 1969; Reger, 1970; Medina and Rodríguez, 1992b).

The ER, Golgi complex, and other cytoplasmic membrane are also subjected to extensive morphological modifications during the spermiogenesis in *M. brachydactyla*. During spermiogenesis, the cytoplasmic membrane systems progressively develop and differentiate into the ER and the Golgi complex. The presence of annulate lamellae during the mid-spermatid stage suggests that the ER could develop from the annulate lamellae themselves, as documented during the spermiogenesis in *Drosophila* sp. (Merisko, 1989). Once the proacrosomal vesicle reaches its maximum size, the ER and the Golgi complex degenerate into a membrane system that occupies the equatorial region of the cell at the end of the mid-spermatid stage. Later, the membrane system together with the mitochondria is pushed toward the apical portion of the late spermatid and gives rise in the spermatozoa to the SO-complex. The origin of the SO-complex (synonymous with membranous organelle (Reger, 1970), nucleo-chondrio-polymicrotubular complex (*complex nucléochondriomique*, Pochon-Masson, 1968), membrane complex (Langreth, 1969; Du et al., 1988; Li, 1995; Wang et al., 1999), membranous lamellar complex (Chiba et al., 1992), and membranous lamellae (Medina and Rodríguez, 1992b)) has been previously attributed to ER cisternae (Langreth, 1969; Du et al., 1988; Li, 1995) or a nuclear and ER origin (Reger, 1970). Aside its origin, the SO-complex is composed of a membrane system, mitochondria, and occasionally microtubules (Krol et al., 1992).

Our observations suggest that the acrosome in *M. brachydactyla* is derived from the vesicles of a putative Golgi complex, similar to that observed in *S. yangtsekiense* (Wang et al., 1999). At the mid-spermatid stage, a cytoplasmic membrane system morphologically similar to a Golgi complex produces two kinds of vesicles that give rise to the acrosome. These results contrast to most of previous morphological studies, in which the origin of the acrosome was ascribed to the ER or nuclear envelope derivatives (Pochon-Masson, 1983). More recently, Tudge (2009) has proposed that the Golgi complex described in *S. yangtsekiense* and *Macrobrachium nipponense* could represent Golgi-like extensions of the ER. However, the Golgi complex has been indirectly demonstrated during the spermiogenesis of *E. sinensis* by means of the detection of two proteins, kinesin KIFC1 and GM130 protein, specifically associated to the Golgi complex (Yu et al., 2009). Since no typical Golgi complex has been described in morphological studies (Du et al., 1988), Yu et al. (2009) proposed that the Golgi complex in *E. sinensis* may be composed of single Golgi stacks. Thus, it seems that if the Golgi complex is present in the spermatids of brachyurans, but it may show a complex morphology, which is difficult to identify.

The Golgi complex produces two kinds of vesicles that contain either light or electron-dense material. Thus, the acrosome is formed from the combination of the proacrosomal vesicle and an electron-dense granule, which are respectively originated by the fusion of the light and electron-dense Golgi vesicles. The electron-dense granule has also been reported in other species (Pochon-Masson, 1968; Langreth, 1969; Medina and Rodríguez, 1992b), but its origin has not been determined. Later, the electron-dense granule migrates toward the apical region of the proacrosomal vesicle and, finally, extends posteriorly surrounding the perforatorium or adjacent areas, as occurs for several species (Pochon-Masson, 1983; Krol et al., 1992). As described for *U. tangeri* (Medina and Rodríguez, 1992b), the operculum develops above the granule as a thin, highly electron-dense band. After the extension of the electron-dense granule, the development of the perforatorium begins in the basal region of the acrosome. A layer of granular material, known as the granular belt (Langreth, 1969; Medina and Rodríguez, 1992b), appears at the base of the proacrosomal vesicle. Simultaneously, an invagination, which will develop into the perforatorium, follows the central axis of the proacrosomal vesicle, from the posterior end toward the apical region. As in *Cancer* sp., the electron-dense granule in *M. brachydactyla* grows posteriorly surrounding the perforatorium up to its base (Langreth, 1969). The last event in the proacrosomal vesicle is the aggregation of the acrosomal materials. In *M. brachydactyla*, this process occurs in two stages. First, the materials condense throughout the proacrosomal vesicle, and second, the materials surround the central axis. However, an outer layer of uncondensed material remains until the expansion of the nucleus, which is the last step of spermatid differentiation. As a result of this two-step aggregation, the acrosome of *M. brachydactyla* contains three layers disposed in a concentric pattern. Previous studies did not describe the aggregation pattern in the acrosome, because those species did not exhibit a three-layered acrosome (Yasuzumi, 1960; Pochon-Masson, 1968; Langreth, 1969; Reger, 1970; Du et al., 1988; Medina and Rodríguez, 1992b; Li, 1995; Wang et al., 1997b; Wang et al., 1999).

In conclusion, the ultrastructural study of spermatogenesis in *M. brachydactyla* has revealed the presence of annulate lamellae in the spermatocyte, early and mid-spermatids, the presence of a putative Golgi complex involved in the acrosome formation, and a two-step aggregation process of the acrosomal contents.

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LITERATURE CITED

Beninger PG, Pennec Ml. 1991. Functional anatomy of scallops. In: Shumway SE, editor. Scallops: Biology, Ecology and Aquaculture. Amsterdam: Elsevier. pp 133–233.

Binford R. 1913. The germ-cells and the process of fertilization in the crab, *Menippe mercenaria*. J Morphol 24:147–201.

Brown GG. 1966. Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus*. J Ultrastruct Res 14:425–440.

Chevaillier P. 1970. Recherches sur la structure et les constituants chimiques des cellules germinales mâles des crustacés décapodes [PhD thesis]. Rennes: Université de Rennes. p 322.

Chiba A, Kon T, Honma Y. 1992. Ultrastructure of the spermatozoa and spermatophores of the zuwai crab, *Chionoecetes opilio* (Majidae, Brachyura). Acta Zool 73:103–108.

Cledón M, Arntz W, Penchaszadeh PE. 2005. Gonadal cycle in an *Adelomelon brasiliana* (Neogastropoda: Volutidae) population of Buenos Aires province, Argentina. Mar Biol 147:439–445.

Du N, Xue L, Lai W. 1988. Studies on the sperm of Chinese mitten-handed crab, *Eriocheir sinensis* (Crustacea, Decapoda). I. Spermatogenesis. Oceanol Limnol Sinica 19:71–75.

Estampador EP. 1949. *Scylla* (Crustacea: Portunidae) II. Comparative studies on spermatogenesis and oogenesis. Philip J Sci 78:95–109.

Fasten N. 1918. Spermatogenesis of the pacific coast edible crab, *Cancer magister* Dana. Biol Bull 34:277–307.

Fasten N. 1924. Comparative stages in the spermatogenesis of various Cancer crabs. J Morphol 39:47–61.

Fasten N. 1926. Spermatogenesis of the black-clawed crab, *Lophopanopeus bellus* (Stimpson) Rathburn. Biol Bull 50:277–293.

Felgenhauer BE, Abele LG. 1991. Morphological diversity of decapoda spermatozoa. In: Bauer RT, Martin JW, editors. Crustacean Sexual Biology. New York: Columbia University Press. pp 322–339.

Freire J, Bernárdez C, Corgos A, Fernández L, González- Gurriarán E, Sampedro MP, Verísimo P. 2002. Management strategies for sustainable invertebrate fisheries in coastal ecosystems of Galicia (NW Spain). Aquatic Ecol 36:41–50.

Goudeau M. 1982. Fertilization in a crab. I. Early events in the ovary, and cytological aspects of the acrosome reaction and gamete contacts. Tissue Cell 14:97–111.

Hinsch GW. 1969. Microtubules in the sperm of the spider crab, *Libinia emarginata* L. J Ultrastruct Res 29:525–534.

Hinsch GW. 1971. Penetration of the oocyte envelope by spermatozoa in the spider crab. J Ultrastruct Res 35:86–97.

Hinsch GW. 1973. Sperm structure of Oxyrhyncha. Can J Zool 51:421–426.

Hinsch GW. 1993. Ultrastructure of spermatogonia, spermatocytes, and sertoli cells in the testis of the crayfish, *Procambarus paeninsulanus*. Tissue Cell 25:737–742.

Hoestlandt H. 1948. Recherches sur la biologie de l'*Eriocheir sinensis* en France. Ann Ins Oceanogr 24:1–116.

Imreh G, Hallberg E. 2000. An integral membrane protein from the nuclear pore complex is also present in the annulate lamellae: Implications for annulate lamella formation. Exp Cell Res 259:180–190.

Jamieson BGM, Tudge CC. 2000. Progress in male gamete ultrastructure and phylogeny. In: Adiyodi KG, Adiyodi RG, editors. Reproductive Biology of Invertebrates. Kerala: Wiley. pp 1–95.

Kessel RG. 1992. Annulate lamellae: A last frontier in cellular organelles. In: Jeon KW, Friedlander M, editors. International Review of Cytology, A Survey of Cell Biology. San Diego, California: Academic Press, Inc. pp 43–120.

Krol RM, Hawkins WE, Overstreet RM. 1992. Reproductive components. In: Harrison FW, Humes AG, editors. Microscopic Anatomy of Invertebrates. New York: Wiley-Liss, Inc. pp 295–343.

Kurtz K, Ausió J, Chiva M. 2009. Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosome-like structures in decapod crustacean sperm nuclei previously described without SNBPs. Tissue Cell 41:334–344.

Kurtz K, Martínez-Soler F, Ausió J, Chiva M. 2008. Histones and nucleosomes in *Cancer* sperm (Decapod: *Crustacea*) previously described as lacking basic DNA-associated proteins: A new model of sperm chromatin. J Cell Biochem 105:574–584.

Langreth SG. 1969. Spermiogenesis in *Cancer* crabs. J Cell Biol 43:575–603.

Li TW. 1995. On spermatogenesis and sperm ultrastructure of blue crab *Portunus trituberculatus* (Crustacea, Decapoda). Acta Zool Sinica 41:41–47.

Medina A. 1992. Structural modifications of sperm from the fiddler crab *Uca tangeri* (Decapoda) during early stages of fertilization. J Crustacean Biol 12:610–614.

Medina A, Rodríguez A. 1992a. Structural changes in sperm from the fiddler crab, *Uca tangeri* (Crustacea, Brachyura), during the acrosome reaction. Mol Reprod Dev 33:195–201.

Medina A, Rodríguez A. 1992b. Spermiogenesis and sperm structure in the crab *Uca tangeri* (Crustacea, Brachyura), with special reference to the acrosome differentiation. Zoomorphology 111: 161–165.

Merisko EM. 1989. Annulate lamellae: An organelle in search of a function. Tissue Cell 21:343–354.

Meusy MJ-J. 1972. La gamétogenèse et la fraction protéique de l'hémolymphe spécifique du sexe femelle chez quelques Crustacés supérieurs: étude descriptive et rôle des glandes androgènes [PhD Thesis]. Paris: Universite Paris VI. p 165.

Mouchet S. 1931. Spermatophores des Crustacés Décapodes Anomures et Brachyoures et castration parasitaire chez quelques Pagures. Ann Sta Océanogr Salammbô VI:1–203.

Nath V. 1932. Spermatid and sperm in *Paratelphusa spinigera*. Q J Microsc Sci 229:543–556.

Neumann V. 1996. Comparative gonopod morphology of the European spider crabs of the genus *Maja* Lamarck 1801. Senckenb Biol 75:143–157.

Neumann V. 1998. A review of the *Maja squinado* (Crustacea: Decapoda: Brachyura) species-complex with a key to the eastern Atlantic and Mediterranean species of genus. J Nat Hist 32:1667–1684.

Patiño R, Redding JM. 2000. Reproductive systems. In: Ostrander GK, editor. The Laboratory Fish. London: Academic Press. pp 489–500.

Pearson PJ, Walker MH. 1975. Alteration of cytochrome-C oxidase activity during spermatogenesis in *Carcinus maenas*. Cell Tissue Res 164:401–410.

Pochon-Masson J. 1962. Le chondriofusome des gamètes males du Crustacé Décapode *Carcinus maenas*. C R Acad Sci Paris 254:4076–4078.

Pochon-Masson J. 1965. L'ultrastructure des épines du spermatozoïde chez les décapodes (Macroures, Anomoures, Brachyoures). C R Acad Sci Paris 260:3762–3764.

Pochon-Masson J. 1968. L'ultrastructure des spermatozoïdes vésicularies chez les crustacés décapodes avant et au cours de leur dévagination expérimentale. I. Brachyoures et Anomoures. Ann Sci Nat Zool 10:1–100.

Pochon-Masson J. 1983. Spermatogenesis and sperm function. In: Adiyodi KG, Adiyodi RG, editors. Reproductive Biology of Invertebrates. Kerala: Wiley. pp 407–449.

Reger JF. 1970. Studies on the fine structure of spermatids and spermatozoa of the crab, *Pinnixia* sp. J Morphol 132:89–100.

Rorandelli R, Paoli F, Cannicci S, Mercati D, Giusti F. 2008. Characteristics and fate of the spermatozoa of *Inachus phalangium* (Decapoda, Majidae): Description of novel sperm structures and evidence for an additional mechanism of sperm competition in Brachyura. J Morphol 269:259–271.

Sainte-Marie G, Sainte-Marie B. 1999a. Reproductive products in the adult snow crab (*Chionoecetes opilio*). I. Observations on spermiogenesis and spermatophore formation in the vas deferens. Can J Zool 77:440–450.

Sainte-Marie G, Sainte-Marie B. 1999b. Reproductive products in the adult snow crab (*Chionoecetes opilio*). II. Multiple types of sperm cells and of spermatophores in the spermathecae of mated females. Can J Zool 77:451–462.

Sasso-Cerri E, De Faria FP, Freymuller E, Miraglia SM. 2004. Testicular morphological changes during the seasonal reproductive cycle in the bullfrog *Rana catesbeiana*. J Exp Zoolog A Comp Exp Biol 301A:249–260.

Simeó CG, Ribes E, Rotllant G. 2009. Internal anatomy and ultrastructure of the male reproductive system of the spider crab Maja *brachydactyla* (Decapoda: Brachyura). Tissue Cell 41:345–361.

Simeó CG, Kurtz K, Rotllant G, Chiva M, Ribes E. Sperm ultrastructure of the spider crab *Maja brachydactyla* (Decapoda: Brachyura). J Morphol (in press).

Sotelo G, Morán P, Posada D. 2008. Genetic identification of the northeastern Atlantic spiny spider crab as *Maja brachydactyla* Balss, 1922. J Crustacean Biol 28:76–81.

Thongkukiatkul A, Jungudomjaroen S, Ratanapahira C. 2008. Spermatogenesis and chromatin condensation in male germ cells of sea cucumber *Holothuria leucospilota* (Clark, 1920). Tissue Cell 40:167–175.

Tudge CC. 2009. Spermatozoal morphology and its bearing on decapod phylogeny. In: Martin JW, Crandall KA, Felder DL, editors. Crustacean Issues 18: Decapod Crustacean Phylogenetics. Boca Raton, FL: CRC Press, Taylor & Francis Group. pp 101–119.

Tudge CC, Justine JL. 1994. The cytoskeletal proteins actin and tubulin in the spermatozoa of 4 decapod crabs (Crustacea, Decapoda). Acta Zool 75:277–285.

Vaughn JC, Locy RD. 1969. Changing nuclear histone patterns during development. III. The deoxyribonucleic acid content of spermatogenic cells in the crab *Emerita analoga*. J Histochem Cytochem 17:591–600.

Vaughn JC, Hinsch GW. 1972. Isolation and characterization of chromatin and DNA from the sperm of the spider crab, *Libinia emarginata*. J Cell Sci 11:131–152.

Vaughn JC, Thomson LA. 1972. A kinetic study of DNA and basic protein metabolism during spermatogenesis in the sand crab, *Emerita analoga*. J Cell Biol 52:322–337.

Wang L, Du N-S, Lai W. 1997a. Mitochondrial ultrastructure during spermatogenesis of *Sinopotamon yangtsekiense* (Crustacea: Decapoda). Acta Zool Sinica 43:113–118.

Wang L, Du N-S, Lai W. 1999. Studies on spermiogenesis of a freshwater crab *Sinopotamon yangtsekiense* (Crustacea Decapoda). Acta Hydrobiol Sinica 23:29–33.

Wang YL, Zhang ZP, Li SJ. 1997b. Ultrastructure of spermatogenesis in the crab *Scylla serrata*. Acta Zool Sinica 43:249–254.

Yasuzumi G. 1960. Spermatogenesis in animals as revealed by electron microscopy: VII. Spermatid differentiation in the crab, *Eriocheir japonicus*. J Biophys Biochem Cytol 7:73–78.

Yu K, Hou L, Zhu J-Q, Ying X-P, Yang W-X. 2009. KIFC1 participates in acrosomal biogenesis, with discussion of its importance for the perforatorium in the Chinese mitten crab *Eriocheir sinensis*. Cell Tissue Res 337:113–123.

Article 3

Títol: Sperm ultrastructure of the spider crab *Maja brachydactyla* (Decapoda: Brachyura)

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Informe de la contribució del doctorand

La hipòtesi de treball i la metodologia a seguir varen estar realitzades pel Dr. Ribes, la Dra. Rotllant i el Dr. Chiva. El doctorand va participar en les dissseccions i els mostrejos dels teixits amb la col·laboració del Dr. E. Ribes. Les mostres foren processades pels Serveis Cientificotècnics de la Universitat de Barcelona. Les imatges de microscòpia electrònica foren realitzades pel Dr. E. Ribes. La descripció i interpretació de les imatges, i la redacció del manuscrit foren realitzades pel doctorant amb la col·laboració dels coautors.

Dra. Guiomar Rotllant Estelrich

Resum

Aquest estudi descriu la morfologia de l'espermatozoide de la cabra de mar, *Maja brachydactyla*, amb especial interès en la localització de l'actina i la tubulina. L'espermatozoide de *M. brachydactyla* és similar en aparença i organització a altres espermatozoides de braquiürs. L'espermatozoide és una cèl·lula globular formada per un acrosoma central, que està envoltat per una capa fina de citoplasma i un nucli en forma de copa amb quatre braços radials laterals. L'acrosoma és l'estructura més voluminosa de l'espermatozoide, i es tracta d'una vesícula esferoïdal formada per tres capes concèntriques de diferent electrodensitat, un perforatori central, i l'opercle en la regió apical. La capa interna de l'acrosoma és la més electrodensa i la intermèdia la menys electrodensa. Una càpsula envolta la capa externa de l'acrosoma. L'opercle és l'estructura més electrodensa de l'espermatozoide, cobreix l'àpex de l'acrosoma i presenta una depressió central amb una protuberància central. El perforatori és una columna romboïdal que creua l'acrosoma des de la seua base fins l'opercle. Associat a la zona basal del perforatori, es troba l'anell electrodens de l'acrosoma. El perforatori conté material granular, composat parcialment per actina. El citoplasma presenta un centríol sota l'acrosoma. Sota la zona apical, el citoplasma forma un anell que envolta l'acrosoma i conté el complex d'estructures i orgànuls (abreviat complex SO, de l'anglès *structures and organelles*), que està format per un sistema de membranes, mitocondris amb poques crestes i poc desenvolupades, i microtúbuls. El centríol i el complex SO reaccionen intensament als anticossos anti-β-tubulina. El nucli conté cromatina poc condensada que s'estén pels braços laterals, en els quals no s'observen microtúbuls. La cromatina està agregada en fibres en algunes regions del nucli, associades generalment al complex SO. L'embolcall nuclear s'uneix amb la membrana plasmàtica donant lloc a una estructura pentalaminar i electrodensa que delimita una gran part de la superfície de l'espermatozoide.

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Sperm Ultrastructure of the Spider Crab *Maja brachydactyla* (Decapoda: Brachyura)

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ABSTRACT This study describes the morphology of the sperm cell of *Maja brachydactyla*, with emphasis on localizing actin and tubulin. The spermatozoon of *M. brachydactyla* is similar in appearance and organization to other brachyuran spermatozoa. The spermatozoon is a globular cell composed of a central acrosome, which is surrounded by a thin layer of cytoplasm and a cup-shaped nucleus with four radiating lateral arms. The acrosome is a subspheroidal vesicle composed of three concentric zones surrounded by a capsule. The acrosome is apically covered by an operculum. The perforatorium penetrates the center of the acrosome and has granular material partially composed of actin. The cytoplasm contains one centriole in the subacrosomal region. A cytoplasmic ring encircles the acrosome in the subapical region of the cell and contains the structures-organelles complex (SO-complex), which is composed of a membrane system, mitochondria with few cristae, and microtubules. In the nucleus, slightly condensed chromatin extends along the lateral arms, in which no microtubules have been observed. Chromatin fibers aggregate in certain areas and are often associated with the SO-complex. During the acrosomal reaction, the acrosome could provide support for the penetration of the sperm nucleus, the SO-complex could serve as an anchor point for chromatin, and the lateral arms could play an important role triggering the acrosomal reaction, while slightly decondensed chromatin may be necessary for the deformation of the nucleus. J. Morphol. 271:407–417, 2010.

KEY WORDS: *Maja brachydactyla*; spermatozoa; morphology; acrosome; microtubules; chromatin

INTRODUCTION

The spermatozoon of Brachyura is generally described as a nonflagellated cell with a globular acrosome, often surrounded by a fine layer of cytoplasm, and a nucleus located in the periphery that extends into several radiating arms (Jamieson and Tudge, 2000). Numerous ultrastructural variations have been described and used as characteristics to validate morphological and molecular-based phylogenetic studies (Jamieson, 1994). For example, the separation between the Gecarcinucidae and Potamidae families in the Potamoidea is confirmed by the presence of a middle acrosomal layer in the sperm of the Potamidae (represented by the subfamily Potamiscinae by Klaus et al., 2009). Similarly, the differences observed between the spermatozoa of *Macropodia longirostris* (Jamieson et al., 1998) and *Inachus phalangium* (Rorandelli et al., 2008) and the rest of Majoidea, including the spider crab *Maja brachydactyla*, support the existence of a taxonomic unit within the Inachinae, as suggested by larval studies (see discussion of Rorandelli et al., 2008).

Apart from its phylogenetic importance, the study of the morphology and composition of brachyuran spermatozoa can contribute to our understanding of the complex mechanisms of gamete fertilization in these animals. Several authors have described that during the first phases of gamete contact, the acrosome undergoes an eversion (Fasten, 1921; Hinsch, 1971; Goudeau, 1982; Nanshan and Luzheng, 1987; Krol et al., 1992; Medina and Rodríguez, 1992a), initiating the motion of the sperm nucleus toward the oocyte. In some studies, the presence of actin in the perforatorium suggests that polymerization of actin could have an active role in the acrosomal eversion (Hernandez et al., 1989). In addition, actin has been found in the lateral arms of the nucleus, and myosin occupies the basal portion of the lateral arms (Perez et al., 1986; Hernandez et al., 1989).

The consistency and composition of the nucleus are also important characteristics. Chromatin in

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the sperm nucleus of most brachyurans appears decondensed and fluid-like (Brown, 1966; Langreth 1969; Hinsch 1969, 1986; Medina and Rodríguez, 1992b). These properties are necessary in nuclei that must undergo mechanic deformation to enter the oocyte (Talbot and Chanmanon, 1980; Goudeau, 1982). Despite its malleability, the chromatin must also possess a minimum consistency to penetrate the oocyte without causing DNA breakage. In this regard, the chromatin close to the acrosome in *I. phalangium* is more electron dense (greater condensation according to Rorandelli et al., 2008) than the chromatin located in the nuclear periphery. In this case, the dense inner chromatin could better resist the mechanical traction exerted during the acrosomal eversion.

Here, we complement the study of spermiogenesis of *M. brachydactyla* (Simeó et al., in press) by describing the morphology of the spermatozoon of this species and the distribution of actin and tubulin.

MATERIALS AND METHODS

Animals

Mature male specimens of *M. brachydactyla* Balss, 1922 were captured in Galicia, Northwest Spain, by artisanal coastal fishery using gillnets between November 2006 and July 2007. Each specimen was anesthetized on ice for at least 10 min, until they did not respond to external stimuli, heart was dissected causing the death of the animal, and testis and vas deferens were then removed. The experimental procedure conforms to the current animal protection regulations (86/609/CEE, RD 1201/2005, and D 214/1997).

Transmission Electron Microscopy

Small pieces of testes and vas deferens were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (0.1 mol L^{-1}, pH 7.4) for 24 h at 4°C. Samples were rinsed in cacodylate buffer (3 times for 10 min and 3 times for 30 min) and postfixed for 1 h and 30 min at 4°C in 1% osmium tetroxide in cacodylate buffer. The samples were then rinsed in cacodylate buffer twice for 5 min and once for 30 min. Fixed tissue samples were dehydrated through graded series of acetone and embedded in Spurr's resin. Ultrathin sections were made using a Leica UCT ultramicrotome and counterstained with uranyl acetate and lead citrate. Observations were made with a Jeol EM-1010 transmission electron microscope at 80 kV. Cellular measurements were made using an image analyzing system (AnalySIS, SIS).

Scanning Electron Microscopy

Fresh testis and vas deferens tissues were dissected in phosphate-buffered saline (PBS), and cell suspensions were placed on glass slides pretreated with poly-L-lysine. Tissue samples were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (0.1 mol L^{-1}, pH 7.4) for 24 h at 4°C. Samples were rinsed in cacodylate buffer (3 times for 10 min and 3 times for 30 min) and postfixed for 1 h and 30 min at 4°C in 1% osmium tetroxide in cacodylate buffer. Then, the samples were rinsed in cacodylate buffer twice for 5 min and once for 30 min. Progressive dehydration of fixed tissue samples was done in an ascending ethanol series. After dehydration, samples were critical point dried and sputter coated with gold–palladium. Observations were made with a Hitachi S-2300 scanning electron microscope at 10-15 kV.

Confocal Immunomicroscopy and Epifluorescence Microscopy

Confocal immunofluorescence was performed based on the technique used by Tudge et al. (1994) with some variations. Fresh testis and vas deferens tissues were dissected in PBS, and cell suspensions were placed on glass slides pretreated with poly-L-lysine, air dried for 2 h, and refrigerated overnight. The slides were then fixed in 3% paraformaldehyde, 60 mmol L^{-1} glucose in phosphate buffer (0.1 mol L^{-1}, pH 7.4) for 20 min at 4°C, washed three times in PBS for 15 min each, and blocked with PBS containing 1% bovine serum albumin for 30 min using a humid chamber. The slides were then incubated overnight at 4°C in the humid chamber with anti-β-tubulin antibody (Amersham Biosciences, Munich, Germany) diluted 1:25 in the same solution used for blocking. Next day, samples were washed several times with PBS containing 0.1% Tween-20, followed by a wash with PBS. The samples were then incubated for 1 h in a humid chamber with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Molecular probes, Camarillo, CA) diluted 1:500 in PBS containing 0.1% Tween-20 and 1% bovine serum albumin. After the secondary antibody incubation, the slides were treated for 20 min with rodaminated phalloidin (Sigma, St. Louis, MO) to highlight actin, and for 5 min with 1% bisbenzimide (Hoechst 33258) in PBS to stain nuclear material. Finally, the slides were washed in PBS, mounted with Immunofluore mounting media (MP Biomedicals, Heidelberg, Germany), and dried overnight at 4°C before observation with a Leica DMRD confocal fluorescence microscope.

RESULTS

General Description of the Spermatozoa

Mature sperm cells are packed into spermatophores in the median vas deferens. The spermatophores of *M. brachydactyla* vary in size and contain between a few and several hundreds of sperm cells, which are separated by an intercellular matrix (Fig. 1A). The mature spermatozoon of *M. brachydactyla* is a star-shaped cell, with a globular body approximately 5 μm long and 7 μm wide and four radiating arms (Fig. 1B). The sperm cell is composed of a complex acrosome, a thin layer of cytoplasm, and a cup-shaped nucleus. The acrosome is the most voluminous structure of the spermatozoon and appears as an electron-dense body that fills the center of the sperm cell (Fig. 1A). The cytoplasm is located between the acrosome and the nucleus. The nucleus is positioned in the periphery of the cell and extends throughout the radiating lateral arms. Both, the acrosome, except in the apical region, and the cytoplasm are surrounded by the nucleus (Fig. 1B).

Acrosome

The acrosome is a subspheroidal, complex vesicle that measures around 4 μm in length and 5 μm in width. It consists of three concentric layers of different electron densities (external, intermediate, and internal acrosomal layers), a central perforatorium, and an operculum in the apical region (Fig.

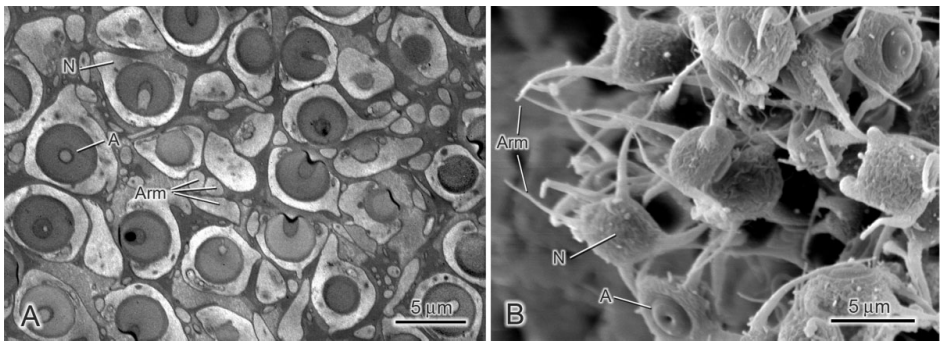

Fig. 1. Partial view of a spermatophore located in the median vas deferens of *Maja brachydactyla* containing ripe spermatozoa. (**A**) Transmission electron micrograph, (**B**) scanning electron micrograph. A, acrosome; Arm, lateral or nuclear arms; N, nucleus.

2A). The internal acrosomal layer is the most electron dense of the three concentric layers, and the intermediate layer is the least electron dense (Fig. 2A,B). A capsule envelopes the external acrosomal layer (Fig. 3C). The capsule is a thin (35 ± 5 nm) lightly electron-dense layer limited by the acrosomal membrane, which is in close association with the nuclear envelope.

The operculum is the most electron-dense structure of the spermatozoon and is centrally depressed (Fig. 2A). In fully mature spermatozoa, the operculum shows a small protuberance in its center (Fig. 4D).

The perforatorium is a central rhomboidal column that crosses the acrosome up to the operculum following the anterior-posterior axis of the cell (Fig. 2A). The basal region of the perforatorium is separated from the cytoplasm by a discontinuous membrane (Fig. 3B). In this region, the thickened ring, an electron-dense band of the acrosome associated with the base of the perforatorium, is also observed (Figs. 2A and 3B). The perforatorium contains granular material in the upper half and small, granular, rod-shaped matter in the basal area (Figs. 2A,C and 3A,B). A positive reaction with rodaminated phalloidin indicates that actin forms part of this material (Fig. 5B).

Cytoplasm

The cytoplasm is a sparse, thin layer between the acrosome and the nucleus and is only noticeable below the perforatorium and around the subapical region of the spermatozoon (Fig. 2A). Only in these regions, the cytoplasm contains the organelles (Fig. 3A). In the basal region of the perforatorium, one centriole is found (Fig. 3A,B). In the subapical region of the spermatozoon, the cytoplasm forms a cytoplasmic ring (Figs. 2C and 4D), which surrounds the operculum and contains a circular aggregate of structures and organelles called the structures-organelles complex (SO-complex), (Figs. 2A,B and 3A). The SO-complex is composed of a membrane system that wraps around an accumulation of microtubules and some mitochondria with few cristae (Fig. 3C-E). Both the centriole and the SO-complex intensely react with anti-β-tubulin antibody (Fig. 5B).

Nucleus

The sperm nucleus of *M. brachydactyla* is a cup-shaped structure with four lateral arms that surrounds the acrosome, with the exception of the operculum (Figs. 2A,C and 4C). The nucleus contains decondensed chromatin that, here, is organized into fibers of 10 nm (Fig. 3E,F). The chromatin fibers extend along the lateral arms (Fig. 3F), as confirmed by their reaction with Hoechst fluorescent dye (Fig. 5B). Occasionally, the chromatin fibers are aggregated in electron-dense areas throughout the nucleus, some of them appearing close to the SO-complex (Fig. 3D-F). The absence of reaction with either rodaminated phalloidin or anti-β-tubulin (Fig. 5B) indicates that no microtubules or any other cellular cytoskeleton components are found in the lateral arms.

The lateral arms develop during the last phases of spermiogenesis. While immature spermatozoa obtained from the transformation zone of the seminiferous tubule contain poorly developed or no lateral arms (Fig. 4A,B), the nucleus of the mature spermatozoa found in the evacuation zone already presents the four, well-developed lateral arms (Fig. 4C). Each lateral arm is approximately 9 µm long (Fig. 4C). Occasionally, the spermatozoa present only three lateral arms (Fig. 5A). In all spermatozoa observed, morphogenesis of the lateral arms, both in position and in length, is remarkably regular.

The nuclear envelope appears as a thick, electron-dense wall (Fig. 3F). However, it presents some discontinuities along the inner edge, where the nuclear envelope is in contact with the acrosome (white arrows in Fig. 3E).

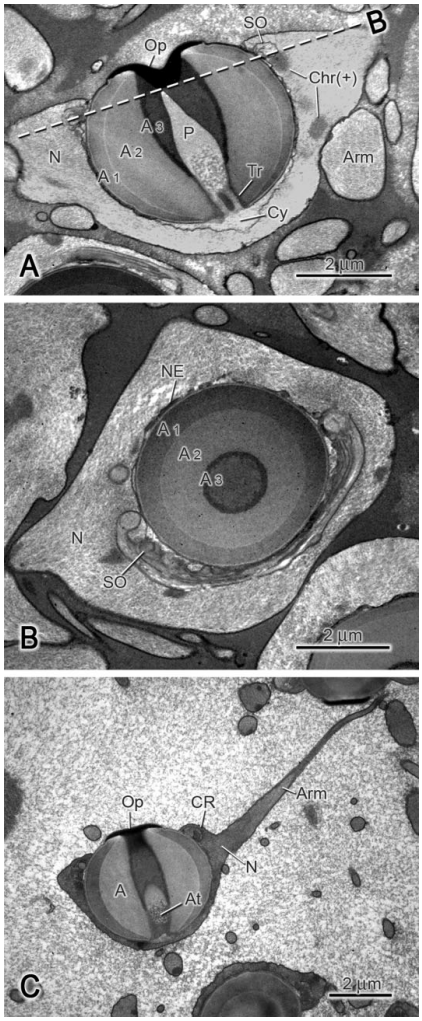

Fig. 2. Transmission electron micrographs of the spermatozoa of *Maja brachydactyla* contained within the spermatophore in the median vas deferens (A, B) and contained within the evacuation zone of the seminiferous tubule in the testis (C). **A**, radial section. **B**, cross section made approximately at the level indicated in A (see dashed line). **C**, radial section. A, acrosome; A1, external acrosomal layer; A2, intermediate acrosomal layer; A3, internal acrosomal layer; Arm, lateral or nuclear arms; At, actin; Chr(+), more densely arranged chromatin; CR, cytoplasmic ring; Cy, cytoplasm; N, nucleus; NE, nuclear envelope; Op, operculum; P, perforatorium; SO, structures-organelles complex; Tr, thickened ring.

DISCUSSION

Terminological Considerations

The different structures of the spermatozoon have been termed according to the most accepted denominations in the literature (Table 1), with the exception of the SO-complex. Although the denominations acrosome, perforatorium, and operculum, including their derivatives, are widely accepted, the terminology of the complex composed of the membrane system, mitochondria, and occasionally the microtubules that encircle the acrosome is highly variable. We believe that previous denominations of this complex are not appropriate because they place too much emphasis on the membrane system but mask the presence of mitochondria and microtubules. Therefore, we propose the novel term SO-complex, in which structures refer to the membrane systems and microtubules, whereas organelles refer to mitochondria. Although the role of the complex is not yet clear, bringing together the different components of the complex under the generic term SO-complex reflects the close relationship observed between the three components during spermiogenesis in *M. brachydactyla* (Simeó et al., in press). In addition, using a generic name may facilitate the morphological descriptions of the brachyuran spermatozoa, addressing the differences of the development of the components between species, as well as further comparisons for taxonomical studies. We are aware that the denomination SO-complex is based on morphological observations, and a more proper term could be found in the future, as more information about its development and function becomes available.

Sperm Morphology of *M. brachydactyla*

The spermatozoon of *M. brachydactyla* exhibits the appearance and general organization of the brachyuran spermatozoon (Jamieson, 1994). The sperm cell is nonflagellated and is composed of a central spheroidal acrosome, surrounded by a thin layer of cytoplasm, and a cup-shaped nucleus with four lateral arms. The spermatozoon shares most of the traits of the spermatozoa of the Majoidea superfamily, such as the broad and centrally depressed operculum, the rhomboidal and short perforatorium, the concentric zonation of the acrosome, and the presence of centrioles (Jamieson et al., 1998; Jamieson and Tudge, 2000). Other characters observed in several Majoidea are absent: the posterior median process (Hinsch, 1969, 1973), the presence of microtubules in the lateral arms (Hinsch, 1969, 1973), and the perforation of the operculum (Jamieson, 1991; 1994; Jamieson and Tudge, 2000). We summarize the presence, absence, and the type of characters used by Jamieson (1994) for cladistic analysis in Table 2, while the relative position and structural elements of the spermatozoon components of *M. brachydactyla* are represented in Fig. 6.

The acrosome is the most prominent component of the spermatozoa in *M. brachydactyla* and follows the general pattern observed in Majoidea

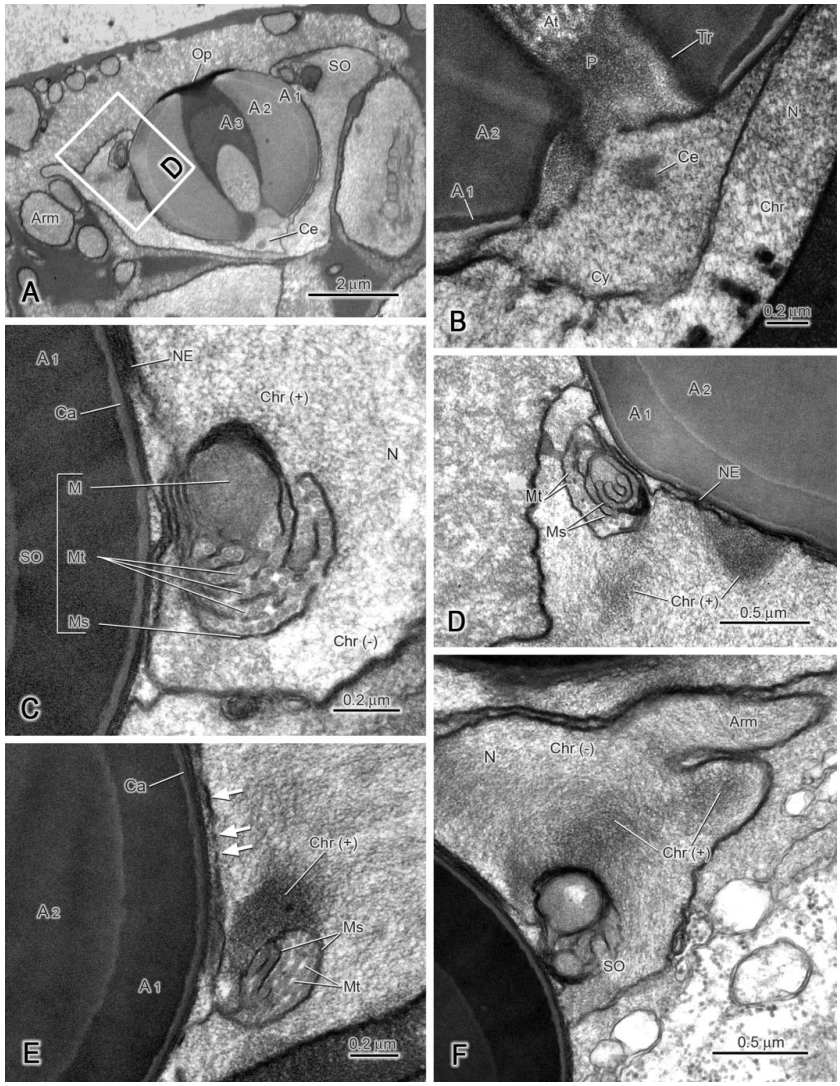

Fig. 3. Transmission electron micrographs of the nucleus and cytoplasm of the spermatozoa of *Maja brachydactyla*. **A**, radial section of the spermatozoon, demonstrating the large size and morphological complexity of the acrosome. **B**, detail of the basal area of the acrosome and the reduced area of cytoplasm where the centriole is found. **C**, transversal section of the structures-organelles complex composed of a series of membranes, microtubules, and some mitochondria. **D**, amplification of area D in image A showing the membrane system that encircles the acrosome. **E**, the SO-complex of the cytoplasm, showing its relation to the densely arranged chromatin fibers; also observe that the nuclear envelope is discontinuous (white arrows). **F**, detail of the nucleus located within the lateral arms. The chromatin appears non-condensed and organized into fibers. A1, external acrosomal layer; A2, intermediate acrosomal layer; A3, internal acrosomal layer; Arm, lateral or nuclear arms; At, actin; Ca, capsule; Ce, centriole; Chr, chromatin; Chr(+), more densely arranged chromatin; Chr(-), less densely arranged chromatin; Cy, cytoplasm; M, mitochondria; Ms, membrane system; Mt, microtubules; N, nucleus; NE, nuclear envelope; Op, operculum; P, perforatorium; SO, structures-organelles complex, Tr, thickened ring.

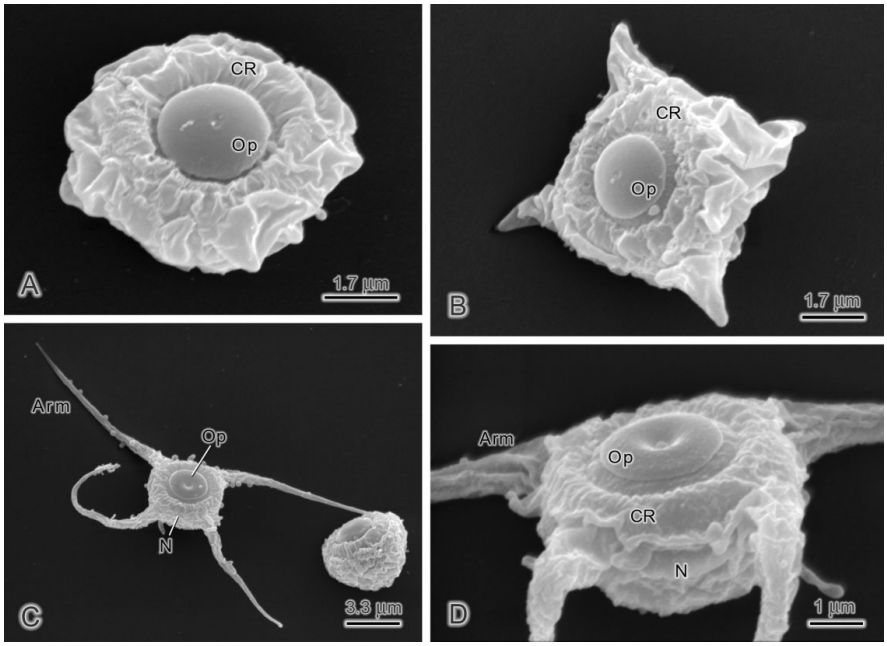

Fig. 4. Scanning electron micrographs documenting the development of the lateral arms at the end of the spermatogenesis in *Maja brachydactyla* observed in the seminiferous tubule of the testis. (**A, B**) Spermatozoon released from the transformation zone. (**C, D**) Mature spermatozoon obtained from the evacuation zone. Arm, lateral or nuclear arms; CR, cytoplasmic ring; N, nucleus; Op, operculum.

(Hinsch, 1973; Chiba et al., 1992; Jamieson et al., 1998). However, the subspheroidal shape of the acrosome contrasts with the strongly depressed acrosome of *M. longirostris* (Jamieson et al., 1998) and *I. phalangium* (Rorandelli et al., 2008). The acrosome is organized into three concentric layers of different electron densities, all surrounded by a capsule, covered apically by the operculum, and

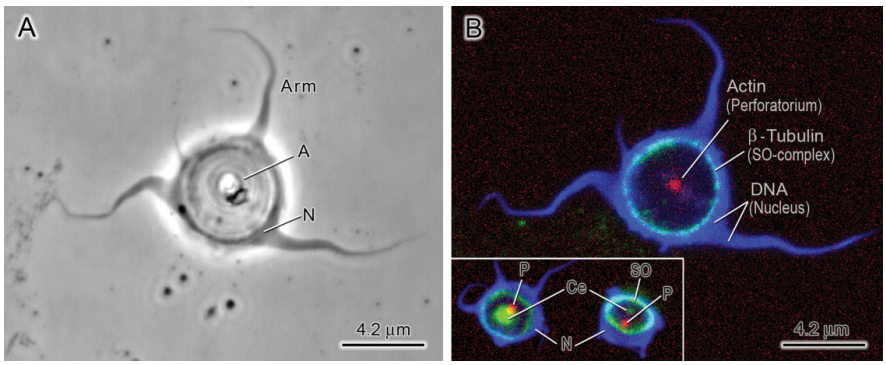

Fig. 5. Mature spermatozoon of *Maja brachydactyla*. (**A**) Phase contrast microscopy, (**B**) confocal fluorescence microscopy. The reaction of the rodaminated phalloidin (red) in the center of the cell indicates the presence of actin in the perforatorium. The anti-β-tubulin antibody (green) binds to a ring that surrounds the acrosome and corresponds to the area occupied by the structures-organelles complex and the area where the centriole(s) is located (see also Fig. 3). The DNA is labeled by Hoechst intercalating dye (blue). A, acrosome; Arm, lateral or nuclear arms; Ce, putative centriole; N, nucleus; P, perforatorium; SO, structures-organelles complex.

TABLE 1. Terminology used in the ultrastructural studies of brachyuran spermatozoa

Terminology used in this study	Terminology used in previous studies	Reference
Acrosome	Head	Yasuzumi, 1960
	Acrosomal region	Brown, 1966
	Vesicle	Pochon-Masson, 1968
	Acrosome	Langreth, 1969; Hinsch, 1969, 1973, 1986, 1988; Du et al., 1988; Jamieson, 1989, 1990, 1991, 1994; Jamieson and Tudge, 1990; Jamieson et al., 1994, 1997, 1998; El-Sherief, 1991; Medina and Rodríguez, 1992b; Li, 1995; Richer de Forges et al., 1997; Anilkumar, 1999[a]; Matos et al., 2000; Cuartas and Sousa, 2007; Benetti et al., 2008; Rorandelli et al., 2008; Klaus et al., 2009
	Capsule	Chevaillier, 1970
	Acrosomal vesicle	Reger, 1970; Wang et al, 1997, 1999
	Acrosomal body	Chiba et al., 1992
	Acrosome vesicle	Shang Guan and Li, 1994; Tudge and Justine, 1994; Tudge et al., 1994
Perforatorium	Tubule	Yasuzumi, 1960
	Acrosomal tubule	Brown, 1966; Hinsch, 1969, 1973, 1986, 1988; Du et al., 1988; El-Sherief, 1991; Chiba et al., 1992; Shang Guan and Li, 1994; Li, 1995; Wang et al., 1999
	Percutor organ	Pochon-Masson, 1968
	Acrosomal core	Langreth, 1969
	Axial rod	Chevaillier, 1970
	Cylinder-shaped invagination	Reger, 1970
	Perforatorium	Jamieson, 1989, 1990, 1991[b], 1994[b]; Jamieson and Tudge, 1990; Medina and Rodríguez, 1992b; Jamieson et al., 1994, 1997, 1998[b]; Wang et al., 1997[c]; Richer de Forges et al., 1997; Anilkumar, 1999; Cuartas and Sousa, 2007; Benetti et al., 2008; Rorandelli et al., 2008; Klaus et al., 2009[b]
	Perforatorial column	Tudge and Justine, 1994; Tudge et al., 1994
	Subacrosomal space	Matos et al., 2000
Operculum	Dense band	Yasuzumi, 1960
	Apical cap	Brown, 1966; Hinsch, 1969, 1973, 1986, 1988; Du et al., 1988; El-Sherief, 1991; Chiba et al., 1992; Shang Guan and Li, 1994; Li, 1995; Wang et al., 1997
	Opercular sphincter	Pochon-Masson, 1968
	Acrosomal cap	Langreth, 1969
	Apical granule	Chevaillier, 1970
	Electron opacities	Reger, 1970
	Operculum	Jamieson, 1989, 1990, 1991, 1994; Jamieson and Tudge, 1990; Medina and Rodríguez, 1992b; Jamieson et al., 1994, 1997, 1998; Tudge and Justine, 1994; Tudge et al., 1994; Richer de Forges et al., 1997; Anilkumar, 1999; Matos et al., 2000; Cuartas and Sousa, 2007; Benetti et al., 2008; Rorandelli et al., 2008; Klaus et al., 2009
	Acrosome cap	Wang et al., 1999
SO-complex	No denomination[d]	Jamieson, 1989, 1990, 1991, 1994; Jamieson and Tudge, 1990; Tudge and Justine, 1994; Tudge et al., 1994; Jamieson et al., 1997; Richer de Forges et al., 1997; Matos et al., 2000; Benetti et al., 2008; Rorandelli et al., 2008
	NCT complex (nucleo-chondrio-polymicrotubular)	Pochon-Masson, 1968
	Membrane complex	Langreth, 1969; Du et al., 1988; Li, 1995; Wang et al., 1999
	Lamellar region	Hinsch, 1969[e], 1973[f], 1986; El-Sherief, 1991
	Lamellar system	Jamieson et al., 1994
	Collier	Chevaillier, 1970
	Membrane remnants	Reger, 1970
	Membranous lamellar complex	Hinsch, 1988[g];Chiba et al., 1992
	Membrane lamellae	Medina and Rodríguez, 1992b
	Cytoplasmic islet	Jamieson et al., 1998

[a]Acrosome is also cited as acrosomal vesicle.
[b]In these works, the perforatorium is also cited as perforatorial chamber or column.
[c]Perforatorium is also cited as acrosomal tubule.
[d]These studies describe the presence of membranes, mitochondria, and microtubules without specific denomination.
[e]Lamellar region is also cited as central region.
[f]Lamellar region is indistinctly cited as lamellar system.
[g]Membranous lamellar complex is also cited as lamellar region.

TABLE 2. Brachyuran spermatozoon characters (Jamieson, 1994) described in Maja brachydactyla

	Spermatozoon character	*Maja brachydactyla*
1	Acrosome length/width	0.88
2	Zonation of the acrosome	Prominently concentric
3	Perforation of the operculum	Perforated, enclosed with apical button
4	Opercular projection	Absent
5	Opercular continuity with the capsule	Discontinuous with capsule
6	Operculum thickness	Moderately thick
7	Operculum width	No extremely wide
8	Periopercular rim	Absent
9	Accessory opercular ring	Absent
10	Subopercular protuberance through operculum	Absent
11	True acrosome ray zone	Absent
12	Outer acrosome zone border with peripheral zone	Absent
13	Anterolateral pole zone of acrosome	Absent
14	Flange-like peripheral extension of lower acrosome zone	Absent
15	Xanthid ring	Absent
16	Subacrosomal chamber of perforatorium	Pre-equatorial
17	Head of perforatorium	Spiked wheel
18	Configurations of wall of perforatorial chamber	Absent
19	Lateral arms (number)	Several (four)
20	Lateral arms (composition)	Nuclear only
21	Centrioles	Present
22	Posterior median process of nucleus	Absent
23	Thickened ring	Present
24	Concentric lamellation of the acrosome	Absent
25	Capsular chambers	Absent
26	Capsular projections	Absent
27	Capsular flange	Absent

centrally penetrated by the perforatorium. In addition, the thickened ring is also observed. Although the three-coat morphology and organization of the acrosome in *M. brachydactyla* is similar to that of other Majoidea, such as *Libinia* sp. (Hinsch, 1969) and *Chionoecetes opilio* (Chiba et al., 1992), it differs from the description of *M. brachydactyla* with only two separate layers given by Tudge and Justine (1994, as *Maja squinado*). The internal acrosomal layer of *M. brachydactyla* spermatozoa may be homologous to the inner acrosome zone described by Tudge and Justine (1994); however, the homology is unclear for the outer acrosome zone of Tudge and Justine (1994) and the external and intermediate acrosomal layers of *M. brachydactyla*.

The presence of actin in brachyuran spermatozoa has been reported in several species with a variable distribution (Tudge et al., 1994; Rorandelli et al., 2008). Actin in *M. brachydactyla*, as in *I. phalangium* (Rorandelli et al., 2008), is restricted to the basal region of the perforatorium,

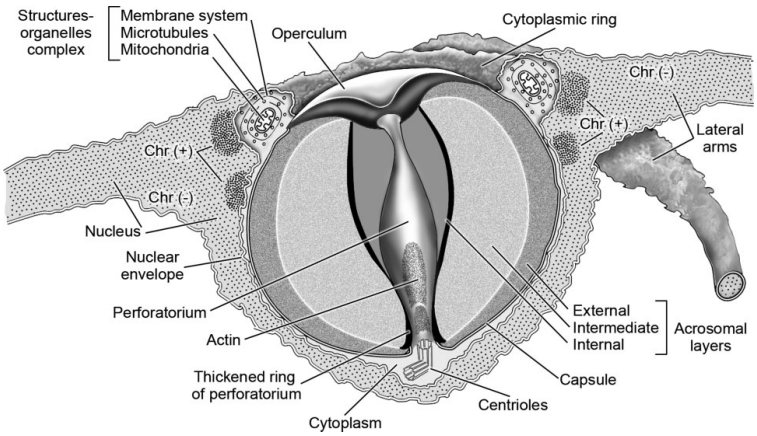

Fig. 6. Schematic reconstruction of the mature spermatozoon of spider crab, *Maja brachydactyla*.

while in *Cancer pagurus* (Tudge et al., 1994), actin is present in the perforatorium as well as in two concentric rings of the acrosome.

The highly reduced cytoplasm of *M. brachydactyla* sperm cells is more apparent in the base of the perforatorium and in the cytoplasmic ring, contrary to the conspicuous cytoplasm observed in the spermatozoa of *Jasus novaehollandiae* (Tudge et al., 1998) and *Pylocheles* sp. (Tudge et al., 2001). The base of the perforatorium contains at least one centriole, as described in *C. opilio* (Chiba et al., 1992), but we do not discard the presence of two centrioles as in other Majoidea (Jamieson et al., 1998). The cytoplasmic ring is filled with the SO-complex, which is composed of a membrane system, microtubules, and very simplified mitochondria with few cristae. The SO-complex of *Carcinus maenas* (Pochon-Masson, 1968), *Cancer* sp. (Langreth, 1969), and *Neodorippe astuta* (Jamieson and Tudge, 1990) has the same components (membranes, mitochondria, and microtubules) as *M. brachydactyla*, while the SO-complex in most brachyuran species only contains membranes and mitochondria (see SO-complex section in Table 1 for references).

The sperm nucleus of *M. brachydactyla* presents an electron-dense complex envelope, derived from the fusion of the outer edge of the plasma membrane and the nuclear envelope during spermiogenesis (Simeó et al., in press), similar to other brachyurans (Hinsch, 1988; Chiba et al., 1992). The nucleus of the sperm cell generally has four radial arms, occasionally only three situated laterally. The number of nuclear arms varies among Majoidea, showing three in some species (Hinsch, 1969; Jamieson et al., 1998), between four and 10 in *C. opilio* (Chiba et al., 1992), and five radial arms with several ventral protrusions in *I. phalangium* (Rorandelli et al., 2008). Although the lateral arms of *L. emarginata* (Hinsch, 1969) contain chromatin and microtubules, the lateral arms of *M. brachydactyla* only seem to contain chromatin; neither microtubules nor β-tubulin were observed using transmission electron microscopy or immunofluorescence microscopy. We did not observe the source for the development of the lateral arms, either during spermiogenesis (Simeó et al., in press) or in mature spermatozoa.

Chevaillier (1970) and Reger et al. (1984) demonstrated in some decapods that the morphology of the nucleus depends on the method of fixation applied. Nevertheless, our methods seem to obtain reproducible results, which we describe and discuss below. The chromatin is noncondensed and is organized into fibers of approximately 10 nm of diameter, similar to the size of a nucleosome (see the discussions by Kurtz et al., 2007; Martínez-Soler et al., 2007b). Indeed, the chromatin does not seem to be organized into superior structures such as the granules or fibers with a diameter of 20 nm or greater observed in other non-crustacean species (Gimenez-Bonafé et al., 2002; Martínez-Soler et al., 2007a; Kurtz et al., 2009b). However, the chromatin is not completely uniform throughout the nucleus, and the chromatin fibers seem to agglutinate in several more electron-dense areas, around the SO-complex and at the base of the lateral arms. We do not reject the possibility that part of the chromatin could be bound to the SO-complex through the nuclear envelope, similar to the anchorage of chromatin to the nuclear envelope, covered by microtubules, observed in other spermiogenesis (Kessel and Spaziani, 1969; Soley, 1997; Martínez-Soler et al., 2007a).

Despite the possible relation between the nuclear lateral arms and the chromatin aggregates, the causes and mechanical elements that determine the development of the four nuclear arms remain unknown. In this regard, the presence and role of the sperm nuclear matrix in the organization of the nucleus in the brachyuran spermatozoon, which has been described in the sperm of mammals (Ward and Coffey, 1990; Nadel et al., 1995; Kramer and Krawetz, 1996), should be investigated in further studies.

Origin and Function of Some Sperm Organelles

The acrosome, including the perforatorium, is the most complex organelle in the nonflagellated sperm of brachyurans and provides the necessary movements for the sperm nucleus to penetrate the oocyte envelope and reach the oocyte cytoplasm (Brown, 1966; Hinsch, 1971; Goudeau, 1982; Medina and Rodríguez, 1992a). In *M. brachydactyla*, the acrosomal layers were formed independent of each other during spermiogenesis (Simeó et al., in press), and the internal acrosomal layer and the perforatorium appear to have a coordinated self-organization in the later phases of spermiogenesis. The internal acrosomal layer originates from an electron-dense vesicle formed in the cytoplasm that later merges with the proacrosomal vesicle. Then, the perforatorium develops from an invagination of the proacrosomal vesicle simultaneously with the elongation of the electron-dense granule, which constitutes the internal acrosomal layer. These facts suggest that the composition and function of the acrosomal layers could be complementary during the acrosomal eversion.

The SO-complex is composed of a membrane system, which is derived from the degeneration of the endoplasmic reticulum and the Golgi complex, along with mitochondria with poorly developed cristae, and microtubules (Simeó et al., in press). Among other functions, the SO-complex could serve as an anchor point for the chromatin and provide the necessary mechanic stability to push the chromatin (by way of frontal traction) toward the oocyte

during the acrosomal eversion (see descriptions of the acrosomal eversion in the studies by Brown, 1966; Hinsch, 1971; Goudeau, 1982; Medina and Rodríguez, 1992a). The different degrees of chromatin condensation observed in the sperm of the Majoidea *I. phalangium* (Rorandelli et al., 2008) could be related to the nuclear traction described during the first stages of fertilization.

In brachyuran spermatozoa, the lateral arms could be involved in triggering the acrosomal reaction. Attachment of the spermatozoa to the oocytes occurs through the operculum and lateral arms and is followed by the acrosomal reaction (Brown, 1966; Hinsch, 1971; Medina, 1992). The lateral arms increase the contact surface between gametes, which may be necessary to provoke an ion transport that triggers the acrosomal reaction, as suggested by experimental activation of the acrosomal reaction using calcium ionophore treatments or rich-calcium solutions (Fasten, 1921; Nanshan and Luzheng, 1987; Medina and Rodríguez, 1992a).

Finally, poor condensation of the sperm chromatin is most likely indispensable to this type of gamete fertilization. The chromatin in the mature sperm of most non-crustacean species is highly compact because of the presence of highly basic DNA-interacting proteins, such as histones (without any post-translational modifications) or other proteins (sperm nuclear basic proteins, protamines) with a high content of arginine or lysine residues (reviewed by Kasinsky, 1989). However, the DNA in the sperm nucleus of *C. pagurus* (Kurtz et al., 2008) and *M. brachydactyla* (Kurtz et al., 2009a) is bound to hyperacetylated histones (histone H4 in *C. pagurus* and histone H3 in *M. brachydactyla*). Hyperacetylation prevents the condensation of chromatin into structures of higher order than nucleosomes (Garcia-Ramirez et al., 1995; reviewed by Zheng and Hayes, 2003; Calestagne-Morelli and Ausió, 2006), but it may also provide resistance to breakage as well as flexibility to the nucleus during the acrosomal reaction.

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LITERATURE CITED

Anilkumar G, Sudha K, Subramonian T. 1999. Spermatophore transfer and sperm structure in the brachyuran crab *Metopograpsus messor* (Decapoda: Grapsidae). J Crustacean Biol 19:361–370.

Benetti AS, Santos DC, Negreiros-Fransozo ML, Scelzo MA. 2008. Spermatozoal ultrastructure in three species of the genus *Uca* Leach, 1814 (Crustacea, Brachyura, Ocypodidae). Micron 39:337–343.

Brown GG. 1966. Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus*. J Ultrastruct Res 14:425–440.

Calestagne-Morelli A, Ausió J. 2006. Long-range histone acetylation: Biological significance, structural implications, and mechanisms. Biochem Cell Biol 84:518–527.

Chevaillier P. 1970. Recherches sur la structure et les constituants chimiques des cellules germinales mâles des crustacés décapodes [PhD thesis]. Rennes: Université de Rennes. p 322.

Chiba A, Kon T, Honma Y. 1992. Ultrastructure of the spermatozoa and spermatophores of the zuwai crab, *Chionoecetes opilio* (Majidae, Brachyura). Acta Zool 73:103–108.

Cuartas EI, Sousa LG. 2007. Ultrastructure study of the spermatophores and spermatozoa in *Uca uruguayensis* (Decapoda, Brachyura, Ocypodidae). Biociências 15:21–28.

Du N, Xue L, Lai W. 1988. Studies on the sperm of Chinese mitten-handed crab, *Eriocheir sinensis* (Crustacea, Decapoda). II. Spermatogenesis. Oceanol Limnol Sinica 19:71–75.

El-Sherief SS. 1991. Fine structure of the sperm and spermatophores of *Portunus pelagicus* (L.) (Decapoda, Brachyura). Crustaceana 61:271–279.

Fasten N. 1921. The explosion of the spermatozoa of the crab *Lophopanopeus bellus* (Stimpson) Rathbun. Biol Bull 41:288–301.

Garcia-Ramirez M, Rocchini C, Ausió J. 1995. Modulation of chromatin folding by histone acetylation. J Biol Chem 270:17923–17928.

Gimenez-Bonafé P, Ribes E, Sautière P, Gonzalez A, Kasinsky H, Kouach M, Sautière P-E, Ausió J, Chiva M. 2002. Chromatin condensation, cysteine-rich protamine, and establishment of disulphide interprotamine bonds during spermiogenesis of *Eledone cirrhosa* (Cephalopoda). Eur J Cell Biol 81:341–349.

Goudeau M. 1982. Fertilization in a crab. I. Early events in the ovary, and cytological aspects of the acrosome reaction and gamete contacts. Tissue Cell 14:97–111.

Hernandez W, Berry D, Baccetti B, Ahluwalia B, Murray SA, Anderson WA. 1989. Remodeling of the nucleocytoplasm as consequence of the acrosomal reaction of the spider crab sperm. J Submicrosc Cytol 21:163–186.

Hinsch GW. 1969. Microtubules in the sperm of the spider crab, *Libinia emarginata* L. J Ultrastruct Res 29:525–534.

Hinsch GW. 1971. Penetration of the oocyte envelope by spermatozoa in the spider crab. J Ultrastruct Res 35:86–97.

Hinsch GW. 1973. Sperm structure of Oxyrhyncha. Can J Zool 51:421–426.

Hinsch GW. 1986. A comparison of sperm morphologies, transfer and sperm mass storage between two species of crab, *Ovalipes ocellatus* and *Libinia emarginata*. Int J Invertebr Repr Dev 10:79–87.

Hinsch GW. 1988. Ultrastructure of the sperm and spermatophores of the golden crab *Geryon fenneri* and a closely related species, the red crab *G. quinquedens*, from the Eastern Gulf of Mexico. J Crustacean Biol 8:340–345.

Jamieson BGM. 1989. Ultrastructural comparison of the spermatozoa of *Ranina ranina* (Oxystomata) and of other crabs exemplified by *Portunus pelagicus* (Brachygnatha) (Crustacea, Brachyura). Zoomorphology 109:103–111.

Jamieson BGM. 1990. The ultrastructure of the spermatozoa of *Petalomera lateralis* (Gray) (Crustacea, Brachyura, Dromiacea) and its phylogenetic significance. Invertebr Reprod Dev 17:39–45.

Jamieson BGM. 1991. UItrastructure and phylogeny of crustacean spermatozoa. Mem Queensl Mus 31:109–142.

Jamieson BGM. 1994. Phylogeny of the Brachyura with particular reference to the Podotremata: Evidence from a review of spermatozoal ultrastructure (Crustacea, Decapoda). Philos Trans R Soc Lond B Biol Sci 345:373–393.

Jamieson BGM, Tudge CC. 1990. Dorippids are Heterotremata: Evidence from ultrastructure of the spermatozoa of *Neodorippe astuta* (Dorippidae) and *Portunus pelagicus* (Portunidae) Brachyura: Decapoda. Mar Biol 106:347–354.

Jamieson BGM, Tudge CC. 2000. Progress in male gamete ultrastructure and phylogeny. In: Adiyodi KG, Adiyodi RG,

editors. Reproductive Biology of Invertebrates. Kerala: John Wiley and Sons. pp 1–95.
Jamieson BGM, Guinot D, Richer de Forges B. 1994. Podotreme affinities of *Raninoides* sp. and *Lyreidus brevifrons*: Evidence from spermatozoal ultrastructure (Crustacea: Brachyura: Raninoidea). Mar Biol 120:239–249.
Jamieson BGM, Scheltinga DM, Richer de Forges B. 1998. An ultrastructural study of spermatozoa of the Majidae with particular reference to the aberrant spermatozoon of *Macropodia longirostris* (Crustacea, Decapoda, Brachyura). Acta Zool 79:193–206.
Jamieson BGM, Guinot D, Tudge CC, Richer de Forges B. 1997. Ultrastructure of the spermatozoa of *Corystes cassivelaunus* (Corystidae), *Platepistoma nanum* (Cancridae) and *Cancer pagurus* (Cancridae) supports recognition of the Corystoidea (Crustacea, Brachyura, Heterotremata). Helgol Meeresunters 51:83–93.
Kasinsky H. 1989. Specificity and distribution of sperm basic proteins. In: Hnilica L, Stein G, Stein J, editors. Histones and Other Basic Nuclear Proteins. Boca Raton, Florida: CRC Press. pp 73–163.
Kessel R, Spaziani E. 1969. Nuclear morphogenesis in rat spermatids. J Cell Biol 160:67a.
Klaus S, Schubart CD, Brandis D. 2009. Ultrastructure of spermatozoa and spermatophores of Old World freshwater crabs (Brachyura: Potamoidea: Gecarcinucidae, Potamidae, and Potamonautidae). J Morphol 270:175–193.
Kramer JA, Krawetz SA. 1996. Nuclear matrix interactions within the sperm genome. J Biol Chem 271:11619–11622.
Krol RM, Hawkins WE, Overstreet RM. 1992. Reproductive components. In: Harrison FW, Humes AG, editors. Microscopic Anatomy of Invertebrates. New York: Wiley-Liss, Inc. pp 295–343.
Kurtz K, Ausió J, Chiva M. 2009a. Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosome-like structures in decapod crustacean sperm nuclei previously described without SNBPs. Tissue Cell 41:334–344.
Kurtz K, Martínez-Soler F, Ausió J, Chiva M. 2007. Acetylation of histone H4 in complex structural transitions of spermiogenic chromatin. J Cell Biochem 102:1432–1441.
Kurtz K, Martínez-Soler F, Ausió J, Chiva M. 2008. Histones and nucleosomes in *Cancer* sperm (Decapod: Crustacea) previously described as lacking basic DNA-associated proteins: A new model of sperm chromatin. J Cell Biochem 105:574–584.
Kurtz K, Saperas N, Ausió J, Chiva M. 2009b. Spermiogenic nuclear protein transitions and chromatin condensation. Proposal for an ancestral model of nuclear spermiogenesis. J Exp Zool B Mol Dev Evol 312B:149–163.
Langreth SG. 1969. Spermiogenesis in *Cancer* crabs. J Cell Biol 43:575–603.
Li TW. 1995. On spermatogenesis and sperm ultrastructure of blue crab *Portunus trituberculatus* (Crustacea, Decapoda). Acta Zool Sinica 41:41–47.
Martínez-Soler F, Kurtz K, Chiva M. 2007a. Sperm nucleomorphogenesis in the cephalopod *Sepia officinalis*. Tissue Cell 39:99–108.
Martínez-Soler F, Kurtz K, Ausió J, Chiva M. 2007b. Transition of nuclear proteins and chromatin structure in spermiogenesis of *Sepia officinalis*. Mol Reprod Dev 74:360–370.
Matos E, Matos P, Corral L, Azevedo C. 2000. Ultrastructure of spermatozoon of the crab *Ucides cordatus* Linaeus (Crustacea, Decapoda) of the northern littoral of Brazil. Rev Bras Zool 17:753–756.
Medina A. 1992. Structural modifications of sperm from the fiddler crab *Uca tangeri* (Decapoda) during early stages of fertilization. J Crustacean Biol 12:610–614.
Medina A, Rodríguez A. 1992a. Structural changes in sperm from the fiddler crab, *Uca tangeri* (Crustacea, Brachyura), during the acrosome reaction. Mol Reprod Dev 33:195–201.
Medina A, Rodríguez A. 1992b. Spermiogenesis and sperm structure in the crab *Uca tangeri* (Crustacea, Brachyura), with special reference to the acrosome differentiation. Zoomorphology 111:161–165.
Nadel B, de Lara J, Finkernagel S, Ward W. 1995. Cell-specific organization of the 5S ribosomal RNA gene cluster DNA loop domains in spermatozoa and somatic cells. Biol Reprod 53:1222–1228.
Nanshan D, Luzheng X. 1987. Induction of acrosome reaction of spermatozoa in the decapoda *Eriocheir sinensis*. Chin J Oceanol Limnol 5:118–123.
Perez RA, Langford GM, Eckberg WR, Anderson WA. 1986. Contractile proteins (actin, myosin) and tubuline are revealed within DNA-containing nucleocytoplasm in mature spermatozoa of *Libinia emarginata* L. J Submicrosc Cytol 18471–480.
Pochon-Masson J. 1968. L'ultrastructure des spermatozoïdes vésicularies chez les crustacés décapodes avant et au cours de leur dévagination expérimentale. I. Brachyoures et Anomoures. Ann Sci Nat Zool 10:1–100.
Reger JF. 1970. Studies on the fine structure of spermatids and spermatozoa of the crab, *Pinnixia* sp. J Morphol 132:89–100.
Reger JF, Escaig F, Pochon-Masson J, Fitzgerald MEC. 1984. Observations on crab, *Carcinus maenas*, spermatozoa following rapid-freeze and conventional fixation techniques. J Ultrastruct Res 89:12–22.
Richer de Forges B, Jamieson BGM, Guinot D, Tudge CC. 1997. Ultrastructure of the spermatozoa of Hymenosomatidae (Crustacea: Brachyura) and the relationships of the family. Mar Biol 130:233–242.
Rorandelli R, Paoli F, Cannicci S, Mercati D, Giusti F. 2008. Characteristics and fate of the spermatozoa of *Inachus phalangium* (Decapoda, Majidae): Description of novel sperm structures and evidence for an additional mechanism of sperm competition in Brachyura. J Morphol 269:259–271.
Shang Guan B, Li SJ. 1994. On ultrastructure of the sperm of *Scylla serrata* (Crustacea, Decapoda, Brachyura). Acta Zool Sinica 40:7–11.
Simeó CG, Kurtz K, Chiva M, Ribes E, Rotllant G. Spermatogenesis of the spider crab, *Maja brachydactyla* (Decapoda: Brachyura). J Morphol (in press).
Soley J. 1997. Nuclear morphogenesis and the role of the manchette during spermiogenesis in the ostrich (*Struthio camelus*). J Anat 190:563–576.
Talbot P, Chanmanon P. 1980. Morphological features of the acrosome reaction of lobster (*Homarus*) sperm and the role of the reaction in generating forward sperm movement. J Ultrastruct Res 70:287–297.
Tudge CC, Justine JL. 1994. The cytoskeletal proteins actin and tubulin in the spermatozoa of 4 decapod crabs (Crustacea, Decapoda). Acta Zool 75:277–285.
Tudge CC, Grellier P, Justine JL. 1994. Actin in the acrosome of the spermatozoa of the crab, *Cancer pagurus* L (Decapoda, Crustacea). Mol Reprod Dev 38:178–186.
Tudge CC, Scheltinga DM, Jamieson BGM. 1998. Spermatozoal ultrastructure in the spiny lobster *Jasus novahollandiae* Holthuis, 1963 (Palinuridae, Palinura, Decapoda). J Morphol 236:117–126.
Tudge CC, Scheltinga DM, Jamieson BGM. 2001. Spermatozoal morphology in the "symmetrical" hermit crab, *Pylocheles (Bathycheles)* sp. (Crustacea, Decapoda, Anomura, Paguroidea, Pylochelidae). Zoosystema 23:117–130.
Wang L, Du N-S, Lai W. 1999. Studies on spermiogenesis of a freshwater crab *Sinopotamon yangtsekiense* (Crustacea Decapoda). Acta Hydrobiol Sinica 23:29–33.
Wang YL, Zhang ZP, Li SJ. 1997. Ultrastructure of spermatogenesis in the crab *Scylla serrata*. Acta Zool Sinica 43:249–254.
Ward WS, Coffey DS. 1990. Specific organization of genes in relation to the sperm nuclear matrix. Biochem Biophys Res Commun 173:20–25.
Yasuzumi G. 1960. Spermatogenesis in animals as revealed by electron microscopy. VII. Spermatid differentiation in the crab, *Eriocheir japonicus*. J Biophys Biochem Cytol 7:73–78.
Zheng C, Hayes JJ. 2003. Structures and interactions of the core histone tail domains. Biopolymers 68:539–546.

Article 4

Títol: L'espermatogènesi en els crancs (Crustacea, Brachyura). Un model atípic de condensació del nucli espermàtic

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Informe de la contribució del doctorand
La hipòtesi de treball i la metodologia a seguir varen estar realitzades pel Dr. Ribes, la Dra. Rotllant i el Dr. Chiva. El doctorand va participar en les dissseccions i els mostrejos dels teixits amb la col·laboració del Dr. E. Ribes, la Dra. G. Rotllant i la Dra. K. Kurtz. Les mostres foren processades pels Serveis Cientificotècnics de la Universitat de Barcelona. Les imatges de microscòpia electrònica foren realitzades pel doctorand i Dr. E. Ribes. La descripció i interpretació de les imatges, i la redacció del manuscrit foren realitzades pel doctorant amb la col·laboració dels coautors. Les aportacions realitzades per la Dra. K. Kurtz a aquest treball han estat utilitzades per a l'obtenció del grau de doctora.

Dra. Guiomar Rotllant Estelrich

Resum

En aquest treball es tracten dues qüestions de la biologia reproductiva dels braquiürs, com ara són el procés d'espermatogènesi i la naturalesa de les proteïnes associades a la cromatina dels espermatozoides utilitzant com a model dues espècies de braquiürs: la cabra de mar, *Maja brachydactyla*, i el bou de mar, *Cancer pagurus*. En aquest treball es realitza la descripció de la morfologia de l'aparell reproductor masculí de *M. brachydactyla* i de la formació dels espermatòfors que són transferits a la femella. A continuació, el procés d'espermatogènesi de *M. brachydactyla* es descriu i compara amb els estudis previs en altres espècies, amb especial atenció a la formació de l'acrosoma. La descripció dels diferents caràcters dels espermatozoides dels braquiürs es complementa amb un estudi específic sobre les proteïnes associades al nucli de l'espermatozoide *C. pagurus*. En resum, els aspectes més rellevants de l'espermatogènesi de la cabra de mar, *M. brachydactyla* són: 1) la formació de l'acrosoma associada a l'activitat del reticle endoplasmàtic i el complex de Golgi, el qual no havia estat observat en altres estudis previs; 2) la formació d'un sistema de estructures (membranes) i orgànuls (mitocondris i microtúbuls) format a partir de la degeneració del reticle endoplasmàtic i el complex de Golgi i que forma un anell al citoplasma sota la zona apical; 3) la fusió de les membranes plasmàtica i nuclear, que donen lloc a una estructura membranosa pentalaminar en algunes regions de la superfície cel·lular i, 4) el baix grau de condensació del nucli de l'espermatozoide. L'estudi de la composició i estructura de la cromatina de l'espermatozoide del bou de mar *C. pagurus* mostra, a diferència del descrit prèviament, la presència d'histones amb diferents graus d'acetilació. Degut a que l'acetilació de les histones disminueix la interacció electrostàtica entre aquestes i el DNA, la cromatina no pot organitzar-se en estructures més complexes, la qual cosa donaria lloc a la aparença poc electrodensa del nucli dels braquiürs. A més, la baixa proporció proteïna/DNA observada indica que entre un 40-50% del DNA del nucli de l'espermatozoide no està associat a histones. El procés especial de la fertilització dels gàmetes dels crustacis decàpodes braquiürs posa una sèrie de restriccions estructurals/funcionals en els nuclis espermàtics. És molt possible que entre els crustacis hagin aparegut diferents solucions evolutives a tals restriccions i que no tots els nuclis dels espermatozoides dels crancs tinguin una composició igual a la de *C. pagurus*. No obstant, la composició d'aquesta cromatina (DNA organitzat en nucleosomes però no en estructures d'ordre superior degut a l'acetilació de la histona H4; i regions amplies del DNA lliure d'histones) permet comprendre, almenys parcialment, perquè el nucli és deformable i suficientment fluid com per a poder fecundar l'oòcit.

Biologia de la reproducció
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L'ESPERMATOGÈNESI EN ELS CRANCS (CRUSTACEA, BRACHYURA). UN MODEL ATÍPIC DE CONDENSACIÓ DEL NUCLI ESPERMÀTIC

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RESUM

Els espermatozoides dels crustacis decàpodes es caracteritzen per tenir un nucli amb cromatina poc condensada, un acrosoma complex i un citoplasma reduït sense flagel. Es disposa d'una àmplia informació sobre la ultraestructura i variabilitat morfològica dels espermatozoides dels crustacis decàpodes i particularment dels braquiürs, ja que la seva especificitat ha permès utilitzar-los com a caràcter filogenètic, però el procés d'espermatogènesi i la naturalesa de les proteïnes associades a la cromatina dels espermatozoides són qüestions encara no resoltes. En aquest treball hem tractat aquestes dues qüestions utilitzant com a model dues espècies de braquiürs: la cabra de mar, *Maja brachydactyla*, i el bou de mar, *Cancer pagurus*. D'aquesta manera, hem realitzat una breu descripció de la morfologia de l'aparell reproductor masculí de *M. brachydactyla*, hem localitzat el lloc on es desenvolupa l'espermatogènesi i hem descrit la formació dels espermatòfors que són transferits a la femella. A continuació, hem comparat el procés d'espermatogènesi de *M. brachydactyla*, amb els estudis fets en altres espècies, considerant de manera preferent la formació de l'acrosoma. Finalment, hem descrit diferents aspectes del nucli dels espermatozoides dels braquiürs, fent un estudi específic de les proteïnes associades al nucli de l'espermatozoide de *C. pagurus*.

Paraules clau: aparell reproductor masculí, espermatogènesi, espermatozoide, cromatina, Brachyura.

SPERMATOGENESIS IN CRABS (CRUSTACEA: BRACHYURA). AN ATYPICAL CONDENSATION MODEL OF THE SPERMATIC NUCLEI

SUMMARY

Decapods crustacean spermatozoa are characterized by a nucleus containing a low condensed chromatin, a complex acrosome and reduced cytoplasm lacking of flagellum. Large information on ultrastructure and morphological variability of the spermatozoa is available in crustacean particularly, in Brachyuran, since their specificity has been used as a phylogenetic character. However, the spermatogenesis and the nature of the proteins associated to chromatin in the nucleus of the spermatozoa are still unclear. In the present study, we have dealt both topics using two brachyuran species as model: the spider crab, *Maja brachydactyla* and the edible crab, *Cancer pagurus*. Thus, we have briefly described the morphology of the male reproductive system of *M. brachydactyla* in order to locate the spermatogenesis process and to describe the formation of the spermatophore transferred to the female. Finally, we have also described and compared with previous studies, the spermatogenesis process of *M. brachydactyla* with special attention to the acrosomal vesicle. We have finished our study describing the different characters presented in the nucleus of the spermatozoa, with special reference to the proteins associated to chromatin in *C. pagurus.*

Key words: male reproductive system, spermatogenesis, spermatozoa, chromatin, Brachyura.

INTRODUCCIÓ

A diferència d'altres grups taxonòmics, els crustacis decàpodes presenten un espermatozoide caracteritzat per la falta de flagel, un nucli amb una cromatina laxa i poc condensada i un acrosoma complex de gran varietat morfològica (Felgenhauer i Abele, 1991). La morfologia de l'espermatozoide dels crustacis decàpodes va despertar gran interès, i va donar lloc lloc a les primeres descripcions de l'espermatogènesi dels braquiürs (Binford, 1913; Fasten, 1918, 1924, 1926; Nath, 1932; Estampador, 1949). La utilització de la microscòpia electrònica va permetre observar la ultraestructura dels diferents components de l'espermatozoide (Yasuzumi, 1960; Langreth, 1969; Reger, 1970; Medina i Rodríguez, 1992b), i va mostrar una gran diversitat morfològica en els espermatozoides dels crustacis decàpodes, la qual cosa ha estat utilitzada per dur a terme reconstruccions filogenètiques, especialment en els braquiürs (Jamieson, 1991*a*, 1994*b*; Jamieson *et al*., 1995). Tanmateix, la utilització de la microscòpia electrònica va demostrar que la cromatina d'aquests espermatozoides era poc electrodensa, i això indicava una falta de compactació del DNA i va obrir les portes a l'estudi de les proteïnes associades a la cromatina en l'espermatozoide (Chevaillier, 1966*a*, 1967, 1968; Vaughn i Locy, 1969; Vaughn i Hinsch, 1972; Vaughn i Thomson, 1972).

Els estudis ultraestructurals de l'espermatogènesi van anar perdent interès al voltant de l'any 1970, i alguns aspectes, com ara l'origen de l'acrosoma, el sistema de membranes de l'espermatozoide, la baixa condensació de la cromatina i la naturale-

sa de les proteïnes associades, van quedar sense resoldre. En el present treball tractem el procés de l'espermatogènesi i l'estudi de les proteïnes associades a la cromatina dels nuclis dels espermatozoides.

L'APARELL REPRODUCTOR DELS BRAQUIÜRS.

La cabra de mar, *Maja brachydactyla*

L'absència del flagel és un dels aspectes més característics en els espermatozoides dels crustacis decàpodes (Felgenhauer i Abele, 1991); per tant, els espermatozoides resten immòbils o tenen una mobilitat reduïda, i és per això que els crustacis decàpodes han desenvolupat diferents estratègies reproductives, que assoleixen la màxima complexitat en els braquiürs. Els mascles dels crustacis decàpodes transfereixen els espermatozoides en estructures anomenades *espermatòfors*, que en els braquiürs estan formats per espermatozoides envoltats per una capa de material amorf, envoltats de grans quantitats de fluids seminals (Hinsch, 1991; Subramonian, 1991). Els espermatòfors i els fluids seminals dels braquiürs són injectats mitjançant el primer parell de pleopodis modificats per a la funció copuladora (gonopodis) als receptacles seminals de la femella, uns allargaments de l'aparell reproductor connectats als ovaris per uns oviductes (Bauer, 1986; Rotllant *et al*., 2007). La fecundació és interna i es produeix aprofitant el pas dels oòcits per una cambra dels receptacles seminals de la femella abans de ser alliberats pels gonopors i adherits als pleopodis (Diesel, 1991).

L'aparell reproductor masculí dels braquiürs consta de dos testicles, dos vasos deferents i dos conductes ejaculadors situats sobre l'hepatopàncrees (vegeu la figura 1A). Els testicles es localitzen en la part anterior del cefalotòrax, i s'estenen anteriorment i lateralment en forma de banyes. En algunes espècies, ambdós testicles estan units per una comissura localitzada entre l'estómac i el cor (Mouchet, 1931). Els braquiürs presenten dos tipus de testicles (Nagao i Munehara, 2003): els de forma lobular, que també es troben en la resta de decàpodes, i estan formats per uns acinis o lòbuls connectats per un conducte col·lector, i els de forma tubular, exclusius dels braquiürs, que estan formats per un únic tub seminífer molt recaragolat. Malgrat les diferents morfologies, la fisiologia del testicle sembla ser equivalent en ambdós casos. Les espermatogònies es troben a la perifèria del lòbul o del tub seminífer i els diferents estadis de l'espermatogènesi ocupen l'espai central. A l'extrem oposat de les espermatogònies es troba generalment la zona col·lectora testicular, que transporta els espermatozoides al conducte deferent. En el cas de *M. brachydactyla*, els testicles s'estenen descrivint un arc des de la base de l'epipodi del maxil·lípede fins a la part anterior del cor. Cada testicle està format per un únic tub seminífer altament recaragolat, i per això ha estat classificat com a testicle tubular (Simeó *et al*., en revisió) (vegeu la figura 1 B). El tub seminífer de *M. brachydactyla* es troba organitzat en tres zones: germinal, de transformació i d'evacuació (Simeó *et al*., en revisió) (vegeu la figura 1 C). La zona germinal està localitzada en un pol del tub, i conté espermatogònies i cèl·lules accessòries. Aquesta zona és una fina capa durant la major part de l'espermatogènesi i augmenta de mida en el moment en què la zona de transformació presenta espermatozoides. La zona de transformació ocupa la zona central i és on es produeix la maduració dels espermatòcits fins arribar als espermatozoides. És en aquesta zona on les cèl·lules gamètiques es troben acompanyades per prominents cèl·lules accessòries

(vegeu la figura 1 C). La zona d'evacuació, al pol oposat al de la zona germinal, conté exclusivament els espermatozoides produïts a la zona de transformació i els trasllada fins al conducte deferent, on són empaquetats en espermatòfors.

El conducte deferent dels braquiürs està situat a la part posterior del cefalotòrax, i es divideix en tres regions: anterior, mitjana i posterior, d'acord amb criteris morfològics i funcionals (Krol *et al*., 1992). El conducte deferent anterior és un tub on es produeix la formació de l'espermatòfor mitjançant les secrecions de l'epiteli de la paret del conducte. Les regions mitjana i posterior del conducte deferent són els llocs d'emmagatzematge dels espermatòfors i de la producció dels fluids seminals que els engloben durant l'ejaculació (Diesel, 1991). Aquestes dues regions presenten expansions laterals o diverticles que augmenten la superfície de secreció i el volum d'emmagatzematge, i assoleixen mides considerables (Mouchet, 1931; Adiyodi i Anilkumar, 1988).

En *M. brachydactyla,* tal com s'ha descrit en la resta de braquiürs, el conducte deferent també s'ha dividit en tres regions (Simeó *et al*., en revisió) (vegeu la figura 1

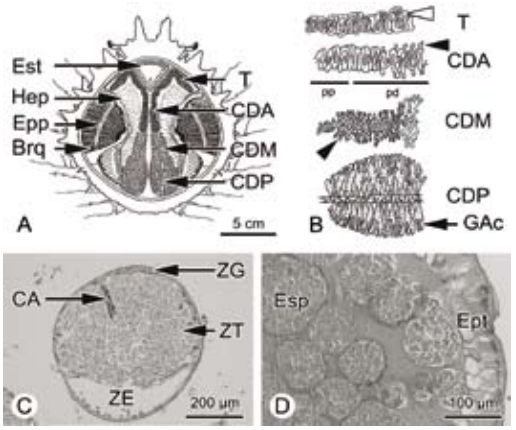

Figura 1**.** *Maja brachydactyla***.** A: Diagrama de l'anatomia interna**; e**s mostra l'aparell reproductor localitzat en el cefalotòrax. B: Diagrama que representa la morfologia externa del testicle amb un únic tub seminífer (cap de fletxa blanca) i del conducte deferent amb la presència de diverticles que van augmentant des del conducte anterior al mitjà (cap de fletxa negra); el conducte deferent posterior és un tub que té associada una glàndula accessòria formada per diverticles molt ramificats. C: Secció transversal del tub seminífer (MO, tinció de Mallory); el tub seminífer es troba dividit en tres zones: germinal, de transformació i d'evacuació. D: Porció distal del conducte deferent anterior (MO, hematoxilina i eosina); els espermatòfors ja formats es troben envoltats per les secrecions produïdes per l'epiteli. Brq: brànquies, CA: cèl·lula accessòria, CDA: conducte deferent anterior, CDM: conducte deferent mitjà, CDP: conducte deferent posterior, Epp: epipodi, Ept: epiteli, Est: estómac, GAc: glàndula accessòria, Hep: hepatopàncrees, pd: porció distal del conducte deferent anterior, pp: porció proximal del conducte deferent anterior, Esp: espermatòfor, T: testicle, ZE: zona d'evacuació, ZG: zona germinal, ZT: zona de transformació.

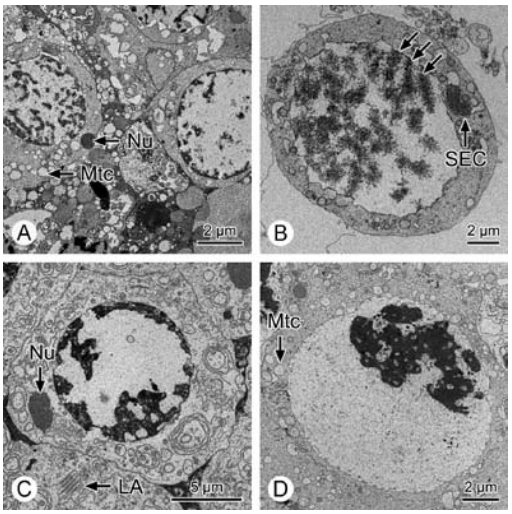

Figura 2**.** *Maja brachydactyla***.** Espermatòcits primaris (TEM)**.** A: Preleptotè (esquerra) i leptotè (dreta). La condensació dels cromosomes augmenta durant el preleptotè i el leptotè. En aquest darrer estadi, el citoplasma conté pocs mitocondris i *nuage*. B: Paquitè. S'observen complexes sinaptonemals (fletxes) en el nucli i la disposició concèntrica del sistema d'endomembranes. C: Diplotè. El sistema d'endomembranes es troba molt desenvolupat i s'observen làmines anellades. El *nuage* assoleix la màxima mida en aquest estadi. D: Diacinesi. Es pot veure una reducció del sistema d'endomembranes. LA: làmines anellades, Mtc: mitocondri, Nu: nuage, SEC: sistema d'endomembranes concèntric.

B). El conducte deferent anterior és un tub doblegat dividit en dues porcions: proximal i distal; la porció proximal, propera al testicle, és un tub llis, mentre que la distal presenta uns petits diverticles aïllats (vegeu la figura 1 B). Al llarg del conducte deferent anterior es produeix la formació de l'espermatòfor, de manera que els espermatozoides provinents dels testicles s'agrupen en petites masses mitjançant dues secrecions de l'epiteli secretor de la paret del conducte deferent (vegeu la figura 1 D). El conducte deferent mitjà és un tub enrotllat helicoïdalment que presenta un gran nombre de diverticles localitzats en un pol del tub (vegeu la figura 1 B); tant a la llum d'aquest conducte com als diverticles es troben grans quantitats d'espermatòfors envoltats per noves secrecions elaborades per l'epiteli d'aquesta regió del conducte (vegeu la figura 1 D). El conducte deferent posterior té associada una gran glàndula accessòria, formada per diverticles altament ramificats que produeixen i emmagatzemen un fluid seminal secretat per l'epiteli de la seva paret (vegeu la figura 1 B), que és abocat al conducte deferent posterior i engloba als espermatòfors provinents del conducte deferent mitjà durant l'ejaculació.

El conducte ejaculador de *M. brachydactyla* està situat entre la musculatura de la coxa de la cinquena pota marxadora. En la seva paret per sota de l'epiteli mostra una capa de musculatura esquelètica molt desenvolupada, que és la responsable de l'extrusió

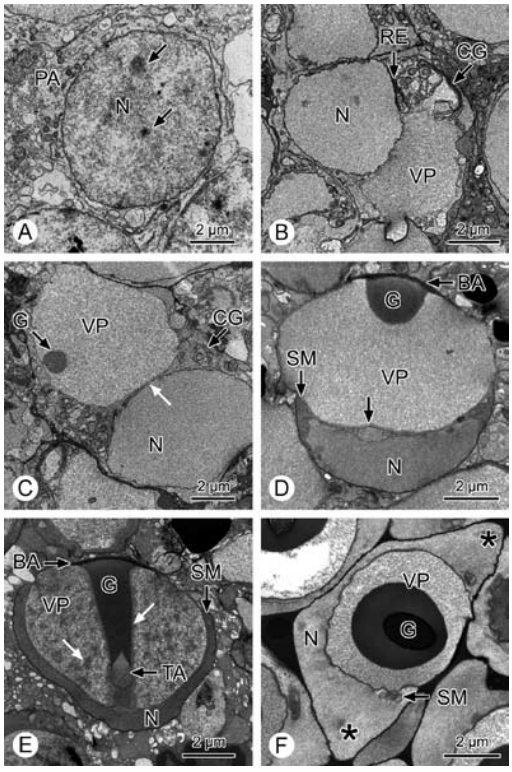

Figura 3. *Maja brachydactyla***.** Espermàtides (TEM). A: Espermàtida primerenca. El nucli es troba desplaçat del centre de la cèl·lula i presenta petits grumolls de cromatina condensada (fletxes), mentre que el citoplasma ho fa en sentit contrari. B: Espermàtida intermèdia. La cromatina s'ha descondensat i mostra un aspecte granular. Al citoplasma, el sistema d'endomembranes s'ha desenvolupat i diferenciat en reticle endoplasmàtic i complex de Golgi, l'activitat dels quals dóna lloc a la vesícula proacrosòmica. C: Espermàtida intermèdia. En una fase més avançada, apareix un gránul electrodens a l'interior de la vesícula proacrosòmica. La fletxa blanca indica la zona on es produeix el trencament de l'embocall nuclear. D: Espermàtida madura. El nucli presenta una forma de mitja lluna i el citoplasma reduït conté un sistema de membranes. La vesícula proacrosòmica mostra un opercle sobre el gránul electrodens en posició apical. A la base de la vesícula observem una capa de material granular a la zona on es formarà el tub acrosòmic (fletxa). E: Espermàtida madura. El nucli s'estén envoltant la vesícula proacrosòmica. Els components de la vesícula es condensen en petits grumolls (fletxes blanques) que s'organitzen al voltant del tub acrosòmic. F: Espermàtida madura. El nucli s'expandeix, desenvolupant les prolongacions laterals o *lateral arm* (asteriscs). Els components de la vesícula proacrosòmica s'organitzen concèntricament al voltant del tub acrosòmic. BA: barret acrosòmic o opercle, CG: complex de Golgi, G: gránul de la vesícula proacrosòmica, N: nucli, PA: pol acrosòmic, RE: reticle endoplasmàtic, SM: sistema de membranes, TA: tub acrosòmic o *perforatium*, VP: vesícula proacrosòmica.

dels espermatòfors i del fluid seminal fins al gonopor (Simeó *et al*., en revisió).

L'ESPERMATOGÈNESI DELS BRAQUIÜRS

L'espermatogènesi de la cabra de mar, *Maja brachydactyla*

L'estudi de l'espermatogènesi de *M. Brachydactyla* s'ha centrat en la zona de transformació del tub seminífer, on podem trobar des dels espermatòcits primaris fins als espermatozoides madurs (Simeó *et al*., en preparació) (vegeu la figura 1 C).

Els espermatòcits primaris són cèl·lules esfèriques amb una alta relació nucli/ citoplasma (vegeu la figura 2). El nucli és esfèric i voluminós i ocupa la zona central de la cèl·lula. La cromatina presenta les diferents figures meiòtiques de la primera divisió meiòtica, com ara els complexos sinaptonemals durant el paquitè (vegeu les fletxes de la vegeu la figura 2 B). El citoplasma conté pocs mitocondris, un complex sistema d'endomembranes i el *nucleolus-like body* o *nuage*. El sistema d'endomembranes es desenvolupa progressivament al llarg dels diferents estadis, i durant el paquitè mostra una característica disposició concèntrica de les membranes amb dilatacions laterals (vegeu la figura 2 B), mentre que durant el diplotè el sistema d'endomembranes es troba altament desenvolupat i s'organitza en estructures conegudes com les làmines anellades i d'altres formacions membranoses (vegeu la figura 2 C). El *nuage* apareix com un cos electrodens que està present en pràcticament tots els estadis d'espermatòcits primaris i és especialment prominent durant el diplotè (vegeu la figura 2 A i C). Després de la diacinesi de la primera divisió meiòtica, els estadis dels espermatòcits secundaris transcorren molt ràpidament i fan difícil l'obtenció de mostres per microscòpia.

Les espermàtides primerenques són cèl·lules esfèriques, amb una relació nucli/ citoplasma alta, que mostren els primers signes de polarització. Així, el nucli es troba lleugerament desplaçat del centre de la cèl·lula en el que serà el pol nuclear de l'espermatozoide, mentre que al pol oposat, el pol acrosòmic, es desenvoluparà la vesícula acrosòmica (vegeu la figura 3 A). El nucli de l'espermàtida primerenca és esfèric i té la cromatina condensada en grumolls. Per altra banda, el citoplasma conté alguns mitocondris i un sistema de membranes poc desenvolupat. El primer canvi que es produeix durant l'espermiogènesi és la descondensació de la cromatina nuclear, que adquireix un aspecte granular homogeni (vegeu la figura 3 B). En el citoplasma, el sistema de membranes constituït pel reticle endoplasmàtic i el complex de Golgi es desenvolupa i s'organitza progressivament, de manera que el complex de Golgi produeix dos tipus de vesícules: les de baixa i les de mitjana electrodensitat, que es fusionen al pol acrosòmic i donen lloc a la vesícula proacrosòmica, que conté material granular homogèniament distribuït. Al llarg de l'espermàtida intermèdia la vesícula proacrosòmica creix paral·lelament al desenvolupament del reticle endoplasmàtic i del complex de Golgi, són desplaçats a la zona equatorial de la cèl·lula entre el nucli i la vesícula proacrosòmica, i s'observa a l'interior de la vesícula l'aparició d'un gränul electrodens (vegeu la figura 3 C). La fase d'espermàtida intermèdia finalitza amb el trencament de les membranes nuclears en la zona equatorial de la cèl·lula i la degeneració del reticle endoplasmàtic. L'espermàtida madura es mostra com una cèl·lula altament polaritzada (vegeu la figura 3 D); el nucli, en el pol nuclear, presenta forma de mitja

Taula 1. *Estudis de l'espermatogènesi en diferents espècies de braquiürs*

Superfamília	*Espècie*	*Procés*	*Tècnica*	*Autor*
Majoidea	*Maja brachydactyla*	Espermatogènesi	ME	(Simeó *et al*., en preparació(*b*))
Cancroidea	*Cancer magister*	Espermatogènesi	MO	Fasten, 1918
	Cancer productus *Cancer oregonensis* *Cancer gracilis*	Espermatogènesi	MO	Fasten, 1924
	Cancer borealis *Cancer irroratus* *Cancer magister* *Cancer productus*	Espermiogènesi	ME	Langreth, 1969
Portunoidea	*Scylla serrata* *Scylla paramamosain* *Scylla oceanica* *Scylla tranquebarica*	Espermatogènesi	MO	Estampador, 1949
Xanthoidea	*Menippe mercenaria*	Espermatogènesi	MO	Binford, 1913
Potamoidea	*Sartorina spinigera*	Espermatogènesi	MO	Nath, 1932
Pinnotheroidea	*Pinnixa sp*	Espermiogènesi	ME	Reger, 1970
Ocypodoidea	*Uca tangeri*	Espermiogènesi	ME	Medina i Rodríguez, 1992*b*
Grapsoidea	*Eriocheir sinensis*	Espermatogènesi	MO	Hoestlandt, 1948
	Eriocheir japonicus	Espermàtides finals	ME	Yasuzumi, 1960

ME: microscòpia electrònica, MO: microscòpia òptica.

lluna, i la vesícula proacrosòmica, en el pol acrosòmic, apareix com un cos esfèric i molt voluminós. En aquesta fase, la vesícula proacrosòmica experimenta una gran transformació morfològica: en la zona apical es veu una fina banda altament electrodensa que donarà lloc al barret acrosòmic o opercle (*acrosomal cap*, *operculum*), mentre que en la zona basal es forma una invaginació de la vesícula, embolcallada d'un material granular electrodens, que donarà lloc al tub acrosòmic (*acrosomal tube*, *perforatium*) (vegeu la fletxa de la figura 3 D). El citoplasma és molt reduït i es troba localitzat entre els extrems del nucli i la vesícula proacrosòmica, i conté un sistema de membranes, produït per la degeneració del reticle endoplasmàtic i el complex de Golgi, associat a mitocondris i microtúbuls. A continuació, en la fase final de l'espermiogènesi, s'observa com el component granular present a l'interior de la vesícula proacrosòmica es va condensant i origina una sèrie de capes concèntriques, de diferent electrodensitat al voltant del tub acrosòmic (vegeu les fletxes blanques de la figura 3 E). En aquesta fase també s'observa com el nucli va formant quatre prolongacions laterals (*lateral arm*) que tenen el seu interior ple de fibres de cromatina (vegeu els asteriscs de la figura 3 F).

L'espermatozoide de *M. brachydactyla* té forma esferoïdal estrellada (vegeu la figura

4 A). L'acrosoma ocupa el centre de la cèl·lula i es troba envoltat basalment i lateralment per una fina franja de citoplasma i per un nucli que ocupa una posició més perifèrica (vegeu la figura 4 B).

L'acrosoma és esfèric, en la part anterior té un opercle de forma lenticular còncava amb una petita protuberància. L'acrosoma està constituït també per tres capes: externa, intermèdia i interna (vegeu la figura 4 B). La formació de cadascuna d'aquestes tres capes té lloc independentment durant l'espermiogènesi. La capa interna que envolta el *perforatorium* s'origina a partir d'un gránul electrodens d'origen citoplasmàtic, mentre que les capes intermèdia i externa es formen a partir de la condensació seriada del contingut de la vesícula proacrosòmica (vegeu les figures 3 C, D, E i F).

El citoplasma, en l'espermatozoide madur, es troba restringit a una zona estreta en forma d'anell que envolta l'acrosoma just per sota de l'opercle; en aquesta regió del citoplasma és on s'observa un sistema de membranes i microtúbuls juntament amb un nombre reduït de petits mitocondris, que tenen una matriu electrodensa amb poques crestes (vegeu la figura 4 B). Una altra zona estreta de citoplasma es localitza entre la zona basal de l'acrosoma i el nucli, i és en aquesta on s'observen un o dos centríols.

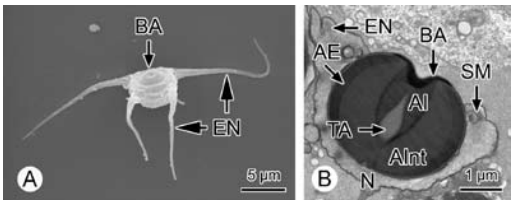

Figura 4. *Maja brachydactyla*. Espermatozoide. A: SEM. L'espermatozoide mostra una forma ovoidal amb quatre llargues prolongacions laterals. B: TEM. El nucli de l'espermatozoide conté cromatina poc condensada. A la base de les prolongacions laterals es troba el sistema de membranes. L'acrosoma és esfèric i està format per tres capes concèntriques. La capa interna envolta el tub acrosòmic. La part apical presenta l'opercle amb una depressió central. AE: capa externa de l'acrosoma, AI: capa interna de l'acrosoma, AInt: capa intermèdia de l'acrosoma, BA: barret acrosòmic o opercle, EN: prolongacions laterals del nucli, SM: sistema de membranes, TA: tub acrosòmic

El nucli s'estén pràcticament per tot el volum cel·lular, excepte en l'espai ocupat per l'acrosoma i l'estreta franja de citoplasma que l'envolta. Les membranes de l'embolcall nuclear se situen properes a la membrana citoplasmàtica i donen lloc a una estructura multimembranosa electrodensa que delimita la superfície de l'espermatozoide. La cromatina és poc condensada i mostra un aspecte fibril·lar homogeni al llarg de tot el nucli, i arriba fins i tot a ocupar l'espai interior de les quatre prolongacions de l'espermatozoide (vegeu la figura 4 B).

L'espermatogènesi en altres espècies de braquiürs

El procés d'espermatogènesi segueix un patró comú en relació al nucli, el citoplasma i el desenvolupament de la vesícula acrosòmica, malgrat la gran variabilitat morfològica dels espermatozoides dels braquiürs estudiats (vegeu la taula 1).

El fet més particular que experimenta el

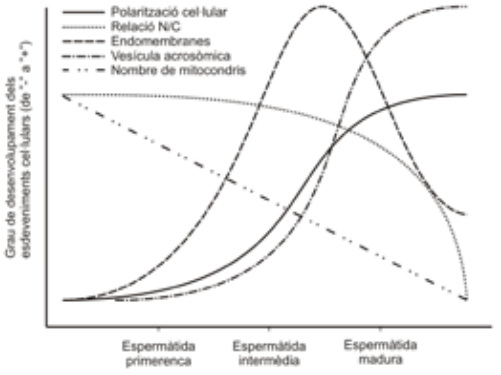

Figura 5. *Maja brachydactyla*. Síntesi dels esdeveniments cel·lulars durant el procés de maduració de les espermàtides.

nucli al llarg de l'espermatogènesi és la descondensació de la cromatina. Les espermàtides primerenques presenten una cromatina condensada en grànuls a la perifèria del nucli (Langreth, 1969; Reger, 1970; Simeó *et al*., en preparació) o un aspecte granular homogeni (Medina i Rodríguez, 1992*b*). Al final de l'espermatogènesi, el nucli de les espermàtides mostra una cromatina poc condensada d'aspecte fibrós. Paral·lelament a la descondensació de la cromatina, es dóna un augment del volum del nucli i un desenvolupament de les prolongacions nuclears (Medina i Rodríguez, 1992*b*). Aquests canvis estan associats a modificacions de les proteïnes que acompanyen la cromatina i que possiblement tenen un paper molt important en el moment de la fertilització del oòcit.

Un altre fet característic és el trencament de les membranes nuclears (Langreth, 1969; Reger, 1970; Medina i Rodríguez, 1992*b*; Simeó *et al*., en preparació), de tal manera que la cromatina queda en contacte, però no barrejada, amb el citoplasma. Aquesta desintegració de les membranes nuclears sol estar associada a la zona basal de l'acrosoma i més específicament, a la base del tub acrosòmic, i facilita el pas del nucli a través del canal format pel tub acrosòmic durant la fertilització.

Les membranes nuclears s'ajunten entre si (Langreth, 1969; Reger, 1970; Medina i Rodríguez, 1992*b*) i amb la membrana plasmàtica per formar un sistema pentalaminar (Reger, 1970).

Al llarg de l'espermiogènesi es produeix una gran reducció del citoplasma en favor de la vesícula acrosòmica (Reger, 1970; Medina i Rodríguez, 1992*b*). El citoplasma és activament abocat fora de la cèl·lula (Langreth, 1969) i fins i tot es desprenen regions senceres dins de vesícules membranoses (Reger, 1970). Els canvis soferts pel citoplasma també afecten els diferents components citoplasmàtics: mitocondris, centríols i el sistema d'endomembranes. Els mitocondris, que són poc nombrosos i presenten unes crestes poc desenvolupades (Medina i Rodríguez, 1992*b*; Simeó *et al*., en preparació), són susceptibles de ser abocats a les cèl·lules accessòries amb la resta del citoplasma (Langreth, 1969). Una característica comuna a totes les espermiogènesis descrites de braquiürs és que els mitocondris que resten en les espermàtides queden embolcallats pel sistema de membranes (Pochon-Masson, 1962; Langreth, 1969; Reger, 1970; Medina i Rodríguez, 1992*b*; Simeó *et al*., en preparació). El centríols, per la seva banda, es localitzen per sota del tub acrosòmic a la base de la vesícula proacrosòmica (Pochon-Masson, 1962). En alguns casos, els centríols presenten signes de degeneració (Langreth, 1969), i poden arribar a desaparèixer en algunes espècies (Reger, 1970). El fet que els centríols es trobin presents o absents en els espermatozoides obre una incògnita sobre la seva funció, i s'ha suggerit que podrien tenir un paper important durant la reacció acrosòmica. El sistema d'endomembranes és un dels grans tòpics en els estudis de l'espermiogènesi dels crustacis decàpodes, i tant l'origen com el desenvolupament i la funció romanen encara incerts. Al llarg de l'espermiogènesi es poden distingir dos tipus de sistemes d'endomembranes: el sistema de membranes i les vesícules citoplasmàtiques. El sistema de membranes ha rebut noms diferents en els treballs realitzats: orgànul membranós *(membranous organelle*, Reger, 1970), *chondriofusome* (Pochon-Masson, 1962), complex membranós (*membrane complex*, Langreth, 1969) i làmines membranoses (*membranous lamellae*, Medina i Rodríguez, 1992*b*). La seva estructura i origen pot variar en les diferents espècies: en *Carcinus maenas* (Pochon-Masson, 1962) està format per fragments de les membranes nuclears, centríols i mitocondris; en *Cancer* sp.

Llista d'estudis sobre la ultraestructura dels espermatozoides dels braquiürs

Superfamília	**Espècie**	**Autor**
Homolodroioidea	*Homolodromia kai*	Jamieson *et al*., 1995; Guinot *et al*., 1998
Dromoidea	*Moreirodromia antillensis*	Brown, 1966, Felgenhauer i Abele, 1991
	Dromidiopsis edwardsi	Jamieson, 1994a, Jamieson *et al*., 1993d, 1994b
	Stimdromia lateralis	Jamieson, 1990, 1991a, 1991b, 1994a, Guinot *et al*., 1994
	Sphaerodromia lamellata	Guinot *et al*., 1998
	Metadynomene tanensis	Jamieson *et al*., 1995; Guinot *et al*., 1998
	Paradynomene tuberculata	Jamieson, 1994a, Jamieson *et al*., 1993b
	Tymolus sp.	Jamieson, 1994a, Jamieson *et al*., 1994a
Homoloidea	*Homola ranunculus*	Guinot *et al*., 1994
	Homologenus levii	Jamieson *et al*., 1993c
	Homolomannia sibogae	Jamieson *et al*., 1993c
	Latreillopsis gracilipes	Jamieson, 1994a, Jamieson *et al*., 1993c, 1994b
	Dagnaudus petterdi	Guinot *et al*., 1994
	Paromola bathyalis	Guinot *et al*., 1994
	Paromolopsis boasi	Jamieson *et al*., 1993c
	Latreillia sp.	Jamieson, 1994a, 1994b
Raninoidea	*Lyreidus brevifrons*	Jamieson, 1994a, Jamieson *et al*., 1994b
	Ranina ranina	Jamieson, 1989a, 1991a, 1991b, 1994a, Guinot *et al*., 1994
	Raninoides sp.	Jamieson, 1994a, 1994b, Jamieson *et al*., 1994b
	Cosmonotus sp.	Jamieson i Tudge, 2000
Cyclodorippoidea	*Xeinostoma richeri*	Jamieson, 1994a, Jamieson *et al*., 1994a
	Cymonomus sp.	Jamieson, 1994a, Jamieson *et al*., 1994a
Dorippoidea	*Neodorippe callida*	Jamieson, 1991b, 1994a, Jamieson i Tudge, 1990, 1991a
	Ethusa indica	Jamieson i Tudge, 2000
Calappoidea	*Calappa hepatica*	Jamieson, 1991a
	Calappa gallus	Jamieson i Tudge, 2000
	Mursia microspina	Jamieson i Tudge, 2000
Leucosioidea	*Iliacantha subglobosa*	Felgenhauer i Abele, 1991
	Bellidilia laevis	Jamieson i Tudge, 2000
	Tanaoa serenei	Jamieson i Tudge, 2000
	Odiomaris pilosus	Forges *et al*., 1997
	Odiomaris estuarius	Forges *et al*., 1997
	Elamena vesca	Jamieson i Tudge, 2000
Majoidea	*Maja brachydactyla*	Tudge i Justine, 1994
	Chionoecetes opilio	Beninger *et al*., 1988; Chiba *et al*., 1992
	Cyrtomaia furici	Jamieson i Tudge, 2000; Jamieson *et al*., 1998
	Grypacheus hyalinus	Jamieson i Tudge, 2000; Jamieson *et al*., 1998
	Macropodia longirostris	Jamieson i Tudge, 2000; Jamieson *et al*., 1998
	Platymaia rebierei	Jamieson i Tudge, 2000; Jamieson *et al*., 1998
	Podochela riisei	Hinsch, 1973
	Stenorhynchus seticornis	Hinsch, 1973
	Inachus phalangium	Rorandelli *et al*., 2008
	Hyastenus diacanthus	Jamieson i Tudge, 2000; Jamieson *et al*., 1998
	Libinia dubia	Hinsch, 1973
	Libinia emarginata	Hinsch, 1969, 1971, 1973, 1986; Vaughn i Hinsch, 1972; Hernandez *et al*., 1989; Murray *et al*., 1991
	Macrocoeloma trispinosum	Hinsch, 1973
	Mithrax sp.	Hinsch, 1973
	Menaethius monoceros	Jamieson, 1991a, 1994a
	Oxypleurodon orbiculatum	Jamieson i Tudge, 2000; Jamieson *et al*., 1998

	Oxypleurodon stuckiae	Jamieson i Tudge, 2000; Jamieson *et al*., 1998
	Pitho lherminieri	Hinsch, 1973
Parthenopoidea	Parthenopidae sp.	Jamieson i Tudge, 2000
	Heterocrytpa granulata	Hinsch, 1973
	Parthenope serratus	Hinsch, 1973
Retroplumoidea	*Retropluma* sp.	Jamieson i Tudge, 2000
Cancroidea	*Corystes cassivelaunus*	Jamieson *et al*., 1997
	Cancer borealis	Langreth, 1965, 1969
	Cancer irroratus	Langreth, 1965, 1969
	Cancer magister	Langreth, 1965, 1969
	Cancer pagurus	Pochon-Masson, 1968; Tudge i Justine, 1994; Tudge *et al*., 1994; Jamieson *et al*., 1997
	Cancer productus	Langreth, 1965, 1969
	Platepistoma nanum	Jamieson *et al*., 1997
Portunoidea	*Portunus pelagicus*	Jamieson, 1989a, 1991a, 1994a, Jamieson i Tudge, 1990; El-Sherief, 1991; Guinot *et al*., 1994
	Callinectes sapidus	Brown, 1966; Felgenhauer i Abele, 1991
	Xaiva sp.	Jamieson i Tudge, 2000
	Caphyra loevis	Jamieson, 1991a, 1994a
	Caphyra rotundifronis	Jamieson, 1991a, 1994a
	Carcinus maenas	Pochon-Masson, 1962, 1968; Chevaillier, 1966b, 1967, 1969; Pearson i Walker, 1975; Goudeau, 1982a, Reger *et al*., 1984
	Ovalipes ocellatus	Hinsch, 1986
	Ovalipes molleri	Jamieson i Tudge, 2000
	Podophthalmus vigil	Jamieson i Tudge, 2000
	Chaceon fenneri	Hinsch, 1988
	Chaceon quinquedens	Hinsch, 1988
Bythograeoidea	*Austinograea alayseae*	Tudge *et al*., 1998
	Bythograea thermydron	Tudge *et al*., 1998
	Segonzacia mesatlantica	Tudge *et al*., 1998
Xanthoidea	*Atergatis floridus*	Jamieson, 1989b, 1989c, 1991a, Jamieson *et al*., 1993a
	Etisus laevimanus	Jamieson, 1989c, 1991a
	Pilodius areolatus	Jamieson, 1989c, 1991a, 1994a
	Liagore rubromaculata	Jamieson, 1989c, 1991a
	Eurypanopeus depressus	Felgenhauer i Abele, 1991; Jamieson i Tudge, 2000
	Eurytium limosum	Felgenhauer i Abele, 1991
	Panopeus obesus	Jamieson i Tudge, 2000
	Ceratoplax sp.	Jamieson i Tudge, 2000
	Hexaplax megalops	Jamieson i Tudge, 2000
	Menippe mercenaria	Brown, 1966
	Eriphia sebana	Jamieson i Tudge, 2000
	Pilumnus semilanatus	Jamieson i Tudge, 2000
	Gonatonotus granulosus	Jamieson i Tudge, 2000
	Harrovia albolineata	Jamieson i Tudge, 2000
	Trapezia cymodoce	Jamieson, 1993a
	Tetralia glaberrima	Jamieson i Tudge, 2000
	Tetralia nigrolineata	Jamieson i Tudge, 2000
	Calocarcinus africanus	Jamieson, 1994a, Jamieson *et al*., 1993a
Goneplacoidea	*Australocarcinus riparius*	Jamieson i Guinot, 1996
	Carcinoplax microphthalmus	Jamieson i Tudge, 2000
	Goneplax sp.	Jamieson i Tudge, 2000
Potamoidea	*Potamon fluviatile*	Tudge i Justine, 1994; Guinot *et al*., 1997
	Potamon ibericum	Guinot *et al*., 1997
	Potamonautes perlatus sidneyii	Jamieson, 1993b, 1994a, 1994b

Gecarcinucoidea	*Holthusiana transversa*	Jamieson i Tudge, 2000
Cryptochiroidea	*Cryptochirus coralliodytes*	Jamieson i Tudge, 2000
	Hapalocarcinus marsupialis	Jamieson i Tudge, 2000
Pinnotheroidea	*Pinnixa* sp.	Reger, 1970
Ocypodoidea	*Mictyris longicarpus*	Jamieson, 1993a, 1994a
	Macrophthalmus crassipes	Jamieson, 1991a, 1994b
	Ocypode ceratophthalmus	Jamieson, 1991a, 1994b
	Uca paradussumieri	Jamieson, 1991a, 1994b
	Uca polita	Jamieson i Tudge, 2000
	Uca pugilator	Jamieson i Tudge, 2000
	Uca tangeri	Medina, 1992; Medina i Rodríguez, 1992a, 1992b
	Uca uruguayensis	Cuartas i Sousa, 2007
Grapsoidea	*Cardisoma carnifex*	Jamieson *et al*., 1996
	Grapsus albolineatus	Jamieson, 1991a
	Cyclograpsus punctatus	Jamieson i Tudge, 2000
	Varuna litterata	Jamieson *et al*., 1996
	Eriocheir japonica	Yasuzumi, 1960
	Eriocheir sinensis	Du *et al*., 1987, 1993
	Armases cinereum	Jamieson i Tudge, 2000
	Chiromantes haematocheir	Honma *et al*., 1992
	Parasesarma erythodactyla	Jamieson, 1991a
	Parasesarma catenatum	Jamieson i Tudge, 2000
	Sesarma reticulatum	Felgenhauer i Abele, 1991; Jamieson i Tudge, 2000

(Langreth, 1969) s'origina a partir de les cisternes del reticle endoplasmàtic lliures de ribosomes, associades a microtúbuls i mitocondris, mentre que en *Pinnixia* sp. (Reger, 1970) està format per les membranes de l' embolcall nuclear i el reticle endoplasmàtic. La funció del sistema de membranes encara no està aclarida. L'origen de les vesícules citoplasmàtiques varia segons les espècies estudiades, ja sigui del reticle endoplasmàtic rugós (Pochon-Masson, 1962), del reticle endoplasmàtic lliss (Langreth, 1969) o del complex de Golgi (Reger, 1970). En *M. brachydactyla*, com s'ha descrit en l'apartat anterior, les vesícules citoplasmàtiques, que en fusionar-se donaran lloc a la vesícula proacrosòmica, deriven fonamentalment del complex de Golgi, mentre que el sistema de membranes s'origina a partir de fragments de membranes del reticle endoplasmàtic i s'associa a microtúbuls i mitocondris (Simeó *et al*., en preparació).

El desenvolupament de la vesícula acrosòmica es dóna en un pol de la cèl·lula (Medina i Rodríguez, 1992*b*; Simeó *et al*., en preparació) que, en augmentar de volum, desplaça el nucli a la perifèria de l'espermàtida, i llavors el citoplasma queda entre el nucli i la vesícula. L'origen de l'acrosoma varia entre les espècies estudiades, i s'han descrit fins al moment els possibles llocs de procedència: el reticle endoplasmàtic (Pochon-Masson, 1962), les vesícules citoplasmàtiques d'origen no determinat (Langreth, 1969) i el reticle endoplasmàtic i el complex de Golgi (Reger, 1970; Simeó *et al*., en preparació). Al llarg de l'espermiogènesi, la vesícula proacrosòmica presenta una regionalització deguda a una reorganització interna dels seus components. El primer signe de diferenciació és l'aparició d'una regió electrodensa a la zona apical de la vesícula (Pochon-Masson, 1962; Langreth, 1969; Medina i Rodríguez, 1992*b*; Simeó *et al*., en preparació) que, en forma de gránul, dóna lloc en alguns casos a l'opercle (Pochon-Masson, 1962; Langreth, 1969). Simultàniament apareix en la zona basal de la vesícula

proacrosòmica una fina capa de material granular que envolta una invaginació de la vesícula, de la qual resultarà el tub acrosòmic (Langreth, 1969; Medina i Rodríguez, 1992*b*; Simeó *et al*., en preparació). Altres components granulars que es dipositen en la vesícula proacrosòmica s'organitzen, durant les fases finals de l'espermiogènesi, generalment en tres capes concèntriques al voltant del tub acrosòmic.

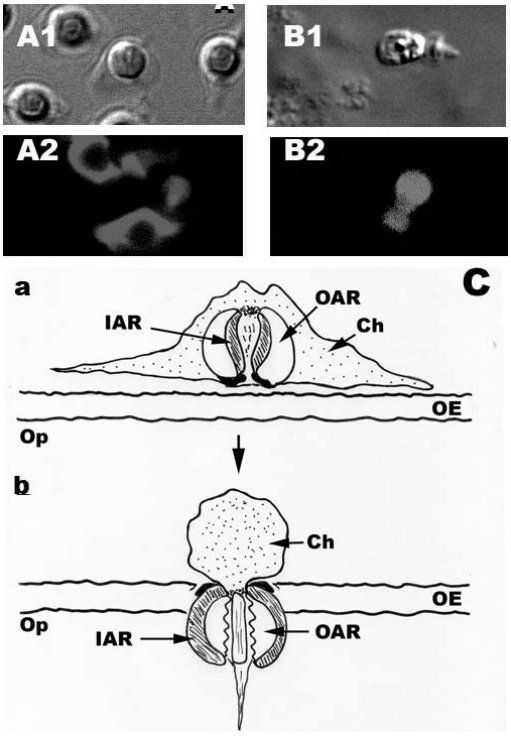

Figura 6. *Cancer pagurus*. Activació dels espermatozoides. A: Espermatozoides intactes de *C. pagurus* observats per microscòpia de contrast de fase (A1), i per tinció del nucli amb el reactiu de Hoechst (A2). B: Espermatozoides activats del mateix animal, observats per contrast de fase (A1) i per tinció del seu nucli amb reactiu de Hoechst. C: Esquema de l'activació de l'espermatozoide quan entra en contacte amb la coberta externa oocitària (a: abans de l'activació; b: durant l'activació). El nucli ha de travessar el canal intern que forma l'acrosoma en la seva eversió. Ch: cromatina, IAR i OAR: regions acrosòmiques interna i externa (observeu el canvi de posició de IAR/OAR després de l'eversió), OE: coberta oocitària, OP: citoplasma de l'oòcit.

L'espermatozoide dels braquiürs, com hem pogut constatar en aquest treball, és una cèl·lula aflagel·lada amb un acrosoma ben desenvolupat i complex, envoltat per una estreta franja de citoplasma i per un nucli amb cromatina poc condensada que ocupa la part més perifèrica de la cèl·lula. El nucli sol tenir prolongacions laterals en un nombre que varia segons les espècies. Els diferents tàxons de braquiürs presenten una gran diversitat morfològica espermàtica, que ha comportat un gran nombre d'estudis de la ultraestructura dels espermatozoides (vegeu la llista més endavant).

EL NUCLI ESPERMÀTIC DELS CRUSTACIS DECÀPODES BRAQUIÜRS

La cromatina espermàtica

El nucli espermàtic dels braquiürs ocupa una posició perifèrica en la cèl·lula i el seu contingut, la cromatina, mostra un aspecte poc electrodens i poc condensat (Chevaillier, 1966*a*, 1967, 1968; Langreth, 1969). Aquest últim aspecte contrasta amb la major part dels espermatozoides d'altres espècies animals, els quals tenen la cromatina molt condensada (apareix amb una elevadíssima electrodensitat en les imatges de microscòpia electrònica), i un volum nuclear molt reduït (vegeu Kurtz *et al*., 2008). Moltes de les propietats del nucli espermàtic dels braquiürs estan relacionades amb el seu particular procés de fertilització. L'espermatozoide dels crustacis és aflagel·lat, i el moviment necessari per a la penetració del nucli espermàtic en el citoplasma oocitari és generat per un procés d'eversió del gränul acrosòmic (Hinsch, 1971; Brown, 1976; Goudeau, 1982*b*; Medina i Rodríguez, 1992*a*). L'eversió de l'acrosoma origina un canal intern i

provoca un arrossegament del nucli cap a l'interior del citoplasma oocitari a través de l'estret canal intern (vegeu la figura 6).

Els nuclis d'aquests espermatozoides han de tenir una consistència suficient per poder ser arrossegats sense trencar-se, i simultàniament una flexibilitat o capacitat de deformació adequada per poder travessar l'estret canal per on entren dins de l'oòcit. Evidentment, els responsables principals d'aquestes propietats són la composició química i l'organització estructural de la cromatina.

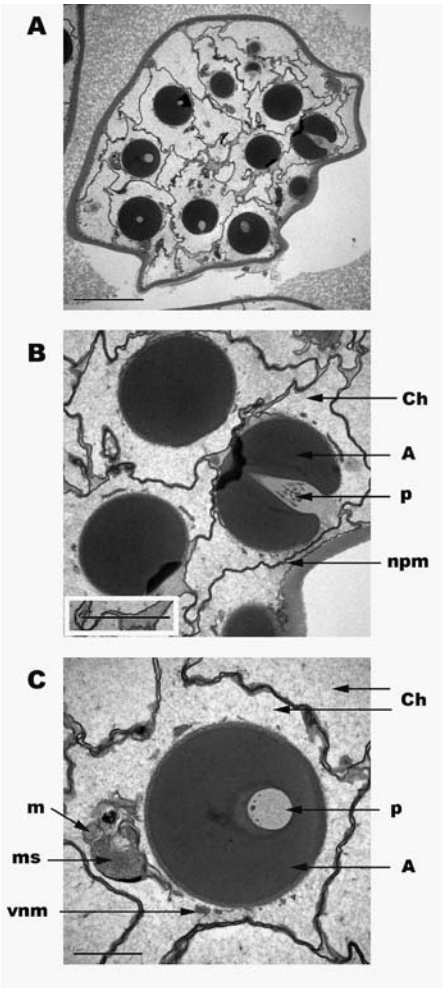

Figura 7. *Cancer pagurus*. Estructura general de l'espermatozoide (TEM). A: Espermatòfor obtingut del conducte deferent. B: Secció meridional d'un espermatozoide. C: Secció tallada equatorialment on es poden observar els orgànuls d'origen citoplasmàtic immersos en la cromatina. A: acrosoma, Ch: cromatina, m: mitocondri, ms: sistema de membranes, npm: membrana nucleocitoplasmàtica, p: *perforatorium*, vnm: membranes vesiculades.

L'anàlisi de les proteïnes de la cromatina espermàtica ha estat un objectiu a assolir força complicat. La major part dels estudis es van fer entre els anys seixanta i vuitanta del segle passat, i posteriorment van ser abandonats. En un article clàssic en aquest camp del coneixement, Bloch (1969) va demostrar a través de tincions histoquímiques que els nuclis dels crustacis decàpodes no contenien histones, ni protamina, ni cap altra proteïna bàsica que interaccionés amb el DNA. Els estudis realitzats per altres autors (la major part dels quals utilitzaven tècniques de tincions histoquímiques i d'altres a partir de cromatina solubilitzada) tampoc van posar de manifest la presència de proteïnes bàsiques (Chevaillier, 1966*a*, 1967, 1968; Vaughn i Locy, 1969; Vaughn i Hinsch, 1972; Vaughn i Thomson, 1972). Vaughn i Hinsch (1972) van descriure en *Libinia emarginata* que l'única fracció de proteïnes que es podia solubilitzar a partir de la cromatina espermàtica, tenia un caràcter acídic. A partir de l'any 1980, es troben molt pocs treballs que tractin de les proteïnes nuclears espermàtiques dels crustacis, i en particular dels braquiürs, i com a conseqüència el tema de la composició i organització de la cromatina espermàtica dels crustacis resta sense ser resolta.

Composició i estructura de la cromatina de *Cancer pagurus*

Els espermatozoides de *C. pagurus* es troben estretament agrupats en els esper-

matòfors (vegeu la figura 7A). En aquesta figura també es pot observar l'espermatozoide d'aquest animal en secció meridional i equatorial (vegeu les figures 7 B i C). Es pot apreciar que la cromatina coexisteix sense una separació física completa (és a dir, a través d'un sistema de membranes) amb mitocondris, microtúbuls i altres components citoplasmàtics.

En un treball recent (Kurtz *et al*., 2008), s'han reexaminat la composició i estructura del nucli espermàtic del decàpode braquiür *C. pagurus*, i s´han obtingut uns resultats

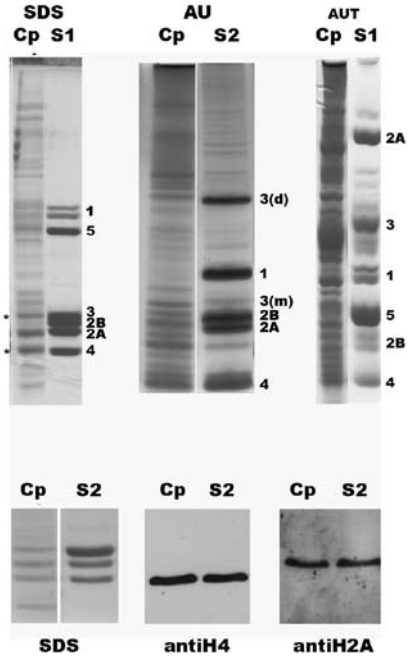

Figura 8. *Cancer pagurus*. Proteïnes bàsiques de l'espermatozoide. Gels de: poliacrilamida/SDS (a dalt, esquerra), de poliacrilamida/acíd acètic/urea (a dalt, mig), i de poliacrilamida/acètic/urea/tritó (a dalt, dreta) de les proteïnes espermàtiques solubilitzades amb 0,4 N HCl. De 1 a 5: histones H1, H2A, H2B, H3, H4 i H5; 3(d): dímer de H3; 3(m): monòmer de H3. A baix: immunodetecció (a partir d'una separació en gel de SDS) de les proteïnes bàsiques dels espermatozoides de *C. pagurus* amb anti-H4 i anti-H2A. Cp: *C. pagurus,* S1: estàndard d'histones d'eritròcit de gallina, S2: estàndard d'histones d'espermatozoide de llampresa.

que no coincideixen amb els dels estudis precedents, però que poden explicar-los, tal com es discuteix breument al final d'aquest apartat.

En primer lloc s'han trobat que les cèl·lules espermàtiques íntegres (alliberades dels espermatòfors) contenen proteïnes que es poden identificar com a histones, perquè manifesten un comportament electroforètic en gels de poliacrilamida i SDS igual al de les histones control, i perquè reaccionen amb els anticossos antihistona (vegeu la figura 8). Aquesta figura també mostra com les histones de l'espermatozoide de *C. pagurus* es resolen en diverses bandes electroforètiques quan són analitzades en gels de poliacrilamida/urea amb o sense tritó. Això suggereix que aquestes histones contenen modificacions postraduccionals (vegeu més endavant).

En segon lloc s'han observat, a través de digestions amb la nucleasa micrococcal, que aquestes histones es troben interaccionant amb el DNA, organitzant-lo en nucleosomes de 165-170 parells de bases (vegeu la figura 9). En aquest experiment també s'han trobat que només el 50-60 % del DNA estaria associat a les histones.

Finalment, s'han estudiat algunes de les característiques d'aquestes histones. És destacable que la histona H4 de la cromatina espermàtica es troba en un estat elevat d'acetilació, i que les histones H2B i H3 també presenten acetilació però en un grau molt menor (vegeu la figura 10). És important tenir present que l'acetilació de les histones disminueix la interacció electrostàtica entre aquestes proteïnes i el DNA i no permet que la cromatina es replegui en estructures més compactes. Una altra característica notable que ha posat en evidència l'estudi esmentat és que la relació proteïna/DNA en el nucli espermàtic és de 1,0 a 1,2 en aquelles especies que condensen la cromatina, però que només té un valor de 0,5 a 0,6 en dues es-

pecies del gènere *Cancer*. Aquesta proporció correspon aproximadament a la proporció de DNA no unit a histones (40-50 %) estimat a partir de les digestions amb la nucleasa micrococcal.

Consideracions respecte als estudis dels altres autors

S'intenta poder respondre la pregunta: per quin motiu els diferents investigadors no van poder observar proteïnes bàsiques interaccionant amb el DNA en el nucli espermàtic madur d'aquestes espècies?

La major part dels treballs citats anteriorment es van efectuar a través de tincions histoquímiques, i alguns pocs a través d'anàlisis electroforètiques. Per un cantó, la baixa proporció relativa entre histones i DNA (així com l'estat d'acetilació) probablement limita la intensitat de la reacció histoquímica (reactiu amb grups amino de les histones) i, per tant, la reacció no arribaria a un llindar mínim per ser detectada. També hem de dir que l'anàlisi electroforètica de les histones no és trivial: els nuclis no es poden purificar (ja que són molt fluids i es trenquen pels mètodes convencionals), i els extractes de proteïnes totals dels espermatozoides produeixen patrons electroforètics molt complexos en els quals la major part de proteïnes provenen del gränul acrosòmic, i en què les histones són minoritàries i no es poden reconèixer. Una última possibilitat a afegir és que actualment es disposa de combinacions d'inhibidors de la proteòlisi molt eficaços, mentre que els investigadors dels anys 1960-1980 no podien controlar adequadament l'activitat proteolítica.

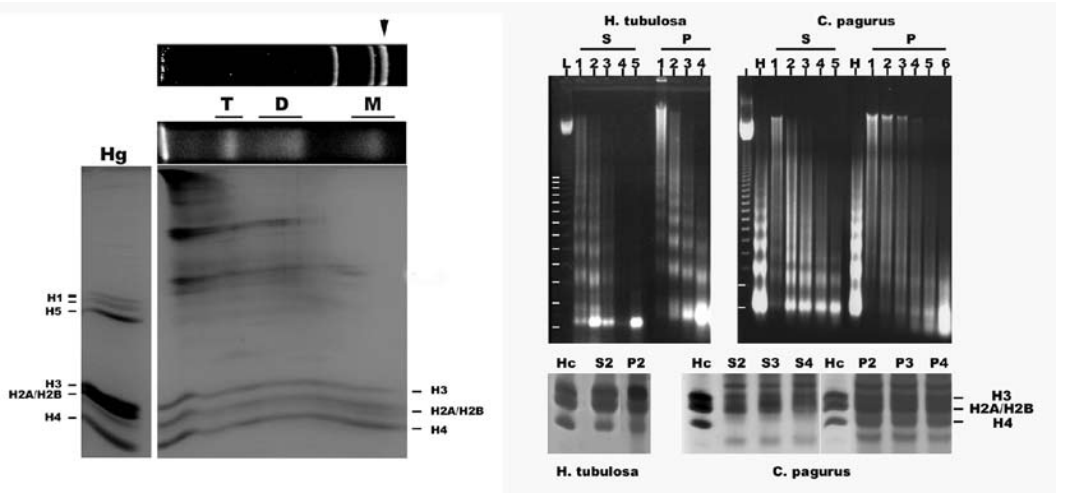

Figura 9. *Cancer pagurus*. Cromatina espermàtica. Esquerra: gel bidimensional que resol les proteïnes contingudes en els fragments de cromatina (obtinguts a partir de la digestió amb nucleasa micrococcal). Observeu que els mononucleosomes (M) i els di i trinucleosomes (D, T) contenen histones (H3, H2A, H2B, H4). Dreta: Gels d'agarosa amb els fragments de DNA que provenen de diferents temps d'incubació amb nucleasa micrococcal de la cromatina espermàtica d'*Holothuria tubulosa* (utilitzada com a control conegut) i de la cromatina espermàtica de *C. pagurus* (cinètiques d'incubació). 1 a 6: 2, 5, 10, 20, 40 i 120 minuts d'incubació; S: sobrenedant de les incubacions; P: sediment; L: estàndard de DNA de 123 parells de bases i dels seus múltiples. A la part de sota dels gels de DNA demostrem que les histones es troben associades als fragments de DNA, tant en el sobrenedant (Si), com en el sediment (Pi) de la reacció.

CONCLUSIONS

Els espermatozoides dels crustacis decàpodes han despertat un gran interès per la seva morfologia poc usual: no tenen flagel i, per tant, no són mòbils o amb escassa mobilitat, fet que contrasta amb el típic model flagel·lat. La informació respecte els diferents aspectes sobre l'espermatogènesi dels crustacis decàpodes és escassa. En aquest treball, hem abordat aquest fet utilitzant els braquiürs com a model per a la descripció morfològica de l'espermatogènesi i l'estudi de les proteïnes associades a la cromatina dels espermatozoides.

Els aspectes més rellevants de l'espermatogènesi són: *a*) La formació d'un complex i voluminós acrosoma, format en el cas de la cabra de mar, *M. brachydactyla*, a partir de vesícules originades principalment pel complex de Golgi; *b*) la formació d'un sistema de membranes a partir del reticle endoplasmàtic, associat a microtúbuls i a pocs mitocondris, que forma un anell en la base de les prolongacions nuclears; *c*) la fusió de les membranes plasmàtica i nuclear, que donen lloc a una estructura membranosa pentalaminar en algunes regions de la superfície cel·lular i *d*) la poca condensació que presenta el nucli de l'espermatozoide, a causa de la baixa proporció i la modificació de les histones associades a la cromatina.

El procés especial de la fertilització dels gàmetes dels crustacis decàpodes braquiürs posa una sèrie de restriccions estructurals i funcionals en els nuclis espermàtics. És molt possible que entre els crustacis hagin aparegut diferents solucions evolutives a tals restriccions i que no tots els nuclis dels espermatozoides dels crancs tinguin una composició igual a la de *C. pagurus*. No

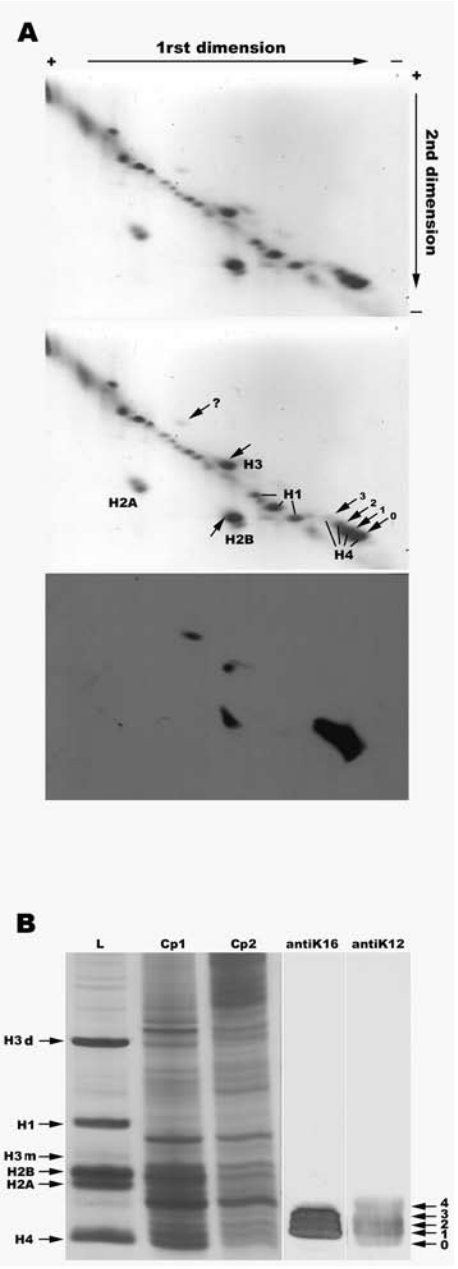

Figura 10. *Cancer pagurus.* Acetilació de les histones de l'espermatozoide. A: Electroforesi bidimensional de les proteïnes extretes dels espermatozoides de *C. pagurus* (a dalt), i de la seva reacció amb l'anticòs antiacetil-lisina (a sota). B: Formes mono, di i triacetilades de la histona H4 demostrades a través de la seva reacció amb anticossos específics contra la H4 acetilada en la lisina 16 i la lisina 12. L: histones estàndard d'espermatozoide de llampresa, Cp: histones de *C. pagurus*.

obstant això, la composició d'aquesta cromatina (DNA organitzat en nucleosomes però no en estructures d'ordre superior a causa de l'acetilació de la histona H4, regions àmplies del DNA lliure d'histones) permet comprendre, almenys parcialment, la deformabilitat i fluïdesa del nucli per poder fecundar l'oòcit.

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BIBLIOGRAFIA

Adiyodi, K. G.; Anilkumar, R. G. (1988). «Accessory sex glands». A: Adiyodi, K. G.; Adiyodi, R. G. [ed.]. *Reproductive Biology of Invertebrates*. Kerala: John Wiley and Sons: 261-318.

Bauer, R. T. (1986). «Phylogenetic trends in sperm transfer and storage complexity in decapod crustaceans». *J. Crustacean Biol*., 6: 313-325.

Beninger, P. G.; Elner, R. W.; Foyle, T. P.; Odense, P. H. (1988). «Functional anatomy of the male reproductive system and the female spermatheca in the snow crab *Chionoecetes opilio* (O. Fabricius) (Decapoda: Majidae) and a hypotheses for fertilization». *J. Crustacean Biol*., 8: 322-332.

Binford, R. (1913). «The germ-cells and the process of fertilization in the crab, *Menippe mercenaria*». *J. Morphol*., 24: 147-201.

Bloch, D. P. (1969). «A catalog of sperm histones». *Genetics*, 61 (supl. 1): 93-111.

Brown, G. G. (1966). «Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus*». *J. Ultra. Res*., 14: 425-440.

— (1976). «Scanning electron-microscopical and other observations of sperm fertilization reactions in *Limulus polyphemus* L (Merostomata-Xiphosura)». *J. Cell. Sci*., 22: 547-562.

Cuartas, E. I.; Sousa, L. G. (2007). «Ultrastructure study of the spermatophores and spermatozoa in *Uca uruguayensis* (Decapoda, Brachyura, Ocypodidae)». *Biociências*, 15: 21-28.

Chevaillier, P. (1966a). «Structure et constitution cytochimique de la capsule du spermatozoïde des Crustacés Décapodes». *C. R. Acad. Sci. Paris*, D262: 1546-1549.

— (1966b). «Contribution à l'étude du complexe ADN-histone dans le spermatozoide du pagure *Eupagurus bernhardus* L. (Crustace Decapode)». *J. Microsc*., 5: 739-758.

— (1967). «Mise en évidence et étude cytochimique d'une protéine basique extranucleaire dans les spermatozoïdes des crustacés decapodes». *J. Cell. Biol*., 32: 547-556.

— (1968). «Etude cytochimique des nucléoprotéins dans le spermatozoide du pagurus *Eupagurus bernhardus* L. (Crustace decapode)». *J. Microsc*., 7: 107-114.

— (1969). «Evolution des constituants nucléaires et cytoplasmaiques au cours de la spermatogenèse chez les Crustacés Décapodes». *C. R. Acad. Sci. Paris*, D269: 2251-2253.

Chiba, A.; Kon, T.; Honma, Y. (1992). «Ultrastructure of the spermatozoa and spermatophores of the zuwai crab, *Chionoecetes opilio* (Majidae, Brachyura)». *Acta Zool*., 73: 103-108.

Diesel, R. (1991). «Sperm competition and the evolution of mating behaviour in Brachyura, with special reference to spider crabs (Decapoda, Majidae)». A: Bauer, R. T.; Martin, J. W. [ed.]. *Crustacean Sexual Biology*. Nova York: Columbia University Press: 145-163.

Du, N. H.; Lai, W.; Xue, L. H. (1987). «Acrosome reaction of the sperm in the chinese mitten-handed crab, *Eriocheir sinensis* (Crustacea, Decapoda)». *Acta Zool. Sinica*, 33: 8-13.

Du, N. S.; Lai, W.; An, Y.; Jiang, H. W. (1993). «Studies on the cytology of fertilization in the chinese mitten-handed crab, *Eriocheir sinensis* (Crustacea, Decapoda)». *Sci. China B*, 36: 288-296.

El-Sherief, S. S. (1991). «Fine structure of the sperm and spermatophores of *Portunus pelagicus* (L.) (Decapoda, Brachyura)». *Crustaceana*, 61: 271-279.

Estampador, E. P. (1949). «Scylla (Crustacea: Portunidae) II. Comparative studies on spermatogenesis and oogenesis». *Philip. Jour. Sci.*, 78: 95-109.

Fasten, N. (1918). «Spermatogenesis of the pacific coast edible crab, *Cancer magister* Dana». *Biol. Bull.*, 34: 277-307.

— (1924). «Comparative stages in the spermatogenesis of various Cancer crabs». *J. Morphol.*, 39: 47-61.

— (1926). «Spermatogenesis of the black-clawed crab, *Lophopanopeus bellus* (Stimpson) Rathburn.». *Biol. Bull.*, 50: 277-293.

Felgenhauer, B. E.; Abele, L. G. (1991). «Morphological diversity of decapoda spermatozoa». A: Bauer, R. T.; Martin, J. W. [ed.]. *Crustacean Sexual Biology.* Nova York: Columbia University Press, 322-339.

Forges, B. R. de; Jamieson, B. G. M.; Guinot, D.; Tudge, C. C. (1997). «Ultrastructure of the spermatozoa of Hymenosomatidae (Crustacea: Brachyura) and the relationships of the family». *Mar. Biol.*, 130: 233-242.

Goudeau, M. (1982a). «Fertilization in a crab. 1: Early events in the ovary, and cytological aspects of the acrosome reaction and gamete contacts». *Tissue & Cell*, 14: 97-111.

— (1982b). «Fertilization in a crab.1. Early events in the ovary, and cytological aspects of the acrosome reaction and gamete contacts». *Tissue & Cell*, 14: 97-111. REPETIT

Guinot, D.; Jamieson, B. G. M.; Deforges, B. R. (1994). «Relationship of Homolidae and Dromiidae -Evidence from spermatozoal ultrastructure (Crustacea, Decapoda)». *Acta Zool.*, 75: 255-267.

Guinot, D.; Jamieson, B. G. M.; Forges, B. R. de; Tudge, C. C. (1998). «Comparative spermatozoal ultrastructure of the three dromiacean families exemplified by *Homolodromia kai* (Homolodromiidae), *Sphaerodromia lamellata* (Dromiidae), and *Dynomene tanensis* (Dynomenidae) (Podotremata : Brachyura)». *J. Crustacean Biol.*, 18: 78-94.

Guinot, D.; Jamieson, B. G. M.; Tudge, C. C. (1997). «Ultrastructure and relationships of spermatozoa of the freshwater crabs *Potamon fluviatile* and *Potamon ibericum* (Crustacea, Brachyura, Potamidae)». *J. Zool.*, 241: 229-244.

Hernandez, W.; Berry, D.; Baccetti, B.; Ahluwalia, B.; Murray, S. A.; Anderson, W. A. (1989). «Remodeling of the nucleocytoplasm as consequence of the acrosomal reaction of the spider crab sperm». *J. Submicrosc. Cytol. Pathol.*, 21: 163-186.

Hinsch, G. W. (1969). «Microtubules in the sperm of the spider crab, *Libinia emarginata* L.». *J. Ultra. Res.*, 29: 525-534.

— (1971). «Penetration of the oocyte envelope by spermatozoa in the spider crab». *J. Ultra. Res.*, 35: 86-97.

— (1973). «Sperm structure of Oxyrhyncha». *Can. J. Zool.*, 51: 421-426.

— (1986). «A comparison of sperm morphologies, transfer and sperm mass storage between two species of crab, *Ovalipes ocellatus* and *Libinia emarginata*». *Int. J. Invertebr. Repr. Dev.*, 10: 79-87.

— (1988). «Ultrastructure of the sperm and spermatophores of the golden crab *Geryon fenneri* and a closely related species, the red crab *G. quinquedens*, from the eastern gulf of Mexico». *J. Crustacean Biol.*, 8: 340-345.

— (1991). «Structure and chemical content of the spermatophores and seminal fluid of reptantian decapods». A: Bauer, R. T.; Martin, J. W. [ed.]. *Crustacean Sexual Biology.* Nova York: Columbia University Press: 290-307.

Honma, Y.; Ogiwara, M.; Chiba, A. (1992). «Studies on gonad maturity in some marine invertebrates XII. Light and electron microscope studies on spermatozoa of the land crab, *Sesarma haematocheir* (de Haan)». *Rpt. Sado Mar. Biol. Sta. Niigata Uni.*, 22: 13-21.

Jamieson, B. G. M. (1989a). «Ultrastructural comparison of the spermatozoa of *Ranina ranina* (Oxystomata) and of other crabs exemplified by *Portunus pelagicus* (Brachygnatha) (Crustacea, Brachyura)». *Zoomorphology*, 109: 103-111.

— (1989b). «A comparison of the spermatozoa of *Oratosquilla stephensoni* and *Squilla mantis* (Crustacea, Stomatopoda) with comments on the phylogeny of the Malacostraca». *Zool. Scr.*, 18: 509-517.

— (1989c). «The ultrastructure of the spermatozoa of 4 species of xanthid crabs (Crustacea, Brachyura, Xanthidae)». *J. Submicrosc. Cytol. Pathol.*, 21: 579-584.

— (1990). «The ultrastructure of the spermatozoa of *Petalomera lateral*i (Gray) (Crustacea, Brachyura, Dromiacea) and its phylogenetic significance». *Invertebr. Reprod. Dev.*, 17: 39-45.

— (1991a). «Sperm and phylogeny in the Brachyura (Crustacea)». A: Bacetti, B. [ed.]. *Comparative spermatology 20 year after.* Nova York: Raven Press: 967-972.

— (1991b). «UItrastructure and phylogeny of crustacean spermatozoa». *Mem. Queensl. Mus.*, 31: 109-142.

— (1993a). «Ultrastructure of the spermatozoon of *Potamonautes perlatus sidneyii* (Heterotremata, Brachyura, Crustacea)». *S. Afr. J. Zool.*, 28: 40-45.

— (1993b). «Spermatological evidence for the taxonomic status of *Trapezia* (Crustacea: Brachyura: Heterotremata)». *Mem. Queensl. Mus*., 33: 225-234.
— (1994a). «Phylogeny of the Brachyura with particular reference to the Podotremata: Evidence from a review of spermatozoal ultrastructure (Crustacea, Decapoda)». *Philos. Trans. R. Soc. Lond. B Biol. Sci*., 345: 373-393.
— (1994b). «Phylogeny of the Brachyura with particular reference to the Podotremata (Crustacea, Decapoda)». A: Bradley, M.; Cummins, J. [ed.]. *Seventh International Symposium on Spermatology.* Cairns (19-20 d'octubre).
Jamieson, B. G. M.; Guinot, D. (1996). «The ultrastructure of the spermatozoon of *Australocarcinus riparis* (Crustacea: Brachyura: Trogoplacinae)». *Mem. Queensl. Mus*., 39: 289-296.
Jamieson, B. G. M.; Guinot, D.; Deforges, B. R. (1993a). «The spermatozoon of *Calocarcinus africanus* (Heterotremata, Brachyura, Crustacea): Ultrastructural synapomorphies with xanthid sperm». *Invertebr. Reprod. Dev*., 24: 189-196.
— (1993b). «The ultrastructure of the spermatozoon of *Paradynomene tuberculata* Sakai, 1963 (Crustacea, Brachyura, Dynomenidae): Synapomorphies with dromiid sperm». *Helgol. Meeresunters*, 47: 311-322.
— (1993c). «Spermatozoal ultrastructure in four genera of Homolidae (Crustacea, Decapoda): Exemplified by *Homologenus* sp., *Latreillopsis* sp., *Homolomannia sibogae* and *Paromolopsis boasi*». *Helgol. Meeresunters*, 47: 323-334.
— (1994b). «Relationships of the Cyclodorippoidea Ortmann -Evidence from Spermatozoal Ultrastructure in the Genera *Xeinostoma*, *Tymolus* and *Cymonomus* (Crustacea, Decapoda)». *Invertebr. Reprod. Dev*., 26: 153-164.
— (1996). «Contrasting spermatozoal ultrastructure in two thoracotreme crabs, *Cardisoma carnifex* (Gecarcinidae) and *Varuna litterata* (Grapsidae) (Crustacea: Brachyura)». *Invertebr. Reprod. Dev*., 29: 111-126.
— (1994a). «Podotreme affinities of Raninoides sp. and *Lyreidus brevifrons*: evidence from spermatozoal ultrastructure (Crustacea: Brachyura: Raninoidea)». *Mar. Biol*., 120: 239-249.
— (1995). «Phylogeny of the Brachyura (Crustacea, Decapoda): evidence from spermatozoal ultrastructure». *Mém. Mus. Natn. Hist. Nat*, 166: 265-283.
Jamieson, B. G. M.; Guinot, D.; Tudge, C. C.; Forges, B. R. de (1997). «Ultrastructure of the spermatozoa of *Corystes cassivelaunus* (Corystidae), *Platepistoma nanum* (Cancridae) and *Cancer pagurus* (Cancridae) supports recognition of the *Corystoidea* (Crustacea, Brachyura, Heterotremata)». *Helgol. Meeresunters*, 51: 83-93.
Jamieson, B. G. M.; Scheltinga, D. M.; Forges, B. R. de (1998). «An ultrastructural study of spermatozoa of the Majidae with particular reference to the aberrant spermatozoon of *Macropodia longirostris* (Crustacea, Decapoda, Brachyura)». *Acta Zool*., 79: 193-206.
Jamieson, B. G. M.; Tudge, C. C. (1990). «Dorippids are Heterotremata: evidence from ultrastructure of the spermatozoa of *Neodorippe astuta* (Dorippidae) and *Portunus pelagicus* (Portunidae) Brachyura: Decapoda». *Mar. Biol*., 106: 347-354.
— (2000). «Crustacea-Decapoda». A: Jamieson, B. G. M. [ed.]. *Reproductive Biology of Invertebrates.* Nova Delhi: Oxford & IBH Publishing: 1-95.
Jamieson, B. G. M.; Tudge, C. C.; Scheltinga, D. M. (1993d). «The ultrastructure of the spermatozoon of *Dromidiopsis edwardsi* Rathburn, 1919 (Crustacea: Brachyura: Dromiidae): Confirmation of a dromiid sperm type». *Aust. J. Zool*., 41: 537-548.
Krol, R. M.; Hawkins, W. E.; Overstreet, R. M. (1992). «Reproductive components». A: Harrison, F. W.; Humes, A. G. [ed.]. *Microscopic Anatomy of Invertebrates.* Nova York: Wiley-Liss, Inc.: 295-343.
Kurtz, K.; Martínez-Soler, F.; Ausió, J.; Chiva, M. (2008). «Histones and nucleosomes in *Cancer* sperm (Decapod: *Crustacea*) previously described as lacking basic DNA-associated proteins: A new model of sperm chromatin». *J. Cell. Biochem.* [En premsa (es pot consultar per Internet)]
Langreth, S. G. (1965). «Ultrastructural observations on sperm of crab *Cancer borealis*». *J. Cell. Biol*., 27: A56-A57.
— (1969). «Spermiogenesis in cancer crabs». *J. Cell. Biol*., 43: 575-603.
Medina, A. (1992). «Structural modifications of sperm from the fiddler crab *Uca tangeri* (Decapoda) during early stages of fertilization». *J. Crustacean Biol*., 12: 610-614.
Medina, A.; Rodríguez, A. (1992a). «Spermiogenesis and sperm structure in the crab *Uca tangeri* (Crustacea, Brachyura), with special reference to the acrosome differentiation». *Zoomorphology*, 111: 161-165.
— (1992b). «Structural changes in sperm from the fiddler crab, *Uca tangeri* (Crustacea, Brachyura), during the acrosome reaction». *Mol. Reprod. Dev*., 33: 195-201.
Mouchet, S. (1931). «Spermatophores des Crustacés Décapodes Anomures et Brachyoures et castration parasitaire chez quelques Pagures». *Ann. Sta. Océanogr. Salammbô*, VI: 1-203.
Murray, S. A.; Gesiotto, J.; Perez, R.; Baccetti, B.; An-

derson, W. A. (1991). «Polyethylene-glycol induced fusion of crustacean sperm and rat-liver cells in culture». A: Baccetti, B. [ed.]. *Comparative Spermatology 20 Years After.* Nova York: Raven Press: 575-579.

Nagao, J.; Munehara, H. (2003). «Annual cycle of testicular maturation in the helmet crab *Telmessus cheiragonus*». *Fish. Sci*., 69: 1200-1208.

Nath, V. (1932). «Spermatid and sperm in *Paratelphusa spinigera*». *Q. J. Microsc. Sci*., 229: 543-556.

Pearson, P. J.; Walker, M. H. (1975). «Alteration of cytochrome-C oxidase activity during spermatogenesis in *Carcinus maenas*». *Cell Tissue Res*., 164: 401-410.

Pochon-Masson, J. (1962). «Le chondriofusome des gamètes males du Crustacé Décapode *Carcinus maenas*». *C. R. Acad. Sci. Paris*, 254: 4076-4078.

— (1968). «L'ultrastructure des spermatozoïdes vésicularies chez les crustacés décapodes avant et au cours de leur dévegination expérimentale. I. Brachyoures et Anomoures». *Ann. Sci. Nat. Zool*., 10: 1-100.

Reger, J. F. (1970). «Studies on the fine structure of spermatids and spermatozoa of the crab, *Pinnixia* sp.». *J. Morphol*., 132: 89-100.

Reger, J. F.; Escaig, F.; Pochon-Masson, J.; Fitzgerald, M. E. C. (1984). «Observations on crab, Carcinus maenas, spermatozoa following rapid-freeze and conventional fixation techniques». *J. Ultra. Res*., 89: 12-22.

Rorandelli, R.; Paoli, F.; Cannicci, S.; Mercati, D.; Giusti, F. (2008). «Characteristics and fate of the spermatozoa of *Inachus phalangium* (Decapoda, Majidae): Description of novel sperm structures and evidence for an additional mechanism of sperm competition in brachyura». *J. Morphol*., 269: 259-271.

Rotllant, G.; González-Gurriarán, E.; Fernández, L.; Benhalima, K.; Ribes, E. (2007). «Ovarian maturation of the multi-spawning spider crab *Maja brachydactyla* (Decapoda: Majidae) with special reference to yolk formation». *Mar. Biol*., 152: 383-394.

Simeó, C. G.; Kurtz, K.; Chiva, M.; Ribes, E.; Rotllant, G. «Spermatogenesis of the spider crab, *Maja brachydactyla* (Decapoda: Brachyura)». [En preparació]

Simeó, C. G.; Ribes, E.; Rotllant, G. «Internal anatomy and ultrastructure of the male reproductive system of the spider crab *Maja brachydactyla* (Decapoda: Brachyura)». [En revisió]

Subramonian, T. (1991). «Spermatophores and sperm transfer in marine crustaceans». *Adv. Mar. Biol*., 29: 129-214.

Tudge, C. C.; Grellier, P.; Justine, J. L. (1994). «Actin in the acrosome of the spermatozoa of the crab, *Cancer pagurus* L (Decapoda, Crustacea)». *Mol. Reprod. Dev*., 38: 178-186.

Tudge, C. C.; Jamieson, B. G. M.; Segonzac, M.; Guinot, D. (1998). «Spermatozoal ultrastructure in three species of hydrothermal vent crab, in the genera *Bythograea*, *Austinograea* and *Segonzacia* (Decapoda, Brachyura, Bythograeidae)». *Invertebr. Reprod. Dev*., 34: 13-23.

Tudge, C. C.; Justine, J. L. (1994). «The cytoskeletal proteins actin and tubulin in the spermatozoa of 4 decapod crabs (Crustacea, Decapoda)». *Acta Zool*., 75: 277-285.

Vaughn, J. C.; Hinsch, G. W. (1972). «Isolation and characterization of chromatin and DNA from the sperm of the spider crab, *Libinia emarginata*». *J. Cell Sci*., 11: 131-152.

Vaughn, J. C.; Locy, R. D. (1969). «Changing nuclear histone patterns during development. III: The deoxyribonucleic acid content of spermatogenic cells in the crab *Emerita analoga*». *J. Histochem. Cytochem*., 17: 591-600.

Vaughn, J. C.; Thomson, L. A. (1972). «A kinetic study of DNA and basic protein metabolism during spermatogenesis in the sand crab, *Emerita analoga*». *J. Cell. Biol*., 52: 322-337.

Yasuzumi, G. (1960). «Spermatogenesis in animals as revealed by electron microscopy: VII. Spermatid differentiation in the crab, *Eriocheir japonicus*». *J. Biophys. Biochem. Cytol., 7: 73-78.*

Article 5

Títol: Identification of *vasa*, a potential marker of primordial germ cells in the spider crab *Maja brachydactyla*, and its expression during early post-embryonic development

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Informe de la contribució del doctorand

La hipòtesi de treball i la metodologia a seguir varen estar seleccionades per la Dra. G. Rotllant i el Dr. K. Andree. Els cultius dels estadis larvaris i juvenils es varen dur a terme segons la metodologia posada a punt per la Dra. Rotllant. El doctorand va participar en les disseccions i els mostrejos dels teixits dels reproductors, i va realitzar els cultius larvaris i els mostrejos d'estadis larvaris i primer juvenil. L'aïllament i clonació del gen, així com el processat de les mostres van ser realitzades pel doctorand amb la col·laboració del Dr. K.B. Andree. La seqüenciació dels diferents fragments es realitzà en un laboratori extern. El tractament i interpretació de les dades, així com la redacció del manuscrit foren realitzades pel doctorant amb la col·laboració dels coautors.

Dra. Guiomar Rotllant Estelrich

Resum

Aquest estudi descriu l'aïllament, seqüenciació i caracterització del gen *vasa* (*Mb vasa*) a la cabra de mar. El gen *vasa*, descrit per primera volta en *Drosophila*, és un gen que s'expressa específicament en les cèl·lules de la línia germinal. Un únic fragment de 865 parells de bases fou amplificat a partir del DNA complementari sintetitzat a partir de RNA extret del testicle. La seqüència deduïda d'aminoàcids resultà en un fragment de 293 aminoàcids, el qual presenta set regions conservades de la família DEAD-box: motius I (SGKT), Ia (PTRELA), Ib (TPGK), II (DEAD), III (SAT), IV (LIV) i V (ARGLD). La comparació de la seqüència d'aminoàcids amb altres homòlegs de *vasa* mostrà una alta similitud (>70%) amb el gen homòleg del *vasa* d'altres crustacis decàpodes. La reconstrucció filogenètica mostrà que el gen aïllat a la cabra de mar s'agrupa consistentment amb el gen *vasa* d'altres espècies. L'expressió del gen aïllat només es detectà en les gònades de la cabra de mar. Per tant, en base a la seqüència deduïda d'aminoàcids, l'anàlisi filogenètic i l'expressió específica del gen en les gònades dels adults, el fragment fou identificat com l'homòleg del gen *vasa* a la cabra de mar i anomenat *Mb vasa*. A més a més, un fragment parcial de 2.210 parells de bases del DNA genòmic de *Mb vasa* fou aïllat per determinar l'estructura del gen. El fragment de DNA genòmic s'organitzà en cinc exons separats per quatre introns. L'exó E1 contenia els motius I i Ia, l'exó E2 contenia els motius Ib, II i III, mentre que l'exó E3 no contingué cap motiu conservat de la família DEAD-box. L'exó E4 contenia els motius IV i V. A més a més, quatre microsatèl·lits es varen detectar en els introns I1 i I2. L'anàlisi de l'expressió de *Mb vasa* durant les primeres etapes del desenvolupament postembrionari (3 estadis larvaris i primer juvenil) es realitzà mitjançant la reacció en cadena de la polimerasa (PCR) quantitativa amb SYBR green com fluorocrom i el gen *β-actina* com control endogen. El nivells d'expressió foren baixos però detectables, i amb un lleuger increment al llarg del desenvolupament larvari. L'expressió després de la muda de metamorfosi al primer juvenil fou significativament major que durant els estadis larvaris. D'aquesta manera, el patró d'expressió de *Mb vasa* durant les primeres etapes de desenvolupament postembrionari s'ajusta a una corba exponencial de creixement, degut bé a un increment en nombre o bé en l'activitat de les cèl·lules germinals, especialment després de la muda de metamorfosi.

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Identification of *vasa*, a potential marker of primordial germ cells in the spider crab *Maja brachydactyla*, and its expression during early post-embryonic development

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A partial sequence of the *vasa* gene (*Mb vasa*) has been isolated from the testis of spider crab (*Maja brachydactyla*). It has been identified as the homologue of *vasa* based on the deduced amino acid sequence, phylogenetic analysis and tissue-specific expression in adult gonads. Quantitative PCR analysis of *Mb vasa* during early post-embryonic development (three larval stages and first juvenile crab) found expression at low, but detectable levels. During larval development, expression levels increased slightly, while expression after moult during metamorphosis to first juvenile crab was significantly higher as compared to all larval stages. Overall, the pattern of expression of *Mb vasa* during early post-embryonic development fits an exponential growth curve, which might be either due to an increase in number or in activity of germ cells, especially after moult to first juvenile instar.

Keywords: gonads; juvenile; larvae; quantitative PCR; reproductive system

Introduction

Germ cells during early development can be identified by several peculiarities, such as cell morphology, the presence of cytoplasmic electron-dense bodies, a differentiated transcriptional and translational regulation and the expression of germ cell-specific genes (Extavour and Akam 2003). Among different germ cell-specific genes, *vasa* is the most widely used and best characterized molecular marker of germ-lines in developmental and evolutionary studies due to its continuous expression in germ-lines through different developmental stages of most metazoans. However, *vasa* mRNA expression, protein localization and expression do not always correspond to the germ-line (Ikenishi 1998; Raz 2000; Extavour and Akam 2003; Rosner et al. 2009). The *vasa* gene encodes for an ATP-dependent RNA helicase belonging to the DEAD-box protein family (Raz 2000). Despite the information available with the structure of *vasa* and its expression through development in many groups of metazoans, its function is still unclear. It is believed that it participates in the transcriptional and post-transcriptional control of several germ cell specific mRNAs such as *oskar* and *nanos* (Saffman and Lasko 1999), development of germ cells, control of gametogenesis and germ cell determination and differentiation in *Drosophila* and vertebrates including mammals (Noce et al. 2001).

In addition to its evolutionary and developmental significance (Extavour and Akam 2003), *vasa* has also been characterized in several species of interest to aquaculture, such as *Litopenaeus vannamei* (Aflalo et al. 2007), *Macrobrachium rosenbergii* (Nakkrasae and Damrongphol 2007), *Crassostrea gigas* (Fabioux et al. 2004b), *Neobenedenia girellae* (Ohashi et al. 2007) and *Thunnus orientalis* (Nagasawa et al. 2009), due to the potential application in controlling their reproduction in captivity. The spider crab (*Maja brachydactyla* Balss, 1922) supports an intensive fishery (Freire et al. 2002) and it has been proposed as a potential species for aquaculture (Andrés et al. 2007, 2008). Some developmental aspects of reproduction, such as the development of germ cells during embryogenesis (Lang 1973, as *Maja squinado*), development of external sexual secondary characteristics (Guerao and Rotllant 2009) and gonad and functional maturity (Sampedro et al. 1999; Corgos and Freire 2006; Rotllant et al. 2007; Simeó et al. 2009, 2010a, 2010b) have already been described. In this study, a fragment of *Mb vasa*, encompassing five exons, has been isolated and its expression during larval development to first juvenile crab has been quantified in order to provide basic

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knowledge to assist advancement of reproduction in captivity and future mass production.

Materials and methods

Tissue collection

Adult males and females of the spider crab *M. brachydactyla* were captured in Galicia (Atlantic NE) and transported under high humidity conditions to IRTA. During dissection, gonads, hepatopancreas, gill tissue and heart were extracted and immediately frozen at −80°C for RNA extraction. Muscle tissue from the pereiopods (walking legs) was extracted and fixed in absolute ethanol at 4°C until being processed for nucleic acid extraction, cloning and sequencing.

Larval culture

Three different batches of newly hatched larvae were collected from the broodstock tank and cultured until first juvenile stage following the recommendations of Andrés et al. (2007). Samples were taken three times during larval development (Andrés et al. 2010; Guerao et al. 2010): zoea I (ZI) at hatching; zoea II (ZII) at intermoult stage, 7 days post hatching (dph), megalopa (M) at intermoult stage, 11 dph; and first juvenile crab (C) just after metamorphosis, at 18 dph. Each sampling day an average of 101 ± 2 mg wet weight of larvae and C were collected, gently rinsed in distilled water, and stored immediately at −80°C until being processed. In addition, the weight of 25 individuals were used as a standard measure for estimation of the number of individuals in each sample for further data normalization of quantitative PCR (qPCR). Wet weight values were obtained by rinsing the collected larvae or juveniles in distilled water and drying them briefly on filter paper.

Additionally, 100 mg of brine shrimp enriched *Artemia* metanauplii, a crustacean used as food during larval culture, were also collected as above for use as a negative control of non-specific amplification of *Mb vasa*.

RNA extraction

Total RNA was extracted from the testis using TRIZOL reagent (Invitrogen, CA, USA) following the manufacturer's instructions with some modifications which included centrifugation at 12,000 × *g* for 10 min at 2–8°C after homogenization with TRIZOL to reduce the excess of lipids and extracellular material and precipitation of RNA using a mixture of 0.25 mL of isopropanol and 0.25 mL of high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) to prevent co-precipitation of proteoglycan and polysaccharide. RNA concentration and purity were determined by measuring the OD at 260 nm and 280 nm in a GeneQuant *pro* spectrophotometer (Amersham Biosciences, Bavaria, Germany), and integrity was visualized by denaturing gel electrophoresis in TAE agarose (Masek et al. 2005).

Cloning and sequencing

One microgram of total RNA was used for first-strand cDNA synthesis by RT PCR using Superscript First-strand Synthesis kit for RT PCR (Invitrogen, CA, USA) according to manufacturer's instructions. In addition, 1 µg of total RNA samples for gene quantification was treated with DNAse I (DNA-*free*, Ambion, TX, USA) according to manufacturer's instructions to remove traces of gDNA.

Isolation of the *vasa* homologue in *M. brachydactyla* was carried out by PCR, using degenerate primers (dg *Vasa* F2 and dg *Vasa* R3, Table 1), which were designed based on the sequence alignment of the following invertebrate *vasa* homologues: *Nematostella vectensis* (accession number: AY730696), *C. gigas* (accession number: AY423380), *Daphnia magna* (accession number: AB193324), *L. vannamei* (accession number: DQ095772), *M. rosenbergii* (accession number: DQ339110) and *Apis mellifera* (accession number: NM 001040255). Amplification was carried out using 1 µL of cDNA from testis, 200 µM of each dNTP, 1 µM of each primer, 1 *Taq* DNA polymerase PCR buffer and 0.5 unit of *Taq* DNA polymerase (Invitrogen) in a final volume of 25 µL. Conditions for PCR were: initial denaturation at 94°C for 3 min; 40 cycles at 94°C for 1 min, 55°C for 45 s and 72°C for 1 min; and a final extension at 72°C for 5 min.

PCR products were cloned into pCR 2.1-TOPO vector (TOPO-TA cloning kit, Invitrogen, CA, USA), and clones containing the inserts were sequenced by an external laboratory (Sistemas Genómicos, Valencia, Spain) using primer walking with standard M13 primers flanking the insertion point in the plasmid and internal primers where necessary (Table 1).

The gDNA was extracted from muscle tissue using DNeasy Blood & Tissue kit (QIAGEN, North Rhine-Westphalia, Germany). Amplification of gDNA was performed by PCR using specific primers based on the partial cDNA sequence (Table 1). Conditions of PCR were as described above, using an annealing temperature of 62°C and a variable polymerization time, depending on the expected fragment size. PCR products were either excised and purified using QIAquick Gel Extraction kit (QIAGEN, North Rhine-Westphalia, Germany), or cloned as described above, and sequenced by primer walking.

Phylogenetic analysis

Amino acid sequence of the partial *Mb vasa* was aligned with *vasa*, *PL10* and *p68* sequences using

Table 1. Primers for cloning, sequencing and quantifing *Mb vasa* gene in *M. brachydactyla*.

Primer	Sense	Sequence (5′ → 3′)	
dg *Vasa* F2	Forward	ACDGGMTCCGGMAAAACGGC	Amplification
dg *Vasa* R3	Reverse	CCAATDCGRTGDACATAY	Amplification
V79 F1	Forward	TTCCTATTGCCAATGCTGCATTAC	Sequencing
V79 R1	Reverse	GCAGGCTTTCCTGGTGTGGCTG	Sequencing
V79 F2r	Reverse	TTAGGAGGAATGTCAGGATTTGC	Sequencing
V79 R2	Reverse	CAGTCTTCGGTAGATCGTAGTTG	Sequencing
SSR1	Reverse	TGTCTTGACTACAGATGGTTAGTC	Sequencing
SSR3	Forward	TTAGTGCTATGCCAGTGATCTGTC	Sequencing
V1120	Forward	CGCAACATTCCCAGAGGATGTG	Sequencing
V200	Forward	TTGCTTGTATTTATGGAGGAGTGG	Sequencing
V79 F2	Forward	GAGAAACTTGTCGAGTATCTGCG	Sequencing/qPCR
V79 R3	Reverse	AACACCTCGAATAT*CCAATCCTC	qPCR
BACTF1	Forward	CACGCCATCCTGCGTCTTGAC	qPCR
BACTR1	Reverse	GACCGTCAGGAAGCTCGTAGG	qPCR

Notes: Asterisk in V79 R3 indicates exon–exon junction to avoid amplification from gDNA traces. BACTF1 and BACTR1 correspond to specific primers used in *β-actin* gene quantification.

Clustal W. Phylogenetic trees were constructed by a minimum evolution analysis with statistical bootstrap support of 1000 replicates using Mega 4.0 software (Tamura et al. 2007).

Expression of Mb vasa in adult tissues

Amplification of *Mb vasa* and *β-actin* from adult gonads, hepatopancreas, gill tissue and heart was carried out by end-point PCR using specific primers (Table 1). Each reaction was carried out using 1 μL of cDNA, 200 μM of each dNTP, 1 μM of each primer, 1 *Taq* DNA polymerase PCR buffer and 0.5 units of *Taq* DNA polymerase (Invitrogen) in a final volume of 25 μL. Conditions for PCR were: initial denaturation at 94°C for 3 min; 40 cycles at 94°C for 1 min, 62°C for 45 s, and 72°C for 30 s; and a final extension at 72°C for 5 min.

Quantitative PCR

Expression of *Mb vasa* throughout larval development and first juvenile crab was estimated by qPCR using the absolute quantification method with SYBR green dye in a ABI 7300 thermal cycler (Applied Biosystems, CA, USA) using *β-actin* as the endogenous control gene. Amplification was carried out by PCR using 1 μL of cDNA, 0.5 μM of each primer and 1 SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) in a final volume of 20 μL. Primers for amplification were specifically designed based on the sequence of *Mb vasa* (Table 1) and *β-actin* (DDBJ/EMBL/GenBank databases accession number: DQ 990372.1; Table 1). Conditions for qPCR were: initial denaturation at 95°C for 5 min; 40 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 20 s; with a final dissociation stage included for melting curve analysis. A total of three assays were run, containing triplicates of each developmental stage and each batch of larvae for both genes. The sample belonging to *Artemia* was run once in triplicate for *Mb vasa* and *β-actin* genes. In addition, two dilutions of plasmid DNA (10^6 and 10^2 molecules μL^{-1}) were incorporated as positive controls in each qPCR.

Number of copies was estimated using a standard curve based on a serial 10-fold dilution (10^6 to 10 molecules·μL^{-1}) of plasmid, which contained the inserts of *Mb vasa* (865 bp) and *β-actin* (570 bp). The slope of the standard curves was described by the following equations for each gene:

$$Mbvasa,\ y = -3.373023x + 37.486584$$
$$\beta{-}actin,\ y = -3.39762x + 39.024899$$

with an efficiency of amplification of 97% for *Mb vasa* and 96% for *β-actin*.

Data analysis

Threshold value for each qPCR was manually adjusted and the number of copies for each sample was recalculated according to the standard curves. Then, expression levels of *Mb vasa* for each developmental stage and batch in each qPCR were normalized to the endogenous control, *β-actin*. Thereafter, the relative expression of *Mb vasa* for a given developmental stage in each sample was normalized per larvae or juvenile, which thereby provides a better estimation of the expression levels of *Mb vasa*.

Statistical analysis of expression levels between developmental stages was carried out using one-way ANOVA followed by a Holm–Sidak test for multiple comparisons. Significant differences were assumed

for $p < 0.05$. Expression levels were fitted to an exponential curve using SigmaPlot (SYSTAT, IL, USA).

Results

cDNA cloning of Mb vasa

A single fragment of cDNA of *M. brachydactyla vasa* (*Mb vasa*) of 865 bp was amplified using degenerate primers dg *Vasa* F2 and dg *Vasa* R3. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number EF607281. The deduced amino acid sequence resulted in a fragment of 293 amino acids, which contain seven conserved motifs found in the DEAD-box protein family (underlined in Figure 1): motif I (SGKT), Ia (PTRELA), Ib (TPGK), II (DEAD), III (SAT), IV (LIV) and V (ARGLD). In addition, two additional motifs, GG doublets and QxxR, observed in other presumptive *vasa* protein sequences were also found. BLAST analysis of the amino acid sequence of *Mb vasa* showed the highest similarity levels related to *vasa* homologues of the brachyuran decapod *Scylla paramamosain* (78%) and the shrimp *L. vannamei* (72%). Phylogenetic reconstruction showed that the putative *Mb vasa* protein sequence was consistently grouped into a *vasa* clade, which was separated from PL10- and p68-related DEAD-box proteins (Figure 2). Among the *vasa* cluster, *Mb vasa* was closely grouped with other decapod crustacean (brachyurans and shrimps) *vasa*-like sequences.

gDNA sequence and structure of Mb vasa

A partial open reading frame of 2210 bp from *Mb vasa* gDNA was isolated using specific primers (Table 1) and these sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number HM068594. This gDNA fragment was organized into five exons separated by four introns (Figures 1 and 3). Exons E1 and E4 showed two DEAD-box family conserved motifs each: I and Ia, and IV and V, respectively. Exon E2 contained three conserved motifs: Ib, II and III, while exon E3 did not show any conserved motifs. As shown in Figure 1, *Mb vasa* gDNA presented four microsatellites located within the introns I1 ($(CCTT)_{12}$, position 256-303; CT_{87}, position 372-545) and I2 ($GTCT_8$, position 1024-1055; GT_{24}, position 1056-1103).

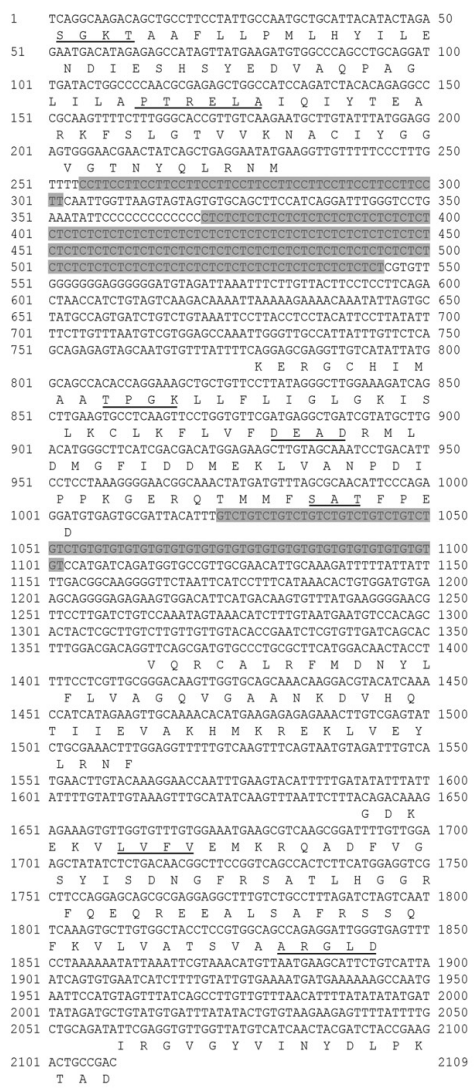

Figure 1. gDNA partial sequence of *Mb vasa* gene. Amino acid residues of coding fragments are below the nucleotide sequence. Underlined amino acid residues correspond to conserved motifs of DEAD-box family proteins. Nucleotides shaded in grey highlight microsatellites within introns.

Expression of Mb vasa in adult tissues

Using specific *Mb vasa* primers, a single fragment of approximate 250 bp was amplified only in testis and ovary from adult individuals (Figure 4). In contrast, *β-actin* was expressed in both somatic and gonad tissues.

Quantification of Mb vasa expression

Mb vasa was expressed in all larval stages and first juvenile crab (Figure 5), but neither *Mb vasa* nor *β-actin* was detected in enriched *Artemia* metanauplii (data not shown). Expression levels were low but detectable, especially in early larval stages

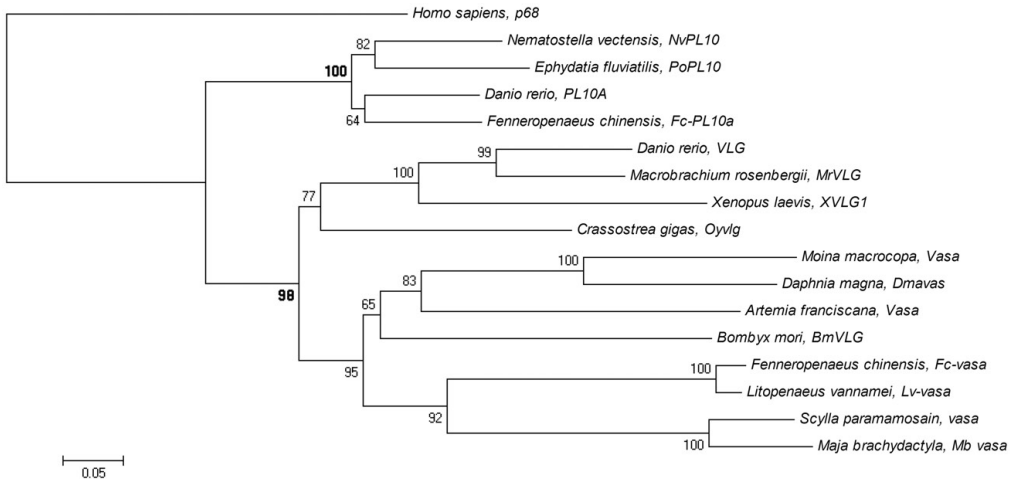

Figure 2. Phylogenetic tree of *vasa* and *PL10* amino acid sequences. *Mb vasa* clusters together with the *vasa* homologues of decapod crustaceans. Phylogenetic analysis generated by a minimum evolution method (1000 trials) and was rooted at its mid-point. Bootstrap values are shown as percentage scores for each node.

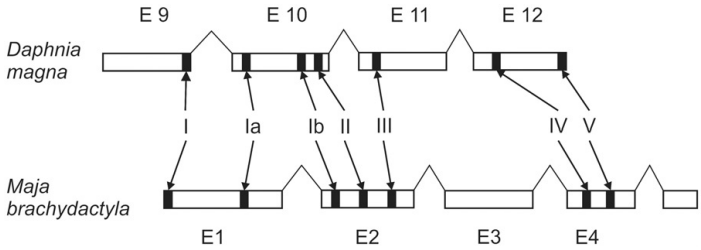

Figure 3. Diagrammatic comparison (not to scale) of gDNA structure of *vasa*-like gene homologues in *D. magna* (*Dmvas*, top, modified from Sagawa et al. 2005) and *M. brachydactyla* (*Mb vasa*, bottom). Conserved motifs are distributed differently between species.

(ZI and ZII). During larval development, relative expression of *Mb vasa* was constant, and increased significantly after metamorphosis to first juvenile crab ($F = 10.777$, $p = 0.003$). Overall, expression of *Mb vasa* during the early post-embryonic development fits an exponential growth curve (Figure 5).

Discussion

Mb vasa isolation

Based on the deduced amino acid sequence, phylogenetic analysis and the specific expression in adult gonads, *Mb vasa* can be considered a true homologue of *vasa* in the spider crab *M. brachydactyla*. The deduced amino acid sequence of *Mb vasa* contains the core region of a DEAD-box found in all proteins from this family. The fragment shows seven out of nine conserved motifs of DEAD-box proteins, organized into two domains: DEXDc and HELICc. Motifs I (SGKT), Ia (PTRELA), II (DEAD), and V (ARGLD) are involved in ATP binding, while motifs Ib (TPGK) and IV (LIV) are involved in RNA binding (Rocak and Linder 2004; Cordin et al. 2006). In addition, *Mb vasa* presents two additional motifs, GG doublet and QxxR, also described in the crystal structure of *vasa* in *Drosophila* (Sengoku et al. 2006). BLAST analysis also reported high similarity rates of *Mb vasa* with *vasa* homologues of *S. paramamosain* (78%, Cheng et al. unpublished data) and *L. vannamei* (72%, Aflalo et al. 2007). The identity of *Mb vasa* was also confirmed using phylogenetic analysis, which demonstrated that its putative protein only clusters with *vasa*-related DEAD-box proteins.

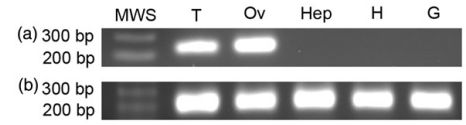

Figure 4. Expression of *Mb vasa* (A) and *β-actin* (B) in adult tissues. *Mb vasa* is restricted to testis and ovary, while *β-actin*, used as endogenous control, is expressed in both somatic and gonad tissues. H, heart; Hep, hepatopancreas, G, gill; MWS, molecular weight standard; Ov, ovary; and T, testis.

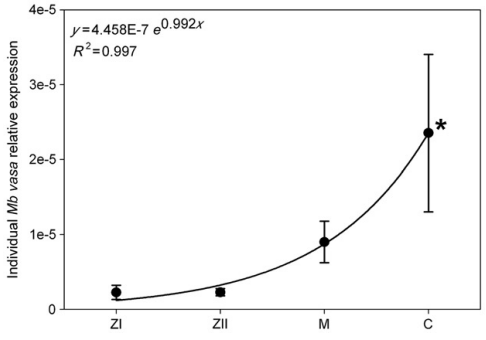

Figure 5. Relative expression levels of *Mb vasa* during larval development and first juvenile crab. *Mb vasa* is already expressed in the newly hatched larvae, and its expression remained constant during successive larval stages. Expression level in first juvenile crab is significantly higher, statistically, than larval stages ($p = 0.003$, $n = 3$). Asterisk denotes significant differences between stages of development. Values are shown as mean and error bars indicate standard deviation. ZI, zoea I; ZII, zoea II, M, megalopa; and CI, first juvenile crab.

Finally, *Mb vasa* is specifically expressed in adult gonads. In most metazoans, expression of *vasa* in gonads occurs exclusively in germ-line cells (Fujimura and Takamura 2000; Kobayashi et al. 2000; Toyooka et al. 2000; Mochizuki et al. 2001; Chang et al. 2002; Cardinali et al. 2004; Fabioux et al. 2004b; Extavour et al. 2005; Xu et al. 2005; Juliano et al. 2006; Sunanaga et al. 2006; Aflalo et al. 2007; Nakkrasae and Damrongphol 2007; Ohashi et al. 2007; Ye et al. 2007; Dill and Seaver 2008; Nagasawa et al. 2009; Özhan-Kizil et al. 2009), as well as in other germ-line related cells, such as nurse cells of hydra (Mochizuki et al. 2001) and oyster gonads (Fabioux et al. 2004b). Currently, *vasa* expression in crustacean adult gonads has been solely ascribed to germ cells (Aflalo et al. 2007; Nakkrasae and Damrongphol 2007; Özhan-Kizil et al. 2009) and it has been selected as a molecular marker of gonad ontogeny during early post-embryonic development in the spider crab.

The sequencing of the partial *Mb vasa* gDNA locus encompassing the isolated cDNA fragment was to aid the design of primers for qPCR between exon–exon junctions. The structure of the gDNA portion revealed that the core region is divided into at least four exons. Exon E1 and exon E2 contain motifs belonging to domain 1, while exon E4 contains motifs of domain 2. This organization differs from those described in *D. magna*, in which motifs of domain 1 are divided into exons 9 and 10, and domain 2 is divided in exons 11 and 12 (Figure 5 and Sagawa et al. 2005), or *Drosophila melanogaster*, where domain 1 is found clustered in one exon while the motifs of domain 2 are in exon 3 (Hoskins et al. 2007). In addition, the partial fragment of *Mb vasa* gDNA showed four microsatellites in two different introns in *M. brachydactyla*, while no microsatellites have been observed in *D. magna* (Sagawa et al. 2005). We do not discard any functional role, since transcription factors can be subjected to regulation by means of microsatellites (Li et al. 2002, 2004).

Mb vasa expression during early post-embryonic development

Due to the accuracy and high sensitivity of qPCR, expression of *vasa*-related genes during embryonic development and larval stages has been recently quantified (Fabioux et al. 2004a; Sagawa et al. 2005; Juliano et al. 2006; Sellars et al. 2007a; Fabioux et al. 2009; Özhan-Kizil et al. 2009; Rosner et al. 2009). Analysis of *vasa* expression confirmed the maternal supply of *vasa* mRNA and the onset of germ cell transcription in studies of embryonic development (Fabioux et al. 2004a; Sagawa et al. 2005; Juliano et al. 2006; Özhan-Kizil et al. 2009). In *D. magna*, qPCR results suggested that *Dmavas* was maternally supplied, while no signal was observed using probes for *in situ* hybridization (Sagawa et al. 2005). In the sea urchin, qPCR analysis also revealed that *Sp-vasa* showed a particular expression pattern (sharp decrease from ovary to egg and slow increase during embryogenesis) that was somewhat contrary to observations from *in situ* hybridization experiments where *Sp-vasa* displayed an early and uniform accumulation and late restriction during embryogenesis like other germ-line determinants (*Sp-ovo* and *Sp-seawi*) as revealed by *in situ* hybridization (Juliano et al. 2006).

In this study, *Mb vasa* was reported at low levels, but detectable in all stages of larval development and first juvenile crab by qPCR. In addition, *Mb vasa* expression was only ascribed to larvae and first juvenile of the spider crab, since specific primers did not cross-react or non-specifically amplify *Artemia* cDNA, a crustacean given as food to crab larvae. Overall, the pattern of the expression level of *Mb vasa* fits an exponential curve, which differs from those patterns previously reported during larval development

(Fabioux et al. 2004a; Sellars et al. 2007a). While *Mb vasa* increased exponentially during larval development and first juvenile crab, in oyster, *Oyvlg* decreased during embryogenesis and became undetectable after the trochophore larva stage, probably due to the reduced number of germ cells compared to the total number of cells in the larva (Fabioux et al. 2004a). Similarly, *MjPL10* expression decreased after hatching, and despite the augmented expression observed in nauplius IV stage, expression levels constantly decreased, being finally undetectable after the PL_{48} stage. Although the decrease in *MjPL10* expression observed during the development of the shrimp *Marsupenaeus japonicus* was also explained by the asymmetric increase of total RNA originated from somatic tissues (Sellars et al. 2007a), it should be noted that *MjPL10* belongs to the PL10 family, a DEAD-box protein highly related to *vasa* (Mochizuki et al. 2001), but not specifically expressed in germ cells.

The cause of the increase of *Mb vasa* expression during the early post-embryonic development is unknown. *Mb vasa* expression in larvae and juveniles might be ascribed to their gonads, assuming that there exists the same tissue-specific expression in larvae as was observed in adults. Thus, the increase of expression levels might be related to an increase of the number of germ cells, since it has been shown that germ cells proliferate continuously during larval and juvenile development in brachyurans (Payen 1974; Lee et al. 1994). We also do not discount that the pattern of *Mb vasa* expression levels could be related to an increase in germ cell activity, since the augmentation of *vasa* expression during embryogenesis in *Parhyale hawaiensis* (Özhan-Kizil et al. 2009) and *D. magna* (Sagawa et al. 2005) has been attributed to increased transcriptional activity rather than the number of germ cells expressing *vasa*.

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References

Aflalo ED, Bakhrat A, Raviv S, Harari D, Sagi A, Abdu U. 2007. Characterization of a *vasa*-like gene from the Pacific white shrimp *Litopenaeus vannamei* and its expression during oogenesis. Molecular Reproduction and Development. 74:172–177.

Andrés M, Estévez A, Anger K, Rotllant G. 2008. Developmental patterns of larval growth in the edible spider crab, *Maja brachydactyla* (Decapoda: Majidae). Journal of Experimental Marine Biology and Ecology. 357:35–40.

Andrés M, Estévez A, Rotllant G. 2007. Growth, survival and biochemical composition of spider crab *Maja brachydactyla* (Balss, 1922) (Decapoda: Majidae) larvae reared under different stocking densities, prey:larva ratios and diets. Aquaculture. 273:494–502.

Andrés M, Gisbert E, Díaz M, Moyano FJ, Estévez A, Rotllant G. 2010. Ontogenetic changes in digestive enzymatic capacities of the spider crab, *Maja brachydactyla* (Decapoda: Majidae). Journal of Experimental Marine Biology and Ecology. 389:75–84.

Cardinali M, Gioacchini G, Candiani S, Pestarino M, Yoshizaki G, Carnevali O. 2004. Hormonal regulation of *vasa*-like messenger RNA expression in the ovary of the marine teleost *Sparus aurata*. Biology of Reproduction. 70:737–743.

Chang CC, Dearden P, Akam M. 2002. Germ line development in the grasshopper *Schistocerca gregaria*: *vasa* as a marker. Developmental Biology. 252:100–118.

Cordin O, Banroques J, Tanner NK, Linder P. 2006. The DEAD-box protein family of RNA helicases. Gene. 367:17–37.

Corgos A, Freire J. 2006. Morphometric and gonad maturity in the spider crab *Maja brachydactyla*: a comparison of methods for estimating size at maturity in species with determinate growth. ICES Journal of Marine Science. 63:851–859.

Dill K, Seaver E. 2008. *Vasa* and *nanos* are coexpressed in somatic and germ line tissue from early embryonic cleavage stages through adulthood in the polychaete *Capitella* sp. I. Development Genes and Evolution. 218:453–463.

Extavour CG, Akam M. 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development. 130:5869–5884.

Extavour CG, Pang K, Matus DQ, Martindale MQ. 2005. *Vasa* and *nanos* expression patterns in a sea anemone and the evolution of bilaterian germ cell specification mechanisms. Evolution and Development. 7:201–215.

Fabioux C, Corporeau C, Quillien V, Favrel P, Huvet A. 2009. *In vivo* RNA interference in oyster-*vasa* silencing inhibits germ cell development. FEBS Journal. 276:2566–2573.

Fabioux C, Huvet A, Lelong C, Robert R, Pouvreau S, Daniel JY, Minguant C, Le Pennec M. 2004a. Oyster *vasa*-like gene as a marker of the germline cell development in *Crassostrea gigas*. Biochemical and Biophysical Research Communications. 320:592–598.

Fabioux C, Pouvreau S, Le Roux F, Huvet A. 2004b. The oyster *vasa*-like gene: a specific marker of the germline in *Crassostrea gigas*. Biochemical and Biophysical Research Communications. 315:897–904.

Freire J, Bernárdez C, Corgos A, Fernández L, González-Gurriarán E, Sampedro MP, Verísimo P. 2002. Management strategies for sustainable invertebrate fisheries in coastal ecosystems of Galicia (NW Spain). Aquatic Ecology. 36:41–50.

Fujimura M, Takamura K. 2000. Characterization of an ascidian DEAD-box gene, *Ci-DEAD1*: specific expression in the germ cells and its mRNA localization in the posterior-most blastomeres in early embryos. Development Genes and Evolution. 210:64–72.

Guerao G, Rotllant G. 2009. Post-larval development and sexual dimorphism of the spider crab *Maja brachydactyla* (Brachyura: Majidae). Scientia Marina. 73:797–808.

Guerao G, Rotllant G, Anger K. 2010. Characterization of larval moulting cycles in *Maja brachydactyla* (Brachyura, Majidae) reared in the laboratory. Aquaculture. 302:106–111.

Hoskins RA, Carlson JW, Kennedy C, Acevedo D, Evans-Holm M, Frise E, Wan KH, Park S, Mendez-Lago M, Rossi F, et al. 2007. Sequence finishing and mapping of *Drosophila melanogaster* heterochromatin. Science. 316:1625–1628.

Ikenishi K. 1998. Germ plasm in *Caenorhabditis elegans*, *Drosophila* and *Xenopus*. Development Growth and Differentiation. 40:1–10.

Juliano CE, Voronina E, Stack C, Aldrich M, Cameron AR, Wessel GM. 2006. Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage. Developmental Biology. 300:406–415.

Kobayashi T, Kajiura-Kobayashi H, Nagahama Y. 2000. Differential expression of *vasa* homologue gene in the germ cells during oogenesis and spermatogenesis in a teleost fish, tilapia, *Oreochromis niloticus*. Mechanisms of Development. 99:139–142.

Lang R. 1973. Die Ontogenese von *Maja squinado* (Crustacea malacostraca, Decapoda, Brachyura), unter besonderer Berücksichtigung der embryonalen Ernährung und der Entwicklung des Darmtraktes. Zoologische Jahrbücher Anatomie Bd. 90:389–449.

Lee TH, Yamauchi M, Yamazaki F. 1994. Sex-differentiation in the crab *Eriocheir japonicus* (Decapoda, Grapsidae). Invertebrate Reproduction and Development. 25:123–137.

Li YC, Korol AB, Fahima T, Beiles A, Nevo E. 2002. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Molecular Ecology. 11:2453–2465.

Li YC, Korol AB, Fahima T, Nevo E. 2004. Microsatellites within genes: structure, function, and evolution. Molecular Biology and Evolution. 21:991–1007.

Masek T, Vopalensky V, Suchomelova P, Pospisek M. 2005. Denaturing RNA electrophoresis in TAE agarose gels. Analytical Biochemistry. 336:46–50.

Mochizuki K, Nishimiya-Fujisawa C, Fujisawa T. 2001. Universal occurrence of the *vasa*-related genes among metazoans and their germline expression in *Hydra*. Development Genes and Evolution. 211:299–308.

Nagasawa K, Takeuchi Y, Miwa M, Higuchi K, Morita T, Mitsuboshi T, Miyaki K, Kadomura K, Yoshizaki G. 2009. cDNA cloning and expression analysis of a vasa-like gene in Pacific bluefin tuna *Thunnus orientalis*. Fisheries Science. 75:71–79.

Nakkrasae LI, Damrongphol P. 2007. A *vasa*-like gene in the giant freshwater prawn, *Macrobrachium rosenbergii*. Molecular Reproduction and Development. 74:835–842.

Noce T, Okamoto-Ito S, Tsunekawa N. 2001. *Vasa* homolog genes in mammalian germ cell development. Cell Structure and Function. 26:131–136.

Ohashi H, Umeda N, Hirazawa N, Ozaki Y, Miura C, Miura T. 2007. Expression of *vasa* (vas)-related genes in germ cells and specific interference with gene functions by double-stranded RNA in the monogenean, *Neobenedenia girellae*. International Journal for Parasitology. 37:515–523.

Özhan-Kizil G, Havemann J, Gerberding M. 2009. Germ cells in the crustacean *Parhyale hawaiensis* depend on *vasa* protein for their maintenance but not for their formation. Developmental Biology. 327:230–239.

Payen G. 1974. Morphogenèse sexuelle de quelques Brachyoures (Cyclométopes) au cours du développement embrionnaire, larvarie et postlarvaire. Bulletin du Museúm National d'Histoire Naturelle. 139:209–262.

Raz E. 2000. The function and regulation of *vasa*-like genes in germ-cell development. Genome Biology. 1:1017.1–1017.6.

Rocak S, Linder P. 2004. DEAD-box proteins: the driving forces behind RNA metabolism. Nature Reviews Molecular Cell Biology. 5:232–241.

Rosner A, Moiseeva E, Rinkevich Y, Lapidot Z, Rinkevich B. 2009. Vasa and the germ line lineage in a colonial urochordate. Developmental Biology. 331:113–128.

Rotllant G, González-Gurriarán E, Fernández L, Benhalima K, Ribes E. 2007. Ovarian maturation of the multi-spawning spider crab *Maja brachydactyla* (Decapoda: Majidae) with special reference to yolk formation. Marine Biology. 152:383–394.

Saffman EE, Lasko P. 1999. Germline development in vertebrates and invertebrates. CMLS Cellular and Molecular Life Sciences. 55:1141–1163.

Sagawa K, Yamagata H, Shiga Y. 2005. Exploring embryonic germ line development in the water flea, *Daphnia magna*, by zinc-finger-containing VASA as a marker. Gene Expression Patterns. 5:669–678.

Sampedro MP, González-Gurriarán E, Freire J, Muiño R. 1999. Morphometry and sexual maturity in the spider crab *Maja squinado* (Decapoda: Majidae) in Galicia, Spain. Journal of Crustacean Biology. 19:578–592.

Sellars MJ, Lyons RE, Grewe PM, Vuocolo T, Leeton L, Coman GJ, Degnan BM, Preston NP. 2007a. A PL10 *vasa*-like gene in the Kuruma shrimp, *Marsupenaeus japonicus*, expressed during development and in adult gonad. Marine Biotechnology. 9:377–387.

Sellars MJ, Vuocolo T, Leeton LA, Coman GJ, Degnan BM, Preston NP. 2007b. Real-time RT-PCR quantification of Kuruma shrimp transcripts: a comparison of relative and absolute quantification procedures. Journal of Biotechnology. 129:391–399.

Sengoku T, Nureki O, Nakamura A, Kobayashi S, Yokoyama S. 2006. Structural basis for RNA unwinding by the DEAD-box protein *Drosophila* Vasa. Cell. 125:287–300.

Simeó CG, Kurtz K, Chiva M, Ribes E, Rotllant G. 2010a. Spermatogenesis of the spider crab, *Maja brachydactyla*

(Decapoda: Brachyura). Journal of Morphology. 271:394–406.

Simeó CG, Kurtz K, Rotllant G, Chiva M, Ribes E. 2010b. Sperm ultrastructure of the spider crab *Maja brachydactyla* (Decapoda: Brachyura). Journal of Morphology. 271:407–417.

Simeó CG, Ribes E, Rotllant G. 2009. Internal anatomy and ultrastructure of the male reproductive system of the spider crab *Maja brachydactyla* (Decapoda: Brachyura). Tissue and Cell. 41:345–361.

Sunanaga T, Saito Y, Kawamura K. 2006. Postembryonic epigenesis of *Vasa*-positive germ cells from aggregated hemoblasts in the colonial ascidian, *Botryllus primigenus*. Development Growth and Differentiation. 48:87–100.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution. 24:1596–1599.

Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, Noce T. 2000. Expression and intracellular localization of mouse *Vasa*-homologue protein during germ cell development. Mechanisms of Development. 93:139–149.

Xu H, Gui J, Hong Y. 2005. Differential expression of *vasa* RNA and protein during spermatogenesis and oogenesis in the gibel carp (*Carassius auratus gibelio*), a bisexually and gynogenetically reproducing vertebrate. Developmental Dynamics. 233:872–882.

Ye D, Lv D, Song P, Peng M, Chen Y, Guo M, Yang Q, Hu Y. 2007. Cloning and characterization of a rice field eel *vasa* -like gene cDNA and its expression in gonads during natural sex transformation. Biochemical Genetics. 45:211–224.

2. Qualitat de les postes en captivitat

Article 6

Títol: The effect of male absence on the larval production of the spider crab *Maja brachydactyla*

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Informe de la contribució del doctorand

La hipòtesi de treball i la metodologia a seguir varen estar realitzades per la Dra. G. Rotllant. El doctorand es va encarregar de la gestió dels reproductors i va realitzar els mostrejos en col·laboració de les coautores i personal de suport de l'IRTA Sant Carles de la Ràpita. El doctorand realitzà les anàlisis bioquímiques proximals i de biomassa de les mostres en col·laboració de la Dra. M. Andrés, Dra. A. Estévez i el personal de laboratori de l'IRTA Sant Carles de la Ràpita. El tractament i interpretació de les dades, així com la redacció del manuscrit foren realitzades pel doctorant amb la col·laboració de les coautores.

Dra. Guiomar Rotllant Estelrich

Resum

L'efecte de l'absència dels mascles en la producció larvària de la cabra de mar es va estudiar mitjançant l'elaboració d'un experiment en el qual les femelles es mantingueren en captivitat en presència (MP) o absència (MA) de mascles. Els reproductors foren capturats a la Ria d'A Coruña i transportats fins les instal·lacions de l'IRTA a Sant Carles de la Ràpita, on es van distribuir aleatòriament en els grups experimentals, amb tres tancs per tractament. Cada tanc del grup MA estava format per set femelles, mentre que els grups de MP estaven formats inicialment per sis femelles i dos mascles. La supervivència de les femelles es va controlar diàriament, però només els mascles es van reposar al llarg de l'experiment per mantenir una proporció de sexes de 3 femelles: 1 mascle. Els tancs dels reproductors tenien un volum de 2.000 litres i estaven connectats a un sistema de recirculació IRTAmar™ per mantenir constants les condicions d'estabulació a 18,5±1,0ºC i 34,8±0,7‰ al llarg dels dos anys de l'experiment. Els reproductors foren alimentats a sacietat alternativament amb musclo fresc (*Mytilus* sp.) i cranc congelat (*Liocarcinus depurator*). El nombre de larves, i quan fou possible, el pes sec i la composició bioquímica proximal de cada grup de larves acabades de descloure es va calcular i les dades foren agrupades per estacions. La supervivència de les femelles a final de l'experiment fou nul·la en el grup MP, mentre què en el grup MA fou d'un 60% aproximadament. La longevitat de les femelles en MP fou significativament menor (T=98,500; p=0,002). Els receptacles seminals de les femelles de MA a final de l'experiment es trobaven buits, en contrast a aquells de les femelles de MP que presentaven abundants quantitats de fluids seminals, espermatòfors i espermatozoides lliures. La producció larvària (z=753,213; p<0,001) fou significativament menor en MA a conseqüència de l'esgotament de les reserves de l'esperma. En relació al pes sec individual i la composició bioquímica de les larves, només el contingut en proteïnes (F=4,544; p=0,037) fou significativament menor en MA. S'observaren diferències significatives estacionals en el pes sec individual (en ambdós tractaments) i en les proteïnes i lípids en el grup MP, la qual cosa suggereix que la composició bioquímica de les larves es veié més afectada pel temps en captivitat que per l'efecte de la presència dels mascles. Tanmateix, considerant que la producció de larves en el grup MP es va estancar durant la darrera part de l'experiment com a conseqüència de la baixa supervivència de les femelles, la presència dels mascles es deuria gestionar per mantenir una producció larvària alta sense amenaçar la supervivència de les femelles. Així, es recomana mantenir les femelles segregades dels mascles, i només transferir els mascles als tancs de les femelles per a la còpula.

The effect of male absence on the larval production of the spider crab *Maja brachydactyla*

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RUNNING TITLE

Larval production of spider crab in male absence

KEYWORDS

Stocking conditions, broodstock, biochemical composition, sperm viability, seminal receptacles.

ABSTRACT

The spider crab *Maja brachydactyla*, Balss can produce three consecutive broods per breeding season in the wild, whereas females in captivity can spawn up to four times in the absence of males. The effect of male absence on the larval production of the spider crab *M. brachydactyla* was studied in a two-year experiment in which females were kept in captivity in the presence (MP) or absence (MA) of males. The broodstock were maintained under natural photoperiod conditions at 18.5±1.0ºC and 34.8±0.7 $g \cdot L^{-1}$. The number of larvae, and when possible, the dry weight (DW) and proximate biochemical composition of each larval batch were calculated and the data grouped seasonally. The larval production (p<0.001) and protein content (p=0.037) were significantly lower in the absence of males. However, considering that the larval production of the MP females decreased due to the low female survival rate, particularly in the last part of the experiment, the presence of males should be managed in order to maintain a high larval production and condition without jeopardizing the survival of females. Therefore, we recommend keeping females segregated from males and transferring males to female tanks only to mate.

1.- Introduction

The reproductive system of brachyuran females has two blinded pouches called seminal receptacles in which sperm and seminal fluids are stored (Diesel, 1991). Therefore, brachyuran females can fertilize their oocytes using stored sperm in consecutive spawns without needing to mate. However, fertilization success in the absence of males may be affected in consecutive spawns due to the decrease in the sperm reserves. In the tanner crab *Chionoecetes bairdi* Rathburn, it was found that the percentage of viable egg clutches in consecutive spawns decreased in relation to the decrease in the number of sperm cells stored in the seminal receptacles (Paul, 1984). In the blue crab *Callinectes sapidus* Rathburn, it was found that the clutch volume and the percentage of developing embryos normally did not vary significantly over the four consecutive broods, suggesting that the fertilization success was not affected as long as the sperm reserves supplied sufficient sperm cells (Darnell, Rittschof, Darnell & McDowell, 2009). Larvae from spawns obtained in the absence of males are generally considered to be normal. No differences in survival and larval development between first and second clutches that used sperm reserves were found in *Mithraculus forceps* A. Milne-Edwards (Penha-Lopes, Rhyne, Figueiredo, Lin & Narciso, 2006). The larval carapace width and survival in starvation were not different between consecutive spawns in *C. sapidus* (Darnell *et al*., 2009). However, in species of aquacultural interest, such as the swimming crab *Portunus trituberculatus* (Miers), commercial hatcheries only use the first spawn of the breeding season because several egg and larval parameters have been found to decrease significantly between the first and second broods (Wu, Cheng, Zeng, Wang & Cui, 2010 and references therein).

The spider crab *Maja brachydactyla* Balss is an important fishery species and has interesting biological characteristics that make it appropriate for aquaculture (González-Gurriarán, Fernández, Freire & Muiño, 1998; Iglesias, Sánchez, Moxica, Fuentes, Otero & Pérez, 2002; Andrés, Estévez & Rotllant, 2007; Marques, Teixeira, Barrento, Anacleto, Carvalho & Nunes, 2010; Verísimo, Bernárdez, González-Gurriarán, Freire, Muiño & Fernández, 2010). *M. brachydactyla* females mate with several males during the migration to deep waters, and the sperm stored in the seminal receptacles can be used in successive spawns if males are not available. Considering the length of the breeding cycle and the incubation time, it has been estimated that there are three to four egg clutches per breeding cycle in the wild; however, up to six

clutches have fully developed in captivity in the absence of males (González-Gurriarán *et al*., 1998; García-Flórez & Fernández-Rueda, 2000).

In the present study, we carried out a two-year experiment in which females of the spider crab were kept either in the presence (MP) or absence (MA) of males to test the hypothesis that the absence of males would have an effect on spider crab larval production. The results allowed us to determine both the time in which females can rely on stored sperm for larval production and the viability of the stored sperm. In addition, we sampled the newly hatched larvae (NHL) to detect the effect of using stored sperm on their biochemical composition. Recommendations for aquaculture practices are also discussed.

2.- Material and methods

2.1.- Broodstock capture and maintenance

Adult *Maja brachydactyla* were captured with coastal fishery vessels off the Galician coast (NE Atlantic) in November 2004, and transported to IRTA (Sant Carles de la Ràpita, Tarragona, Spain) in high humidity containers at 8±2ºC. After one month of acclimation to captive conditions, the broodstock were distributed and reared for two years (from 1 January, 2005 to 31 December, 2006) in 2,000-L tanks connected to a recirculation unit (IRTAMar®; Ingesom, Castelló, Spain) with constant conditions of temperature (18.5±1.0ºC) and salinity (34.8±0.7gL^{-1}), and a natural photoperiod. The broodstock were fed *ad libitum* with a combination of fresh and frozen mussels (*Mytilus* sp.) and frozen crab (*Liocarcinus depurator* Linnaeus).

In this study we used primiparous (only a few epibionts on the top of their carapace (González-Gurriarán *et al*., 1998)) adult females (carapace length=161.6±6.7 mm; body weight=1177.40±155.60 g; mean± standard deviation). Two treatments were run in triplicate as follows: male absence – MA – (7 females per tank), and male presence – MP – (8 individuals per tank; initial sex ratio of 3F:1M; F, female; M, male). Only males that died during the experiment were replaced in order to keep the sex ratio as close as possible to 3F:1M. Female mortality was monitored daily throughout the experimental period for each treatment, and female survival (as %) was determined in seasons.

The seminal receptacles were extracted and fixed in Bouin's fluid for 24 to 48 h in order to observe them under light microscopy at the end of the experiment. Samples were processed following Simeó, Ribes & Rotllant (2009), and 3-µm sections were stained with Harris's hematoxylin-eosin dye and pho-

tographed using an Olympus DP70 (Olympus, Hamburg, Germany) camera connected to a Leica DM LB light microscope (Leica Microsystems, Wetzlar, Hessen, Germany).

2.2.- Larval collection

A larval batch was defined as the presence of actively swimming newly hatched larvae (NHL) in a broodstock tank. When possible, larval collection and proximate biochemical analysis were carried out following Andrés, Estévez, Simeó & Rotllant (2010). The larval contents of each biochemical component were calculated as $\mu g \cdot mg\ DW^{-1}$.

2.3.- Statistical analysis

The data sets were analyzed and plotted using the SigmaPlot 9 and SigmaStat 3 software packages (Systat Software Inc., Chicago, Illinois, USA). Data from tanks belonging to the same treatment were pooled and grouped in seasons (3 months per season) for statistical analysis. The life span of females was compared by means of a Mann-Whitney Rank Sum test, and the total larval production between treatments was compared using a z- test. Differences in the larval DW and proximate biochemical contents between treatments (absence vs. presence of males) and time in captivity (number of seasons in captivity) were evaluated using a two-way ANOVA analysis. Only data from winter 2005 to spring 2006 were used so that interactions between factors could be detected. Thus, changes of full data sets in terms of larval DW and biochemical composition were evaluated using a one way ANOVA analysis. Post-hoc comparisons among groups were performed with the Holm-Sidak test. The normal distribution of data was checked with a Kolmogorov-Smirnov normality test. For the data values that were still not normally distributed after transformation, a Kruskal-Wallis one way ANOVA on ranks was applied. Statistically significant differences ($p<0.05$) were indicated in tables and figures by different letters.

3.- Results

3.1.- Broodstock

The survival of females decreased after the fourth season (during the second year in captivity). In the MA group survival was more than 60%, but in the MP group there was total mortality at the end of the experiment (Fig. 1). The life span of females in MA (7.2±1.3 seasons in captivity) was significantly longer (p=0.002) than in MP (5.2±0.9 seasons in captivity).

The female seminal receptacles were dissected at the end of the experiment and it was found that those from the MA group were thinner than those from the MP group (Fig. 2). In MA, the seminal receptacles only contained small amounts of secretions, whereas in MP these receptacles were filled with a mixture of seminal secretions, spermatophores (masses of spermatozoa surrounded by an acellular wall in which spermatozoa are transferred to the female) and free spermatozoa.

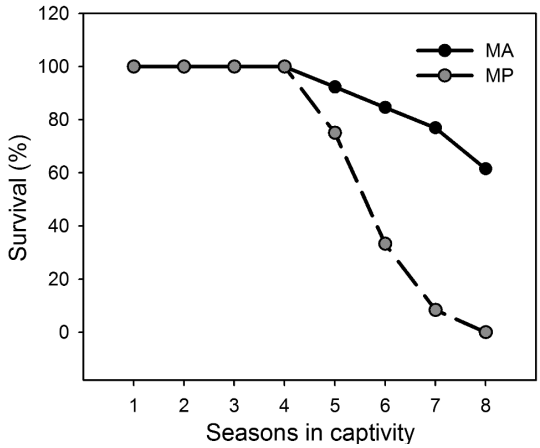

Fig. 1. Female survival of *Maja brachydactyla* Balss kept for two years in captivity in two treatments: MA (male absence) and MP (male presence).

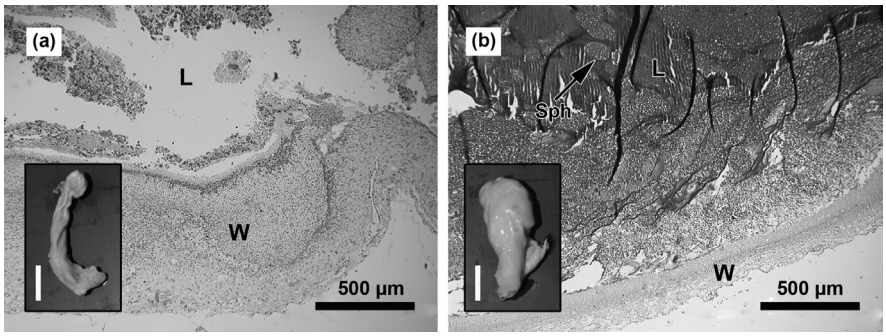

Fig. 2. Longitudinal sections of the seminal receptacles of female *Maja brachydactyla* Balss in the (a) absence and (b) presence of males. Photographs in the left corner of (a) and (b) show the external appearance of the seminal receptacles. The white scale bar in the photographs indicates 1 cm. L, lumen, Sph, spermatophore, W, wall of the seminal receptacle.

3.2.- Larvae

Larval production was significantly higher (p<0.001) in the MP group, which produced 3.9 million NHL, while the MA group only produced 1.9 million larvae. Seasonal variation in the larval production of the MP group showed maximum values in the second and fourth seasons (ca. 850,000 NHL) and also in the sixth season (ca. 700,000 NHL) in captivity (Fig. 3). After the seventh season, in which larval production decreased greatly, no more NHL were produced. In MA, larval production was concentrated in the second season (ca. 1.2 million NHL), and it remained at around 100,000 NHL in the following seasons until the seventh season, when no more larvae were obtained.

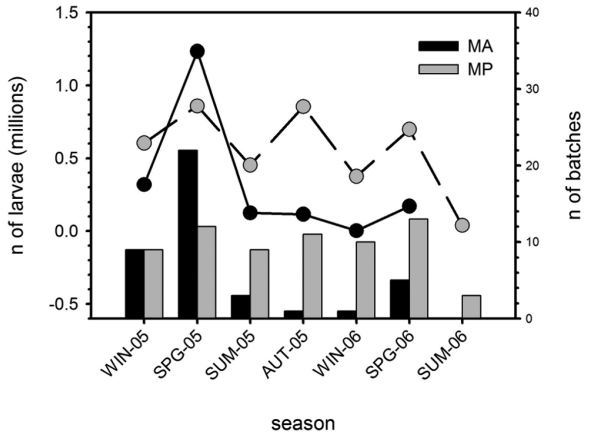

Fig. 3. Number of newly hatched larvae (NHL) per batch obtained in captivity from *Maja brachydactyla* Balss females over two years in the two treatments: MA (male absence) and MP (male presence). Circles indicate the number of NHL and bars indicate the number of batches.

The DW of the NHL was not affected by the absence of males (p=0.107). However, the DW was affected by the time in captivity (p<0.001), and a significant decrease in larval DW was observed in both groups (MA, p=0.028 and MP, p<0.001) (Table 1). The interaction between the two factors was not significant (p=0.888).

The PR content of the NHL was affected by the absence of males (p=0.037), and it was significantly higher in the MP group. Time in captivity also significantly affected the PR content of NHL (p=0.029), although the interaction between factors was not significant (p=0.386). Unlike the DW, the PR content of NHL increased during the first four seasons. The fourth season showed the highest PR content in both treatments, which then decreased (Table 1). Differences in relation to time in captivity were only significant in the MP group (p<0.001).

Table 1. Dry weight, protein, carbohydrate and lipid content of newly hatched larvae of *Maja brachydactyla* Balss obtained in the presence or absence of males during the experiment. n indicates the number of batches. The number of batches used for the biochemical analyses is only indicated for the PR analysis. Data are shown as mean±S.D. Different superscripts within a column indicate significant differences ($p<0.05$).

Seasons in captivity	DW (µg)				PR (µg·mg DW^{-1})				CH (µg·mg DW^{-1})		LP (µg·mg DW^{-1})	
	MA	n	MP	n	MA	n	MP	n	MA	MP	MA	MP
1	$94±9^{ab}$	7	$99±12^{a}$	8	299±62	7	$306±40^{b}$	8	18±7	18±5	50±18	$80±14^{ab}$
2	$104±5^{a}$	9	$97±10^{ac}$	7	283±55	9	$304±64^{b}$	7	18±2	18±3	76±10	$64±9^{b}$
3	$91±20^{ab}$	3	$90±5^{ac}$	8	244±80	3	$374±116^{ac}$	8	16±1	21±7	69±23	$85±21^{ab}$
4	$85±0^{ab}$	1	$76±13^{bc}$	11	368±0	1	$489±112^{a}$	11	16±0	18±6	67±0	$98±29^{a}$
5	$77±0^{ab}$	1	$65±14^{b}$	8	240±0	1	$280±95^{b}$	8	22±0	26±10	61±0	$68±19^{ab}$
6	$80±18^{b}$	4	$75±9^{bc}$	10	261±38	4	$260±58^{b}$	10	18±3	17±4	54±16	$65±25^{b}$
7			$65±6^{b}$	3			$309±60^{b}$	3		23±4		$53±16^{b}$

Abbreviations: CH, carbohydrate, DW, dry weight, LP, lipid, MA, male absence, MP, male presence, PR, protein

The carbohydrate (CH) content of the NHL was not affected by the absence of males (p=0.412) or the time in captivity (p=0.552). The interaction between the factors was also not significant (p=0.889). The seasonal changes in the CH content of the NHL showed an irregular pattern (Table 1).

The larval LP content was affected by the absence of males (p=0.016), and the LP content of NHL from the MP group was significantly higher than that of the NHL from the MA group. However, neither the effect of time in captivity (p=0.210) nor the interaction between factors (p=0.384) showed statistical differences. Seasonal variations in the LP content were only significant in the MP group (p=0.003), and showed a similar pattern to the PR content, with a significantly higher LP content in the fourth season than in the second, sixth and seventh seasons in captivity (Table 1).

4.- Discussion

The effect of male absence on the reproductive success during consecutive spawns varies greatly between species. In *Telmessus cheiragonus* White and *P. trituberculatus*, the number of eggs did not differ between the first and second spawns, even when they occurred in different years (Nagao & Munehara, 2007; Wu *et al*., 2010). The clutch volume in *C. sapidus* decreased significantly in the fifth spawn, but not in the four previous broods that were spawned in the same breeding season (Darnell *et al*., 2009). However, the number of females with viable eggs decreased over one breeding season in the mud crab *Rhithropanopeus harrisii* (Gould), which has four spawns per season (Morgan, Goy & Costlow Jr, 1983), and over two consecutive seasons in *C. bairdi*, which only has one egg clutch per season (Paul, 1984). In the present study, we tested the hypothesis that the absence of males would have an effect on spider crab larval production. As the number of NHL can be used as an estimate of fertilization success, we expected that the number of NHL produced in MP would be greater than that produced in MA. The significantly higher number of NHL produced in MP (3.9 million) corroborates this hypothesis. The seasonal pattern of larval production also shows that (1) the number of NHL in MP was always higher than in MA, with the single exception of the second season, and (2) larval production in MP lasted one more season than in MA. These results suggest that the sperm reserves were consumed during the successive spawns in MA, which would explain the emptiness of the seminal receptacles in the females of the MA group at the end of the experiment (Figure 2).

Newly hatched larvae from eggs fertilized with stored sperm are generally considered viable; however, in the second brood of *P. trituberculatus*, several biometric and biochemical parameters were found to be significantly lower (Cheung, 1968; Morgan *et al*., 1983; Haddon & Wear, 1993; Penha-Lopes *et al*., 2006; Darnell *et al*., 2009; Wu *et al*., 2010). In our study, only the PR content of the NHL showed significant differences between treatments, which suggests that the absence of males in the rearing tank had little influence on the larval composition. Furthermore, since the DW, and PR and LP content were all affected by the spawning season, it seems likely that the time in captivity had more effect on the larval composition than the absence of males. Indeed, the seasonal pattern of the larval DW and composition was similar in both treatments. These results are similar to those reported by Andrés *et al*. (2010), who also observed significant monthly variations in the DW, and PR and LP content of the NHL. Only the photoperiod had been modified in the two experiments, suggesting that the photoperiod plays an important role in controlling the reproduction of the spider crab.

Brachyuran sperm remains viable in the seminal receptacles during one breeding season for many species, and the stored sperm may fertilize from one to several egg clutches, depending on the species (Nagao & Munehara, 2007). However, stored sperm successfully fertilized the second egg clutch in *C. bairdi*, *Chionoecetes opilio* (O. Fabricius) and *T. cheiragonus*, which only carry one egg clutch per year (Paul, 1984; Sainte-Marie, 1993; Nagao & Munehara, 2007). The sperm of *Uca lactea* (De Haan) and *C. sapidus*, which spawn several times during one breeding season, was also viable after two years of storage in the female seminal receptacles (Yamaguchi, 1998; Darnell *et al*., 2009). The viability of stored sperm in the spider crab is evidenced by the presence of NHL in the MA group; hence, we can conclude that stored sperm remain viable for at least six seasons.

The survival of females in the presence of males was nil at the end of the experiment, while in the absence of males it exceeded 60%, indicating that females have a significantly longer life span in the absence of males. In addition, the larval production of the MP group decreased greatly in the last part of the experiment when female survival reached its lowest levels. These results suggest that the presence of males has a negative effect on female fitness, and consequently, on seed production. Similarly, female mortality of the signal crayfish caused a decrease in the juvenile production when females were kept during two consecutive reproductive cycles in the presence of males (Celada, Antolin, Carral, Perez & Saez-Royuela, 2007). In the aqua-

cultural context, reducing the mortality of females would extend the production lifetime of the broodstock, and would therefore optimize hatchery resources. Male agonistic behavior is one possible cause of female mortality, which has been observed in other brachyuran species in captivity (Stevcic, 1971; Diesel, 1991; Rondeau & Sainte-Marie, 2001). Lost walking legs and male mate-guarding were occasionally observed in this experiment, and male harassment and take-over attempts have been reported in other Majoidea (Hinsch, 1968; Schöne, 1968). Our results show that it is necessary to manage the presence of males to maintain the high larval production and condition without jeopardizing the survival of females. Therefore, we recommend keeping females segregated from males and transferring males to female tanks only to mate.

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6.- References

Andrés M, Estévez A, Rotllant G (2007) Growth, survival and biochemical composition of spider crab *Maja brachydactyla* (Balss, 1922) (Decapoda: Majidae) larvae reared under different stocking densities, prey:larva ratios and diets. Aquaculture, 273, 494-502.

Andrés M, Estévez A, Simeó CG, Rotllant G (2010) Annual variation in the biochemical composition of newly hatched larvae of *Maja brachydactyla* in captivity. Aquaculture, 310, 99-105.

Celada JD, Antolin JI, Carral JM, Perez JR, Saez-Royuela M (2007) Effects of breeder reuse on the reproductive potential of the signal crayfish (*Pacifastacus leniusculus* Dana. Astacidae) in culture. Aquac Int, 15, 37-42.

Cheung TS (1968) Trans-molt retention of sperm in the female stone crab, *Menippe mercenaria* (Say). Crustaceana, 15, 117-120.

Darnell MZ, Rittschof D, Darnell KM, McDowell RE (2009) Lifetime reproductive potential of female blue crabs *Callinectes sapidus* in North Carolina, USA. Mar Ecol Prog Ser, 394, 153-163.

Diesel R (1991) Sperm competition and the evolution of mating behaviour in Brachyura, with special reference to spider crabs (Decapoda, Majidae). In: Crustacean Sexual Biology (ed. by Bauer RT, Martin JW). Columbia University Press, New York, pp. 145-163.

García-Flórez L, Fernández-Rueda P (2000) Reproductive biology of spider crab females (*Maja brachydactyla*) off the coast of Asturias (north-west Spain). Journal of the Marine Biological Association of the United Kingdom, 80, 1071-1076.

González-Gurriarán E, Fernández L, Freire J, Muiño R (1998) Mating and role of seminal receptacles in the reproductive biology of the spider crab *Maja squinado* (Decapoda: Majidae). J Exp Mar Bio Ecol, 220, 269-285.

Haddon M, Wear RG (1993) Seasonal incidence of egg-bearing in the New Zealand paddle crab *Ovalipes catharus* (Crustacea: Brachyura), and its production of multiple egg batches. N Z J Mar Freshwater Res, 27, 287-293.

Hinsch GW (1968) Reproductive behavior in the spider crab, *Libinia emarginata* (L.). Biol Bull, 135, 273-278.

Iglesias J, Sánchez FJ, Moxica C, Fuentes L, Otero JJ, Pérez JL (2002) Datos preliminares sobre el cultivo de larvas y juveniles de centolla *Maja squinado* Herbst, 1788 en el Centro Oceanográfico de Vigo del Instituto Español de Oceanografía. Bol Inst Esp Oceanogr, 18, 25- 30.

Marques A, Teixeira B, Barrento S, Anacleto P, Carvalho ML, Nunes ML (2010) Chemical composition of Atlantic spider crab *Maja brachydactyla*: Human health implications. J Food Compos Anal, 23, 230-237.

Morgan SG, Goy JW, Costlow Jr JD (1983) Multiple ovipositions from single matings in the mud crab *Rhithropanopeus harrisii*. J Crustacean Biol, 3, 542-547.

Nagao J, Munehara H (2007) Characteristics of broods fertilized with fresh or stored sperm in the helmet crab *Telmessus cheiragonus*. J Crustacean Biol, 27, 565-569.

Paul AJ (1984) Mating frequency and viability of stored sperm in the tanner crab *Chionoecetes bairdi* (Decapoda, Majidae). J Crustacean Biol, 4, 375-381.

Penha-Lopes G, Rhyne AL, Figueiredo J, Lin J, Narciso L (2006) Can larvae produced from stored sperm in the ornamental crab *Mithraculus forceps* (A. Milne Edwards, 1875)(Decapoda: Brachyura: Majidae) be used in aquaculture? Aquaculture, 257, 282-286.

Rondeau A, Sainte-Marie B (2001) Variable mate-guarding time and sperm allocation by male snow crabs (*Chionoecetes opilio*) in response to sexual competition, and their impact on the mating success of females. Biol Bull, 201, 204-217.

Sainte-Marie B (1993) Reproductive cycle and fecundity of primiparous and multiparous female snow crab, *Chionoecetes opilio*, in the northwest Gulf of Saint Lawrence. Can J Fish Aquat Sci, 50, 2147-2156.

Schöne H (1968) Agonistic and sexual display in aquatic and semi-terrestrial Brachyuran crabs. Am Zool, 8, 641-654.

Simeó CG, Ribes E, Rotllant G (2009) Internal anatomy and ultrastructure of the male reproductive system of the spider crab *Maja brachydactyla* (Decapoda: Brachyura). Tissue and Cell, 41, 345-361.

Stevcic Z (1971) Laboratory observations on the aggregations of the spiny spider crab (*Maja squinado* herbst). Anim Behav, 19, 18-25.

Verísimo P, Bernárdez C, González-Gurriarán E, Freire J, Muiño R, Fernández L (2010) Changes between consecutive broods in the fecundity of the spider crab, *Maja brachydactyla*. ICES J Mar Sci, 68, 472-478.

Wu X, Cheng Y, Zeng C, Wang C, Cui Z (2010) Reproductive performance and offspring quality of the first and the second brood of female swimming crab, *Portunus trituberculatus*. Aquaculture, 303, 94-100.

Yamaguchi T (1998) Longevity of sperm of the fiddler crab *Uca lactea* (De Haan, 1835)(Decapoda, Brachyura, Ocypodidae). Crustaceana, 71, 712-713.

Article 7

Títol: Effect of photoperiod on larval production of the spider crab *Maja brachydactyla*

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Informe de la contribució del doctorand

La hipòtesi de treball i la metodologia a seguir varen estar realitzades per la Dra. G. Rotllant, la Dra. A. Estévez i el doctorant. El doctorand es va encarregar de la gestió dels reproductors i va realitzar els mostrejos en col·laboració amb el personal de suport de l'IRTA Sant Carles de la Ràpita. El doctorand realitzà les anàlisis bioquímiques proximals i de biomassa de les mostres amb la col·laboració del personal de suport de l'IRTA Sant Carles de la Ràpita. El tractament i interpretació de les dades, així com la redacció del manuscrit foren realitzades pel doctorant amb la col·laboració de les coautores.

Dra. Guiomar Rotllant Estelrich

Resum

L'efecte del fotoperíode en la reproducció de la cabra de mar, *Maja brachydactyla*, s'estudià mitjançant dos experiments de dos anys de duració cadascun, un amb fotoperíodes constats (CP) i un altre amb els fotoperíodes desfasats (DP). Els reproductors foren capturats a la Ria d'A Coruña i transportats fins a l'IRTA - Sant Carles de la Ràpita, on es van distribuir aleatòriament en els grups experimentals, amb dues rèpliques per tractament. Els reproductors utilitzats a l'experiment CP es mantingueren sota un dels tres règims de llum: 8L (8hL, llum: 16hD, foscor), 12L (12hL:12hD) i 16L (16hL:8hD). Durant l'experiment DP, els reproductors es mantingueren sota fotoperíodes naturals després d'una aclimatació amb un fotoperíode constant 8L durant 3 i 6 mesos, corresponents als grups 3M i 6M. Un grup control (CTRL) es mantingué sempre en fotoperíode natural. A cada tanc es van distribuir inicialment sis femelles i dos mascles. Només els mascles es van reposar al llarg de l'experiment per mantenir una proporció de sexes de 3 femelles: 1 mascle. La supervivència de les femelles es va controlar diàriament i les dades es van agrupar per estacions. Els tancs dels reproductors tenien un volum de 2.000 litres i estaven connectats a un sistema de recirculació IRTAmar™ per mantenir constants les condicions d'estabulació a 18,2±0,6ºC i 34,9±0,9‰ a l'experiment CP; i 18,8±1,1ºC i 34,7±0,9‰ a l'experiment DP. Els reproductors foren alimentats a sacietat alternativament amb musclo fresc (*Mytilus* sp.) i cranc congelat (*Liocarcinus depurator*). Al llarg dels experiments, s'anotà el nombre de larves acabades de descloure, i quan fou possible, es prengueren mostres per les anàlisis de biomassa i bioquímica (pes sec, proteïna, PR; carbohidrats, CH i lípids, LP). La supervivència final de les femelles a l'experiment CP fou similar en els tres tractaments: 42% en 8L i 16L, i 33% en 12L. A l'experiment CP, el nombre de larves acabades de descloure varià significativament entre els tractaments: la producció del grup 8L fou major que la resta dels grups, què deixaren de produir larves després de la primavera (16L) i tardor (12L) del primer any. No es detectaren diferències significatives en el pes sec individual, ni la composició bioquímica de les larves acabades de descloure entre tractaments ni estacions. El percentatge de femelles vives a final de l'experiment DP fou del 8% en els grups CTRL i 3M, mentre que al tractament 6M fou del 58%. La màxima producció larvària es registrà en la primavera del primer any en el CTRL, en l'hivern del primer any en el grup 3M, i durant el període d'aclimatació en el grup 6M. Aquests resultats indiquen que el patró estacional de larves acabades de descloure no varià amb la manipulació del fotoperíode, ja que la màxima producció larvària es correspon a la primavera natural. La composició bioquímica de les larves mostrà diferències significatives entre tractaments en algunes estacions: contingut de PR en la

primavera (F=3,529; p=0,039), contingut de CH en hivern (F=6,116; p=0,005) i primavera (F=14,422; p=<0,001) i contingut de LP en primavera (F=7,109; p=0,002). Tanmateix, aquestes diferències no foren significatives quan les dades s'organitzaren d'acord amb les estacions naturals. En resum, aquests resultats suggereixen que el fotoperíode controla la reproducció d'aquesta espècie, i què els dies curts podrien ser necessaris per induir la posta i l'eclosió larvària. Tanmateix, la manipulació del fotoperíode és insuficient per modificar el comportament reproductiu d'aquesta espècie.

Effect of photoperiod on larval production of the spider crab *Maja brachydactyla*

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KEYWORDS
Larval production, stocking conditions, broodstock, biochemical composition.

ABSTRACT
Two experiments, one with a constant photoperiod (CP) and one with a delayed photoperiod (DP), were carried out to assess the effect of the photoperiod on the reproduction of the spider crab *Maja brachydactyla*. In the CP experiment, the broodstock were kept under three different constant light regimes: 8L (8h light:16h dark), 12L (12hL:12hD) and 16L (16hL:8hD). In the DP experiment the broodstock groups were kept under natural photoperiod regimes after an acclimation period with a constant 8L photoperiod during 3 or 6 months, corresponding to the 3M and 6M groups respectively. A control group was always kept in a natural photoperiod. In both experiments, the broodstock were kept at a constant temperature of 18ºC for two years. Throughout the experiments we recorded the number of newly hatched larvae (NHL) per batch, and when possible, samples were collected for larval biochemical analyses (dry weight, DW; protein, PR; carbohydrate, CH; and lipid, LP content). In the CP experiment, the number of NHL varied significantly among treatments: the 8L group resulted in more NHL and a similar production pattern to that observed in the wild and in captivity. These results indicate that the photoperiod effectively controls reproduction success, and short days might be necessary to trigger spawning and larval release in this species. The DW, PR, CH and LP content of NHL were not significantly different among treatments or seasons. In the DP experiment, the seasonal pattern of NHL production and larval composition did not vary with the changes in photoperiod, which indicates that the adjustments to the photoperiod made in this study did not modify the reproductive behavior of this species.

1.- Introduction

The photoperiod has been reported to be one of the environmental factors that most influences reproduction in decapod crustaceans (Sastry, 1987); however, its effects are still unclear. Among brachyurans, temperature has been cited as the most important environmental factor for reproduction, and photoperiod has been found to play an important role in the final maturation of the ovaries and oviposition in the swimming crab *Portunus trituberculatus* (Sulkin et al., 1976; McConaugha et al., 1980; Goy et al., 1985; Hamasaki, 2002; Hamasaki et al., 2004; Zeng, 2007; Kim et al., 2010). With the aim of producing larvae all year round, off-season larval production and consequently reproduction have been assayed in several species of cultured decapod crustaceans (Hamasaki, 2002; Matsuda et al., 2002; Aktas et al., 2003; Hamasaki, 2003; Karplus et al., 2003; Smith et al., 2003; Sachlikidis et al., 2005; Zeng, 2007; Kim et al., 2010). Off-season induction has been successfully achieved in many brachyuran species by modifying the temperature, although a combination of increasing the temperature and a long photoperiod was required for *P. trituberculatus* (reported in Hamasaki, 2002). The larvae obtained off-season were similar to those obtained in the natural breeding season (McConaugha et al., 1980; Zeng, 2007), although some carry-over effects were observed, such as the reduction of one larval stage in *Callinectes sapidus* (Sulkin et al., 1976), and hatching in the prezoea stage in *Scylla paramamosain* (Hamasaki, 2002). In other decapods, such as the spiny lobster *Jasus edwardsii,* several biochemical components of the phyllosoma stage changed significantly when the larvae were obtained after photothermal manipulation (Smith et al., 2003).

The breeding season of the spider crab *Maja brachydactyla* on the Galician coast (NE Atlantic) begins in March and extends until September (González-Gurriarán et al., 1998). During this period, the females produce between two and three consecutive broods that correspond to two peaks of ovigerous females that carry late stage eggs in early spring and summer. The last hatch is at the end of the breeding season in September. Evidence suggests that the photoperiod could play an important role in the reproduction of the spider crab: (1) the breeding season of this species has a similar duration from the British Islands to Senegal despite the geographical variations in sea temperature (ca. 8ºC between Brittany and Galicia, González-Gurriarán et al., 1993; González-Gurriarán et al., 1998); and (2) larval production during one year in captivity with the broodstock kept at a constant temperature and natural

variations in the photoperiod followed a similar pattern to that observed in the wild (Andrés et al., 2010). Seasonal changes in the larval biochemical composition were also observed and attributed to the effect of the photoperiod. Therefore, our hypothesis was that the photoperiod influences the larval production of *M. brachydactyla*. We tested this hypothesis with two experiments. In the first experiment the spider crab broodstock were reared at a constant temperature and under three different constant photoperiods. In the second experiment we attempted to modify the reproductive behavior of the spider crabs by delaying the natural photoperiod at the beginning of the year. Newly hatched larvae were sampled throughout the experiments to determine whether the biochemical composition differed as a result of the treatments.

2.- Material and methods

2.1.- Broodstock capture and maintenance

Adult *Maja brachydactyla* were captured with coastal fishery vessels off the Galician coast (NE Atlantic), transported to IRTA (Sant Carles de la Ràpita, Tarragona, Spain) in high humidity containers at 8±2ºC, and acclimated during one month to captive conditions before starting the two experiments with constant (CP) and delayed (DP) photoperiods. The CP experiment was carried out in 2006 and 2007, and the DP experiment was carried out in 2008 and 2009.

For each experiment, a total of 36 adult females (CP: carapace length (CL) =150.7±8.7 mm, body weight (BW) =1015.16±203.69 g; DP: CL=142.0±7.8 mm, BW=886.89±137.75 g, mean± standard deviation) and 12 adult males (CP: CL =150.7±7.8 mm, BW =992.61±326.08 g; DP: CL=149.1±7.0 mm, BW=1118.07±145.73 g) were randomly distributed in six tanks, two tanks per treatment, thus obtaining a sex ratio of 3F:1M (F, female: M, male). The tanks were connected to a recirculation unit with a constant temperature (CP: 18.2±0.6ºC; DP: 18.8±1.1ºC) and salinity (CP: 34.8±0.9‰; DP: 34.7±0.9‰). The broodstock were fed *ad libitum* with a combination of fresh and frozen mussels (*Mytilus* sp.) and frozen crab (*Liocarcinus depurator*).

In order to control the hours of light, each tank was covered with an opaque canvas and independently illuminated with two daylight fluorescent tubes, so that the photoperiod was controlled and adjusted manually. Each tank of the CP experiment was assigned to one of the constant light regimes: 8L (8hL:16hD), 12L (12hL:12hD) and 16L (16hL:8hD) photoperiods. In the DP

experiment, the photoperiod for the control group (CTRL) followed natural variations from the beginning of the experiment in January 2008. In the 3M and 6M delayed photoperiod treatments the broodstock groups were acclimated to the experimental conditions by applying a short light regime (constant 8hL:16hD) from January 2008 for three and six months respectively. After the acclimation periods, the 3M and 6M groups were kept under natural photoperiod variations and adjusted manually to local light hours. As the acclimation periods lasted for different times, the experimental period of the 3M group began in March 2008, and the experimental period of the 6M group began in June 2008 (Figure 1).

	2008												2009											
Month	J	F	M	Ap	My	Jn	Jl	Au	S	O	N	D	J	F	M	Ap	My	Jn	Jl	Au	S	O	N	D
CTRL	WIN			SPG			AUT			SUM			WIN			SPG			AUT			SUM		
3M	-3 months			WIN			SPG			AUT			SUM			WIN			SPG			AUT		
6M	-6 months			-3 months			WIN			SPG			AUT			SUM			WIN			SPG		

Fig. 1. Experimental design of the DP experiment. The pattern of experimental seasons during the DP experiment is shown according to the modifications in the photoperiod. The CTRL group was kept under a natural photoperiod. During the acclimation period the 3M and 6M groups were subjected (highlighted in grey) to a constant photoperiod of 8hL:16hD (L, light; D, dark) during 3 and 6 months respectively. Then the photoperiod pattern of the CTRL group was applied. Abbreviations: AUT, autumn; SPG, spring; SUM, summer; WIN, winter.

Female survival (%) was monitored daily throughout the experiments and only males that died during the experiment were replaced in order to maintain the 3F:1M sex ratio (F, female: M, male). Female survival in the CP experiment decreased during the first year of captivity and at the end of the experiment it was 42% in the 8L and 16L photoperiod treatments, and 33% in the 12L photoperiod treatment. In the DP experiment, female survival in the CTRL and 3M groups decreased continuously from the very start and was 8% at the end of the experiment. Survival in the 6M group decreased after the winter of 2009 and was 58% at the end of the experiment.

2.2.- *Larval collection and proximate biochemical analysis*

A larval batch was defined as the presence of actively swimming newly hatched larvae (NHL) in a broodstock tank. Larval collection and proximate biochemical analysis were carried out as in Andrés et al. (2010). The larval content of each biochemical component was calculated as μg·mg DW^{-1}.

2.3.- Data and statistical analysis

For both experiments, the data sets from tanks belonging to the same treatment were pooled and grouped seasonally. Data were analyzed and plotted

using the SigmaPlot 9 and SigmaStat 3 software packages (Systat Software Inc., USA). In the CP experiment, larval production was compared using a chi-square test, while the larval biomass (DW and proximate biochemical contents) obtained during the first year of the experiment was evaluated using a two-way ANOVA in order to detect differences between the factors treatment and season. Seasonal differences in larval biomass in the 8L group were also evaluated using a one-way ANOVA. Data from the DP experiment were arranged and plotted following the experimental seasons. As the 3M and 6M groups did not produce larvae after autumn and summer of the first year, only differences between treatments of the first experimental year were analyzed using a one way ANOVA. The normal distribution of the data was checked with a Kolmogorov-Smirnov normality test, and a Kruskal-Wallis one way ANOVA on ranks was applied to the data values that were not normally distributed. Post-hoc comparisons among groups were performed using either the Holm-Sidak test or Dunn's test. Statistically significant differences ($p<0.05$) were indicated in tables and figures by different letters.

3.- Results

3.1.- CP Experiment

A total of 2.2 million NHL were obtained under 8L photoperiod conditions, whereas the 12L and 16L photoperiods produced 1.1 million NHL (Figure 2a). The differences in NHL production were statistically significant ($p<0.001$). Larval production was highest in the spring of the first year for all groups and it decreased considerably afterwards. In the 8L group, NHL were produced until the summer of the second year in captivity. In the 12L and 16L groups, larval production ended after autumn and spring respectively of the first year.

The larval biochemical composition did not show significant differences related to season or photoperiod length for any of the parameters analyzed: DW, PR, CH and LP (Table 1). The DW of NHL was not affected by the daylength ($p=0.229$) or season ($p=0.788$), and the seasonal differences in the NHL production observed in the 8L group were not significant ($p=0.119$). Similarly, daylength ($p=0.737$) and season ($p=0.290$) did not have any effect on PR, not even in the 8L group ($p=0.460$). The CH content of the NHL was similar and not significantly different in any of the treatments ($p=0.696$) or seasons ($p=0.903$), including the 8L group ($p=0.321$). Finally, the LP content of the NHL was not affected by season ($p=0.817$) or daylength ($p=0.236$). However, the LP content of the NHL from the 8L group varied seasonally, although the differences were not significant ($p=0.171$).

3.2.- DP Experiment

The larval production in the CTRL group peaked in spring (1.9 million NHL), followed by a seasonal reduction until the autumn of the second year (Figure 1b). In the 3M group, the highest number of larvae was obtained in winter (2.6 million NHL), production then decreased until the autumn of the first year. In the 6M group, larval production during the experimental period was highest in the experimental spring (0.6 million). However, the highest larval production occurred during the acclimation period (2.6 million). The 6M group did not produce any larvae after the summer of the first year.

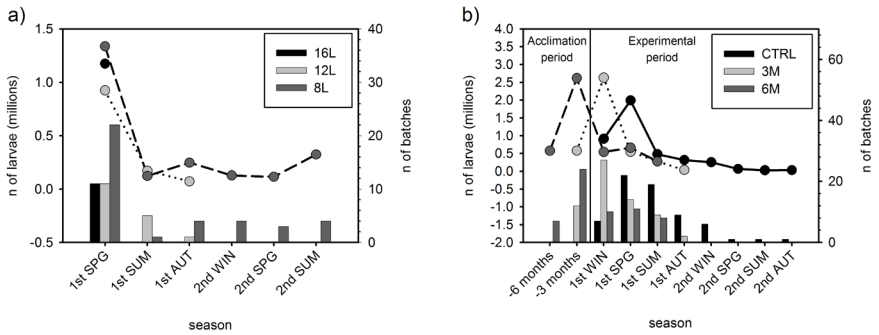

Fig. 2. Seasonal variation in the number of NHL of *Maja brachydactyla* obtained per season in the (a) CP experiment and (b) DP experiment. Circles indicate the number of NHL and bars indicate the number of batches. Abbreviations: AUT, autumn; SPG, spring; SUM, summer; WIN, winter.

NHL had a similar individual DW in all seasons and treatments, and consequently no significant differences were detected (Table 2). Larval PR content was similar among the treatments during the entire experimental period and only showed significant differences in spring (p=0.039). The larvae in the CTRL group obtained the highest PR levels. The CH content of the NHL varied significantly in winter (p=0.005) and spring (p<0.001), although these differences were not observed afterwards. The LP content of the NHL only varied significantly in spring (p<0.002); however, in this case, the NHL obtained in the CTRL group showed the lowest LP content.

4.- Discussion

Although published data show large variability between species, the photoperiod is believed to be an important environmental factor that governs the reproduction of decapod crustaceans (Sastry, 1987). In some decapods the

Table 1. Seasonal variation in dry weight (DW, in µg), protein (PR), carbohydrate (CH) and lipid (LP) content of NHL (µg·mg DW^{-1}) obtained under 16L (16hL: 8hD; L, light; D, dark), 12L (12hL: 12hD) and 8L (8hL: 16hD) photoperiods in the CP experiment. n indicates the number of batches. The number of batches used for the biochemical analyses is only indicated for the PR analysis. Abbreviations: AUT, autumn; SPG, spring; SUM, summer, WIN, winter.

	DW						PR						CH			LP		
Season	16L	n	12L	n	8L	n	16L	n	12L	n	8L	n	16L	12L	8L	16L	12L	8L
1st SPG	77±10	10	81±10	10	75±14	22	186±82	6	235±113	8	196±85	19	12±6	12±9	13±8	65±19	59±17	65±19
1st SUM			77±25	4	67±0	1			236±73	2	80±0	1		14±1	4±0		39±0	55±0
1st AUT			78±0	1	89±12	4			236±0	1	202±45	3		13±0	13±1		72±0	49±8
2nd WIN					80±17	3					231±64	3			15±7			45±8
2nd SPG					90±17	3					297±47	2			18±5			53±8
2nd SUM					66±14	4					223±33	4			19±4			54±6

Data are shown as mean±S.D.

photoperiod is not necessary for triggering gonadal maturation (Carmona-Osalde et al., 2002), whereas in others it is the primary reproductive cue (Sachlikidis et al., 2005). Previous studies suggest that the photoperiod has little influence on the reproduction of most brachyuran species, although these studies were mainly focused on off-season larval production (Sulkin et al., 1976; McConaugha et al., 1980; Goy et al., 1985; Hamasaki, 2002; Zeng, 2007). However, the photoperiod was found to have a significant effect on the number of ovigerous females in *P. trituberculatus* (Kim et al., 2010) as well as in other temperate spiny lobsters (Matsuda et al., 2002; Sachlikidis et al., 2005). Since a higher number of ovigerous females results in a higher number of NHL, we considered the number of NHL to be the best estimate for studying the effects of the photoperiod in the CP experiment in order to avoid stressing the females (to check egg laying implies opening the abdomen of females weekly). Thus, the statistically significant differences in NHL production obtained under different photoperiods corroborate the hypothesis that photoperiod plays a role in the reproduction of the spider crab *M. brachydactyla*. In addition, the pattern of larval production observed in the 8L group during the first year was similar to that observed in the wild (González-Gurriarán et al., 1998) and in captivity under a natural photoperiod (Andrés et al., 2010). This suggests that a short photoperiod might be required to trigger different aspects of spider crab reproduction. These results contrast with those observed in other temperate crab and lobster species, which require long-day photoperiods for reproduction and spawning (Quackenbush, 1994; Matsuda et al., 2002; Hamasaki et al., 2004; Sachlikidis et al., 2005; Kim et al., 2010). This difference could be explained by the fact that the first gonadal maturation of *M. brachydactyla* occurs during the last part of the year (September to December) when the photoperiod is decreasing from ca. 12 hours to 8 hours of light (González-Gurriarán et al., 1998).

Neither seasonal or daylength associated differences were found in the DW, PR, CH or LP content of the NHL. Andrés et al. (2010) reported significant changes in the composition (DW, PR and LP content) of spider crab larvae produced over an entire year and suggested that natural variations in the photoperiods could cause these changes. In the present study the broodstock were kept under a constant photoperiod, and consequently no significant seasonal differences in larval composition were observed.

Using short photoperiods in aquaculture production of spider crabs could be a useful strategy since it reduces illumination costs and could also help to obtain homogeneous groups of NHL during the production cycle. However, it

Table 2. Seasonal variation in dry weight (DW, in µg), protein (PR), carbohydrate (CH) and lipid (LP) content of NHL (µg·mg DW^{-1}) obtained under a natural photoperiod (CTRL), and the photoperiod delayed 3 months (3M) and 6 months (6M) of the DP experiment. n indicates the number of batches. The number of batches used for the biochemical analyses is only indicated in the PR analysis. Abbreviations: AUT, autumn; SPG, spring; SUM, summer, WIN, winter.

		DW						PR						CH			LP		
Period	Season	CTRL	n	3M	n	6M	n	CTRL	n	3M	n	6M	n	CTRL	3M	6M	CTRL	3M	6M
Acclimation	-6 months					88 ±9	7					160 ±2	7			17 ±9			52 ±1
	-3 months			91 ±3	9	93 ±8	23			149 ±3	9	165 ±3	23		12 ±6	12 ±7		43 ±8	41 ±8
Experimental	1st WIN	88 ±8	7	92 ±8	26	84 ±2	8	189 ±2	7	165 ±5	25	165 ±5	7	12 ±5[b]	12 ±6[b]	25 ±17[a]	53 ±1	43 ±8	46 ±1
	1st SPG	87 ±7	21	86 ±6	13	91 ±7	11	190 ±3[a]	21	160 ±3[b]	12	177 ±3[ab]	11	12 ±5[b]	18 ±3[a]	20 ±4[a]	43 ±5[b]	49 ±8[a]	52 ±9[a]
	1st SUM	86 ±1	11	88 ±7	8	92 ±2	6	158 ±4	11	200 ±3	8	186 ±4	6	19 ±3	20 ±4	13 ±7	47 ±1	57 ±1	52 ±1
	1st AUT	88 ±9	9	86 ±2	2			202 ±3	8	175 ±3	2			19 ±2	17 ±2		56 ±1	52 ±1	
	2nd WIN	89 ±5	5	86 ±2	2	92 ±2	6	195 ±2	5					24 ±3			48 ±8		
	2nd SPG	93 ±0	1					170 ±0	1					21 ±0			40 ±0		
	2nd SUM	67 ±0	1					131 ±0	1					12 ±0			28 ±0		
	2nd AUT	74 ±0	1					265 ±0	1					4 ±0			24 ±0		

Data are shown as mean±S.D. Different letters in superscript within the same row indicate significant differences among seasons (ANOVA, $p<0.05$).

would be necessary to determine possible carry-over effects of photoperiod manipulation on larval and juvenile quality.

Off-season reproduction and larval production in brachyurans has been achieved by modifying the temperature and photoperiod and by eyestalk ablation (Sulkin et al., 1976; McConaugha et al., 1980; Goy et al., 1985; Hamasaki, 2002; Hamasaki et al., 2004; Zeng, 2007; Kim et al., 2010). In general, temperature has been found to be the primary cue, although the photoperiod increased the number of ovigerous females in *P. trituberculatus* (Kim et al., 2010). Based on the results of the CP experiment, we tried to manipulate the reproductive behavior of the spider crab using delayed photoperiods in order to postpone the spring peak of NHL until the following seasons. However, the highest NHL peaks in the 3M and 6M groups occurred respectively one and two seasons before their experimental springs, that is, the spring according to the time in captivity. As it has been shown that there are significant seasonal variations in larval composition over a year (Andrés et al., 2010) we expected that there would be no differences among treatments if data were organized according to experimental seasons. However, PR, CH and LP varied significantly among treatments in several seasons, which shows that the delayed photoperiods did not affect reproduction. Indeed, no significant seasonal differences among treatments were detected when data were analyzed according to the time in captivity. It seems that the manipulation of the photoperiod alone is not enough to modify the reproductive behavior of the spider crab. It would therefore be interesting to study the role temperature plays in the reproduction of the spider crab and its relation to the photoperiod. It is possible that, as occurred in *P. trituberculatus*, a certain combination of temperature and photoperiod might be required to induce off-season reproduction. We also do not discard the possibility that the acclimation period could have played a part in the effect of the modified photoperiod. Karplus et al. (2003) showed that the acclimation period played a key role in off-season induction of the first spawning in sexually mature females of the Australian freshwater crayfish *Cherax quadricarinatus*. Considering the results of the CP experiment, it would be of interest to use long photoperiods to arrest reproduction, and then apply natural photoperiods beginning with a short photoperiod to trigger the reproductive cycle.

5.- Conclusions

1. The photoperiod influences the reproduction of the spider crab. A short photoperiod seems to be required to trigger reproduction.

2. Delaying the photoperiod is not enough to modify the reproductive behavior of the spider crab in order to extend the use of hatchery facilities over the year.

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7.- References

Aktas, M., Kumlu, M., Eroldogan, O.T., 2003. Off-season maturation and spawning of *Penaeus semisulcatus* by eyestalk ablation and/or temperature-photoperiod regimes. Aquaculture. 228, 361-370.

Andrés, M., Estévez, A., Simeó, C.G., Rotllant, G., 2010. Annual variation in the biochemical composition of newly hatched larvae of *Maja brachydactyla* in captivity. Aquaculture. 310, 99-105.

Carmona-Osalde, C., Rodriguez-Serna, M., Olvera-Novoa, M.A., 2002. The influence of the absence of light on the onset of first maturity and egg laying in the crayfish *Procambarus* (*Austrocambarus*) *llamasi* (Villalobos, 1955). Aquaculture. 212, 289-298.

González-Gurriarán, E., Fernández, L., Freire, J., Muiño, R., 1998. Mating and role of seminal receptacles in the reproductive biology of the spider crab *Maja squinado* (Decapoda: Majidae). J Exp Mar Bio Ecol. 220, 269-285.

González-Gurriarán, E., Fernández, L., Freire, J., Muiño, R., Parapar, J., 1993. Reproduction of the spider crab *Maja squinado* (Brachyura: Majidae) in the Southern Galician coast (NW Spain). ICES, Shellfish Committee. K:19, 15.

Goy, J.W., Morgan, S.G., Costlow Jr, J.D., 1985. Studies on the reproductive biology of the mud crab, *Rhithropanopeus harrisii* (Gould): induction of spawning during the non-breeding season (Decapoda, Brachyura). Crustaceana, 83-87.

Hamasaki, K., 2002. Effects of temperature on the survival, spawning and egg incubation period of overwintering mud crab broodstock, S*cylla paramamosain* (Brachyura: Portunidae). Suisan Zoshoku. 50, 301-308.

Hamasaki, K., 2003. Effects of temperature on the egg incubation period, survival and developmental period of larvae of the mud crab *Scylla serrata* (Forskål) (Brachyura: Portunidae) reared in the laboratory. Aquaculture. 219, 561-572.

Hamasaki, K., Imai, H., Akiyama, N., Fukunaga, K., 2004. Ovarian development and induced oviposition of the overwintering swimming crab *Portunus trituberculatus* (Brachyura: Portunidae) reared in the laboratory. Fish Sci. 70, 988-995.

Karplus, I., Gideon, H., Barki, A., 2003. Shifting the natural spring-summer breeding season of the Australian freshwater crayfish *Cherax quadricarinatus* into the winter by environmental manipulations. Aquaculture. 220, 277-286.

Kim, D.H., Kim, S.K., Choi, J.H., Kim, B.R., Seo, H.C., Jang, I.K., 2010. The effects of manipulating water temperature, photoperiod, and eyestalk ablation on gonad maturation of the swimming crab, P*ortunus trituberculatus*. Crustaceana. 83, 129-141.

Matsuda, H., Takenouchi, T., Yamakawa, T., 2002. Effects of photoperiod and temperature on ovarian development and spawning of the Japanese spiny lobster *Panulirus japonicus*. Aquaculture. 205, 385-398.

McConaugha, J.R., McNally, K., Goy, J.W., Costlow, J.D., 1980. Winter induced mating in the stone crab, *Menippe mercenaria*. Proc World Maricult Soc. 11, 544-547.

Quackenbush, L.S., 1994. Lobster reproduction: a review. Crustaceana. 67, 82-94.

Sachlikidis, N.G., Jones, C.M., Seymour, J.E., 2005. Reproductive cues in *Panulirus ornatus*. N Z J Mar Freshwater Res. 39, 305-310.

Sastry, A.N., 1987. Ecological aspects of reproduction. in: Vernberg, F.J., Vernberg, W.B. (Eds.), The Biology of Crustacea. Environmental Adaptations. Academic Press, New York, pp. 179-269.

Smith, E.G., Ritar, A.J., Carter, C.G., Dunstan, G.A., Brown, M.R., 2003. Morphological and biochemical characteristics of phyllosoma after photothermal manipulation of reproduction in broodstock of the spiny lobster, *Jasus edwardsii*. Aquaculture. 220, 299-311.

Sulkin, S.D., Branscomb, E.S., Miller, R.E., 1976. Induced winter spawning and culture of larvae of the blue crab, *Callinectes sapidus* Rathbun. Aquaculture. 8, 103-113.

Zeng, C., 2007. Induced out of season spawning of the mud crab, *Scylla paramamosain* (Estampador) and effects of temperature on embryo development. Aquac Res. 38, 1478-1485.