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Development of the female reproductive system in the freshwater crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae)

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Abstract. The differentiation of the female reproductive system from a macroscopic and microscopic point of view was studied in *Cherax quadricarinatus*. For this characterization, 184 females were dissected and processed for the histological analysis. From the differentiation of the ovary up to its maturity, three ovarian morphotypes could be distinguished macroscopically: parallel strands without any contact between them, an H-shaped ovary, and a Y-shaped ovary. These morphotypes were compared within the Astacida. Four ovarian developmental stages were recognized based on ovary color, and the histological structure and relative proportion of cellular types. The post-spawning ovary was also characterized. The components of the female reproductive system sheath were described and its modifications in the ovary and oviducts were determined and compared. Theoretical aspects of the study of sexual differentiation in *C. quadricarinatus* were discussed within a phylogenetic framework.

Additional key words: "red claw," ovary, functional morphology, phylogeny

Members of *Cherax quadricarinatus* Von Martens 1898 (Parastacidae) are large, freshwater crayfish native to northwest Queensland and the Northern Territory of Australia, and are intensively cultured in Australia and many other countries in southern Asia, North and South America, and Africa (Lawrence & Jones 2002; Edgerton 2005). In accordance with its importance for aquaculture, many biological aspects of its culture have been studied, including growth, nutrition, and reproduction (see López Greco et al. 2007, for a review). Within the complex framework that represents reproduction, oogenesis is one of the most important processes.

Oogenesis is an energetically expensive reproductive process, which can be divided into several phases. The latter phases of oogenesis, which are periods characterized by the accumulation of yolk proteins in the growing oocytes and by significant increases in the oocyte diameter, are referred to as primary and secondary vitellogenesis (Meusy & Charniaux-Cotton 1984; Tsukimura 2001). During these phases, many yolk proteins are synthesized within the oocytes, while vitellogenin is transported from the hepatopancreas through the hemolymph to developing oocytes, sequestered by the growing oocytes, and modified by means of the addition of polysaccharides and lipids. This is the common form of yolk, named vitellin, which is stored in oocytes and is the nutrient source of developing embryos (Abdu et al. 2000; see Tsukimura 2001 for a review).

Within the Astacida, the ovarian cycle of mature females has been studied morphologically, physiologically, and biochemically in *Procambarus clarkii* Girard 1853 (Kulkarni et al. 1991), in the "Gilgie," *Cherax quinquecarinatus* Gray 1845 (Beatty et al. 2005), and in *C. quadricarinatus* (Sagi et al. 1996; Abdu et al. 2000). Although the changes in the mature ovary have been elucidated in *C. quadricarinatus* (Abdu et al. 2000), the sexual differentiation and onset of sexual maturity in females has not yet been studied for this species nor others within the Astacida. Although the structural features of the gonads are considered relevant characters in addressing phylogenetic relationships among crustacean taxa (Ando & Makioka 1992, 1998; Vogt 2002), only *P. clarkii* has been studied (Ando & Makioka 1998) in relation to the macroscopic and microscopic architecture of the female reproductive system.

The objective of this study was to characterize the differentiation of the female reproductive system and the onset of maturity in the freshwater "red claw" crayfish, *C. quadricarinatus,* from both a macroscopic and a microscopic point of view, and to compare these features within the Astacida.

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Methods

For characterization of the reproductive system, 184 females (0.09–108 g) of *Cherax quadricarinatus* were dissected and processed for histological analysis. Females weighing >2 g (21-mm carapace length [CL]) were purchased from a local dealer (San Mateo farm, Entre Ríos, Argentina) while females <2 g were obtained from our laboratory stock as described. Five ovigerous females (52.10 ± 11.05 g) were selected and maintained in individual glass aquaria (60 × 40 × 30 cm) containing 20 L of dechlorinated tap water (pH 7.4, hardness: 80 mg L^{-1}, as $CaCO_3$ equivalents), under continuous aeration, at a temperature of 26°–27°C and a 14:10 photoperiod, in accordance with Jones (1995a). They were fed daily *ad libitum* on freshwater aquarium weed (*Elodea* sp.) and commercial Tetradiskus granules (Tetra: Melle, Germany) until independence of the hatched juveniles (Levi et al. 1999). At juvenile Stage III, they were separated from their mothers and maintained under the conditions indicated previously, adding small PVC tubes and onion bag mesh that were used as shelters (Jones 1995b). Every week, during nursery rearing, juveniles were observed under a stereomicroscope to check whether they could be sexed by the presence of the paired genital openings on the basis of the third (females), fifth (males), or both (intersex) pair(s) of walking legs. In females, sex could be determined by 0.09–0.10 g, a weight that corresponds to juvenile Stages V–VII (Vazquez et al. 2004). They were then collected for morphological analysis of their reproductive system. For the analysis of the post-spawning ovary, two ovigerous females (<48 h post-spawning) were also processed.

All animals were weighed (BW) (precision: 0.1 mg) and their CL and post-orbital length were measured (Swiss Confederation, Digital Caliper, precision: 0.01 mm) following Austin (1995). After being cold anesthetized at −20°C for 15 min, the carapace was removed and the gonads were inspected, and the relative size, form, and color were recorded. They were quickly dissected and fixed in Bouin's solution for 4 h at room temperature. Gonads were then sequentially passed through 90% and 96% ethanol for 20 min each, 96% ethanol–butylic alcohol (1:1 v/v) for 30 min, and 100% butylic alcohol for 30 min, and then embedded in paraffin.

Histological sections, 5–6 μm thick, were cut with a Carl Zeiss ultramicrotome (Gberkochen, Baden, Württemberg, Germany) and stained with hematoxylin–eosin, periodic acid Schiff (PAS), Alcian blue (AB), and Masson-Trichrome (TM), according to López Greco et al. (2007). The sections were examined and photographed with a Carl Zeiss, Axioimager A1 microscope. No less than three slides from each female were inspected under light microscopy. Primary, intermediate, and secondary oocytes were identified following López Greco et al. (1997) and Abdu et al. (2000) and then counted. The proportion of each cell type was estimated and the mean oocyte diameter was measured by means of an × 8 Zeiss microscopic ocular lens, calibrated against a Leitz Wetzlar plate (Leitz, Stuttgart, Germany) with 10 μm spacing on a representative section of each ovary. All oocytes with a visible nucleus were measured. Mean ± standard error was calculated for each cellular type using the program Statistica 6.0 (Statsoft [Cia], Tulsa, OK). The presence and position of the oogonia, follicular cells, and empty follicles were recorded, and the microscopic structures of the ovarian sheath and oviducts were also characterized.

Results

The ovary in *Cherax quadricarinatus* is a sac-like structure where the proliferative zone, containing mainly oogonia, is located in the center of the gonad, next to the ovarian lumen, while oocytes surround the oogonia in a more peripheral position. The ovarian lumen is seen in all ovarian stages although it is larger in the earlier stages. The ovary sheath is composed of three layers: the innermost layer is the ovarian epithelium, a monolayer of flat to cubic cells that delimits the ovary lumen and develops folds that surround the follicles containing secondary oocytes, giving rise to a kind of "oogenetic pouch" (*sensu* Ando & Makioka 1998). The middle layer is the muscular tunica that is composed of three or four layers of muscular cells, while the outermost layer consists of a single layer of connective tissue. The proliferative zone is located on the ovarian epithelium and the growing oocytes are located between the ovarian epithelium and the connective external layer (Fig. 1).

From initial differentiation of the ovary, at 0.09 g BW, up to its maturity, three ovarian morphotypes can be macroscopically distinguished: parallel strands (without contact) ovary, an H-shaped ovary, and a Y-shaped ovary. Based on the ovary color, the histological structure, and the relative proportion of cellular types, four ovarian developmental stages could be recognized:

Stage I: This stage represents the onset of ovarian differentiation. From 0.09 g BW (7 mm CL) up to 2 g (21 mm), the ovary is transparent. It can be seen as two parallel strands (mainly in the smallest juveniles, 0.09–0.2 g). These cords later interconnect medially to form an H-shaped ovary in crayfish 0.2 g/10 mm and larger. The transverse commissure between the right and the

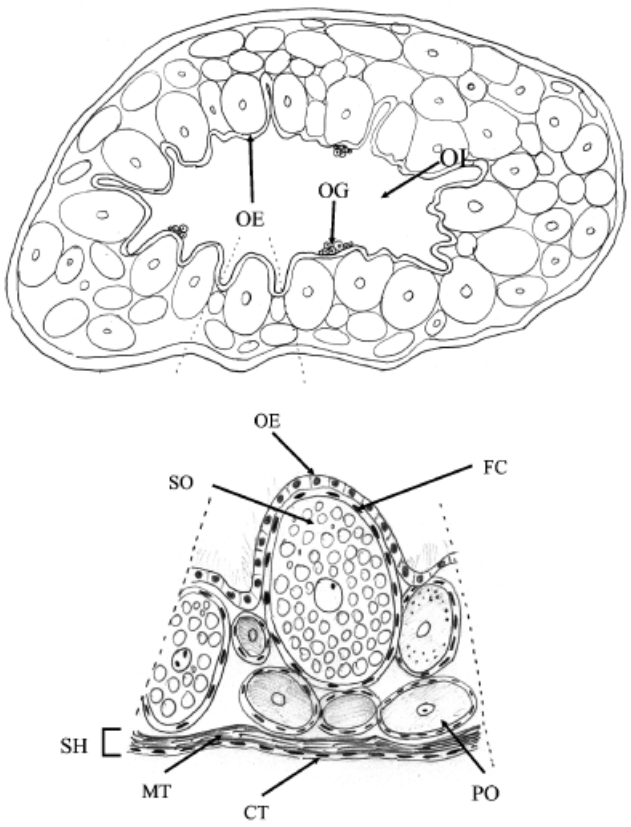

Fig. 1. Schematic drawing of the ovary. The ovary is surrounded by the sheath. All oocytes are surrounded by the follicular cells and the secondary oocytes are surrounded mainly by the ovarian epithelium. Oogonia are in the ovarian epithelium near the lumen. ct, connective tissue; fc, follicular cells; mt, muscular tunica; oe, ovarian epithelium; og, oogonium; ol, ovary lumen; po, primary oocyte; sh, sheath of the ovary; so, secondary oocyte.

left part of the H-shaped ovary is located just posterior to the stomach and defines the anterior and posterior ovarian lobes. At this stage, oogonia arranged in "nests" of five to ten cells and primary oocytes (mean size: $122.26 \pm 2.89\,\mu m$) are found in the ovary. The cytoplasm of primary oocytes is homogeneous and acidophilic. Round basophilic follicular cells are found surrounding both oogonia and oocytes (Table 1, Fig. 2).

Stage II: The ovary appears as a cream to pale orange color and is H-shaped. At this stage, the differentiation of a pair of structures defined as "connectors" (*sensu* Vazquez & López Greco 2007) becomes evident (Fig. 3). Although the most representative cell types of this ovary are the primary oocytes, the first intermediate oocytes appear. They are recognized by the presence of small yolk platelets in their periphery and a diameter similar to the primary oocytes (Table 1, Fig.

Table 1. Characterization of the ovarian developmental stages. CL, carapace length (mm); POL, post-orbital carapace length (mm). All variables are expressed as means ± standard error; for oocyte diameter, range is also given.

Ovarian stage	Form: color	CL (mm)	POL (mm)	Weight of female (g)	Oogonia (μm)	Proportion of cell types (primary: intermediate: secondary oocytes)	Primary oocytes (μm)	Intermediate oocytes (μm)	Secondary oocytes (μm)
I	Parallel strands or H: transparent	16.70 ± 0.71	13.00 ± 1.31	0.09–0.2 or 0.2–2.00	11.18 ± 0.33	100:0:0	122.66 ± 2.97 (25.91–512.50)	—	—
II	H: Cream to pale orange	30.06 ± 0.80	21.24 ± 0.52	2–8	13.60 ± 0.22	97:3:0	218.84 ± 4.70 (36.82–543.75)	193.29 ± 15.19 (111.82–275.00)	—
III	H: orange–orange with some green oocytes	36.94 ± 0.80	26.03 ± 0.60	6–18	14.32 ± 0.23	43:45:12	109.07 ± 9.01 (36.82–350.00)	220.75 ± 18.63 (122.73–331.25)	458.79 ± 31.05 (181.25–919.36)
IV	Y: olive green	54.34 ± 1.64	38.47 ± 1.12	> 18	—	11:16:73	235.19 ± 12.24 (112.50–403.22)	351.97 ± 17.53 (106.25–580.64)	1057.6 ± 21.77 (506.25–1629.03)

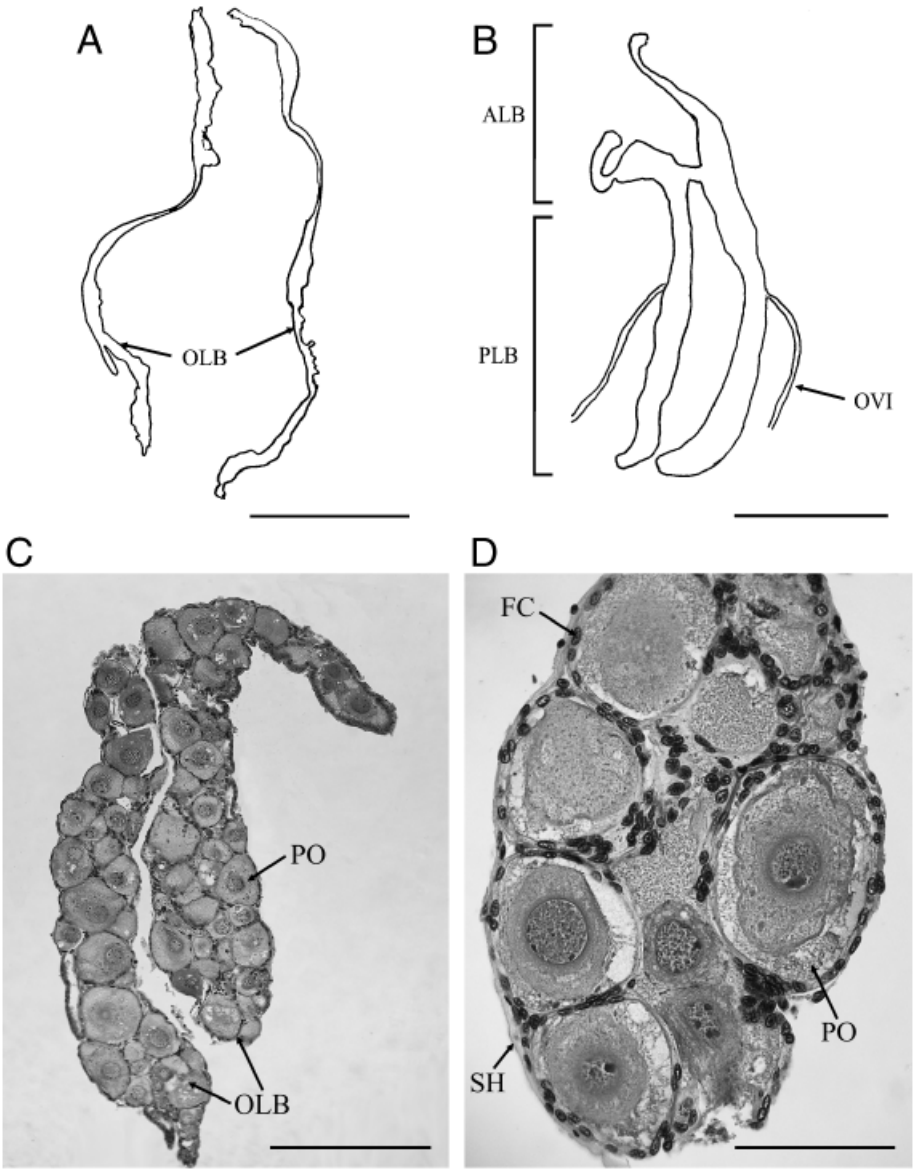

Fig. 2. A, B. Schematic drawing of a Stage I ovary as two parallel strands and an H shape, respectively (scale bars, 130 and 510 μm, respectively). **C, D.** Histological view through a longitudinal and a transversal section stained with hematoxylin–eosin and Masson-Trichrome, respectively (scale bars, 195 and 78 μm, respectively). alb, anterior ovarian lobes; fc, follicular cells; olb, ovarian lobes; ovi, oviduct; plb, posterior ovarian lobes; po, primary oocytes; sh, sheath of the ovary.

3). Among these, both oocytes with homogeneous and heterogeneous cytoplasm are observed. The heterogeneity is related to the presence of mucopolysaccharides (PAS+) (Fig. 3). The presence and shape of the oogonia and follicular cells are similar to that in Stage I. The Stage II ovary is larger than that of Stage I.

Stage III: The H-shaped ovary is bright orange or orange with some, macroscopically visible, olive green oocytes. The connectors are clearly differentiated from the anterior ovarian lobes containing oocytes. Primary, intermediate, and a small proportion of secondary oocytes can be distinguished (Table 1, Fig. 4). Secondary oocytes are the largest cells within the ovary; they are surrounded by flat and pycnotic follicular cells and have an eosinophilic cytoplasm containing yolk droplets and globules. The presence of the first secondary oocytes defined this stage as the beginning of sexual maturity in *C. quadricarinatus*. The Stage III ovary is larger than that of Stage II.

Stage IV: This final stage of ovarian differentiation is represented by a large, and full, olive-green Y-shaped structure, due to the posterior lobes coalescing and extending into the pleon (Fig. 5). The posterior lobes partially maintain the paired condition although they seem to fuse in their most posterior edge (Fig. 5A,B). Secondary oocytes represent the main and largest cellular type in this stage (Table 1, Fig. 5C,D). At this stage, oogonia are rarely observed. This ovarian type is the fully

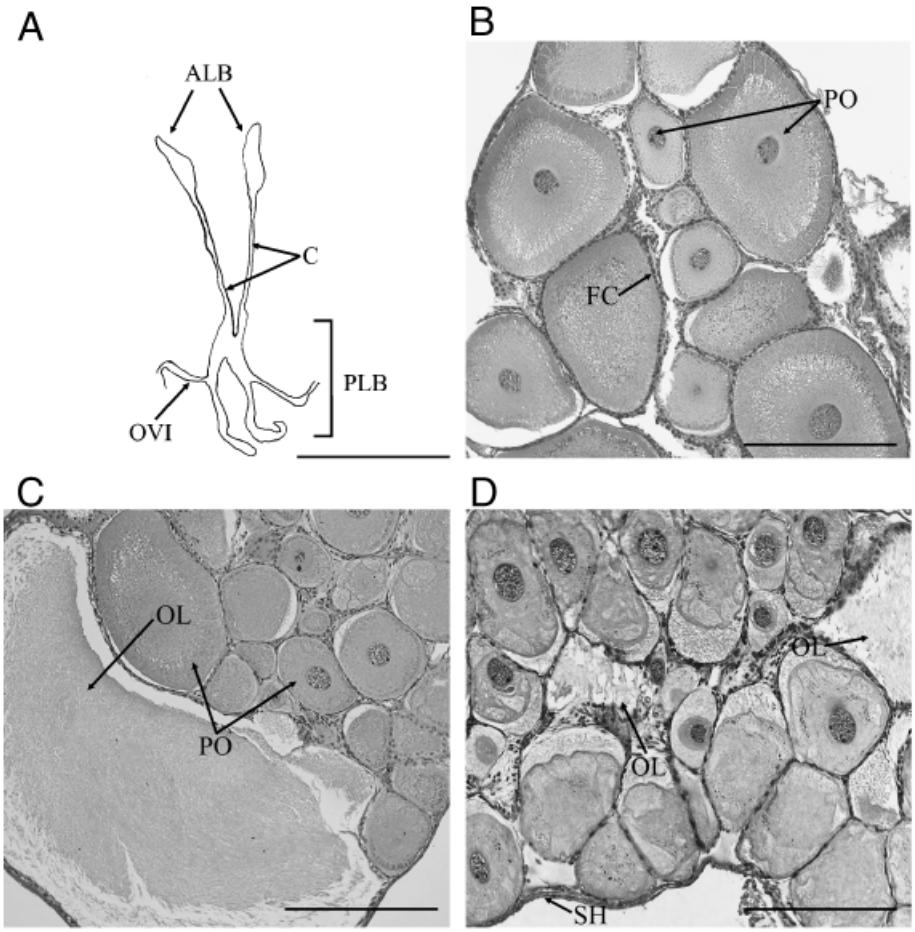

Fig. 3. A. Schematic drawing of a Stage II ovary as an H shape with differentiated connectors (scale bar, 1200 μm). **B–D.** Histological cross-section of the ovary (stained with hematoxylin–eosin). Scale bars, 140, 220, and 210 μm, respectively. alb, anterior ovarian lobes; c, connectors; fc, follicular cells; ol, ovary lumen; ovi, oviduct; plb, posterior ovarian lobes; po, primary oocytes; sh, sheath of the ovary.

mature ovary of *C. quadricarinatus,* beginning at 18 g BW/40 mm CL.

The post-spawning ovary is a Y-shaped pale-orange structure, which appears to be swollen. Although primary oocytes dominate, some intermediate and secondary oocytes are distinguishable (Fig. 6). The lumen of the ovary is clearly seen. The most characteristic structures of this stage are the empty oogenetic pouches, delimited by the ovarian epithelium. They look like ''flowers'' among the oocytes (Fig. 6). The middle layer of the ovarian wall is thicker than in other ovarian stages.

The oviducts are short, straight, isodiametric, and transparent tubular structures that extend laterally from the middle part of the ovary and connect with the gonopores located on the bases of the third pair of walking legs, while the paired connectors represent a transparent anatomical differentiation of the anterior ovarian lobes. The lumen of both structures is wide enough to allow the passage of only one oocyte at a time. Histologically, both structures are composed of three layers: the inner layer is composed of a folded monolayer of cubic/cylindrical and tall secretory cells, continuous with the ovarian epithelium; the folded middle layer is composed of three or four layers of muscular cells while the outer layer is formed by a monolayer of connective tissue. Although the connectors are a differentiated zone of the ovary, non-germinative cells are observed inside (Fig. 7).

Discussion

The general structural organization of the ovary in *Cherax quadricarinatus* has a relatively simple design and is similar to that described in other decapod crustaceans (Krol et al. 1992; Ando & Makioka 1998; Kronenberger et al. 2004). This study outlines the macroscopic and microscopic changes of the ovary from its differentiation until the onset of maturity, including the different sizes and proportions of cellular stages that allow characterization of an ovarian maturation scale for this species.

In crabs, marine lobsters, and many anomurans, the ovary is H-shaped with a ''bridge'' situated behind the gastric mill (Johnson 1980; Talbot 1981;

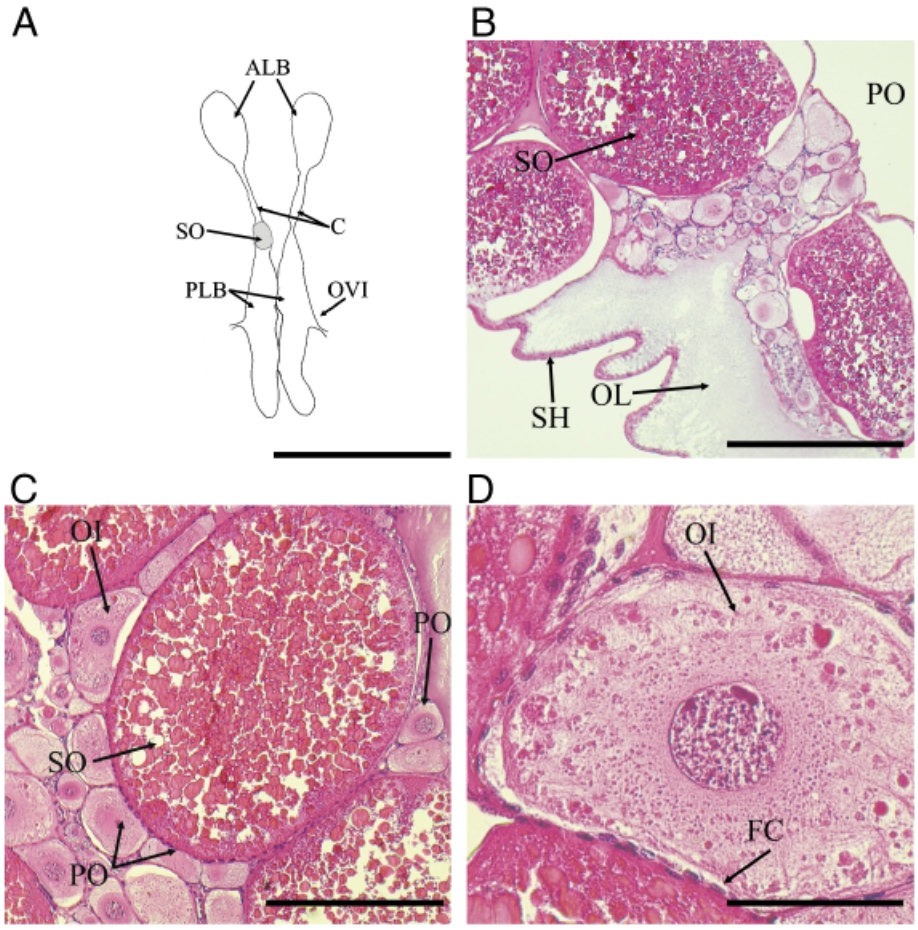

Fig. 4. A. Schematic drawing of a Stage III ovary. Scale bar, 4.3 mm. **B–D.** Histological cross-section of the ovary (stained with hematoxylin–eosin). Scale bars, 142, 100, 40 µm, respectively. alb, anterior ovarian lobes; c, connector; fc, follicular cells; io, intermediary oocytes; ol, ovary lumen; ovi, oviduct; plb, posterior ovarian lobes; po, primary oocytes; sh, sheath of the ovary; so, secondary oocytes.

Beninger et al. 1988; Krol et al. 1992; Elorza & Dupré 1999; Kronenberger et al. 2004). This bridge connects the left and right cords, defining an anterior and posterior portion, which correspond to the anterior and posterior ovarian lobes. In *C. quadricarinatus,* we observed the ovaries consisting of parallel cords during the early maturation phase, passing through a transition stage consisting of a differentiated H-shaped structure, and then acquiring a Y shape in a more advanced phase. A fusion of posterior lobes may take place during maturation from the H-shaped to the Y-shaped ovary.

Within freshwater crayfishes, a mature Y-shaped ovary is characteristic of the Astacidae and Cambaridae (Ando & Makioka 1998; Vogt 2002), while at least two patterns can be observed within the Parastacidae. One pattern is that reported here for *C. quadricarinatus;* a second is characteristic of some other South American members of the Parastacidae (e.g., *Samastacus, Parastacus*) in which ovaries consist of two lobes longitudinally connected by connective tissue (Rudolph 1995, 2002; de Almeida & Buckup 1999; Rudolph & Almeida 2000; Rudolph et al. 2001).

The Y morphotype found in *C. quadricarinatus,* in its most advanced maturation stage, also differs from the Y-shaped gonads of astacid and cambarid females, described by Ando & Makioka (1998) and Vogt (2002), in which the anterior lobes are intimately related to the posterior lobe, forming a trilobed ovary. The oviducts of *C. quadricarinatus* are straight like other studied members of the Parastacidae (Rudolph 1995, 2002; de Almeida & Buckup 1999; Rudolph & Almeida 2000; Rudolph et al. 2001), and contrast with the proximal folded one of *Procambarus clarkii* (Ando & Makioka 1998). The microscopic pattern of the oviduct in *C. quadricarinatus* is similar to that in *Parastacus brasiliensis* Von Martens 1869 where the folded wall is probably necessary to withstand the extruding pressure of the oocytes during egg laying (de Almeida & Buckup 2000).

This study describes for the first time the presence of structures called "connectors," which characterize the ovary of *C. quadricarinatus*. The connectors are structures with a tubular aspect that lack germinal cells. They would facilitate the evacuation of the mature oocytes from the anterior lobes in an individual

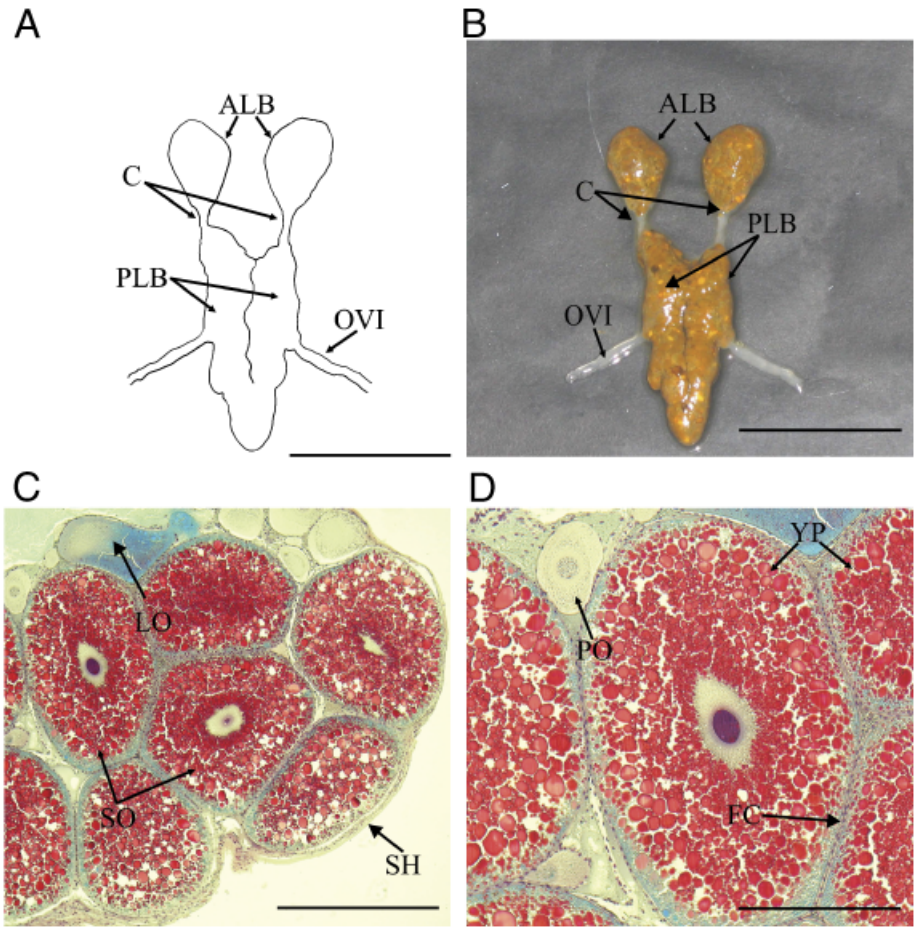

Fig. 5. A. Schematic drawing of a Stage IV ovary. Scale bar, 16.2 mm. **B.** General view of the green olive ovary. Scale bar, 12.2 mm. **C, D.** Cross-section of the ovary (stained with Masson-Trichrome). Scale bars, 489 and 217 μm, respectively. alb, anterior ovarian lobes; c, connectors; fc, follicular cells; ol, ovary lumen; ovi, oviduct; plb, posterior ovarian lobes; po, primary oocytes; sh, sheath of the ovary; so, secondary oocytes; yp, yolk platelets.

and aligned manner. The mature oocytes of *C. quadricarinatus* are large, reaching 2.2 mm in length (Jones 1995a; Abdu et al. 2000). The presence of connectors would optimize the complete evacuation of the ovary, replacing the highly developed muscular net around the follicles that characterizes the ovarian sheath of the marine lobsters *Homarus americanus* H. Milne Edwards 1837 and *Jasus frontalis* Milne-Edwards 1836 (Talbot 1981; Elorza & Dupré 1999; Dupré 2003). Unlike *H. americanus* (Talbot 1981) and *J. frontalis* (Elorza & Dupré 1999), the ovary wall of *C. quadricarinatus* does not present a thick muscular layer involved in ovulation, but instead it has a thin and distended muscular layer similar to that observed in *P. clarkii* (Ando & Makioka 1998), *Aegla platensis* Schmitt 1942 (Sokolowitz et al. 2007), and many other decapods (Krol et al. 1992). The presence of non-germinal areas within a decapod ovary has not been reported previously.

The inner layers of both connectors and oviducts are highly secretory epithelia that could function in lubrication and/or the modification of the oocyte envelopes. This epithelium type has also been reported in the oviduct walls of *A. platensis* (Sokolowitz et al. 2007), *Galathea intermedia* Liljeborg 1851 (Kronenberger et al. 2004), and *P. clarkii* (Ando & Makioka 1998). Ando & Makioka (1998) proposed that this kind of epithelium in the oviduct wall could be related to fertilization that could occur inside the oviduct. Because fertilization in *C. quadricarinatus* occurs out of the female reproductive system (external fertilization), we consider that this is not the role of the oviduct secretions in *C. quadricarinatus*.

In *C. quadricarinatus,* during sexual differentiation and the onset of sexual maturity the ovaries are characterized by a significant increase in the size of the oocytes and changes in the relative proportions of primary to secondary oocytes (see Sokolowitz et al. 2007 for a review). Both the size and the histological pattern of the oocytes are in accordance with the eight stages reported by Abdu et al. (2000). According to the biochemical characterization of ovary maturation (Abdu et al. 2000; Rodríguez-González et al. 2005), primary oocytes typically present a polypeptidic profile of 65–95 kDa, while secondary oocytes contain polypeptides of relatively higher

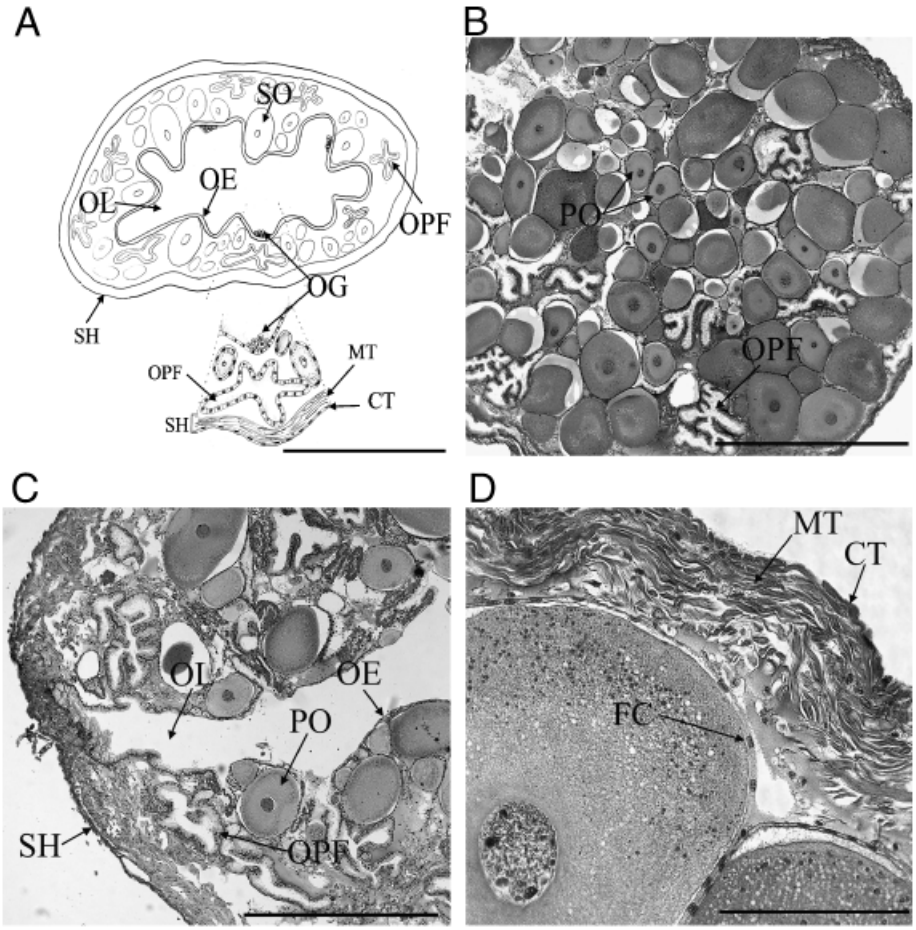

Fig. 6. A. Schematic drawing of the post-spawning ovary. Scale bar, 30 mm. **B, C.** Histological cross-section of the ovary (stained with Masson-Trichrome [TM]) showing the presence of empty oogenic pouches. Scale bars, 392 and 223 μm, respectively. **D.** Detail of a primary oocyte contacting the ovarian sheath (stained with TM). Scale bar, 43 μm. ct, connective tissue; fc, follicular cells; mt, muscular tunica; oe, ovarian epithelium; og, oogonium; ol, ovary lumen; opf, oogenetic pouch like a "flower"; po, primary oocyte; sh, sheath of the ovary; so, secondary oocyte.

molecular masses (>100 kDa). As in other crustacean species, the presence of secondary oocytes indicates the onset of sexual maturity. The secondary oocytes of *C. quadricarinatus* reach sizes similar to those of other freshwater crayfishes (Rudolph & Iracabal 1994; Ando & Makioka 1998; Brian et al. 2001; Rudolph & Rojas 2003; Beatty et al. 2005). These large oocyte sizes are related to a low relative fecundity, which is compensated for by rapid growth of juveniles and a high tolerance to different ranges of water and temperature conditions (Brian et al. 2001).

When comparing the ovarian microscopic structure of *C. quadricarinatus* (present study) with that of *P. clarkii* (Ando & Makioka 1998), some features are worth noting. The ovarian epithelium of *P. clarkii* surrounds both primary and secondary oocytes, while in *C. quadricarinatus* it only surrounds the latter ones, mainly when they are protruding into the ovarian lumen. Furthermore, no follicles exist in *P. clarkii,* although oocytes are surrounded by the ovarian epithelium "that sometimes look like follicles" according to Ando & Makioka (1998). In *C. quadricarinatus,* the follicular cells surrounding oocytes (primary and secondary) are seen in all ovarian stages, while in secondary oocytes the ovarian epithelium is located immediately outside the follicular cells. At ovulation, each empty ovarian epithelium surrounding an ovulated secondary oocyte resembles a "flower," while the oocyte *plus* its follicular cells are found within the ovarian lumen or within the connectors, and then transferred into the oviduct and oviposited through female gonopores. The mature ovaries of the South American members of the Parastacidae, *Parastacus nicoletti* Philippi 1882, *Parastacus brasiliensis, Parastacus defossus* Faxon 1898, *Parastacus varicosus* Faxon 1898, and *Samastacus spinifrons* Philippi 1882 show the presence of follicular cells surrounding primary and secondary oocytes (Rudolph 1995, 2002; de Almeida & Buckup 1997, 1999; Rudolph et al. 2001).

Such differences between *P. clarkii* and members of the Parastacidae, including *C. quadricarinatus,* need further study, because they could represent different patterns within the Astacida. Recently, clear differences in the spermatophore structure within the Astacida, including differences between South American parastacids and *Cherax* spp., have been recognized (Noro et al. 2006; López Greco & Lo Nostro

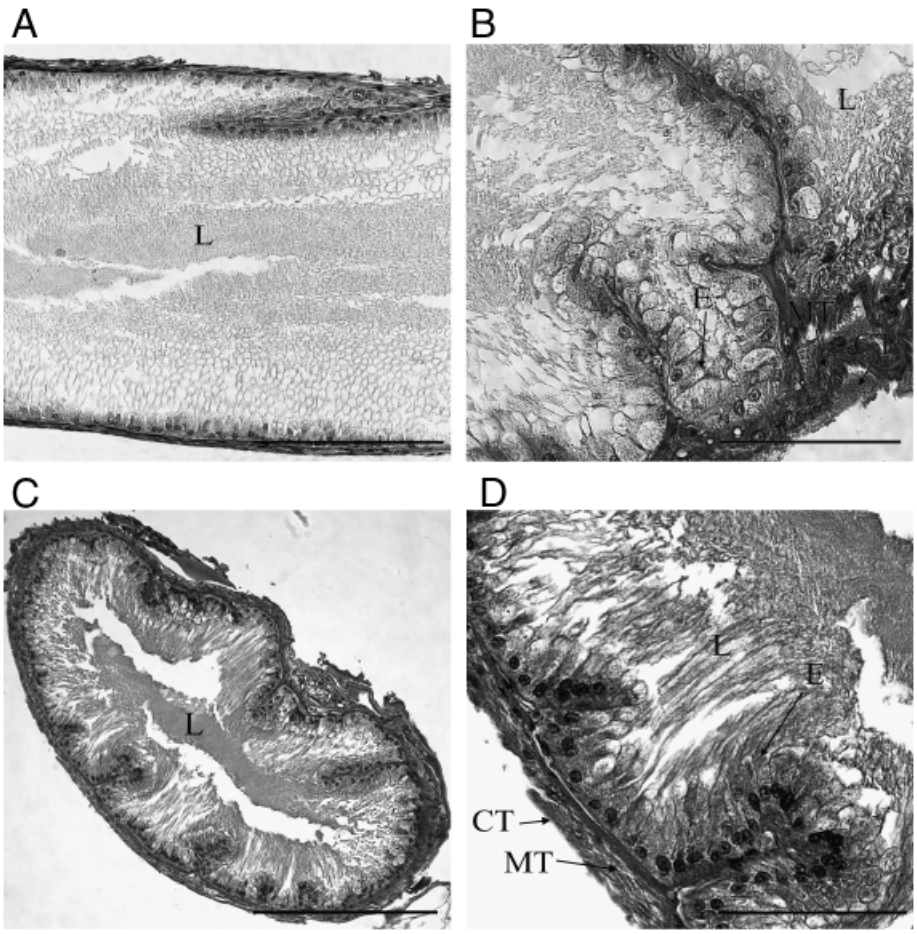

Fig. 7. A. General view of connectors in a histological longitudinal section of the ovary. Scale bar, 140 μm. **B.** Detail of the connector sheath. Scale bar, 36 μm. **C.** General view of oviducts in a histological cross-section. Scale bar, 150 μm. **D.** Details of the oviduct wall. Scale bar, 40 μm. A and B are stained with Masson-Trichrome, while C and D are stained with periodic acid-Schiff. ct, connective tissue; e, epithelium; l, lumen; mt, muscular tunica.

2008). This fact reinforces the idea that gonad structure, in addition to sperm morphology and strategies of transferring sperm from male to female, may be a useful tool in accepting or rejecting hypotheses about decapod evolution (Bauer 1986; Hinsch 1991; Jamieson 1991; Jamieson & Tudge 2000; Tirelli et al. 2007).

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