REPRODUCTIVE ORGANOGENESIS IN THE APPLE SNAIL *POMACEA CANALICULATA* (LAMARCK, 1822), WITH REFERENCE TO THE EFFECTS OF XENOBIOTICS

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ABSTRACT

The complex array of male and female reproductive organs in *Pomacea canaliculata* develops from the gonoductal and copulatory primordia. These primordia are present in both sexes, but while the gonoductal primordium (or 'gonoduct') is present in hatchlings, the copulatory primordium only appears in juveniles at the 5-mm (shell length) stage. The proximal portion of the gonoduct will produce the gonad, while the more distal portions will produce the accessory sexual organs (the visceral vas deferens, pallial seminal vesicle, prostate and genital papilla in males, and the visceral oviduct, uterus and vagina in females). The gonadal portion of the gonoduct differentiates histologically into either the testis or the ovary at the 10-mm stage, while the more distal portions remain undifferentiated and the copulatory primordium remains approximately the same size in both sexes. The copulatory rudiment becomes larger in juvenile males at the 15-mm stage and rudiments of all adult components have appeared by then. At this stage, the pallial gonoduct differentiates into either the pallial seminal vesicle and prostate, or the uterus and vagina. Subadults at the 20-mm stage possess either some mature spermatogenic nests or some large oocytes. Crossing of the genital papilla over the rectum, a crucial event in male differentiation, has already occurred at this stage. Sexual differentiation of the operculum and the initiation of copulatory behaviour occur between the 15- and 20-mm stages, while oviposition begins *c*. 2 weeks later. Full development of gonoduct derivatives is reached at the 25-mm stage in both sexes. However, the copulatory structures remain rudimentary in females, even though they may still develop to some extent later on. These findings not only indicate two distinct origins of reproductive organs, but also several developmental stages during which different control mechanisms may operate. This has implications for understanding potential targets and responses to endocrine-disrupting xenobiotics.

INTRODUCTION

Interest in ampullariid development may be traced to Semper's (1862) monograph on *Pila polita* (Deshayes, 1830) (as *Ampullaria polita*). This was continued by studies on three *Pomacea* species (Brooks & McGlone, 1908; Fernando, 1931; Hylton Scott, 1934, 1958), *Pila globosa* (Swainson, 1822; Ranjah, 1942) and *Marisa cornuarietis* (Linnaeus, 1758; Demian & Yousif, 1973a, b, c, d, 1975). Recently, there has been a revival of interest in pre-hatching development of ampullariids (Heras, Garin & Pollero, 1998; Koch, Winik & Castro-Vazquez, 2009; Sun *et al.*, 2010). However, this is the first study of post-hatching reproductive organogenesis in an ampullariid snail. It is important to document critical developmental moments and sites at which sexually differentiating factors, trophic hormones, neurohormones and/or endocrine disruptors may act, and which may be significant for assessing the impact of environmental pollutants. Also, this study may shed light on problems of homology, as well as behavioural-functional issues such as the relation of copulation and fertilization to oviposition.

Snails in the family Ampullariidae (Caenogastropoda) are unique among caenogastropods in that the male copulatory organs are located at the inner right side of the mantle edge (Hyman, 1967). The ampullariid copulatory organs are particularly complex and they reach their greatest complexity in the genera *Pomacea* and *Marisa* (Berthold, 1989). The existence of rudimentary male copulatory organs has been observed in females from the ampullariid genera *Lanistes*, *Pila*, *Turbinicola*, *Marisa* and *Pomacea* (Bouvier, 1888; Sachwatkin, 1920; Hägler, 1923; Prashad, 1925; Hylton Scott, 1958; Andrews, 1964;

Thiengo, 1987, 1989; Keawjam & Upatham, 1990; Thiengo, Borda & Araújo, 1993; Schulte-Oehlmann *et al.*, 1994, 1995).

The adult male reproductive structures of *Pomacea canaliculata* (Lamarck, 1822) include the testis, vas deferens, pallial seminal vesicle, prostate and genital papilla, and a separate copulatory apparatus on the right mantle edge which comprises the penial pouch (enclosing a penial bulb and three distinct portions of the penis) and penial sheath with its glands (Gamarra-Luques *et al.*, 2006; Giraud-Billoud, Gamarra-Luques & Castro-Vazquez, 2013). Female reproductive structures include the ovary, oviduct, uterus (a complex organ including the seminal receptacle, copulatory bursa, albumen and capsule glands) and vagina, as well as the rudimentary copulatory apparatus (Hylton Scott, 1958; Andrews, 1964; Catalán de Canelada & Moreno, 1984; Catalán, Fernandez & Winik, 2002).

It will be shown in this paper that the primordial gonoduct (henceforth, 'the gonoduct') present in hatchlings will differentiate into either the male or the female set of organs indicated above. The copulatory primordium will appear in both sexes and will become fully developed in males, but will remain more or less rudimentary in females.

MATERIAL AND METHODS

Animals from a cultured strain of *Pomacea canaliculata* were used. The original stock was collected at Rosedal Lake (Palermo, Buenos Aires, Argentina) and ethanol-preserved voucher specimens of the original population and of the cultured strain were deposited in the collections of Museo Argentino de Ciencias Naturales in Buenos Aires, Argentina (MACN-In 35707 and MACN-In 36046, respectively). Our colony bears haplotype A, a widespread haplotype in both the original and introduced ranges of *P. canaliculata* (Hayes *et al.*, 2008). Water temperature was maintained between 23 and 25°C and artificial lighting was provided for 14 h per day. Aquarium water was changed three times per week.

To construct a standard growth curve for hatchlings under laboratory conditions, 20–30 egg clutches were collected from the culture room on three occasions and incubated in Petri dishes at 23–25°C. Hatching occurred *c*.14 days later (Koch *et al.*, 2009); 120 of the hatchlings were collected at random and distributed in 56 × 38 × 20 cm aquaria, where water level was kept *c*. 4 cm deep, with 20 hatchlings per aquarium. Shell length (defined as the distance between the apex and the most distant part of the aperture) was measured at weekly intervals with calipers to the nearest 0.1 mm. Since Estebenet & Cazzaniga (1992) have reported a negative effect of crowding on snail growth, snail density was progressively reduced: day 10, 14 animals/aquarium; day 30, 12 animals/aquarium; day 40, 10 animals/aquarium. Animals were fed *ad libitum* on fresh lettuce during the first 2 weeks post-hatching and thereafter a mixed diet of lettuce, fish food pellets (Peishe Car Shulet®, Argentina) and toilet paper. Changes in the shape of the operculum (Cazzaniga, 1990; Estebenet, Martín & Burela, 2006) and the occurrence of reproductive behaviours (copulation and spawning) were recorded daily from Monday to Friday. Egg clutches were incubated in Petri dishes in the snail-culture room and fertility of each was recorded. No animals died during the observation period.

Individuals reared in the conditions described above were sampled for morphological observations (at least 10 snails per group) when reaching shell lengths of 2–3, 5, 10, 15, 20 and 25 mm, when they were, respectively, 0, 10, 20, 30, 40 or 50 days old. Shells of the animals were cracked before fixation in dilute Bouin's fluid at 4°C for 1 week (Vega *et al.*, 2007), or in modified Raillet-Henry's fluid at room temperature for at least 30 days (Thiengo, 1987). All dissections were carried out after fixation under a Zeiss Stemi SV11stereoscopic microscope.

Selected samples were prepared for routine histology (see below) or were critical-point dried, mounted on aluminium stubs, coated with gold and examined with a LEO 1450VP scanning electron microscope (SEM).

At least two undissected animals of each size/age were fixed in dilute Bouin's fluid, dehydrated via a graded ethanol series and embedded in a resin-paraffin mixture, m.p. 56–58°C. Afterwards, they were serially sectioned and stained with either Heidenhain's iron haematoxylin or Harris's haematoxylin and eosin (Clark, 1981).

The presence of motile sperm was determined in males 15, 20 and 25 mm in length ($n \geq 3$ each) with live testis squashes in 0.6% NaCl, and observed under a Zeiss Axioskop 2 using phase contrast.

Finally, 14 females 30–40 mm long and 4–6 months old were dissected after fixation in Raillet-Henry's fluid, and selected pieces were prepared for SEM as described above.

RESULTS

Age, shell length and expression of reproductive behaviours

Increase in shell length follows a smooth sigmoidal curve (Fig. 1). Opercula become convex in juvenile males (Fig. 2) during week 5 (i.e. at *c*.17.7 mm mean shell length; range
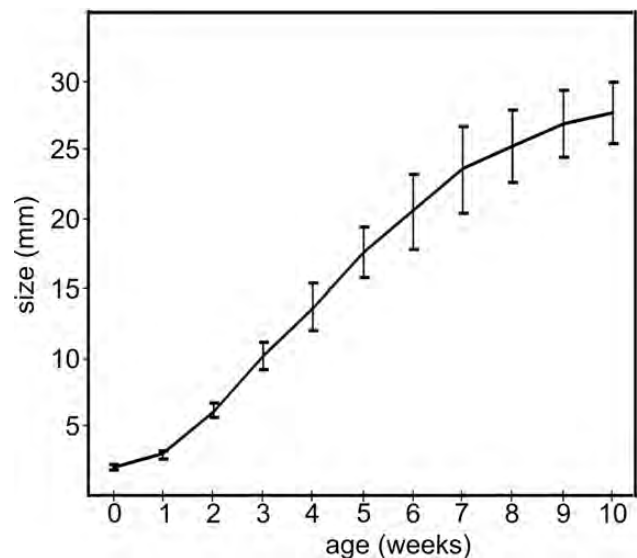

Figure 1. Growth curve (shell length) of *Pomacea canaliculata* from birth to maturity under culture conditions. Each point indicates mean ± standard deviation (SD). Weeks 0 and 1, $n = 120$ per week; weeks 2–4, $n = 84$ per week; weeks 5–7, $n = 72$ per week; weeks 8–10, $n = 60$ per week.
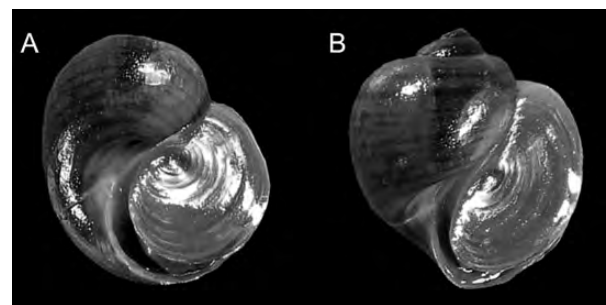

Figure 2. Opercular morphology of *Pomacea canaliculata*. Male (**A**) and female (**B**) with convex and concave opercula, respectively. Shell length = 20 mm.

Table 1. Summary of changes in the gonoductal and copulatory primordia from hatching to maturity of *Pomacea canaliculata*.

Snail size (mm)	Gonoductal primordium			Copulatory primordium
	Gonadal portion	Visceral portion	Pallial portion	
2–3	Undifferentiated epithelioid cord	Epithelioid cord	Thin duct	No copulatory primordium
5	Undifferentiated epithelioid cord	Thin duct	Proximally enlarged loop, continued as a thin duct	Pear-shaped copulatory primordium
10	Early differentiation (testicular tubules are much wider than the ovarian tubules; germ cells become distinct in both sexes)	Thin duct	Proximally enlarged loop, continued as a thin duct	Elongating ridge with a proximal and a distal promontory (penial complex and penial sheath rudiments, respectively)
15	M: partly empty testicular tubules; some motile sperm present	M: Thin duct	M: distally enlarging duct; genital papilla crossing over rectum; prostate duct with tributaries	M: developing penial complex; small penial sheath with T-sulcus, lacking glands
	F: ovarian tubules with slender lumina and oogenic cells	F: Thin duct	F: differentiated uterine components and enlarging vagina with some mucosal folds	F: unchanged
20	M: replenished testicular tubules; few mature germ cell nests; motile sperm present	M: Thin duct	M: differentiated seminal vesicle, prostate and genital papilla	M: developed penial complex; growing penial sheath with full glandular complement
	F: ovarian tubules with some dilated lumina and rather large oocytes	F: Thin duct	F: uterus with differentiated seminal receptacle, albumen and capsule glands; thicker vagina with folded mucosa	F: elongated ridge with two promontories (penial complex and penial sheath)
25	M: adult testis	M: Thin duct	M: adult seminal vesicle, prostate and genital papilla	M: adult copulatory apparatus
	F: adult ovary	F: Thin duct	F: adult, proximally larger vagina	F: elongated ridge with two promontories or a flattened dome with small penial sheath

Abbreviations: M, male; F, female.

13.3–21.5 mm) and copulatory behaviours are also observed for the first time. Spawning behaviour begins 2 weeks later (i.e. at *c*. 23.6 mm mean shell length; range 17.5–29.6 mm) and the majority of eggs (>80%) in the first deposited egg clutches are fertile. Developmental changes in the three portions of the gonoduct and the copulatory primordium are summarized in Table 1.

Hatchlings (2–3 mm in shell length, 0 days old)

Hatchlings (Fig. 3) externally resemble the adults, but differ in the possession of a transparent shell, a reddish-pink midgut gland, incompletely pigmented mantle and head-foot, and comparatively shorter and blunter cephalic tentacles. At this stage, the gonadal portion of the gonoduct is composed of interconnected cell cords embedded in connective tissue along the columellar aspect of the visceral hump (Fig. 3C). These cords are continuous with the visceral portion of the gonoduct, which similarly forms a solid cord at this stage, near the base of the visceral hump. At the base of the mantle cavity, the visceral gonoduct is continuous with the tube-like pallial gonoduct (Fig. 3D). Once the gonoduct enters the mantle cavity wall, it turns to the right and crosses the epitaenium (Fig. 3A), a raised tissue presumably dividing water currents in the mantle cavity, which is already present at birth. After crossing the epitaenium, the pallial gonoduct extends forward along the left side of the rectum. The gonoduct terminates distally in the mantle cavity, approximately midway between the anus and the point where it crosses the epitaenium. No copulatory primordium is present (Fig. 3B).

Juveniles with undifferentiated gonads (5 mm length, 10 days old)

The shell is still fully transparent at this stage (Fig. 4). However, many darkly pigmented spots have appeared over the external epithelium, particularly that of the body whorl and the head-foot region, and the midgut gland has acquired its adult dark-greenish colour, highlighting the whitish threads of perivascular urate tissue that penetrate the gland (Vega *et al*., 2007; Giraud-Billoud *et al*., 2008).

The undifferentiated gonadal gonoduct ramifies as solid cords of epithelioid cells on the surface of the midgut gland. Surrounding the cords is a layer of connective tissue of varying thickness, with numerous, large, glycogen-filled 'Leydig's cells' (Voltzow, 1994) that appear empty in light microscopy preparations. The gonoductal lumen, which had been present only in the pallial portion, now expands through most of the visceral portion of the gonoduct. The gonoduct forms a loop where it becomes incorporated in the rear wall of the mantle cavity (Fig. 4C) and from there crosses the epitaenium to the right and then turns parallel to the rectum. The pallial seminal vesicle (males) or the uterus (females) will develop at this loop in the rear mantle cavity wall. At this stage, the pallial gonoduct is slightly thicker and longer than in hatchlings (Fig. 4A).
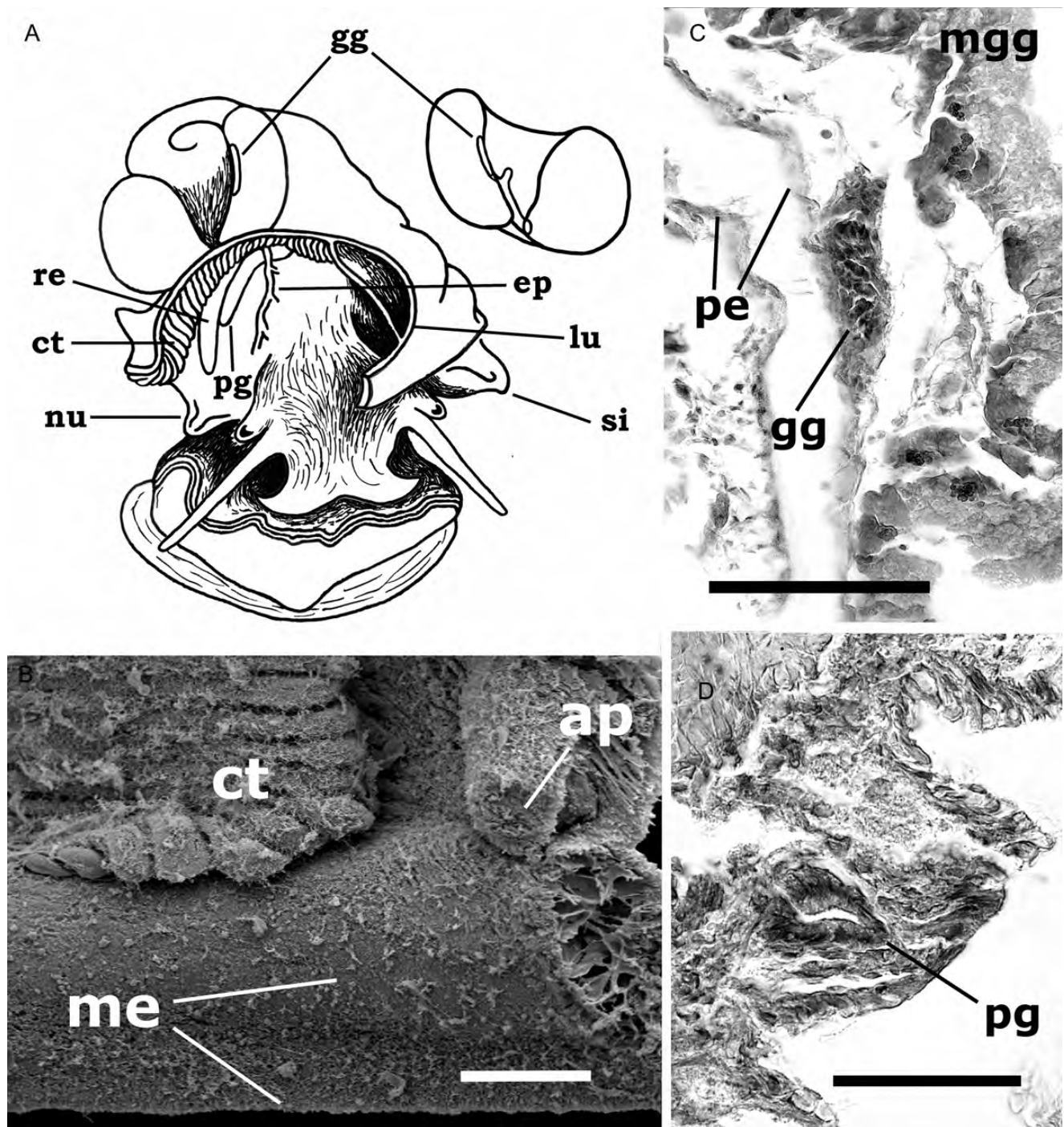
Figure 3. Hatchlings (2–3 mm long, 0 days old) of *Pomacea canaliculata*. **A.** Schematic drawing of whole hatchling removed from shell. Inset illustrates first whorl cut and turned to show gonadal gonoduct as a solid cord on columellar surface of midgut gland; mantle cavity has been opened to show undifferentiated pallial gonoduct. **B.** SEM of inner mantle edge showing presumptive location of copulatory primordium between ctenidium and te anal papilla. **C.** Histological section of gonadal gonoduct as a solid epithelioid cord overlying midgut gland (iron haematoxylin). **D.** Histological section of pallial gonoduct with lumen at rear of mantle cavity (haematoxylin-eosin). Abbreviations: ap, anal papilla; ct, ctenidium; ep, epitaenium; gg, gonadal gonoduct; lu, lung; me, inner mantle edge; mgg, midgut gland; nu, right nuchal lobe; pe, pallial epithelium; pg, pallial gonoduct; re, rectum; si, siphon. Scale bars: **B** = 50 μm; **C** = 100 μm; **D** = 50 μm.

Also at this stage, the copulatory rudiment is first seen as a pear-shaped structure extending between the rectum and the anterior end of the ctenidium (Fig. 4B) and is present in both males and females. It is formed by a compact mass of tissue covered by a columnar epithelium (Fig. 4D, E); a short inner duct (Fig. 4B, E) opens on its ctenidial side (Fig. 4B). Although we did not follow the development of this duct, it is likely that it gives rise to the penial spermiduct and the penial pouch in males.
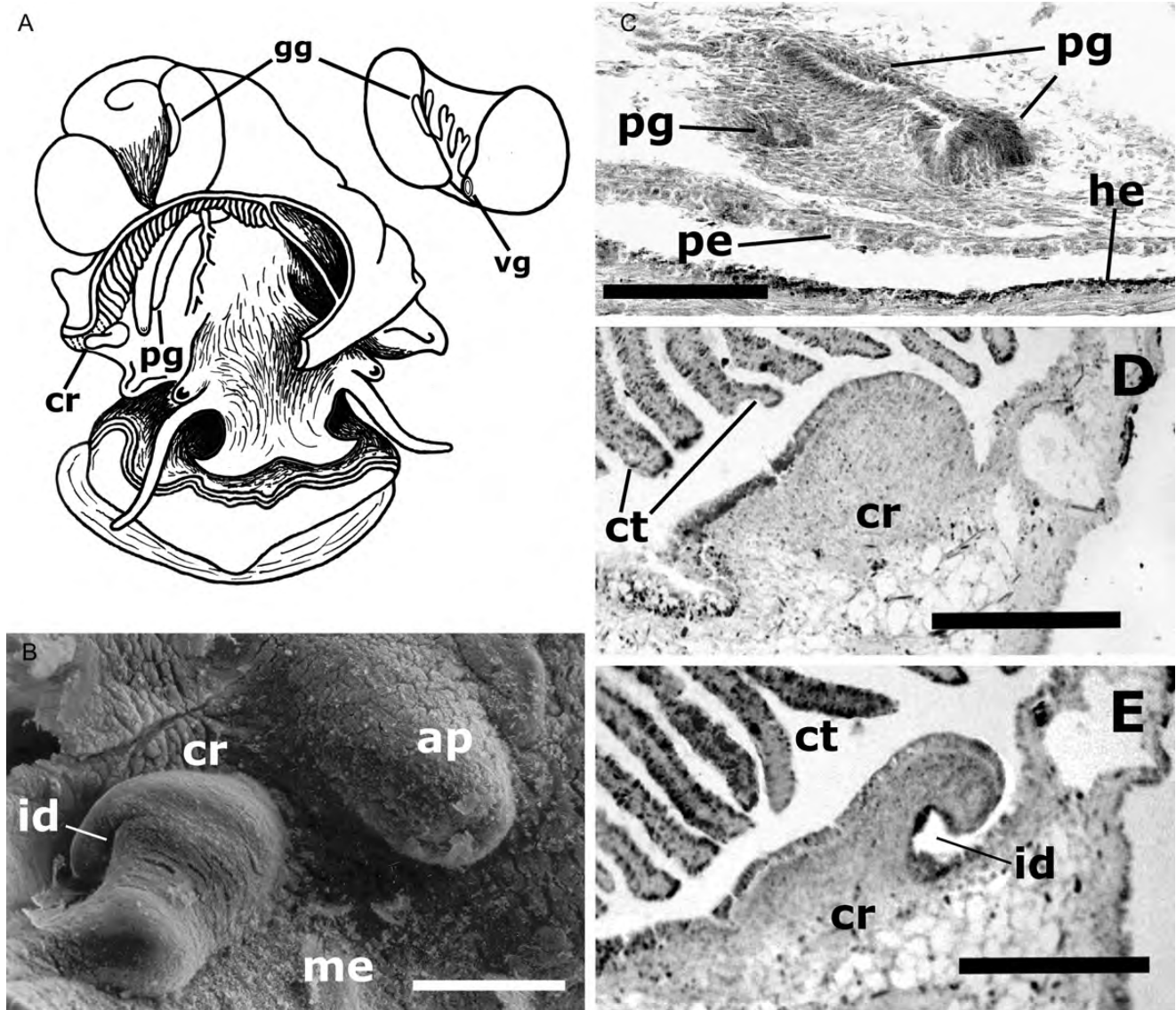
Figure 4. Juveniles of *Pomacea canaliculata* with undifferentiated gonad (5 mm long, 10 days old). **A.** Schematic drawing of whole juvenile removed from shell. Pallial gonoduct has increased in size and copulatory rudiment has appeared as a pear-shaped structure between anal papilla and ctenidium. **B.** SEM of copulatory rudiment showing the infolding on its right side. **C.** Histological section through loop in pallial gonoduct within rear wall of mantle cavity (haematoxylin-eosin). **D.** Longitudinal section through copulatory rudiment (iron haematoxylin). **E.** Longitudinal section through copulatory rudiment showing infolding continued as short duct (section parallel to that in **C**; iron haematoxylin). Abbreviations: ap, anal papilla; cr, copulatory rudiment; ct, ctenidium; gg, gonadal gonoduct; he, head; id, inner duct; me, inner mantle edge; pe, pallial epithelium; pg, pallial gonoduct; vg, visceral gonoduct. Scale bars: **B** = 50 μm; **C** = 100 μm; **D** = 100 μm.

Juveniles with sexually differentiated gonads but undifferentiated pallial gonoduct (10 mm long, 20 days old)

At this stage (Fig. 5) the darkly pigmented spots on the external epithelium have enlarged and merged, becoming a continuous black lining over the body whorl and beneath the transparent shell.

The gonadal gonoduct is becoming sexually differentiated at this stage. In males, the widened testis tubules (Fig. 5C) can be recognized under the stereomicroscope mainly on the first whorls. Histologically, testis tubules form an almost continuous layer over the midgut gland and show a variable number of free germ cells (Fig. 5C). In females, ovarian tubules with narrow lumina have spread over the columellar aspect of the midgut gland and some large germ cells can be distinguished (Fig. 5D).

The visceral portion of the gonoduct remains a thin duct in both sexes. However, a widening of the lumen occurs in the initial loop of the pallial gonoduct within the mantle cavity rear wall. This widening is sometimes more conspicuous in females and is visible macroscopically. The pallial gonoduct gives rise to the pallial seminal vesicle in males and to the uterus in females, two organs that will be mostly or totally located within the visceral sac in adults (Hylton Scott, 1958; Andrews, 1964). However, we consider these rudiments as the first part of the pallial gonoduct, since they are formed within the rear wall of the mantle cavity in both sexes. The distal pallial gonoduct is a thin duct located close to the rectum at this stage and its tip is now closer to the anal papilla (Fig. 5A).

The copulatory rudiment has elongated and is mostly covered by the ctenidial leaflets (Fig. 5B). It possesses a more
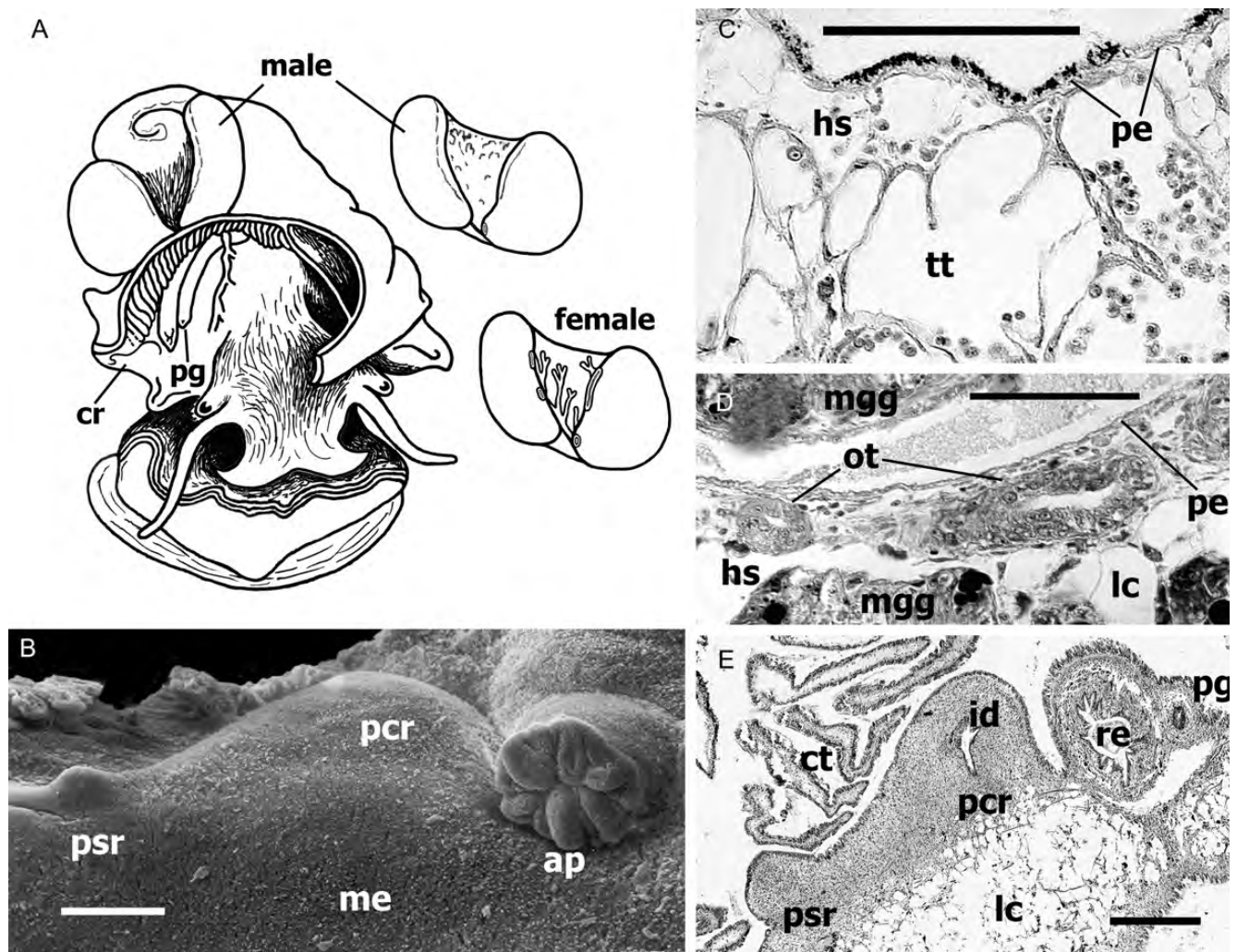
Figure 5. Juveniles of *Pomacea canaliculata* with differentiated gonad but undifferentiated pallial gonoduct (10 mm long, 20 days old). **A.** Schematic drawing of whole juvenile removed from shell showing growing pallial gonoduct and elongated copulatory rudiment. Insets illustrate first whorl cut and turned to show the testicular tubules forming an almost continuous layer in males, and the ramifying ovarian tubules in females. **B.** SEM of copulatory rudiment in a male, showing rudiments of penial sheath and penial complex; ctenidial leaflets have been removed. **C.** Histological section through testicular tubules showing scattered spermatocytes (iron haematoxylin). **D.** Histological section through ovarian tubules with slender lumina overlying midgut gland (iron haematoxylin). **E.** Longitudinal section through copulatory rudiment in a male, with rudiments of th penial sheath and penial complex (iron haematoxylin). Abbreviations: ap, anal papilla; cr, copulatory rudiment; ct, ctenidium; hs, haemocoelic spaces; lc, Leydig cells; me, inner mantle edge; mgg, midgut gland; ot, ovarian tubules; pe, pallial epithelium; pcr, penial complex rudiment; pg, pallial gonoduct; psr, penial sheath rudiment; re, rectum; tt, testicular tubules. Scale bars = 100 μm.

or less pronounced small promontory at the front (the penial sheath rudiment) and a wider, flattened dome at the rear (the penial complex rudiment) where the inner duct has now expanded and ends in a small irregular cavity (Fig. 5E).

Juveniles with differentiated gonad and pallial gonoduct (15 mm long, 30 days old)

At this stage (Figs 6–8), the external pigmentation is similar to that of adults. The testis is a yellowish-white layer extending over the columellar aspect of the dark midgut gland, predominantly in the first whorls (Fig. 6A). Groups of spermatogenic cells are seen in testis tubules (Fig. 6D) and motile sperm are present in testis squashes. Occasional large nuclei of Sertoli cells (Gamarra-Luques *et al.*, 2006) are first recognized at this stage (Fig. 6D). Tubular branches of the female gonad have

grown and ramified (Fig. 7A) and some large oogenic cells are visible (Fig. 8A).

The visceral gonoduct forms a slender duct lined by a ciliated columnar epithelium. It descends along the columella and joins the first part of the pallial gonoduct, i.e. the pallial seminal vesicle in males and the uterus in females.

Distally, the male pallial gonoduct gives rise to the prostate and genital papilla. The prostate begins as a rather thin duct at the base of the mantle cavity, extending first along the left of the epitaenium, then crossing it and turning forward, accompanying the left side of the rectum. The first tributaries of the prostate main duct develop at this time, predominating in the distal part of the prostate along the rectum (Fig. 6E). As a continuation of the prostate main duct, a small promontory (the genital papilla) has formed close to the anal papilla (Fig. 6B, C). The genital papilla is a simple tube with no tributaries at this stage (Fig. 6G).
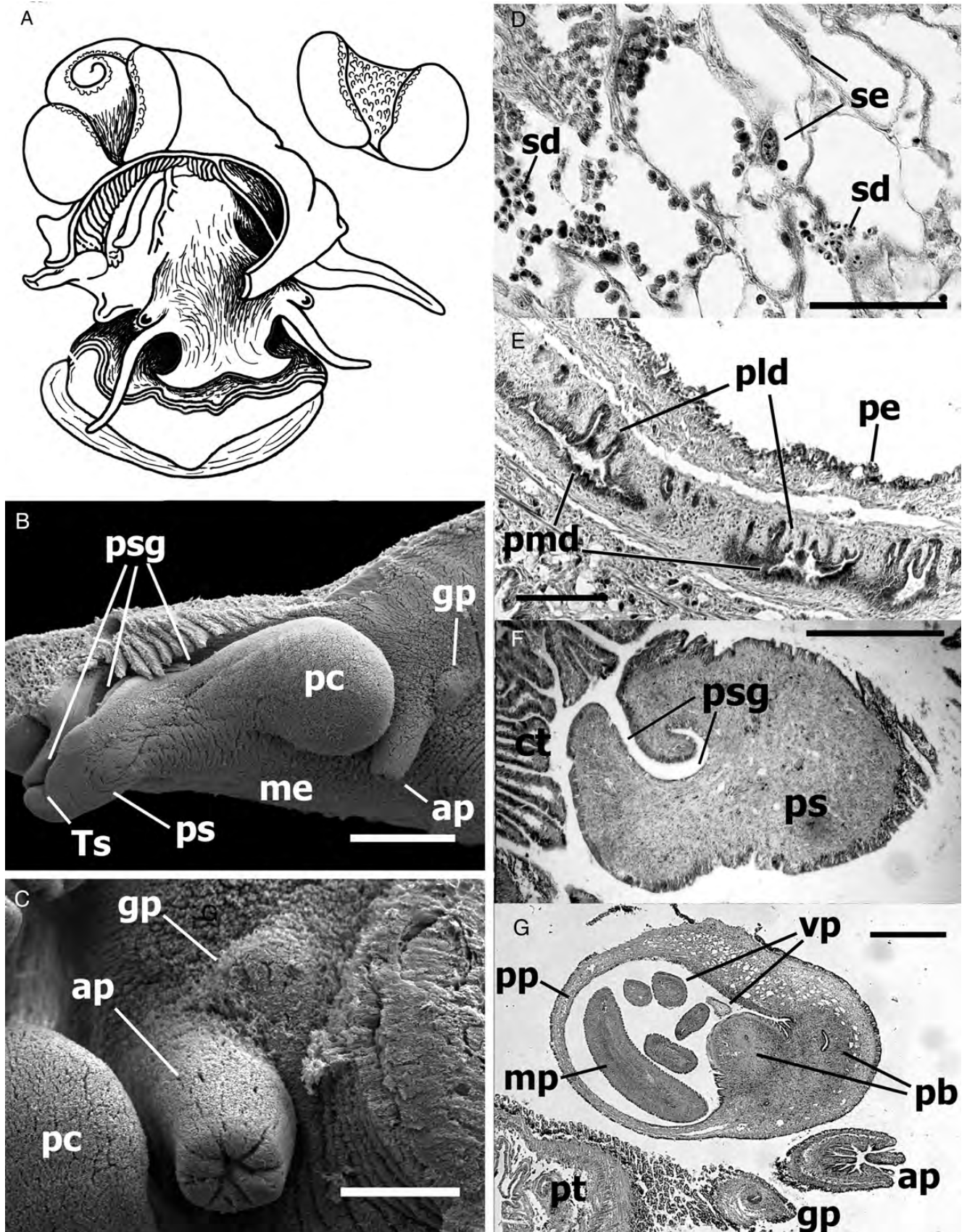
Figure 6. Juvenile males of *Pomacea canaliculata* (15 mm long, 30 days old). **A.** Schematic drawing of whole juvenile removed from shell showing testis tubules on initial whorls, palial gonoduct (prostate), genital papilla and copulatory apparatus. Note that the genital papilla has started to grow over the rectum. **B.** SEM of copulatory apparatus; ctenidial leaflets have been trimmed. **C.** SEM showing a close up of genital papilla
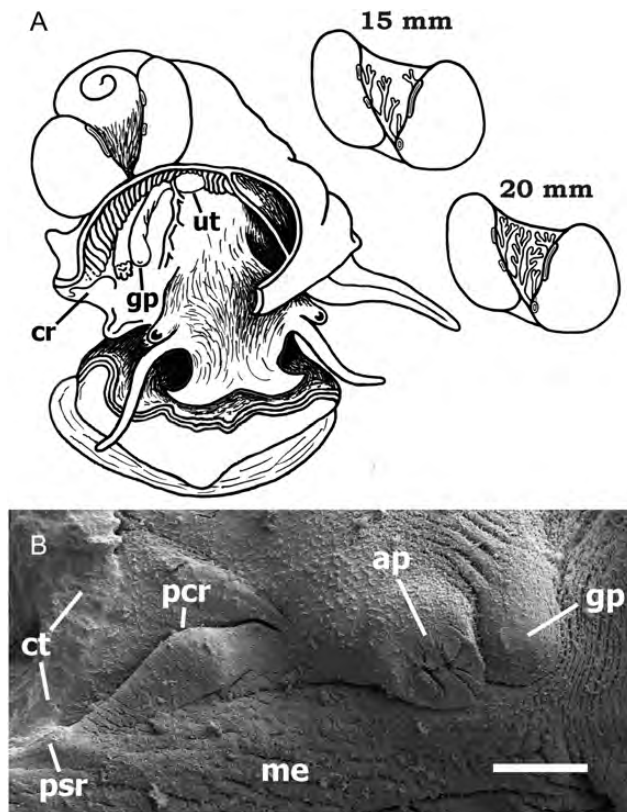
Figure 7. Females of *Pomacea canaliculata*, either juvenile (15 mm long, 30 days old) or subadult (20 mm long, 40 days old). **A.** Schematic drawing of whole juvenile at 15-mm stage removed from shell, showing developing pallial oviduct (with genital papilla, vagina and uterus) and copulatory rudiment. Insets illustrate first whorl cut and turned to show development of ovarian tubules across columellar aspect at 15-mm and 20-mm stages. **B.** SEM of the copulatory rudiment (15-mm female), with rudiments of penial sheath and the penial complex. Abbreviations: ap, anal papilla; cr, copulatory rudiment; ct, ctenidium; gp, genital papilla, me, inner mantle edge; pcr, penial complex rudiment; psr, penial sheath rudiment; ut, uterus. Scale bar = 300 μm.

In females, the pallial gonoduct is much more differentiated than in males. The proximal part has developed as an ovoid uterus bulging in the mantle's rear wall (Fig. 7A). The seminal receptacle can be recognized as a thin, convoluted duct, the albumen gland shows the first outgrowths of the parenchymal tubules, and the capsule gland has at least five of the six whorls (Fig. 8B) it will possess in adult life (Catalán de Canelada & Moreno, 1984; Catalán *et al.*, 2002; Hayes *et al.*, 2012). The distal whorl of the capsule gland is followed anteriorly by the vagina, a rather flattened duct lined by a ciliated columnar epithelium (Fig. 8C). The vagina is thinner proximally than distally and terminates to the right of the anal papilla (Fig. 7A), separated from the copulatory rudiment (Fig. 7B).

The copulatory rudiments of males and females have begun to develop differently at this stage (Figs 6A, B and 7A, B,

respectively). A penial pouch containing the developing penial complex and a growing penial sheath are apparent in males. In histological sections (Fig. 6G), the penial spermiduct penetrates three distinct regions as in adult snails: the penial bulb, and the muscular and vermiform portions of the penis (Giraud-Billoud *et al.*, 2013). However, the bulb spermiduct forms two distinct volutions that are not yet contained within the thick, muscular envelope that will develop later; the spermiduct within the muscular penis is not yet coiled. The T-shaped sulcus of the penial sheath is already present (Fig. 6B) (Giraud-Billoud *et al.*, 2013). In females, the copulatory rudiment remains similar to that in 10-mm juveniles (Fig. 7B).

Subadults (20 mm long, 40 days old)

At this stage (Figs 8–10), the testis is approaching adult proportions in males (Fig. 9A) and a whitish, arborescent ovary extends over the columellar aspect of the midgut gland in females (Fig. 7A). Microscopically, numerous spermatogenic nests are visible in the testis and some contain mature eupyrene sperm (Fig. 9D). In females, oocytes and oogonia are visible in the ovary (Fig. 10A).

In males, the pallial seminal vesicle has attained adult morphology, forming a cone adjoining the proximal end of the prostate. Its longitudinally folded lumen is lined by a ciliated columnar epithelium (not shown) and is continuous with the prostate's main duct. Tributaries of the prostate main duct have continued to expand (Fig. 9E), making the prostate thicker than in younger specimens (Fig. 6E). However, no tributaries to the main duct have yet appeared in the genital papilla.

In females, the uterus has grown, particularly through the ramifying and growth of the albumen gland parenchymal tubules (Fig. 10B), and it now bulges into both the mantle and renal cavities (Andrews, 1964). Distal to the capsule gland, the vagina has grown thicker and wider, and is internally lined with multiple longitudinal folds (Fig. 10C).

In males, the penial complex is similar to that of an adult, except that the penial bulb (Fig. 9B, G) has not yet developed into the thick, globular muscle typical of adult males (Gamarra-Luques *et al.*, 2006). The penial sheath is still smaller than in adult males but it shows the full glandular complement of the accessory outer and medial glands (Fig. 9F). These subadults engage in copulatory behaviours typical of adults (mounting and sheath intromission) (Burela & Martín, 2009), but apparently no sperm transfer occurs and no eggs are laid by females at this stage. In females, the copulatory rudiment may have enlarged somewhat with growth and the promontory formed by the penial sheath rudiment may be more developed, but they remain similar to that of 10-mm female juveniles.

Adults (25 mm, 50 days old)

The gross anatomy of gonoduct derivatives are fully developed as in mature males or females (Andrews, 1964; Thiengo *et al.*, 1993; Catalán *et al.*, 2002; Gamarra-Luques *et al.*, 2006; Hayes *et al.*, 2012; Giraud-Billoud *et al.*, 2013; Fig. 11). Histologically, the gonads possess abundant mature gametes in both sexes,

growing over rectum (same animal as in **B**). **D.** Histological section of testis tubules showing germ cells up to the round spermatid stage and large Sertoli cells (iron haematoxylin). **E.** Histological section of prostate with lateral ducts emerging from central lumen (iron haematoxylin). **F.** Histological section of basal penial sheath, showing no development of outer gland (iron haematoxylin). **G.** Histological section of penial complex showing penial pouch, penial bulb (not fully developed), muscular penis and coiled, vermiform penis (iron haematoxylin). Abbreviations: ap, anal papilla; ct, ctenidium; gp, genital papilla; me, inner mantle edge; mp, muscular penis; pb, penial bulb; pc, penial complex; pe, pallial epithelium; pld, prostate lateral ducts; pmd, prostate main duct; pp, penial pouch; ps, penial sheath; psg, penial sheath groove; psp, penial sheath's medial gland; pt, prostate; sd, spermatids; se, Sertoli cells; Ts, T-sulcus; vp, vermiform penis. Scale bars: **B** = 300 μm; **C** = 200 μm, **D**, **E** = 100 μm; **F** = 50 μm; **G** = 200 μm.
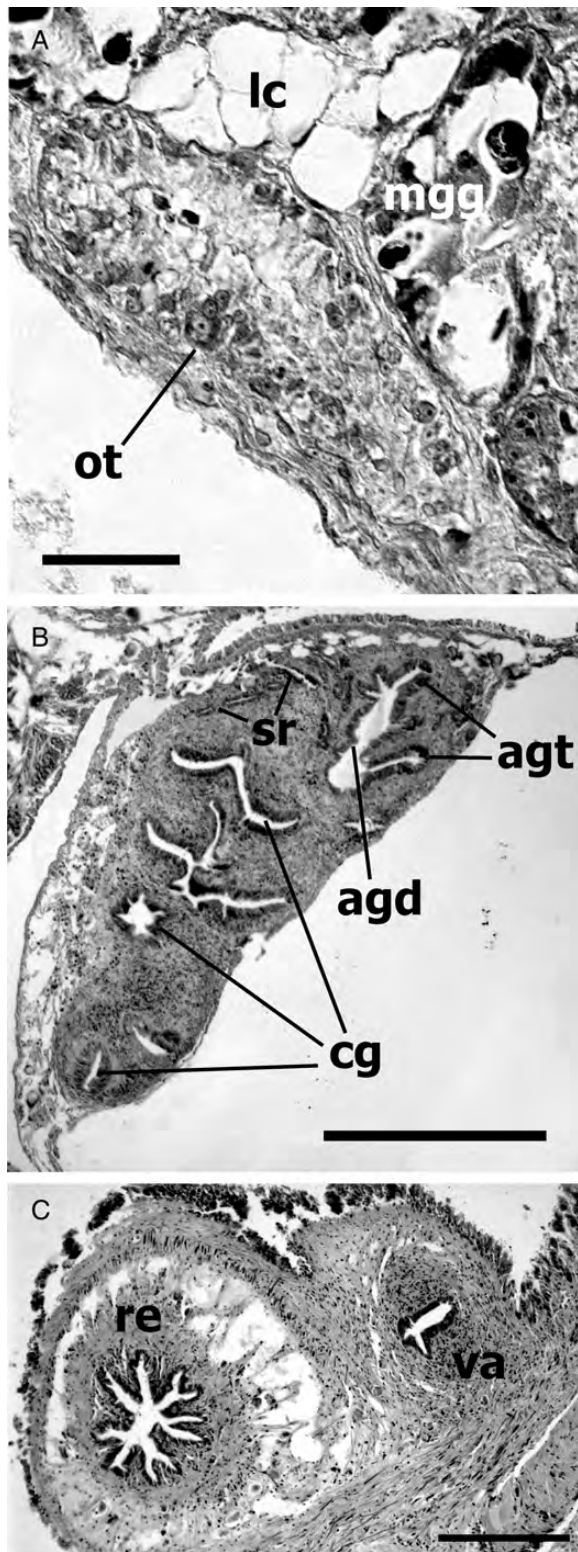
Figure 8. Juvenile females of *Pomacea canaliculata* (15 mm long, 30 days old) (iron haematoxylin). **A.** Histological section of ovarian tubule showing oogenic cells. **B.** Histological section of uterus, showing seminal receptacle, albumen gland duct with emerging parenchymal tubules, and capsule gland. **C.** Histological section of vagina midway between epitaenium and anus, showing slit-like vaginal lumen. Abbreviations: agd, albumen gland duct; agt, albumen gland tubules; cg, capsule gland; lc, Leydig cells; mgg, midgut gland; ot, ovarian tubules; re, rectum; sr, seminal receptacle; va, vagina. Scale bars = 50 μm.

and while Sertoli cells form a rather continuous envelope around each testicular tubule (Fig. 11A), ovarian tubular cells are small and scanty and many oogenic cells lie directly on the basal membrane (Fig. 11B). Histological features of the other reproductive organs (not shown) are as reported elsewhere for mature males and females of the species (Andrews, 1964; Moreno & Catalán de Canelada, 1985; Catalán *et al.*, 2002; Gamarra-Luques *et al.*, 2006; Hayes *et al.*, 2012; Giraud-Billoud *et al.*, 2013).

The copulatory apparatus is fully developed in males, while the female homologue may have remained as in the preceding stage or the proximal promontory may have extended as a flattened dome, as sometimes found in older females (see below).

Spawning is first seen at this stage, and the majority of deposited eggs are fertile.

The copulatory apparatus in fully grown females (30 mm or longer, 120–180 days old)

Development and growth of the female copulatory rudiment continues to varying degrees (Figs 12, 13). In 7 out of 14 animals, a small (<1 mm high) but distinct penial sheath is found, bearing a distal gland at the tip, while the rest of the copulatory apparatus appears as a flattened dome between the anal papilla and the anterior ctenidial tip (Fig. 12). However, the entire copulatory apparatus measures ≤4 mm in length and may be completely covered by the ctenidial leaflets.

In other cases, however, the flattened dome develops into a bulging penial bulb and a penial pouch. Under scanning electron microscopy the penial pouch may be a simple, round structure (Fig. 13A, B) or it may become more elongated and curved, terminating as a blunt cone (Fig. 13C) beside the anal papilla. These more developed penial pouches may be transparent in fresh material, with the coiled penis visible within (see Fig. 13D). In these more developed individuals, the penial sheath also possesses the penial sheath groove, the distinct indentation of the T-sulcus (Gamarra-Luques *et al.*, 2006), and both a medial and a distal gland (Fig. 13E) (Giraud-Billoud *et al.*, 2013). In the most extreme examples among older females, the penis and sheath structures are fully formed as in adult males, even though the whole apparatus remains minute (4 mm between the sheath's tip and the proximal end of the penial pouch) (Fig. 13F, G).

DISCUSSION

Derivatives of the gonoduct and the copulatory primordium

The current results are consistent with our preliminary observations (Gamarra-Luques *et al.*, 2006) that most male and female genital organs develop from a single primordial gonoduct, and that the copulatory apparatus develops from a separate, mantle-edge primordium which occurs in both sexes but remains rudimentary to varying degrees in females.

The gonadal portion of the gonoduct (an epithelioid cord-like structure, Table 1) lies on the columellar surface of the visceral hump in hatchlings. Ranjah (1942) described the origin of the gonadal primordium in *Pila globosa* as a thickening of the roof of the pericardial coelom, but he did not follow his observations until hatching, so that our observations cannot be sequentially related to his. Also, since the gonadal portion of the gonoduct is a solid structure in hatchlings of *Pomacea canaliculata*, the lumina of the adult gonadal tubules should be regarded as a secondary development and not as remnants of the embryonic coelom in this species.
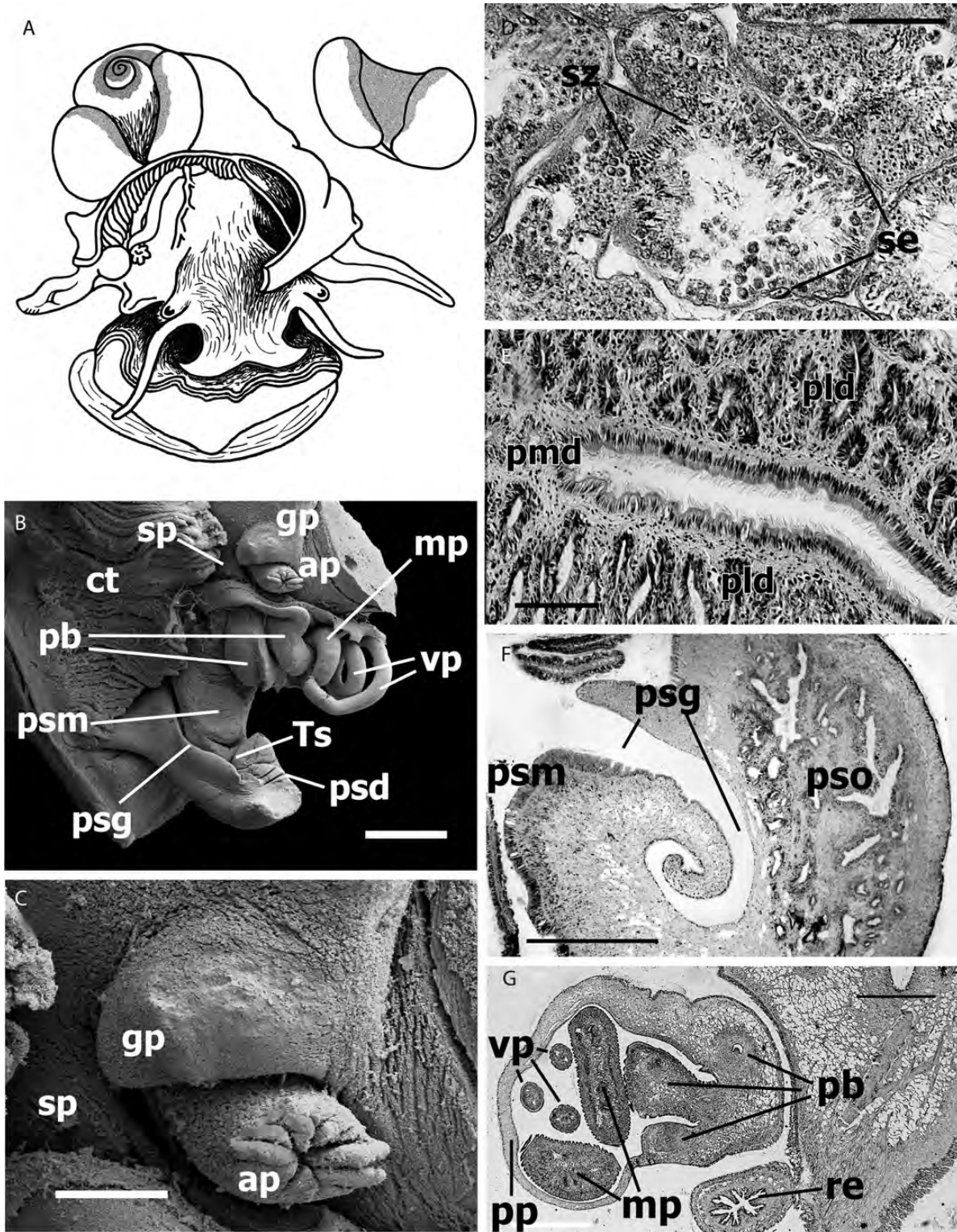
Figure 9. Subadult males of *Pomacea canaliculata* (20 mm long, 40 days old). **A.** Schematic drawing of whole juvenile removed from shell, showing genital papilla crossing rectum and fully-formed copulatory apparatus. Inset illustrates first whorl cut and turned to show the testis as a thick layer on the columellar aspect. **B.** SEM of copulatory apparatus. The penial pouch has been removed to reveal the other components of the penial complex (penis tip broken). **C.** SEM showing detail of genital papilla crossing rectum towards sperm pit. **D.** Histological section of testis tubules with mature sperm and several large Sertoli nuclei. **E.** Histological section of prostate showing ciliated main duct, with lateral outgrowths and tubular acini. **F.** Histological section of basal penial sheath, showing developing outer gland and medial gland. **G.** Histological section of penial complex. Note that penial bulb is not yet fully developed. Abbreviations: ap, anal papilla; ct, ctenidium; gp, genital papilla; mp, muscular penis; pb, penial bulb; pld, prostate lateral ducts; pmd, prostate main duct; pp, penial pouch; psd, penial sheath distal gland; psg, penial sheath groove; psm, penial sheath medial gland; pso, penial sheath outer gland; re, rectum; se, Sertoli cells; sp, sperm pit; sz, mature sperm; Ts, T-sulcus; vp, vermiform penis. Scale bars: **B** = 300 μm; **C** = 200 μm; **D** = 50 μm; **E** = 1000 μm; **F** = 200 μm.
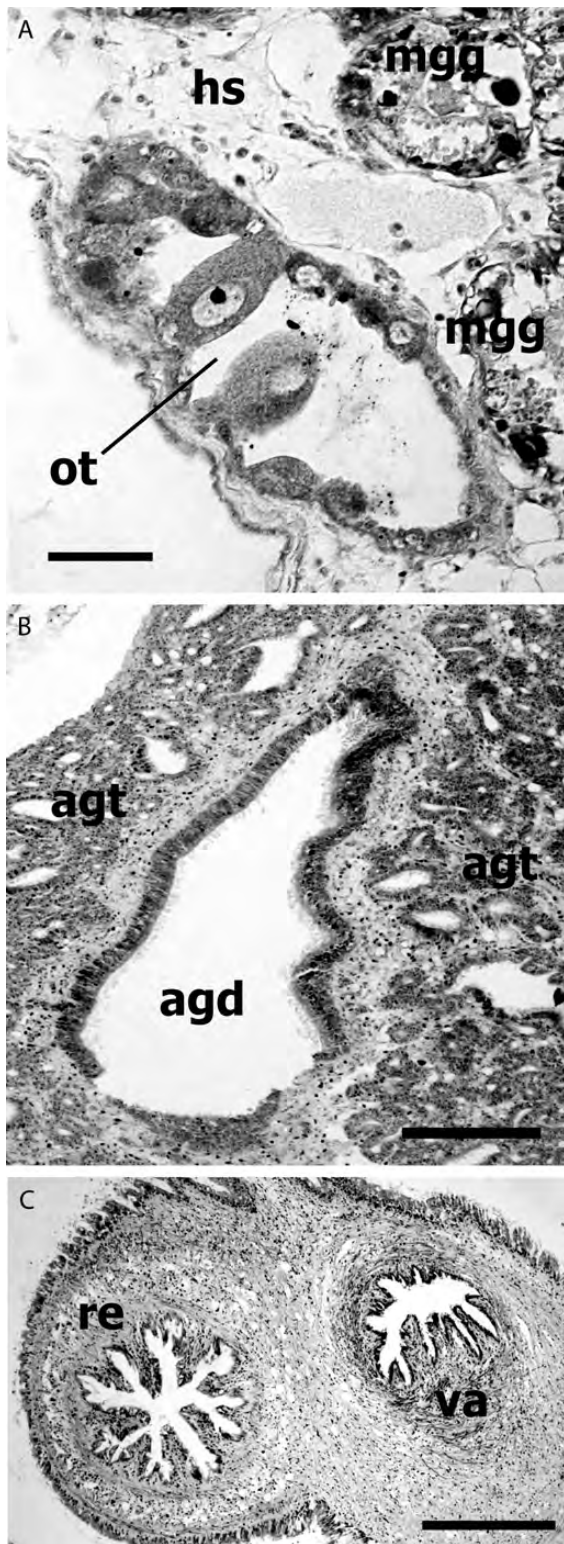
Figure 10. Subadult females of *Pomacea canaliculata* (20 mm long, 40 days old) (iron haematoxylin). **A.** Histological section of ovarian tubule with large lumen and germ cells at different stages of development. **B.** Histological section of ciliated albumen gland duct embedded within parenchymal tissue. **C.** Histological section of vagina midway between epitaenium and anus, showing enlarged, longitudinally folded lumen. Abbreviations: agd, albumen gland duct; agt, albumen gland tubules; hs, haemocoelic spaces; mgg, midgut gland; ot, ovarian tubules; re, rectum; va, vagina. Scale bars = 50 μm.

Homologies of the genital system

Reproductive organogenesis can give important insight into questions of homology of the genital organs. For example, derivatives of the gonadal and visceral gonoduct in *P. canaliculata* correspond to the gonad and visceral vas deferens (='renal' vas deferens) of other Caenogastropoda. The transition from the testis tubules to the vas deferens occurs without any intermediate structure such as the (visceral) seminal vesicle of sorbeoconchan caenogastropods, which is a convoluted initial part of the visceral vas deferens (Hyman, 1967; Ponder & Lindberg, 1997) and no indication of a visceral seminal vesicle was observed during development in *P. canaliculata*. However, this species does possess a seminal vesicle formed within the rear wall of the pallial cavity at the 10 mm stage, which is here identified as the 'pallial seminal vesicle'. This vesicle also occurs in other ampullariids (Simone, 2004, 2011) and is not homologous with the (visceral) seminal vesicle of sorbeoconchans.

The pallial gonoduct derivatives distal to the pallial seminal vesicle in *P. canaliculata* seem homologous to the prostate and pallial vas deferens found in the Sorbeoconcha (Ponder & Lindberg, 1997) and in basal ampullariids (Berthold, 1989). A tube-like pallial gonoduct is already present at the time of hatching (Fig. 3D) and the prostate develops from this part of the gonoduct (Fig. 6A, E). The pallial gonoduct ends in the pallial opening of the genital papilla. Consequently, the pallial spermiduct is not anatomically continuous with the penis, and sperm are transferred from the genital papilla to the so-called sperm pit (Berthold, 1989; Fig. 9C), which is also present in *Marisa cornuarietis* (Berthold, 1989; Schulte-Oehlmann *et al.*, 1994). Sperm are passed from the sperm pit to the penis by the muscular action of the penial bulb (Giraud-Billoud *et al.*, 2013). The male genital papilla appears rather late in development (15 mm juveniles, Fig. 6A–C) as a distal continuity of the pallial gonoduct, later crossing over the rectum (20-mm juveniles, Fig. 9A–C). This crossing-over is a crucial event in male differentiation as it brings the papilla into contact with the sperm pit, which is necessary for sperm transfer to the penis to occur. The adult genital papilla is histologically different from the prostate (not shown) and an interesting question is whether this structure might be homologous with the penis of sorbeoconchan gastropods. Testing of this hypothesis may require the examination of more basal members of the family.

It is clear, however, that the genital papilla of *P. canaliculata* is not homologous with the 'vas deferens papilla' described by Schulte-Oehlmann *et al.* (1995) in *M. cornuarietis*. In fact, the genital papilla of *P. canaliculata* is derived from the most distal part of the gonoduct, while that of *M. cornuarietis* develops from the copulatory rudiment.

There are no homologous structures of the ampullariid copulatory organs in other gastropod families, and a copulatory apparatus derived from the mantle edge is an apomorphy of the Ampullariidae (Berthold, 1989).

It was observed here that the female pallial gonoduct develops rapidly from a simple widening of the gonoduct at the entrance to the pallial cavity (10-mm long juveniles) into a small but seemingly complete uterus, with recognizable seminal receptacle, albumen and capsule glands, by the 15-mm stage (Fig. 8B). Distally, the female pallial gonoduct gives rise to the vagina (Fig. 7A), which is much longer than that found in other caenogastropods.

The operculum, onset of copulatory behaviour, fertilization and oviposition

The shape and dimensions of the operculum are sexually dimorphic in *P. canaliculata* (Cazzaniga, 1990; Estebenet *et al.*,
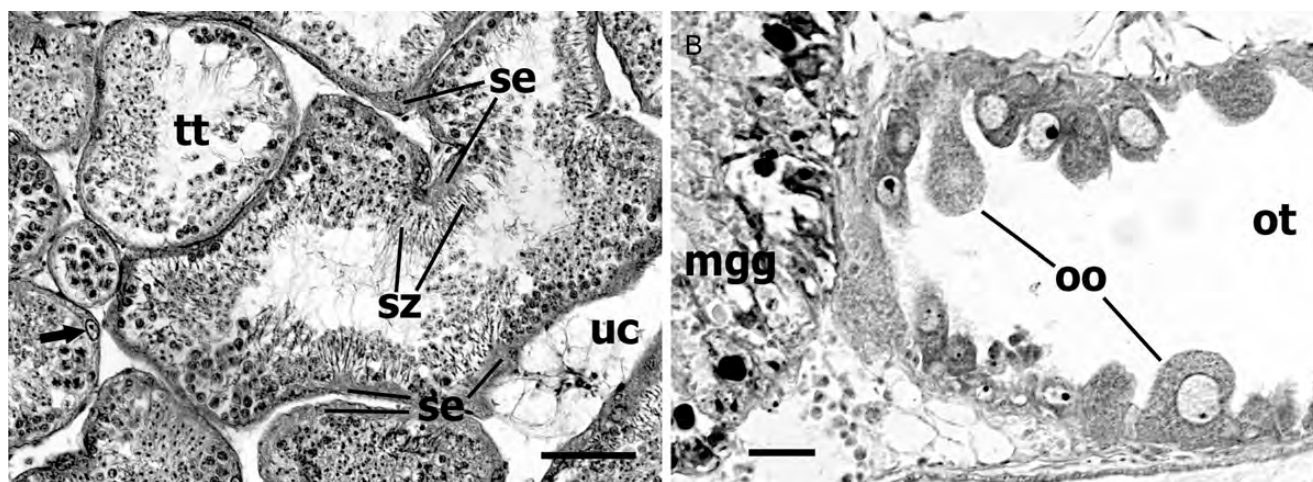
Figure 11. Adult gonads of *Pomacea canaliculata* (25 mm long, 50 days old). **A.** Histological section of large testis tubules delimited by a band of Sertoli cells with mature sperm; black arrow indicates a large Sertoli nucleus. **B.** Histological section of ovarian tubule with large lumen and oogenic cells at different stages, including large pedunculate oocytes. Abbreviations: mgg, midgut gland; oo, oocytes; ot, ovarian tubules; se, Sertoli cells; sz, mature sperm; tt, testis tubules; uc, perivascular urate cells. Scale bars = 50 μm.
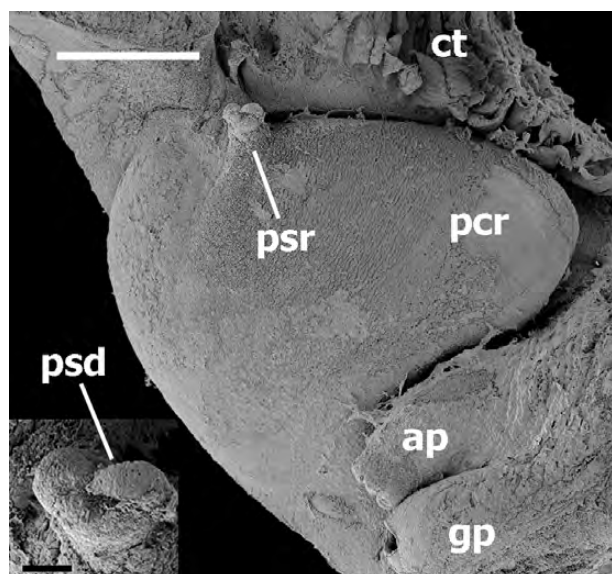

Figure 12. Copulatory rudiment in fully grown females of *Pomacea canaliculata* (≥30 mm long, 120–180 days old). SEM of copulatory rudiment in a female 120 days old; ctenidial leaflets have been trimmed. Inset shows detail of penial sheath tip with developing distal gland . Abbreviations: ap, anal papilla; ct, ctenidium; gp, genital papilla; pcr, penial complex rudiment; psd, penial sheath distal gland; psr, penial sheath rudiment. Scale bar = 1000 μm; inset = 100 μm.

2006): it is slightly concave in juveniles irrespective of gender and remains concave in females, but most of its surface becomes convex in males after week 4 or 5 (i.e. between the 15- and 20-mm stages). The display of copulatory behaviour also starts at this time. This change in shape of the operculum occurs in a matter of hours and, as any other secondary sexual character, may be the result of hormone-regulated morphogenetic processes.

Oviposition behaviour starts between the 20- and 25-mm stages, i.e. *c.* 2 weeks after copulatory behaviour has started. The majority of eggs are fertile in the first deposited egg clutches, which confirms the view that oviposition is determined by the existence of fertilized eggs in this species (Estebenet & Cazzaniga, 1998; Gamarra-Luques *et al.*, 2006). The 2-week delay between the beginning of copulation and oviposition may be related to the incomplete development of the penial bulb in 20-mm males, since this bulb seems critical for the transfer of sperm from the sperm pit to the penis (Giraud-Billoud *et al.*, 2013).

Protandric hermaphroditism?

The presence of a rudimentary penis and its sheath in females was considered by Bouvier (1888) as indicative of hermaphroditic ancestry. However, Sachwatkin (1920), Prashad (1925) and Hylton Scott (1958) did not find any evidence of a hermaphroditic gonad, nor of a transition from one sex to the other, in adult ampullariids. Notwithstanding, Keawjam & Upatham (1990) suggested the possibility of protandric hermaphroditism in *P. canaliculata* from Thailand, probably due to the fact that males are usually smaller than females in the field (Estebenet & Martín, 2003; Estebenet *et al.*, 2006). In the current study, no indication of a hermaphroditic gonad was observed, as the gonadal primordium differentiates into either the testis or the ovary at the 10-mm stage and is presumably genetically determined (Yusa, 2007). In addition, the pallial gonoduct differentiates during the next stage (15 mm) into either male (Fig. 6A, E) or female structures (Fig. 8B, C) in all cases studied here, and we have never observed transitional structures in any individual, regardless of developmental stage, dissected in our laboratory for various purposes over the course of several years (Castro-Vazquez *et al.*, unpubl.).

The copulatory apparatus develops at the same rate in both sexes from birth to the 10-mm stage. From then on, the copulatory apparatus will grow rapidly in males (Fig. 5A, B, E) until it reaches adult size; in females, however, the copulatory apparatus will only continue to grow at a very slow rate (Figs 7, 12, 13).

The effects of xenobiotics

Studies on this subject have been made in ampullariids that have been exposed at different ages, from hatching to adulthood (Schulte-Oehlmann *et al.*, 2000; Tillmann *et al.*, 2001; Oehlmann *et al.*, 2006), or in adult animals exposed for
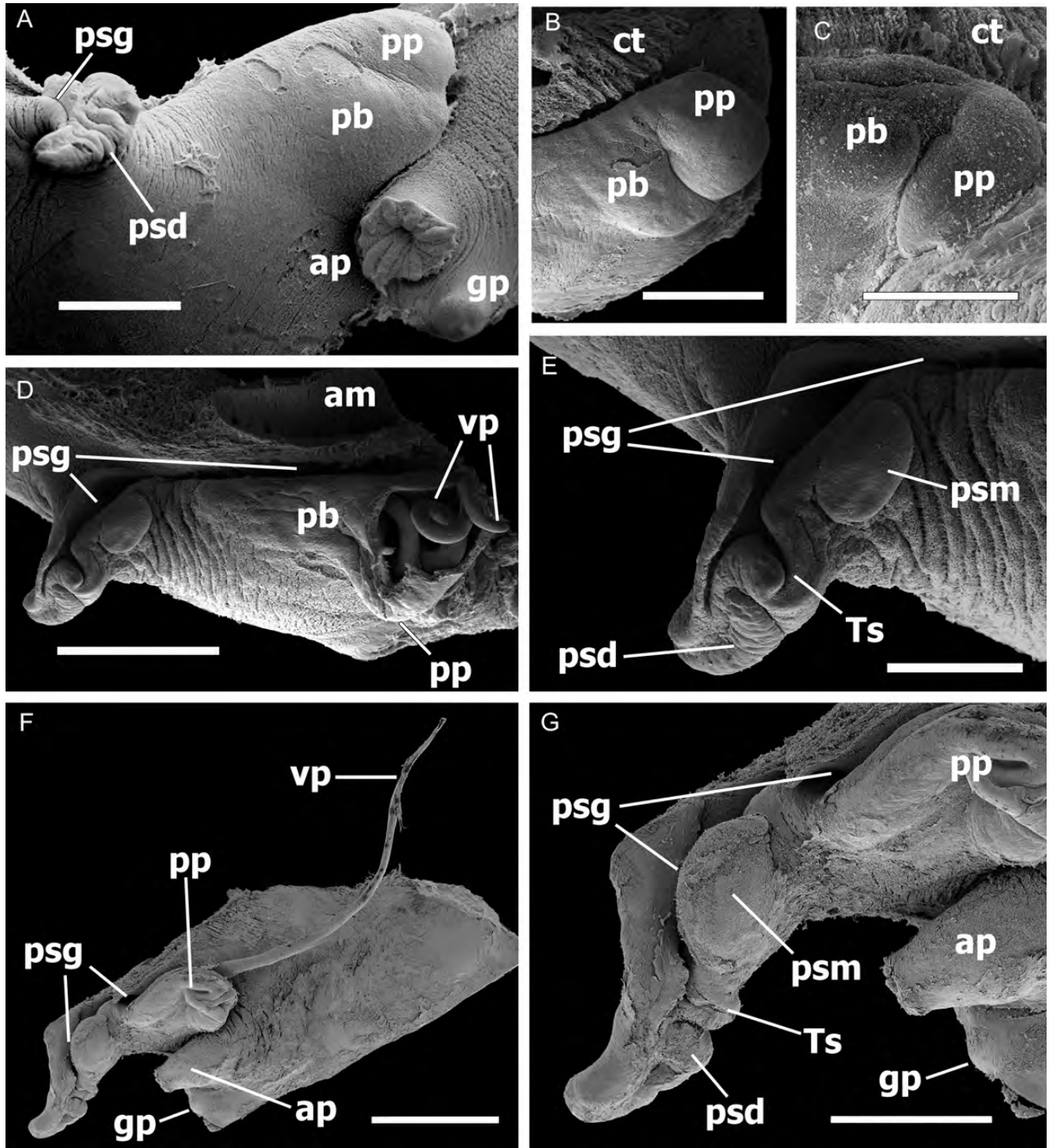
Figure 13. Copulatory rudiment in fully grown females of *Pomacea canaliculata* ($\geq$30 mm long, 120–180 days old). SEMs showing advanced development of the penial sheath and penial complex. **A.** Bulging penial bulb within penial pouch; penial sheath with distal gland. **B.** Penial complex showing a distinct penial bulb and a rounded penial pouch. **C.** Penial pouch has developed further and has bent forward as a blunt cone. **D.** Conical penial pouch opened to show coiled penis within (penis tip broken). **E.** Detail of penial sheath, showing corrugated surface of the distal gland, a fully developed T-sulcus, and a large, flattened medial gland (same animal as in **D**). **F.** Maximum development of copulatory rudiment observed in this study. Penis removed from penial pouch through pouch orifice and uncoiled before fixation. **G.** Detail of penial sheath (same animal as in **F**), showing penial sheath distal gland and medial gland, T-sulcus and penial sheath groove. Abbreviations: am, insertion surface of the adductor muscle; ap, anal papilla; ct, ctenidium; gp, genital papilla; pb, penial bulb; pp, penial pouch; psg, penial sheath groove; psd, penial sheath distal gland; psm, penial sheath medial gland; Ts, T-sulcus; vp, vermiform penis. Scale bars: **A–D** = 1000 μm; **E** = 400 μm; **F** = 2000 μm; **G** = 500 μm.

different durations (Schulte-Oehlmann *et al.*, 1994, 1995; Takeda, 2000; Tillmann *et al.*, 2001; Oehlmann *et al.*, 2006). At least five developmental periods can be distinguished on the basis of the present study, during which different control mechanisms may be acting, each with the potential for generating different responses to xenobiotics: (1) juveniles from birth to 5 mm long, during which the rudiments of both the gonoduct and the copulatory apparatus become fully established; (2) juveniles from 5 to 10 mm long, when gonadal differentiation occurs; (3) juveniles from 10 to15 mm, when the pallial gonoduct differentiates in both sexes and the copulatory apparatus rapidly grows in males; (4) subadults from 15 to 20 mm, when the gonads and accessory structures develop, as well as the physiological mechanisms of copulation and (5) adults from 20 to 25 mm, when both fertile sperm and mature oocytes are produced, with repeated and long copulations resulting in fertile eggs that are laid on a regular basis (Albrecht, Carreño & Castro-Vazquez, 1996). Full consideration of these stages will help to understand the hormonal control of development and potential targets for endocrine disruptors that may be useful in controlling populations of this invasive snail.

In addition, studies of female masculinization (='imposex'; Smith, 1971) in ampullariids must consider that masculinizing substances such as tributyltin (TBT) and triphenyltin (TPT) act on the copulatory rudiment normally present in females near the right mantle edge (Schulte-Oehlmann *et al.*, 1995, 2000) and which is independent from the pallial gonoduct derivatives. In this respect, ampullariids are different from other caenogastropods and from vetigastropods where masculinizing xenobiotics induce the extension of the pallial gonoduct as a pallial vas deferens and the appearance of a small penis (or penes) on the right side of the female neck (e.g. Gibbs & Bryan, 1986; Gibbs *et al.*, 1987; Bauer *et al.*, 1997; Matthiessen & Gibbs, 1998; Oehlmann *et al.*, 2000; Tillmann *et al.*, 2001; Shi *et al.*, 2005; Titley-O'Neal, Munkittrick & MacDonald, 2011).

Age of the individual also must be considered when assessing the effects of exposure, as the copulatory rudiment will continue to develop in females after they have reached sexual maturity (Figs 12, 13). For instance, the development of the female copulatory rudiment after TBT treatment reported by Takeda (2000) is similar in magnitude to that reported here in untreated females, 120–180 days old (e.g. compare Fig. 3A of Takeda, 2000 with Fig. 13F of the present study). In Takeda's (2000) study, female *P. canaliculata* of unreported age were exposed to TBT or testosterone in water for 3–8 months without an experimental control group of unexposed females of the same age. A detailed study of the influence of age and exposure to TBT on development of the copulatory rudiment is in preparation (available in abstract form, Giraud-Billoud *et al.*, 2010a, b).

Furthermore, we have shown that the pallial gonoduct derivatives of *P. canaliculata* end at the genital papilla, and are not continuous with the copulatory apparatus (Fig. 9). This is in contrast with the interpretation that a 'vas deferens' enters the base of the copulatory apparatus in TBT-treated females of this species (Takeda, 2000: Fig. 3A); indeed, the 'vas deferens' in that figure seems to be a haemolymphatic vessel.

It should also be noted that the occurrence of imposex in four of the six ampullariid species quoted in the current lists of Titley-O'Neal *et al.* (2011) and Shi *et al.* (2005) is erroneous, since the inclusion of *Pila globosa*, *Pila polita* (as *Ampullaria polita*), *Pila cinerea* (as *A. cinerea*) and *Pomacea maculata* (as *A. gigas*) refers to the natural occurrence of the copulatory rudiment in females of these species (Semper, 1862; Sachwatkin, 1920; Hägler, 1923; Prashad, 1925) and not to the '. . . superimposition of male characters on to . . . females'

(Smith, 1971: 377). For the remaining two species, the case for the xenobiotic induction of imposex in *M. cornuarietis* is experimentally well supported (Schulte-Oehlmann *et al.*, 1995, 2000, 2004; Janer *et al.*, 2006), while the case of *P. canaliculata* (Takeda, 2000) is subject to the objections mentioned above.

This is the first report on post-hatching reproductive organogenesis in an ampullariid species. Our conclusions will have to be compared with studies in other ampullariids, particularly those in the more basal African and Asian genera in which a significant diversity may be found, but also in other neotropical species including additional populations of *P. canaliculata*.

REFERENCES

ALBRECHT, E.A., CARREÑO, N.B. & CASTRO-VAZQUEZ, A. 1996. A quantitative study of copulation and spawning in the South American applesnail, *Pomacea canaliculata* (Prosobranchia: Ampullariidae). *Veliger*, **39**: 142–147.

ANDREWS, E. 1964. The functional anatomy and histology of the reproductive system of some pilid gastropod molluscs. *Proceedings of the Malacological Society of London*, **36**: 121–140.

BAUER, B., FIORONI, P., SCHULTE-OEHLMANN, U., OEHLMANN, J. & KALBFUS, W. 1997. The use of *Littorina littorea* for tributyltin (TBT) effect monitoring—results from the German TBT survey 1994/1995 and laboratory experiments. *Environmental Pollution*, **96**: 299–309.

BERTHOLD, T. 1989. Comparative conchology and functional morphology of the copulatory organ of the Ampullariidae (Gastropoda, Monotocardia) and their bearing upon phylogeny and palaeontology. *Abhandlungen naturwissenschaftlicher Vereins in Hamburg*, **28**: 141–164.

BOUVIER, M.E.L. 1888. Étude sur l'organisation des Ampullaires. *Mémoires de la Societé Philomatique, Paris*, Volume du Centenaire: 63–85.

BROOKS, W. & MCGLONE, B. 1908. The origin of the lung of *Ampullaria. Carnegie Institution of Washington Publication*, **102**: 95–104.

BURELA, S. & MARTÍN, P.R. 2009. Sequential pathways in the mating behavior of the apple snail *Pomacea canaliculata* (Caenogastropoda: Ampullariidae). *Malacologia*, **51**: 157–164.

CATALÁN, N., FERNANDEZ, S. & WINIK, B. 2002. Oviductal structure and provision of egg envelops in the apple snail *Pomacea canaliculata* (Gastropoda, Prosobranchia, Ampullariidae). *Biocell*, **26**: 91–100.

CATALÁN DE CANELADA, N.M.Y. & MORENO, A.R. 1984. Descripción del tracto intrauterino en el gasterópodo *Ampullaria canaliculata*. *Neotropica*, **30**: 153–160.

CAZZANIGA, N. 1990. Sexual dimorphism in *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Veliger*, **33**: 384–388.

CLARK, G. 1981. *Staining procedures*. Williams & Wilkins, Baltimore, MD.

DEMIAN, E.S. & YOUSIF, F. 1973a. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda, Ampullariidae). II. Development of the alimentary system. *Malacologia*, **12**: 151–174.

DEMIAN, E.S. & YOUSIF, F. 1973b. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). I. General outlines of development. *Malacologia*, **12**: 123–150.

DEMIAN, E.S. & YOUSIF, F. 1973c. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). III. Development of the circulatory and renal systems. *Malacologia*, **12**: 175–194.

DEMIAN, E.S. & YOUSIF, F. 1973d. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). IV. Development of the shell gland, mantle and respiratory organs. *Malacologia*, **12**: 195–211.

DEMIAN, E.S. & YOUSIF, F. 1975. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). V. Development of the nervous system. *Malacologia*, **15**: 29–42.

ESTEBENET, A. & CAZZANIGA, N. 1992. Growth and demography of *Pomacea canaliculata* (Gastropoda: Ampullariidae) under laboratory conditions. *Malacological Review*, **25**: 1–12.

ESTEBENET, A.L. & CAZZANIGA, N.J. 1998. Sex-related differential growth in *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Journal of Molluscan Studies*, **64**: 119–123.

ESTEBENET, A.L. & MARTÍN, P.R. 2003. Shell interpopulation variation and its origin in *Pomacea canaliculata* (Gastropoda: Ampullariidae) from Southern Pampas, Argentina. *Journal of Molluscan Studies*, **69**: 301–310.

ESTEBENET, A.L., MARTÍN, P.R. & BURELA, S. 2006. Conchological variation in *Pomacea canaliculata* and other South American Ampullariidae (Caenogastropoda, Architaenioglossa). *Biocell*, **30**: 329–335.

FERNANDO, W. 1931. The development of the kidney in *Ampullaria (Pila) gigas*. *Proceedings of the Zoological Society of London*, **62**: 745–750.

GAMARRA-LUQUES, C., WINIK, B., VEGA, I.A., ALBRECHT, E.A., CATALÁN, N. & CASTRO-VAZQUEZ, A. 2006. An integrative view to structure, function, ontogeny and phylogenetical significance of the male genital system in *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Biocell*, **30**: 345–357.

GIBBS, P. & BRYAN, G. 1986. Reproductive failure in populations of the dog-whelk, *Nucella lapillus*, caused by imposex induced by tributyltin from antifouling paints. *Journal of the Marine Biological Association of the United Kingdom*, **66**: 767–777.

GIBBS, P., BRYAN, G., PASCOE, P. & BURT, G. 1987. The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *Journal of the Marine Biological Association of the United Kingdom*, **67**: 507–523.

GIRAUD-BILLOUD, M., GAMARRA-LUQUES, C. & CASTRO-VAZQUEZ, A. 2013. Functional anatomy of male copulatory organs of *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Zoomorphology*, doi 10.1007/s00435-012-0183-y.

GIRAUD-BILLOUD, M., KOCH, E., VEGA, I.A., GAMARRA-LUQUES, C. & CASTRO-VAZQUEZ, A. 2008. Urate cells and tissues in the South American apple-snail *Pomacea canaliculata*. *Journal of Molluscan Studies*, **74**: 259–266.

GIRAUD-BILLOUD, M., VEGA, I.A., CARRIÓN, A.M. & CASTRO-VAZQUEZ, A. 2010a. A quantitative study of the androtrophic effect ('imposex') of an organotin and of other metal compounds on females of an ampullariid snail. *Biocell*, **34**: 48, abstract.

GIRAUD-BILLOUD, M., VEGA, I.A., CARRIÓN, A.M. & CASTRO-VAZQUEZ, A. 2010b. Rudimentary male copulatory organs in females of an ampullariid snail. *Biocell*, **34**: 49, abstract.

HÄGLER, K. 1923. Anatomie von *Pachylabra (Ampullaria) cinerea* Reeve: I. Teil. Mit Einschluss einer kurzen Besprechung der wichstigen Literatur über die Ampullariidae. *Acta Zoologica*, **4**: 313–424.

HAYES, K.A., COWIE, R.H., THIENGO, S.C. & STRONG, E.E. 2012. Comparing apples to apples: clarifying the identities of two highly invasive Neotropical Ampullariidae. *Zoological Journal of the Linnean Society*, **166**: 723–753.

HAYES, K.A., JOSHI, R.C., THIENGO, S.C. & COWIE, R.H. 2008. Out of South America: multiple origins of non native apple snails in Asia. *Diversity and Distributions*, **14**: 701–712.

HERAS, H., GARIN, C. & POLLERO, R. 1998. Biochemical composition and energy sources during embryo development and in early juveniles of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). *Journal of Experimental Zoology*, **280**: 375–383.

HYLTON SCOTT, M. 1934. Sobre el desarrollo embrionario de *Ampullaria canaliculata*. *Revista del Museo de La Plata*, **34**: 373–385.

HYLTON SCOTT, M.I. 1958. Estudio morfológico y taxonómico de los ampulláridos de la República Argentina. *Revista del Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia'*, **3**: 233–333.

HYMAN, L. 1967. *The invertebrates*. McGraw-Hill, New York.

JANER, G., LYSSIMACHOU, A., BACHMANN, J., OEHLMANN, J., SCHULTE-OEHLMANN, U. & PORTE, C. 2006. Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: the effect of xenoandrogenic compounds. *Steroids*, **71**: 435–444.

KEAWJAM, R. & UPATHAM, E. 1990. Shell morphology, reproductive anatomy and genetic patterns of three species of apple snails of the genus *Pomacea* in Thailand. *Journal of Medical and Applied Malacology*, **2**: 45–57.

KOCH, E., WINIK, B.C. & CASTRO-VAZQUEZ, A. 2009. Development beyond the gastrula stage and digestive organogenesis in the apple-snail *Pomacea canaliculata* (Architaenioglossa, Ampullariidae). *Biocell*, **33**: 49–65.

MATTHIESSEN, P. & GIBBS, P. 1998. Critical appraisal of the evidence for tributyltin mediated endocrine disruption in mollusks. *Environmental Toxicology and Chemistry*, **17**: 37–43.

MORENO, A. & CATALÁN DE CANELADA, N. 1985. Descripción histológica e histoquímica del receptáculo seminal en el gasterópodo *Ampullaria canaliculata*. *Neotropica*, **31**: 27–37.

OEHLMANN, J., SCHULTE-OEHLMANN, U., BACHMANN, J., OETKEN, M., LUTZ, I., KLOAS, W. & TERNES, T.A. 2006. Bisphenol A induces superfeminization in the ramshorn snail *Marisa cornuarietis* (Gastropoda: Prosobranchia) at environmentally relevant concentrations. *Environmental Health Perspectives*, **114**(Suppl. 1): 127–133.

OEHLMANN, J., SCHULTE-OEHLMANN, U., TILLMANN, M. & MARKERT, B. 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. *Ecotoxicology*, **9**: 383–397.

PONDER, W.F. & LINDBERG, D.R. 1997. Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. *Zoological Journal of the Linnean Society*, **119**: 83–265.

PRASHAD, B. 1925. Anatomy of the common Indian apple-snail, *Pila globosa*. *Memoirs of the Indian Museum*, **8**: 91–154.

RANJAH, A. 1942. The embryology of the Indian apple-snail, *Pila globosa* (Swainson) (Mollusca, Gastropoda). *Memoirs of the Indian Museum*, **44**: 217–322.

SACHWATKIN, V. 1920. Das Urogenitalsystem von *Ampullaria gigas* Spix. *Acta Zoologica*, **1**: 67–130.

SCHULTE-OEHLMANN, U., BETTIN, C., FIORONI, P., OEHLMANN, J. & STROBEN, E. 1995. *Marisa cornuarietis* (Gastropoda, Prosobranchia): a potential TBT bioindicator for freshwater environments. *Ecotoxicology*, **4**: 372–384.

SCHULTE-OEHLMANN, U., FIORONI, P., OEHLMANN, J. & STROBEN, E. 1994. The genital system of *Marisa cornuarietis* (Gastropoda, Ampullariidae)–a morphological and histological analysis. *Zoologische Beiträge*, **36**: 59–81.

SCHULTE-OEHLMANN, U., OETKEN, M., BACHMANN, J. & OEHLMANN, J. 2004. Effects of ethinyloestradiol and methyltestosterone in prosobranch snails. In: *Pharmaceuticals in the environment: sources, fate, effects and risks* (K. Kümmerer, ed.), pp. 233–347. Springer, Berlin, Germany.

SCHULTE-OEHLMANN, U., TILLMANN, M., MARKERT, B., OEHLMANN, J., WATERMANN, B. & SCHERF, S. 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: Triphenyltin as a xeno-androgen. *Ecotoxicology*, **9**: 399–412.

SEMPER, C. 1862. *Entwickelungsgeschichte der* Ampullaria polita *Deshayes nebst Mittheilungen über die Entwickelungsgeschichte einiger andern Gastropoden aus den Tropen*. C. van der Post Jr., Utretch.

SHI, H.H., HUANG, C.J., ZHU, S.X., YU, X.J. & XIE, W.Y. 2005. Generalized system of imposex and reproductive failure in female gastropods of coastal waters of mainland China. *Marine Ecology Progress Series*, **304**: 179–189.

SIMONE, L.R.L. 2004. Comparative morphology and phylogeny of representatives of the superfamilies of architaenioglossans and the Annulariidae (Mollusca, Caenogastropoda). *Arquivos do Museu Nacional, Rio de Janeiro*, **62**: 387–504.

SIMONE, L.R.L. 2011. Phylogeny of the Caenogastropoda (Mollusca), based on comparative morphology. *Arquivos de Zoologia (Museu de Zoologia da Universidade de Sao Paulo)*, **42**: 161–323.

SMITH, B.S. 1971. Sexuality in the American mud snail, *Nassarius obsoletus* Say. *Proceedings of the Malacological Society of London*, **39**: 377–378.

SUN, J., ZHANG, Y., THIYAGARAJAN, V., QIAN, P. & QIU, J. 2010. Protein expression during the embryonic development of a gastropod. *Proteomics*, **10**: 2701–2711.

TAKEDA, N. 2000. Development of a penis from the vestigial penis in the female apple snail, *Pomacea canaliculata*. *Biological Bulletin*, **199**: 316–320.

THIENGO, S. 1987. Observations on the morphology of *Pomacea lineata* (Spix, 1827) (Mollusca, Ampullariidae). *Memórias do Instituto Oswaldo Cruz*, **82**: 563–570.

THIENGO, S. 1989. On *Pomacea sordida* (Swainson, 1823) (Prosobranchia, Ampullariidae). *Memórias do Instituto Oswaldo Cruz*, **84**: 351–355.

THIENGO, S., BORDA, C. & ARAÚJO, J. 1993. On *Pomacea canaliculata* (Lamarck, 1822) (Mollusca; Pilidae: Ampullariidae). *Memórias do Instituto Oswaldo Cruz*, **88**: 67–71.

TILLMANN, M., SCHULTE-OEHLMANN, U., DUFT, M., MARKERT, B. & OEHLMANN, J. 2001. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part III: Cyproterone acetate and vinclozolin as antiandrogens. *Ecotoxicology*, **10**: 373–388.

TITLEY-O'NEAL, C.P., MUNKITTRICK, K.R. & MACDONALD, B.A. 2011. The effects of organotin on female gastropods. *Journal of Environmental Monitoring*, **13**: 2360–2388.

VEGA, I.A., GIRAUD-BILLOUD, M., KOCH, E., GAMARRA-LUQUES, C. & CASTRO-VAZQUEZ, A. 2007. Uric acid accumulation within intracellular crystalloid corpuscles of the midgut gland in *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Veliger*, **48**: 276–283.

VOLTZOW, J. 1994. Gastropoda: Prosobranchia. In: *Microscopic anatomy of invertebrates*, Vol. 5 (F. Harrison & A. Kohn, eds), pp. 111–252. Wiley-Liss, New York.

YUSA, Y. 2007. Nuclear sex-determining genes cause large sex-ratio variation in the apple snail *Pomacea canaliculata*. *Genetics*, **175**: 179–184.