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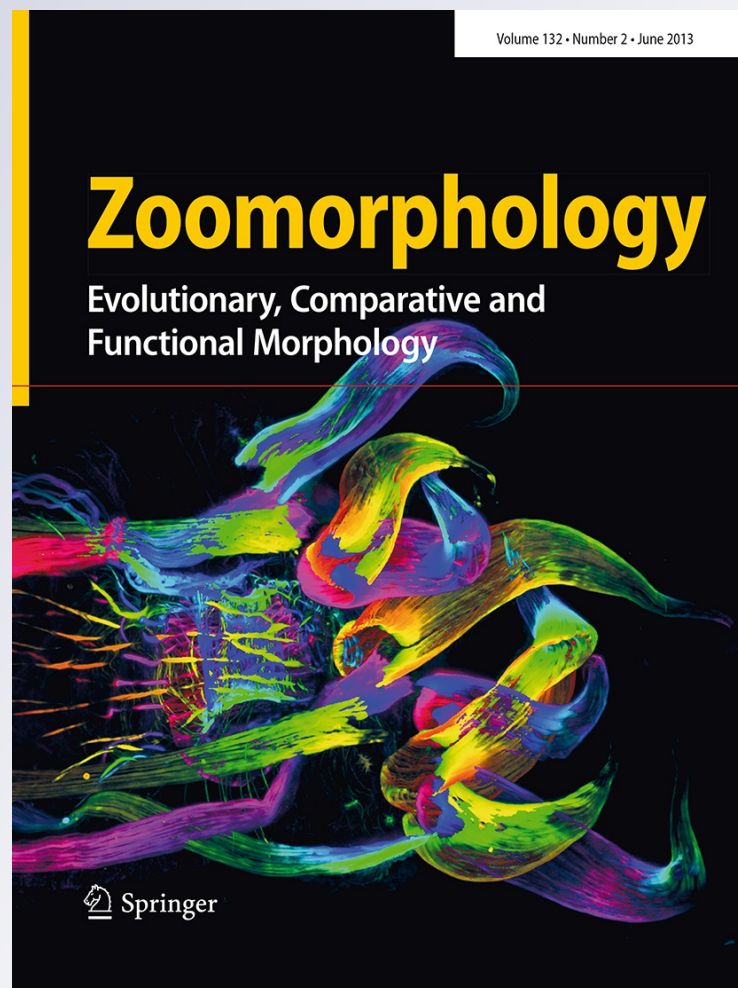
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## Functional anatomy of male copulatory organs of *Pomacea canaliculata* (Caenogastropoda, Ampullariidae)

Maximiliano Giraud-Billoud · Carlos Gamarra-Luques · Alfredo Castro-Vazquez

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**Abstract** This study was aimed to investigate the functional morphology of copulation and sperm transfer in the invasive snail *Pomacea canaliculata*. Three-dimensional renderings of the male copulatory apparatus were made and showed elaborate systems for innervation and for hemolymph supply and drainage. A key component of the male copulatory apparatus is the penial sheath, which shows three specialized glands; the medial and distal glands may participate in adherence to the mantle cavity wall of the female during copulation. The outer gland has an epithelium composed of columnar cells with branched microvilli, mucous goblet cells and large granular secretory cells containing intragranular crystalloids, which produce an exocrine secretion during copulation. The interaction of male/female copulatory organs was studied in dissections of snap-frozen pairs. Sperm are left in the sperm pit, at the end of the pallial spermiduct. Afterwards, the muscular action of the penial bulb takes the sperm up to the vermiform penis, which slides from the penial pouch into the central groove of the penial sheath, and it later emerges through a T-shaped sulcus of this structure and enters the female vagina. Then, it climbs through the capsule duct, and its tip reaches the proximity of the seminal receptacle.

A model of copulation and sperm transfer is presented on the basis of the new findings and on published literature.

**Keywords** Gastropods · Snail reproduction · Genital system · Apple snail

### Introduction

Ampullariidae are freshwater snails distributed in humid tropics and subtropics (Berthold 1991) and also in temperate regions and have become major pests of agricultural (e.g., rice and taro) in wetlands in southeastern Asia, continental United States of America and Hawaii (Cowie 2002; Carlsson et al. 2004; Joshi and Sebastian 2006; Rawlings et al. 2007; Hayes et al. 2008). In the invaded areas, apple snails are also intermediate hosts for some human parasites such as *Angiostrongylus* and *Echinostoma* species (WHO 1995; Thiengo et al. 2004; Damborenea et al. 2006; Hollingsworth and Cowie 2006; Vega et al. 2006; Lv et al. 2006, 2009; Saijuntha et al. 2011). Because of these features, *Pomacea canaliculata* (Lamarck 1822) has been listed among the ‘100 of the world’s worst invasive alien species’ (Lowe et al. 2000).

Several aspects of the reproductive biology of *P. canaliculata* have been studied (e.g., Estebenet and Cazzaniga 1993; Albrecht et al. 1999, 2004; Estoy et al. 2002a, b; Gamarra-Luques et al. 2006; Burela and Martín 2007, 2009, 2011; Tamburi and Martín 2009;), but a complete understanding is still elusive and hampers the development of efficient management strategies for this invasive species (Joshi 2007).

*P. canaliculata* shows one of the longest copulatory interactions recorded among caenogastropods, and the first 8 h of copulation is critical to transfer enough sperm to

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ensure the fertilization of a virgin female (Burela and Martín 2011). Also, the gross morphology of both male and female organs involved in copulation is remarkably complex in this species as compared with other caenogastropod taxa (Berthold 1989, 1991; Thiengo et al. 1993; Catalán et al. 2002; Cowie et al. 2006; Gamarra-Luques et al. 2006; Burela and Martín 2007, 2009, 2011).

In males, the copulatory apparatus is constituted by a penial sheath with three glands (medial, distal and outer glands) and a penial complex (penial bulb and penis, the latter enclosed within the penial pouch, Gamarra-Luques et al. 2006). During copulation, the erectile penial sheath is introduced into the female's mantle cavity (Andrews 1964; Albrecht et al. 1996; Burela and Martín 2009) conveying the long and vermiform penis to the female gonopore (Gamarra-Luques et al. 2006). The rather long vagina is continued by a uterus (Blainville 1822; Hylton-Scott 1958), a complex organ which includes the albumen and capsule glands, the seminal receptacle and the copulatory bursa (Catalán de Canelada and Moreno 1984; Catalán et al. 2002; Hayes et al. 2012).

Inside the female, how the male and female organs interact during copulation is not directly observable, and two different hypotheses have been advanced regarding how the sperm reach the site of fertilization (the seminal receptacle). One of them holds that sperm are deposited in the vagina and that they are transported to the seminal receptacle by their swimming movements and by the cilia of the central channel, a specialized sector of the capsule gland (Catalán et al. 2002), while the other one holds that the penis climbs through the central channel until its tip reaches the proximity of the seminal receptacle (Gamarra-Luques et al. 2006).

In the current work, a three-dimensional (3D) rendering and a histological and ultrastructural study of the copulatory apparatus of male *P. canaliculata* were made. Also, snap-frozen copulating pairs were dissected to disclose how the male and female organs interact during copulation, and how (and to which extent) the penis penetrates the female genital tract for sperm transfer.

## Materials and methods

### Animals and culturing conditions

Adult males and females of *P. canaliculata* from a cultured strain were used for this study. The original stock was collected at the Rosedale Lake (Palermo, Buenos Aires, Argentina). Voucher specimens (alcohol preserved) of the original population, and of the cultured strain, were deposited at the collection of Museo Argentino de Ciencias Naturales (Buenos Aires, Argentina; lots MACN-In 35707

and MACN-In 36046, respectively). Culturing conditions were performed at room relative humidity around 80 %, temperature was regulated at 23–26 °C, and artificial lighting was provided 14 h per day. The animals were maintained in aquaria containing 2 L of tap water, and the water was changed thrice weekly. Snails were fed ad libitum with a mixed diet made mostly of fresh lettuce and that was supplemented weekly with carp food pellets, powdered dried eggs and toilet paper. When was needed, sex was determined externally by the shape of the operculum (Cazzaniga 1990; Estebenet and Martín 2002) and confirmed at sacrifice.

### Light and electron microscopy

The penial sheath glands were obtained from three male snails that were placed in 4 °C water for at least 5 min (to minimize pain and relax the animals) before the shell was cracked. Then, 1–2 mm thick slices of the penial sheath were obtained and fixed in a 4 % paraformaldehyde—2.5 % glutaraldehyde mixture in 0.1 M sodium phosphate buffer, pH 7.4 for 5 h and washed three times (10 min each) with the same buffer. The samples were postfixed overnight with 1 % osmium tetroxide and 2 % uranyl acetate for 45 min and subsequently dehydrated via graded ethanol and acetone and finally embedded in Spurr's resin. For light microscopy, 1- $\mu$ m sections of these blocks were stained with 1 % toluidine blue in 1 % potassium carbonate for 30 s. Digital light micrographs were obtained using an AxioCam HRc mounted on a Zeiss Imager Z.1 microscope. Ultrathin sections from the same blocks were obtained for transmission electron microscopy, and the contrasted sections (uranyl acetate and lead citrate) were examined with a Zeiss 900 microscope.

For scanning electron microscopy, selected pieces of dissection from the male copulatory structures and the female uterus were fixed in Railliet-Henry's fluid (distilled water 930 ml, sodium chloride 6 g, 40 % formalin 50 ml and acetic acid 20 ml) for 7 days. Afterwards, pieces were serially dehydrated in ethanol, passed through acetone and then critical point dried, mounted on aluminum stubs, coated with gold and examined in a LEO 1450VP scanning electron microscope.

### 3D renderings

After the relaxing procedure and shell cracking, one male was dissected to obtain the copulatory apparatus and the penial complex was separated from the penial sheath. These pieces were fixed in Bouin's fluid, subsequently dehydrated and embedded in a 1:1 resin–paraffin mixture (Histoplast<sup>®</sup>). Then, the blocks were serially sectioned with a rotary microtome (Microm<sup>®</sup> HM 325) with disposable

steel blades, obtaining 1470 (penial sheath) and 880 (penial complex) 10 µm serial sections. These sections were stained with Harris' hematoxylin and eosin and photographed. Images of one out of four sections were aligned and ordered manually, and the 3D-rendering of the structures was performed with Reconstruct software version 1.1.0.1 (Fiala 2005), downloaded from Synapse Web, Kristen M. Harris, PI, <http://synapses.clm.utexas.edu/>. Preparation of the PDF-3D-model was accomplished following published procedures (Ruthensteiner 2008; Ruthensteiner and Heß 2008) using the 3D components of Adobe Acrobat 9 Pro Extended software. The preservation of the specimens was also adequate for histological studies.

#### Dissection of snap-frozen copulating pairs

Sixteen male/female pairs were placed separately in 4-liter aquaria (5 cm deep water) and observed until copulation started (i.e., until the female became passive after penial sheath intromission into the mantle cavity; Burela and Martín 2009). 2, 4, 6 and 8 h after the start of the copulation ( $n = 4$  per group), the copulating pairs were rapidly removed from the aquarium and snap-frozen by dropping them in ethyl ether at  $-85\text{ }^{\circ}\text{C}$ . Then, each frozen couple was immersed in 40 % formaldehyde at  $4\text{ }^{\circ}\text{C}$  for 2 h. After that, the penial sheath was held in place by a needle piercing both the sheath and the female body, while the rest of the male body was removed. Then, the female, plus the penis and its sheath, were kept in 40 % formaldehyde at  $4\text{ }^{\circ}\text{C}$  for 72 h. Finally, the whole piece was transferred to 70 % ethanol and kept there until dissections were performed. Photographs were taken with an AxioCam HRc on a Zeiss stereomicroscope.

## Results

### 3D-rendering of the copulatory apparatus

#### *Penial complex*

The genital papilla opens into a mucosal pit (termed the 'sperm pit', Fig. 1d) where sperm coming through the pallial spermiduct are deposited. From there, the sperm should pass into the penial spermiduct, which begins as a U-shaped duct contained within the bulb itself, but soon becomes coiled within a muscular extension of the bulb (Fig. 1d). The muscular extension is continued by the vermiform penis, which is extremely coiled when at rest, and is contained within the membranous penial pouch (Fig. 1a–d). During copulation, the vermiform penis slides through an opening of the penial pouch and extends along the central groove of the penial sheath, which is the main

erectile organ. This groove ends into a superficial T-shaped sulcus (Fig. 3c), through which the penis leaves the sheath and enters into the female vagina.

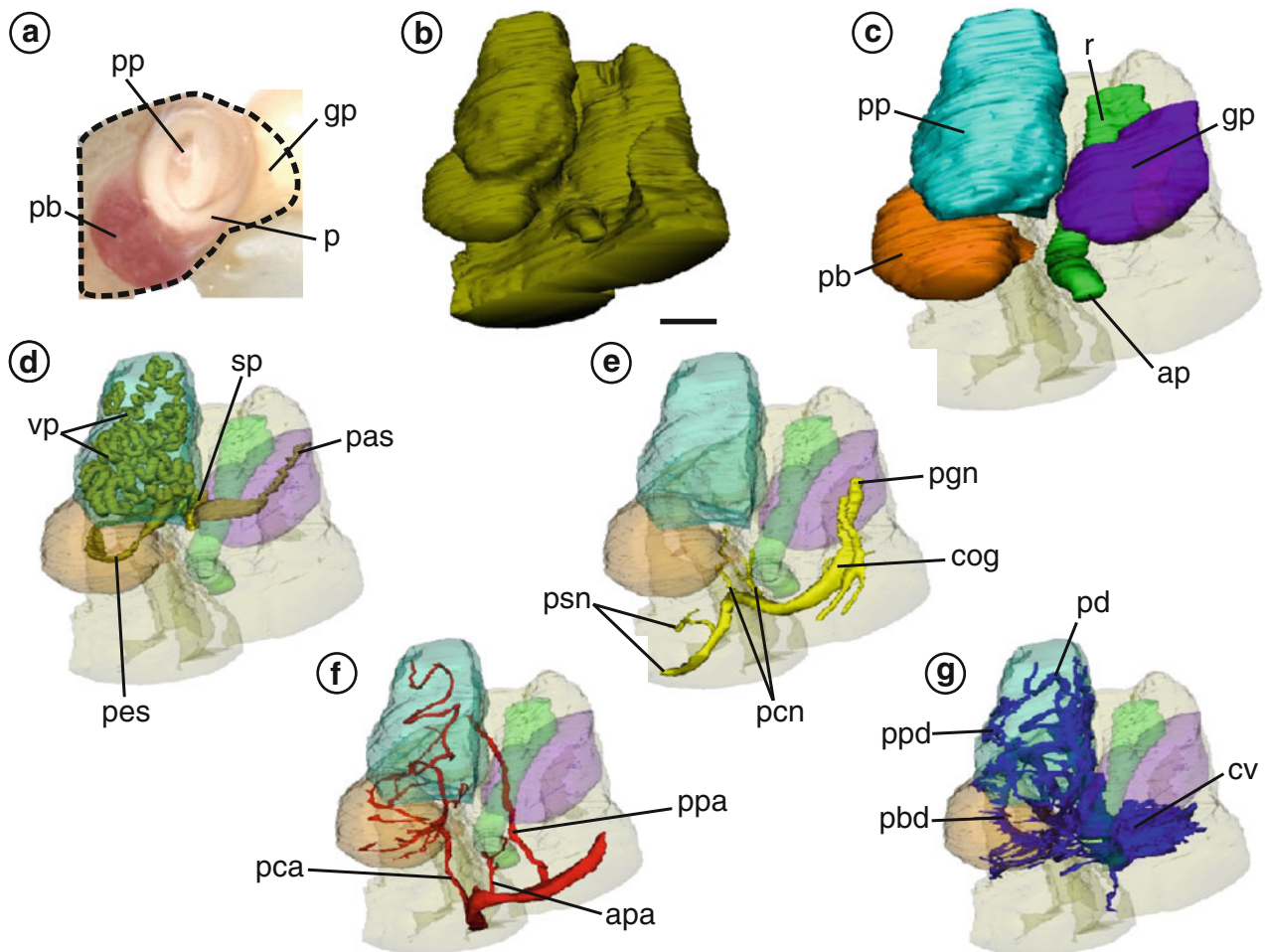
The innervation of the copulatory apparatus (penial complex and penial sheath) is provided by a nerve arising from the right pleural ganglion and shows a ganglion on its course (termed the 'copulatory ganglion') and does not occur in females. The copulatory ganglion is located deep in the muscular mass between right nuchal lobe and the anus. Interestingly, the left pleural ganglion gives off a similar nerve innervating the siphon in both males and females, but no ganglion is interposed on its course. The copulatory ganglion first originates nerves to the mantle edge, rectum, penial complex and finally gives off two nerves directed to the penial sheath (Fig. 1e).

The hemolymph supply is provided by an artery that runs through the mantle edge and gives off branches to the periprostatic and perirectal sinuses, the anal papilla, and to the penial bulb and penis (Fig. 1f). This artery ensures the hemolymph influx into the penial complex while the efflux is carried out by a draining system which separately collects the hemolymph from the penial bulb (Figs. 1g, 2c), the penis (Figs. 1g, 2d), the penial pouch (Figs. 1g, 2e) and the rectum (Fig. 1g). All these drainage vessels converge into a central vein that leaves the penial complex and runs along the mantle edge and finally debouches into the afferent vessel to the lung and ctenidium (Fig. 2a, b).

#### *Penial sheath*

The 3D-surface rendering of the male penial sheath shows a central groove, which ends into the superficial T-shaped sulcus (Fig. 3a–c) and the three sheath's glands. Two of these glands (the medial and distal glands) are located superficially in the dorsal aspect of the sheath, while the outer gland is immersed into the base of the sheath, and its excretory duct opens at the ventral and basal aspect of the penial sheath (Fig. 3d). We could not confirm in this study the previously reported small ducts directed from the outer gland toward the sheath's groove (Gamarra-Luques et al. 2006).

Innervation of the penial sheath is provided by two nerves originating in the copulatory ganglion. These nerves enter the sheath at the right side of its base. Both nerves divide after entering the sheath into a major branch that bends toward the sheath's tip and a smaller one that penetrates the outer gland. One of the nerves penetrating the outer gland ends within the gland, while the other (the thicker one) traverses the entire gland and finally leaves the sheath and continues to the left within the mantle edge. The major branches innervating the sheath's distal regions divide into numerous small nerves constituting an intricate nervous plexus underlying the T-sulcus and the distal gland



**Fig. 1** Male penial complex of *Pomacea canaliculata* (PDF 3D model available as Online Resource 1). **a** Detail of the anatomical piece of the penial complex used for reconstruction. **b** Dorsal surface. **c** Main components: penial bulb, penial pouch with penis, genital papilla and rectum with anal papilla. **d** End of pallial spermiduct and sperm pit. **e** Copulatory ganglion and nerves. **f** Hemolymph supply. **g** Hemolymph drainage. Abbreviations: *ap* anal papilla; *apa*

papilla artery; *cog* copulatory ganglion; *cv* central vein; *gp* genital papilla; *p* penis; *pas* pallial spermiduct; *pb* penial bulb; *pbd* penial bulb drainage; *pca* penial complex artery; *pcn* penial complex nerves; *pd* penial drainage; *pes* penial spermiduct; *pgn* pleural ganglion nerve; *pp* penial pouch; *ppa* periprostatic and perirectal artery; *ppd* penial pouch drainage; *psn* penial sheath nerves; *r* rectum; *sp* sperm pit; *vp* vermiform penis. Scale bar = 1 mm

(Fig. 3e). Comparatively, the nerve supply to the medial gland is scanty.

Hemolymph supply is provided by an artery coming from the mantle edge and enters the penial sheath from the right (close to the nerves) and gives off 9 branches within the sheath. The first branch and the recurrent fourth and fifth branches supply the outer gland. The second supplies the T-sulcus. The third is a small branch that supplies the medial gland, while the remaining branches (the sixth to ninth ones) supply the T-sulcus and the more distal parts of the sheath, including the fibromuscular erectile tissue and the distal gland (Fig. 3f).

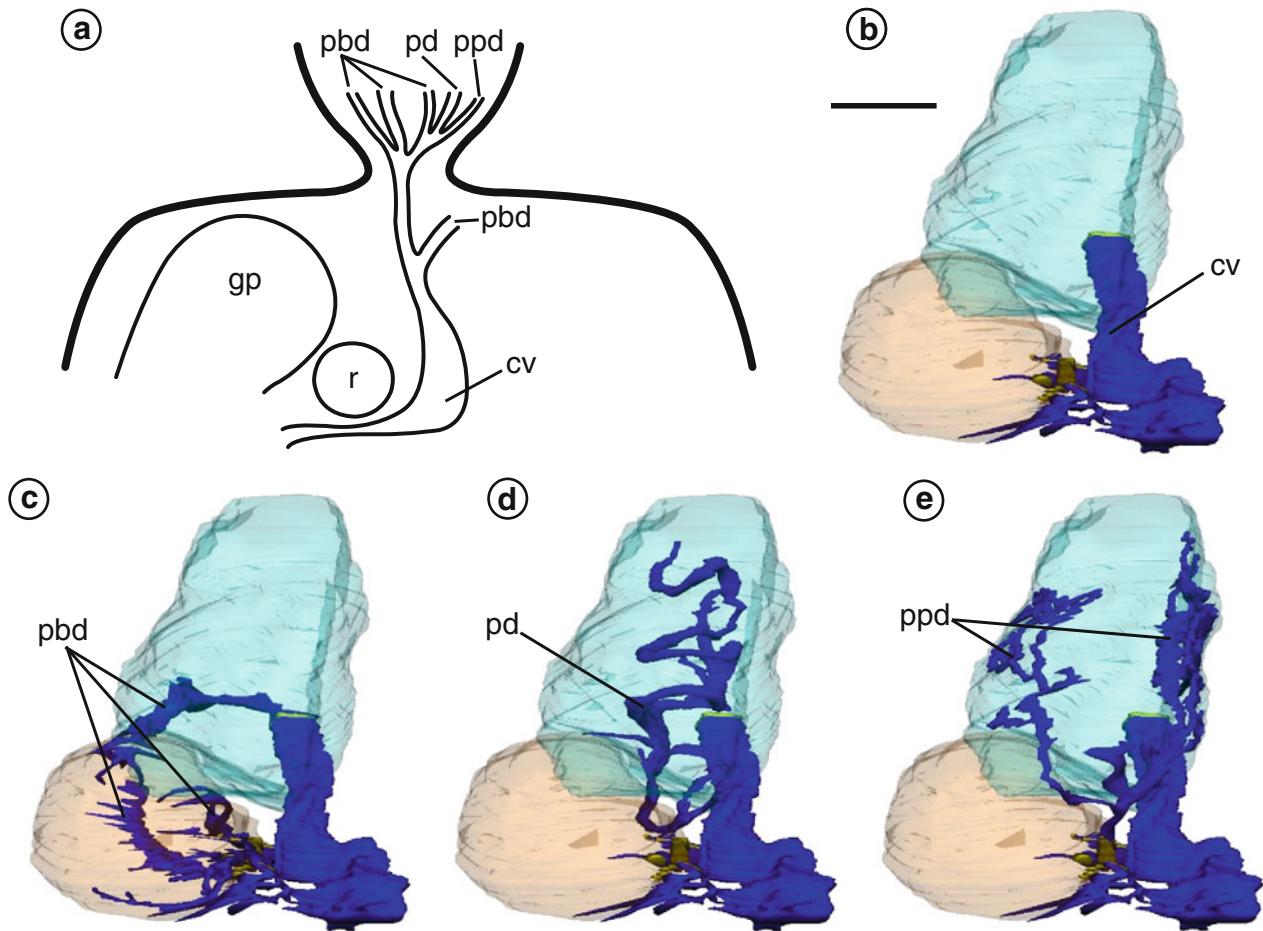
Hemolymph drainage is accomplished by a confluent system of veins running through the dorsal region of the penial sheath and communicates with large venous sinuses located anterodorsally to the outer gland and finally communicates with the ctenidial hemocoelic spaces (Fig. 3g).

## Glandular morphology

### Medial and distal glands

They are located in the dorsal aspect of the penial sheath and are serial sets of mucosal plies with no secretory ducts. The medial gland is located close or within the sheath's groove and thus may not be superficially apparent in fixed material under the stereoscopic microscope, while the distal one is located near the sheath's tip (Fig. 3d).

The epithelium of the mucosal plies is a partly submerged epithelium (sensu Welsch and Storch 1976; Bigatti et al. 2010) composed of a superficial, an intermediate and a deep layer. This kind of epithelium is commonly referred to as an epithelium and a subepithelium (as in Voltzow 1994) but is indeed a single epithelium above an irregularly



**Fig. 2** Sectors of the hemolymph drainage from the penial complex of *P. canaliculata* (PDF 3D model available as Online Resource 2). **a** Drainage from the penial complex, diagram showing the main sectors. **b** Rendering of the central common drainage. **c**, **d** and

**e** Drainage from the penial bulb, penis and penial pouch, respectively. Abbreviations: *cv* central vein; *gp* genital papilla; *pbd* penial bulb drainage; *pd* penial drainage; *ppd* penis pouch drainage; *r* rectum. Scale bar = 1 mm

organized basal membrane that also surrounds each cell or group of cells in the deep layer.

The superficial stratum is composed of microvillar cells, mucous cells and the necks of the bottle-shaped granular cells whose perikarya are found in the deep stratum.

Microvillar cells are tall columnar cells that are crowned by tufts composed of numerous microvilli (Figs. 4a, b, 5a, b). When viewed under the scanning electron microscopy, the tips of these microvilli appear each one covered by a secretion, which should be produced by secretory cells in the same epithelium (see below). The apical cytoplasm of microvillar cells is full of vesicles (Fig. 5b, d), while the cytoplasm below the nucleus shows numerous mitochondria with tubular cristae (Fig. 5e).

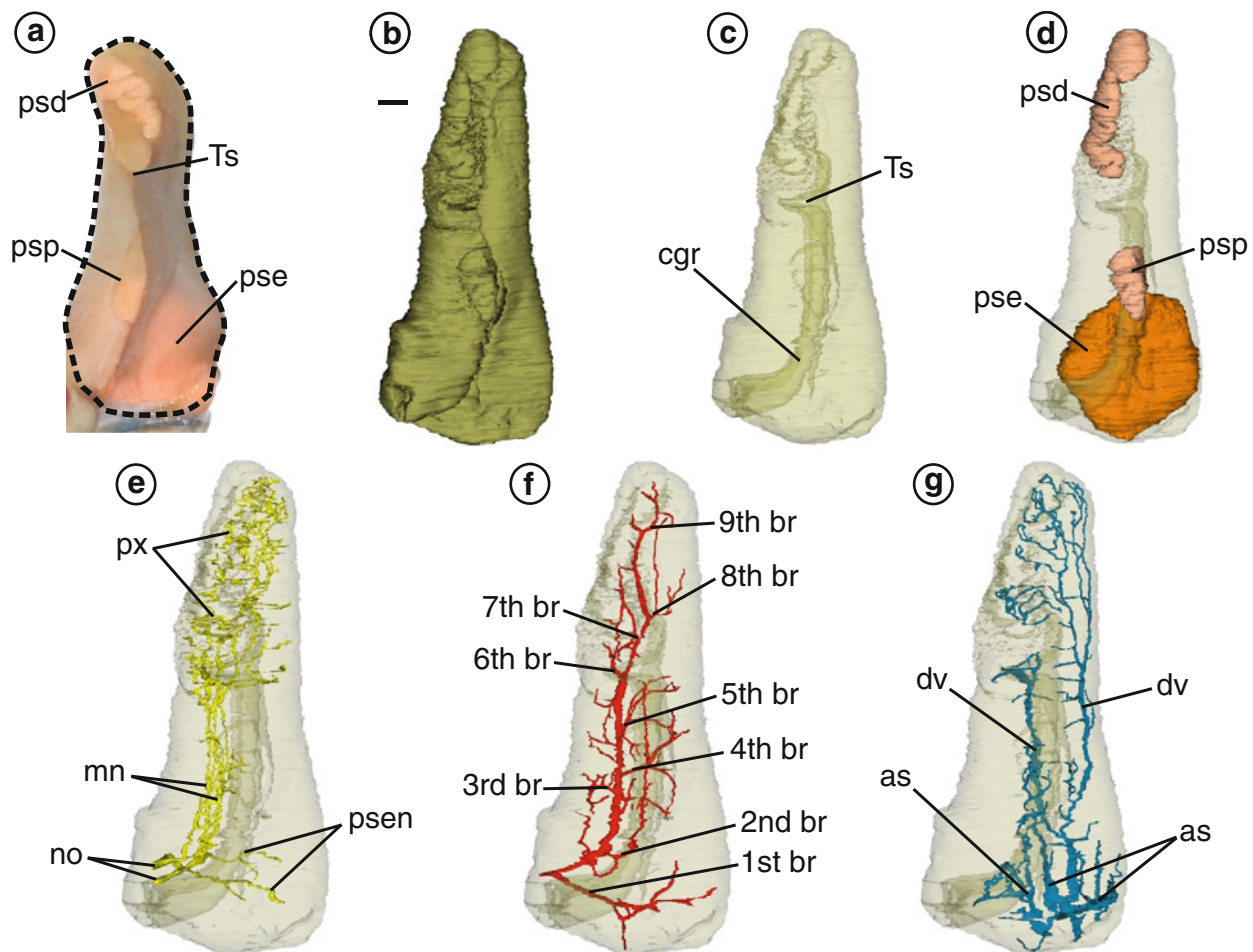
Mucous cells are metachromatic when stained with toluidine blue (Fig. 4b) and some of them are slightly protruding into the deep stratum (thus traversing the intermediate stratum, Fig. 4a, b; see also below). These

cells have clear unobscured nuclei and a foamy cytoplasm.

The necks of the bottle-shaped cells are full of granules which are basophilic when stained with toluidine blue and appear as large membrane-bound granules (1–2  $\mu\text{m}$  wide) in transmission electron microscopy. These granules are composed of a microgranular matrix of low electron density (Fig. 5g). Exocytosis of granules is seen in the apices of these necks (Fig. 5b, d).

The intermediate stratum is a narrow and irregular space separating the superficial stratum from the deep one. Hemocoelic spaces and bands of connective tissue are found separated by a basal membrane from the necks of granular cells and from the bodies of mucous cells that traverse this stratum. In these glands, we have not observed muscle fibers as those seen in similar epithelia (Catalán et al. 2002).

The deep stratum contains the long and wide perikarya of granular cells, which are loaded with the same large granules



**Fig. 3** Male penial sheath of *Pomacea canaliculata* (PDF 3D model available as Online Resource 3). **a** Anatomical piece of the penial sheath used for reconstruction. **b** Dorsal surface. **c** Central groove and T-sulcus. **d** Location of distal, proximal and outer glands. **e** Innervation. **f** Hemolymph supply. **g** Hemolymph drainage. Abbreviations: *as*

anterodorsal sinuses; *cgr* central groove; *br* arterial branch; *dv* dorsal veins; *mn* main nerves; *no* nerve origins; *psd* distal gland; *pse* outer gland; *psen* outer gland nerves; *psp* medial gland; *px* plexus; *Ts* T-sulcus. Scale bar = 1 mm

found in the necks of these cells. The nuclei show heterochromatic clumps and a nucleolus (Fig. 5c). The perikarya form bundles perpendicular to the superficial stratum (Fig. 4c) and also contain profiles of the rough endoplasmic reticulum, free ribosomes and Golgi complexes (Fig. 5f).

#### Outer gland

It is orange-yellowish in fresh preparations and is located within the sheath's base (Fig. 3d). Its large secretory duct opens in a depression at the front of the sheath's insertion, near the mantle edge. The tubulo-alveoli of this gland are also formed by a partly submerged epithelium in which short ciliated columnar cells, metachromatic mucous cells and granular cells are found (Fig. 4d, e).

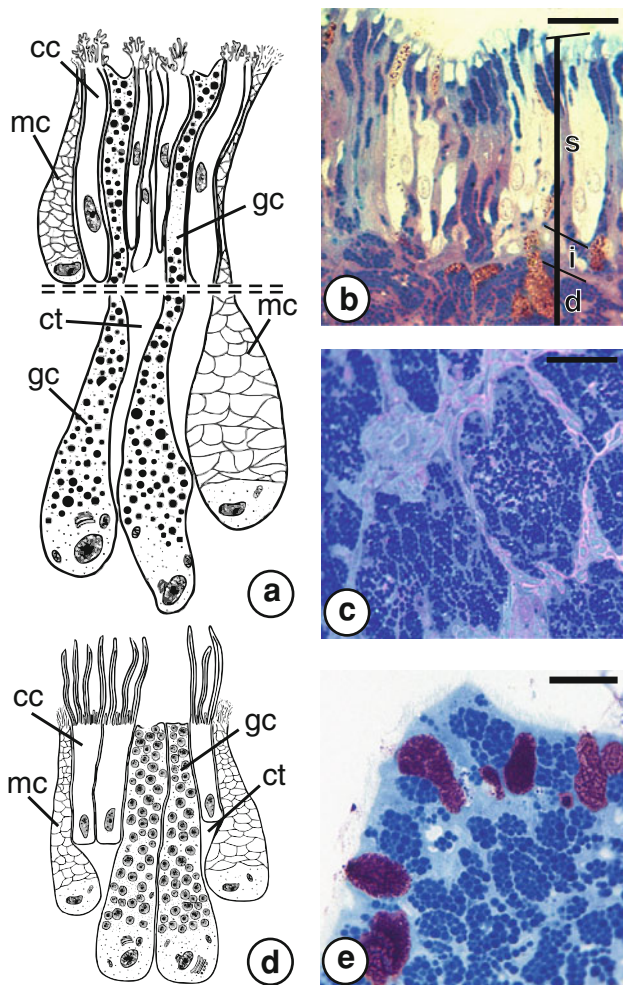
The apex of columnar cells shows both cilia and short microvilli (Fig. 6a–c). Again, some mucous cells protrude

toward the deep stratum, which is not as thick as that in the medial and distal gland. However, some of the granular cells are short, and their conspicuous nuclei (Fig. 6d) are found in the superficial stratum, while other cells traverse the intermediate stratum and extend into the deep stratum (Fig. 4d, e). Both short and long granular cells show mitochondria with tubular cristae (Fig. 6g), rough endoplasmic reticulum and free ribosomes (Fig. 6e–h), and Golgi complexes (Fig. 6h). A remarkable feature of these cells is their very large granules (~2–4 μm wide) which contain a microgranular matrix and conspicuous crystalloids (Fig. 6d, e, g). Also, these granules are exocytosed at the apex (Fig. 6c).

#### Dissection of snap-frozen copulating pairs

Even though these pairs were snap-frozen, it is still possible that some retraction of the structures involved in





**Fig. 4** **a** Diagram of the epithelium of the medial and distal glands. **b** Superficial and intermediate strata and upper part of the deep stratum of the distal gland. **c** Transversal sections of epithelial columns in the deep stratum of the distal gland. **d** Diagram of the epithelium of the outer gland. **e** General view of the outer gland epithelium. Abbreviations: *cc* columnar cell; *ct* connective tissue; *d* deep stratum; *gc* granular cell; *i* intermediate stratum; *mc* mucous cell; *s* superficial stratum. Toluidine blue staining; scale bars = 50  $\mu$ m

copulation may have occurred, and this should be kept in mind when interpreting the following observations.

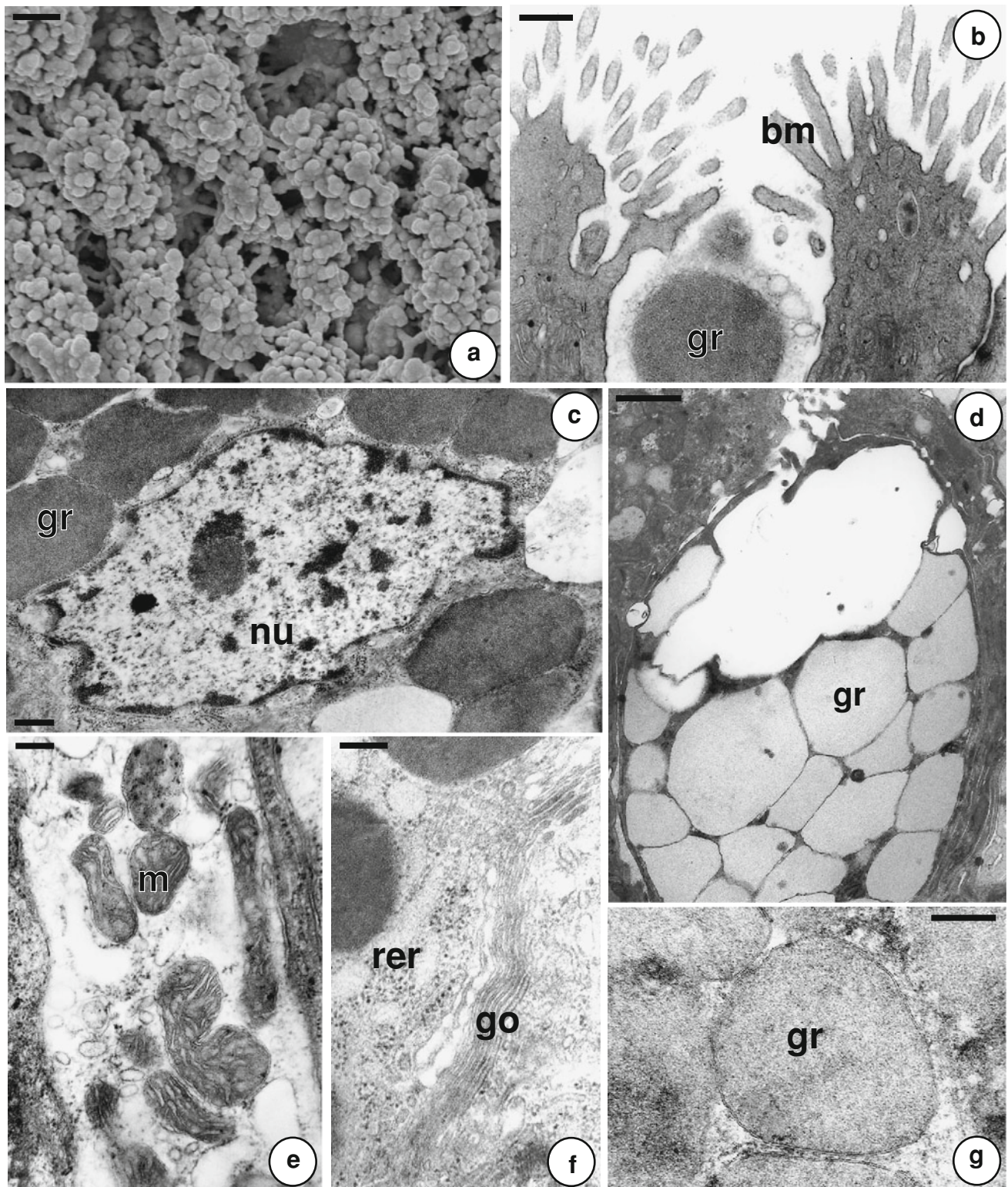
Dissections showed that the penial sheath first extends along the right border of the mantle cavity roof until reaching the proximal part of the epitaenium. When doing that, the sheath becomes a flattened tape, and the medial and distal glands become elongated together with the sheath (not shown in figures). Afterwards, the T-shaped sulcus attaches to the female gonopore. By that time, the penis has already slid throughout the central groove of the penial sheath and its tip can then leave the sheath through the T-sulcus and enter the vagina, while the more distal part of the penial sheath may retract (Fig. 7). Afterwards, the penis traverses the whole length of the vagina (Fig. 7)

until reaching the first whorl of the capsule gland, which is located on the anterior (pallial) surface of the uterus (Figs. 9a, 10). From there, the penis reaches the central channel of the capsule gland (sensu Catalán et al. 2002) around which the whorls of the capsule gland are organized (Fig. 8). The progression of the penis may be assisted by the ciliated surfaces of the vagina and the capsule gland duct (Fig. 9b, c). Once in there the penis is able to climb the whole length of the central channel (Fig. 10). Climbing of the penis could be followed until the proximity of the seminal receptacle (Figs. 8, 11).

The timing of the interaction of the male and female organs was studied at 2, 4, 6 and 8 h after sheath insertion (Table 1). At 2 h, the penial tip was found either within the vagina (3 cases) or within the first part of the central channel of the capsule gland (1 case). At 4 h, the penial tip had already progressed in or beyond the last part of the capsule gland in 3 cases, while it was still in the first part of the gland in the fourth case. At 8 h, both the penis and sheath were already retracting: In one case, the penis was still lodged in the vagina, while the penis had already retracted into the sheath in the other 3 cases; in one of the latter cases, the sheath was hanging loose in the mantle cavity.

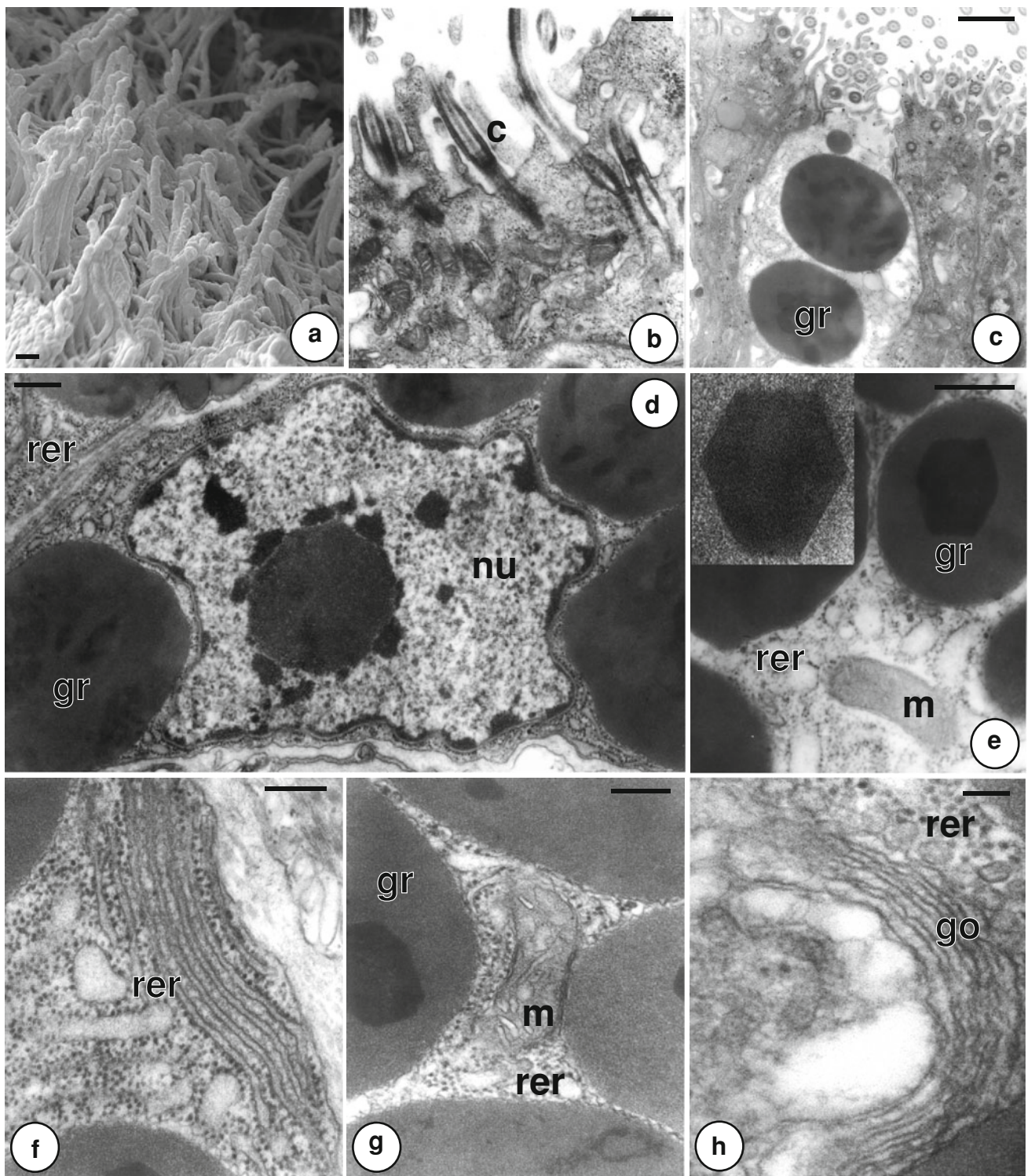
## Discussion

The evolution of the male copulatory apparatus in Neotropical Ampullariidae has resulted in closure of the penial spermiduct (that is an open channel in the African and Asian genera) and in the development of a penial complex separate from an erectile penial sheath, which has also developed a set of conspicuous glands (Sachwatkin 1920; Hägler 1923; Thiengo 1987, 1989; Berthold 1989; Thiengo et al. 1993; Simone 2004). This evolutionary trend has reached its outmost development in the Neotropical genera *Marisa* and *Pomacea* (Berthold 1989). Even though the evolution of the female tract in the family Ampullariidae has not received a similar attention, the evolutionary trend toward complexity has resulted in *Pomacea canaliculata* in a convoluted uterine tract (Andrews 1964; Catalán de Canelada and Moreno 1984; Catalán et al. 2001; Hayes et al. 2012) (Fig. 8). The albumen gland conveys its secretion into a dorsoventrally flattened duct which connects in turn through a narrow opening with a spiral capsule gland duct. There are six capsule gland revolutions also made of a flattened duct, which reminds somehow the steps of a winding staircase. However, a central channel of the capsule gland bypasses the glandular revolutions as the newel of the winding staircase, which has been termed either ‘central ventral channel’ or ‘ventral channel’ by other authors (Andrews 1964; Hayes et al. 2012). This



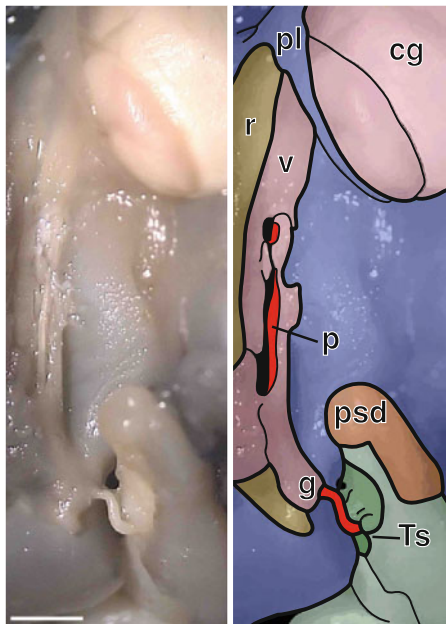
**Fig. 5** Electron microscopy of the distal gland of the penial sheath. **a** Scanning electron microscopy of microvilli on the epithelial surface; *scale bar* = 1  $\mu\text{m}$ . **b** Transmission electron microscopy of the branched microvilli, *scale bar* = 0.5  $\mu\text{m}$ . **c** Nuclei of a granular cell; *scale bar* = 0.5  $\mu\text{m}$ . **d** Apex of a granular cell; *scale bar* = 1  $\mu\text{m}$ . **e** Mitochondria with tubular cristae in a granular cell.

**f** A Golgi complex in the perikaryon of a granular cell. **g** Detail showing the microgranular matrix of granules; *scale bars* for **d**, **e**, **f** and **g** = 0.25  $\mu\text{m}$ . Abbreviations: *bm*: branched microvilli; *gr*: granule; *go*: Golgi complex; *m*: mitochondria; *nu*: nucleus; *rer*: rough endoplasmic reticulum



**Fig. 6** Electron microscopy of the outer gland of the penial sheath. **a** Scanning electron microscopy of cilia on the epithelial surface; *scale bar* = 1  $\mu\text{m}$ . **b** Transmission electron microscopy of the epithelial apices showing cilia and microvilli; *scale bar* = 0.5  $\mu\text{m}$ . **c** Apex of a granular cell with granules being exocytosed; *scale bar* = 1  $\mu\text{m}$ . **d** Nucleus of a granular cell; *scale bar* = 0.5  $\mu\text{m}$ . **e** Crystalloid bearing granules (the inset shows detail of a crystalloid);

*scale bar* = 0.5  $\mu\text{m}$ . **f** Rough endoplasmic reticulum and free ribosomes in the perikaryon of a granular cell; *scale bar* = 0.25  $\mu\text{m}$ . **g** A mitochondrion with tubular cristae, rough endoplasmic reticulum and granules in the perikaryon of a granular cell; *scale bar* = 0.25  $\mu\text{m}$ . **h** A Golgi complex; *scale bar* = 0.1  $\mu\text{m}$ . Abbreviations: *c*: cilia; *gr*: granule; *go*: Golgi complex; *m*: mitochondria; *nu*: nuclei; *rer*: rough endoplasmic reticulum

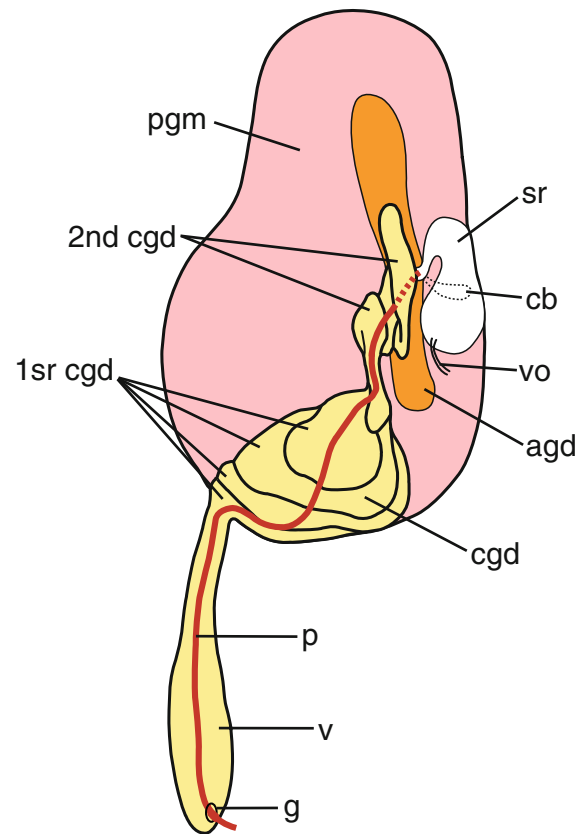


**Fig. 7** Dissection of a snap-frozen copulating pair. The sheath has been slightly separated from the vagina to show the penis leaving the sheath (between the turgid borders of the T-sulcus) and entering the vagina. The latter has been partially opened to show the penis within. The sheath distal to the T-sulcus has already retracted. Abbreviations: *p* penis, *psd*: penial sheath distal gland, *ps* penial sheath, *v* vagina, *r* rectum, *cg* capsule gland, *g* gonopore, *pl* pallial ligament. Scale bar = 250  $\mu$ m

channel was also studied by Catalán et al. (2002) and they called it ‘sperm channel,’ since they thought was the pathway for the ascent of sperm, an interpretation that is challenged by our current findings. We are calling this functionally significant structure the ‘central channel’ because of its position respective to the revolutions of the capsule gland.

At the behavioral level, a model of copulatory interactions in *P. canaliculata* has been published (Burela and Martín 2009). We will here present a theoretical model of copulation and sperm transfer in *P. canaliculata*, which is intended to supplement that behavioral model and that integrates our findings with those of other authors.

The penial sheath is a wriggling structure that penetrates into the female’s mantle cavity in search of the female gonopore, which is a tiny spot on the right margin of the roof of the mantle cavity. The sheath becomes fully extended through the right and posterior border of the mantle cavity, toward the posterior end of the epitaenium. In doing that, the sheath and its glands become a flattened tape attached to the mantle cavity roof, to which they adhere by the increased surface (at the nanoscale level) provided by the branched microvilli and also by the secretion of the medial and distal glands. An increased surface at the nanoscale level is also involved in adhesion



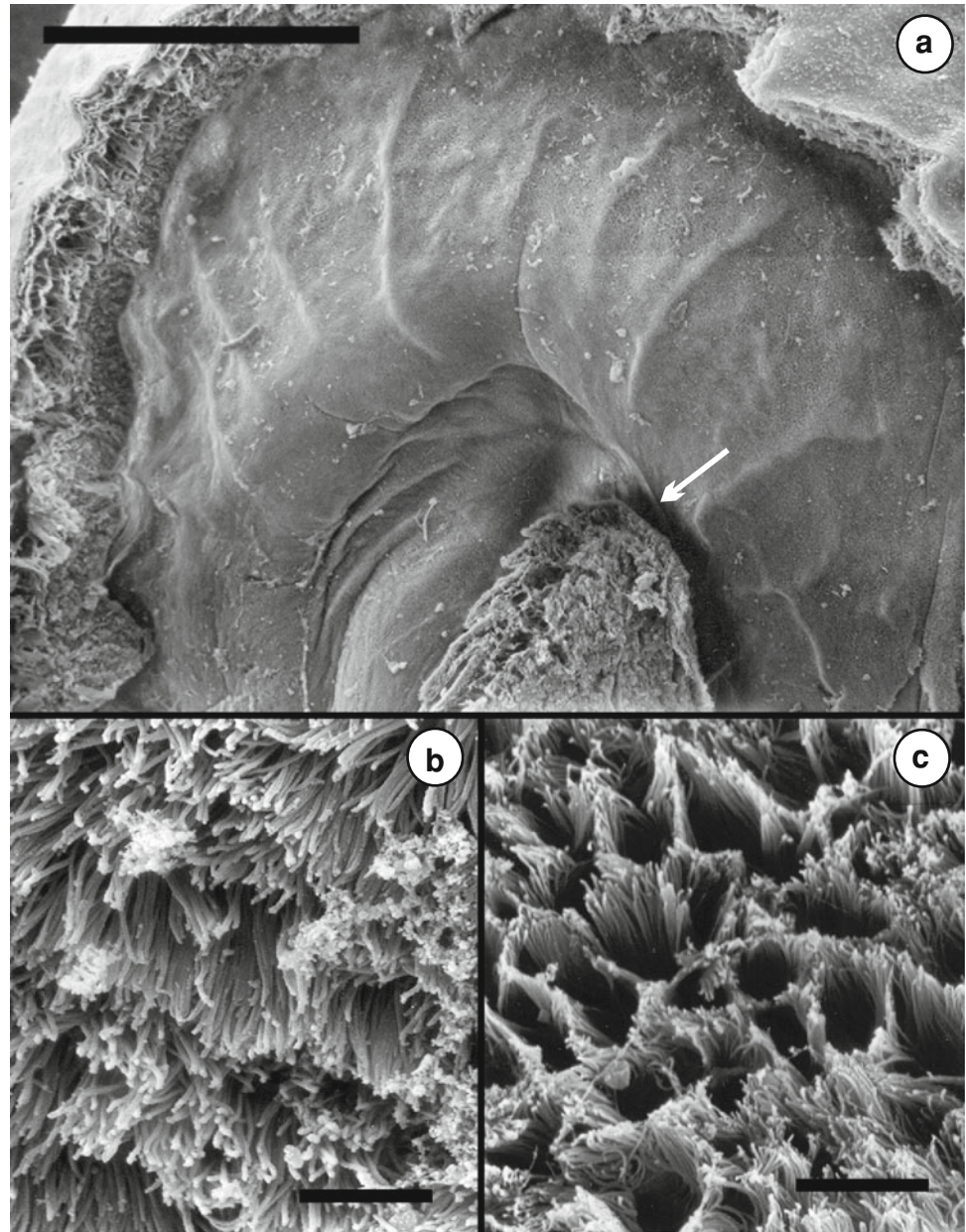
**Fig. 8** Diagram of the uterus and vagina of *Pomacea canaliculata* (adapted from Catalán et al. 2002). The penis reaches the proximity of the seminal receptacle. Abbreviations: *agd* albumen gland duct; *cb* copulatrix bursa; *cgd* capsule gland duct (1st and 2nd set of whorls); *g* gonopore; *pgm* parenchymal glandular mass; *p* penis; *sr* seminal receptacle; *v* vagina; *vo* visceral oviduct

of the toe setae to substrates in geckonid lizards (Ruibal and Ernst 1965; Autumn and Peattie 2002; Autumn et al. 2002; Huber et al. 2005). However, it should be noted that the adhesion in geckos takes place on dry surfaces (Stark et al. 2012) and that nothing similar to the glandular secretion of *P. canaliculata* participates in these lizards.

The T-sulcus of the penial sheath becomes in contact with the female gonopore. It is intriguing how the precise location of the gonopore is found by the T-sulcus, and one is tempted to speculate that some sensory receptor (perhaps a chemoceptor) may participate in orientation. Indeed, the rich innervation underlying the sulcus suggests a neural participation.

It may be noted that there is not a clear sectoring of draining vessels in the sheath, as compared with that of the penial complex. Therefore, one may wonder how the sheath becomes fully extended during copulation. It may be hypothesized that the tonus of the basal part of the fibromuscular mass may increase as compared with that at the tip, which is in fact suggested by the thinning of the distal part of the sheath at the beginning of copulation. Also, the

**Fig. 9** Scanning electron microscopy of the first whorl of the capsule gland. **a** The lid at the anterior (pallial) surface of the uterus has been removed, to show the entrance to the central channel (*arrow*). **b** Cilia on the surface of the vaginal epithelium. **c** Cilia on the surface of central channel; the pits correspond to non-ciliated mucous cells. *Scale bars*  $a = 1\text{ mm}$ ,  $b$  and  $c = 10\ \mu\text{m}$

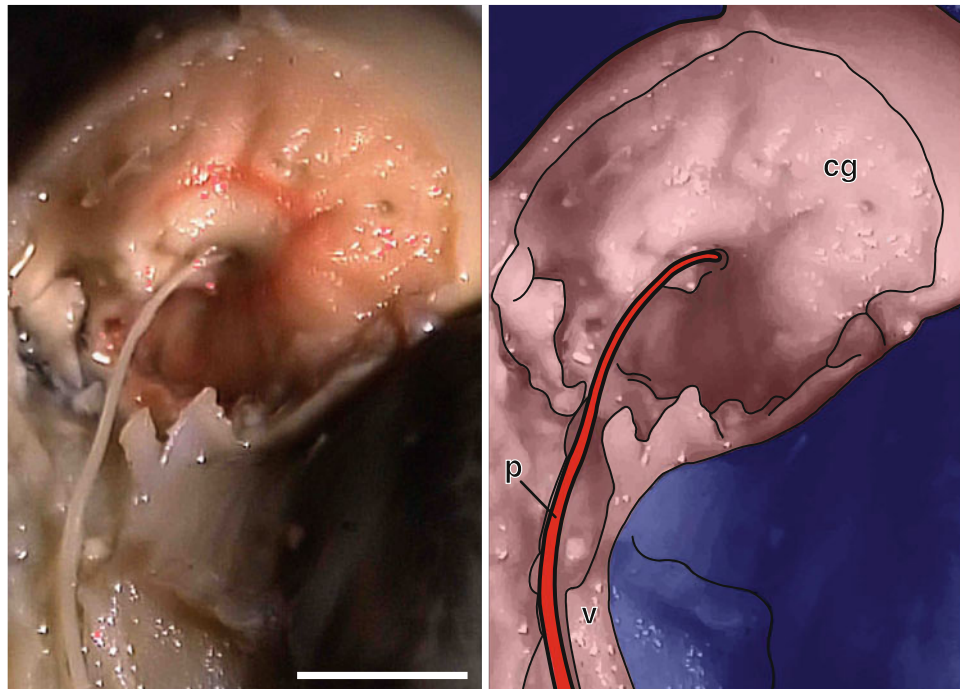


fully active outer gland (Burela and Martín 2007), which becomes turgid during copulation, may hamper hemolymph drainage from the sheath, since it receives a rich hemolymph supply and occupies most of the sheath's base.

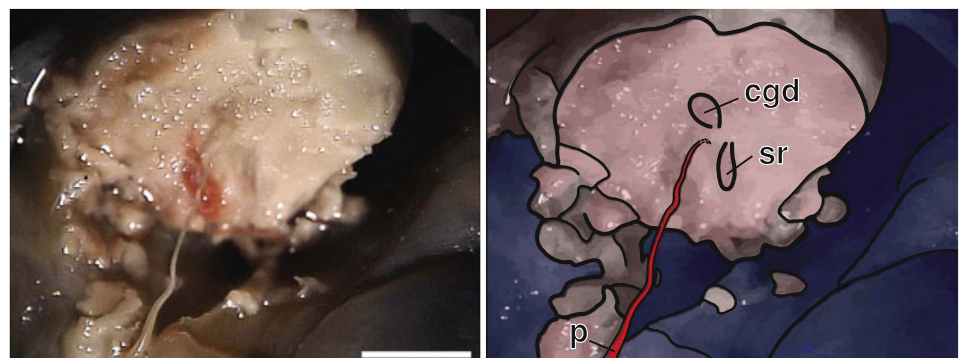
Once the penis traverses the gonopore, penial progression along the vagina is favored by the pumping action of the penial bulb and by the cilia of the vaginal mucosa. Pumping by the penial bulb should also take up sperm from the sperm pit. Eventually, the penis enters the central channel of the capsule gland. As mentioned above, this channel traverses the tortuous capsule gland in a rather direct way (i.e., as 'the newel of a winding staircase') and thereby reaches the proximity of the duct leading to the seminal receptacle.

The role of the seminal receptacle as the site of fertilization is uncontested (Hylton-Scott 1958; Andrews 1964; Catalán et al. 2002; Hayes et al. 2012). This receptacle is composed of a bulb and a U-shaped duct (Hylton-Scott 1958; Andrews 1964) that joins the proximal end of the capsule gland (Fig. 8). This bulb receives the oocytes conveyed through the visceral oviduct and also shows sperm whose heads are orderly arranged as a palisade. *P. canaliculata* displays both fertilizing sperm and two types of parasperm (Catalán et al. 1997; Winik et al. 2001, 2009), and apyrene parasperm are found side by side with eupyrene sperm in the seminal receptacle (Catalán de Canelada 2007). Parasperm are thought to have a role in sperm competition (Buckland-Nicks 1998; Till-Bottraud et al.

**Fig. 10** Dissection of a snap-frozen copulating pair. The vagina has been opened throughout, and the pallial lid of the capsule gland has been removed as in Fig. 9. The penis is seen entering the central channel. Abbreviations: *p* penis, *cg* capsule gland, *v* vagina. Scale bar = 250  $\mu$ m



**Fig. 11** Dissection of a snap-frozen copulating pair. Most of the capsule gland and surrounding albumen parenchyma have been removed to show the penis penetrating the central channel (between the last whorl of the capsule gland and the duct of the seminal receptacle). Abbreviations: *p* penis, *cgd* capsule gland duct, *sr* seminal receptacle. Scale bar = 250  $\mu$ m



**Table 1** Timing of events during copulation in *Pomacea canaliculata*

Events	2 h	4 h	6 h	8 h	Total
Penial sheath inserted into mantle cavity	–	–	–	1	1
T-sulcus attached to gonopore	–	–	–	2	2
Penial tip within vagina	3	–	–	1	4
Penial tip within first whorls of capsule gland	1	1	3	–	5
Penial tip within last whorls of capsule gland or beyond	–	3	1	–	4

*N* = 16 (4 couples per group)

2005), and in the case of *P. canaliculata*, it has been shown that continuous mating with a second male displaces sperm deposited by a former one (Yusa 2004), and parasperm may have a role in such displacement. Burela and Martín (2011) have found that no fertilization of virgin females occurs if copulation is interrupted 2 h after penial sheath insertion and have speculated that only parasperm were

transferred during that time. We believe, however, it is unlikely that sperm and parasperm were partitioned during copulation, since their spermatogenic nests where they are generated are placed side by side within the testis tubules, and in fact, the results of Burela and Martín (2011) can better be explained by the timing of penis progression within the female tract, since the penis had not reached the

vicinity of the seminal receptacle by 2 h after sheath insertion (Table 1).

Several authors (Hylton-Scott 1958; Andrews 1964; Hayes et al. 2012) have proposed that sperm are first transferred to the copulatory bursa and from there to the seminal receptacle. We propose a direct transfer from the central channel of the capsule gland to the seminal receptacle as an alternative hypothesis. Indeed, both ducts leading to the copulatory bursa and the seminal receptacle open side by side at the proximal end of the central channel of the capsule gland, and therefore, it is not obvious whether sperm will enter either one or the other. However, sperm are not orderly arranged in the copulatory bursa, and they coexist there with numerous phagocytic hemocytes (Andrews 1964), so we suggest that ‘copulatory bursa’ is a misnomer and that it may be the site of sperm resorption, a function served by the ‘ingesting glands’ of other caenogastropods (Ponder and Lindberg 1997). However, further studies are needed to clarify this point.

It is intriguing that Andrews (1964) found no parasperm in the copulatory bursa. It is possible that the highly motile parasperm were dislodging fertile sperm from the sacculs of the seminal receptacle and that only the dislodged sperm were transferred for resorption into the ‘copulatory’ bursa.

Regarding the timing of copulation and sperm transfer, our results essentially agree with Burela and Martín (2011) who reported that sperm transfer occurs between 2 and 10 h after penial sheath insertion. These authors have reported 12 h as the mean duration of copulation, and we are reporting here that both the penis and the penial sheath are already retracting to some extent by 8 h after penial sheath insertion. Therefore, one may wonder which the functional significance is of the prolonged attachment after sperm transfer is completed. It is possible that it may play a role in sperm competition, by impeding insemination by other males, as it has been shown in other animal species (Dickinson 1986; Suter and Parkhill 1990; Stockley 1997).

The mitochondria with tubular cristae in all the penial sheath’s glands, as well as the intragranular crystalloids in the outer gland, are notable findings. The latter are similar to Reinke’s crystalloids found in androgen-secreting cells in the mammalian testis (Janko and Sandberg 1970). Both those mitochondria and Reinke’s crystalloids have long been considered peculiar of steroid-producing cells (Fawcett et al. 1969) even though a mechanistic model of how they are related to steroid production has not been worked out. Radioimmunoassayable progesterone has been found in extracts of the distal half of the penial sheath, which contains both the medial and distal glands, while radioimmunoassayable androstenedione has been found in extracts of the proximal half, which contains the outer gland (Gamarra-Luques et al. 2006). A paracrine androtrophic role for these steroids has been proposed

(Gamarra-Luques et al. 2006). In the current study, hemocoelic spaces were found associated with the epithelial bundles in the deep stratum of both the medial and distal glands, although the latter had a larger hemolymph supply than the former. Also, the outer gland receives a profuse hemolymph supply in *P. canaliculata*, and this gland releases a voluminous secretion during copulation, that is, eaten by the female partner (Burela and Martín 2007). The possible occurrence of steroids in the outer gland secretion and its role after ingestion by the female partner will have to be studied.

Finally, we may say that the emerging picture of copulatory interactions and of the structures involved in sperm transfer in *P. canaliculata* looks as an interwoven fabric, albeit with loose borders of speculation that will warrant future studies. It is expected that all these efforts will help the development of rational control strategies for the populations of this highly invasive snail.

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