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Workshop: Biology of Ampullariidae

An integrative view to structure, function, ontogeny and phylogenetical significance of the male genital system in *Pomacea canaliculata* (Caenogastropoda, Ampullariidae)

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The knowledge of the reproductive biology of *Pomacea canaliculata* (Lamarck 1822) is particularly important in the context of its impact as an invasive species in Asia and many Pacific islands (Cowie, 2002). Several studies on the structure of the genital system of *P. canaliculata* and of other Neotropical ampullariids have been published (see Hylton-Scott, 1957; Andrews, 1964; Martín, 1980; Berthold, 1989; and Thiengo *et al*., 1993, for early references). The present work is focused on the male reproductive system of this species, and it integrates those early works with more contemporary studies, mainly (but not exclusively) performed with ultrastructural and biochemical techniques. The emerging picture impresses as if the male genital system of *Pomacea canaliculata* was one of the best studied among freshwater Caenogastropod species, as much as from functional, ontogenetical and phylogenetical perspectives.

Copulation, and its role in spawning induction and in sperm competition

Copulation occurs under water in these snails, and these behavior has been described thoroughly (Andrews, 1964; Albrecht *et al*., 1996). Copulatory episodes has been observed about three times *per* week in isolated, reproductively active couples (Albrecht *et al*., 1996) and each episode lasted 10-18 h, during which the female may be observed crawling and feeding while transporting the apparently resting male attached to her shell. The relation of copulation to spawning has been a matter of speculation: though Bachmann (1960) and Andrews (1964) have suggested that spawning in this species may be directly induced by the stimuli of copulation, later authors have shown that the copulation frequency almost doubles that of spawning and that some spawnings are not directly preceded by copulation (Estebenet and Cazzaniga, 1993; Albrecht *et al*., 1996). However, virgin females do not start spawning until the first fertilization occurs (Estebenet and Cazzaniga, 1998).

It is puzzling which would be the biological significance of these lengthy and repeated copulations. It should first be considered that sperm transfer may occur either continuously or in repeated short episodes during hours. And also, since Yusa (2004) has shown

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that continuous mating with a second male displaces sperm deposited by a former one, it is certainly possible that those lengthy and repeated copulatory episodes may have a role in sperm competition in this species.

Sex determination and differentiation, and phylogenesis of the male copulatory apparatus

The sex ratio in wild populations of this snail is 0.5 (Yusa and Suzuki, 2003), but little is known of the mechanisms determining individual sex in this species. In contrast with an early report (von Brandt *et al*., 1990) there appear to be no dimorphic sex chromosomes neither in *P. canaliculata* (Mercado-Laczkó and Lopretto, 1998) nor in other *Pomacea* species (Kawano *et al*., 1990; Diupotex-Chong *et al*., 2004).

An interesting insight to the problem of sex determination in *P. canaliculata* was provided by Yusa and Suzuki (2003) who observed that the sex ratio was highly variable among broods, ranging continuously from almost exclusively males to almost exclusively females. Moreover, they found that sex ratios were similar between broods from the same mating pair, suggesting that sex ratio is a family trait, and they have suggested that a polyfactorial genetic determination of sex in *P. canaliculata* would be the most likely mechanism to explain their findings. More recents studies suggest the involvement of either a small number of sex-determining genes or a more complicated system such as sex-ratio or sex-determining polygenes that act non additively (Yusa, 2005).

Regarding ontogenetic development of the genital system, it is generally accepted that the prosobranch genital tract, which will develop a continuous duct from the upper surface of the midgut gland to the anterior border of the pallial cavity, is brought about by the merging of three primordia,: (1) the gonadal sector of the gonoduct, that originates as an unpaired thickening at the roof of the pericardial coelom; (2) the left rudimentary kidney also of mesodermal origin, which originates the visceral gonoduct; and (3) the efferent duct of the left rudimentary kidney, which is ectodermal in origin and gives rise to the pallial gonoduct. This basic composition has been described by Ranjah (1942) for *Pila globosa* and there is no reason to believe that it may be different in *Pomacea* species. The resulting gonoduct of *P. canaliculata* is a single one and it differentiates to either a male or a female tract when juveniles are 10 mm long (Gamarra-Luques, *pers. obs.*)

In sexually undifferentiated juvenile Ampullariidae (Gamarra-Luques, *pers. obs.*), the genital tract is a single arborescent structure extending over the inner surface of the visceral mass. It follows down the columella as a single duct, until it reaches the mantle epithelium and turns right, just beneath the epithelium, and side by side to the ctenidium. It then crosses the epitaenium (or "mantle ligament") and later bends forward, parallel to the rectum, until it ends in the mantle cavity, approximately at midway between the epitaenium and the anus.

In males, the part of the gonoduct near the shell apex (gonadal duct) enlarges and develops as the testis, causing some displacement and reduction of the midgut gland (Vega *et al.*, 2005), while the visceral duct develops as the *vas deferens* and the seminal vesicle. The pallial section of the gonoduct develops as the prostate, whose main duct grows distally, reaches the proximity of the anus, and crosses to the right over the rectum, to end in the so-called sperm pit (Fig. 1). The part of the prostate that crosses over the rectum is structurally different to that of the rest of the organ and it is identified as the genital papilla. In females, the apical part of the gonoduct (gonadal duct) develops as the ovary (a thin, ramified tubular layer on the columellar surface

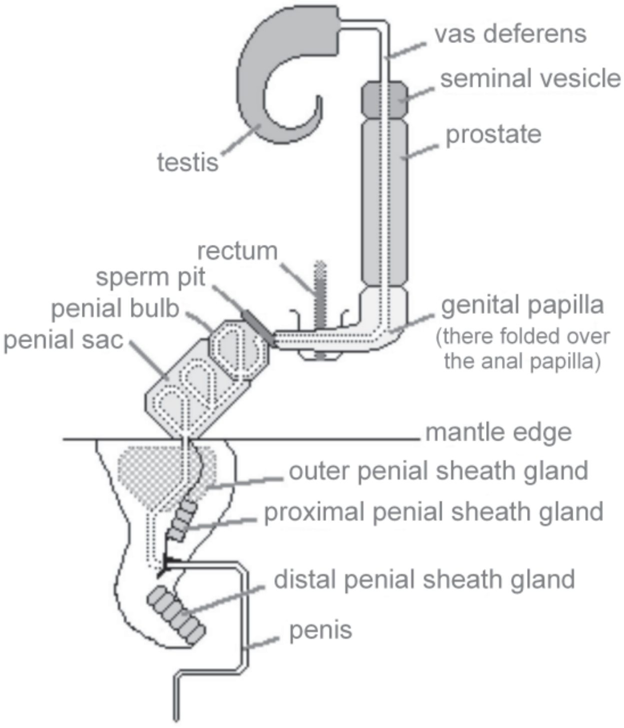

FIGURE 1. Schematic representation of the organs composing the male genital system of *Pomacea canaliculata* (modified from Ghesquiere, 2005, with permission).

of the midgut gland) while the visceral part develops as the oviduct. The pallial section of the gonoduct gives origin to the albumen gland/capsule gland complex (sometimes referred to as the "uterine gland", Catalán *et al*., 2002) and the vagina. Notably, there are no female genital structures crossing over the rectum such as the male genital papilla, and therefore, the adult vaginal orifice lies to the left of the rectum.

Besides the male gonoduct derivatives which produce and convey sperms, the male genital system of Ampullariidae includes a set of structures serving penetration of the female vagina and which derive from the mantle edge. This mantle edge primordium that gives rise to this "copulatory apparatus" does not develops fully in normal females but it remains vestigial, and interestingly, it may be the target for masculinizing xenobiotics (see below).

The evolutionary trend towards complexity of the copulatory apparatus seems associated to the optimization of sperm transfer, since analogous adaptations appear in other Caenogastropod freshwaters families (in the Viviparidae, also in the superfamily Ampullarioidea, as well as in the fairly unrelated Bythiniidae). In Ampullariidae, in particular, evolution has been towards the acquisition of a closed sperm channel, and of a bipartite copulatory apparatus (comprising both a penial complex and a penial sheath). This complexity reaches its maximum in the Neotropical section of this family, particularly in the genera *Marisa* and *Pomacea,* with the acquisition of the proximal sheath gland (Berthold, 1989; see below). Simone (2004) has also confirmed the occurrence of these structures in several Neotropical Ampullariidae.

Gonoduct derivatives: the testis and spermatogenesis

The growth and progressive branching of the gonoduct thus formed results proximally (*i.e*., near the shell apex) in a sponge-like testicular mass[1]. The labyrinth within that sponge is made up of tubules of similar width, and which are lined by the spermatogenic epithelium (Winik, 1982; Albrecht, 1998). In the adult, the *vas deferens* originates as a slender tube at the lower end of the testicular labyrinth. There are muscular cells surrounding the seminipherous tubules, which may help mature sperms to traverse the tubular labyrinth to enter the *vas deferens*.

The spermatogenic epithelium is organized in nests of germinal cells, each one being in a single developmental stage. However, tubular sections show nests in different stages, each one presumably descendant of a single spermatogonial cell (Fig. 2). Three-dimensionally, Sertoli's cells are flattened ones (its shape best reminding a fried egg) whose cytoplasms line the tubules externally; their large nuclei appear ovoid in sections and have one or more conspicuous nucleoli (Fig. 2). Cytoplasmic extensions of Sertoli's cells are in contact with the germinal cells until advanced stages of development (Winik *et. al.*, 1994). Mature eupyrene sperms before spermiation are organized with their spiral heads pointing perpendicularly to the band of Sertoli cell's cytoplasm that lines the tubules externally (Fig. 2), while their single flagella are free in the tubular lumen. A conspicuous feature of Sertoli cell's cytoplasm is the great development of their smooth endoplasmic reticulum (Winik *et al*., 1994).

As it is the case of many Caenogastropod species, the testis of *P. canaliculata* also develops paraspermatic cells (Fig. 3). An apyrene class of parasperms (*i.e.*, devoid of any chromatin remnants, and therefore, infertile cells) is produced in the testis although at an apparently lower rate than that of eupyrene fertile sperms (Winik *et al.*, 2001). Apyrene parasperms are the consequence of a particular spermatogenic process, with no maturation division, and which can be traced back to gonial stages. Mature parasperms of this class have a long fusiform body and are propelled by three to five flagella. Another feature of these snails is that they also generate a small number of an oligopyrene paraspermatic cell, *i.e.*, parasperms that do not loose completely their chromatin. Oligopyrene parasperms are also produced by a special spermatogenic process, again with no maturation division (Winik, *pers. obs.*). As for other invertebrate taxa, the exact role of paraspermatic cells in this snail is just a matter of speculation: it is notable that with the single known exception of an abyssal echinoid (Eckelbarger *et al.*, 1989), parasperms are only present in species in which internal fertilization occurs.

Seasonal changes in reproductive activity are a common feature among Ampullariidae (Bachmann, 1960; Andrews, 1964; Fausto Filho, 1962, 1965; Burky, 1974; Milward-de-Andrade *et al.*, 1978; Lum-Kong and Kenny, 1989; Estebenet and Cazzaniga, 1992, 1993; Estebenet and Martín, 2002; Albrecht *et al.*, 1996, 1999,

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[1] Light microscopy observations were made either in Bouin-fixed or in Zencker-fixed material. Bouin-fixed material was stained with either Harris' hematoxylin and eosin or Heindenhain's iron hematoxylin. Zencker-fixed material was stained with Harris' hematoxylin and counterstained with light green and eosin (referred to as the trichrome stain).

2004) and they are conspicuous in *P. canaliculata* populations inhabiting non-tropical environments. Albrecht (1998) studied histological regression of the testis in controlled conditions in which the animals were exposed during 30 days to a water temperature (18°C) that induces an arrest in copulatory activity. In such conditions, both eu- and paraspermatogenesis stop, and the tubules become disorganized. Notably, the nuclei of Sertoli's cells become polymorphic, some of them being smaller than during the reproductive season while other become gigantic ones. "Brushes" of eupyrene sperms remain attached to the external tubular layer. Another notable feature is the formation of rather large and compact interstitial masses among the tubules.

In *P. canaliculata* there is a specialized perivascular tissue arranged as sleeves surrounding arterioles and some venules. It is made up of clear vacuolated cells arranged radially around the luminal section. These vessels and their surrounding tissue can be recognized in fresh material as whitish nervures both on the surface (once the pigmented mantle epithelium is removed) and within the mass of the testis. In all respects, this specialized perivascular tissue appears similar to that found in the midgut gland and other organs. Their clear radiated cells are loaded with intracellular crystalloids containing mostly uric acid and protein (Vega *et al.*, 2006). Besides those perivascular sleeves, the tubules are only separated by flattened hemolymphatic spaces. Cells of

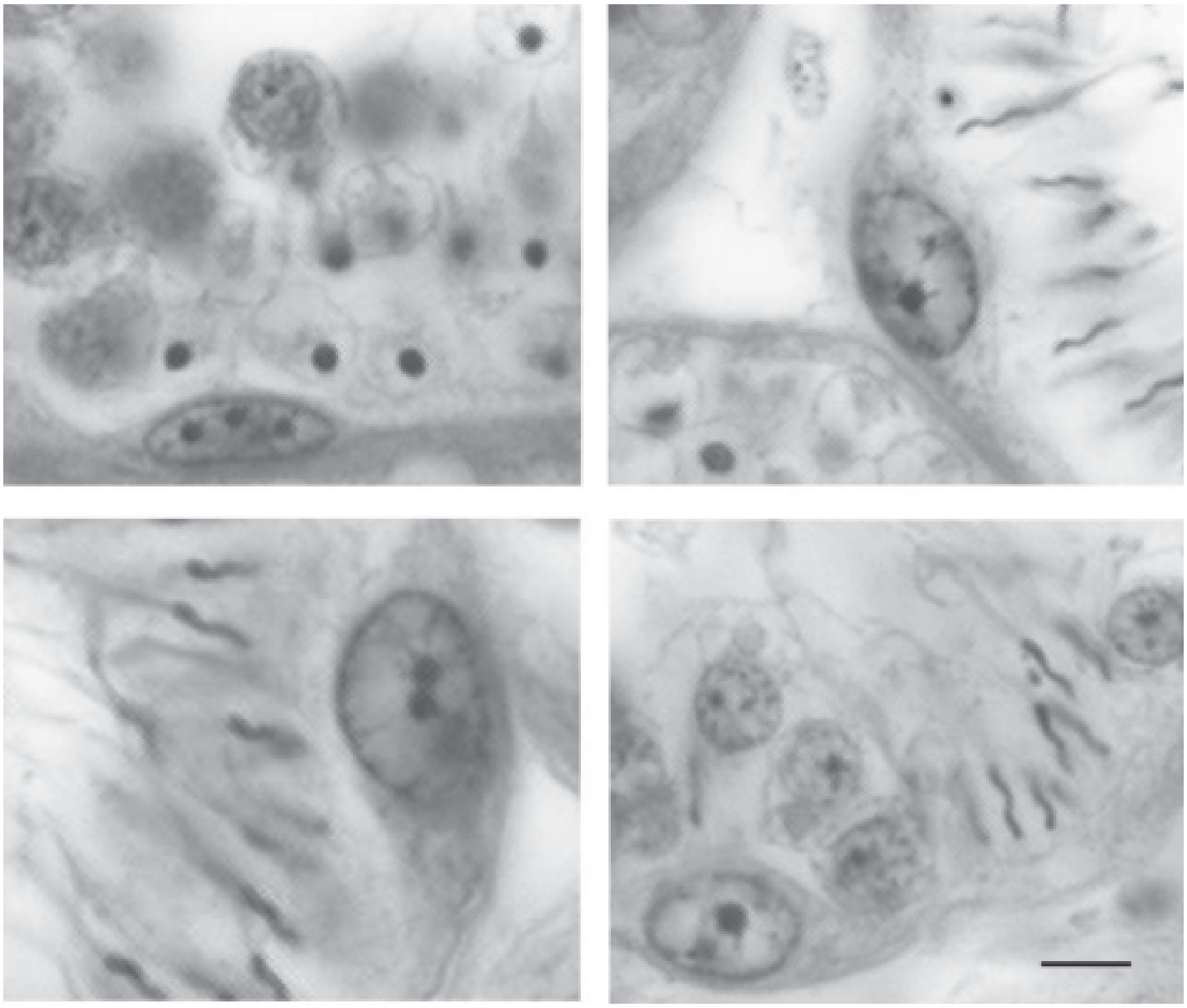

FIGURE 2. Large nuclei of Sertoli´s cells (bearing 1-3 nucleoli) which are associated to isogenic groups of euspermatogenic cells in different stages of development (Heindenhain's iron hematoxylin; the bar indicates 10 µm): clear nuclei with a single nucleolus (spermatocytes), round dark nuclei (spermatides), dark helicoidal nuclei (eupyrene sperms heads).

varying appearance have also been observed in the intertubular space of *P. canaliculata* in light microscopy preparations (Winik, 1982) but they appear as migrating hemolymphatic cells, and not epithelioid interstitial cells, in the sense of vertebrate-like Leydig's cells. Indeed, nothing similar to these androgen secreting interstitial cells of vertebrates have been observed in the course of the many electron microscopy observations of the testis of *P. canaliculata* (Winik *et al.*, 1994; Catalán *et al.*, 1997; Winik *et al.*, 2001).

Gonoduct derivatives other than the testis

The visceral part of the gonoduct develops distally and close to the inner surface of the visceral hump (*i.e.*, the surface surrounding the columella). In the adult, the *vas deferens* leaves the testis and descends close to the columella in the proximity of some nerves and vessels. It is a slender tube embedded in the connective tissue and muscular layers underlying the mantle epithelium, and it is made up of a columnar epithelium composed mostly of ciliated cells.

Just before leaving the visceral hump, this slender duct enlarges in what it is called the seminal vesicle, which is also lined by a columnar epithelium plied in large cristae. The muscular layers surrounding it are very thin and the seminal vesicle is easily torn down in dissections. The seminal vesicle cuffs the proximal end of the prostate, which is the next gonoduct derivative, and which appears after the gonoduct detaches from the columella, crosses over the adductor muscle and slides beneath the floor of the mantle cavity. The prostate is a derivative of the pallial gonoduct.

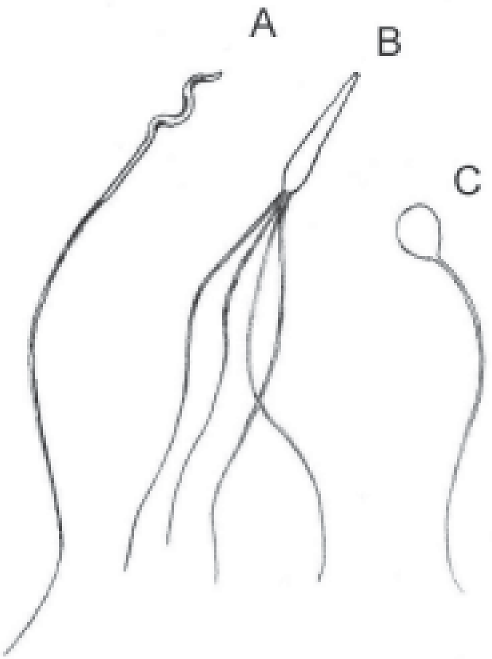

FIGURE 3. Outlines of the three sperm types found in *P. canaliculata*. *A*. Eupyrene sperm. *B*. Apyrene parasperm. *C*. Oligopyrene parasperm (Winik, pers. obs.).

The adult prostate is a rather thick and cylindrical parenchymal organ, running from left to right, beneath the epithelial floor of the mantle cavity. In sections, the ductal epithelium seems composed of both mucous and ciliated cells, and of a subepithelial zone surrounding each epithelial duct, which is composed of radially arranged, granule-bearing elongated cells. This interpretation has been challenged by electron microscopy observations, in the sense that the prostate has a single layered but complex epithelium which reminds that of the main duct of the female's albumen gland-capsule gland complex (also a derivative of the pallial gonoduct), where the albumen-secreting cells have a similar shape and arrangement and their necks are interspersed with both ciliated and neuroendocrine-like cells in the epithelial zone lining the ductal lumina, while their nucleus containing bodies lie in the "subepithelial" zone, actually the deep zone of the single layered epithelium (Catalán *et al.*, 2002). After crossing the epitaenium, the prostate gets close to the ctenidium, with which it continues side by side until reaching the rectum. It then joins the genital papilla, and organ crossing over the rectum (and therefore lying on it), and which is distinctly recognized in trichrome-stained sections, as composed by mostly parallel tubes, with a poor development of the "subepithelial zone". Tubules of the genital papilla end up in the same duct of the prostate (Gamarra-Luques, *pers. obs.*), so that both the prostate and the genital papilla appear macroscopically as a single cylindrical organ, whose continuous inner duct ends in the so-called sperm pit.

Puzzlingly enough, it is not common to see sperms within either the *vas deferens*, the seminal vesicle or the main prostate duct, even in animals that were copulating. So, it is possible that sperms transfer episode/s occupy only a small part of the prolonged copulations that are typical of this species (10-18 hours, Andrews, 1964; Albrecht *et al.*, 1996).

The copulatory apparatus: the penial complex

The bipartite copulatory apparatus of Ampullariidae (composed of the penial complex and the penial sheath) is not a derivative of the gonoduct, but of a separate primordium on the right edge of the mantle, and it is the most peculiar aspect of the genital system of these snails (Fig. 4).

As a part of the penial complex, a significant acquisition of Neotropical Ampullariidae is a tube-like sperm channel which runs within a long and coiled penis (Fig. 5). In *Pomacea*, the penis has a muscular bulb at its base and the whole is contained within a membranous penial sac (Hylton-Scott, 1957; Andrews, 1964; Berthold, 1989). The only open part of the for sperm pathway is the so-called sperm pit, where the duct of the genital papilla ends. It is at this sperm pit where the proximal end of the penial sperm duct takes up sperms, presumably by a sucking action of the penial muscular bulb.

In *P. canaliculata,* the penial sperm duct runs through three distinct regions (Fig. 5*A*). The first loop of the duct is wide and it is contained within the thick muscular wall of the bulb itself; afterwards, a coiled duct runs within a highly muscular and cylindrical extension of the bulb; and beyond that point, the vermiform penis starts, which shows a scarce muscular development. The sperm duct within the vermiform penis is accompanied by two venules and a tissue made up of clear vacuolated cells (Fig. 5*B*). The penis becomes progressively thinner at its terminal end, and the sperm duct ends without any conspicuous structure at its tip. An opening of the penial sac communicates with the groove of the penial sheath, and the slender part of the penis passes through this opening, its distal part lying within the groove, even in animals that were not copulating.

The copulatory apparatus: the penial sheath and its glands

The penial sheath is much developed in the genera *Pomacea* and *Marisa*. In *P. canaliculata* it is a spongy organ mainly made up of connective and muscular fibers delimiting hemolymphatic spaces (Martín, 1980). Conventionally, we will refer to a dorsal and ventral aspect of the sheath, according to their position at rest, considering the normal situation of this organ into the mantle cavity (see Fig. 4). Also at rest, the proximal and anterior half of the sheath is rolled around the deep groove mentioned above, (Hylton-Scott, 1957; Winik, 1982; Thiengo *et al.*, 1993; Fig. 4*A*), which extends along the basal and medial portions, at the dorsal aspect of the sheath.

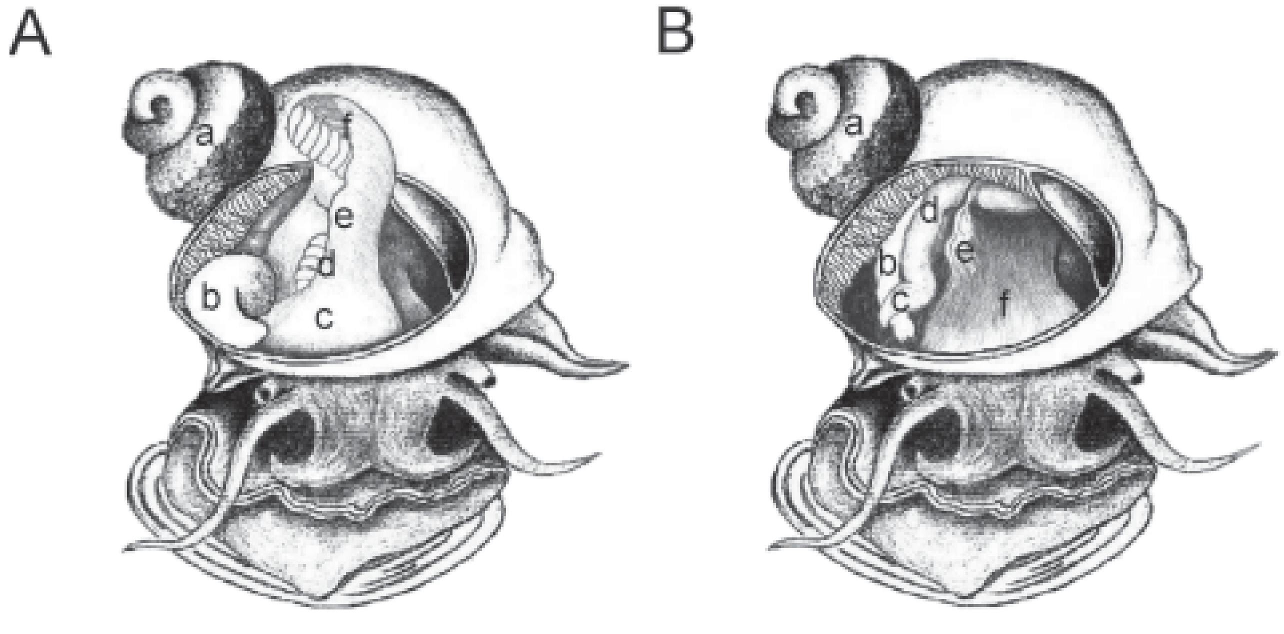

FIGURE 4. Schematic view of a dissected mature male in which the shell has been removed. The testis is shown contrasting over the dark background of the midgut gland; also, on both sides of the head, the nuchal lobes are shown (the larger left one functions as siphon). **Panel *A*** shows the copulatory apparatus when at rest (the roof of the mantle cavity has been partly removed, and both the gill and the opened lung encircle the opening, through which the tip of the penial sheath stretches out): *a*, testis; *b*, penial complex; *c*, broad base of the penial sheath, including the outer sheath gland (not seen); *d*, proximal sheath's gland; *e*, T-shaped sulcus; *f*. distal sheath's gland. **Panel *B*** shows the organs on the floor of the mantle cavity (both the penial complex and the penial sheath has been removed): *a*, testis; *b*, rectum; *c*, genital papilla; *d*, prostate; *e*, epitaenium; *f*, neck.

There are three distinct glands in the penial sheath. The most conspicuous is the basal penial sheath gland (“hypobranchial gland” of some early authors, *e.g.*, Hägler, 1923; named “outer gland” more recently, *e.g.*, Berthold, 1989). Its orange color can be recognized by transparency. It has a main duct with an opening on the sheath’s ventral aspect, while several smaller ducts have their openings within the proximal part of the groove. The main duct is the result of the simultaneous confluence of many smaller and mostly unbranched ducts. It has long been recognized that a sticky mucous secretion is freed through the ventral opening during copulation, and Andrews (1964) has postulated a deterrent effect of this secretion on other males attracted by the same female. The secretion conveyed by the previously unrecognized smaller ducts to the groove is more likely to have a lubricating action for penis sliding, however. The ductal epithelium in all parts of the basal gland is also a single layered but complex one (*i.e.*, with a “subepithelial zone”), reminding that of the prostate.

The remaining two glands on the penial sheath have no ducts. One of them is located deeply in the penial sheath groove and appears as an oval-shaped, somewhat whitish, and well delimited corrugated surface on the right bank of the groove. This gland is a synapomorphy of the genera *Pomacea* and *Marisa* (Berthold, 1989; Simone, 2004), and Andrews (1964) designated it as the “proximal” sheath’s gland, as opposed to the “distal” gland (the third aforementioned gland). The distal gland can be distinguished as a large whitish corrugated surface, located at the tip of the sheath. Both corrugated surfaces are underlain by hemolymphatic spaces and by an orange-colored layer of granule bearing, elongated epithelioid cells (Gamarra-Luques *et al.*, 2003), that may be analog to the “subepithelial” cells found in the prostate, the genital papilla and the sheath’s basal gland.

We have postulated that they might be related to a paracrine or endocrine secretion to the nearby hemolymphatic spaces that would be involved in sustaining genital growth in this species (Gamarra-Luques *et al.*, 2003).

Besides both the proximal and distal glands, and their whitish corrugated surfaces, another zone with thick and swollen plies can be seen delimiting a T-shaped sulcus at approximately midway between the base and the tip of the sheath (Figs. 1 and 4): it is the distal end of the aforementioned sheath’s groove. The thick plies are continued with countless smaller ones that cover a

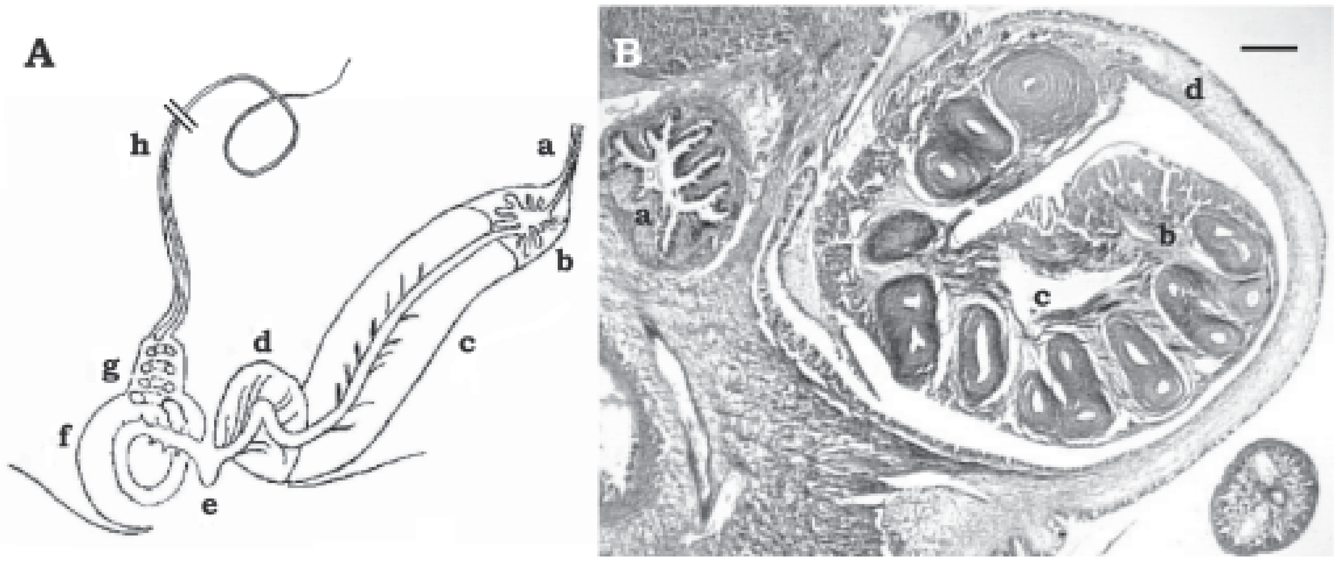

FIGURE 5. Panel *A*. General arrangement of the *vas deferens* (*a*), the seminal vesicle (*b*), the prostate (*c*) and the genital papilla (*d*) which discharges into the sperm pit (*e*). From there, the penial bulb (*f*) recovers the sperms, presumably through a sucking action. The bulb contains a curved part of the sperm duct. After it, a muscular extension of the bulb (*g*) contains the coiled part of the duct. Afterwards, the vermiform part of the penis (*h*) starts (not at scale schematic drawing; in general the structures have been shortened, and the penial sac was not drawn). **Panel *B*.** Section through part of the penial complex (Heindenhain’s iron hematoxylin, the bar is 200 µm): *a*, rectum; *b*, free end of the muscular extension containing multiple sections of the coiled part of the penial duct; *c*, central vein within the muscular extension of the penial bulb; *d*, penial sac; *e*, vermiform penis, with the central sperm duct and two lateral veins.

significant part of the dorsal aspect of the sheath. All these plies are not associated with underlying epithelioid cells and are likely to facilitate fixation during copulation, since the T-shaped sulcus affixes to the female's genital pore during copulation (Gamarra-Luques *pers. obs.*).

A fourth and extended exocrine gland is the mucous external cover of the penial sheath. It is a single layered, columnar epithelium mainly composed of mucous and ciliated cells. However, a third and conspicuous cell type may be observed on the ventral side cover. It is an elongated cell, whose finely granular cytoplasm stains with light green, and which may also be seen in the underlying connective tissue. In the latter case, they are disposed perpendicularly to the epithelial surface. When included in the surface epithelium, they sometimes show an extrusion of the apical cytoplasm. We have not yet been able to observe this cells under the electron microscope, but we are tempted to suggest that they are sensory receptor neurons. It should be kept in mind that the ventral surface of the sheath is exposed to the mantle cavity's water currents, both of the female's mantle cavity during copulation, and of the male's mantle cavity when at rest.

Innervation of the copulatory apparatus

Most monotocardians have their penis as an outgrowth on the right side of the neck and their innervation is provided by the right pedal ganglion (Hyman, 1967). However, members of the superfamily Ampullarioidea differ from this generalized plan: while the penis of the Viviparidae results from the transformation of the right tentacle and is cerebrally innervated, the copulatory apparatus of the Ampullariidae derives from the mantle edge and is innervated from the right pleural ganglion (Berthold, 1989).

In *P. canaliculata* there is a rather thick nerve that leaves the right pleural ganglion and goes towards the copulatory apparatus (Hylton-Scott, 1957). Also, as observed by Gamarra-Luques (*pers. obs.*), a conspicuous ganglion occurs within the mass of the right foot retractor muscle, below the right nuchal lobe and the anus, and all the innervation of the bipartite copulatory apparatus derives from this copulatory ganglion. No homologous ganglion was found in the females, and also, the nerve that leaves the left pleural ganglion to innervate the siphon (an adaptation of the left nuchal lobe) is much less apparent in both sexes. Microdissections do not show any ganglion on the left side either.

A tridimensional reconstruction of the nervous branching originating in the copulatory ganglion (Gamarra-Luques, *pers. obs.*) was made with the trichrome stain mentioned above[1] (see footnote 1) which shows the nervous tracts as faintly bluish. We have failed so far in impregnating *P. canaliculata* nervous tissues with more specific myelin dyes such as Luxol Fast Blue (Klüver and Barrera, 1953). Also, demonstration of the nerve terminals, both the motor terminals on the muscular fibers and the sensory terminals on the mucosal folds and in the possibly sensory cells on the external cover, is still wanting.

After the initial branching at the exit of the copulatory ganglion, branches are left at the base of the copulatory apparatus, both for the penial complex and for the sheath's basal gland. A thick nerve then enters the penial sheath, ramifying into three complex and interconnected plexuses: one ends in the vicinity of the proximal sheath's gland, apparently innervating it, the second appears to innervate the T-shaped sulcus where the sheath groove ends, and the third ends in the vicinity of the apical sheath's gland, near the tip of the sheath. Many groups of neurons are distributed along the nerves' surfaces, as irregularly distributed secondary ganglia; the nerve branches do not appear to become more slender as soon as they are further away of the copulatory ganglion, probably because they receive a substantial amount of fibers from the secondary ganglia and from many small groups of neurons found in the connective tissue underlying the outer covering epithelium.

The complexity of the sheath's innervation warrants that future studies of the sheath's fine innervation will be much interesting. It will be specially interesting to disclose if there are sensory receptors such as the suggested intraepithelial "neurons" that stain with light green.

Source, chemical nature and receptors of androtrophic hormones[2]

The attention to possible steroid actions in gastropods has come not from physiological experiments but from ecotoxicological observations (see Oetken *et al.*, 2004, for a recent review of the latter). Particularly in ampullariid snails, Tillmann *et al*. (2001) and Schulte-Oehlmann *et al*. (2000; 2004) have shown in *Marisa cornuarietis* that xenobiotics may induce abnormal development of the female vestigial penial complex and sheath. This abnormal development, often called imposex when originated by endocrine disruptors, has been described in many invertebrates (Oetken *et al.*, 2004) and has been attributed to the exposure to environmental pollutants, particularly to tributyltin (TBT, an anti-fouling component of boat-bottom paints, Matthiessen and Gibbs, 1998).

In particular, the carefully designed studies of Tillmann *et al*. (2001) and Schulte-Oehlmann *et al*. (2000; 2004) in *M. cornuarietis* have also shown that these androtrophic effects of organotins are shared with methyltestosterone (a synthetic androgen agonist) and ethinylestradiol (a synthetic estrogen agonist). Furthermore, they have shown that the effects of both methyltestosterone and ethinylestradiol may be blocked by cyproterone acetate (a competitive androgen receptor antagonist) which suggest that all these compounds may be acting on or through a receptor similar to the vertebrate androgen receptor.

It is likely, therefore, that the development and growth of copulatory organs in the male may be controlled by some androtrophin/s whose effects are mimicked in the female by the already mentioned exogenous compounds (androgens, estrogens and organotins). It is notable, however, that the androtrophic effects of these substances are apparently restricted to the ampullariid copulatory apparatus (with only a marginal effect on the genital papilla), suggesting that the growth of the gonoduct derivatives may be controlled through mechanisms different to those controlling the copulatory organs, which are derived from the mantle edge.

There is evidence in the literature suggesting that the endogenous androtrophin in ampullariid snails may be testosterone, as in vertebrates (Takeda, 2000a, 2000b). Testicular testosterone levels reported by Takeda (2000b) are rather low (actually they are within the range of those found in the plasma of rodents, Buzzio and Castro-Vazquez, 2002), but are similar to those found in the testis of another mollusk, *Octopus vulgaris* (D'Aniello *et al*., 1996). Notwithstanding with Takeda's findings, we have been unable to detect steroids in testicular extracts from *P. canaliculata*, using both specific and sensitive radioimmunoassays (RIAs)[3] for either progesterone, testosterone, androstenedione or estradiol (Gamarra-Luques *et al.*, 2004). However, one should be cautious to interpret these data, in view of the fact that testosterone may be esterified with fatty acids in gastropod mollusks (Gooding and LeBlanc, 2001; Gooding *et al*., 2003) and that it may then escape detection by antibodies. Indeed, the occurrence of free testosterone has been reported in other mollusks: *e.g.*, in the above mentioned study (D'Aniello *et al*., 1996) where it was conclusively shown (through radioimmunoassays, immunoenzymatic assays and high performance liquid chromatography) that authentic testosterone occurs in the testis of *Octopus vulgaris* (Cephalopoda, Octopodidae). Unfortunately, the report of Takeda (2000b) does not mention the methodology used in *P. canaliculata*, so we are unable to critically assess his work.

On the other hand, we did detect progesterone in *P. canaliculata* (in extracts from the midgut gland, the prostate, the penial complex and the proximal part of the penial sheath, which includes the sheath's basal gland). Androstenedione was also detected in all those organs plus the distal part of the penial sheath (which includes both the proximal and distal sheath's glands). When the esterified/free cholesterol ratio was studied, only the midgut gland showed a ratio (0.27) that would suggest that cholesterol may be accumulated there for steroid biosynthesis; the other studied organs showed ratios of 0.05 or less. As a working hypothesis we have suggested (Gamarra-Luques *et al.*, 2004) that (1) the midgut gland may be the main steroid producing organ in *P. canaliculata*; (2) that both progesterone and androstenedione may be acting as androtrophins in this snail; and (3) that these hormones may originate in the male accessory organs, where they would act paracrinally after conversion from an hemolymph-borne precursor originated in the midgut gland.

Beyond the possible androtrophic role of steroids in ampullariid snails, it should be reminded that the comparative endocrinology of invertebrates has shown the importance of neuropeptide hormones in their regulatory processes (*e.g.*, Thorndyke and Goldsworthy, 1988) and particularly, APGWamide and related peptides have been implicated in the control of several reproductive processes in gastropods, including penial growth (see Kuroki *et al.*, 1990; Chen and Walker, 1992; Croll and VanMinnen, 1992; Griffond *et al.*, 1992; Li *et al.*, 1992; van Golen *et al.*, 1995; de Lange *et al.*, 1997,

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[2] In this discussion we will use the terms *androtrophic hormone* or *androtrophin* to refer to any hormonal substance stimulating the growth and development of a male (or male-like) genital organ except the gonad, mainly because these terms do not imply any preconceived idea regarding the chemical nature of these hormones, that may be either peptides or steroids.

[3] The progesterone RIA used has been previously described and validated (Cutrera *et al*., 1998). Minimum measurable progesterone levels were 100-210 ng/g of tissue. The androstenedione RIA has been developed in our laboratory and does not show cross-reactivity with either testosterone, 17β-estradiol, progesterone or androstanediol (among other steroids). Minimum measurable androstenedione levels were 2.1-5.3 ng/g of tissue. Both testosterone and17β-estradiol were measured using *Testosterone RIA DSL-4100* and *Ultra-Sensitive Estradiol RIA DSL-4800*, both provided by Diagnostic Systems Laboratories, Inc; Webster, TX. Minimum measurable levels were 2 and 0.2 ng/g of tissue, for testosterone and 17β-estradiol, respectively.

1998; Fan *et al.*, 1997; McCrohan and Croll, 1997; de Lange and van Minnen, 1998; Koene *et al.*, 2000; Ohtani *et al.*, 2002; Morgan *et al.*, 2002; Oberdörster *et al.*, 2005). The latter authors have proposed a dual mechanism for seasonal androtrophic regulation, implying both a neuropeptide component, and a steroid-producing component regulated by the former. In Fig. 6 we have extended this hypothesis (originally formulated for the eastern mud snail *Ilyanassa obsoleta*, Neogastropoda, Nassariidae) to explain the regulation of male organ growth in ampullariid snails, and we have integrated it with the hypothesis of the origin of steroid hormones that we have formulated above.

Regarding the site/s of steroid production in *P. canaliculata*, it is intriguing that the only cells in the male reproductive system that shows a conspicuous development of the smooth endoplasmic reticulum (SER) are testicular Sertoli's cells (Winik *et al.*, 1994). No cells with a conspicuously developed SER have been observed in its midgut gland either (Koch *et al.*, 2005). This is intriguing if we consider that SER is the site of conversion of pregnenolone to both androgens and estrogens in vertebrate steroid-producing cells (Fawcett, *et al.*, 1969). Besides that, we have already mentioned that the testis of *P. canaliculata* seems devoid of anything similar to the androgen-secreting interstitial cells of vertebrates (Leydig's cells). Indeed, cells located in the intertubular spaces of the testis in *P. canaliculata* appear to be hemolymphatic infiltrating cells (Winik, 1982). And the same may be said of the large groups of interstitial cells that are observed when testicular regression is induced by low water temperature (Albrecht, 1998) or endocrine disruptors (Tillmann *et al.*, 2001).

Finally, regarding the nature of the steroid receptors involved, it should be mentioned that Thornton *et al*. (2003) have isolated an estrogen receptor ortholog from *Aplysia californica* (Opistobranchia, Aplysiidae) and have proposed that a protein similar to vertebrate estrogen receptors may be the ancestor of all steroid receptors in Bilateria. However, this *Aplysia* receptor activates transcription constitutively (*i.e.*, it is not ligand-activated), and these authors have proposed, upon phylogenetic analysis, that steroid-activation ability may have been lost in the opistobranch lineage but was probably intact in other Bilateria (including non-opistobranch gastropod lineages).

However, it should not be overlooked that the functioning of steroid receptors in invertebrate taxa may not be as hormone-specific as in mammals (Tillmann *et al.*, 2001; Schulte-Oehlmann *et al.*, 2004).

Future perspectives

Our current knowledge of the male genital system of *P. canaliculata* provides an apt starting platform for many further studies of its reproductive biology. Some of these interesting and unexplored aspects are highlighted below:

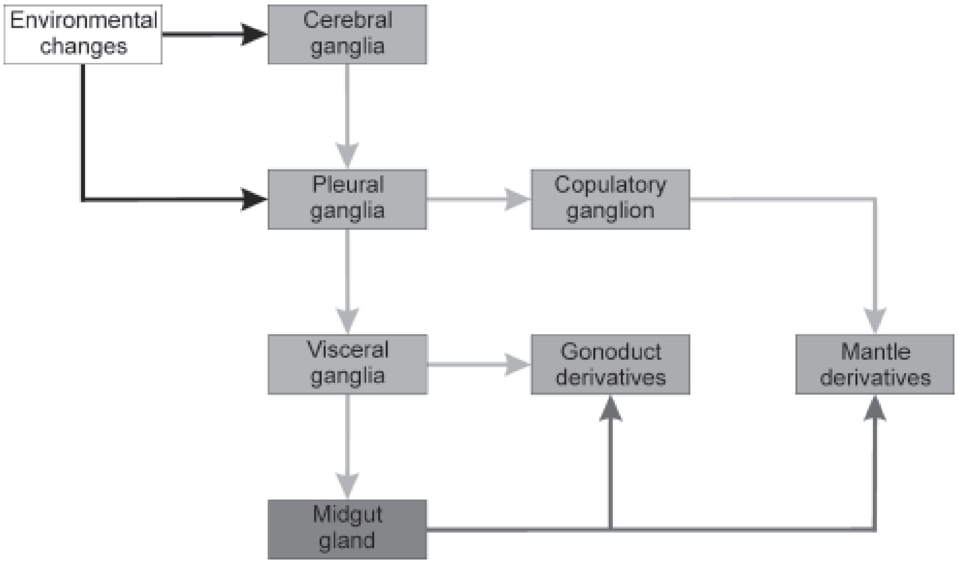

FIGURE 6. Hypothetical interrelations between the nervous system (light blue) and the midgut gland as an endocrine organ (red), and their influence on the gonoduct and mantle derivatives (orange).

1. The mechanisms of sex determination and of those bringing about the differentiation of a single primordial gonoduct into a fully adult, either male or female genital system are, at present, just a matter of speculation. The exploration of the presumably many factors involved may open a potentially wide research field.

2. Another unexplored aspect of male function is the control of spermatogenesis in adult snails, particularly of the factors determining at the cellular level the seasonality of this process and the differential generation of either eusperms or the two types of parasperms. A related intriguing question is which is the functional role played by parasperms.

3. The hormonal control of genital organs growth is poorly understood at present. Presumably, both neuropeptides and steroids may be involved as androtrophic hormones. Therefore, knowledge of the occurrence, location and seasonal changes of neuropeptides in the ganglia innervating the genital system (particularly, the rich innervation of the penial sheath) may be important to disclose the androtrophic role of peptides. On its part, localization of the site/s of steroid production and identification of the steroidal androtrophins and their precise sites and mechanisms of action appears as a potentially fruitful research area.

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