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REGULAR ARTICLE

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Pallial oviduct of *Pomacea canaliculata* (Gastropoda): ultrastructural studies of the parenchymal cellular types involved in the metabolism of perivitellins

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Abstract Seasonal variations in the morphology of the parenchymal mass and function of the albumen gland/capsule gland complex have been studied in *Pomacea canaliculata*, together with the cellular types involved in the synthesis and secretion of perivitellin fluid components. The two major parenchymal cell types, albumen secretory cells (AS) and labyrinthic cells (LC), undergo seasonal variations throughout the annual reproductive cycle, which is divided into three periods. Both cellular types show maximal development and structural complexity during the reproductive period (spring and summer). AS cells have a well-developed Golgi complex and rough endoplasmic reticulum and their secretory granules show electron-dense particles of about 20 nm (probably galactogen). These cells are uniquely involved in ovorubin and PV2 perivitellin synthesis and their secretory granules are the single storage site for these two major perivitellins, as revealed by immunoelectron microscopy. AS also possess calcium deposits that infiltrate the cytoplasmic matrix. The luminal surfaces of LC exhibit long cilia intermingled with sparce short microvilli. Basally, the plasma membrane shows deep irregular folds that extend through the cytoplasm up to the subapical region. Calcium deposits infiltrate the cytoplasm and accumulate in the extracellular space of the basal labyrinth. Nerve terminals seem to be involved in the regulation of parenchymal cell secretion. At the post-reproductive period, AS markedly change their aspect following the release of most of the secretory granules into the acinar lumen. LC decrease in volume, the number of their cilia decreases, their cytoplasmic folds are much thinner and their extracellular spaces lack calcium particles. At the pre-reproductive period (winter), AS and LC recover and prepare for the subsequent period.

Keywords Mollusc · Perivitellogenesis · Electron (Mollusca) microscopy · Carotenoprotein · *Snail, Pomacea canaliculata*

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Introduction

The apple snail *Pomacea canaliculata* (Lamarck 1822) is an amphibious, dioecious, freshwater gastropod widely distributed in American tropical and subtropical regions. This species was introduced into Southeast Asia and the Pacific Islands, where it became a pest in rice crops. It is also a vector of human meningoencephalitis, which is rapidly expanding worldwide (Mochida 1991). Estebenet and Cazzaniga (1992) have suggested that *P. canaliculata* has one or more reproductive periods during its lifespan depending upon climate conditions. Recorded lifespan fecundities range from 1,300 to 11,000 eggs per female distributed in 8–57 egg masses (Estebenet and Martín 2002).

The female reproductive system of *P. canaliculata* has an organization similar to that of other prosobranch gastropods (Hylton Scott 1957; Andrews 1964; Meenakshi et al. 1974; Carvalho Thiengo 1987, 1989, 1993; Keawjam 1987; Buckland-Nicks and Chia 1990; Hinsch and Vermeire 1990a,b; Schulte-Oehlmann et al. 1994). It consists of a single tree-branched ovary closely attached to the digestive gland followed by a complex oviduct that opens onto the female genital pore localized on the mantle edge. The ovi-

duct, according to its ontogenetic origin, can be divided into three sections: the ovarian, renal and pallial segments.

The name of the different segments of the pallial oviduct of prosobranch gastropods varies in the literature. In general terms, we have followed the terminology used by Andrews (1964) and Meenakshi et al. (1974). In *P. canaliculata*, the pallial oviduct is constituted by a seminal receptaculum, a bursa copulatrix and an albumen gland/capsule gland complex that merges into a large pinkish-red ovoid-shaped structure (Catalán et al. 2002). The albumen gland is found not only in the genital tract of prosobranchs but also in many, if not all, hermaphroditic species of fresh water and terrestrial pulmonate gastropods (de Jong-Brink 1969; Nieland and Goudsmit 1969; Courtot and Gomot 1982; Wijsman and Wijck-Batenburg 1987; Zubiaga et al. 1989; Gómez et al. 1998; Morishita et al. 1998).

The albumen gland/capsule gland complex of *P. canaliculata* consists of a parenchymal mass that encloses two main glandular ducts: the albumen gland duct and the capsule gland duct (Catalán et al. 2002). This glandular complex not only synthesizes and secretes the perivitellin fluid (PVF) that surrounds the fertilized oocytes but is also involved in the formation of the external calcareous capsule of the eggs (Catalán et al. 2002; Heras and Pollero 2002; Dreon et al. 2002, 2003). We have studied the PVF of eggs during embryogenesis and have shown that it provides energy and structural precursors for the developing embryo (Heras et al. 1998). Two lipoproteins, perivitellin-1 (PV1) or ovorubin (Cheesman 1958) and perivitellin-2 (PV2), plus one lipoprotein fraction (PV3) have been isolated; ovorubin and PV2 are the major egg proteins (Garin et al. 1996).

At present, little information is available concerning the fine structure of the pallial oviduct in *P. canaliculata* or of the mechanisms involved in the production of the egg envelopes. In a previous study, we have provided preliminary descriptions of the major cell types in the albumen gland/capsule gland complex (Catalán et al. 2002) and have shown that the inorganic component of the pallial oviduct is composed of calcium salts (Catalán et al. 2004). Although the main components of the PVF have been identified (Heras et al. 1998), the relationship between these components and the cells involved in their synthesis and secretion has not yet been elucidated. Taking into account the relevance of this fluid as an energetic and structural source for the developing embryo, we have focused, in the present work, on the parenchymal ultrastructural organization of the albumen gland/capsule gland complex and on the immunocytochemical identification of the cellular types involved in the synthesis and secretion of PVF components. Morphological and functional seasonal variations have also been analyzed.

Materials and methods

Electron microscopy

Adult females of *P. canaliculata* were collected monthly from streams or ponds in Tucumán, Argentina. Shells were cracked with scissors and the snails were carefully removed. Pallial oviducts were dissected and thin slices of tissue were prepared for routine transmission electron microscopy. Samples were fixed in Karnovsky's fluid buffered with a 0.1 M phosphate buffer (pH 7.4) for 3 h, washed in the same buffer, transferred to 1% osmium tetroxide in phosphate buffer, rinsed in distilled water and treated with an aqueous solution of 2% uranyl acetate for 40 min. Fixed tissues were then gradually dehydrated in a series of alcohols of increasing strength, followed by acetone. Infiltration by the embedding medium was performed by the gradual replacement of the solvent with Spurr resin for 2 months, in the following proportion: 100% acetone:plastic resin 4:1 (v/v); 3:1 (v/v); 2:1 (v/v); 1:1 (v/v); 1:2 (v/v); pure Spurr resin. Ultrathin sections mounted on copper grids were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 transmission electron microscope.

X-ray microprobe analysis of the electron-dense granules was performed on ultrathin sections of parenchyme by using a transmission electron microscope coupled with an energy-dispersive X-ray spectrometer (Phillips).

Anti-PV1 and anti-PV2 serum preparation

Ovorubin (PV1) and perivitellin 2 (PV2) were isolated and purified by using the methods described by Garin et al. (1996) and Dreon et al. (2002, 2003). Antibodies directed against purified PV1 and PV2 were prepared in rabbits. Animals were given multiple subcutaneous injections of approximately 1.5 mg each protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, Mo., USA). A booster injection of 1.5 mg antigen mixed with Freund's incomplete adjuvant was administered after 4 weeks. Two weeks later, rabbits were bled by cardiac puncture. Blood was allowed to clot overnight (4°C) and, after centrifugation, the serum obtained was stored at −70°C and used for the immunochemical techniques. The specificity of antisera was checked by immunoblotting against tissue homogenates (stomach, gut, muscle, lung, gonad-digestive gland, albumen gland/capsule gland complex and haemolymph) and against each other as described by Dreon et al. (2002, 2003). In order to eliminate slight cross-reactivity between anti-PV1 and anti-PV2 antibodies, both antisera were purified by affinity chromatography with an Econo Pac Serum IgG Purification Kit (Bio Rad Laboratories).

Western blots were used to check specificity; proteins were separated by SDS-polyacrylamide gel electrophoresis and electroblotted for 1.5 h at 12 V (Trans-Blot SD Semi Dry Transfer Cell, Bio Rad, Hercules, Calif., USA) onto nitrocellulose membranes by using a 39 mM TRIS, 48 mM Gly, pH 9.2, 20% MeOH buffer. After a blocking step overnight at 4°C with 3% (w/v) non-fat dry milk in 10 mM TRIS-HCl, pH 7.4, 0.15 M NaCl, the membranes were incubated for 2 h with anti-PV1 and anti-PV2 serum diluted (1:1,000) in 10 mM TRIS-HCl, pH 7.4, 0.15 M NaCl. Specific antigens were detected by goat anti-rabbit IgG/horseradish peroxidase conjugate (Bio Rad Laboratories) diluted 1:3,000. Immunoreactivity was visualized by electrochemiluminescence.

Immunocytochemistry

Parenchymal samples of the albumen gland-capsule gland complex were fixed by immersion with a solution consisting of 4% paraformaldehyde plus 0.8% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. Dehydration was carried out at increasing concentrations of ethanol followed by gradual infiltration with the acrylic resin LR-White mixed with the solvent as follows: 100% ethanol:LR-White 2:1 (v/v) for 1 h and then 1:1 (v/v) overnight followed by embedding in pure LR-White.

Ultrathin sections mounted on nickel grids were treated with 1% bovine serum albumin (BSA) buffered in a 0.01 M phosphate buffer saline (PBS), pH 7.4, and then incubated with rabbit anti-PV1 used as primary antibody. After repeated rinses in PBS, sections were incubated with the secondary antibody (goat anti-rabbit IgG; Pelco, Redding, Calif., USA) conjugated with 10-nm colloidal gold particles. All the steps of the procedure were also performed with the rabbit anti-PV2. Labelled sections were then post-stained in uranyl acetate and lead citrate before examination with an electron microscope. Control sections were treated in the same way but with no primary antibody.

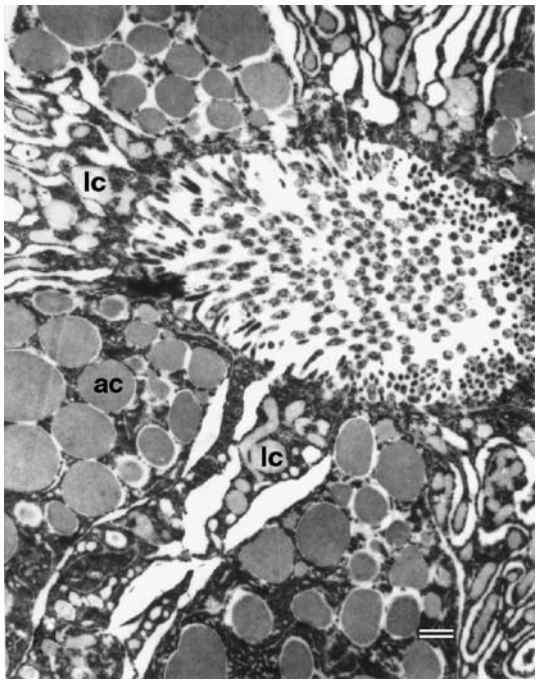

Fig. 1 Acinus of the parenchymal mass formed by albumen secretory cells (*ac*) interdispersed between labyrinthic cells (*lc*). *Bar* 1 μm

Results

Seasonal variations of the parenchymal cells

In our latitude, *P. canaliculata* has a seasonal reproduction with three different periods that we have termed pre-reproductive, reproductive and post-reproductive. All show specific functional characteristics related to structural variations of the cells of the albumen gland/capsule gland complex. The most voluminous and conspicuous component of this glandular complex is the parenchymal mass, which is composed of tubular-acinous adenomeres formed by two cell types: albumen secretory cells interdispersed between labyrinthic cells (Fig. 1).

During the reproductive period that occurs in spring and summer (September to March), the parenchymal cells reach their maximal development and structural complexity.

The albumen secretory cells, which are voluminous and pyramidal in shape, have short microvilli on their apical surfaces (Fig. 2). Laterally, adherens and septate junctions allow the attachment of cells to each other and to the labyrinthic cells, contributing to the structural unity of the adenomere (Figs. 3, 11). They show the typical ultrastructural organization of cells that synthesize and secrete large amounts of complex macromolecules of a glycoprotein nature. The nuclei, which are basally and eccentrically located, are electron-lucid and exhibit an irregular profile and a prominent nucleolus (Fig. 2). The rough endoplasmic reticulum is well-developed. Its dilated cisternae, with numerous ribosomes attached to their membranes, are arranged linearly or concentrically and possess fine granular material of moderate to low electron density (Fig. 4). The Golgi complex is formed by numerous dictyosomes in whose cisternae small electron-dense particles of about 20 nm in diameter can be observed (Fig. 5). The product of the biosynthetic activity of the rough endoplasmic reticulum and the Golgi complex is packed into large spherical or ovoid secretory granules. During the period of maximal

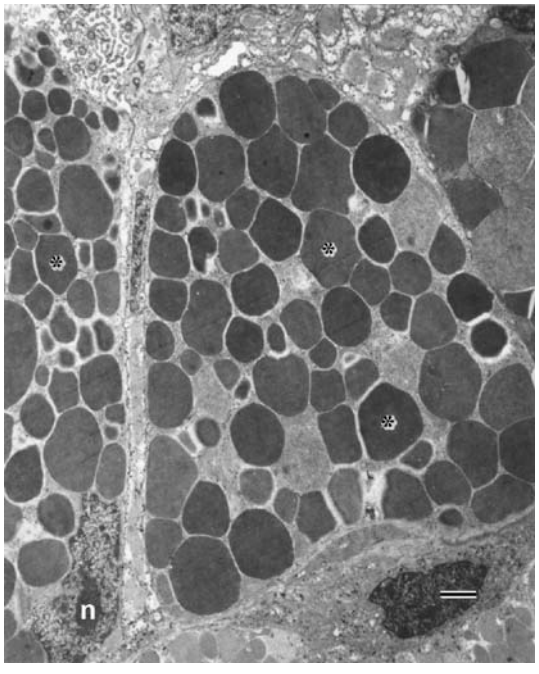

Fig. 2 Albumen secretory cell during the reproductive period. The nucleus (*n*) is peripherally and basally located. Numerous polyhedrical secretory granules fill the cytoplasm (*asterisks*). *Bar* 1 μm

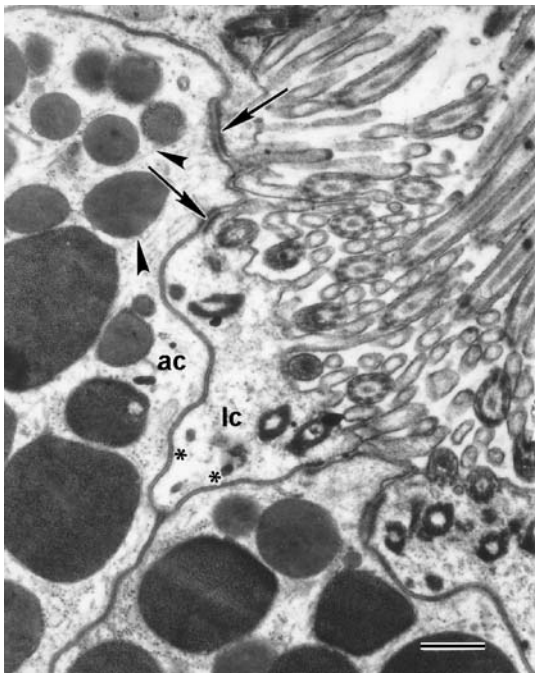

Fig. 3 Acinus of the parenchymal mass. Apical region of the albumen secretory cells (*ac*) and labyrinthic cells (*lc*). Cilia and microvilli outline the acinar lumen. Laterally, cells are joined by adherens (*arrows*) and septate (*asterisks*) junctions (*arrowheads* microtubules closely associated to secretory granules). *Bar* 0.5 μm

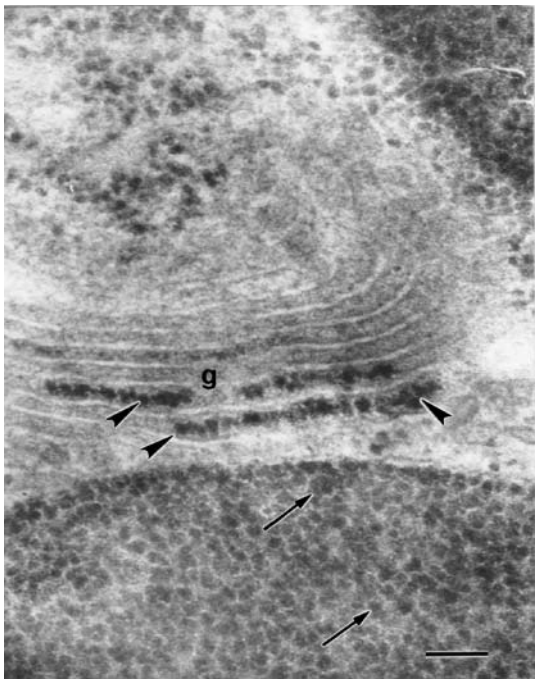

Fig. 5 Albumen secretory cell during the reproductive period (*g* Golgi complex). Note the electron-dense particles within the cisternae (*arrowheads*). The same type of particle can be observed in the mature secretory granules (*arrows*). *Bar* 100 nm

cellular activity, these granules acquire a polyhedral shape attributable to the presence of neighbouring granules. They have a homogeneous matrix of moderate electron density on which electron-dense particles, similar in size and shape to those observed in the Golgi cisternae, can be seen. These particles progressively accumulate and finally fill the granular matrix (Fig. 6). Some granules also have a spherical electron-dense core

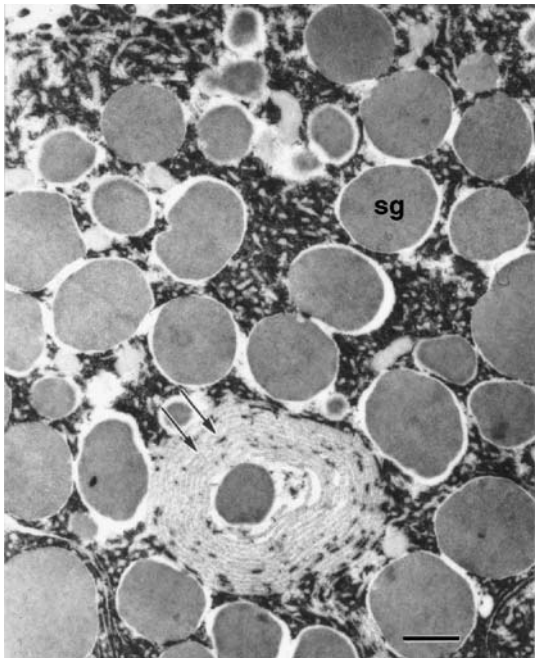

Fig. 4 Albumen secretory cell during the reproductive period. The rough endoplasmic reticulum (*arrows*) is arranged in a concentrical pattern. Note the electron-dense calcium deposits that infiltrate the cytoplasmic matrix (*sg* secretory granules). *Bar* 1 μm

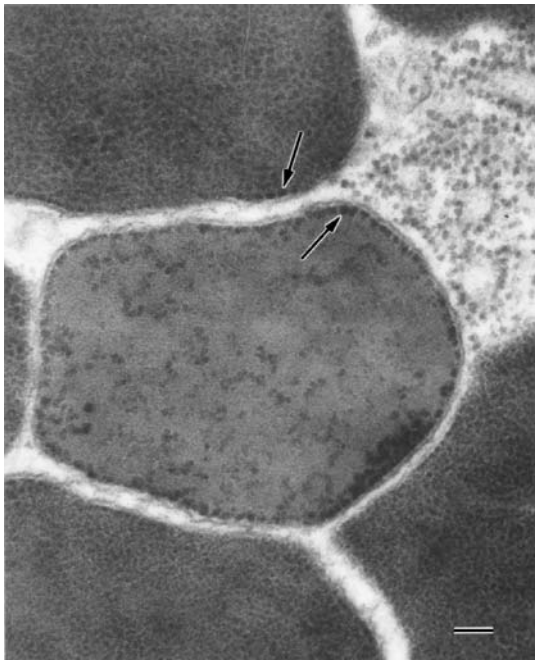

Fig. 6 Albumen secretory cell. Note the polyhedrical shape of the secretory granules, which exhibit a homogeneous matrix and electron-dense particles of about 20 nm in diameter (*arrows*). *Bar* 100 nm

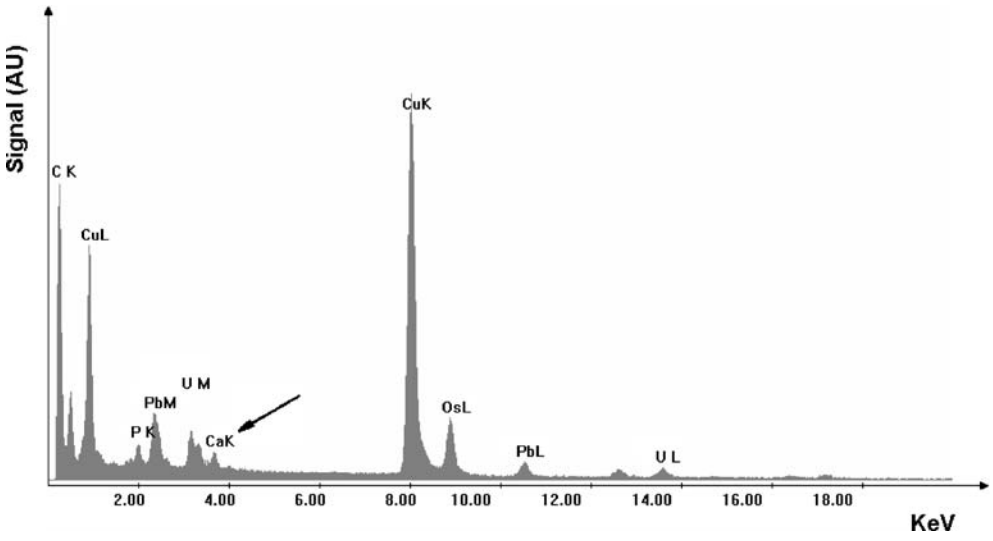

Fig. 7 Energy dispersive X-ray spectrum of a micro-area exhibiting electron-dense deposits in an ultrathin section of parenchymal mass (*arrow* Ca peak). Other elements in the sample are P and C; U, Pb, Os and Cu correspond to elements introduced during sample processing (*K*, *L*, *M* specific spectral lines of the respective elements)

that is eccentrically located. The secretory granules associated with microtubules in the cytoplasm reach the cell apical region and release their contents into the acinar lumen (Fig. 3). Small spheroidal mitochondria are scattered throughout the cytoplasm. Another remarkable characteristic of the albumen secretory cells at this reproductive period is the presence of electron-dense calcium deposits that infiltrate the cytoplasmic matrix, organelles and secretory granules to various degrees (Fig. 4). The calcic nature of these deposits has been previously demonstrated by histochemistry by the Von Kossa technique (Catalán et al. 2004). To characterize these deposits further, we have performed an electron microscope analysis of the electron-dense material with an energy dispersive X-ray spectrometer. Fig. 7 shows the major elemental composition of the samples was demonstrated to be Ca, P and C.

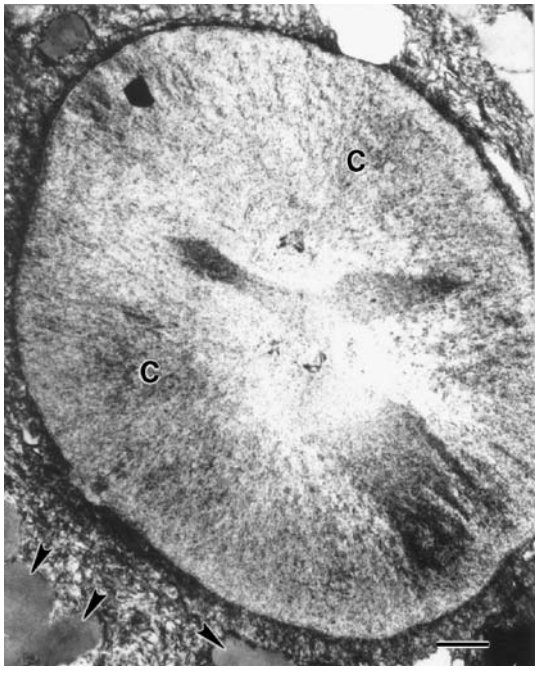

Fig. 8 Albumen secretory cell during the reproductive period. Note the conspicuous intracellular calcipherites (*c*). Electron-dense calcic deposits infiltrate the cytoplasm (*arrowheads* secretory granules). *Bar* 1 μm

Calcium salts within the albumen secretory cell are usually found as fibrous aggregates or as large compact spherical or ovoid structures called calcipherites (Fig. 8).

During the reproductive period, the labyrinthic cells, which are columnar or pyramidal in shape, exhibit long cilia intermingled with a few short microvilli at their luminal surfaces. Basally, the plasma membrane shows deep irregular folds that extend through the cellular cytoplasm up to the subapical region, forming a conspicuous and complex basal labyrinth. This labyrinth determines the formation of multiple cytoplasmic compartments that house numerous elongated mitochondria. The ovoidal electron-lucid nuclei are located in the basolateral region of the cell (Fig. 9). Basal bodies, ciliar rootlets, mitochondria and multivesicular bodies can be seen in the apical cytoplasm.

Electron-dense calcium deposits can be observed not only infiltrating the cellular cytoplasm and nucleus (Fig. 9), but also in the extracellular space of the basal labyrinth (Fig. 10) or as tiny particles (Fig. 11). According to our observations, these calcium deposits in parenchymal cells are part of a normal physiological process in *P. canaliculata*, characteristic of the reproductive period. When the calcic electron-dense material saturates the extracellular space, the basal labyrinth folds are clearly outlined.

Fibres and nerve terminals with electron-lucid and dense-core vesicles can be clearly seen in close proximity to the folds of the labyrinthic cells (Fig. 11). Finally, the granular contents of the albumen secretory cells and the calcic material pass from the adenomere lumen (Fig. 12) and ultimately reach the main albumen gland duct.

In the autumn, during the short post-reproductive period (April-May), the albumen secretory cells markedly change their aspect, as most secretory granules have been released into the acinar lumen. Nuclei show a greater electron density. Organelles involved in biosynthesis processes are poorly

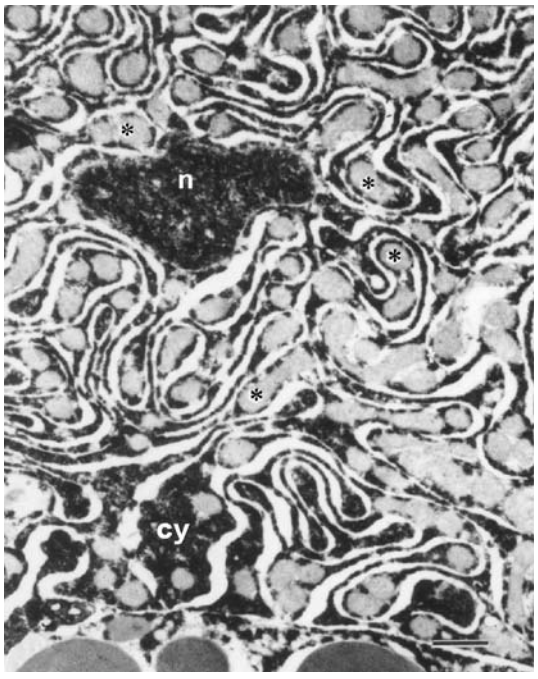

Fig. 9 Labyrinthic cell during the reproductive period (*asterisks* mitochondria). Electron-dense calcium deposits infiltrate the whole cytoplasmic matrix (*cy*). Note the completely calcified nucleus (*n*). *Bar* 1 μm

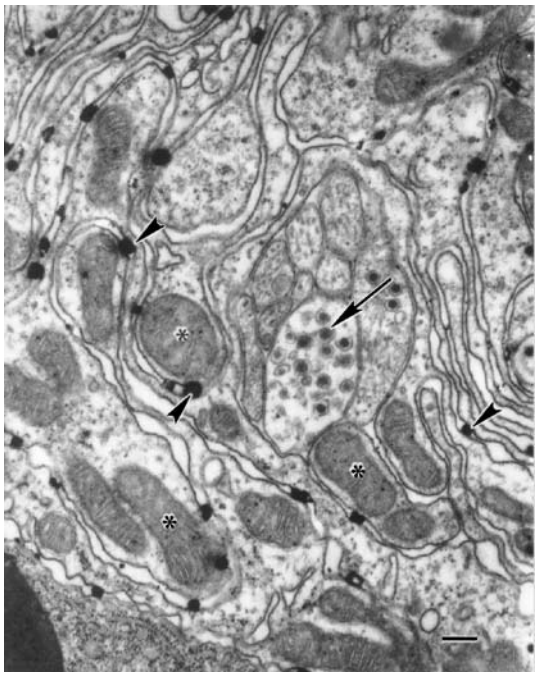

Fig. 11 Labyrinthic cell. Note tiny calcium particles (*arrowheads*) in the complex extracellular spaces of the basal labyrinth. Nerve terminals with neurosecretory vesicles (*arrow*) lie in close proximity to the cytoplasmic folds (*asterisks* mitochondria). *Bar* 0.25 μm

developed. Lysosomes, glycogen granules and lipids are typical structures in the cytoplasm (Fig. 13). During this period, the labyrinthic cells are the main acinous components. Like the albumen secretory cells, the labyrinthic cells have also markedly decreased in volume. They exhibit an apical cytoplasmic band that maintains adenomere integrity via junction complexes. Cilia are reduced in number. The basal labyrinth cytoplasmic folds are much thinner, outlining

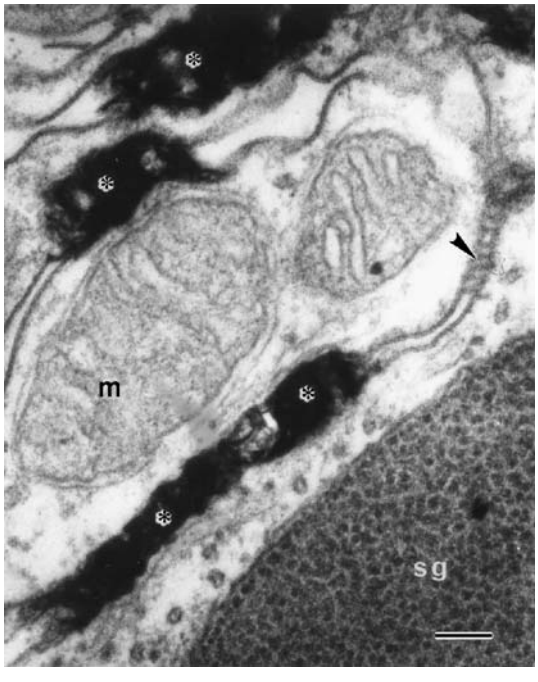

Fig. 10 Reproductive period (*arrowheads* septate junction between a labyrinthic cell and an albumen secretory cell). Note the electron-dense calcium deposits (*asterisks*) in the dilated extracellular spaces (*sg* secretory granule, *m* mitochondrion). *Bar* 100 nm

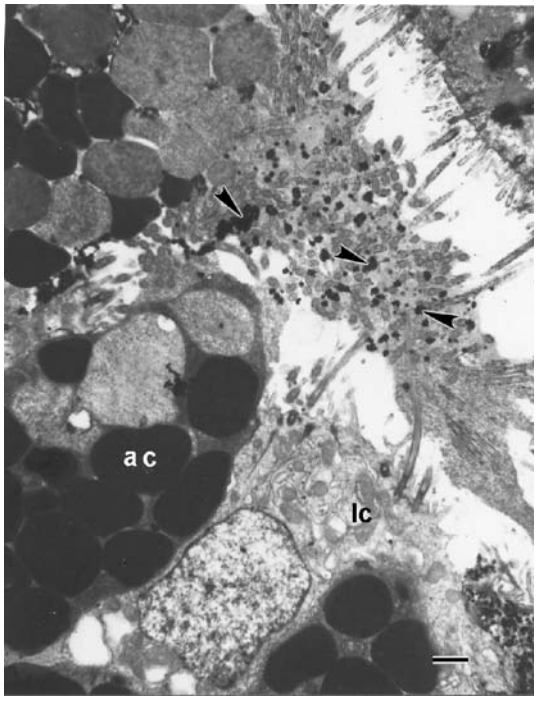

Fig. 12 Reproductive period. Note the presence of several calcic particles (*arrowheads*) in the acinar lumen secretion (*ac* albumen secretory cells, *lc* labyrinthic cell). *Bar* 1 μm

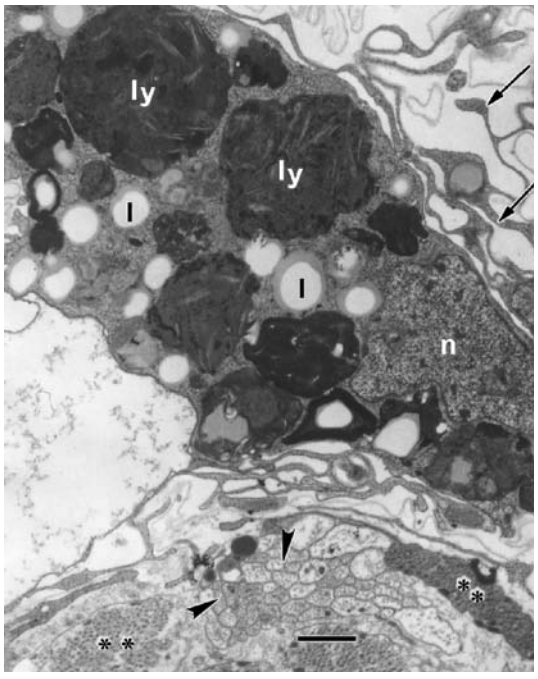

Fig. 13 Post-reproductive period. The albumen secretory cell exhibits voluminous lysosomes (*ly*) and lipid droplets (*l*) in the cytoplasm. Note the absence of secretory granules and the increase of the nuclear (*n*) electron density (*arrows* cytoplasmic folds of labyrinthic cells). A conspicuous neuro-muscular complex (*arrowheads*) can be observed in the interstice (*asterisks* muscular fibres, *arrowheads* nerve fibres). *Bar* 1 μm

Fig. 14 Post-reproductive period. The labyrinthic cells exhibit thin cytoplasmic folds (*arrows*). Note the dilated extracellular spaces (*asterisks*) with no electron-dense calcic particles. Cilia (*arrowheads*) are reduced in number. *Bar* 2 μm

a dilated extracellular space with no electron-dense calcic particles (Fig. 14). Multivesicular bodies, lysosomes and mitochondria can be observed in the cytoplasm.

During the pre-reproductive period, when the lowest water temperatures are recorded (May-August), a reorganization of the biosynthetic organelles in the albumen secretory cells begins. A progressive development of the rough endoplasmic reticulum, Golgi cisternae and nuclei with a prominent nucleolus is observed (Fig. 15). Secretory granules increase in number and size and, consequently, so does the cellular volume. Labyrinthic cells also show a process of cellular recovery together with a reorganization of their structural components, although no circulating calcic particles can as yet be seen in the extracellular space of the basal labyrinth (data not shown).

Immunocytochemical determination of parenchymal cells involved in perivitellin synthesis

The parenchymal cellular types involved in the synthesis and secretion of perivitellins were identified by the colloidal gold technique for immunoelectron microscopy during the reproductive period. Large quantities of colloidal gold particles were observed in the granules of the albumen secretory cells when an antibody against PV1 or ovorubin was used (Fig. 16). Anti-PV2 antibody immunolabelling also gave positive results in the same granules, although with a much lower colloidal gold particle density (Fig. 17). Other immunoreactive structures observed in the

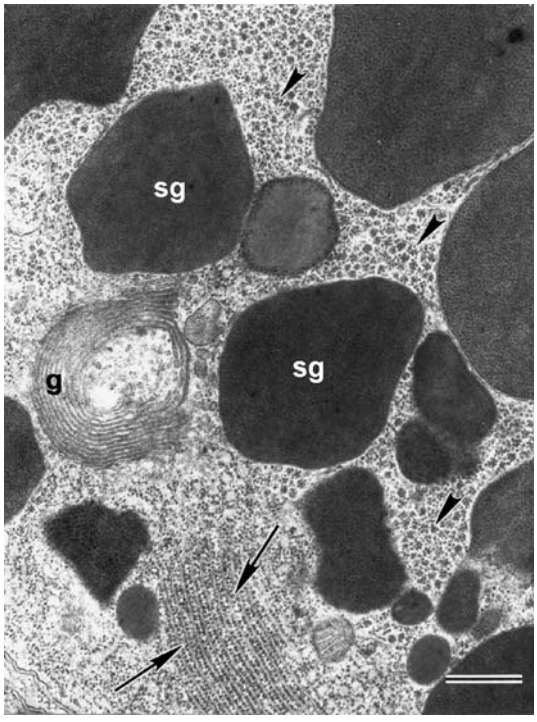

Fig. 15 Pre-reproductive period. The albumen secretory cell exhibits secretory granules (*sg*) of various sizes and shapes scattered throughout the cytoplasm. Note the development of the rough endoplasmic reticulum (*arrows*) and the Golgi complex (*g*). Glycogen particles fill the cytoplasmic matrix (*arrowheads*). *Bar* 0.5 μm

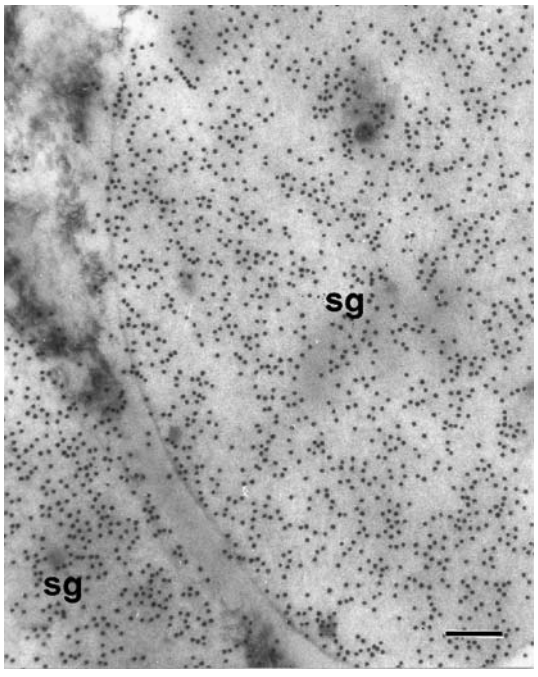

Fig. 16 Immunocytochemistry of an albumen secretory cell at the electron-microscope level. Secretory granules (*sg*) exhibited a high density of colloidal gold particles when an antibody against PV1 was used. *Bar* 100 nm

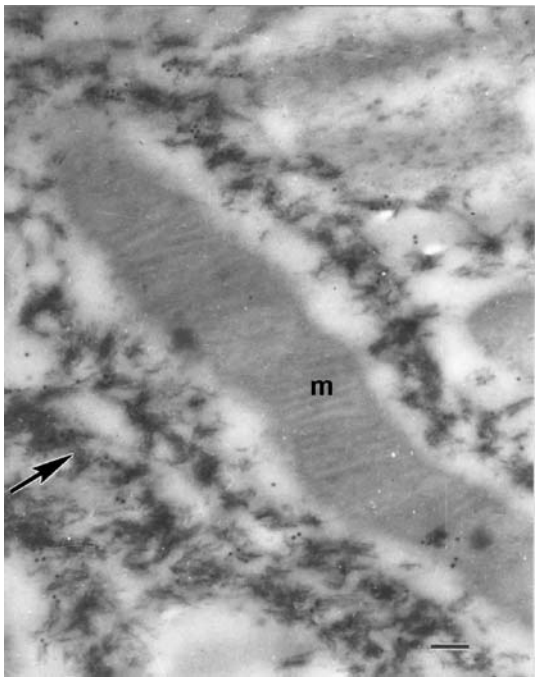

Fig. 18 Immunocytochemistry at the electron-microscope level. The unlabelled labyrinthic cell serves as control for immunostaining background when an antibody against PV1 is used (*m* mitochondrion, *arrow* calcium-containing material). *Bar* 100 nm

albumen secretory cells included the rough endoplasmic reticulum and the Golgi complex. The labyrinthic cells were not reactive and served as a control for immunostaining background (Fig. 18). The absence of colloidal gold particles in the biosynthetic organelles and granules of the albumen secretory cells, when the primary antibody was omitted, confirmed the specificity of the immunostaining (Fig. 19).

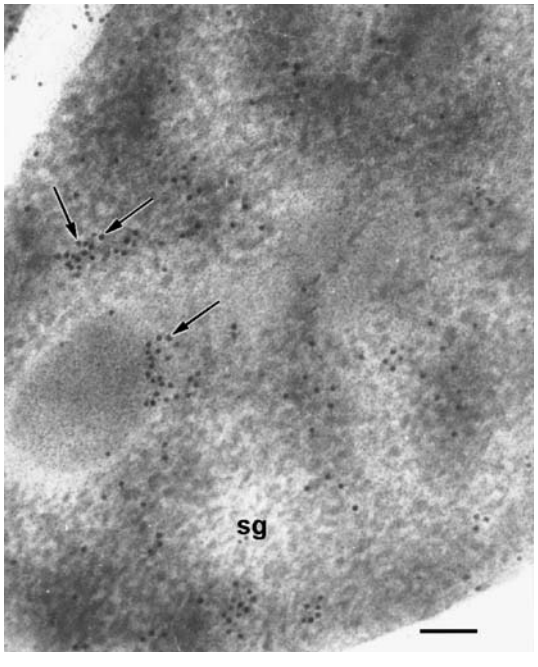

Fig. 17 Immunocytochemistry of an albumen secretory cell at the electron-microscope level. The immunolabelled secretory granule (*sg*) exhibited few colloidal gold particles (*arrows*) when an antibody against PV2 was used. *Bar* 100 nm

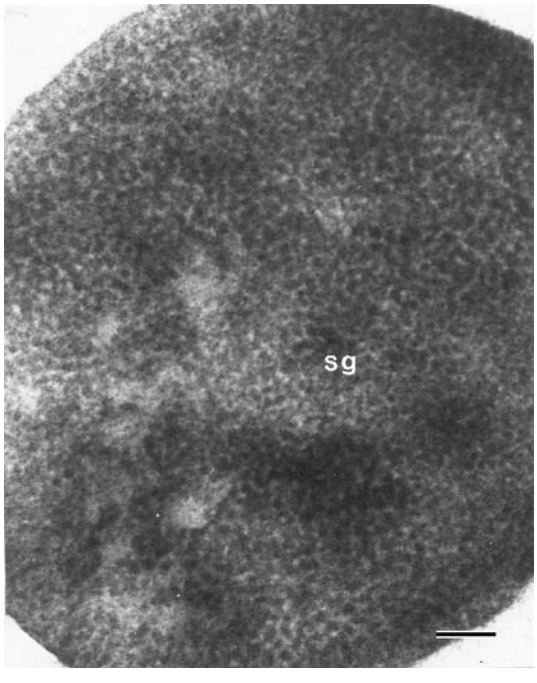

Fig. 19 Immunocytochemistry at the electron-microscope level. Control section of an albumen secretory cell. The primary antibody was omitted. Note the complete absence of colloidal gold particles in the secretory granule (*sg*). *Bar* 100 nm

Discussion

Anatomical considerations

The pallial oviduct of *P. canaliculata* is structurally the most complex segment of the female reproductive tract and is also functionally the most relevant, as it not only provides the various egg envelopes, but is also involved in the transport and storage of sperm and in oocyte fertilization. Our studies at the ultrastructural level have demonstrated that the glandular parenchymal mass has two main cell types, the albumen secretory cells and the labyrinthic non-secretory ciliated cells, both of which exhibit morphological changes during the sexual cycle (Catalán et al. 1996; Winik et al. 1998). The parenchymal mass of this species corresponds to the structure that Meenakshi et al. (1974) and Meenakshi and Watabe (1977) have termed albumen gland tissue in *P. paludosa* and *P. urceus* and to the structure that Vermeire and Hinsch (1984) have called the albumen gland proper in *P. paludosa*. In *P. paludosa*, Vermeire and Hinsch (1984) have only reported the presence of secretory cells as components of the albumen gland proper. Moreover, in numerous pulmonate gastropods such as *Biomphalaria glabrata* (de Jong-Brink 1969), *Stagnicola elodes* (Rudolph 1983), *Helix pomatia* (Nieland and Goudsmit 1969), *Arion subfuscus* (Zubiaga 1989; Gómez et al. 1998) and *Deroceras laeve* (Els 1974), the albumen gland is composed of tubules consisting of two cell types, viz. secretory cells and ciliated non-secretory centrotubular cells, arranged around a central lumen. The former might be homologous to albumen secretory cells, but the latter is not strictly equivalent in morphology and function to the labyrinthic cells found in *P. canaliculata*, as they are not involved in the active transport of calcium. This function might be closely associated with the prominent system of cytoplasmic folds with mitochondria in the basal labyrinth and the membranes of this labyrinth most probably have calcium pumps.

The oviposition of voluminous egg clusters that characterize the reproductive period of ampullarid snails demands the mobilization of large quantities of calcium to the PVF and mainly to the calcareous egg shells (Meenakshi et al. 1974; Meenakshi and Watabe 1977; Catalán et al. 2003, 2004). The calcium carbonate stored in the parenchymal mass, mostly as large calcipherites, might be degraded by an unknown mechanism into tiny particles that move from the parenchymal interstice to the complex extracellular space of the basal labyrinth, saturating it to various degrees during the reproductive period. Cells with similar characteristics have been described in the female duct epithelium of the spermoviduct of pulmonates (Zubiaga et al. 1989; Tompa and Wilbur 1977).

The other parenchymal cell type of *P. canaliculata*, viz. the albumen secretory cell, might be the main cell responsible for the synthesis and secretion of the PVF. The products released by the secretory cells of the albumen gland duct and of the capsule gland duct might also contribute to this fluid (Winik et al. 1998; Catalán et al. 2002, 2004). In agreement with reports in *Helisoma duryi* (Khien et al. 2001) and *Limnaea stagnalis* (Wijsman and Wijck-Batenburg 1987), the importance of the PVF in *P. canaliculata* is apparent because it represents the major nutrient source for the developing embryo, since the oocytes themselves contain little vitellogenic protein (Garin et al. 1996).

The albumen secretory cells acquire their maximal development during the reproductive period; thus, the albumen gland/capsule gland complex becomes enlarged at this stage (Catalán et al. 2001). This is probably attributable to cell hypertrophy rather than hyperplasia, as studies in the albumen gland of the pulmonate *Lymnaea stagnalis* have shown (Wijsman and Wijck-Batenburg 1987). The ultrastructural characteristics of these cells correspond to those found in cells with a highly active secretory pathway, as is evidenced by the remarkable development of the rough endoplasmic reticulum and the Golgi complex. A similar development of these biosynthetic organelles has been observed in the albumen gland secretory cells of several pulmonates (Nieland and Goudsmit 1969; de Jong-Brink 1969; Zubiaga et al. 1989; Gómez et al. 1998).

During the post-reproductive period, the albumen gland suffers an involution process that is also reflected in the biosynthetic cellular organelles, which exhibit poor development. A similar process has been described in the albumen gland of *Biomphalaria glabrata* (de Jong-Brink 1973). During the pre-reproductive period the parenchymal cells prepare their organelles for the next reproductive period, when maximal synthetic activity takes place.

Previous histochemical and biochemical studies have shown that, in addition to proteins, the albumen gland of several gastropods produces galactogen as a component of the PVF (Nieland and Goudsmit 1969; de Jong-Brink 1969; Goudsmit 1975; Okotore et al. 1982; Wijsman and Wijck-Batenburg 1987; Gómez et al. 1998; Wijdenes et al. 1983; Khien et al. 2001). Other authors mention calcium as an additional element in this fluid (Meenakshi and Watabe 1977; Turner and McCabe 1990) and minor amounts of lipids have been reported (Garin et al. 1996).

In *P. canaliculata* and in other species, the synthesis and release of the PVF components may be under the control of a nervous mechanism. The presence of neuronal terminals in close association with the parenchymal cells supports this idea. Secretory activities of the albumen gland in pulmonate gastropods are highly regulated, the coordination of PVF synthesis, storage and secretion processes being necessary during the egg-producing cycle. In these gastropods, polysaccharide synthesis in the albumen gland appears to be regulated by an endocrine factor (ecdysteroid) from the dorsal bodies and possibly by neuroendocrine factor(s) from the cerebral ganglia (Miksys and Saleuddin 1985; Mukai et al. 2004); the release of PVF from albumen secretory cells might be under the control of biogenic monoamines. Indeed, the albumen gland of a variety of pulmonate gastropods is reported to possess a network of catecholaminergic nerve fibres (Brisson and Collin 1977; Hartwig et al. 1980; Croll et al. 1999).

The presence of clear and dense-cored vesicles in the parenchymal nervous terminals of the albumen gland-capsule

gland complex of *P. canaliculata* suggests the existence of more than one type of neurotransmitter. Recently, Mukai et al. (2004) have provided experimental evidence that strongly suggests that PVF secretion is mediated by a dopamine D1-like receptor. Further studies are necessary to elucidate the types of neurotransmitters present in the albumen gland/capsule gland complex of this species.

Perivitellin synthesis during the reproductive cycle

Our ultrastructural and immunocytochemical results performed on the albumen gland/capsule gland complex of *P. canaliculata* strengthens previous studies on perivitellin synthesis carried out by Dreon et al. (2002, 2003) who have demonstrated that the albumen gland of this species is the only site for PV1 and PV2 synthesis and storage; no extra-gland synthesis, circulation or accumulation has been found in this snail. The intense immunolabelling observed in the granules of the albumen secretory cells when we have used an anti-PV1 antibody allows us to infer that these cells are the primary site of ovorubin synthesis in *P. canaliculata*. The significantly lower immunolabelling of the same granules when we have incubated sections with an anti-PV2 antibody is in agreement with the lower concentration of this perivitellin (30 mg/g and 1 mg/g tissue protein for ovorubin and PV2, respectively, as determined by ELISA in the albumen gland/capsule gland complex; Dreon et al. 2002, 2003). The specific location of the label, when using both antibodies, in the endoplasmic reticulum and Golgi cisternae has confirmed the involvement of these organelles in perivitellin synthesis and secretion.

The perivitellins stored in the secretory granules constitute a structure that, at the ultrastructural level, appears as a homogeneous matrix in which 20-nm particles are prominent. Similar particles have been described in the granules of the secretory cells of the albumen glands of a variety of pulmonates, such as *Helix pomatia* (Nieland and Goudsmit 1969), *Limax maximus* (Van Minen et al. 1983), *Arion subfuscus* (Gómez et al. 1998) and *Lymnaea stagnalis* (Wijsman and Wijck-Batenburg 1987). Based on biochemical and ultrastructural studies, several authors have reported that these particles correspond to galactogen (Grainger 1951; Nieland and Goudsmit 1969; Meenakshi and Scheer 1968; Okotore et al. 1982; Wijdenes et al. 1983; Goudsmit 1975; Gómez et al. 1998). Galactogen, which is a high molecular weight homopolymer of galactose, is known to be synthesized in the trans-cisternae and trans-Golgi network. In the same Golgi compartments of the albumen secretory cells of *P. canaliculata*, we have observed particles identical in morphology, size and electron density to those found in the secretory granules. We therefore suggest that these particles correspond to galactogen and that the secretory granules store perivitellins together with this polysaccharide. In addition, we have observed that the secretory granules of *P. canaliculata* exhibit a variable and increasing concentration of particles, suggesting that galactogen synthesis is completed in these granules.

To our knowledge, this is the first report of the seasonal-reproductive variations of the parenchymal cells of the albumen gland/capsule gland complex in *P. canaliculata* and of the identification of the cells involved in perivitellin synthesis.

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