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Ultrastructure of the oviductal mucosa of *Leptodactylus chaquensis*. Analysis of the preovulatory and postovulatory periods

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Summary

In the present study we analysed the ultrastructural characteristics of the oviductal mucosa of *Leptodactylus chaquensis* during the preovulatory period and immediately after ovulation. Epithelial secretory cells, ciliated cells, basal cells and glandular secretory cells are described. During the preovulatory period, the oviduct exhibits its maximum degree of development at both the epithelial and the glandular levels, with numerous secretory cells that contain a large number of secretory granules whose contents are released into the oviductal lumen by apocrine and exocytotic secretory processes. The secretory cells present throughout the oviduct display considerable variability in the characteristics of their secretory granules, which show different shapes, sizes, organization of the material contained and electron density. The different cell types are distributed following a characteristic pattern for each oviductal zone, thus creating an ultrastructural mosaic along the oviduct. During the postovulatory period, the number of secretory cells decreases and the remaining ones exhibit a marked reduction in secretory granules. Ciliated cells show a typical ultrastructural organization that is not modified throughout the reproductive cycle. Basal cells, located at the basal region of the epithelium, are characterized by their heterochromatic nuclei and electron-lucent cytoplasm, while glandular secretory cells exhibit oval, round or polyhedric granules, most of them with a prominent core. Our results, which indicate a high heterogeneity of secretory cell contents, allow us to suggest differential synthesis and secretion of specific products in each oviductal zone.

Keywords: Amphibian, *Leptodactylus chaquensis*, Oviduct; Reproductive cycle, Ultrastructure

Introduction

Leptodactylus chaquensis, like most amphibian species, has a cyclic reproductive activity. In this cycle two periods can be identified, a breeding period and a post-reproductive one. During the breeding period, in the spring to summer months, both ovary and oviduct reach their maximum development and functionality (Hedrick & Nishihara, 1991; Winik *et al.*, 1999; Fernández & Ramos, 2003). The post-reproductive period, during the fall to winter months, is characterized by the recovery of the organs involved in reproduction (Fernández & Ramos, 2003).

In the oviduct, from the structural viewpoint, three main zones can be identified: the pars recta (PR), the pars convoluta (PC), and the ovisac. The most cephalic portion is the PR, which is connected with the pleuroperitoneal cavity through an opening called the ostium. The final portion of the PR and the beginning of the convoluted tract constitute the intermediate proximal zone (IPZ), a transition segment between two anatomically and functionally different zones (Hedrick & Nishihara, 1991; Winik *et al.*, 1999). Subsequent to this segment is the main body of the PC, which is divided into two zones: the pars preconvoluta

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(PPC), constituted by short tight convolutions that occupy almost two-thirds of the oviduct, and the pars convoluta (pc) itself, formed by long wide convolutions that, identical to those of the PPC, are arranged perpendicularly to the oviductal axis. The latter zone dilates into the ovisac, where the oocytes accumulate before oviposition (Alcaide de Pucci, 1991b; Fernández & Ramos, 2003).

In numerous amphibian species the acquisition of fertilizability by the oocytes has been proved to depend on their contact with the products secreted by the oviduct (Elinson, 1974; Miceli *et al.*, 1978; Ishihara *et al.*, 1984; Katagiri, 1987). This duct produces two types of secretion. Firstly, a low viscosity aqueous product secreted by the PR of *Rhinella arenarum* [syn. *Bufo arenarum*] that contains a proteolytic enzyme that induces modifications in the oocyte vitelline envelope at the structural and molecular level, making it sensitive to acrosomal enzymes and penetrable by sperm (Miceli *et al.*, 1978, 1987). Similar results were reported for *Rana japonica*, *Xenopus laevis* and *Bufo japonicus* (Yoshizaki & Katagiri, 1981, Takamune *et al.*, 1986; Hardy & Hedrick, 1992). And secondly, a highly viscous material secreted by the PC that is sequentially deposited around the oocytes during their transit through this portion of the oviduct forming the jelly coats (Barbieri & Budeguer de Atenor, 1973; Yoshizaki, 1985; Hedrick & Nishihara, 1991; Fernández & Ramos, 2003).

Different biological functions, all of them related to fertilization, have been assigned to these secretions (Del Pino, 1973; Elinson, 1974; Takamune *et al.*, 1986; Miceli *et al.*, 1987; Hardy & Hedrick, 1992; Xiang *et al.*, 2004; Medina *et al.*, 2012). However, there are few studies that focus on the oviductal morphology of anurans and they are limited to some species and to some periods of the reproductive cycle (Shivers & James, 1970; Yoshizaki & Katagiri, 1981; Yoshizaki, 1985). Only the *R. arenarum* oviduct has been studied at the histological, ultrastructural and functional level throughout the reproductive cycle (Fernández *et al.*, 1989; Winik *et al.*, 1999; Fernández & Ramos, 2003; Medina *et al.*, 2007; Crespo *et al.*, 2009; Medina *et al.*, 2012). With respect to *L. chaquensis*, at present the structural organization (Alcaide de Pucci, 1991b) and the histochemical analysis (Alcaide de Pucci, 1991a) of the oviduct during the preovulatory period have been studied by optical microscopy. Alcaide de Pucci (1991b) showed that the oviductal mucosa forms longitudinal folds along the axis of the tube and that it is composed of a pseudostratified epithelium and a single tubule-acinous glandular layer that begins to be observable at the end of the PR and increases its thickness toward the pc. This oviductal epithelium is constituted by secretory cells, ciliated and basal cells.

Since morphological analyses provide the bases for functional studies, the present work aims at studying the ultrastructural changes in the characteristics and arrangement of the different cells present in the oviductal mucosa of *L. chaquensis* throughout the reproductive cycle.

Materials and methods

Animals

Sexually mature *L. chaquensis* females, 35–55 g in body weight, were collected in the locality of Agua Azul, Leales Department, Tucumán, Argentina. The animals were used immediately after capture or kept in boxes with appropriate humidity at 25 ± 1°C for brief time periods (2–4 days).

For electron microscopic studies we used specimens both from the preovulatory period ($n = 4$ different animals) and after ovulation ($n = 4$ different animals), selected according to the characteristics of the ovary. The females from the preovulatory period showed an ovarian mass of 8.64 ± 1.87 g together with a prevalence of fully grown oocytes, while those in the postovulatory period exhibited rudimentary ovaries that weighed 1.85 ± 0.71 g with a population of small- and medium-sized previtellogenic oocytes.

The experimental procedures were conducted in accordance with the *Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research* (Herpetological Animal Care and Use Committee of the American Society of Ichthyologists and Herpetologists, 2004).

Oviducts

In order to systematize the morphological description and analyse the changes at the subcellular level, the oviduct was dissected carefully and separated into PR and PC. The latter was divided into three zones: (1) IPZ; (2) PPC; and (3) pc.

Ultrastructural studies

Oviduct samples were fixed in 4% glutaraldehyde buffered in 0.1 M phosphate buffer, pH 7.4, for 3 h at 4–5°C, rinsed in the same buffer and postfixed overnight in 1% osmium tetroxide with phosphate buffer. Then, the samples were dehydrated in an increasing graded ethanol series and embedded in Spurr resin (Pelco Co.). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (TEM) Zeiss EM 109.

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Results

Preovulatory period

Epithelial secretory cells

Before ovulation, epithelial secretory cells (ESC) that exhibit numerous short microvilli oriented toward the oviductal lumen (Fig. 1*A*) can be seen as large columnar cells attached to the basal lamina. A remarkable morphological characteristic is the presence of complex interdigitations of the lateral plasma membranes (Fig. 1*A*–inset). The electron-lucent nuclei, located in the middle or basal zone, are usually ovoid shaped and present deep indentations. In the cytoplasm we can observe a rough endoplasmic reticulum showing in its cisternae a finely granular material of moderate electron density (Fig. 1*B*), a smooth endoplasmic reticulum and a well developed Golgi complex, numerous mitochondria and glycogen granules and scarce lysosomes and lipid droplets (Fig. 1*B*). There are also filaments and cytoplasmic microtubules. The most noticeable ultrastructural aspect of the secretory cells (SC) during this period is the presence of abundant secretory granules (SG) exhibiting size, shape and electron density characteristics peculiar to the region of the oviduct in which they are localized. In the PR we can observe only one type of ESC that contained SG of round or oval shape, variable size and medium to high electron density (Fig. 1*C*).

The greatest heterogeneity in cell types is found at the level of the IPZ. Cells with electron-lucent SG (Fig. 2*A*) containing fibrillar material arranged in parallel (Fig. 2*A* – inset) can be seen, others with SG of moderate electron density (Fig. 2*B*) containing polymorphic high-electron-density aggregates (Fig. 2*B* – inset) and a third type with electron-lucent SG (Fig. 2*C*) containing fibrillar material irregularly arranged (Fig. 2*C* – inset).

In the SC present in the PPC, granules with a wide variety of electron densities and arrangements of the material co-exist (Fig. 3*A*). An outstanding feature is the presence of granules with a paracrystalline organization (Fig. 3*B*) whose content is distributed uniformly in small aggregates of high electron density (Fig. 3*B* – inset) or concentrated in larger aggregates.

The SG stored in the SC of the pc, which show a rounded shape, are homogeneous in size, and the material they contain is of medium to high electron density (Fig. 4*A*). The secretion products are released mainly by exocytosis toward the oviductal lumen, where they appear as flocculent material (Fig. 4*A*). Cells with apical cytoplasmic protrusions (Fig. 4*B*) are common in this period. Intact SG released into the oviductal lumen can also be seen in intimate contact with the cilia (Fig. 4*C*).

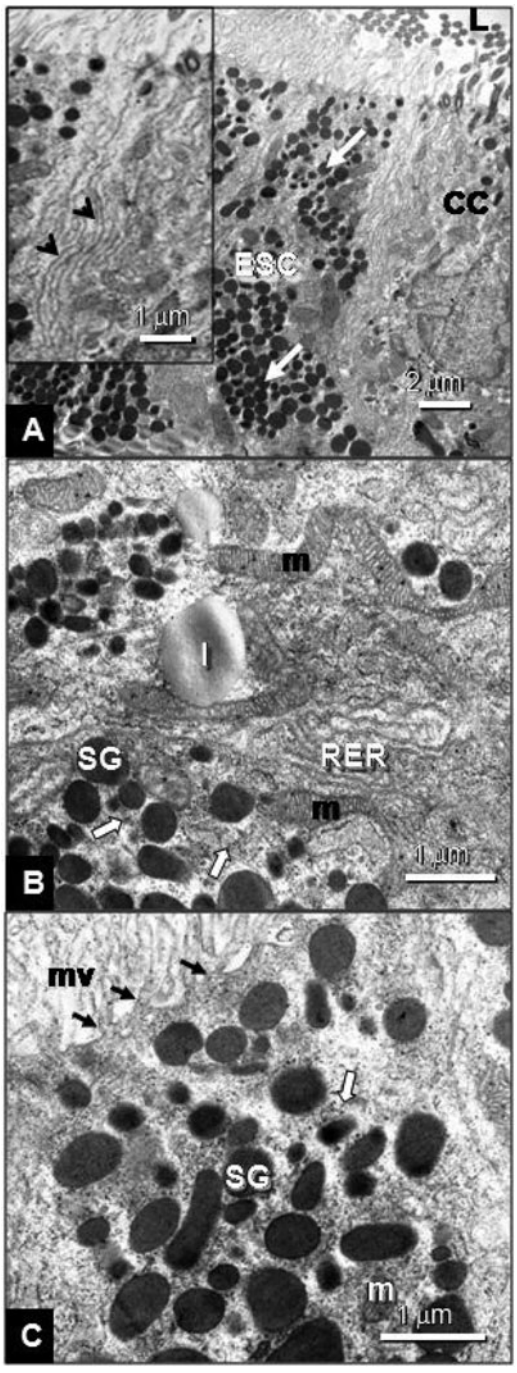

Figure 1 Oviductal mucosa of the pars recta (PR) in the preovulatory period. (*A*) Epithelial secretory cell (ESC) with secretory granules of medium to high electron density (arrows). CC, ciliated cell; L, oviductal lumen. Inset: Detail of interdigitations (arrowheads) of the ESC and CC lateral plasma membranes. (*B*) Cytoplasm of the ESC: mitochondria (m), rough endoplasmic reticulum (RER), lipid droplets (1) and glycogen granules (hollow arrows) among the secretory granules (SG). (*C*) Higher magnification of the ESC showing mitochondria (m), glycogen granules (hollow arrow) and secretory granules (SG). The surface plasma membrane shows well developed microvilli (mv) projecting into the oviductal lumen.

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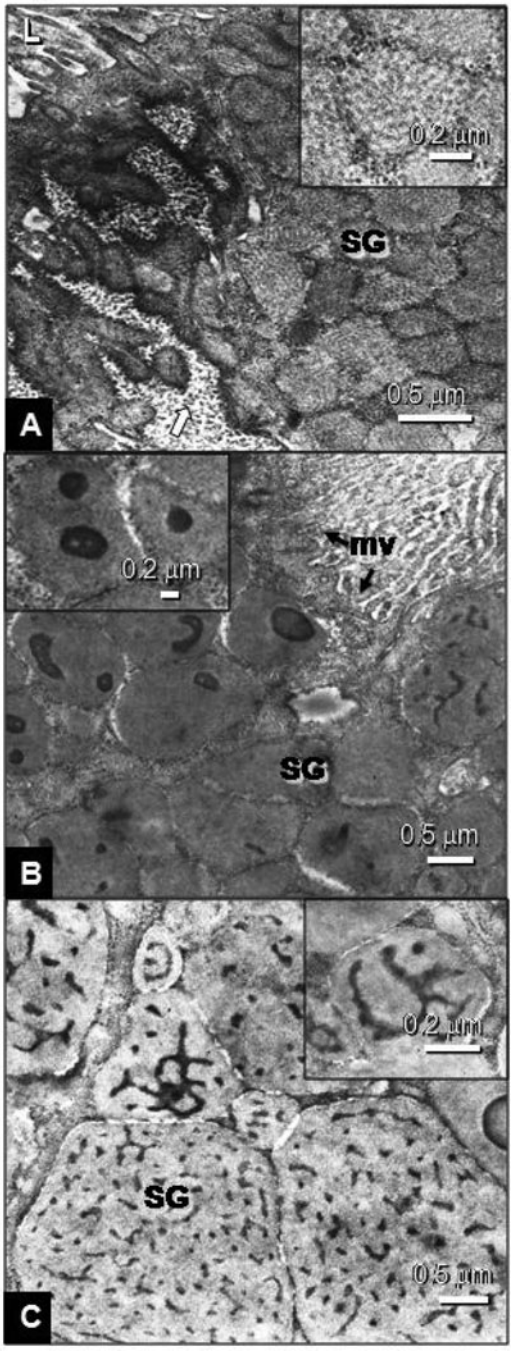

Figure 2 Epithelial secretory cell of the intermediate proximal zone (IPZ) in the preovulatory period. (*A*) Secretory granules (SG), electron lucent, with fibrillar material arranged in parallel. L, oviductal lumen; hollow arrow, glycogen granules. Inset: Details of the organization of the granules. (*B*) Secretory granules (SG) of moderate electron density with polymorphic aggregates of high electron density. mv, microvilli in the oviductal lumen. Inset: Detail of granules. (*C*) Secretory granules (SG), electron lucent, with fibrillar material irregularly arranged. Inset: Detail of the granules.

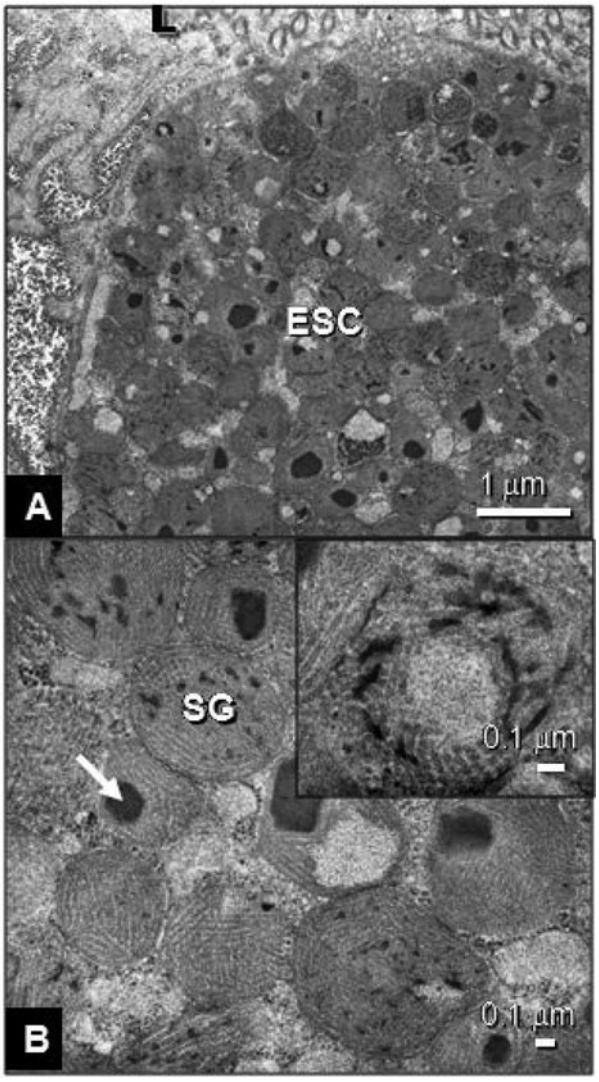

Figure 3 Epithelial secretory cell (ESC) of the pars preconvoluta (PPC) in the preovulatory period. (*A*) ESC with a mosaic of granules of different electron densities and different organization of the material. L, oviductal lumen. (*B*) Secretory granules (SG) of paracrystalline organization (arrow). Inset: Detail of their arrangement.

Ciliated cells

Ciliated cells (CC) are mostly located in the apical and lateral edges of the epithelial folds where they are alternately arranged between the ESC. Before ovulation, they show a particular morphology as they acquire the shape of a triangular wedge due to the pressure exerted on them by the SC (Fig. 1*A*). On the luminal surface they show sparse short microvilli arranged between the cilia (Fig. 5*A*). Their cytoplasm presents numerous mitochondria and abundant glycogen granules usually with an apical localization close to the ciliary basal bodies (Fig. 5*A*).

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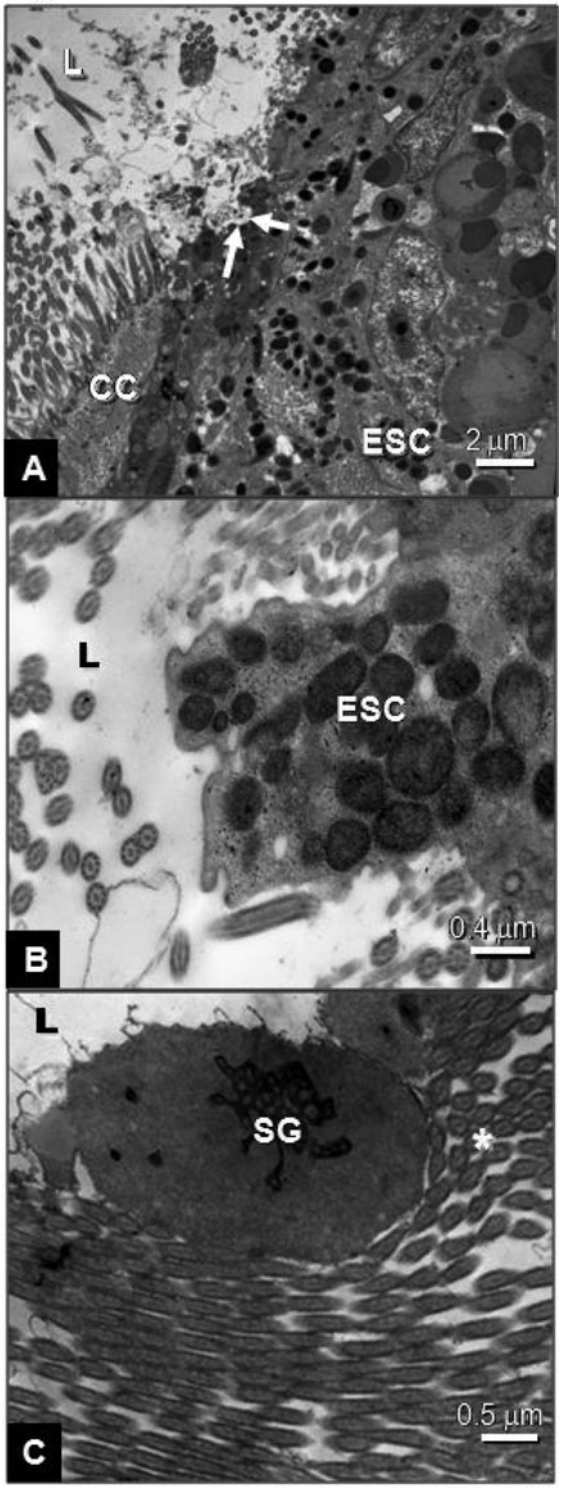

Figure 4 Oviductal mucosa of the pars convoluta (pc) in the preovulatory period. (*A*) Epithelial secretory cell (ESC) with granules of medium to high electron density. Secretion of cell products by exocytosis (arrows). CC, ciliated cell; L, oviductal lumen. (*B*) Protrusion of an ESC containing high-electron-density granules. L, oviductal lumen. (*C*) Secretory granule (SG) displaced by the cilia (asterisk) in the oviductal lumen (L).

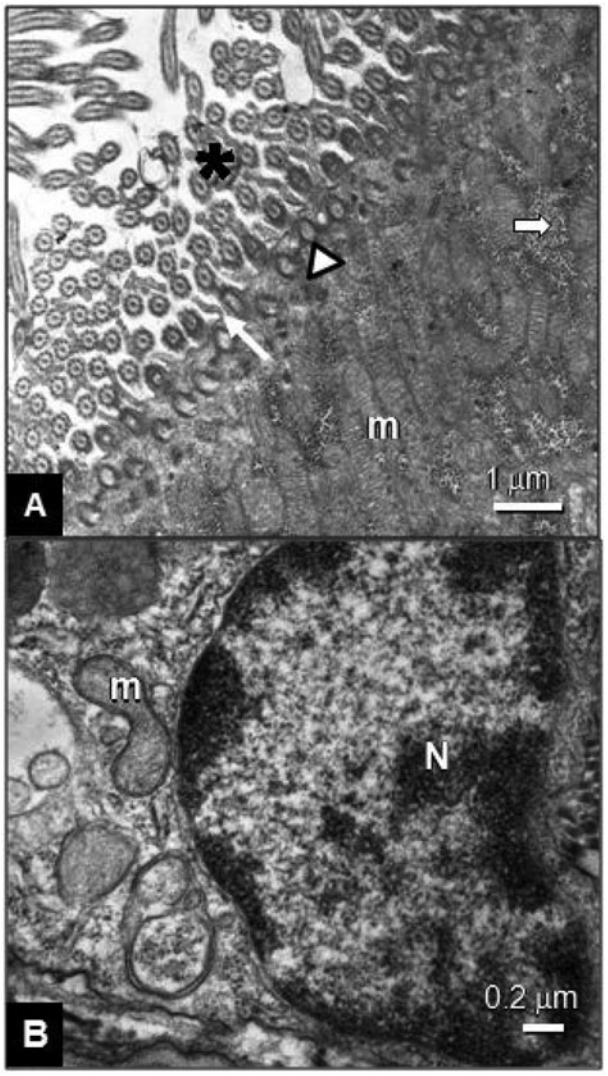

Figure 5 (*A*) Ciliated cell of the oviduct in the preovulatory period showing numerous mitochondria (m) with pronounced crests located in the proximity of the ciliary basal bodies (hollow arrowhead), glycogen granules (arrowhead), microvilli (arrow) and cilia (asterisk). (*B*) Basal cell located in the basal zone of the epithelial layer with heterochromatic nucleus (N). m, mitochondria.

Basal cells

These cells, which are located in the basal zone of the epithelium, very close to the basal lamina and sometimes in contact with it, are small and present thin cytoplasmic prolongations. Their nuclei are more heterochromatic than those of the ESC or CC and characteristically the nucleus/cytoplasm ratio is high. In the electron-lucent cytoplasm a few organelles can be seen (Fig. 5*B*).

Glandular secretory cells

The gland layer along the PC shows a progressive increase in thickness in the cephalocaudal direction

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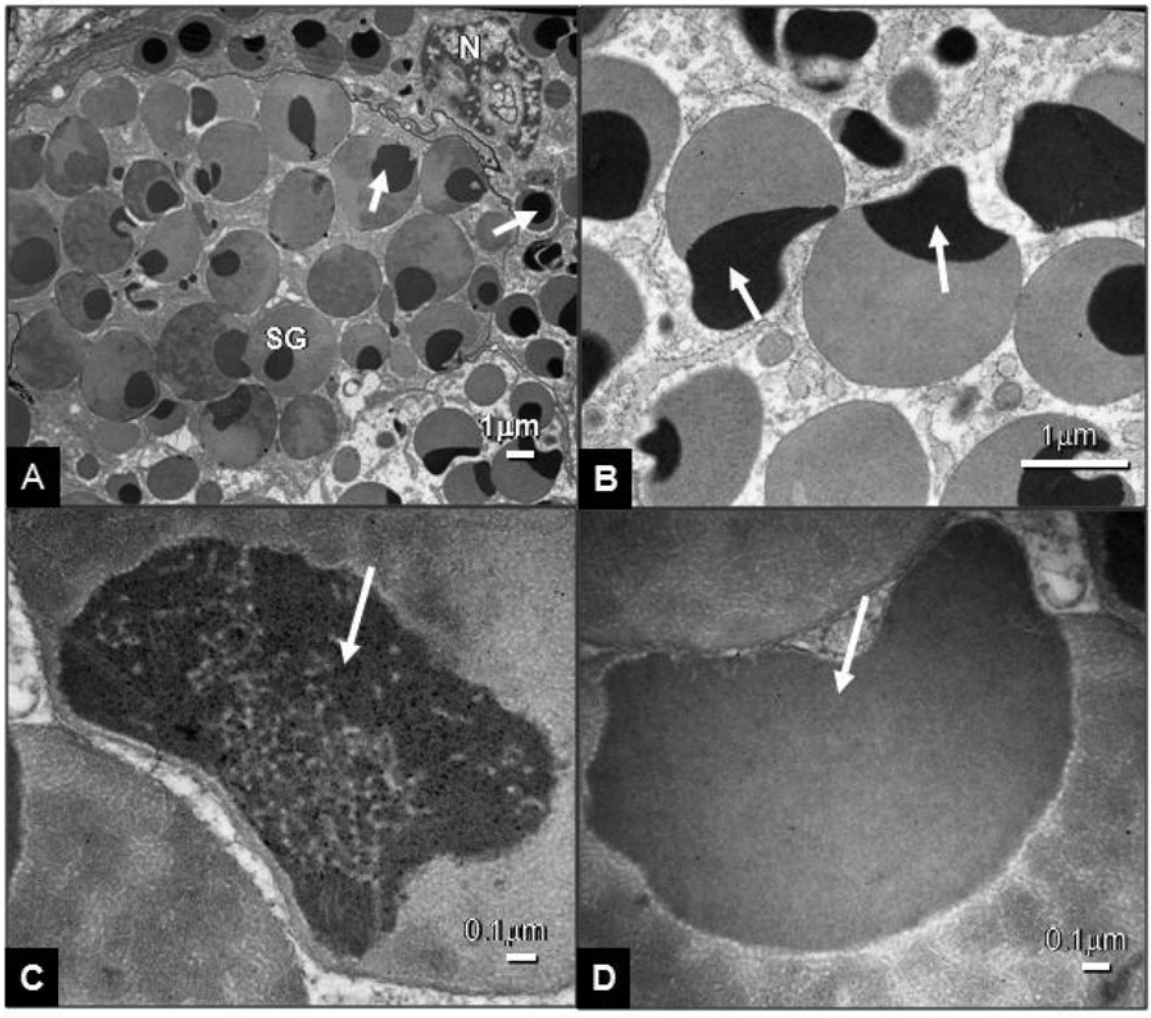

Figure 6 Oviductal glandular cells in the preovulatory period. (*A*) Large secretory granules (SG) of moderate electron density, several of them with prominent cores (arrows). N, nucleus. (*B*) Core (arrows) of SG with different sizes and shapes. (*C*) Detail of the core (arrow) with granular aspect. (*D*) Detail of the core (arrow) with homogeneous aspect.

although, in general lines, it keeps the same morphology in all the zones analysed. During this period, the thickness of the glandular layer is the highest in the reproductive cycle. The simple tubular glands are made up of voluminous glandular secretory cells (GSC) characterized by the presence of numerous SG of different shapes: round, oval or polyhedric, all of them showing moderate electron density (Fig. 6*A*). In many of them the presence of a prominent core with considerable variations in size and shape can be seen (Fig. 6*B*). Images at a higher magnification revealed that the material contained in some cores showed a granular aspect with components with different electron densities irregularly distributed (Fig. 6*C*), while in others they showed a homogeneous aspect with high or medium electron density (Fig. 6*D*). The endoplasmic reticulum, the cisternae of the Golgi complex and the mitochondria, as well as other organelles, are distributed among the SG, which during the process of secretion, release their content into the lumen by exocytosis. The nuclei of these cells are polymorphic (Fig. 6*A*).

Postovulatory period

During this period, there is in the ESC an increase in the number and size of lysosomes and lipid droplets (Fig. 7*A*) and, although no modifications in cell distribution are observed at the epithelial level, the volume of the ESC in the oviduct decreased markedly by the complete or partial release of SG (Figs. 7*A*, *B*). When the pressure on the CC decreases, they increased their volume, and their characteristics and organization can be seen with greater detail (Fig. 7*B*). The nucleus of the CC is voluminous, with an elongated shape, and it is usually located in the mid

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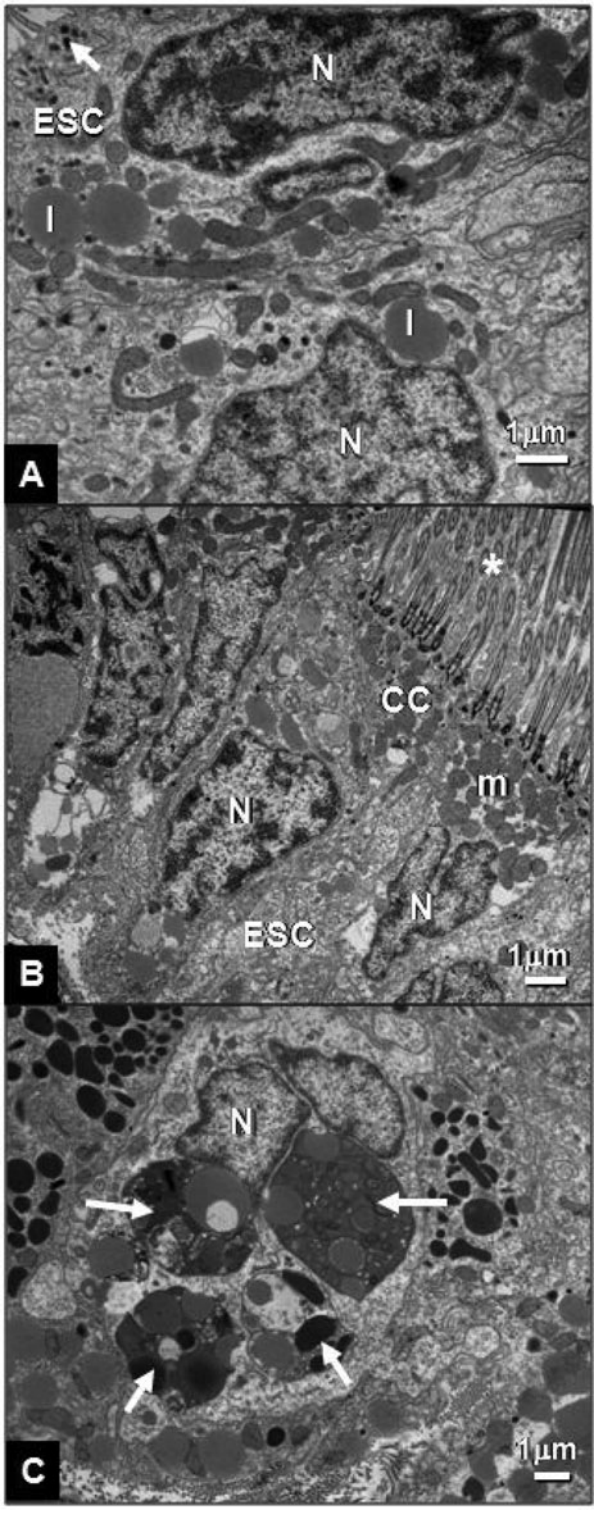

Figure 7 Oviduct in the postovulatory period. (*A*) Epithelial secretory cell (ESC) with numerous lipid droplets (1) and scarce secretory granules (arrows). N, nucleus. (*B*) Ciliated cell (CC) with numerous mitochondria (m) located in the apical zone, cilia (asterisk) and nucleus (N). ESC, epithelial secretory cell. (*C*) Prominent heterolysosomes (arrow) in cell located in the basal region of the epithelium. N, nucleus.

portion of the cell (Fig. 7*B*). During this period, in the basal region of the epithelium another type of cells can be found, characterized by prominent heterolysosomes with different sizes and electron densities (Fig. 7*C*). The GSC are characterized by a decrease in volume due to the reduction in the number of SG as a result of the process of secretion.

Discussion

The results of the present work show that, along the oviduct, the SG of the SC present different shapes, sizes, organization of the material contained and electron densities. The homogeneity of the electron density of the SG of the ESC in the PR can be attributed to a single type of contents. In agreement with this observation, histochemical light microscopic studies have shown the clear predominance of neutral glycoconjugates in the SG of these cells (Alcaide de Pucci, 1991a). In addition, in *B. japonicus*, Takamune & Katagiri (1987) isolated from the SG of the PR a single proteolytic enzyme known as oviductin (Hardy & Hedrick, 1992). The heterogeneity of the electron densities of the SG of the ESC present at both the IPZ and the PPC can be attributed to the presence of contents of diverse chemical nature, glycoproteins, glycosaminoglycans and proteoglycans mostly acidic, with phosphate, carboxyl, sulphate and sialylated radicals revealed by histochemical techniques. As an exception, the cells of the pc present SG with homogeneous acidic and predominantly sulphated contents (Alcaide de Pucci, 1991a). In *R. arenarum* a wide variety in the secretion profile of proteins, glycoconjugates and ions in each segment of the PC has been demonstrated (Medina *et al.*, 2000; Crespo *et al.*, 2009).

With respect to the ultrastructure of the epithelium of the PR of *L. chaquensis*, aspects analogous to those reported were observed for *R. arenarum* (Fernández *et al.*, 1989), *R. japonica* (Yoshizaki & Katagiri, 1981) and *B. japonicus* (Katagiri *et al.*, 1982) although it must be pointed out that in the last two species the studies were performed only at the ovulatory stage.

With respect to the PC, the particular ultrastructural complexity observed in *L. chaquensis* is comparable only with *R. arenarum* (Winik *et al.*, 1999) and shows clear differences with *X. laevis* (Yoshizaki, 1985). In this species the organization of this portion of the oviduct involves only two types of ESC, one present at the tip and the other at the bottom of the crests.

In our species the ESC alternate with the CC, which at the ultrastructural level show a large number of mitochondria in the apical area, in close proximity to numerous glycogen granules. The purpose of this

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arrangement would be the regulation of the ciliary activity, which acquires a fundamental importance during the ovulatory period. During the ovulatory period, the movement of the cilia and the peristaltic movements of the duct would be responsible for the propulsion and rotation of the oocytes, thus facilitating their contact with the PR secretion and the sequential deposition of the components of the jelly coats secreted by the PC.

As a distinctive feature, the lateral membranes of the ESC and CC show complex interdigitations, thus increasing the contact surface. This morphological characteristic would facilitate communication and exchange of fluids between adjoining cells as well as the intercellular cohesion required to allow the distension of the oviductal lumen necessary for the transit of the oocytes without cell damage.

The other type of epithelial cells, the basal cells observed in the PC, present ultrastructural characteristics similar to the ones described in *R. arenarum* (Winik *et al.*, 1999). This type of cells has also been observed in the oviduct of different mammalian species and in primates it was suggested that they may play a phagocytic role (Odor, 1982). In this species under study, as in *R. arenarum* (Winik *et al.*, 1999), the localization and the scarce differentiation of these cells allow us to suggest their participation in the renewal of the ESC during the postovulatory period.

The GSC are also involved in the secretion of the components of the jelly coats. These large cells have SG characterized by prominent cores that vary considerably in the arrangement and electron density of their material. Histochemical studies revealed differences in the content of these cells according to the zone analysed. Thus, while at the level of the IPZ cells with only acid components and others with only neutral components coexist, towards the distal zone of the PC all the GSC reveal the total prevalence of neutral components (Alcaide de Pucci, 1991a).

In the present study we also observed ESC that contained granules with a paracrystalline organization. In bacteria and eukaryote cells from animal and vegetable organisms, these structures are related to protein storage (Theil, 1987; Frazier *et al.*, 1993). In *L. chaquensis* a similar function could be attributed to them. This hypothesis would be supported by investigations carried out in our laboratory that allowed us to determine an abundant protein secretion into the oviductal lumen of *R. arenarum* during the preovulatory and postovulatory periods (Crespo *et al.*, 2009).

In the lumen, before ovulation, we can see abundant heterogeneous material made up of intact SG from the GSC and flocculent material released by exocytosis from the ESC. Besides the images of exocytosis, toward the lumen of the duct, we can often find apical cytoplasmic protrusions that release their content probably by apocrine processes. These data indicate that a wide range of mechanisms of secretion would be involved in the release of different components into the oviductal lumen.

During the postovulatory period there is a marked diminution in the thickness of the epithelial and glandular layers as a consequence of a remarkable reduction in the number of SC. Observations show that the remaining SC exhibit a scarce amount of SG and a concomitant decrease in cell volume. During this period, cells that contain heterolysosomes are localized in the basal region of the epithelium. These organelles present in neutrophilic granulocytes and macrophages (Van Manen *et al.*, 2005) as well as other phagocytic cells of the mononuclear phagocytic system (Sakai *et al.*, 2001) and Sertoli cells (Nistal *et al.*, 1991) are important for the destruction of foreign organic substances and particles internalized by phagocytosis or pinocytosis. In the oviduct the presence of these heterolysosomes suggests processes of intracellular digestion that allow the recycling of basic macromolecular components for the proliferation and recovery of the SC.

The present results show that different SC types are distributed following an ultrastructural pattern, probably related to the synthesis and differential secretion of specific products at both the intra- and interzonal level.

In our laboratory, experiments are in progress to analyse the organic and inorganic composition of oviductal secretions throughout the *L. chaquensis* reproductive cycle.

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