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Morphological and histochemical studies of Bidder's organ in *Rhinella schneideri* (Amphibia: Anura) males

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Abstract
Bufonids have an organ that produces female germ cells in both sexes, known as the Bidder's organ (BO). In males, BO is located in the anterior pole of the testis and it has been compared to a rudimentary ovary. It has been demonstrated that in some species the bidderian follicles can accumulate vitellogenin in males, while in other species, the development of follicles is inhibited by the differentiation of the corresponding gonad. This study describes the anatomical, histochemical and ultrastructural aspects of the BO in males of the neotropical frog *Rhinella schneideri* during the breeding season. A topographic model has also been built using three-dimensional (3D) reconstruction. BO has an irregular shape with lobes varying in size and number. There is no physical barrier between the BO and male gonads and, for this reason, female cells are in intimate association with seminiferous locules. Histologically, two distinct regions are observed in the BO: the cortex, and medulla. In the cortex, bidderian oocytes are surrounded by follicle cells forming bidderian follicles, which are in pre-vitellogenic stage. The ooplasm of bidderian oocytes is rich in cytoplasmic organelles. Microvilosities are formed in the oolemma, where the follicular cells are juxtaposed with oocytes, and amorphous extracellular material is deposited. Lipofuscin and myelin bodies occur in the medulla as a result of the cellular degradation. Pigmented cells were also detected in the medullar region. The oogonias observed in the BO periphery, and the significant amount of degenerating oocytes in the cortex, showed the renewal capacity of BO cells in *R. schneideri* males. The BO anatomical pattern in *R. schneideri* is similar to that observed for most species of bufonids. This work describes for the first time specific aspects related to the morphological description with emphasis on architecture, morphometry and histochemistry.

Keywords: *Anura, morphology, histochemistry, Bidder's organ,* Rhinella schneideri

Introduction

Bufonids (except some *Dendrophryniscus*) are characterized for the presence of a structure called Bidder's organ (BO), a rudimentary ovary capable of producing female germ cells (Spengel 1876; McDiarmid 1971; Petrini & Zaccanti 1998). BO develops early during the larval life in both sexes and before the differentiation of the gonads (Beccari 1925; Vitale-Calpe 1969; Petrini & Zaccanti 1998). The anterior portion of the gonadal primordium develops into the BO and the posterior portion differentiates into either ovary or testis (Petrini & Zaccanti 1998). In some species such as *Bufo bufo*, *B. ictericus* and *B. vulgaris*, BO is present in adults of both sexes (Ponse 1927; Farias et al. 2002; Falconi et al. 2007). However, this organ remains in *B. marinus* and *B. lentiginosus* adult males only (King 1908; Brown et al. 2002).

In males, BO is located at the cranial portion of the testis and has been used as a character in systematic studies of the group (Duellman & Trueb 1999). Studies have suggested that BO development is inhibited by the differentiation of the corresponding gonad (Tanimura & Iwasawa 1986; Duellman & Trueb 1999). In fact, some authors (e.g. Orr 1986; Tanimura & Iwasawa 1986) reported that the BOs develop into a functional ovary only after removing testes or blocking gonads experimentally (Brown et al. 2002).

Histological analyses have demonstrated that the BO is composed of bidderian follicles at several stages of development in male toads (Farias et al.

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2002). However, the degree of development of the bidderian follicles can vary among the different species. In some species such as *B. ictericus*, *B. marinus* and *B. japonicus formosus*, the males have only previtellogenic follicles in their BO (Tanimura & Iwasawa 1986; Brown et al. 2002; Farias et al. 2002). However, vitellogenic follicles were detected in males of *Rhinella arenarum* under normal conditions (Scaia et al. 2011). The production of female germ cells by the BO may represent a morphological reproduction strategy in bufonids. For many authors, BO is a vestigial structure that may become functional under certain circumstances (Vitale-Calpe 1969; Pancak-Roessler & Norris 1991; Duellman & Trueb 1999). The ability to produce the succeeding generation is an essential feature to maintain the species in the environment (Tanimura & Iwasawa 1986; Duellman & Trueb 1999). Bufonids are particularly interesting and important to the study of sex differentiation.

BO differentiation has been previously described by several authors (Beccari 1925; Vitale-Calpe 1969; Petrini & Zaccanti 1998; Falconi et al. 2007), but the morphological aspects of the BO in adults remain scarce for many bufonids. In adulthood, the reproductive organs are at their developmental peak, which facilitates the investigation of the reproductive traits of different species. For example, the BO morphology of *Rhinella schneideri*, a member of a clade restricted to South America, is still unknown. This species is widely distributed in South America, occupying even disturbed areas (Cochran 1955), which are often affected by a variety of compounds that interfere with the growth of germinal tissue. Information about the morphology of reproductive structures is relevant in designing efficient strategies for conservation of anuran species, understanding how the biotic and abiotic factors can interfere with their normal reproduction. For bufonids, this is especially true due to the presence of the BO producing female cells in males. Thus, this study aimed at evaluating BO morphological, histochemical and ultrastructural aspects in *R. schneideri* males during the breeding season, using routine histochemical techniques for light and electron microscopy. In addition, a topographic model of the BO was built using three-dimensional (3D) reconstruction methods.

Materials and methods

Animals collected

Fifteen *Rhinella schneideri* adult males were collected from permanent ponds and wetlands in a non-agricultural area, in southeastern Brazil (20° 47′07.05″; 49°02′42.09″W) during the reproductive season, at night. The animals were transported to the laboratory, immediately anaesthetized, and euthanized with benzocaine (5 g/l water). Male gonads were weighed, measured, and analysed to confirm that toads were at the same stage of sexual development. All experimental procedures were authorized by the Ethics Committee of the São Paulo State University (Protocol #040/2011 CEUA) and followed the NIH Guide for the Care and Use of Laboratory Animals.

Histological and histochemical analyses

The toads were dissected exposing the BO for macroscopic analysis and photo documentation under a dissecting microscope (Leica MZ16), coupled to an image capture system (Image Manager – IM50).

For histological analysis, the BO and testes were removed and fixed for 24 h in a Karnovsky fixative solution (0.1M Sörensen buffer phosphate, phosphate buffer pH 7.2, containing 5% paraformaldehyde and 2.5% glutaraldehyde), at 4°C. Subsequently, the material was dehydrated in increasing ethanol series and embedded in glycol methacrylate resin (Historesin Leica®). Sections of 2 μm were obtained with a microtome (RM 2265, Leica, Switzerland) and stained with haematoxylin–eosin, Gömori trichrome, and silver ion impregnation. BO general morphology was observed and described under a microscope (Leica DM4000 B) coupled with an image capture system (Leica DFC 285). The distinct bidderian follicles were quantified to assess the occurrence of the oocytes (young follicles, late follicles and follicles in degeneration). A total of 250 histological sections of the germinal epithelium were analysed using the Image Pro-plus software v. 6.0, through manual counting. The G-test of goodness-of-fit and the Yates correction (Sokal & Rohlf 1995) were used to test the difference in the amount of cells. This test was implemented with the code provided by Prof. Peter Hurd, available at http://www.psych.ualberta.ca/~phurd/cruft/g.test.r. All analyses used the R v. 2.11.1 software (R Development Core Team 2010).

For histochemical analysis of lipofuscin detection, sections were incubated in Schmorl's solution, composed of 75 ml of 1% ferric chloride, 10 ml of potassium ferricyanide, and 15 ml of distilled water, for 15 min. Then, sections were immersed in 1% neutral red, followed by 1% eosin.

Three-dimensional reconstruction

The tissue mass containing the BO, testes, and fat bodies was embedded in historesin. The 3-µm thick serial sections were stained with haematoxylin–eosin. Images were captured and processed using the Reconstruct 3.2 software (Build 743; Digivision-SIS, San Diego, CA). After image alignment, regions containing BO, testes and fat body were isolated in each image and later processed to obtain the interface limit of each section, generating a 3D model. The model allows an improved visualization of the topographical anatomy of the organ, as well as its limits and connections with male gonad.

Ultrastructural analysis

Tissue samples were fixed in 3% glutaraldehyde and 0.25% tannic acid in Millonig buffer pH 7.3, for 2 h at 25°C. Posteriorly, samples were post-fixed in 1% osmium tetroxide diluted in the same buffer for 1 h, dehydrated in acetone, and embedded in Araldite (Cotta-Pereira et al. 1976). Ultrathin sections (50–75 nm) were contrasted with 2% uranyl acetate for 20 min (Watson 1958), and lead citrate in 1N sodium hydroxide solution (Venable & Coggeshall 1965) for 8 min, and examined under a Leo-Zeiss EM – 906 electron microscope operating at 80 kV.

Results

BOs are paired structures, located in the cranial portion of *Rhinella schneideri* testes (Figure 1A). Male toads have well-developed testes with smaller yellow–brown BO weighing on average 0.05 g (± 0.002 g). The 3D model showed that the BO shape is irregular, with projections of different sizes (Figure 2). Some projections are adhered to the testes, so that the bidderian tissue is associated with testicular tissue (Figure 2D). There is no physical barrier between the BO and male gonads. Thus, the bidderian oocytes are close to seminiferous locules with spermatocysts at different stages (Figure 1B). Externally, the organ is covered by a thin capsule of connective tissue (Figure 3B).

Histological arrangement showed two distinct regions of the BO: the cortex and medulla (Figure 3A–D). The centrally located medulla is smaller than the cortex (Figure 3C). It receives the blood vessels and has abundant collagen fibers synthesized by fibroblast (Figure 3C–E). Lipofuscin granules are detected in the same region, and pigment cells occur dispersed in the tissue (Figure 3E,F).

Most of the cell population in the medullar region is represented by somatic cells, which have highly basophilic nuclei, due to chromatin compaction, well-defined nucleoli, and are relatively small and highly electron-dense when compared to germ cells. Somatic cells surrounding oocytes are called follicular cells because they constitute the follicular layer of the bidderian oocytes (Figure 4C). Follicular cells are mostly flat, but may be round when attached to oocytes in advanced developmental stages. These cells differ not only in shape, but also in the degree of electron density, both in the nucleus and cytoplasm (Figure 4G). Dark and clear follicular cells are associated with the oocytes by cytoplasmic communications while their boundary is easily visualized due to differences in colour (Figure 4G). Nonetheless, all other features and structures are shared. They have many organelles, such as

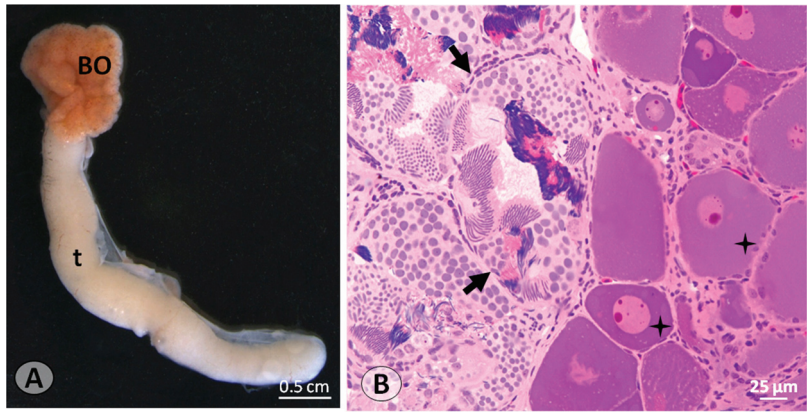

Figure 1. BO of *Rhinella schneideri* male. (A) BO macroscopic aspect showing its localization in the cranial portion of the testis (t). (B) Histological section of the region between BO and testis. Seminiferous locules (arrow) are in direct contact with bidderian oocytes (✦). Staining: haematoxylin–eosin.

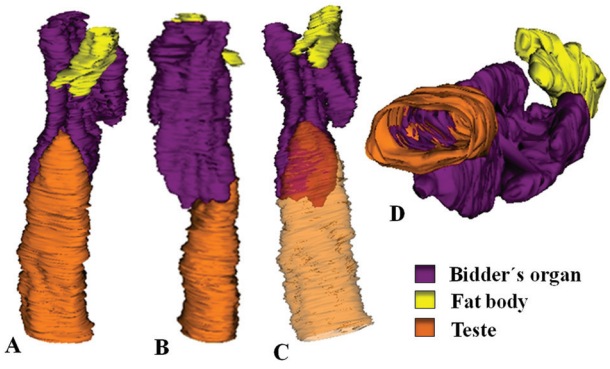

Figure 2. Three-dimensional reconstruction (3D) of the complex formed by BO, testes, and fat body. (A, B) Ventral and dorsal view of the BO and testes, respectively. (C) BO overview through the transparency of the testes. Irregular lobes with different sizes can be seen in the 3D model. (D) Internal view of the complex, showing the arrangement of testicular and bidderian tissue.

mitochondria, well-developed endoplasmic reticulum, and some vesicles (Figure 4D,E). Follicular cells are attached to the follicular layer by focal adhesions, connecting the cytoplasm of the two cells (Figure 4D,E).

Follicles of different stages are found in the BO cortex. In general, bidderian follicles are large structures with a diameter ranging from 68.763 to 172.394 μm and mean area varying from 3108.2 to 16,887.354 μm^2. Bidderian follicles have an either oval or rounded acidophilus nucleus with well-delimited nuclear membrane, which consists of many nuclear pore complexes (Figure 4B). Furthermore, the nucleus has one or more well-defined nucleoli with intense basophilia and uniform electron density (Figures 4B and 5E,F).

The ooplasm of bidderian oocytes is rich in mitochondria and other well-developed organelles, such as endoplasmic reticulum and Golgi complex. There are also many lipid droplets, which should participate in yolk metabolism (Figure 4B). Microvilosities are formed in the oolema as a result of oocyte development, where follicular cells are juxtaposed with oocytes (Figure 4D,E). Amorphous extracellular material is deposited along the microvilosities to the subsequent formation of the zona pellucida (Figure 4E). This material is moderately electron-dense and mainly composed of proteins and proteoglycans. Electron microscopy also revealed myelinic bodies that originated from the degradation of intracellular material (Figure 4F).

Oogonias were observed near the germinal layer at the periphery of the organ (Figures 4A and 5A,B). They have irregular and elliptical shape, very lobed and slightly acidophilic nucleus and abundant nuage material. The nucleus has one or more small, highly basophilic and uniformly electron-dense nucleoli (Figure 4A). Oogonias are separated from the cortex by the connective tissue and can be arranged into nests of multiple cells (Figures 5A,B). Prophase oocytes (POs) originate from the oogonias mitoses and are compartmentalized into asynchronous nests in the BO periphery (Figure 5C). Unlike oogonia, these round cells have spherical nucleus, with moderate chromatin compaction and a slight increase in cytoplasmic basophilia, but the nucleus remains quite basophilic. After that, POs are individualized and originate young follicles (Figure 5D).

The atretic and degenerating oocytes, also found in the BO, were the most common cell type in the organ (68.9% of the bidderian oocytes; Figure 6). Degenerating oocytes are larger cells (± 172.394 μm average diameter) and have nuclear disintegration with acidophilic and impregnated areas in the cytoplasm (Figure 5F). At this stage, the follicle cells surrounding oocytes are less flattened and partially invade the oocyte membrane. Subsequently, somatic cells invaginate towards the interior of the oocyte and promote follicular atresia in advanced stages of degeneration (Figure 5G).

Discussion

The general morphology and anatomy of the BO in *Rhinella schneideri* males are similar to that found in other species of bufonids, such as *Bufo ictericus*, *B. marinus*, *Bufo japonicus* (Moriguchi et al. 1991; Tanimura & Iwasawa 1992; Farias et al. 2002) and *Bufo woodhousii* (Pancak-Roessler & Norris 1991). In these species, the BO is a small organ located in the cranial portion of the testes and has the typical morphology of an undeveloped ovary with pre-vitellogenic oocytes in successive developmental stages. However, distinctive features, which have not been reported for other species, such as pigment cells, lipofuscin granules and the 3D arrangement of the BO were observed.

In most male bufonids, the presence of vitellogenic oocytes in the BO is known to occur only after castration, which suggests that testes are necessary to suppress vitellogenin accumulation (Pancak-Roessler & Norris 1991; Zaccanti 1994; Brown et al. 2002). *B. marinus* male frogs exposed to agricultural sites also exhibited some bidderian follicles in early or late vitellogenesis because agricultural compounds act as endocrine disruptors, interfering with the normal development of the reproductive organs in males (McCoy et al. 2008). However, there is little information regarding the morphological aspects of BO in bufonids under natural conditions. In *R. arenarum*, for example,

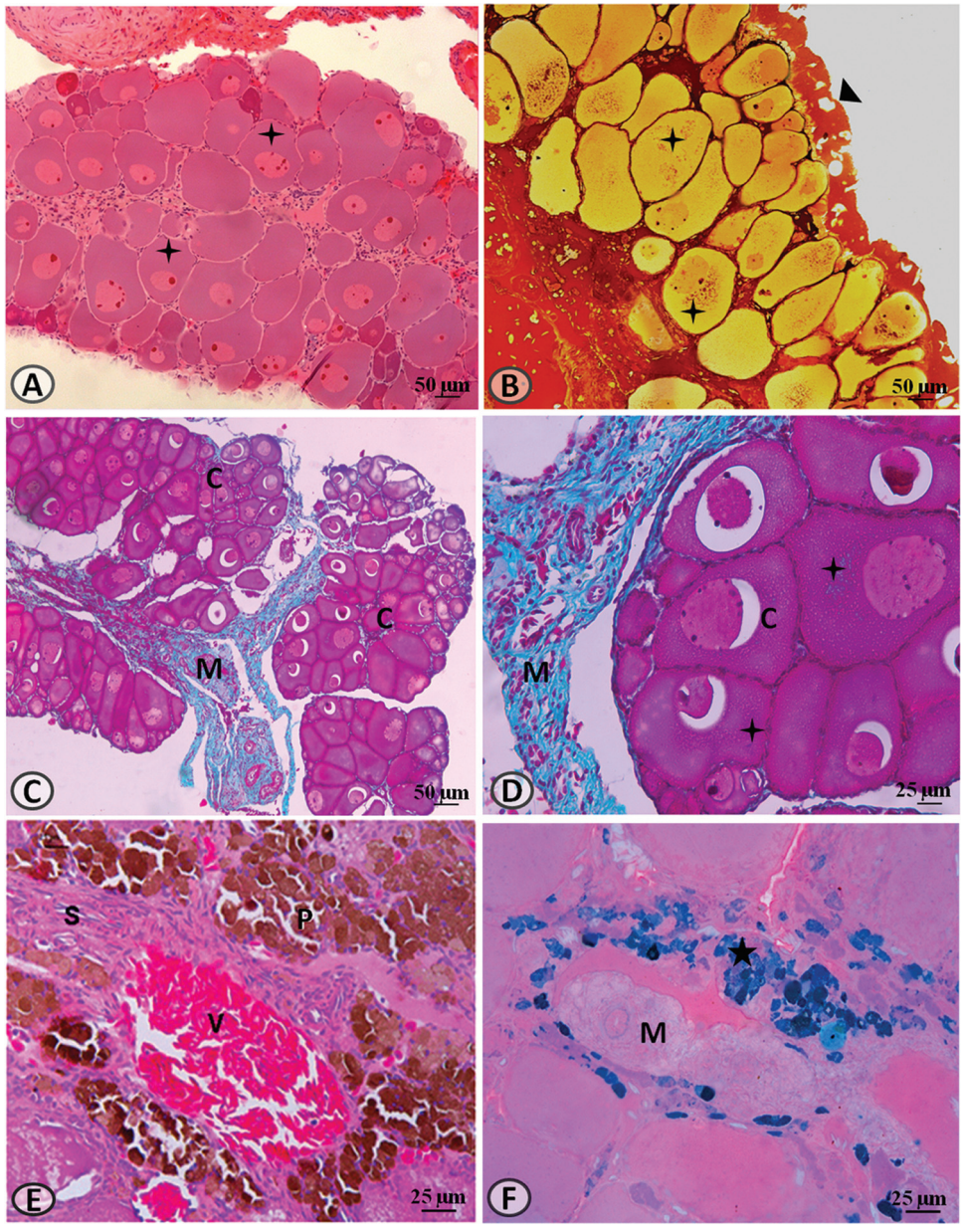

Figure 3. Histological sections of the BO of *Rhinella schneideri*. (A) Bidderian follicles (+) disposed in the cortical region in several development stages. (B) A thin capsule of connective tissue covers the BO externally (arrowhead); (+) bidderian follicles. (C, D) Cortical (C) and medullar (M) regions in the BO. Bidderian follicles (+) in several developmental stages are distributed in the cortex. Medulla (M), rich in collagen fibres (in blue), is observed using the Gömori trichrome technique. (E) Blood vessel (V), pigment cells (P) and somatic cells (S) found in the BO medulla. (F) Lipofuscin granules (star) are also detected in the medullar region (M). Staining: haematoxylin–eosin (A, F), Gömori trichrome (C, D), silver ion impregnation (B) and acidic ferrocyanide (F).

vitellogenic oocytes were reported in the BO of males (Scaia et al. 2011). We believe that the development of oocytes must be linked to the physiological status of the animals and, thus, the development stages of bidderian oocytes could vary naturally among bufonids species. Nevertheless, it is a consensus that oogenesis is not completed in the BO of males.

Although the bidderian oogenesis is interrupted during the pre-vitellogenic stage in *R. schneideri* males, it was noticed that bidderian oocytes have high cell activity. This can be inferred based on the large amount of organelles, such as mitochondria, Golgi complexes, and well-developed endoplasmic reticulum in the ooplasm. Moreover, large, prominent nucleoli and many micronucleoli occur in the

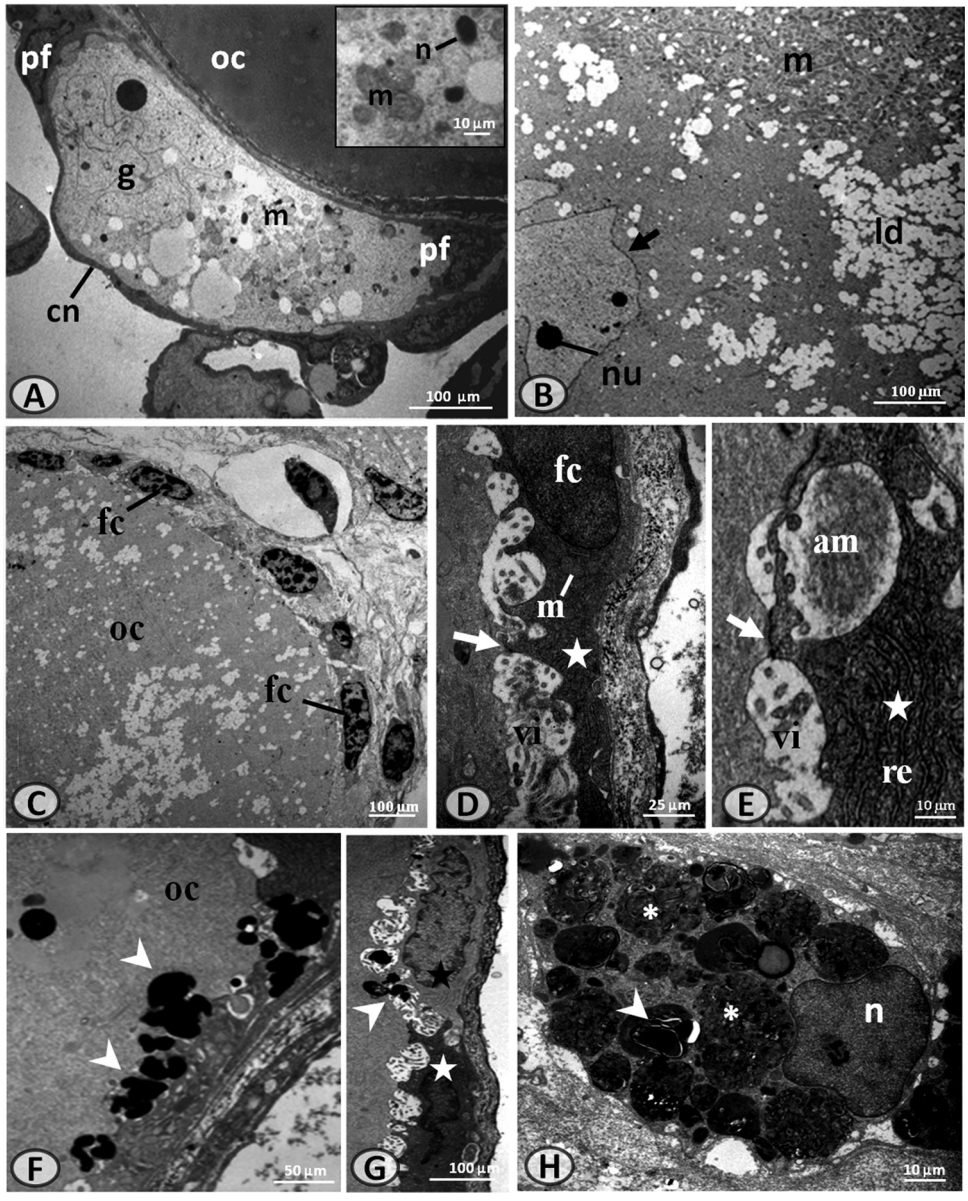

Figure 4. Electron micrographs of the BO. (A) Single oogonium involved by connective tissue (cn) in the peripheral region of the organ. The nucleus is very lobed and the nucleolus is uniformly electron-dense (nu). Nuage material (n) within the oogonia is mainly associated with mitochondria (m). pf, prefollicle cell. (B) Bidderian oocytes showing the nuclear pore complexes delimiting the nuclear membrane (arrow). Cytoplasm contains many mitochondria (m) and lipid droplets (ld). (C) Bidderian follicle, formed by oocytes (oc) surrounded by many follicular cells (fc). (D, E) Region of adhesion between the oocyte and follicular cells (fc). Focal adhesion (white arrow) can be seen in this region, along the microvilosities (vi) in the periphery of oocytes. Amorphous material (am) is accumulated along the villi. Endoplasmic reticulum (re) is observed in cytoplasm of follicle cell. (F) Myeloid bodies (arrowhead) in the periphery of oocyte. (G) Follicular cells with distinct electron density in the nucleus and cytoplasm. Dark (white star) and clear follicular cell (dark star) communicate through cytoplasmic connections (white arrow). (H) Pigmented cells, showing the large nucleus (n) and heterogeneous granules in the cytoplasm (*).

nucleus, indicating intense metabolic activity. The presence of many nucleoli formed by amplified ribosomal DNA genes is common to amphibians (Brown & Dawid 1968; Amaldi et al. 1973; Scheer & Dabawall 1985). The deposition of amorphous extracellular material was observed in the surroundings of oocytes, within the microvilosities. This material is rich in glycoproteins and essential for the formation of the zona pellucida. Amorphous material has also been reported in *R. icterica* BO, but covering the entire oocyte membrane (Farias et al. 2002) and not deposited inside of the microvilosities as in *R. schneideri*. Degenerated and atretic oocytes abound in the BO, probably due to the lack

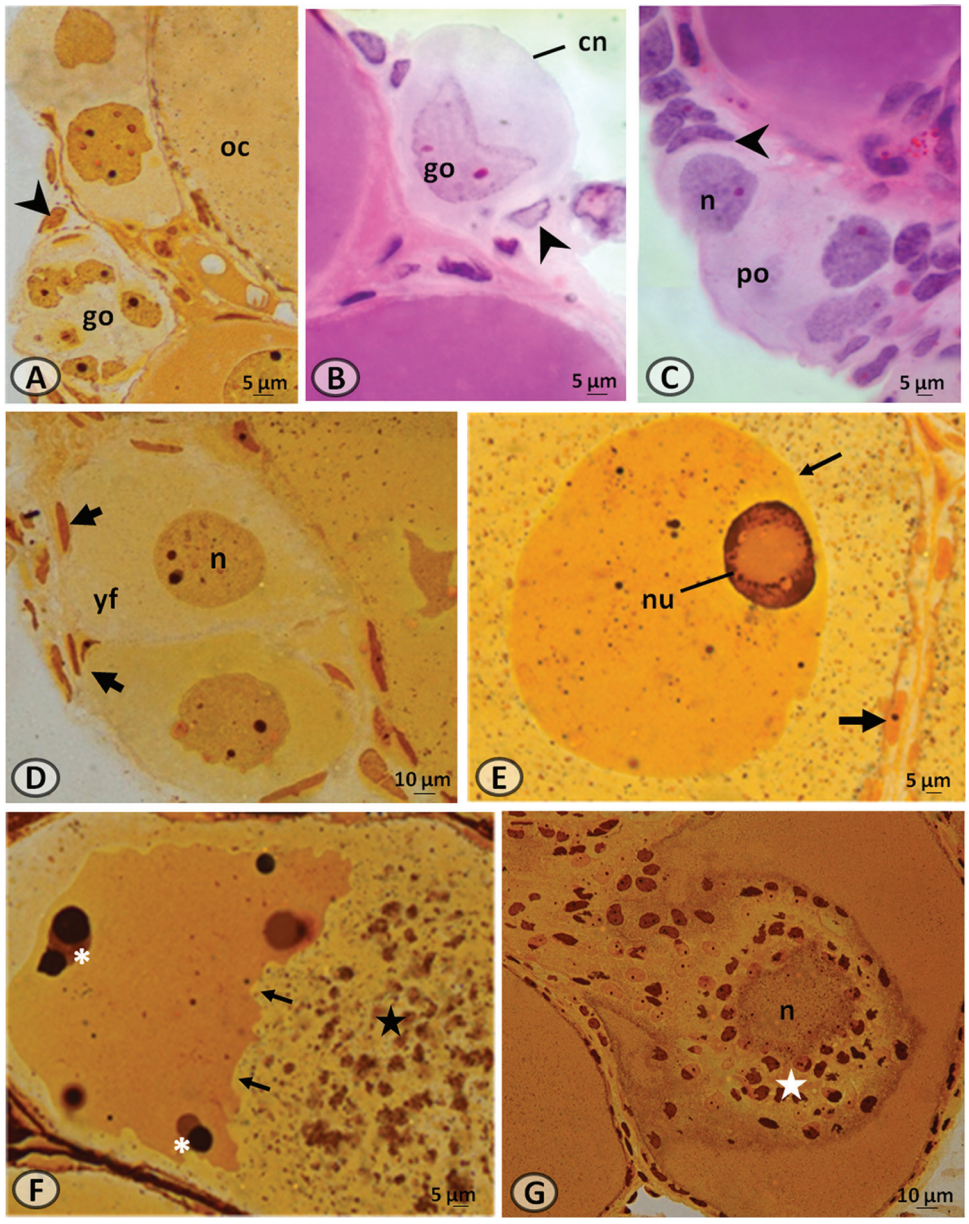

Figure 5. Different developmental stages of germ cells in the BO. (A) Nest of oogonia (go) accompanied by prefollicle cell (arrowhead); oc, oocyte. (B) Simple oogonia (go), separated from the bidderian epithelium by connective tissue (cn). (C) Prophase oocytes (po), organized into nest; n, nucleus. (D) Young follicles (yf) surrounded by a few follicular cells (arrow), constituting the new follicular structures. Cytoplasm and nucleus are distinctly delimited now. (E) Follicles with increased volume of the nucleolus (nu) and nucleus (thin arrow), which have well-delimited aspect. Flattened follicular cells (large arrow) surrounding these oocytes. (F, G) Oocytes in degeneration. (F) The nucleus loses its spherical form (thin arrow), and nuclear material is accumulated in the ooplasm (star). Nucleoli are more fragmented (*). (G) Oocyte in advanced degeneration, showing the invagination of somatic cells (white star) into the oocyte. The nucleus (n) is almost disintegrated. Staining: silver ion impregnation (A, D–G) and heamatoxylin–eosin (B, C).

of stimuli to develop the oocytes. Some authors show that the end of development of bidderian oocytes in males is marked by the degeneration of large previtellogenic oocytes (Zaccanti & Gardenghi 1968; Zaccanti et al. 1971; Brown et al. 2002; Farias et al. 2002). Atretic oocytes are also identified in the BO of other bufonids, such as *B. marinus* (Brown et al. 2002) and *R. arenarum* (Scaia et al. 2011). The absence of a barrier between the BO and the testes can cause the interruption of oocyte development because hormonal regulation in male testes may influence the BOs, contributing to the degeneration of feminine cells. Plasma androgens, such as testosterone, may block ovarian or Bidder estradiol

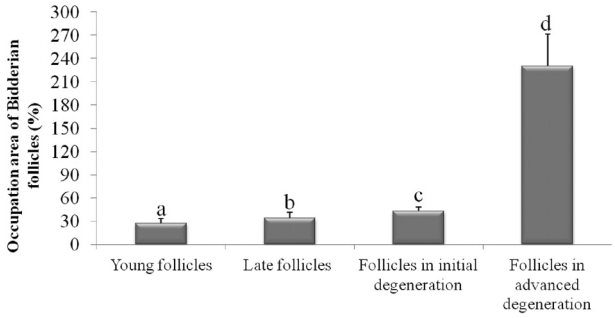

Figure 6. Area occupied by bidderian follicles in *Rhinella schneideri* males. The figure shows the area occupied by different oocytes in the BO. Oocytes in late degeneration are the most common cells in the BO. Different letters indicate statistical significance ($P < 0.05$). Statistical values: $P < 0.0001$ (a–d).

production and/or denying estradiol to the BOs by certain processes, as implied by BO atrophy after administration of testosterone (Deb & Chatterjee 1963). Thus, the presence of testes may in some way inhibit the BO from collecting enough estradiol to take part in vitellogenesis (Calisi 2005). Echeverria (1990) also reports that the degradation of old oocytes in the BO permits a qualitative levelling with the production of young oocytes. In *R. schneideri*, even in the presence of intense oocyte degeneration, oogonia were constant in the BO, showing cell renewal capacity.

A great amount of blood vessels, collagen fibres and reticular fibres is observed in the medullar region. In addition, pigmented cells are observed in the same BO region of *R. schneideri.* These structural components and pigmented cells are also reported in the BO of other species, such as *B. ictericus* (Farias et al. 2002). Generally, pigmented cells in amphibians are associated to an extracutaneous pigmentary system composed of melanin-containing cells in various tissues and organs (Agius & Agbede 1984; Moresco & Oliveira 2009; Franco-Belussi et al. 2011; Oliveira & Franco-Belussi 2012). Some species of frogs have intense pigmentation in male gonads while others have no pigment cells (Zieri et al. 2007; Franco-Belussi et al. 2009, 2011; Moresco & Oliveira 2009). The functional role of the pigment cells in these organs has not been defined yet, although several hypotheses have been proposed (Gallone et al. 2002), including cytoprotective functions (Fenoglio et al. 2005; McGraw 2005). In general, the presence of pigment cells in the BO of bufonids is not well reported; however, it may be considered an adaptive characteristic of the different species to the environment.

Many lipofuscin granules were also found in the medulla and around the bidderian oocytes. Histochemical analysis to detect lipofuscin has not yet been performed on this organ. The presence of these granules indicates the occurrence of autophagocytosis and renovation of intracellular components. Lipofuscin is an intralysosomal, polymeric substance, primarily composed of cross-linked protein residues, resulting from iron-catalysed oxidative processes. Lipofuscin accumulation in postmitotic cells is inevitable because it is not degradable and cannot be removed via exocytosis (Pickford 1953). Although lysosomal degradation is imperfect in all cells, only postmitotic and slowly dividing cells accumulate lipofuscin. Actively proliferating cells, both *in vivo* and *in vitro*, efficiently dilute lipofuscin during successive divisions (Terman 2001). However, lipofuscin accumulates when the proliferation of normal mitotic active cells is inhibited (Jahani et al. 1985; Terman & Brunk 1998). Bidderian oocytes frequently divide in the BO, but oogenesis inhibition causes follicle degeneration in males. The interruption of oocyte development and the high number of atretic oocytes may have been the contributing factor for lipofuscin accumulation around the cell and in the medulla.

The results show that the BO of *R. schneideri* males is similar to a young ovary and has successive germ cells, including pre-vitellogenic oocytes at different developmental stages. Although the BO is known as a vestigial structure in males, we demonstrated that its cells have intense activity and that it is not appropriate to designate the BO as a rudimentary ovary. Furthermore, we believe that both the morphology and developmental stage of bidderian oocytes may vary among bufonids according to the physiological and adaptive characteristics of each species. The reproductive biology of amphibians brings valuable information that could be used in conservation plans, mainly for neotropical species, which have been widely threatened in recent years.

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