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CELLULAR AND MOLECULAR BIOLOGY

Gametogenesis and reproductive dynamics of *Scinax acuminatus* (Anura: Hylidae): morphological, histological and immunohistochemical analysis

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Abstract: To characterize the reproductive biology of *Scinax acuminatus* and contribute to the natural history of this species, the morphology of the reproductive system of males and females was analyzed at anatomical, histological and immunohistochemical levels. The individuals were collected fortnightly between August and December (2016) and January to December (2018). The anatomy of the reproductive system was analyzed in a stereoscopic microscope, and histological preparations staining with hematoxylin-eosin and Masson's trichromic, PAS and Coomassie Blue was performed as well. To characterize the meiotic-active cells in the testes, immunostaining with the PCNA proliferation protein was performed. There were found females with ovaries with oocytes in different stages of maturity and post-ovulatory females. Males presented continuous spermatogenesis, which could be confirmed by the immunostaining of PCNA in spermatogonia during the cycle. The results of this work serve as a basis for the characterization of the reproductive cycle in *S. acuminatus* and provide background information on the analysis of spermatogenic activity by IHQ from the study of the immunodetection of the PCNA cell proliferation protein. Future studies will focus on the evaluation of cell death processes during the reproductive cycle in the studied species to compare with those obtained in terms of cell proliferation.

Key words: amphibians, ovaries, reproduction, testicles.

INTRODUCTION

Anuran amphibians have a great diversity of reproductive modes among vertebrates (Haddad & Prado 2005, Wells 2007, Crump 2015); this complexity is due to the fact that the reproduction strategies of the species are determined by the combination of morphological, physiological, behavioral and environmental factors (Toledo & Haddad 2005, Wells 2007, Curi et al. 2014). Generally, tropical and subtropical species have continuous cycles, while temperate species have discontinuous or potentially continuous cycles (Haddad & Prado 2005).

In recent years, the reproductive biology of Neotropical anurans has been studied in Argentine in some species such as: *Rhinella bergi* (Schaefer & Kehr 2006), *Leptodactylus chaquensis* (Schaefer et al. 2006) *Scinax nasicus* (Hamman et al. 2009), *Argenteohyla siemersi pederseni* (Cajade et al. 2010), *Boana pulchella* and *Boana punctata* (Antoniazzi et al. 2019); and with emphasis on folliculogenesis and spermatogenesis in *Ceratophrys ornata* (Carezzano et al. 2013), *Dendropsophus sanborni* (Curi et al. 2014), *Hypsiboas punctatus* (Brunetti

et al. 2014), *Rhinella arenarum* (Scaia 2015, Scaia et al. 2019).

It has been recently proposed that in the toad cell proliferation in germline is higher during the reproductive season and that cysts in late stages of spermatogenesis (spermatocytes and spermatids) are removed from the testes by apoptosis immediately after the breeding, suggesting that according to different environmental or physiological cues (Scaia 2015).

The proliferation of germline has been studied in *R. arenarum* (Scaia 2015, Scaia et al. 2019) and, in more detail, in *Pelophylax esculentus* (Ferrara et al. 2010). In *P. esculentus* seasonal variation of proliferations seems to be associated with changes in the proliferating cell nuclear antigen PCNA (Chianese et al. 2015). As a consequence, maximum cell proliferation during the reproductive season is associated with high levels of PCNA.

Although there are studies on reproductive biology in the Hylidae family, there are few who analyze cell proliferation during the reproductive cycle with emphasis on gametogenesis. For these reasons, the present analysis of the reproductive biology of *S. acuminatus* will contribute data on gametogenesis during the reproductive cycle in female and male; and the spermatogenic activity of the testicles from the detection of proliferating cells from immunostaining with PCNA.

S. acuminatus (Cope 1862), commonly known as chaqueña snout frog, is a more jumping than walker frog related to natural and anthropic environments, and inhabits humid areas with low vegetation, near estuaries and other bodies of water. It is distributed in 7 provinces of Argentina: Chaco, Corrientes, Entre Ríos, Formosa, Salta, Santa Fé, and Misiones (Zaracho et al. 2012). It is also present in Bolivia, Brazil, and Paraguay.

This work aims to provide data on the reproductive biology of *S. acuminatus*, from the morphological, histological, and immunohistochemical analysis of the reproductive system in males and females. This would provide more information on gonadal structures for the later comparison of the reproductive system of other anuran species of the same family.

MATERIALS AND METHODS

Study area

Samplings were carried out every two weeks between August and December 2016 and from January to December of 2018, 10 km NE near the city of Corrientes, (Paraje Perichón: 27 ° 26'36.6 "S 58 ° 45'14.0" W). Phytogeographically, the sampling site is located in the Humid Chaco (Cabrera 1976). The climate is subtropical or mesothermal (Carnevali 1994), with an average annual temperature gradient that oscillates from north to south between 21 ºC and 19, 5 ºC, with January being the warmest month (annual average, 27 ºC) and July the coldest one (annual average, 14 ºC). In addition, rains are abundant, frequent and irregular throughout the year, presenting their minimum level in winter (Bruniard 1999, Carnevali 1994), with an average annual rainfall of more than 1,500 mm in the northeast and 1,000 mm in the southeast of the province.

Methodology for capture and processing material

The manual capture of a total of 22 individuals of *S. acuminatus* was carried out. The animals were taken out to the laboratory in plastic bags. Then, the euthanasia of the specimens was carried out with a 2% lidocaine overdose, following the protocol established in the Guide for Animal Euthanasia proposed by the IACUC (The

Institutional Animal Care and Use Committee) under the rules of the ethics committee in force in resolution CIC400-MED-2017. The specimens collected are in the didactic collection of the Laboratory of Herpetology - UNNE.

Morphological analysis of the gonadal system

The snout-cloaca length of each specimen was recorded with a precision caliper of 0.01mm. For the macroscopic analysis of the gonadal system, we proceeded to the dissection of the specimens and subsequent observation under a stereoscopic microscope, and isolation of the reproductive system. In the case of males, both testicles were taken, and the maximum length and width were measured to estimate the average size of the gonads with a digital caliber of 0.01mm precision. In females, the presence and number of follicles were analyzed, differentiating them by type. For the determination, the classification proposed by Valdez Toledo & Pisanó (1980) was followed: Previtellogenic oocytes: translucent, with no accumulation of vitelline platelets in the cytoplasm; vitellogenic oocytes: whitish coloration without pigmentation, with onset of vitellogenesis; post-vitellogenic oocytes: greater than the previous ones, with well-differentiated animal and vegetal poles. The presence-absence of post-ovulatory bodies was also recorded. The mature females were identified by the presence of post-vitellogenic oocytes.

Histological analysis

For the tissue analysis of the reproductive system in males and females, histological preparations were made following the conventional techniques of dehydration, inclusion in paraffin, cuts in a microtome, and coloration. Dehydration was performed in increasing concentrations of ethyl alcohol (70, 80 and 96%) and butyl alcohol (100%) for 25 to 45 minutes, depending on the sample size. Inclusion in butyl-paraffin (50-50%) was performed for 24 hours and pure paraffin overnight. After this, the block-paraffins were made, and the samples were oriented to obtain transverse or longitudinal sections of 5 to 7 microns. These were obtained with a Spencer manual rotary microtome. The samples were stained with Hematoxylin-Eosin (H-E) and Masson's Trichromic for general cytological and histological characterization. At the histochemical level, PAS (Periodic acid-Schiff-haematoxylin) reactions were applied for the detection of neutral glycosaminoglycans (Kiernan 1999), and Coomassie blue staining was performed for the determination of proteins. In the case of males, samples with spermatozoa in the seminiferous tubules were considered mature. The images were captured using the LEICA DM4000B microscope, and an image capture system supported by the LASZ LEICA Inc® program. The camera is coupled to a trinocular microscope model LEICA® DCC-380X®.

Immunohistochemical studies to determine the immunodetection of PCNA

The immunodetection of PCNA was only analyzed in testicles because it wanted to highlight the possibility of detecting a continuous cycle without the need to analyze the presence or absence of sperm. In the case of females, this analysis was not necessary given that the presence of follicles in different phases of folliculogenesis corroborates their reproductive activity.

An anti-PCNA antibody made in mice was used (Santa Cruz Biotechnology PCNA Antibody (sc-7907, FL-261)) in order to establish the immunodetection of the PCNA cell proliferation protein in the germline cells during spermatogenesis in testes at different stages of the reproductive cycle, and then defining the cycle as continuous. The antibody was incubated at a working dilution of 1:50, for 120 minutes at

37 °C, and subsequently revealed according to the indirect protocol of "L-streptoavidin-biotin" - HRP (DAKO K0690). PCNA is a phylogenetically conserved protein and said antibody had been tested in many species of anurans, birds, and mammals (Carou et al. 2017, Olea et al. 2018, 2020, Scaia 2019).

RESULTS

Females

From the macroscopic observations in the stereoscopic microscope (Figure 1a-b), the presence of oocytes at different stages of follicular development was identified: from previtellogenic to postvitellogenic oocytes, which shows the reproductive status of the collected females.

Ovaries

The ovaries of the studied specimens have a racemic structure due to the presence of oocytes in different stages of oogenesis. They are located near the kidneys, and are linked to the wall of the body by the mesovaries. Mesovaries are formed by a coelomic mesothelial lining of dense non-patterned connective tissue, with conspicuous vascularization and the presence of nervous tissue (Figure 1c).

These organs are pigmented, observing by naked eye a darker region (given by the presence of pigments) and a lighter region, (determined by the absence of pigments). These zones are coincident with the animal-vegetal polarity found in the oocytes (Figure 1a-b). At the histological level, the peritoneal lining forms a simple flat to cubic epithelium covering and little underlying connective tissue (Figure 1d-g).

Ovarian follicles

Oogenesis begins with the proliferation of oogonia, which is located towards the periphery of the ovary. In this study, oocytes could be distinguished in the three stages of development: previtellogenesis, vitellogenesis, and post-vitellogenesis. They are described below:

The previtellogenic oocytes were observed in all the collected females (even in the post-ovulatory ones) (Figure 1b-d-e). In these follicles, the cover is formed by flat follicular cells and another layer of cells that build the tecal up (Figure 1e). Also, an irregular nucleus is evidenced, which is located towards the central zone of the oocyte and has a large size in comparison with the cytoplasm (Figure 1e).

The vitellogenic oocytes, like the previtellogenic ones, present a layer of follicular cells closely-attached to the tecal cells (Figure 1d-e). These were found in all the collected females and were recognizable by their large size. Its cytoplasm is very abundant and shows a progressive accumulation of yolk in the form of vitelline platelets that surround the nucleus (Figure 1d), moving it to the periphery (animal pole), and leaving the area where the vitelline platelets are most abundant (vegetative pole). Subsequently, vitelline platelets increase in number and size. At this time it can be considered that the follicle is in postvitellogenic stage or ovulatory stage (Figure 1g): oocytes have increased considerably in size, and cells from the follicular layer became much flattened, and external teak surrounded the entire oocyte (Figure 1g). It is also found surrounding the follicle, a thin layer of connective tissue with fibroblasts (these are large and very elongated cells with a bulky nucleus), blood vessels and collagen fibers (structures not present in the image). Atresic follicles have also been observed in different degrees of development (Figure 1f-h) in the histological sections of all the females analyzed.

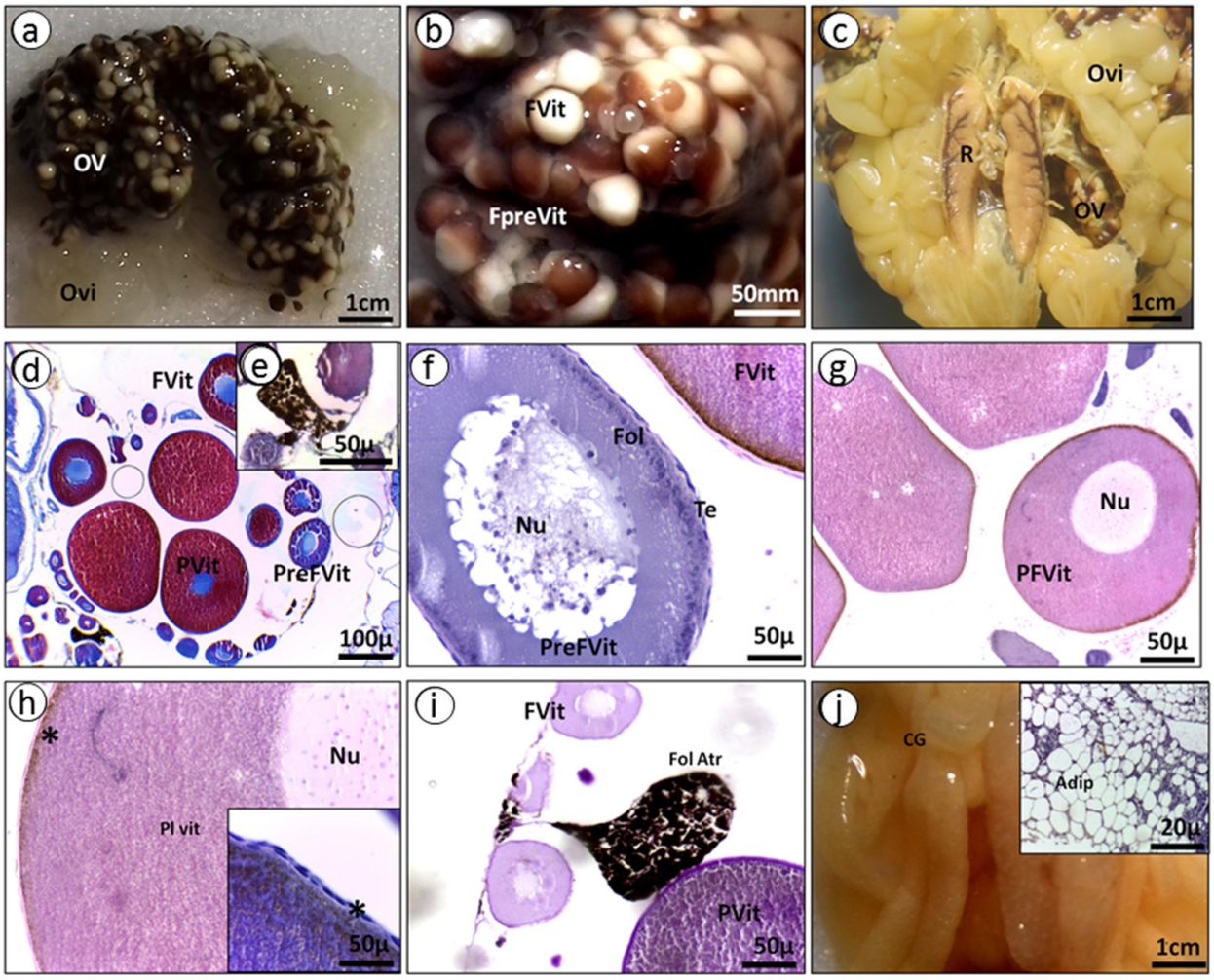

Figure 1. a) Detail of the ovary and oviduct of *S. acuminatus.* b) Detail of the cell types present in the ovary. c) Relationship between the position of the kidneys and the ovary and its vascularization. d) Transversal section of the ovary at the reproductive stage (Staining: Masson's Trichrome). e) Detail of a pre-vitellogenic follicle (Staining: PAS). f) Atretic pre-vitellogenic follicle. g) Detail of a vitellogenic follicle (H-E Staining). h) Detail of post-vitellogenic follicle (Staining: H-E). i) Detail of an atretic and vitellogenic follicle (Staining: H-E) j) Fat bodies and detail in histological section (Staining: H-E). References: arrow: revetment epithelium. Ov: ovary. Ovi: oviduct. R: kidney. Fvit: vitellogenic follicle. FpreVit: Pre-vitellogenic follicle. Pvit: post-vitellogenic. Fol Atr: atretic follicle. Pl vit: viteline platelets. Nu: nucleus. Fol: follicular cells (*). Te: tecal cells, CG: fat body. Adip: adipocytes. Mes: mesovarium.

Fat bodies

Fat bodies are structures that have an intimate topographical and functional relationship with the ovaries and testes. They are structured in sparkling-looking lobes and could present macroscopical color variations from yellow to orange, despite being in the same reproductive period (Figure 1i). Histologically, the adipose tissue corresponds to white fat, for which peripheral and flattened nuclei (Figure 1i).

Males: Testicles

The testicles of *S. acuminatus* were characterized as paired organs without external pigmentation, yellowish and ovoid, with an average size of 2.52 mm long x 1.31 mm wide. Kidneys and fat bodies are associated with the testes (Figure

2a-b). Histologically, it can be seen that they are surrounded by a tunica albuginea composed of dense connective tissue. Smooth muscle and blood vessels are seen in the interstitial tissue (Figure 2c).

Seminiferous tubules

It was possible to observe in all the males analyzed that the testes contain different cellular types organized in seminiferous tubules, where each tubule or cyst presents clusters or groupings of germline cells in the same stage of differentiation: spermatogonia at the edge of the epithelium (Figure 2d), spermatocytes I, II (Figure 2d-f); spermatids are attached to Sertoli cells (Figure 2f). Through the process of spermiogenesis, spermatids finally differentiate into sperm and separate from the Sertoli cell and located within or close to the lumen of the tubule (Figure 2e-f).

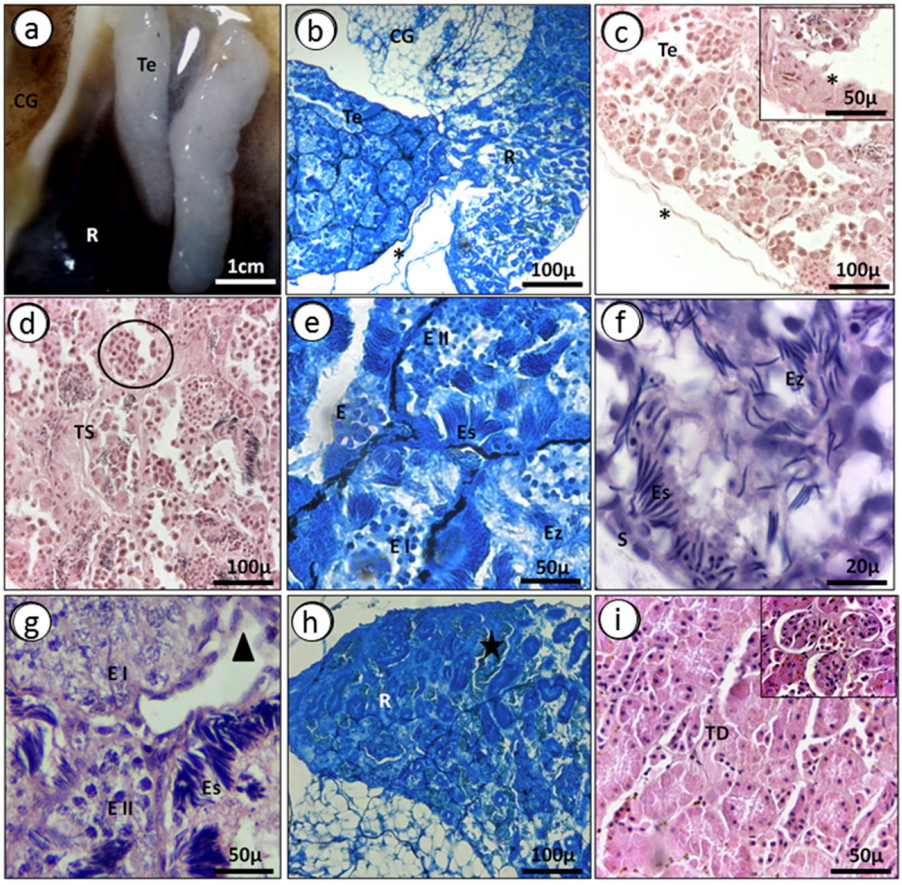

Figure 2. a) Detail of the testes with tunica albuginea without pigmentation, kidneys and fat bodies in *S. acuminatus*. b) Sagittal section of testicles, kidneys and fat body (Staining: Coomassie blue). c) Transversal section of the testicle with detail of the tunica albuginea (Staining: H-E). d) Detail of the seminiferous tubules with cysts (Staining: H-E). e) Detail of the cell types each spermatogenic cyst and seminiferous tubules surrounded by melanocytes (Staining: Coomassie blue). f) Detail of plume of spermatids attached to the Sertoli cell (Staining: PAS). g) Detail of the interstitial cells and Leydig cells (Staining: H-E). h) Transversal section of the kidney, with emphasis on the convoluted tubules that are part of the sexual segment (Staining: Coomassie blue). i) Detail of the convoluted tubules of the hypertrophied sexual segment with positive PAS cells and detail of the genital glomerulus (Staining: PAS). References: Te: testicle. CG: fat bodies. R: kidney. Asterisk: tunica albuginea. TS: seminiferous tubule. Circle: cistos. Me: melanocytes. E: spermatogonia. EI: primary spermatocytes. EII: secondary spermatocytes. Esp: spermatids. Ez: sperm. S: Sertoli cells. Star: hypertrophied contiguous tubules. TD: distal contiguous tubules. Arrowhead: Leydig cell.

Among the seminiferous tubules, there is interstitial tissue composed of Leydig cells, fibroblasts, blood vessels, and melanocytes (Figure 2e-g).

Kidneys sexual segment

The sperm is formed inside the seminiferous tubules and is transported inside the intratesticular ducts to the efferent ducts. Then, they are transported out of the testicle by ducts, which over time are connected to the genital-renal corpuscle (Figure 2h). The convoluted tubules of the sexual segment of the kidney show hypertrophy, and Coomassie Blue, PAS, and positive PCNA cells can be evidenced as well (Figure 2b-I, Figure 3d). These structures were not observable in the kidneys of the females.

Immunodetection of PCNA protein in testicles and kidney

The organ changes and the stage of differentiation of germline cells were significant in showing cell proliferation in both sperm line and kidney. In the case of the testes, the proliferating cells correspond to spermatogonia in the mitotic and meiotic division and some Sertoli cells associated with sperm plumes. All these PCNA positive cells were found in all the males analyzed (Figure 3a-c). In the kidney, the PCNA positive cells correspond to the hypertrophied tubules associated with the genital glomerulus (Figure 3d).

DISCUSSION

The present work showed that *S. acuminatus* has a reproductive system similar to the one described for other species anurans of the Hylidae family. The species has a potentially continuous cycle; of active spermatogenesis as other species of the same genus from the southeast of Brazil and other regions of our country such as *S. centralis, S. fuscovarius* (de

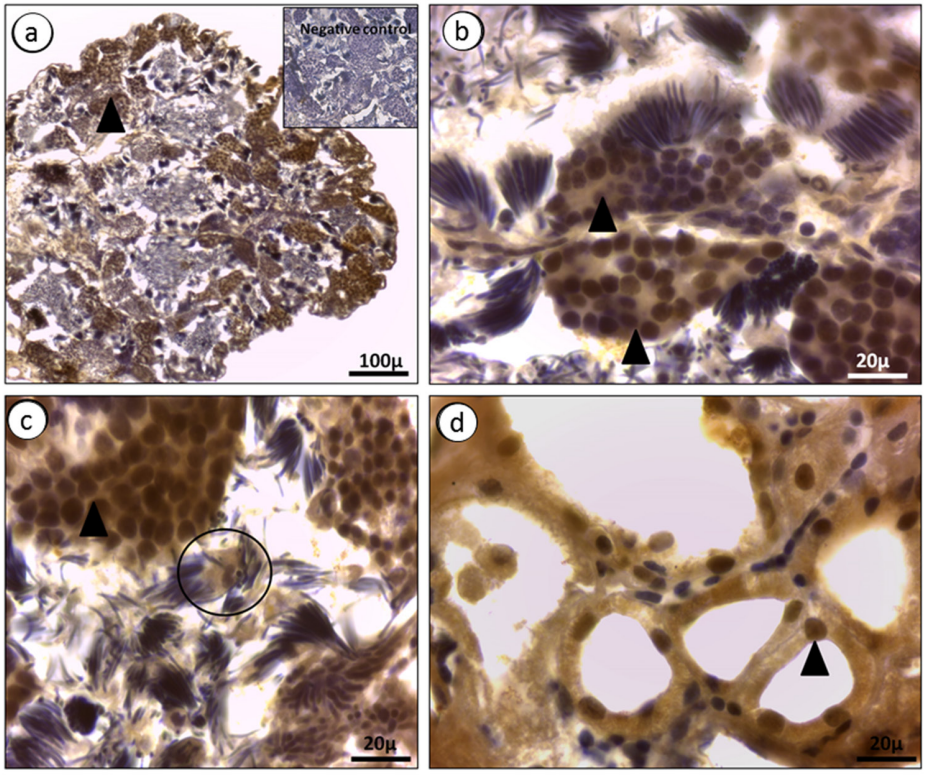

Figure 3. a) Expression of the PCNA cell proliferation protein in the transversal section of the testicle of *S. acuminatus*. b-c) Details of the PCNA positive spermatogenic cells line (circle). d) Expression of the PCNA cell proliferation protein in a cross section of the kidney of *S. acuminatus*. References: Arrow: PCNA positive cells.

Oliveira et al. 2003, Alcantara et al. 2007, Crump 2015).

The location and external characteristics of both ovaries and testicles of the analyzed specimens coincide with that described by other authors for the anurans (Cajade et al. 2010, Carezzano et al. 2013, Curi et al. 2014, Dias et al. 2017, Montezol et al. 2018). In all cases, those organs were characterized as paired, associated with fat bodies, with great vascularization, and located in the abdominal cavity. In the case of males, the testes are ovoid without pigmentation in the tunica albuginea. The lack of testes pigmentation in males (tunica albuginea and medulla) is a common condition among anurans (Duellman & Trueb 1986, Curi et al. 2014). In this work, the variation of the testicular collation between the tunica albuginea and the medulla was revealed, observing the isolated presence of melanocytes in the medulla, while the tunica albuginea lacks pigments.

Histologically, it was observed that spermatogenesis in *S. acuminatus* is similar to that described for other anurans (de Oliveira et al. 2002, 2003, 2004, Curi et al. 2014, Scaia et al. 2019). Spermatocytes are usually observed in the prophase of the first meiotic division, with different levels of chromosomal condensation. At the end of the spermatogenesis, the sperm remain united in the bundles, and they are still supported by the Sertoli cells. By the time they are released to the light of the seminiferous loculus, the arrangement dissolves and sperm remain in the spermatic tubule until the definitive release. The arrangement of the spermatogenic line in a locule, consisting of cysts formed by cells in the same stage of differentiation, has already been observed in other anurans. This demonstrates how the organization of structure is conservated among taxa since it is also described in the rest of the anamniotes.

Additionally, analysis of the PCNA cell proliferation protein allowed continuous spermatogenesis to be evidenced in this species because positive spermatogonia for PCNA could be observed in all the months analyzed. This allows us to confirm the continuous potentiality spermatogenic activity in *S. acuminatus*.

Regarding the male kidney, the secondary sex structures previously identified in *Rana catesbeiana* (Rheubert et al. 2017) increase in the height of the epithelium and become hypertrophied as the production of sperm increases. This relationship, and taking into account what is proposed for *R. catesbeiana*, provides new evidence that the collecting ducts of the kidney of *S. acuminatus* can function as secondary sex structures. However, the actual function of the secretions remains unknown.

In the case of females, the ovary of *S. acuminatus* exhibits the same structural and functional pattern as the rest of the anurans. The shape of the ovaries varies according to the reproductive moment in which the female is found, as we described in the present work. Regarding oocyte pigmentation in females, pigmentation was observed in the outer covering of the ovary, which coincides with a histological study of the *S. fuscovarius* gonads, carried out by de Oliveira & de Souza Santos (2004). The pigmentation found was attributed to the function of protecting the reproductive organs from ultraviolet radiation (de Oliveira & de Souza Santos 2004).

The histological analysis revealed different cell types: previtellogenic, vitellogenic, and post-vitellogenic follicles. Regarding folliculogenesis, the presence of post-vitellogenic oocytes during almost the entire study period gives us an indication that females may be prepared to oviposition and do so when environmental conditions permit. With the stains used (H-E and PAS-H-E), it was possible to observe that

the yolk showed variations in its coloration, presenting darker pigmentation near the animal pole and clearer colorations in the vegetal pole. These results agree with what has been proposed by other authors for folliculogenesis in anurans (Pucci Alcaide et al. 2012, Curi et al. 2014). Vitellogenesis is continuous, and the yolk occupies all the cytoplasm progressively. The oocyte and its nucleus experience an increase in size during follicular development, especially in the oocytes of the first and second growth phases. This relationship between the nucleus and the size of the oocyte was described in *Rana cyanophlyctis* (Pancharatna & Saidapur 1985).

Some of the analyzed ovaries also presented atretic follicles, in both previtellogenic and vitellogenic state. This atretic process that happens during the ovulation, when not all the follicles reach the oviduct, is common in the ovaries of the anurans (Pucci Alcaide et al. 2012, Curi et al. 2014).

The results of this work give a basis for the characterization of the reproductive cycle in *S. acuminatus* and provide background information on the analysis of spermatogenic activity by IHQ from the study of the immunodetection of the PCNA cell proliferation protein. Future studies will focus on the evaluation of cell death processes during the reproductive cycle in the studied species to compare and correlate them with those obtained in terms of cell proliferation.

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The authors' contributions were as follows: Olea Gabriela and Lombardo Daniel designed the experiments, analyzed the data and drafted the manuscript; Olea Gabriela, Cheij Esteban, Cuzziol Boccioni Ana Paula, Rodriguez Florencia and Céspedez Jorge performed the experiments. We declare that we are aware of this submission and agree to be listed as co-authors. Besides, we declare that there are not conflicts of interest.