Apoptosis, Proliferation and Presence of Estradiol Receptors in the Testes and Bidder's Organ of the Toad *Rhinella Arenarum* (Amphibia, Anura)

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ABSTRACT The dynamic equilibrium between spermatogonial proliferation and testicular apoptosis determines the progression of spermatogenesis in amphibians. Estrogens and their receptors play a central role in regulating spermatogenesis in vertebrates, and in some species of anurans, estradiol (E_2) is involved in the regulation of spermatogonial proliferation and apoptosis of germ cells. Bidder's organ (BO) is a structure characteristic of Bufonidae that has historically been compared to an undeveloped ovary. In adult *Rhinella arenarum* males, BO is one of the main sources of plasma E_2. The aim of this study was 1) to describe the seasonal variations in testicular apoptosis, spermatogonial proliferation, and cellular proliferation in BO; and 2) to analyze the presence and localization of estrogen receptor β (ERβ) in the testes and BO of *R. arenarum*. Testicular fragments and BOs from animals collected during the year were labeled with 5-bromo-2′-deoxyuridine (BrdU) and BrdU incorporation was determined using immunohistochemistry. Apoptosis in testicular sections was detected using the TUNEL method, and ERβ localization was assessed using immunohistochemistry in testes and BOs. The results indicate that spermatogonial proliferation is highest during the reproductive season and that cysts of spermatocytes and spermatids undergo apoptosis during the postreproductive season. Furthermore, the proliferation of follicular cells is highest during the reproductive and postreproductive seasons. ERβ was primarily detected by immunolocalization in Sertoli cells, follicular cells, and oocytes. Taken together, these results suggest that cysts that do not form spermatozoa are removed from testes by apoptosis and that estrogens regulate both spermatogenesis and oogenesis in adult males of *R. arenarum*. J. Morphol. 277:412–423, 2016.

KEY WORDS: reproduction; amphibians; estrogens; spermatogenesis; Bufonidae

INTRODUCTION

During spermatogenesis, spermatogonia proliferate, and differentiate into meiotic spermatocytes, spermatids, and mature spermatozoa. In amphibians, the unit of spermatogenesis is the spermatocyst, or cyst, which is formed when Sertoli cells enclose one spermatogonium within cytoplasmic processes (Wake, 1969; Lofts, 1974; Duellman and Trueb 1994; Pudney, 1995). In seasonal breeders, spermatogenesis transitions from recrudescence to quiescence, and this process is intimately related to the dynamic balance between cell proliferation and apoptosis of the germ cells. Amphibians have various reproductive cycles, with continuous and discontinuous cycles being the two extremes (Van Oordt, 1960). In continuous reproductive cycles, spermatogonia and spermatocytes are constantly being produced, while in discontinuous cycles, primary spermatogonia lose their mitotic capacity when spermatocytes start their differentiation; for this reason, spermatozoa are present in the testes only during a particular season. Generally, tropical and subtropical species have continuous cycles, while temperate species have discontinuous or potentially continuous cycles (Rastogi et al., 1976; Lofts, 1987). Therefore, it is useful to analyze the seasonality of cellular proliferation and apoptosis of the germ cells to understand the progression of the spermatogenic cycle during spermatogenesis.

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Spermatogonial proliferation has been studied in *Pelophylax esculentus* (Chieffi et al., 2000a,b, Ferrara et al., 2004). In this species, proto-oncogene products were suggested to play a regulatory role in spermatogenesis and cellular proliferation during the breeding season (Chieffi et al., 1995; Chieffi et al., 2000a; Ferrara et al., 2004; Raucci and Di Fiore, 2007). Estrogens and their receptors play a central role in regulating spermatogonial proliferation and apoptosis of germ cells. Indeed, it has been suggested that estradiol (E_2) regulates spermatogonial proliferation in the testes of *P. esculentus* (Minucci et al., 1997; Cobellis et al., 1999; Chieffi et al., 2000b) but not of other species of anurans such as *Rhinella arenarum* (Scaia et al., 2015) and *Lithobates pipiens* (Tsai et al., 2003).

Testicular apoptosis is an essential process maintaining testicular homeostasis because it determines the equilibrium between the number of germ cells and the supporting capacity of the Sertoli cells. The seasonality of testicular apoptosis was studied in different groups of amphibians such as *Cynops pyrrhogaster* (Yazawa et al., 1999), *Triturus marmoratus* (Ricote et al., 2002), *Ichthyophis tricolor* (Smita et al., 2005), and *Lithobates catesbeianus* (Sasso-Cerri et al., 2004, 2006). However, there is little evidence regarding the role of estrogens in regulating testicular apoptosis in amphibians. It has recently been demonstrated that in vitro treatment with E_2 induces apoptosis of spermatogenic cysts during the reproductive season in *R. arenarum* (Scaia et al., 2015).

The aforementioned evidence suggests that E_2 might play an essential role in the progression of spermatogenesis in anurans by regulating either cellular proliferation or apoptosis of germ cells. In *P. esculentus*, the presence of estrogen receptor β (ERβ) was demonstrated by immunohistochemistry and Western-blot analysis (Stabile et al., 2006), but unfortunately, this is the only study that has analyzed the presence and localization of estrogen receptors in amphibian testes.

Rhinella arenarum is a South American toad with a dissociated reproductive pattern characterized by low levels of plasma androgens during the reproductive season due to a decrease in the activity of cytochrome P450 17α-hydroxylase-$C_{17\text{-}20}$ lyase (Canosa and Ceballos, 2002; Fernández Solari et al., 2002; Canosa et al., 2003). In this species, the spermatogenic wave starts in spring (October), when there is a high number of spermatogonia but few spermatocytes and spermatids. The number of spermatocyte cysts and spermatids increases during the end of summer and the beginning of autumn (February and March). The highest number of spermatozoa occurs during the end of winter (September) (Burgos and Mancini, 1948). Thus, it is not clear what happens to spermatocyte cysts and spermatids that do not complete spermatogenesis. There are two possibilities: immature cysts either remain quiescent until next year or they undergo apoptosis. Then, mature spermatozoa will be produced by a new wave of spermatogonia in the following year.

Testes of *R. arenarum* do not express aromatase activity (Canosa et al., 1998; Canosa and Ceballos, 2001), and the main source of E_2 is Bidder's organ (BO) (Scaia et al., 2011; Scaia et al., 2013). BO is a structure characteristic of Bufonidae that has historically been compared to a rudimentary ovary and that is maintained in male adults of several species. The presence of steroidogenic enzymes in BO has been reported in several species, such as *Duttaphrynus melanostictus* (Ghosh et al., 1984), *Anaxyrus woodhousii* (Pancak-Roessler and Norris, 1991), and *Bufo bufo* (Colombo and Colombo Belvedere, 1980). In *R. arenarum*, BO expresses several steroidogenic enzymes, including aromatase, and it is able to produce E_2 from endogenous substrates (Scaia et al., 2011, 2013). Furthermore, plasma E_2-levels are low during the prereproductive season and gradually increase during the reproductive season, reaching their peak value during the postreproductive season (Scaia et al., 2013). The weight and total aromatase activity of BO are also low in the prereproductive season and increase during the reproductive season (Scaia et al., 2011). The lack of a significant correlation between total aromatase activity and total BO-weight suggests that the contribution to plasma E_2 is not related to the weight of the organ but rather to other aspects such as the number of follicular cells or the aromatase activity per follicular cell (Scaia et al., 2013).

It is likely that BO can be affected by environmental conditions such as agricultural contaminants, because BO-malformations and reproductive abnormalities have been reported in adults of *Rhinella marina* collected from areas with intense agricultural activities (McCoy et al., 2008). Therefore, when considering the fact that BO in *R. arenarum* expresses aromatase, it is important to recall that there is growing evidence suggesting that some endocrine disruptors present in the environment, such as atrazine, have estrogenic effects (Hayes et al., 2002a,b; Fan et al., 2007; Hayes et al., 2010). Because of the vulnerability of BO to environmental contaminants, and considering that BO is the main source of plasma E_2 in *R. arenarum* and that this hormone regulates testicular apoptosis and modulates testicular steroidogenesis, studying BO may contribute to our understanding of anuran testicular functionality.

In the present study, we describe seasonal variations in spermatogonial proliferation and testicular apoptosis in *R. arenarum* as well as the localization of the estrogen receptor in the testes. We also analyze the proliferation of follicular cells

during the year and the localization of estrogen receptor in BOs.

MATERIALS AND METHODS

Animals

Adult *R. arenarum* males (Hensel, 1867) were collected between 2012 and 2014 in a nonagricultural area near Buenos Aires City during prereproductive (May–August, $n = 21$), reproductive (September–December, $n = 20$), and postreproductive (January–April, $n = 18$) seasons (Canosa et al., 2003). Animals were kept under natural light and temperature conditions for two weeks with free access to water and were fed with crickets and zophobas (*Zophobas morio*). Toads were deeply anesthetized by immersion in 1% tricaine methanesulfonate, according to Gentz (2007), resulting in their death due to the long immersion in the anaesthetic. This procedure was carried out in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction and with the approval of Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio (Protocol N° 21), Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.

Tissue Collection and Incubations

Testes and BOs were rapidly dissected and placed in Petri dishes with sterile L15 medium (Sigma-Aldrich) containing 10% charcoal-stripped fetal bovine serum, 60 mmol l^{-1} glucose, 10 mmol l^{-1} HEPES, antibiotics (penicillin, 100 IU/ml, streptomycin, 0.1 mg/ml) and an antimycotic (amphotericin B, 0.25 mg/ml). Testes were used to analyze seasonal variations in apoptosis ($n = 4$ for each season) and in cellular proliferation ($n = 5$ for each season). BOs were used to analyze seasonal variations in cellular proliferation (prereproductive, $n = 5$; reproductive, $n = 3$; postreproductive, $n = 3$). Furthermore, both testes and BOs were used to determine the presence and the localization of ERβ during the year (prereproductive: $n = 4$; reproductive: $n = 5$; postreproductive: $n = 3$ for testes and $n = 3$ for each season for BOs). Testes were sliced into homogenous portions of approximately 2 mm. BOs were analyzed as whole organs.

Histological Procedures

Testicular fragments and BOs were fixed in Bouin's solution for 24 h, dehydrated, and embedded in paraffin-histoplast (50:50, w/w). Organs were cut in serial sections (7 μm), deparaffinized, hydrated and then treated to determine apoptosis, cellular proliferation or the presence of ERβ using immunohistochemistry. For the analysis of cell types and their organization, sections from each specimen were stained with hematoxylin-eosin.

Apoptosis Determination

Testicular serial sections were analyzed for DNA fragmentation using an ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore), as described in Scaia et al. (2015). Briefly, sections were treated with proteinase K (20 μg/μl, Millipore) for 15 min and incubated for 1 h with Tdt-enzyme at 37°C. After the enzymatic reaction was stopped, sections were incubated with anti-digoxigenin antibody labeled with FITC for 30 min, and then nuclei were counterstained with propidium iodide (P-1304, Molecular Probes). Negative controls were processed using the same steps described above but without the Tdt-enzyme incubation. Positive control sections were incubated with a DNase solution for 30 min at 37°C after proteinase K treatment and then incubated as described above. Sections were examined using a confocal laser microscope (Olympus-FV300 attached to an Olympus Bx-61 microscope), and images were acquired using Fluoview v.5 software. The number of TUNEL-positive cells in all cysts from each lobule, taking 5 lobules per section, was counted with ImageJ 1.49 software (Schneider et al, 2012). The stages of spermatogenesis were defined according to Rastogi et al. (1976). The results were expressed as the mean number of apoptotic nuclei in each treatment for each season ± SE.

Proliferation Assay

Cellular proliferation was determined in freshly isolated testicular fragments and in BOs by incubation in 4 ml of sterile L15-medium containing 1 mmol l^{-1} of 5-bromo-2-deoxyuridine (BrdU). After 1 h at 37°C, organs were processed for immunohistochemistry as described above, and immunohistochemical staining was performed as described by Scaia et al. (2015). **S**ections of testicular fragments and of BOs collected during the year were incubated overnight at 4°C with a primary monoclonal anti-BrdU antibody (1:100; GE Healthcare, UK) and then were incubated with a secondary rabbit anti-mouse polyclonal antibody conjugated with biotin for 1 h at room temperature (1:500; Santa Cruz Biotechnology). Sections were then treated with a streptavidin-biotinylated horseradish peroxidase complex for 45 min at room temperature (1:400; GE Healthcare, UK). Immunohistochemical staining was visualized with a 3,3′-diaminobenzidine solution (DAKO North America) and counterstained with hematoxylin. Negative controls were processed as described above, but the primary anti-BrdU antibody was omitted. Finally, sections were examined using a Leica DM2000 microscope, and images were taken with the built-in high-definition digital camera (Leica ICC50), which captures 3-megapixel color images. Cellular proliferation in the testes was expressed as the percentage of BrdU-positive cysts among all the cysts in each lobule, with 5 lobules per 5 sections being measured. Cell proliferation in BOs was expressed as the percentage of BrdU-positive follicular cells of all the follicular cells in each follicle, from 5 sections. The results from each season are expressed as the average value of BrdU-positive percentages (cysts or follicular cells) ±SE.

Presence of Estrogen Receptor B

The presence of ERβ was determined by immunohistochemistry in sections of testicular fragments and of BOs collected during the year. Endogenous biotin was first blocked with an avidin–biotin blocking kit (Vector Laboratories). Sections were incubated overnight at 4°C with a primary polyclonal rabbit anti-ERβ antibody (1:50 dilution in 1.5% BSA; Santa Cruz Biotechnology). The antibody selected has previously been used to localize ERβ in the testes of *P. esculentus* and its specificity was confirmed by Western-blot analysis, with a specific immunoreactive band (molecular weight of 55 kDa, Stabile et al., 2006). Sections were then incubated with a goat anti-rabbit secondary polyclonal antibody conjugated with biotin (1:500; DAKO Cytomation, Denmark) for 1 h at room temperature. Sections were treated with a streptavidin-biotinylated horseradish peroxidase complex for 45 min at room temperature (1:400; GE Healthcare, UK). Negative controls were processed using same steps, but the primary antibody was omitted. Immunohistochemical staining was visualized, counterstained, and examined as described above.

Statistical Analysis

Seasonal variations in testicular cellular proliferation were analyzed and compared using one-way Analysis of Variance (ANOVA), with *Season* as the main factor. *A* posteriori tests of differences among means were performed using Tukey's test. Seasonal variations in testicular apoptosis and in cellular proliferation in BOs were analyzed by the nonparametric Kruskal–Wallis test because the data did not meet criteria of homoscedasticity.

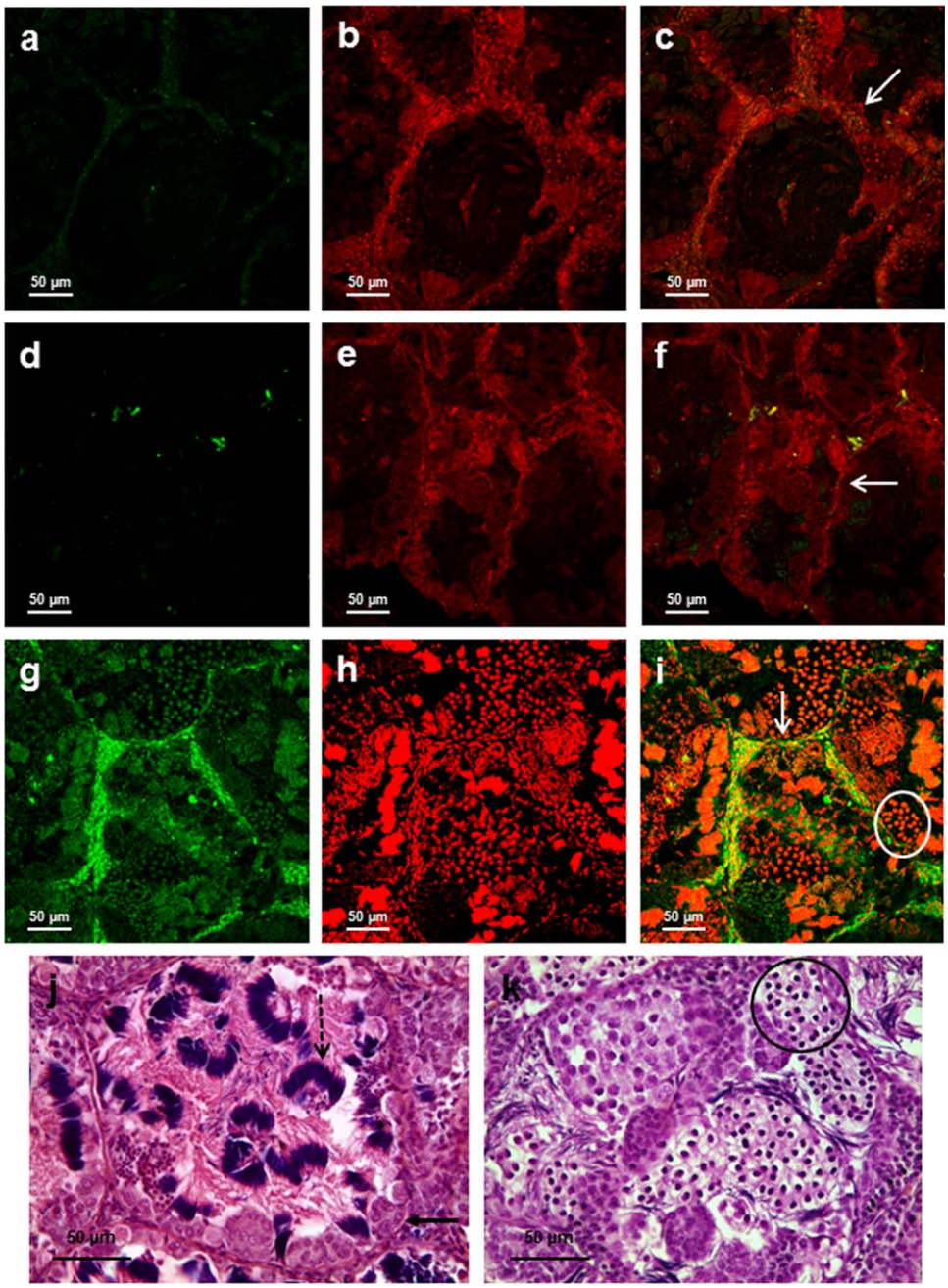

Fig. 1. *Rhinella arenarum*, seasonal variations in apoptosis in the testes. (**a**, **d**, **g**) Apoptosis was determined with the ApopTag Fluorescein In Situ Apoptosis Detection Kit. (**b**, **e**, **h**) Sections were counterstained with propidium iodide. (**c**, **f**, **i**) Merge. Testicular fragments were sampled during (a–**c**) the prereproductive season, (d–f) the reproductive season and (g–i) the postreproductive season. For panels (c), (f) and (i), arrows indicate interstitial compartments delimiting each testicular lobule. In panel (i), the circle indicates a cyst of spermatocytes. (**j**) Hematoxylin-eosin shows a testicular lobule that contains spermatozoa (dashed arrow) and cysts of spermatogonia (closed arrow). (**k**) Hematoxylin-eosin shows a testicular lobule with cysts in the late spermatogenic stages. The circle indicates a cyst of spermatocytes.

RESULTS

Figure 1 compares testicular tissue stained for apoptotic nuclei from animals from the prereproductive season, reproductive season and postreproductive season. During the prereproductive (Fig. 1a–c) and the reproductive (Fig. 1d–f) seasons, we detected only few TUNEL-positive nuclei, while there was a high number of positively stained nuclei in animals from the postreproductive season (Fig. 1g–i). Figure 1 also shows the standard histology of testicular lobules in *R. arenarum*, with spermatozoa, spermatogonial cysts, and spermatocytes (Fig.

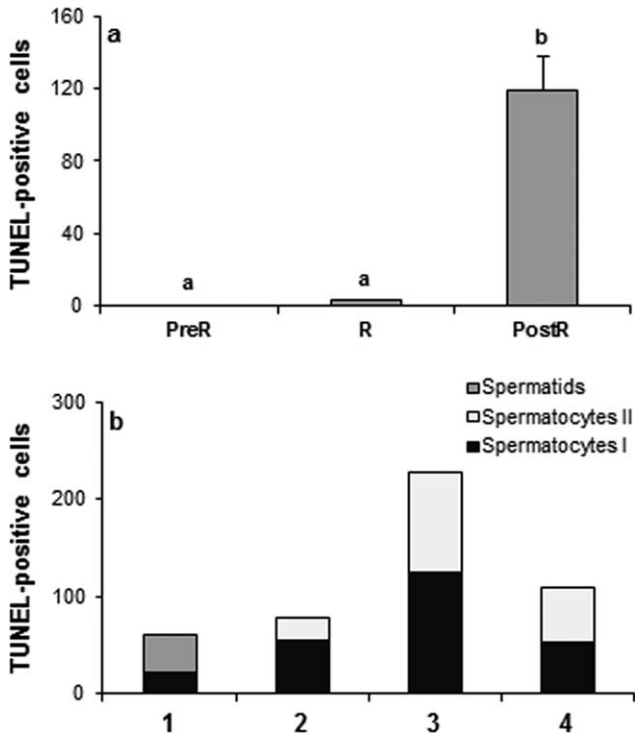

Fig. 2. *Rhinella arenarum*, quantification of apoptotic nuclei in the testes. (**a**) Seasonal variations in testicular apoptosis. Values are expressed as the means of four animals per season ± SE. Different letters indicate significant differences. (**b**) Apoptosis in different spermatogenic stages, defined according to Rastogi et al. (1976); 1–4 refer to the number of toads from the postreproductive season.

1j,k). The quantification of apoptotic nuclei indicated negligible apoptosis in the prereproductive and reproductive season. It also showed that apoptosis of the germ cells occurred almost exclusively during the postreproductive season (Fig. 2a, $P < 0.001$, $H = 8.77$, $df = 2$). Furthermore, analysis of the cellular types during different seasons indicated that all apoptotic cells from the postreproductive season corresponded to spermatocyte Types I and II and spermatids, and that no apoptotic spermatogonia were detected (Fig. 2b). These results show that apoptosis of the germ cells occurs during the postreproductive season and exclusively in late-stage spermatogenic cysts. Notably, no apoptosis was observed in lobules in the negative control sections, which were not incubated with Tdt-enzyme. Moreover, positive control sections were pretreated with DNAse solution and showed high levels of apoptosis, in cysts of spermatocytes and spermatogonia (see supporting information Fig. S1).

To study seasonal variations in cellular proliferation, testes fragments and BOs were incubated with BrdU. The analysis of testicular fragments showed high proliferation of spermatogonia in fragments from the reproductive season (Fig. 3a), while low proliferation was observed in samples collected during the prereproductive and postreproductive seasons (Fig. 3b,c). No immunostaining was observed in the negative controls (Fig. 3d). When results were expressed as the percentage of immunopositive spermatogonia, the proliferation of spermatogonia occurred mostly during the reproductive season (Fig. 3e, $P = 0.0016$, $F = 11.56$, $df = 14$).

High BO proliferation was detected in follicular cells in organs from the reproductive (Fig. 4 a,b) and postreproductive season (Fig. 4c). Figure 4a shows a BO adjacent to a testis; the simultaneous proliferation of spermatogonia and follicular cells can be observed. Notably, proliferating flat and round follicular cells were detected surrounding oocytes in previtellogenic follicles (Fig. 4b). However, BOs from animals collected during the prereproductive season showed negligible proliferation of follicular cells; instead, an intense immunopositivity was detected in the oocyte nuclei (Fig. 4d). No immunostaining was observed in the negative controls (Fig. 4e). The quantification of these results indicates that the proliferation of follicular cells is negligible in the prereproductive season and that it occurs exclusively during the reproductive and postreproductive seasons (Fig. 4f, $P = 0.05$, $H = 5.14$, $df = 2$).

In toad testes, ERβ immunoreactivity was localized in the interstitial tissue (Fig. 5a), in the nuclei of spermatogonia and in spermatocytes from only a few cysts (Fig. 5a,c). However, the most intense immunoreactivity was localized in the cytoplasm of Sertoli cells, adjacent to spermatozoa (Fig. 5b) and surrounding cysts of spermatogonia Types I and II (Fig. 5d,e). No immunoreactivity was found in negative controls performed without the primary antibody (Fig. 5f). Notably, after analyzing the testes of 12 animals, no differences were detected in regard to the localization of ERβ among seasons.

Finally, immunoreactivity for ERβ was found in previtellogenic and vitellogenic follicles of BOs. In previtellogenic follicles, ERβ was localized in the periphery of the ooplasm, but not in follicular cells (Fig. 6a–c). Immunoreactivity for ERβ in the ooplasm in BO and in Sertoli cells adjacent to spermatozoa is also shown in Figure 6a. Moreover, in early vitellogenic follicles in BOs taken from animals collected during the prereproductive season, nuclear and perinuclear immunoreactivity was detected in oocytes, while cytoplasmatic immunoreactivity was detected in follicular cells (Fig. 6d). No immunoreactivity was found in negative controls that were not treated with ERβ antibody (Fig. 6e).

DISCUSSION

Rhinella arenarum is a species with a continuous spermatogenic cycle because its testes never undergo a quiescent period. The spermatogenic wave starts in October, when testes have their

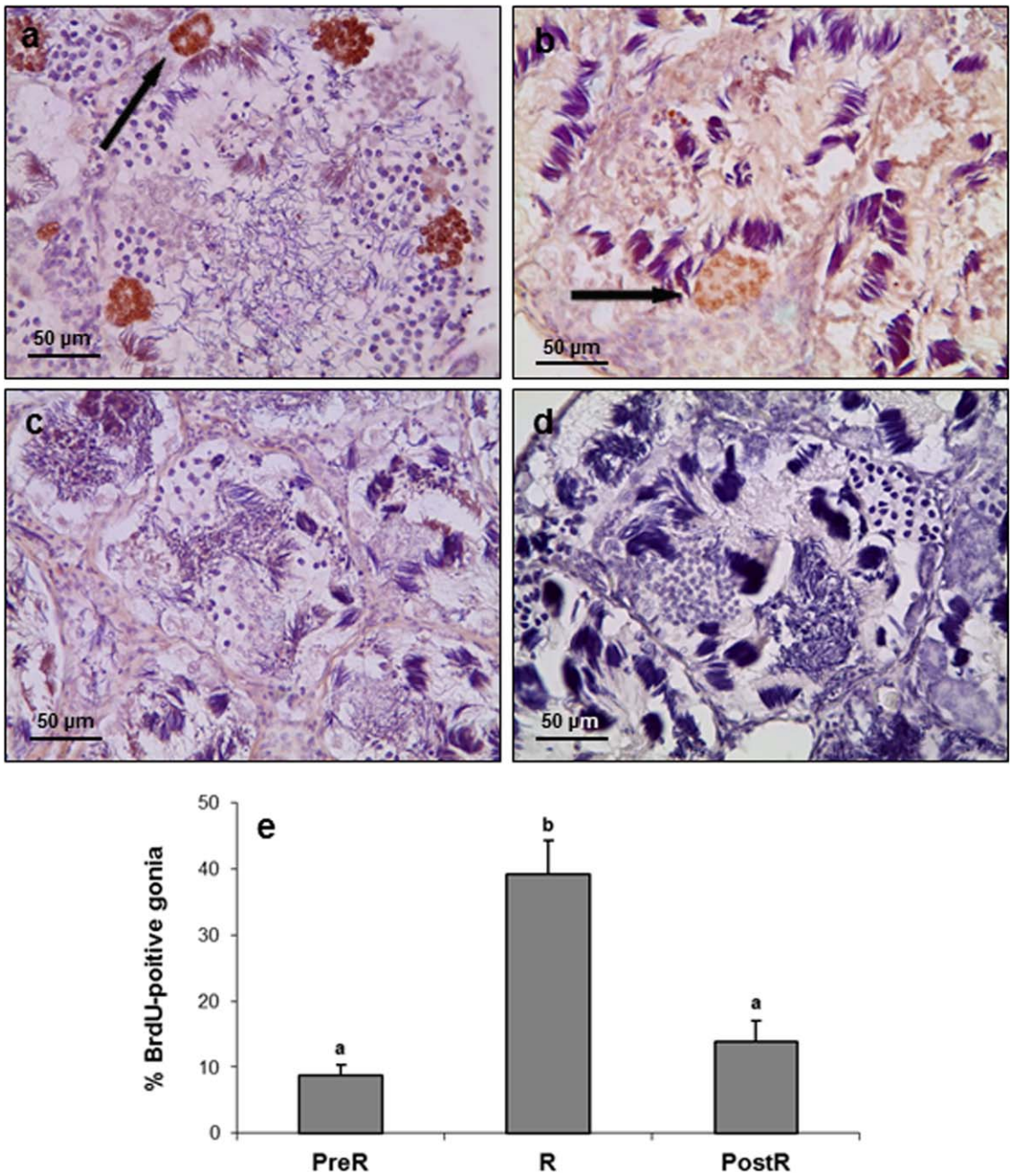

Fig. 3. *Rhinella arenarum*, seasonal variations in cell proliferation in the testes determined with BrdU labeling and counterstained with hematoxylin. The proliferation of spermatogonia during (**a**) the reproductive season, (**b**) the prereproductive season and (**c**) the postreproductive season. Arrows indicate cysts of proliferating spermatogonia. (**d**) Negative control with no primary antibody. (**e**) The proliferation rate is expressed as the percentage of immunopositive cysts related to the whole number of cysts in each spermatogonial stage. Values are expressed as the means of 5 lobules from 5 animals from each season ± SE. Different letters indicate significant differences.

highest number of spermatogonial cysts, and finishes in September of the following year, when spermatozoa are prevalent (Burgos and Mancini, 1948). Because testes also have spermatocyte cysts once the spermatogenic wave finishes, there are at least two possible scenarios: spermatocyte cysts and spermatids remain inactive until the following year, or they undergo apoptosis, and spermatozoa are result of a new wave of spermatogenesis. Because of this background, it is important to study seasonal variations in apoptosis and the proliferation of germ cells for these to contribute to our understanding of the progression of spermatogenesis in amphibians.

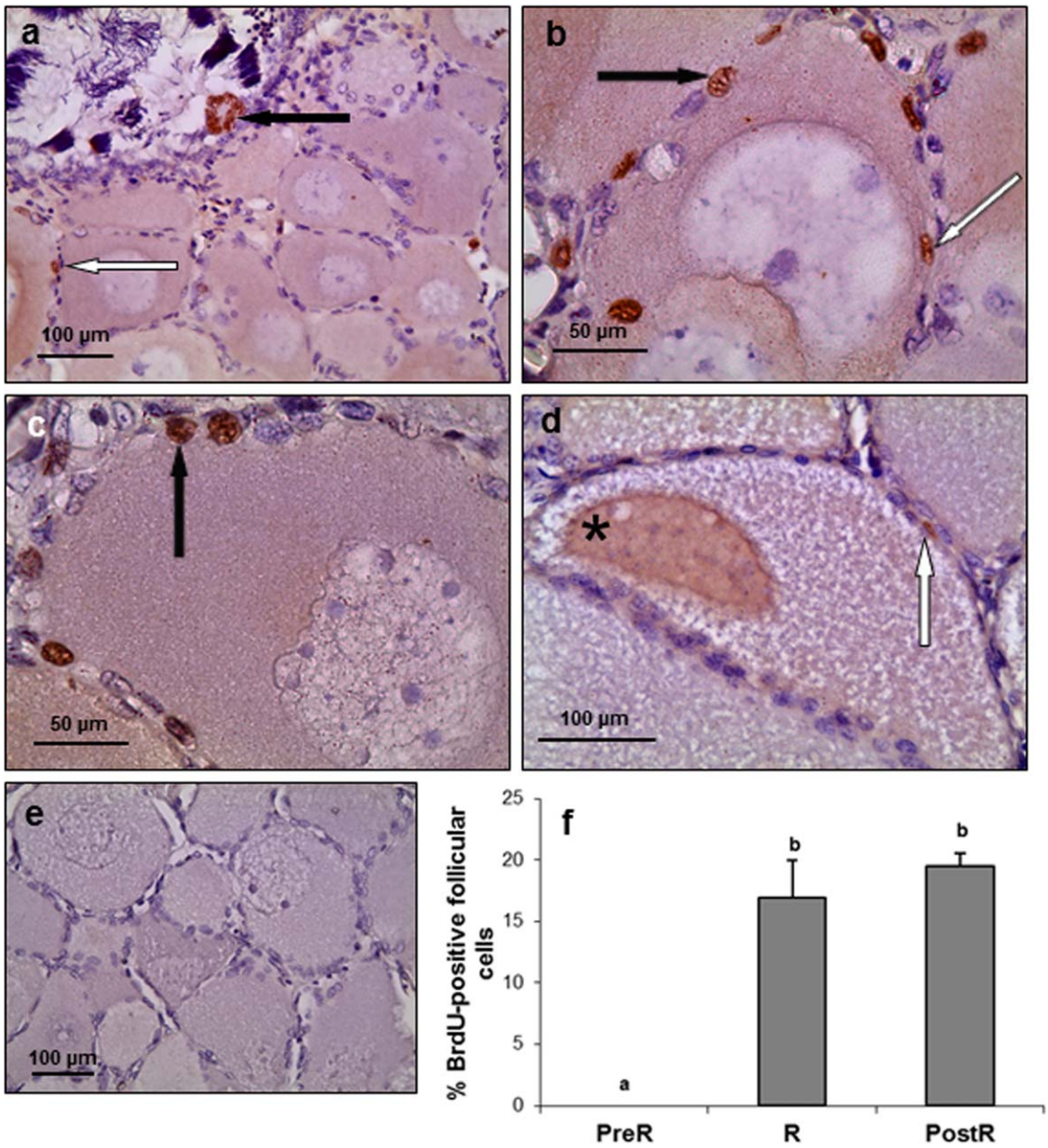

Fig. 4. *Rhinella arenarum*, seasonal variations in the proliferation of follicular cells in BO determined with BrdU labeling and counterstained with hematoxylin. (**a**) A detailed image of immunopositive spermatogonia in testes (black arrow) and an immunopositive follicular cell in BO (white arrow) from an animal collected during the reproductive season. (**b**) Section of BOs from the reproductive season. In previtellogenic follicles, both round (black arrow) and flat (white arrow) immunopositive follicular cells were detected. (**c**) A section of BO from the postreproductive season with an immunopositive follicular cell indicated by an arrow. (**d**) Detail of a Bidderian follicle from the prereproductive season showing immunoreactivity for BrdU in the nucleus of an oocyte (asterisk) and a follicular cell (white arrow). (**e**) Negative control with no primary antibody. (**f**) The proliferation rate is expressed as the percentage of immunopositive follicular cells related to the total number of follicular cells in each Bidderian follicle. Values are expressed as the mean of 5 sections ± SE. Prereproductive (PreR): $n = 5$; reproductive (R): $n = 3$; postreproductive (PostR): $n = 3$. Different letters indicate significant differences.

Seasonal variations in the apoptosis of germ cells and in spermatogonial proliferation have been described in only a few amphibian species. For instance, in urodeles such as *C. pyrrhogaster*, low temperatures induce the apoptosis of secondary spermatogonia, and testes lack spermatocytes during the winter, which corresponds to a quiescent period (Yazawa et al., 1999). In contrast, in the case of *T. marmoratus*, high levels of apoptosis were observed in testes during both winter and summer (Ricote et al., 2002). The seasonality of testicular apoptosis in caecilians has been studied in *I.*

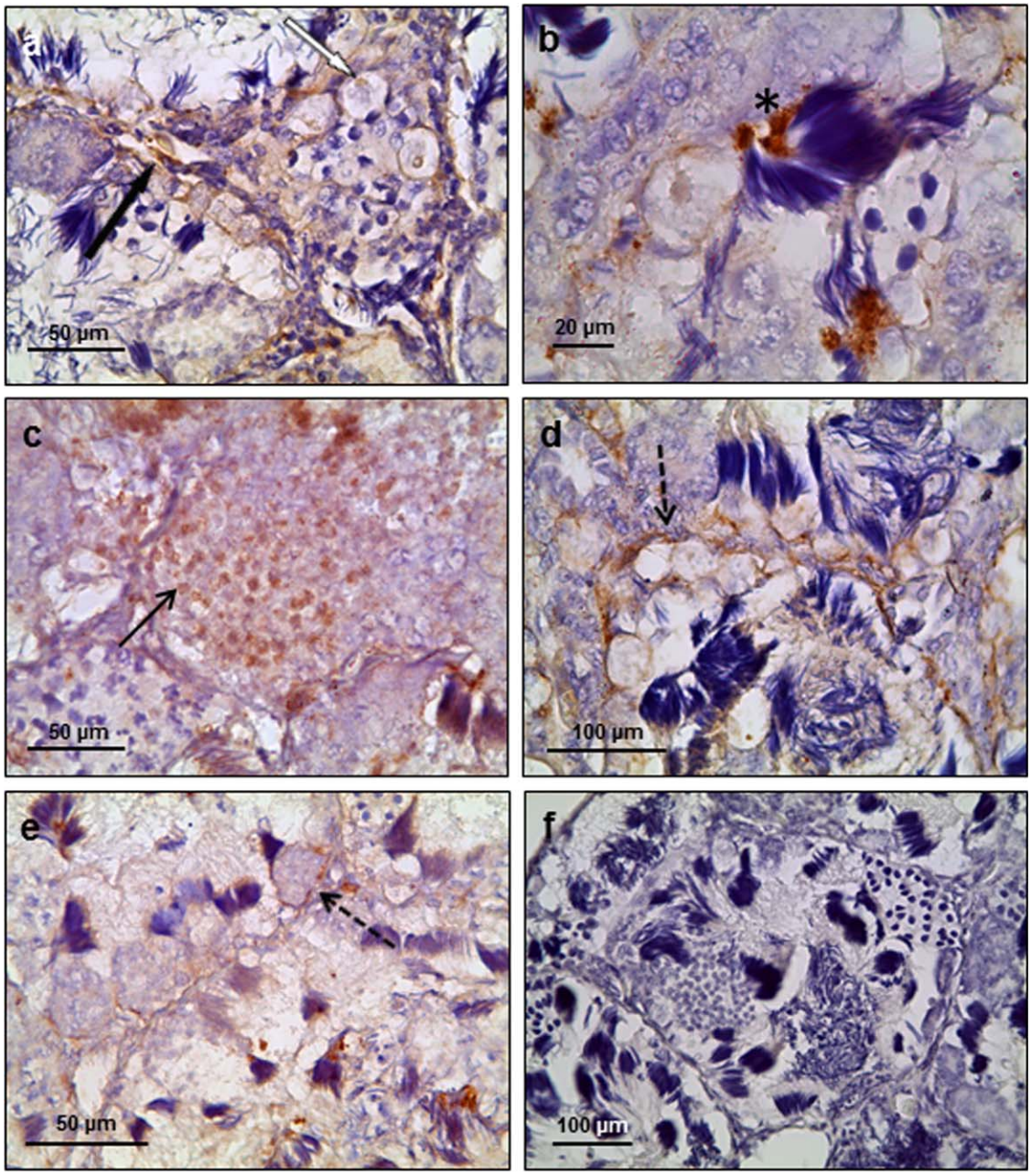

Fig. 5. *Rhinella arenarum*, immunohistochemistry for ERβ in the testes. Immunoreactivity was detected in (**a**) the interstitial tissue (black arrow), the nucleus of spermatogonia (white arrow), (**b**) the cytoplasm of Sertoli cells adjacent to spermatozoa (asterisk), (**c**) the nucleus of spermatocytes (open arrow), and (**d**) the cytoplasm of Sertoli cells surrounding cysts of spermatogonia I and (**e**) II (dashed arrows). (**f**) Negative control with no primary antibody.

tricolor, and apoptosis seems to be the mechanism of germ cell reduction during the regression phase (Smita et al., 2005). In anurans, seasonal variations in the apoptosis of germ cells have been analyzed in *L. catesbeianus*. In males of this species, there is a high production of spermatocytes during the winter, followed by the completion of meiosis, spermiogenesis, and spermiation during spring (breeding season). This is followed by active spermiogenesis/spermiation and early spermatogenesis during the summer (Sasso-Cerri et al., 2004). This last phase at the end of the breeding season is characterized by high levels of apoptosis (Sasso-Cerri et al., 2006). The results of this research show that testicular apoptosis occurs during the postreproductive season in late spermatogenic-stage cells such as spermatocyte Types I and II and spermatids. However, unlike what has been described for *T. marmoratus* and

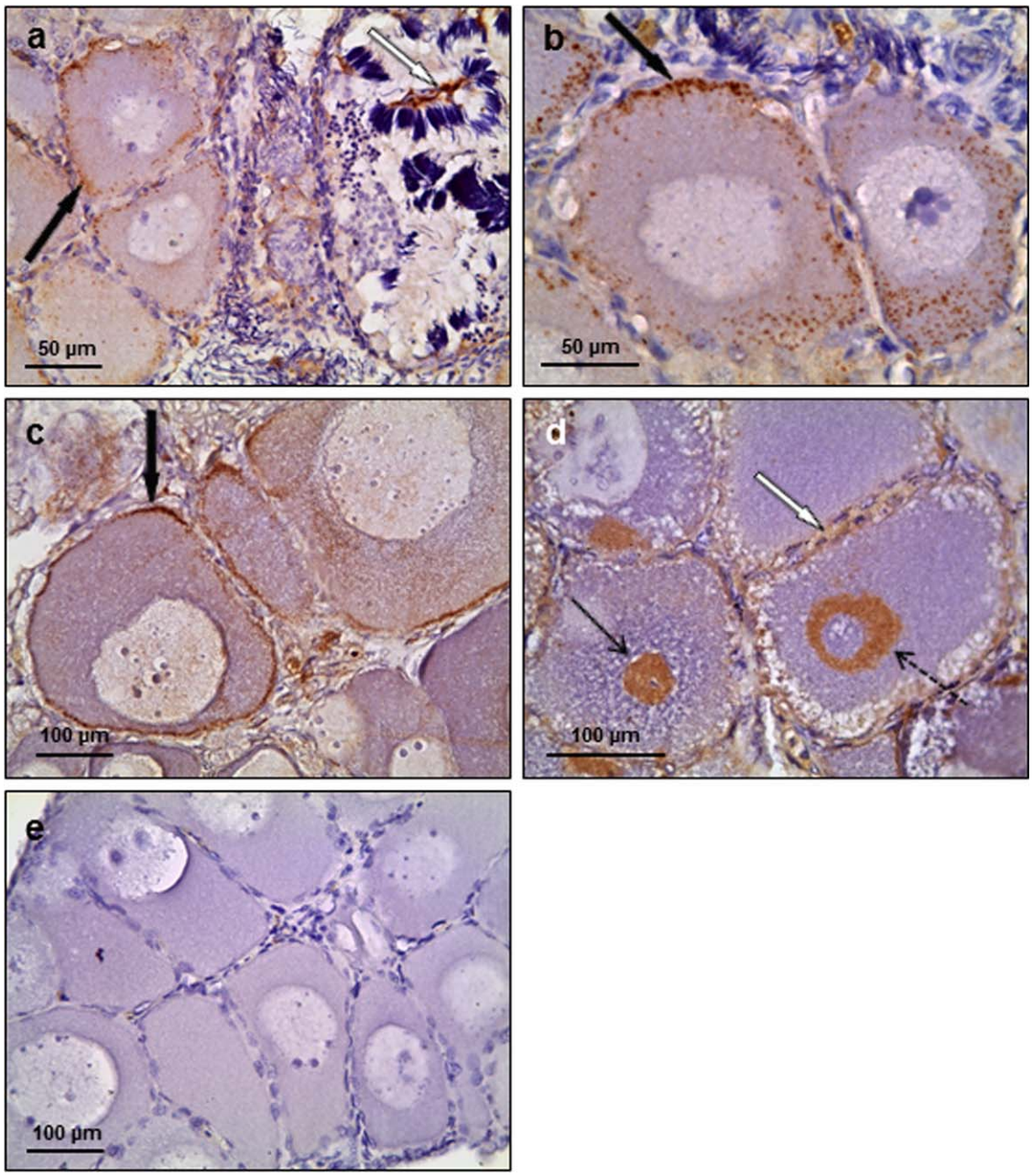

Fig. 6. *Rhinella arenarum*, immunohistochemistry for ERβ in BOs. (**a**, **b**) Immunoreactivity was detected in the periphery of ooplasm in previtellogenic follicles of BOs from animals collected during the reproductive season (black arrow). The white arrow shows immunoreactivity in Sertoli cells of the testicular lobule adjacent to BO. (**c**) Immunoreactivity in the periphery of the ooplasm was also detected in previtellogenic follicles from the prereproductive season. (**d**) Nuclear (black arrow) and perinuclear (dashed arrow) immunoreactivity was detected in oocytes of early vitellogenic follicles of BOs from animals collected in the prereproductive season. Immunoreactivity was also detected in the cytoplasm of follicular cells (white arrow). (**e**) Negative control with no primary antibody.

C. pyrrhogaster, apoptotic spermatogonia were not detected in *R. arenarum*. On the contrary, spermatogonial proliferation occurs mostly in the reproductive season in this species. These results are consistent with evidence reported in *P. esculentus* because in both species, proliferation is at its maximum during the breeding season (Raucci and Di Fiore, 2007).

In most vertebrates, estrogens play a central role in regulating spermatogenesis and testicular function. Particularly in amphibians, estradiol (E_2) has been suggested to regulate spermatogonial proliferation in the testes. In *P. esculentus*, estrogens are essential for regulating proliferation, because in vitro treatment with E_2 stimulates spermatogonial mitosis (Minucci et al., 1997;

Cobellis et al., 1999; Chieffi et al., 2000b; Stabile et al., 2006). However, in other anuran species, E_2 does not seem to be involved in the control of germ cell proliferation. In *R. arenarum*, in vitro treatment with E_2 did not stimulate spermatogonial proliferation (Scaia et al., 2015), while in vivo exposure of *L. pipiens* adults to E_2 delayed the formation of Type II spermatogonia (Tsai et al., 2003).

Taking into account that plasma E_2 levels are highest during the postreproductive season (Scaia et al., 2011), the results presented here are consistent with previous evidence that suggests in vitro treatment with E_2 induces the apoptosis of spermatogenic cysts (Scaia et al., 2015). Notably, the seasonal variations described here are consistent with those reported in *L. catesbeianus*, with apoptosis being highest during the summer in both species (Sasso-Cerri et al., 2006). These results also suggest that cysts in late spermatogenesis do not remain quiescent until the following year but are removed by apoptosis instead, and that spermatozoa from the following year are produced during a new wave of spermatogenesis.

While in *P. esculentus*, belonging to Ranidae, plasma E_2 is produced in the testes (Varriale et al., 1986; Fasano et al., 1989), in *R. arenarum*, plasma E_2 does not have a testicular origin and is mainly produced by BO (Scaia et al. 2013). Seasonal variations in the activity of aromatase in BO indicate that the total activity is lower during the prereproductive season and, because aromatase is expressed in follicular cells surrounding Bidderian follicles, this could be related to the number of follicular cells (Scaia et al., 2011, 2013). The present study aims to analyze for the first time, cellular proliferation in the follicles of BO. The results of this study show proliferation in both the flat and round follicular cells surrounding Bidderian oocytes. Both types of follicular cells have been recently described in the BO of *Rhinella schneideri* (Freitas et al., 2015). These authors have suggested that although most follicular cells are flat, round cells can be found surrounding oocytes in advanced developmental stages. However, here we report the presence of both round and flat follicular cells in previtellogenic follicles, suggesting that there could be interspecific differences in the distribution of these two types of follicular cells in BO. Our results also show that follicular cell proliferation occurs almost exclusively during the reproductive and postreproductive seasons, when plasma levels of E_2 and total aromatase activity are increased, thus suggesting that the contribution of BOs to plasma E_2 could be related to the number of follicular cells proliferating in each follicle. In the prereproductive season, there is very low proliferation in follicular cells, but there is an intense immunoreactivity to BrdU in oocyte nuclei. These results suggest that DNA synthesis could occur in Bidderian oocytes of animals during the prereproductive season, probably related to DNA duplication during interphase, prior to prophase I. However, more studies regarding nuclear morphology are necessary to clarify this matter.

Although estrogens are important for the regulation of amphibian spermatogenesis (Minucci et al., 1997; Cobellis et al., 1999; Chieffi et al., 2000b; Scaia et al., 2015), there are very few studies on estrogen receptors. ERβ has been studied in relation to spermatogonial mitosis in the testes of *P. esculentus* and *L. catesbeianus* (Stabile et al., 2006; Caneguim et al., 2013). As a consequence, and taking into account that E_2 induces apoptosis and modulates testicular steroidogenesis in *R. arenarum* (Scaia et al., 2015), we analyzed the localization of estrogen receptors through immunohistochemistry, making use of an antibody previously used in the testes of *P. esculentus* (Stabile et al., 2006). Similar to evidence on *P. esculentus*, the results of this study showed ERβ in both the interstitial and germinal tissues. However, both species differ in regard to the localization in the germinal cells. In *R. arenarum* ERβ was found mostly in Sertoli cells, while in *P. esculentus*, ERβ was localized in spermatogonia, spermatocytes, spermatids, and Sertoli cells. Moreover, in *P. esculentus* previous research suggest that immunoreactivity in spermatogonia, spermatocytes, and spermatids is less intense in testes during the prereproductive season. In *R. arenarum*, although ERβ was found in the nuclei of some spermatogonia and spermatocytes, immunoreactivity was detected, in most cases, in Sertoli cells, either surrounding spermatogenic cysts or associated to spermatozoa. It is worth mentioning that no seasonal differences were detected in the localization of the receptor. Considered together, these results suggest that the apoptotic effect of E_2 in the testes of *R. arenarum* could be mediated by ERβ localized in Sertoli cells. Nevertheless, more studies analyzing the presence of other estrogen receptors such as ERα and GPR30 could clarify the relationship between estrogens and the regulation of spermatogenesis.

The present study showed that in BOs, previtellogenic follicles have ERβ localized in the periphery of the ooplasm, while oocytes in early vitellogenesis show nuclear and perinuclear immunoreactivity. ERβ was also found in the cytoplasm of follicular cells from early vitellogenic follicles but not previtellogenic follicles. As follicular cells in the BO produce E_2, the results here could suggest a possible autocrine effect of E_2. Furthermore, this is not the first report of steroid receptors in the ooplasm of oocytes. Androgen receptors have been localized in the cytoplasm of oogonia and oocytes in rodents, suggesting that androgens could be involved in the regulation of oogenesis (Galas et al., 2003). ERβ was also detected in the

ovaries of mice and pigs, despite the fact that ooplasm localization was only detected in mice (Zhao et al., 2008). Similarly, the presence of ERβ in the ooplasm and in oocyte nuclei in BO suggests that E_2 could regulate Bidderian oogenesis. To our knowledge, ours is the first study describing estrogen receptors in amphibian oocytes and the presence of steroid receptors in BOs of bufonids. McCoy et al. (2008) suggested that malformations and highly developed BOs in animals from agricultural areas were the consequence of a defect in testicular function because the testes were not properly inhibiting BO development and vitellogenesis. The results of the present study suggest that highly developed BOs might be the consequence of an increased intra-oocyte E_2 level and/or of the interaction of estrogenic compounds with Bidderian estrogen receptors. However, further research on the presence of other estrogen receptors and an in vitro approach could clarify the role of estrogens in regulating oogenesis and steroidogenesis in BO.

In conclusion, this study shows that spermatogonial proliferation is higher during the reproductive season and that cysts in late stages of spermatogenesis (spermatocytes and spermatids) are removed from the testes by apoptosis during the postreproductive season. Regarding BO, our results suggest that the proliferation of follicular cells is higher during the reproductive and postreproductive seasons, when plasma E_2 levels achieve maximal values. In addition, our results also show that ERβ is located in the cytoplasm of Sertoli cells in testes, as well as in the ooplasm, in the nuclei of oocytes and in the cytoplasm of follicular cells in the BO. Altogether, the findings of this study suggest that seasonal variations in testicular apoptosis and cellular proliferation could explain the progression of spermatogenesis and that estrogens may regulate both spermatogenesis and oogenesis in the testes and BOs of *R. arenarum* adult males.

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