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Immunoexpression of aromatase and estrogen receptors β in stem spermatogonia of bullfrogs indicates a role of estrogen in the seasonal spermatogonial mitotic activity

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ABSTRACT

Bullfrog stem spermatogonia, also named primordial germ cells (PGCs), show strong testosterone immunolabeling in winter, but no or weak testosterone immunoexpression in summer. Thus, the role of testosterone in these cells needs to be clarified. In this study, we proposed to evaluate whether PGCs express aromatase and estrogen receptors, and verify a possible role of estrogen in PGCs seasonal proliferation. Testes of male adult bullfrogs, collected in winter (WG) and summer (SG), were fixed and embedded in historesin, for quantitative analysis, or paraffin for immunohistochemistry (IHC). The number of haematoxylin/eosin stained PGCs/lobular area was obtained. Proliferating cell nuclear antigen (PCNA), aromatase, estrogen receptor β (ERβ) and PCNA/ERβ double immunolabeling were detected by IHC. The number of PCNA-positive PGCs and the histological score (HSCORE) of aromatase and ERβ immunolabeled PGCs were obtained. Although the number of PGCs increased significantly in WG, a high number of PCNA-positive PGCs was observed in summer. Moreover, aromatase and ERβ HSCORE was higher in SG than WG. The results indicate that PGCs express a seasonal proliferative activity; the low mitotic activity in winter is related to the maximal limit of germ cells which can be supported in the large lobules. In SG, the increased ERβ and aromatase HSCORE suggests that testosterone is converted into estrogen from winter to summer. Moreover, the parallelism between the high PGCs mitotic activity and ERβ immunoexpression suggest a participation of estrogen in the control of the PGCs seasonal proliferative activity which guarantee the formation of new germ cysts from summer to next autumn.

1. Introduction

Additionally to endogenous factors, temperature and photoperiod are also responsible for regulating the seasonal reproductive cycle in amphibians [25,35]. In bullfrogs, *Lithobates catesbeianus* (Shaw, 1802), previously named *Rana catesbeiana*, spermatogenesis and steroidogenesis change according to seasons [38,39]. During the quiescent reproductive period – winter – the large seminiferous lobules are filled with numerous cysts of spermatogonia and type I spermatocytes. In contrast, spermiogenesis and spermiation takes place in spring/summer and the lobule diameters decrease. Thus, from summer to next autumn, a new spermatogenic process initiates in the post-spermiation lobules leading to the seminiferous lobules increase in autumn/winter [37,38]. Additionally to spermatogenesis, steroidogenesis also undergoes seasonal changes in bullfrogs [38,39]. In summer, the small lobules containing mainly spermatids and spermatozoa are surrounded by a developed interstitial tissue containing Leydig-like cells with numerous lipid inclusions [38] and strongly testosterone immunolabeled cytoplasm [39]. Otherwise, in winter, the large lobules are surrounded by a thin layer of interstitial tissue in which a weak testosterone immunolabeling is observed in the fibroblast-like cells [39]. Thus, the way by which intratesticular testosterone concentration is maintained during the period of low steroidogenic activity has been questioned [16,33]. A strong testosterone immunolabeling has been observed in the cytoplasm of bullfrogs primordial germ cells – PGCs (stem spermatogonia) during winter [39]. Moreover, androgen receptors (ARs) and sex-hormone binding globulin (SHBG) have also been immunohistochemically detected in these cells during this seasonal period, confirming that

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testosterone is maintained by AR and/or SHBG and plays a role in PGCs [4].

In rats, studies have demonstrated that testosterone inhibits the process of spermatogonial differentiation [28]. Excess [44] or even physiological levels [28,43] of intratesticular testosterone could inhibit the spermatogonial proliferation and differentiation. In non-mammalian vertebrates, as frogs, testosterone also seems to inhibit the mitotic activity of spermatogonia [47]. Thus, it has been speculated that testosterone plays a role in the PGCs proliferative control avoiding the formation of new spermatogonial cysts in winter when the number of germ cells reach a maximal limit to be supported within the large seminiferous lobules [4,39].

In summer, while the developed interstitial tissue contains strong testosterone-positive Leydig-like cells, PGCs show a weak or none testosterone immunoexpression [39]. In the testes, the irreversible conversion of testosterone into estrogen by aromatase, also called CYP19, has been well documented [45]. The expression of aromatase has been demonstrated in germ cells of different mammal species confirming the existence of an additional source of estrogens in the testes [5,23,30]. The effective biological function of estrogen depends on its binding to the estrogen receptors (ERs): ERα and ERβ [5]. While ERα is found in Leydig cells and in epithelial cells of deferens ducts from several species, ERβ is widely expressed throughout the male genital system [18,19]. Thus, considering that testosterone is converted into estrogen by aromatase and that estrogen has a role during spermatogenesis of different vertebrates [1,3,34,40,48], the absent or weak testosterone immunoexpression in PGCs during summer could be due to testosterone conversion into estrogen by aromatase [39].

Studies have demonstrated an important role of estrogen on the spermatogenic process, including gonocyte [24] and spermatogonial [21,22] proliferation, spermatocytes maturation, spermatids differentiation [36] and spermatozoa maturation [26] and release [12]. In amphibians, it has been demonstrated that estrogen acts in the early spermatogenic process [3,34] and stimulates the mitotic activity of primordial spermatogonia of *Rana esculenta* [31,32]. In this regard, it has been demonstrated that spermatogonial proliferation can be triggered by an estrogen-mediated mechanism which involves the Fos protein activity [13,14].

Considering that steroid hormones seem to play a role in the spermatogonial proliferation, we purposed to investigate PGCs mitotic activity in bullfrogs during two distinct seasonal spermatogenic periods – winter and summer – and correlate it with the ERβ immunoexpression. With the aim to verify whether testosterone is converted into estrogen in these cells, the immunoexpression of aromatase in PGCs was also investigated.

2. Materials and methods

2.1. Animals

The principles of laboratory animals care (NIH publication 85–23, 1985) and national laws on animal use were observed. This study was authorized by Ethical Committee for Animal Research of the Federal University of São Paulo (UNIFESP), Brazil.

Ten adult male bullfrogs (*L. catesbeianus*) were maintained in the Aquaculture Center of the Veterinary School of São Paulo State University (Jaboticabal, São Paulo, Brazil; latitude: 21°15′17″S and longitude: 48°19′20″W) under natural thermic (20 °C/winter to 36 °C/summer) and photoperiod conditions. The bullfrogs (250 g) were fed housefly larva together with a commercial diet (Nutrimex, Mexico) twice a day. They were collected in February/summer and July/winter and distributed into two groups: summer (SG; $n = 5$) and winter (WG; $n = 5$), respectively.

2.2. Histological procedures

The animals were anaesthetized with 10% chloral hydrate. The testes were removed, sectioned in the equatorial region and immersed in 4% formaldehyde freshly prepared from paraformaldehyde (Merck; Germany) buffered at pH 7.4 in 0.1 M sodium phosphate. Some formaldehyde-fixed testicular pieces were embedded in glycol methacrylate [7], while other pieces were dehydrated and embedded in paraffin. The paraffin sections were subjected to immunohistochemical reactions, and the glycol methacrylate sections to PAS method or stained by H.E. for morphological and morphometrical analyses, respectively.

2.3. Immunohistochemical (IHC) reactions

Paraffin sections (6 μm thick) adhered to silanized slides were immersed in 0.001 M sodium citrate buffer pH 6.0 and maintained at 90 °C for 20 min in a microwave oven for antigen recovery. After inactivation of endogenous peroxidase in 3% hydrogen peroxide for 20 min, the slides were washed in 50 mM phosphate-buffered saline plus 200 mM of sodium chloride (PBS) pH 7.3 and, then, incubated with 2.5% horse serum (Vector Laboratories, Burlingame, California, USA) for 1 h, for detection of proliferating cell nuclear antigen (PCNA) antibodies, or with background sniper (Biocare Medical) for 15 min, for detection of aromatase (CYP19) and estrogen receptor β (ERβ).

2.3.1. PCNA

The slides were incubated overnight with primary mouse antibodies anti-PCNA (1:100; Biocare Medical, USA; CM152C) in a humidified chamber at 4 °C. After incubation with biotinylated anti-rabbit/mouse secondary antibodies (Vector Laboratories) for 35 min, the slides were incubated with Vectastain® Elite® ABC reagent (Vector Laboratories) for 35 min. The peroxidase activity was revealed with 0.06% 3,3′-diaminobenzidine (DAB; Sigma, USA) plus 100 μL of hydrogen peroxide and the nuclei were counterstained with Carazzi's hematoxylin. For negative controls, the incubation step with primary antibody was replaced by incubation in mouse non-immune serum.

2.3.2. ERβ and CYP19

The sections were incubated overnight with the primary rabbit antibodies anti-ERβ (1:150; Upstate, USA; 06–629) and primary goat antibodies anti-CYP19 (1:200; Santa Cruz Biotechnology, USA; H-18; sc-14244) in a humidified chamber at 4 °C. For anti-ERβ, the reaction was amplified using a Mach 4 Universal HRP-Polymer kit (Biocare Medical; USA). According to the manufacturer, the sections were incubated in mouse probe reagent for 15 min and, subsequently, they were incubated with HRP-Polymer for 30 min. For anti-CYP19, the sections were incubated with biotinylated link universal (LSAB+; Dako; USA) for 30 min, and, subsequently, they were incubated with streptavidin-HRP (LSAB+; Dako; USA) for 30 min. After washes in PBS, both reactions were revealed by Betazoid DAB (Biocare Medical; USA), and the sections were counterstained with Carazzi's haematoxylin. For the negative controls, all steps were performed following the same protocol, except that the step of incubation in the primary antibodies was replaced by incubation in rabbit or goat non-immune serum. With the aim to confirm the specificity of antibody used for aromatase detection, newborn rat calvariae sections was used as positive control.

2.4. Double immunolabeling for detection of ERβ and PCNA

Testicular paraffin sections from SG bullfrogs adhered to silanized slides were immersed in 0.001 M sodium citrate buffer pH

6.0 and maintained at 90 °C for 20 min in a microwave oven for antigen recovery. After inactivation of endogenous peroxidase in 3% hydrogen peroxide for 20 min, the slides were washed in PBS pH 7.3, and incubated with background sniper (Biocare Medical) for 15 min. The sections were incubated overnight with the primary rabbit antibodies anti-ERβ (1:150; Upstate, USA; 06–629) in a humidified chamber at 4 °C, and incubated with biotinylated link universal (LSAB+; Dako; USA) for 30 min. After incubation with streptavidin-HRP (LSAB+; Dako; USA) for 30 min, the slides were washed in PBS and they were revealed by Betazoid DAB (Biocare Medical; USA) for 2 min and washed in tap water for 5 min. Subsequently, the slides were incubated with 2% BSA plus 1% sodium azide for 1 h and, then, they were incubated overnight with primary mouse antibodies anti-PCNA (1:200; Biocare Medical, USA; CM152C) in a humidified chamber at 4 °C. After incubation with EasyLink One (EasyPath, Brazil; EP-12–20502) for 15 min, the reaction was revealed by Vector® VIP substrate kit for peroxidase (Vector, USA; SK-4600) for 20 min, and the sections were counterstained with haematoxylin.

2.5. Morphometrical analysis

2.5.1. Number of H.E. stained PGCs/mm^2 of seminiferous lobule

In five animals per group (SG and WG), seventeen fields from three non-serial glycol methacrylate sections stained by H.E. were captured using an Olympus camera (DP71) attached to an Olympus microscope (BX-51) at 695 × magnification. In each field, the seminiferous lobule areas were measured using the Image Pro-Express 6.0 software (Olympus) and the number of PGCs was counted. Thus, the number of PGCs per lobular area (mm^2) was calculated.

2.5.2. Number of PCNA-positive PGCs/mm^2 of seminiferous lobule

The number of PCNA-positive PGCs was counted in ten fields from three non-serial paraffin sections subjected to PCNA IHC reaction in five animals per group. The capture of images and the number of PGCs was obtained as described above.

2.6. Semiquantitative evaluation of ERβ and CYP19 immunoexpression

Histological score (HSCORE) values of ERβ and CYP19 immunoexpression were obtained by a semiquantitative analysis in which intensity and the distribution of specific ERβ and CYP19 antigen, respectively, in PGCs were evaluated by a double-blind test carried out by two observers. Three non-serial testicular sections were analyzed; from each section, 20 fields per animal were randomly selected and captured using an Olympus camera (DP71) attached to an Olympus microscope (BX-51) at 695×. Values were recorded as percentages of positively stained PGCs in each of four intensity categories which were denoted as 0 (no staining), 1+(weak), 2+(moderate), 3+(strong). For each testis, a HSCORE value was derived by summing the percentages of PGCs stained at each intensity category and multiplying that value by the weighted intensity of the staining using the following formula: HSCORE = ΣP_i $(i + 1)$, where P_i is the corresponding percentage of PGCs (according to the intensity score), and $i = 0$, 1, 2 or 3, which represents the intensity score [17,27].

2.7. Protein extraction

Bullfrog frozen testes were homogenized directly into lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), containing 5 ng/mL of each protease inhibitors – Pepstatin, Leupeptin, Aprotinin, Antipain and Chymostatin). The crude extracts were clarified by centrifugation at 10,000 rpm for 20 min. Protein concentrations were determined by Bradford assay (Bio-Rad).

2.8. Western Blot analysis

Total protein extracts (~30 μg) were mixed with an equivalent volume of Laemmli buffer and were boiled for 5 min at 95 °C before electrophoresis. The samples were subjected to SDS–PAGE (12% polyacrylamide). After electrophoresis, proteins were transferred to nitrocellulose membrane (GE healthcare). The membranes were treated for 4 h with blocking solution (10%, for PCNA, or 5%, for CYP19, no fat dry milk) in 1 × PBS/0,1% Tween 20 (PBS/T) and then were incubated overnight at 4 °C with the primary antibodies which were diluted in 1% and 0.5% blocking solution in PBS/T, respectively: anti-PCNA (1:500; Biocare Medical, USA; CM152C) and anti-CYP19 (1:200; Santa Cruz Biotechnology, USA; H-18; sc-14244). After washes in PBS/T, membranes were incubated with 1:7500 anti-mouse (Sigma–Aldrich, USA, A9044) and anti-goat (Santa Cruz Biotechnology, USA; sc-2768) peroxidase antibodies, diluted with 1% (for PCNA) and 0.5% (for CYP19) blocking solution in PBS/T for 1 h (at room temperature) and the reactions were detected using the enhanced chemiluminescence (ECL) system.

2.9. Statistical analysis

Data were analyzed using the software SigmaStat 3.2 version. According to the distribution of morphometric data, the differences between groups were analyzed by *t*-Student or Kruskall–Wallis test. The significance level was set as $p \leqslant 0.05$.

3. Results

3.1. Histological evaluation

The testes from WG bullfrogs showed large seminiferous lobules filled mainly with spermatocytes cysts. The thin interstitial tissue showed scarce fibroblast-like cells (Fig. 1a). In SG, the testes showed mainly small lobules containing cysts of early and elongated spermatids as well as spermatozoa. In this group, the interstitial tissue was more developed than WG and showed numerous Leydig-like interstitial cells (Fig. 1b). In the testicular sections from both group animals, PGCs – the largest cell types – showed a large polymorphic nucleus and evident nucleoli; these cells were located in the lobular periphery and were surrounded by Sertoli cells (Fig. 1a and 1b; insets).

In each WG and SG animal, PCNA immunoexpression was observed in the nuclei of PGCs, spermatogonia and spermatocytes (Fig. 1c and 1d). However, PCNA-positive PGCs were more frequent in the testicular sections from SG (Fig. 1d) than WG (Fig. 1c) bullfrogs. PCNA-positive nuclei were not found in the negative controls (Fig. 1e).

ERβ immunoexpression was observed in the PGCs as well as in the other cells of germinal lineage (Fig. 2a and 2b). Whereas none/weak ERβ immunoexpression was found in WG PGCs (Fig. 2a); a moderate/strong ERβ immunoexpression was usually observed in SG PGCs (Fig. 2b). A strong cytoplasmic ERβ and nuclear PCNA immunoexpression were observed in the double immunolabeled PGCs from SG bullfrogs (Fig. 2g and 2h). In the sections used as negative controls ERβ immunoexpression was not found (Fig. 2i).

The immunoexpression of CYP19 was weak/moderate in the WG PGCs (Fig. 2c and 2d) while, in SG, these cells were usually strongly immunolabeled (Fig. 2e and 2f). CYP19 immunostaining was not found in the sections used as negative controls (Fig. 2j).

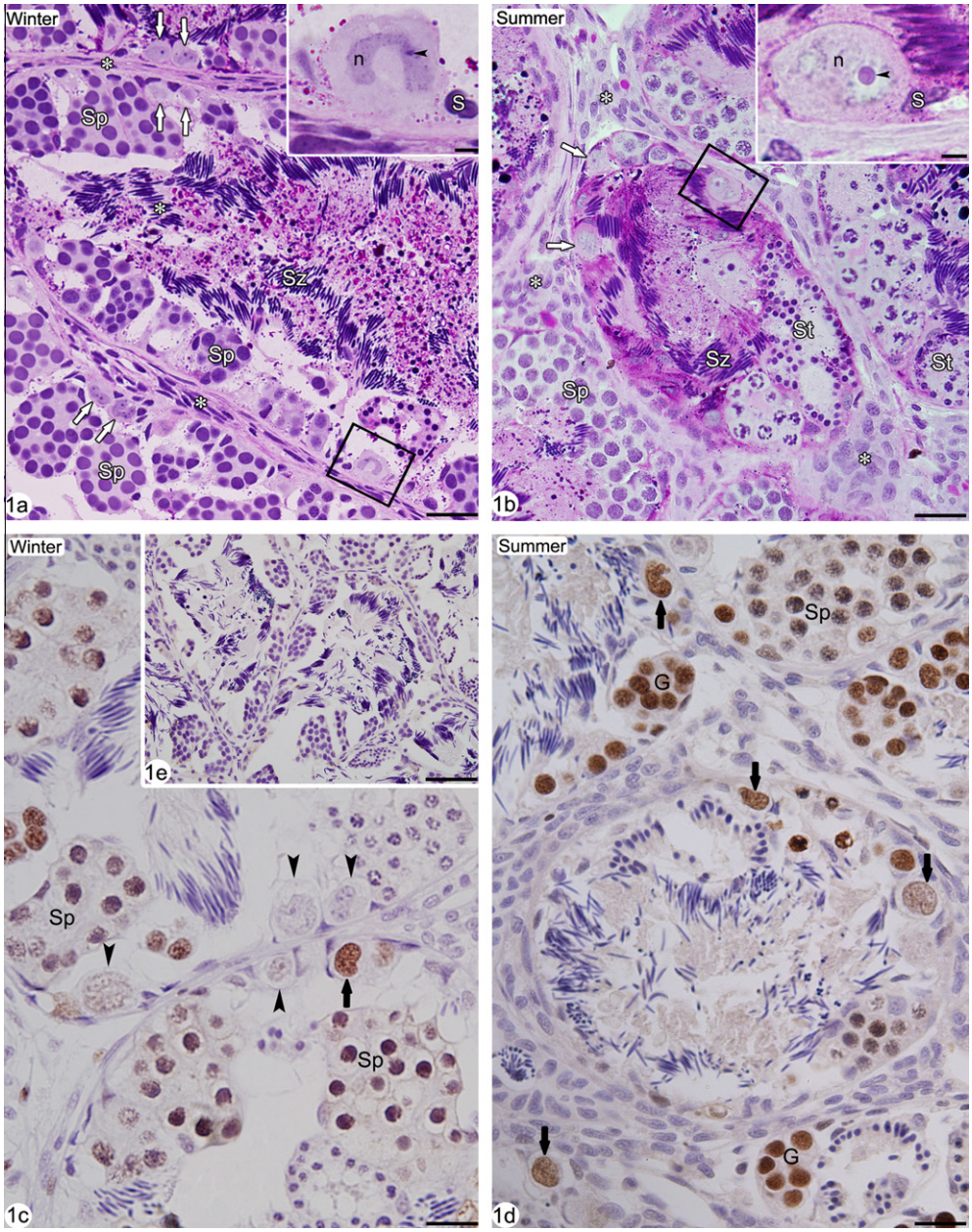

Fig. 1. Photomicrographs of seminiferous lobules of bullfrogs from WG (1a, 1c and 1e) and SG (1b and 1d) submitted to PAS method (1a and 1b), PCNA immunohistochemistry (1c and 1d) and respective negative control (1e). In 1a and 1b, seminiferous lobules are surrounded by interstitial tissue (asterisks). PGCs (arrows) with large polymorphous nucleus (n) and nucleoli (arrowhead) are surrounded by Sertoli cells (S). (Sp) Spermatocytes; (St) Spermatids; (Sz) Spermatozoa. In 1c and 1d, PCNA-immunolabeled PGCs (arrows), spermatogonia (G) and spermatocytes (Sp) are observed. Some PGCs are not immunolabeled (arrowheads) in WG (Fig. 1c). In 1e, PCNA immunolabeling is not observed. Bars: 1a and 1b (35 μm); 1c and 1d (15 μm); 1e (70 μm); insets (5 μm).

Otherwise, numerous CYP19-positive osteoblasts were observed in the bone surface of rat calvariae (Fig. 2k).

3.2. Morphometrical analyses

According to Fig. 3, the number of PGCs per seminiferous lobules area (mm^2) in WG was significantly higher than SG. However, a significant increase in the number of PCNA-positive PGCs/mm^2 was observed in SG (Fig. 4).

3.3. Hscore

Either ERβ or CYP19 HSCORE was higher in SG in comparison to WG; however, only the increase in the ERβ HSCORE was statistically significant (Figs. 5 and 6).

3.4. Western Blot

The results of the Western blot analysis, which was used to assess the specificity of the antibodies in the bullfrog testes, are shown in Fig. 7. All primary antibodies are polyclonal antibodies against antigens of human origin. In this study, Western blot analyses of protein extracts demonstrated two CYP19 immunoreactive bands at 55 kDa and a PCNA immunoreactive band at 35 kDa whose molecular weights are similar to that described for human proteins.

4. Discussion

The immunohistochemical and morphometrical results indicate that PGCs mitotic activity changes according to seasonality and

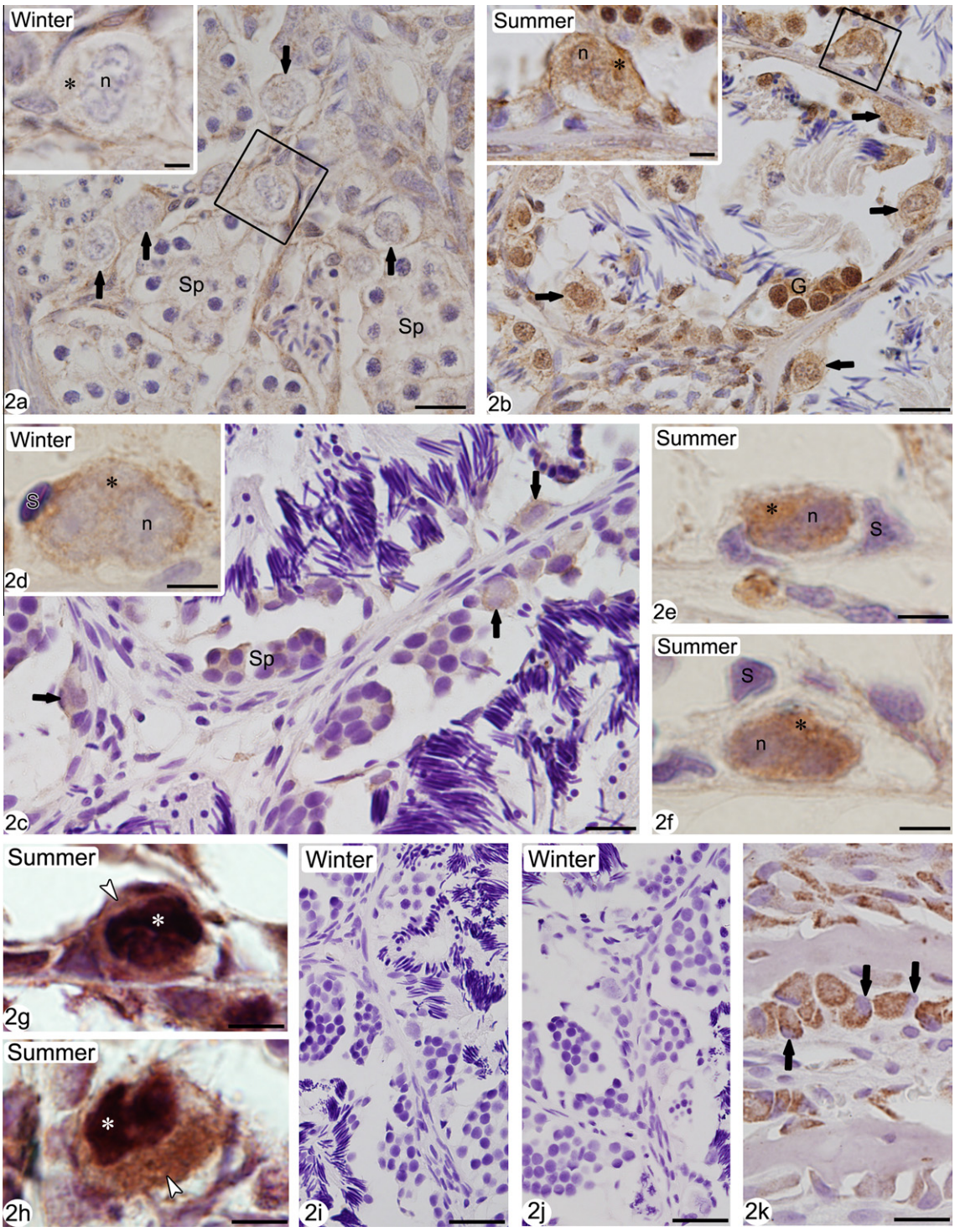

Fig. 2. Photomicrographs of portions of seminiferous lobules of bullfrogs from WG (2a, 2c, 2d, 2i and 2j) and SG (2b, 2e–2h) submitted to ERβ (2a and 2b), CYP19 (2c–2f) immunohistochemistry and respective negative controls (2i and 2j). ERβ/PCNA double immunolabeling (2 g and 2 h). Newborn rat calvariae section used as positive control to CYP19 immunohistochemistry (2 k). In 2a and 2b, weak (2a) and strong (2b) ERβ immunoexpression in PGCs (arrows), spermatogonia (G) and spermatocytes (Sp). (n) Nucleus. In 2c (winter), note a moderate CYP19 immunolabeling in spermatocytes (Sp) and PGCs (arrows). In 2d–2f, PGCs show moderate (2d) and strong (2e, 2f) CYP19 immunoexpression (asterisks). (n) Nucleus; (S) Sertoli cells. In 2g and 2 h, double immunolabeling for detection of PCNA (asterisks) and ERβ (arrowheads) in PGCs. In 2i and 2j, no ERβ (2i) and CYP19 (2j) immunostaining is observed. In 2k, CYP19-positive osteoblasts are observed (arrows). Bars: 2a–2c and 2k (20 μm); 2d–2 h (6 μm); 2i and 2j (40 μm); insets (5 μm).

seems to be regulated by steroid hormones (testosterone and estrogen).

The antibodies immunospecificity against human PCNA and CYP19 used in the IHC reactions was validated since Western blot analysis showed that PCNA and CYP19 immunoreactive bands were of similar size to those observed in other species [6,9,10,20]. Moreover, in the sections of calvariae bone, the osteoblasts, another site of high aromatase expression [45], showed enhanced and specific CYP19 immunolabeling. Regarding ERβ immunoblotting, as demonstrated in our previous study, the bullfrog testicular protein ex-

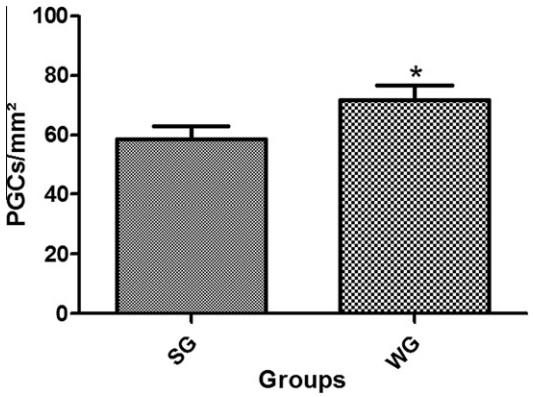

Fig. 3. Number of primordial germ cells (PGCs) per lobular area (mm^2) in the animals collected during summer (SG) and winter (WG). Values expressed as mean ± SEM. $^*p \leqslant 0.05$.

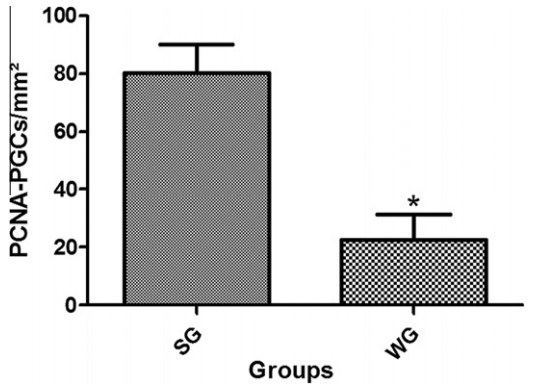

Fig. 4. Number of PCNA-positive primordial germ cells (PCNA-PGCs) per lobular area (mm^2) in the animals collected during summer (SG) and winter (WG). Values expressed as mean ± SEM. $^*p \leqslant 0.05$.

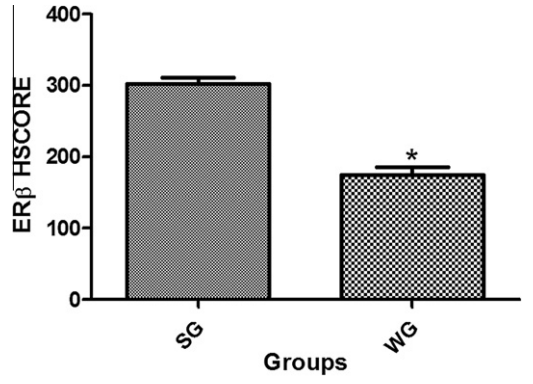

Fig. 5. Semiquantitative values (HSCORE) of ERβ primordial germ cells (PGCs) from animals collected during summer (SG) and winter (WG). Values expressed as mean ± SEM. $^*p \leqslant 0.05$.

tracts yielded two immunoreactive bands of approximately 55 kDa which are also coincident to other species [4].

The morphometrical results showed that the number of PGCs/mm^2 of seminiferous lobule from WG animals was significantly higher than in SG. This increase was due to the PGCs mitotic activity during summer since the number of PCNA-positive PGCs increased significantly in this period. This result is in agreement

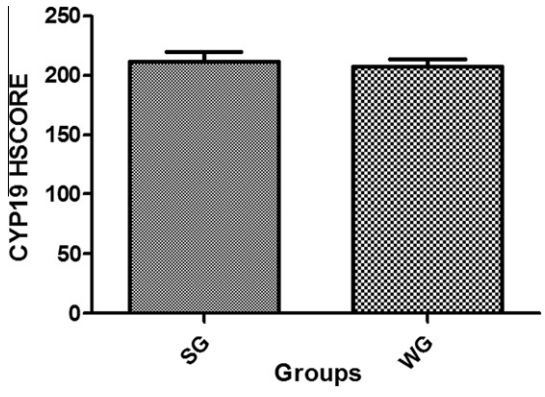

Fig. 6. Semiquantitative values (HSCORE) of CYP19 primordial germ cells (PGCs) from animals collected during summer (SG) and winter (WG). Values expressed as mean ± SEM.

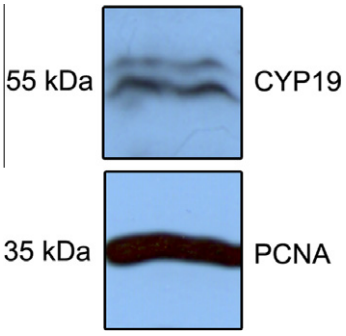

Fig. 7. Western blot analysis of CYP19 and PCNA proteins in the testicular extract of *L. catesbeianus*. CYP19 and PCNA immunoreactive band are observed at 55 and 35 kDa, respectively.

with previous study in which distinct phases of the cyclic spermatogenic processes has been demonstrated during the seasonal periods [38]. Thus, in winter, the large seminiferous lobules of *L. catesbeianus* show germ cysts of spermatogonia and type I spermatocytes while, in spring/summer, spermiogenesis and spermiation takes place and the lobule diameters decrease. Moreover, a new spermatogenic process initiates, from PGCs mitotic activity, in the post-spermiation lobules; then, the seminiferous lobules increase and reach maximal diameters in autumn/winter [38]. Additionally to spermatogenesis, steroidogenesis also undergoes seasonal changes [38,39]. While in winter the large lobules are surrounded by a thin layer of interstitial tissue containing fibroblast-like cells, in summer, the small lobules containing mainly spermatids and spermatozoa are surrounded by a developed interstitial tissue containing Leydig-like cells. Regarding the maximal enlargement of the seminiferous lobules in winter, it is not clear the mechanism by which PGCs mitotic activity is down regulated when the number of germ cells reaches a maximal limit to be supported within the lobules. It is important to emphasize that, in winter, although the scarce interstitial tissue is weakly testosterone immunolabeled, a strong testosterone immunoexpression has been demonstrated in PGCs during this period, but not in summer [39]. Moreover, AR and SBHG were also detected in the WG bullfrogs PGCs cytoplasm, suggesting that testosterone is maintained by these proteins and has a role in these cells [4]. It has been suggested that spermatogonial proliferation and differentiation in rodent testes could be inhibited by physiological levels [28,43] or even an excess [44] of intratesticular testosterone. In frogs, van Oordt and Basu [47] have also demonstrated that testosterone ex-

erts an inhibitory effect in the spermatogonial proliferation. Thus, our findings are in agreement with these previous studies since the number of PCNA-positive PGCs of WG frogs was significantly decreased when compared to the SG animals. In catfish, as in amphibians, PGCs and germ cysts are surrounded by Sertoli cells. Thus, the space required by the growing of the germ cysts from spermatogonia is primarily defined by Sertoli cells proliferation [41]. So, Sertoli cell-germ cells paracrine control together with a possible participation of testosterone can be related to the control of the mitotic activity of these cells in winter, avoiding an excessive formation of new spermatogonia cysts in the maximally enlarged seminiferous lobules.

An opposite process may occur in summer since an intense mitotic activity of PGCs, confirmed by the high number of PCNA-positive PGCs, was demonstrated in SG. During spring and summer, the seminiferous lobule diameters decrease and late spermatids are released into the excretory ducts, indicating spermiation [38]. Thus, an intense PGCs proliferative activity is necessary for the spermatogenic recrudescence from summer to next winter. In bullfrogs maintained under controlled temperature (26°C), the total duration of spermatogenesis is 40 days, and only spermatogonial process (mitosis) lasts around 3 weeks [42]. However, as spermatogenesis in these species changes seasonally, the total duration of spermatogenesis, specifically spermatogonial mitosis, would be different if the animals are maintained under environmental natural conditions, for example at summer (36 °C). In *R. esculenta*, a high number of PCNA-immunopositive spermatogonia has also been observed during breeding period [9]. In summer, photoperiod and temperature induces spermatogonial proliferation [10]. Additionally to environmental stimuli, endogenous factors may be directly related to the spermatogenesis control in these species. Numerous studies have demonstrated that estrogen plays an important role on spermatogenesis, including germ cell proliferation, differentiation and cell death by apoptosis [4,40]. In this study, additionally to other cells of germinal lineage, estrogen receptors (ERβ) was also detected in PGCs, indicating a direct participation of estrogen in these cells, as previously demonstrated [4]. Estrogen is derived from testosterone via aromatase (cytochrome P450 aromatase enzyme, also called CYP19) that catalyzes irreversibly the conversion of androgen into estrogen [45]. Studies have demonstrated the presence of aromatase in rat germ cells, confirming the existence of an additional estrogen source in the testes [5]. To our knowledge, CYP19 was detected for the first time in the cytoplasm of PGCs of anurans. The presence of this enzyme together with testosterone and ERβ in these cells indicates that testosterone is converted into estrogen. This is reinforced by the fact that a similar strong testosterone immunolabeling in PGCs, such as observed in WG, was not found in SG [39], but a strong ERβ and CYP19 immunolabeling in PGCs was observed in SG, indicating that testosterone could have been converted into estrogen between winter and summer.

The presence of ERβ in the male reproductive system has been well documented in germ cells, including spermatogonia of different mammal species [2,5,40,48] and also of amphibians [1,4,46]. Estrogens seem to participate in the control of spermatogenesis and, in murine, this hormone induces gonocytes proliferation [24]. Studies *in vitro* have demonstrated that estrogen may stimulate DNA synthesis in type A spermatogonia [49]. In anurans, estrogen plays a role during early spermatogenesis [3,34] and stimulates primordial spermatogonia mitotic activity in *R. esculenta* [8,15,29]. Estrogen induces the phosphorylation of members of mitogen-activated protein kinases (MAPKs) family named extracellular signal-regulated kinases (ERK1 and ERK2) and stimulates the spermatogonial proliferation via ERK1/2 pathways [8,10]. In addition to MAPKs, estrogen stimulates c-fos activity, a proto-oncogene, in spermatogonia nuclei of *R. esculenta* [14]. In this specie, the nuclear localization of Fos has been demonstrated during the recrudescent spermatogenic period [11]. Thus, spermatogonial proliferation may be triggered by an estradiol mediated mechanism involving Fos transport into the nucleus [14]. In the present study, the HSCORE of ERβ immunolabeled PGCs was significantly higher in SG than WG. Moreover, this high immunoexpression was coincident to the PCNA immunolabeling, as revealed by the double immunohistochemistry, and also to the mitotic index of PGCs; thus, estrogen can be directly related to the induction of PGCs proliferation in summer.

In conclusion, the presence of testosterone, estrogen receptors and aromatase in PGCs indicates that testosterone is converted into estrogen in these cells. PGCs proliferative activity undergoes seasonal changes; the low PGCs mitotic activity in winter is related to the maintenance of the maximal limit of germ cells to be supported in the large seminiferous lobules. On the other hand, the intense PGCs mitotic activity in summer is responsible for the spermatogenic recrudescence and seems to be an estrogen-dependent process. Thus, the seasonal spermatogenesis in *L. catesbeianus* depends on the spermatogonial stem cell activities which seem to be seasonally regulated by steroid hormones.

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