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# Testosterone-immunopositive primordial germ cells in the testis of the bullfrog, *Rana catesbeiana*

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## Abstract

In amphibia, steroidogenesis remains quiescent in distinct seasonal periods, but the mechanism by which spermatogenesis is maintained under low steroidogenic conditions is not clear. In the present study, testosterone location in the testes of Rana catesbeiana was investigated immunohistochemically during breeding (summer) and nonbreeding (winter) periods. In winter, the scarce interstitial tissue exhibited occasional testosterone immunopositivity in the interstitial cells but the cytoplasm of primordial germ cells (PG cells) was clearly immunopositive. By contrast, in summer, PG cells contained little or no immunoreactivity whereas strong immunolabelling was present in the well-developed interstitial tissue. These results suggest that PG cells could retain testosterone during winter. This androgen reservoir could be involved in the control of early spermatogenesis in winter and/or to guarantee spermiogenesis and spermiation in the next spring/summer. The weak or negative immunoreaction in the summer PG cells might reflect consumption of androgen reservoir by the intense spermatogenic activity from spring to summer. Thus, besides acting as stem cells, PG cells of R. catesbeiana could exert an androgen regulatory role during seasonal spermatogenesis.
**Key words** amphibia; androgen; immunohistochemistry; interstitial cells; seasonal spermatogenesis; stem cells.

## Introduction

In amphibians, the seasonal reproductive cycle is controlled by endogenous and external factors, namely temperature and photoperiod (Lofts, 1974; Rastogi et al. 1976; Pierantoni et al. 1984; Paniagua et al. 1990); thus, spermatogenesis and steroidogenesis vary according to the season. In the bullfrog *Rana catesbeiana*, during autumn and winter, the interstitial tissue is very sparse, whereas the seminiferous lobules contain numerous germ cysts at early spermatogenic stage (meiosis I). Both spermiogenesis and interstitial tissue increase gradually from winter to spring. In summer, when spermiogenesis and spermiation take place, the interstitial tissue reaches its maximal density and the interstitial cells contain numerous lipid inclusions. Therefore we have assumed that in winter (nonbreeding) early spermatogenesis is related to quiescent interstitial tissue whereas in summer (breeding) accentuated spermiogenesis and spermiation is related to active steroidogenesis (Sasso-Cerri et al. 2004). Spermiogenesis and spermiation are androgen-dependent processes in amphibians (Rastogi et al. 1976; Fraile et al. 1989; Paolucci et al. 1992). In urodeles, testosterone seems to be an important intratesticular factor for early spermatogenesis (Callard, 1992). The finding that the interstitial tissue is quiescent during the nonbreeding period raises the question of the source of testosterone for the maintenance of germ cells. Minucci et al. (1990) has suggested that, during testicular recrudescence in anurans, the testis may retain androgens for the maintenance of spermatogenesis. Furthermore, a testicular local positive feedback for androgen production may maintain testosterone within the testis during the period of low androgen biosynthesis (Pierantoni et al. 1984; Fasano et al. 1989). Androgen-binding protein (ABP) has been detected in the germinal compartment of

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amphibian testes (Singh & Callard, 1989) and it has been suggested that this protein could maintain adequate concentration of testosterone for the spermatogenic process during the period of testicular quiescence (Paniagua et al. 1990).

The hormonal control of spermatogenesis has been investigated in various vertebrates (van Oordt & Basu, 1960; Rastogi et al. 1976; Lecouteux et al. 1985; Fraile et al. 1989; Callard, 1992; Johnston et al. 2004), and studies detecting androgen receptors (AR) (Callard et al. 1985; Callard, 1992; Vornberger et al. 1994) and ABP (Felden et al. 1992; Gerard, 1995) have contributed to the understanding of hormonal control of both Sertoli and germ cells. In nonmammalian vertebrates, the presence of organelles characteristic of steroid synthesis in Sertoli cells (Guraya, 1976; Pudney & Callard, 1984; Fraile et al. 1989) has suggested that these cells can exert steroidogenic activity (Callard et al. 1985). In urodeles, AR have been detected in follicular (Sertoli) cells, primordial germ cells (PG cells), spermatogonia and spermatocytes (Arenas et al. 2001). Therefore, except for the interstitial cells, no testosterone expression has been detected immunohistochemically in Sertoli or germ cells of urodela (Fraile et al. 1989). There are no immunohistochemical reports concerning the *in situ* expression of testosterone in the testes of anurans. In the present study we proposed to investigate, immunohistochemically, the location of testosterone in the testes of *R. catesbeiana* during nonbreeding (winter) and breeding (summer) periods.

## Materials and methods

### Animals

Six male bullfrogs (*Rana catesbeiana*) were provided by the Aquaculture Center of the Veterinary School of Jaboticabal (São Paulo State University, Brazil). The animals were fed housefly larvae together with a commercial diet (Nutrimex, Mexico) twice a day. Adult animals weighing 250 g were investigated during winter (July; 20 °C) and summer (February; 36 °C) and were divided into two groups – GW and GS, respectively, containing three animals each. The study was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, 1985). The protocol of this study was approved by the Ethical Committee for Animal Research of the Federal University of São Paulo, Brazil.

### Histological procedure

The testes were perfusion-fixed with Bouin's fluid. Fifteen minutes before perfusion, the frogs were intraperitoneally injected with heparin (125 IU $kg^{-1}$ bw). The animals were anaesthetised with intraperitoneal injection of thiopental (Thiopentax; Cristália, SP, Brazil) and the thorax and abdomen were opened to expose the heart and testis. The vascular system was perfused via the heart first with Ringer's saline to remove the blood and then with Bouin's fluid for 20–30 min. The testes were removed, sectioned, immersed in the same fixative for 15 h, dehydrated and embedded in paraffin.

### Testosterone immunohistochemistry

Sections (6 μm thick) of testis adhered to silanized slides were immersed in 0.001 M sodium citrate buffer pH 6.0 and maintained at 90 °C for 40 min in a microwave oven for antigen recovery. The slides were washed, treated with 3% hydrogen peroxide to inactivate endogenous peroxidase, washed in 0.01 M phosphate-buffered saline (PBS) pH 7.2 then were incubated with antirabbit testosterone-BSA (ICN Biomedicals Inc., USA) diluted 1 : 1000 in PBS for 3 h in a humidified chamber at room temperature. The sections were washed in PBS and the immunoreaction detected by the Labelled StreptAvidin-Biotin system (LSAB-plus kit; DAKO Corporation, USA). Sections were incubated with multilink solution containing biotinylated mouse/rabbit/goat antibodies for 30 min at room temperature, washed in PBS, then incubated with streptavidin-peroxidase complex for 15 min at room temperature. Peroxidase activity was revealed by DAB (Diaminobenzidine; Sigma, USA) and hydrogen peroxide. Nuclei were counterstained with Mayer's or Harris's hematoxylin.

Sections of human testis submitted to the same immunohistochemical protocol were used as positive control. As negative control, the immunoreaction was performed by omitting the incubation of the frog testicular sections in the primary antibody.

## Results

In winter testes, few interstitial cells were present and the seminiferous lobules contained mainly PG cells, primary spermatocytes and late spermatids. The scarce interstitial tissue showed a sparse immunostaining to testosterone in the interstitial cells (Fig. 1A–D), whereas

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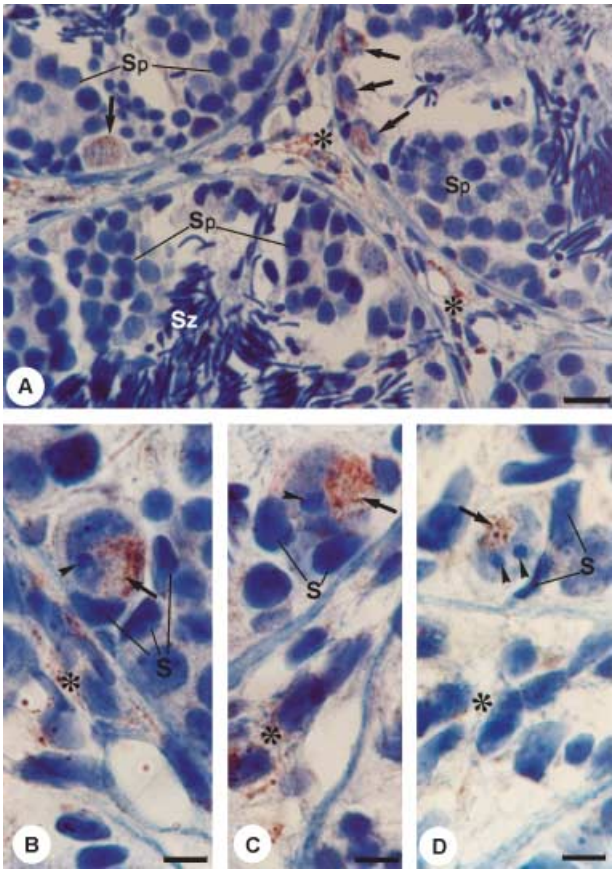


**Fig. 1** Light micrographs of testicular sections submitted to the immunohistochemistry for testosterone detection in winter group frogs. (A) The scarce interstitial tissue exhibits occasional immunolabeled interstitial cells (asterisks). In the seminiferous lobules, containing cysts of primary spermatocytes (Sp) and late spermatids (Sz), immunolabelling is evident in the PG cells (arrows). Bar, 33 μm. (B–D) Primordial germ cells surrounded by Sertoli cell nuclei (S) exhibit irregular nucleus with prominent nucleoli (arrowheads) and positive immunoreaction in the cytoplasm (arrows). Interstitial tissue (asterisks). Bars, 7 μm.

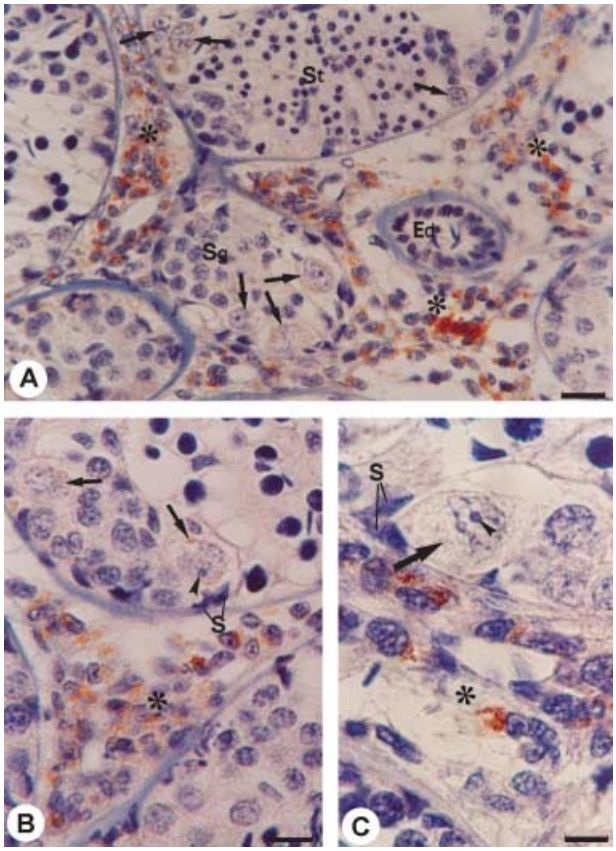


**Fig. 2** Light micrographs of testicular sections submitted to the immunohistochemistry for testosterone detection in the summer group frogs. (A) The developed interstitial tissue exhibits numerous interstitial cells strongly stained by the immunoreaction (asterisks). In the seminiferous lobule exhibiting cysts of round spermatids (St) and in the postspermiation lobule containing cyst of spermatogonia (Sg), the primordial germ cells show scarce or negative testosterone immunolabelling (arrows). Excretory duct (Ed), Bars, 40 μm. (B, C) PG cells with irregular nucleus and evident nucleolus (arrowheads) exhibit scarce (B; thin arrows) or negative (C; thick arrow) immunoreaction. A strong immunopositivity is observed in the interstitial cells (asterisks) adjacent to the seminiferous lobules. Sertoli cell nuclei (S). Bars: B, 13 μm; C, 7 μm.

a strong immunolabelling, characterised by brown–yellow stain, was observed in the cytoplasm of the PG cells in all samples analysed. These large cells, located next to the basal lamina, had an irregular nucleus with one or two nucleoli and were often surrounded by Sertoli cell nuclei (Fig. 1B–D).

In summer testes (Fig. 2A–C), the interstitial tissue was well developed and the seminiferous lobules contained germ cysts in late stages of spermatogenesis. Early spermatogenesis characterised by the presence of cysts of spermatogonia was also observed in the postspermiation lobules. In comparison to the winter group, PG cells were negative to the immunoreaction, except for some in which weak immunostaining of the cytoplasm was present; in contrast, numerous interstitial cells were strongly stained around the seminiferous lobules and excretory ducts.

In the human testicular sections, used as positive control, an intense immunolabelling was observed in the Leydig cells. No immunostaining was observed in the frog testicular sections used as negative control (data not illustrated).

## Discussion

Testosterone immunostaining was detected in the interstitial cells of all animals of both winter and

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summer group frogs. The presence of immunostaining in the poorly developed interstitial tissue in winter suggests that the interstitial cells exert a low steroidogenic activity during this period or maintain a small amount of the testosterone which was secreted during the previous summer. The source of testosterone for the maintenance of germ cells during the period of interstitial quiescence is unknown. ABP has been detected in the seminiferous lobules of amphibians (Singh & Callard, 1989), so ABP in the lobules could retain testosterone for the maintenance of spermatogenesis during testicular quiescence (Paniagua et al. 1990). In urodele testes, testosterone immunostaining has not previously been detected in cells of the germinal compartment (Fraile et al. 1989). The present study reports, for the first time, immunohistochemical detection of testosterone in the cytoplasm of testicular PG cells. These cells are large, well defined and located close to basal lamina of the seminiferous lobules (Sasso-Cerri et al. 2004). Immunostaining (similar to that observed in PG cells) was not observed in the other germ cells.

Androgen biosynthesis by PG cells has not been demonstrated in vertebrate testes and the action of testosterone on the spermatogonial development is not clear (Meachem et al. 2001). In mice, there is evidence that androgenic control of spermatogenesis must be mediated by Sertoli cells (Johnston et al. 2004), raising the question of the presence of AR in the germ cells. By contrast, abundant AR have been detected in the parts of testes containing stem cells and spermatogonia of fish and urodela (Callard, 1992) and AR have now been demonstrated in PG cells and spermatogonia of urodela (Arenas et al. 2001), as well as in spermatogonia of rainbow trout (Takeo & Yamashita, 2001). ABP has been also detected in the parts of urodele testes containing cysts of premeiotic stages (Singh & Callard, 1989), and Nakamura et al. (1994) have detected albumin (a protein that weakly binds testosterone in the plasma and interstitial fluid) in the spermatogonia of anurans. We therefore suggest that PG cells of *R. catesbeiana* can bind and retain testosterone during the winter and that this hormone could play a role in the control of these stem cells. In fish and urodeles, androgens appear to control premeiotic stages of spermatogenesis (Callard, 1992). In rats, testosterone inhibits spermatogonia differentiation (Meistrich & Shetty, 2003), and, in frogs, it inhibits spermatogonia mitotic activity (van Oordt & Basu, 1960). Thus, testosterone within PG cells might control mitotic activity of these cells, maintaining the maximal number of germ cells in the lobules during winter. This hypothesis is consonant with the fact that the seminiferous lobules of *R. catesbeiana* in winter are filled, predominantly, with cysts of primary spermatocytes and exhibit maximal diameters (Sasso-Cerri et al. 2004).

In addition to spermiogenesis and spermiation, testosterone appears to be also required by meiotic activity in seasonal breeders (Roelants et al. 2002). Thus, having in mind that the interstitial tissue is quiescent in winter when early spermatogenesis occurs, it is possible that PG cells maintain an androgen reservoir to guarantee meiosis completion and spermiogenesis in the next spring and become depleted of testosterone immunostaining during summer. In bullfrogs, an accentuated spermatogenic process appears to begin in the lobules after spermiation in spring/summer and continues until winter, when the lobules are maximally developed (Sasso-Cerri et al. 2004). Considering that this new spermatogenic cycle may be due to PG cells mitotic activity, testosterone in PG cells would be diluted after successive mitoses from spring to summer, explaining the negative or scarce immunoreaction observed in the summer PG cells. Another consideration is that, in the testes, aromatase can convert androgens into estrogens (van Pelt et al. 1999; Carreau, 2001). The role of estrogens during spermatogenesis has been confirmed in many vertebrates (Callard, 1992; Polzonetti-Magni et al. 1998; van Pelt et al. 1999; Arenas et al. 2001; Carpino et al. 2001). In amphibians, estrogens act during early spermatogenesis (Callard, 1992; Polzonetti-Magni et al. 1998) and stimulate mitosis of primary spermatogonia (Minucci et al. 1997; d'Istria et al. 2003). Thus, the testosterone present in the winter PG cells might be converted into estrogen between spring and summer. Future studies to detect aromatase activity, ABP and receptors for androgen and estrogen would clarify the role of testosterone in the PG cells.

In conclusion, the testosterone immunopositivity in the PG cells during winter suggests that these cells could retain testosterone during the period of low steroidogenic activity. Thus, a possible androgen regulatory role could be attributed to the PG cells during seasonal spermatogenesis.

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