General and Comparative Endocrinology 221 (2015) 244–254

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Effect of estradiol on apoptosis, proliferation and steroidogenic enzymes in the testes of the toad *Rhinella arenarum* (Amphibia, Anura)

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ARTICLE INFO

Article history:
Available online 9 January 2015

Keywords:
Toad
Estradiol
Testes
Steroidogenesis
Apoptosis
Proliferation

ABSTRACT

Estrogens inhibit androgen production and this negative action on amphibian steroidogenesis could be related to the regulation of steroidogenic enzymes. Estrogens are also involved in the regulation of amphibian spermatogenesis by controlling testicular apoptosis and spermatogonial proliferation. The Bidder's organ (BO) is a structure characteristic from the Bufonidae family and in adult males of *Rhinella arenarum* it is one of the main sources of plasma estradiol (E_2). The purpose of this study is to analyze the effect of E_2 on testicular steroidogenic enzymes, apoptosis and proliferation in the toad *R. arenarum*. For this purpose, testicular fragments were treated during 24 h with or without 2 or 20 nM of E_2. After treatments, the activities of cytochrome P450 17α-hydroxylase-C_{17-20} lyase (CypP450c17) and 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/I) were measured by the transformation of radioactive substrates into products, and CypP450c17 expression was determined by Western blot analysis. Apoptosis in testicular sections was detected with a commercial fluorescent kit based on TUNEL method, and proliferation was evaluated by BrdU incorporation. Results indicate that E_2 has no effect on CypP450c17 protein levels or enzymatic activity, while it reduces 3β-HSD/I activity during the post reproductive season. Furthermore, although E_2 has no effect on apoptosis during the pre and the post reproductive seasons, it stimulates testicular apoptosis during the reproductive season, mostly in spermatocytes. Finally, E_2 has no effect on testicular proliferation all year long. Taken together, these results suggest that E_2 is involved in the regulation of testicular steroidogenesis and spermatogenesis.

1. Introduction

As in other vertebrates, amphibian testes are organized in two compartments, the interstitial and the germinal ones, with the seminiferous component organized in seminiferous lobules (Duellman and Trueb, 1994; Wake, 1969). In anamniotes, the unit of spermatogenesis is the spermatocyst or cyst that is formed when Sertoli cells enclose one spermatogonium with cytoplasmic processes (Duellman and Trueb, 1994; Lofts, 1974; Pudney, 1995; Wake, 1969). In the cysts, spermatogonia proliferate and differentiate into meiotic spermatocytes, spermatids and mature spermatozoa.

The dynamic balance between cell proliferation and apoptosis, mainly regulated by hormones and environmental factors, determines the rate of the spermatogenic process. Apoptosis is important because it determines the equilibrium between the number of germ cells and the supporting capacity of Sertoli cells. In this context, the study of the variations in amphibian spermatogenesis is intimately related not only to proliferation of the germ cells, but also to apoptosis.

Several authors have studied the hormonal regulation of spermatogenesis. In mammals, follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone regulate cell survival, and excess or deprivation of these hormones can lead to testicular apoptosis (see review by Schlatt and Ehmcke, 2014; Shaha, 2008). It is now accepted that estrogens are essential for spermatogenesis (Shaha et al., 2010) and *in vitro* and *in vivo* studies demonstrate that they induce apoptosis in germ cells via the extrinsic pathway

Abbreviations: E_2, estradiol; CypP450c17, cytochrome P450 17α-hydroxylase-C_{17-20} lyase; 3β-HSD/I, 3β-hydroxysteroid dehydrogenase/isomerase; RIA, radioimmunoassay; BO, Bidder's organ.

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http://dx.doi.org/10.1016/j.ygcen.2014.12.016
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through an upregulation of Fas/FasL (Mishra and Shaha, 2005; Nair and Shaha, 2003). In non-mammalian vertebrates, gonadotropins and steroids have also been proved to regulate spermatogenesis (see the review by Chianese et al., 2011; Meccariello et al., 2014). Particularly in amphibians, androgens increase testes size in *Rhinella arenarum* (Penhos, 1956) and spermatogenesis in *Anaxyrus fowleri* but suppress spermatogenesis at spermatogonia II stage in several species of the genus Rana and in *Duttaphrynus melanostictus* (for a review of evidences until 1980, see Rastogi and Iela, 1980). In *Pelophylax esculentus*, gonadotropins stimulate spermatogonial proliferation and also androgen production, while androgens induce spermatid formation and also stimulate spermatogonial mitosis (Minucci et al., 1992). Moreover, during the reproductive season of *P. esculentus*, there is a strong relationship between high levels of testosterone, the mitotic activity of the germinal epithelium and the expression of c-kit, which is indispensable for spermatogenesis and spermatogonial proliferation (Raucci and di Fiore, 2007). Furthermore, in *Lithobates pipiens* corticosterone induces a reduction in spermatid formation, while 5α-dihydrotestosterone reduces the amount of secondary spermatogonia and spermatids, and stimulates spermatocytes formation (Tsai et al., 2003).

Estrogens are also involved in the regulation of amphibian spermatogenesis by acting in the early spermatogenic process (Callard, 1992; Polzonetti-Magni et al., 1998). In *Lithobates catesbeianus* estrogens play an important role in the control of primordial germ cells during the seasonal spermatogenesis (Caneguim et al., 2013a, 2013b). Moreover, in juveniles *Xenopus laevis* estradiol (E_2) induces an acceleration of spermatogenesis (Hu et al., 2008). In *L. pipiens*, the action of chronic estradiol may be both inhibitory and stimulatory, depending on the stages of spermatogenesis, since chronic treatment retards the formation of spermatocytes and secondary spermatogonia, and accelerates the latter stages of spermatogenesis (Tsai et al., 2003). Besides, in *P. esculentus* there is a strong mitogenic activity of spermatogonia in the period of the year characterized by high concentrations of estradiol (Fasano et al., 1989; Rastogi et al., 1985; Varriale et al., 1986). In this species, estradiol promotes spermatogonial proliferation through the activity of Fos protein and the activation of mitogen-activated protein kinase in spermatogonia (Chieffi et al., 1995, 2000; Cobellis et al., 1999; Minucci et al., 1997) and proliferation is inhibited by melatonin (dIstria et al., 2003). Moreover, estradiol regulates the expression of Fra-1, which is located in peritubular myoid cells, efferent ducts and blood vessels and may be involved in sperm transport. Estradiol is also involved in sperm release in this species, since impairment of estrogen activity affects the detachment of spermatozoa from the Sertoli cells (Cobellis et al., 2005, 2008). Taken together, these results suggest that anuran spermatogenesis is a complex process in which each stage can be independently regulated by different steroids.

It has also been suggested that estrogens inhibit androgen production in several amphibians, since estradiol treatment decreases plasma testosterone concentrations in *X. laevis* and *P. esculentus* (Fasano et al., 1991; Hecker et al., 2005; Pierantoni et al., 1986). The negative action of estradiol on amphibian steroidogenesis could be related to the regulation of steroidogenic enzymes. In the rainbow trout, E_2 treatment decreases the expression of cytochrome P450 17-hydroxylase-C_{17-20}-lyase (CypP450c17), 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/I), and 11β-hydroxylase (Govoroun et al., 2001) while in *P. esculentus* it inhibits the activity of 17-hydroxylase (Fasano et al., 1991). Moreover, estradiol levels increase in males of *P. esculentus* in the early summer, following a decline in testosterone levels (Polzonetti-Magni et al., 1984) and authors speculated that estradiol inhibits GnRH release and LH secretion, leading to a decrease in androgen levels. It has been demonstrated that in *P. esculentus* the expression of testicular kisspeptin receptor is estradiol dependent and also that kisspeptins modulate the expression of testicular estrogen receptor alpha (Chianese et al., 2013) and upregulate the GnRH system and the expression of testicular estrogen receptor beta and PCNA (Chianese et al., 2015). These results suggest that kisspeptins and their receptors are involved in the regulation of estradiol-dependent testicular functions such as proliferative activity and spermatogenesis.

The toad *R. arenarum* is a species with an extensive reproductive season (Gallardo, 1974). Spermatogenic wave starts in October, when there is a great number of spermatogonia and scarce spermatocytes and spermatids (Burgos and Mancini, 1948). This is a species with a dissociated reproductive pattern, since the reproductive behavior in males is associated with low levels of plasma androgens due to a decrease in the activity of CypP450c17 (Canosa and Ceballos, 2002a; Canosa et al., 2003; Fernández Solari et al., 2002).

In species from the Ranidae family, it has been demonstrated that plasma estradiol is produced by the testes (Fasano et al., 1989; Varriale et al., 1986). However, in *R. arenarum* testes do not express aromatase activity and the main source of plasma estradiol in the toad is the Bidder organ (BO), a structure which is characteristic from the Bufonidae family (Scaia et al., 2011, 2013). Plasma estradiol levels are low during the pre-reproductive season and they increase gradually during the reproductive season, reaching the highest values during the post-reproductive season (Scaia et al., 2013).

Although the effect of estradiol on testicular physiology has been investigated in frogs, the role of estradiol on testicular steroidogenesis and spermatogenesis in bufonids still remains unexplored. In the present study we analyze the relationship between estradiol and testicular steroidogenic enzymes, apoptosis and proliferation in *R. arenarum*.

2. Materials and methods

2.1. Animals

Adult male toads of *R. arenarum* were collected during 2012–2014 in a non-agricultural area near Buenos Aires City, during pre-reproductive (PreR, May–August), reproductive (R, September–December) and post-reproductive (PostR, January–April) seasons (Canosa et al., 2003). Animals were maintained under natural conditions of light and temperature, with free access to water and fed with crickets and zophobas (*Zophobas morio*). Toads were deeply anesthetized by immersion in 1% tricaine methanesulfonate (MS222) according to Gentz (2007), their death being the result of the ensuing surgery. This procedure is 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, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.

2.2. Collection and processing of plasma

Approximately 1 ml blood samples were obtained with heparinized syringes by cardiac puncture and plasma were separated at 4 °C by centrifugation for 10 min at 3500 rpm. Moreover, 20 μl of plasma were treated overnight with 10 volumes of methanol, then evaporated and re-dissolved in radioimmunoassay (RIA) buffer for E_2 determinations (10 mM phosphate buffer, 0.1% w/v gelatin, 140 mM sodium chloride, 0.1% w/v sodium azide, pH 7.4).

2.3. Tissue collection and incubations

Testes were rapidly dissected and placed in Petri dishes with sterile L15 medium and employed to analyze the effect of E_2 on CypP450c17 activity (PreR: $n = 6$, R: $n = 7$, PostR: $n = 7$) and CypP450c17 protein synthesis (PreR: $n = 5$, R: $n = 5$, PostR: $n = 5$). Besides, organs from 16 animals (PreR: $n = 6$, R: $n = 4$, PostR: $n = 5$) were used to analyze the effect of E_2 on 3β-HSD/I activity. Moreover, 12 animals (4 from each season) and 15 animals (5 from each season) were employed to analyze the effect of E_2 on testicular apoptosis and cellular proliferation, respectively. Testes were cut into homogeneous slices of approximately 2 mm. Slices from both testes were first pooled and then randomly separated into different groups, so that each testis was equally represented in all treatments. For the determination of enzymatic activities, one group was immediately processed to determine initial activities and the initial amount of CypP450c17 protein (T0). The other groups were incubated in multiwell plates for 24 h at 28 °C in 4 ml L15 medium alone (control), with 2 nM E_2 or with 20 nM E_2. These concentrations correspond to E_2 plasma levels and the concentration in which testicular E_2 receptors are saturated, respectively. These conditions were determined in preliminary experiments from the laboratory.

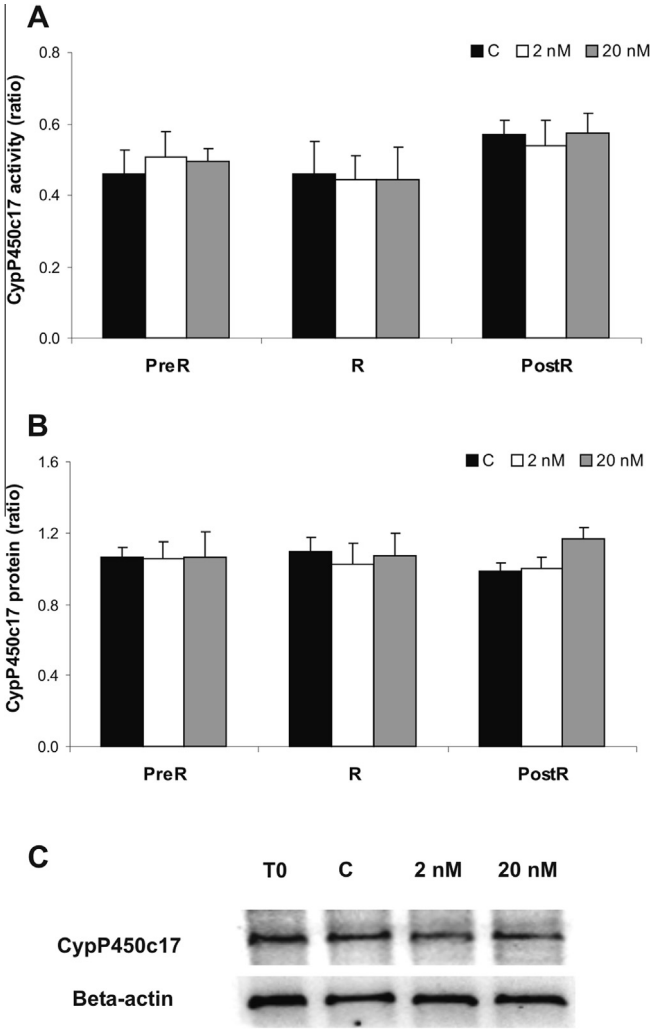

Fig. 1. (a) Effect of E_2 on CypP450c17 activity. Enzymatic activities were determined by transformation of [^{3}H]pregnenolone to [^{3}H]17-hydroxypregnenolone and [^{3}H]dehydroepiandrosterone, in the presence of NADPH. Values of enzymatic activity after E_2 treatment were referred to initial values, and results are expressed as the mean ± SE. There are no statistical differences in the activity among seasons ($p = 0.0986$) and no significant effect of E_2 ($p = 0.9955$). (b) Effect of E_2 on CypP450c17 protein level. Freshly isolated testicular fragments (T0), 24 h control and E_2 treated fragments were processed as described in Section 2 and Western blot analysis was performed in the homogenates. Results are expressed as means of the different experiments ± SE. There are no statistical differences among seasons ($p = 0.9917$) and no significant effect of E_2 ($p = 0.6209$). (c) Membrane corresponding to one representative experiment.

2.4. Enzymatic assays

Testicular fragments were homogenized in 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA and 3 mM $MgCl_2$ to determine the activity of CypP450c17, and an aliquot of each homogenate was stored at −20 °C for Western blot analysis. CypP450c17 activity was determined according to Fernández Solari et al. (2002). To determine 3β-HSD/I activity, fragments were homogenized in 10 mM Tris–Cl buffer, pH 7.4, containing 0.1 mM EDTA, 0.25 M sucrose and 0.4 mM β-mercaptoethanol. 3βHSD/I activity was assayed according to Pozzi et al. (1997). Enzymatic activities were expressed as means ± SE of the ratio between the 24-h activities and time zero values.

2.5. Western blot analysis

To detect changes in the expression of CypP450c17, the amount of protein was semi-quantified by Western blot (Tesone et al.,

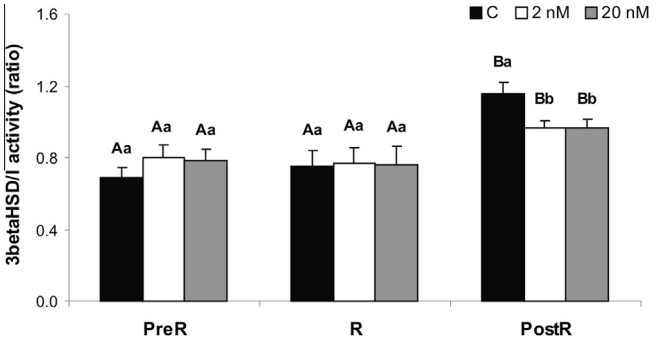

Fig. 2. Effect of E_2 on 3β-HSD/I activity. Enzymatic activity was determined by the transformation of [^{3}H]pregnenolone to [^{3}H]progesterone in the presence of NAD^+. Values of enzymatic activity after E_2 treatment were referred to initial values, and results are expressed as the mean ± SE. Upper case refer to statistical significance among seasons ($p < 0.0001$), and lower case refer to significance among treatments (PreR: $p = 0.4076$; R: $p = 0.9953$; PostR: $p = 0.017$).

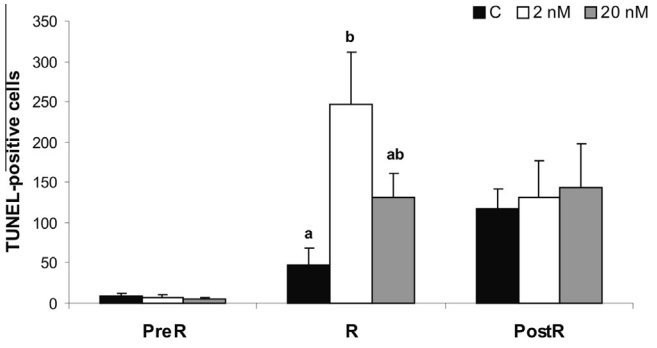

Fig. 3. Effect of E_2 on apoptosis. Apoptosis was determined with the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit. The number of TUNEL-positive cells in each section was counted with ImageJ software. Sections were co-stained with propidium iodide for nuclear detection. Values are expressed as means of 4 animals in each season ± SE. Different letters indicate significant differences (PreR: $p = 0.8378$; R: $p = 0.0280$; PostR: $p = 0.9119$).

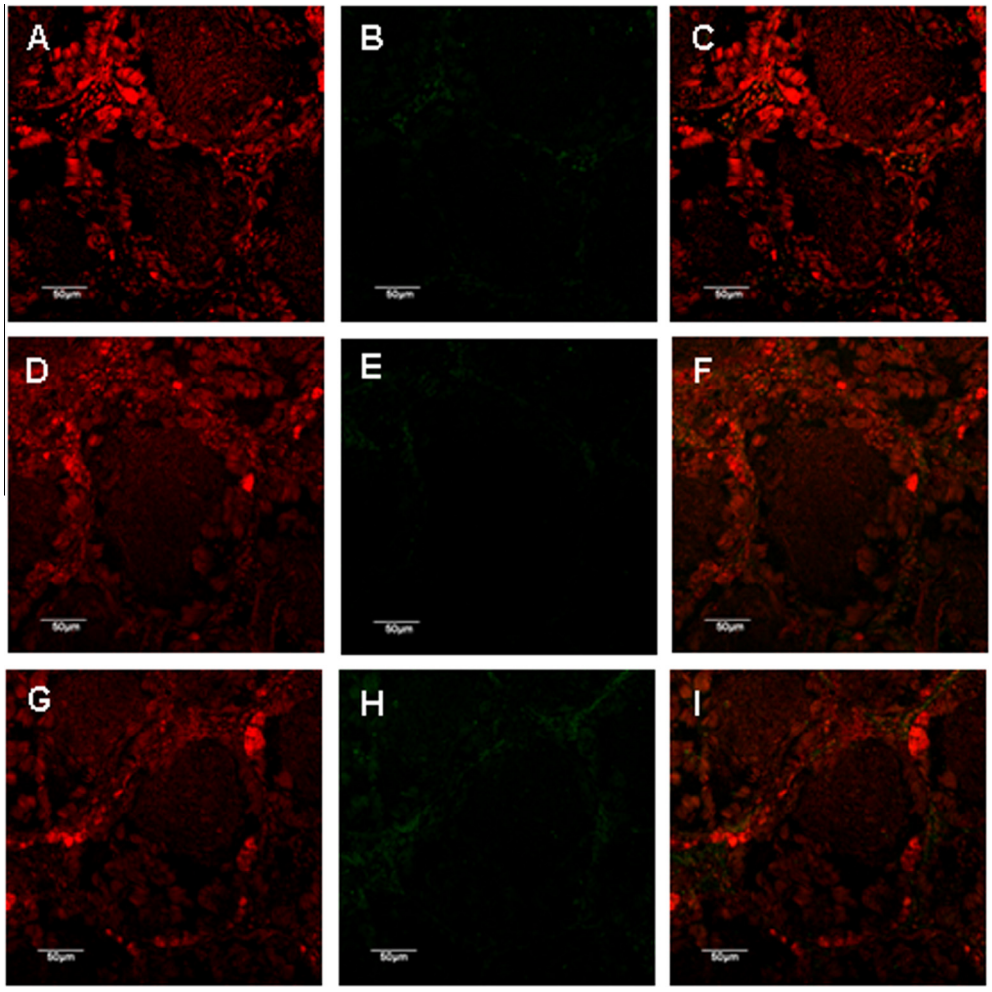

Fig. 4. Effect of E_2 on apoptosis during the PreR season. Apoptosis was labeled with fluorescein (B, E, H) and sections were co-stained with propidium iodide (A, D, G). Merge (C, F, I). Control sections (A–C). Testicular fragments treated with 2 nM E_2 (D–F). Testicular fragments treated with 20 nM E_2 (G–I).

2012). For this purpose, 80 μg of proteins mixed with sample buffer (40 mM Tris–HCl, 2% sodium lauryl sulfate w/v, 4% glycerol v/v, 0.001% bromophenol blue w/v, 2 mM β-mercaptoethanol, pH 6.8) were electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) during 140 min at 130 V. Proteins were then transferred to polyvinylidene difluoride membranes (Thermo Scientific, IL, USA) at 300 mA for 90 min. Membranes were then incubated for 10 min in phosphate buffered solution containing 0.1% Tween-20 (PBS-T) and 3% hydrogen peroxide and blocked for 1 h at room temperature in PBS-T containing 5% low-fat powdered milk. Incubations with primary antibodies were performed overnight at 4 °C in PBS-T with 0.4 μg/ml rabbit polyclonal anti mouse CypP450c17 antibody (Santa Cruz Biotechnology, CA, USA) or 0.4 μg/ml mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology, CA, USA). Secondary antibodies were goat anti-rabbit (0.152 μg/ml, Chemicon International, USA) and goat anti-mouse (0.1 μg/ml, Santa Cruz Biotechnology, CA, USA) conjugated to horse radish peroxidase. Immunopositive bands were visualized by enhanced chemiluminescent detection using 1.25 mM luminol, 0.198 mM cumaric acid, 0.038% (v/v) hydrogen peroxide (100 vol) in 100 mM Tris–HCl buffer, in a Fujifilm LAS-1000 chemiluminescent detection system. Densitometric analysis of the bands was performed with the Image Gauge software (Fuji Photo Film Co. Ltd, Tokio, Japan). The intensity of the CypP450c17 immunoreactive bands was normalized to the intensity of the β-actin immunoreactive band, to account for loading differences. These results were expressed as means ± SE of the ratio between the 24-h and time zero values.

2.6. Radioimmunoassay of estradiol

Plasma estradiol was determined according to Scaia et al. (2013). Measurements were done using [2,4,6,7-^{3}H] E_2 (70.0 Ci/mmol; Perkin-Elmer Life Science, MA, USA) and sheep anti-estradiol (final dilution of 1:250,000 Colorado State University, CO). The standard curve was performed with radioinert E_2 (Sigma Chemical Co., St. Louis, MO, USA) in concentrations ranging from 6.25 to 800 pg/ml. The intra and inter-assay coefficients of variation were 6.7% and 11.5%, respectively. Charcoal-dextran method was employed to separate bound and free hormones. Radioactivity in the supernatant (bound) was determined by liquid scintillation counter and scintillation counting was carried out with Wallac 1409 DSA equipment (Wallac Co., Turku, Finland). The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac Co., Turku, Finland). The amount of E_2 was expressed as pg per ml of plasma.

2.7. Histology, proliferation assay and apoptosis

Cellular proliferation was determined after estradiol treatments by the incubation with 1 mM of 5-bromo-2-deoxyuridine (BrdU)

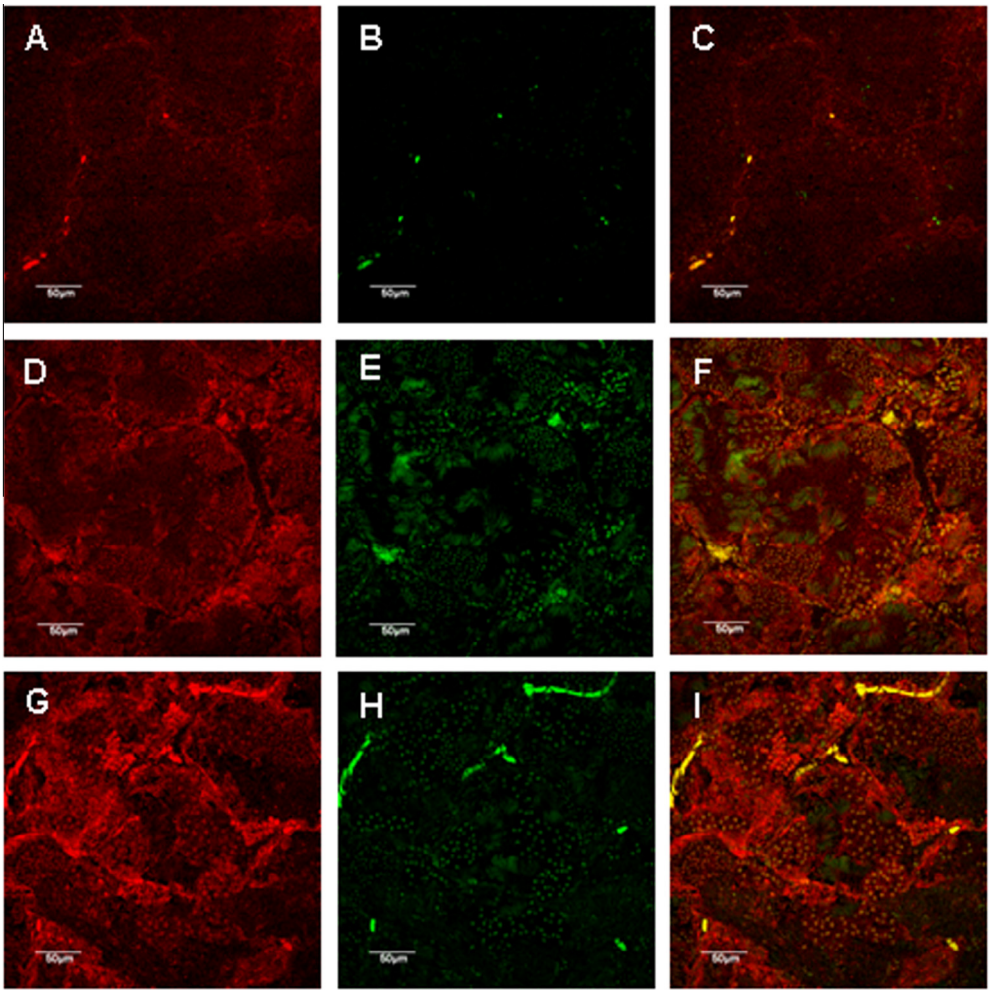

Fig. 5. Effect of E_2 on apoptosis during the R season. Apoptosis was labeled with fluorescein (B, E, H) and sections were co-stained with propidium iodide (A, D, G). Merge (C, F, I). Control sections (A–C). Testicular fragments treated with 2 nM E_2 (D–F). Testicular fragments treated with 20 nM E_2 (G–I).

during 1 h at 37 °C and organs were processed for immunohistochemistry. Testicular fragments were transferred into Bouin's solution, dehydrated and embedded in paraffin-histoplast (50:50, w/w). Serial sections were cut at 7 μm, deparaffinized, hydrated and immunohistochemical techniques were carried out after blocking biotin with the avidin–biotin blocking kit (Vector Laboratories Inc., Burlingame, CA). Immunohistochemical staining was performed in testicular fragments from 5 animals collected from each season, employing a monoclonal anti-BrdU antibody (1:100; GE Healthcare, Buckinghamshire, UK). Sections were then incubated with rabbit anti-mouse secondary polyclonal antibody conjugated with biotin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and with streptavidin–biotinylated horseradish peroxidase complex (1:400; GE Healthcare, UK). Immunohistochemical staining was visualized with 3,3′-diaminobenzidine solution (DAKO North America, Inc., Carpinteria, CA) and counterstained with hematoxylin. The average value ± SE was obtained from 5 animals from each season. Sections were examined using a Leica DM2000 microscope and images were captured with an incorporated digital camera. The proliferation activity was expressed as a percentage of BrdU-positive cysts among all the cysts in 5 lobules/section.

To study the effect of E_2 on testicular apoptosis, serial sections from 4 animals collected in each season were cut at 7 μm, deparaffinized, hydrated and then analyzed for DNA fragmentation using the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Millipore, USA), according to the manufactureŕs instructions. This technique is based on TUNEL method. Briefly, sections were treated with proteinase K (20 μg/μl, Millipore, USA) 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 stained with propidium iodide. Negative controls were performed in the absence of Tdt enzyme, and positive control sections were incubated with a DNAse solution for 30 min at 37 °C. Sections were examined using a Confocal Microscope (Olympus-FV300/BX61) and images were captured with an incorporated camera. The number of TUNEL-positive cells in each section was counted with ImageJ software, and the stages of spermatogenesis were defined according to Rastogi et al. (1976). Results were expressed as the mean number of apoptotic nucleus in each treatment for each season.

2.8. Statistical analysis

CypP450c17 activity and CypP450c17 expression were analyzed and compared by using two-way ANOVA, taking *Season* and *Treatment* as two fixed factors. The effect of E_2 on 3β-HSD/I activity was analyzed and compared by using one-way ANOVA for each season, corrected by Bonferroni, and *a posteriori* contrast by Tukey's test. Estradiol effect on apoptosis and proliferation was analyzed and

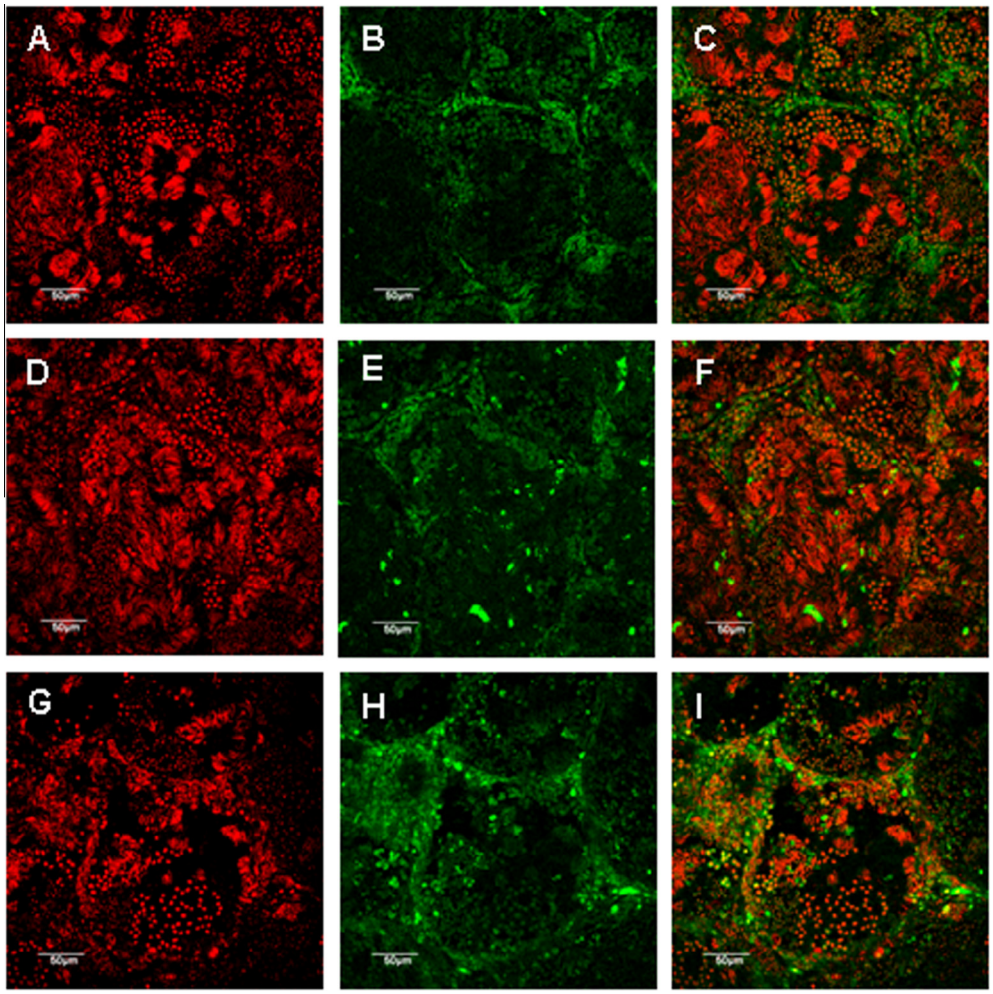

Fig. 6. Effect of E_2 on apoptosis during the PostR season. Apoptosis was labeled with fluorescein (B, E, H) and sections were co-stained with propidium iodide (A, D, G). Merge (C, F, I). Control sections (A–C). Testicular fragments treated with 2 nM E_2 (D–F). Testicular fragments treated with 20 nM E_2 (G–I).

compared by using one-way ANOVA for each season and *a posteriori* contrast by Tukey's test.

3. Results

In order to study the effect of estrogens on two steroidogenic enzymes, fragments of testes were incubated during 24 h and, because of the great variability among toads, data were expressed related to initial activity. Fig. 1 shows that after 24-h incubations there are no statistical differences in the activity of CypP450c17 among seasons and that there is no significant effect of E_2 on CypP450c17 activity during the year ($p = 0.0986$, $p = 0.9955$, respectively). To study the effect of E_2 on CypP450c17 expression, Western blot analysis was performed. A specific band was detected in toad testis homogenates with an apparent molecular weight of 55 kDa. This value is similar to that obtained with mouse ovary homogenates, which were used as a positive control (see Fig. 1 Supplementary material). Results show that there are no significant differences in the expression of CypP450c17 among seasons and no significant effect of E_2 on CypP450c17 expression during the year (Fig. 1, $p = 0.9917$, $p = 0.6209$, respectively). Furthermore, Fig. 2 shows that 3β-HSD/I activity after 24-h incubation is higher during the PostR season than during the other seasons (PreR: 0.760 ± 0.035; R: 0.762 ± 0.047; PostR: 1.030 ± 0.034, $p < 0.0001$). Moreover, there is no significant effect of E_2 on 3β-HSD/I activity during the PreR and R season (Fig. 2, $p = 0.4076$ and $p = 0.9953$, respectively). However, during the PostR season both concentrations of estradiol inhibit 3β-HSD/I activity (Fig. 2, C: 1.158 ± 0.062; 2 nM: 0.966 ± 0.041; 20 nM: 0.966 ± 0.048, $p = 0.017$).

As regards the effect of E_2 on testicular apoptosis, Fig. 3 indicates that during the PreR season, and after 24-h treatment, apoptosis is almost negligible in all the experimental conditions (C: 8 ± 2.17, 2 nM: 6.5 ± 1.97, 20 nM: 4.75 ± 1.55, $p = 0.8378$). This figure also indicates an increase of apoptosis after estradiol treatment during the R season, although statistical significance is only observed with the 2 nM concentration (C: 47 ± 10.8, 2 nM: 246.3 ± 32.3, 20 nM: 131 ± 15.3, $p = 0.0280$). Finally, Fig. 3 also indicates that high apoptosis is detected in controls as well as in estradiol treated fragments from the PostR animals (C: 116.5 ± 12.6, 2 nM: 131 ± 22.9, 20 nM: 143 ± 27.1, $p = 0.9119$). The low apoptotic rate after 24-h during the PreR season can be clearly observed in Fig. 4. Moreover, TUNEL-positive nuclei were detected in estradiol-treated testes from the R season (Fig. 5). Fig. 6 shows the high number of apoptotic nuclei that were detected in all the conditions during the PostR season. Furthermore, the analysis of the cellular types in the different seasons indicates that most of the apoptotic cells in the R season correspond to spermatocytes, and very few apoptotic spermatids and no apoptotic spermatogonia were detected (Fig. 7). The apoptotic rate in the PreR is so low, that apoptosis in spermatogonia is almost negligible. In the PostR season very few apoptotic spermatogonia were detected, but

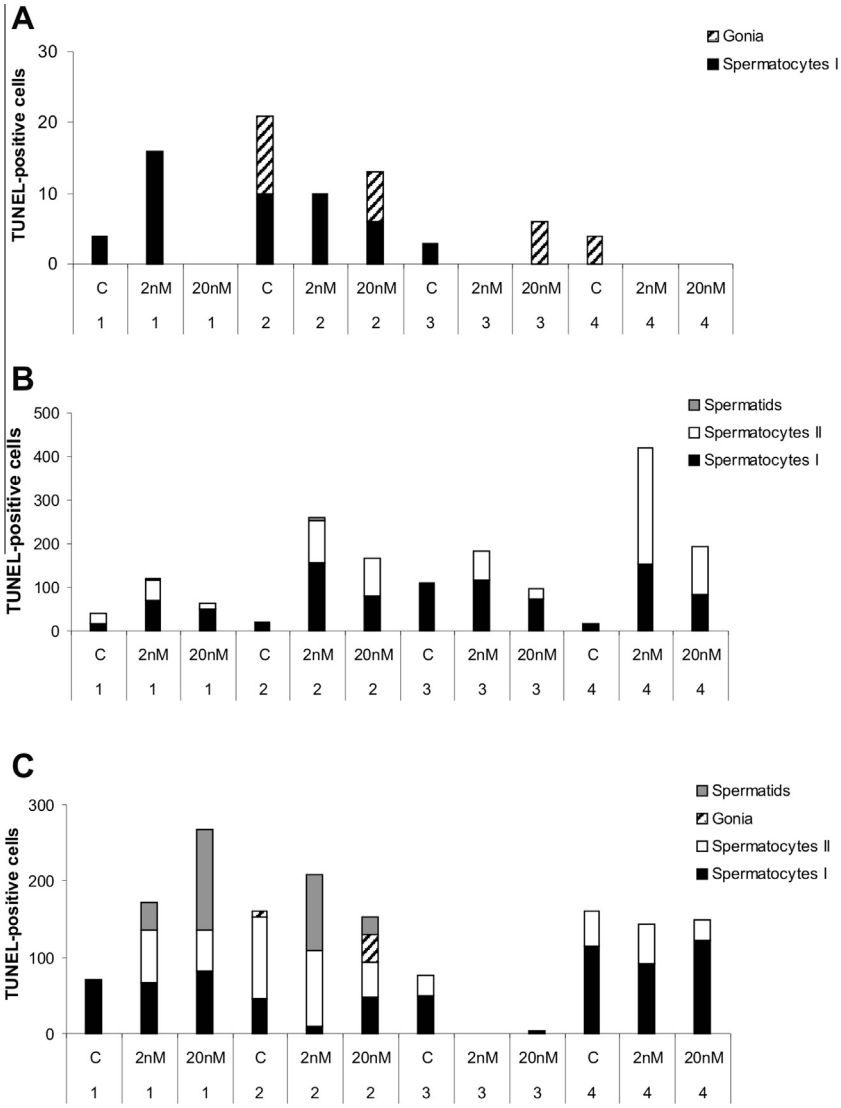

Fig. 7. Effect of E_2 on apoptosis in different spermatogenic stages. The number of TUNEL-positive cells in each section was counted with ImageJ software and data from all animals is represented in each graph. 1–4 refer to the number of toads. (A) Pre reproductive season. (B) Reproductive season. (C) Post reproductive season.

only in one treatment in one animal. Taken together, these results indicate that E_2 induces apoptosis in spermatocytes only during the R season. Moreover, although apoptosis was detected in very few spermatogonia, they represent a very low number and most of the apoptotic nuclei correspond to spermatocytes. It is worth mentioning that no apoptosis was detected in lobules in negative control sections, while positive control sections showed high levels of apoptosis (see fig. 2 Supplementary material).

To study the effect of E_2 on proliferation after 24-h treatment, testes fragments were incubated with BrdU and BrdU incorporation was determined by immunohistochemistry. During the breeding, proliferation was detected in all the conditions (Fig. 8D–F), and high magnification shows that proliferative cysts correspond to spermatogonia (Fig. 8J). No proliferation was detected during the PreR and PostR seasons (Fig. 8A–C, G–I) and no immunostaining was observed in negative controls (Fig. 8K). Furthermore, when results are expressed as the percentage of immunopositive spermatogonia, there were no significant differences between control and treated fragments during the year (C: 10 ± 3.7, 2 nM: 3.1 ± 1.3, 20 nM: 2.8 ± 2 for the PreR season, $p = 0.095$; C: 28.7 ± 9.6, 2 nM: 14.9 ± 6.1, 20 nM: 15.4 ± 5.2 for the R season, $p = 0.3418$; C: 14.5 ± 5.7, 2 nM: 8 ± 4.8, 20 nM: 11 ± 5.3 for the PostR season, $p = 0.632$, Fig. 9). These results indicate that under these experimental conditions, E_2 has no effect on testicular proliferation during the year.

4. Discussion

In *Pelophylax nigromaculata*, chronic exposure to microcystin-LR provokes testicular abnormalities by the induction of aromatase (Jia et al., 2014). Moreover, in *Pleurodeles waltl*, aromatase inhibition produces male-biased sex ratios, suggesting a role of estrogens in the determination of sex ratios (Chardard and Dournon, 1999). Therefore, it is important to know the effect of these hormones in the gonadal function of one species belonging to the Bufonidae family. This family is characterized for having the BO, a structure highly susceptible of being influenced by the environmental conditions (McCoy et al., 2008). Moreover, in several species the BO has steroidogenic activity (Ghosh et al., 1984; Pancak-Roessler and Norris, 1991) and it is able to produce estradiol (Colombo and Colombo Belvedere, 1980). In *R. arenarum*, this organ has been recently proposed as the main source of plasma estradiol (Scaia et al., 2011, 2013).

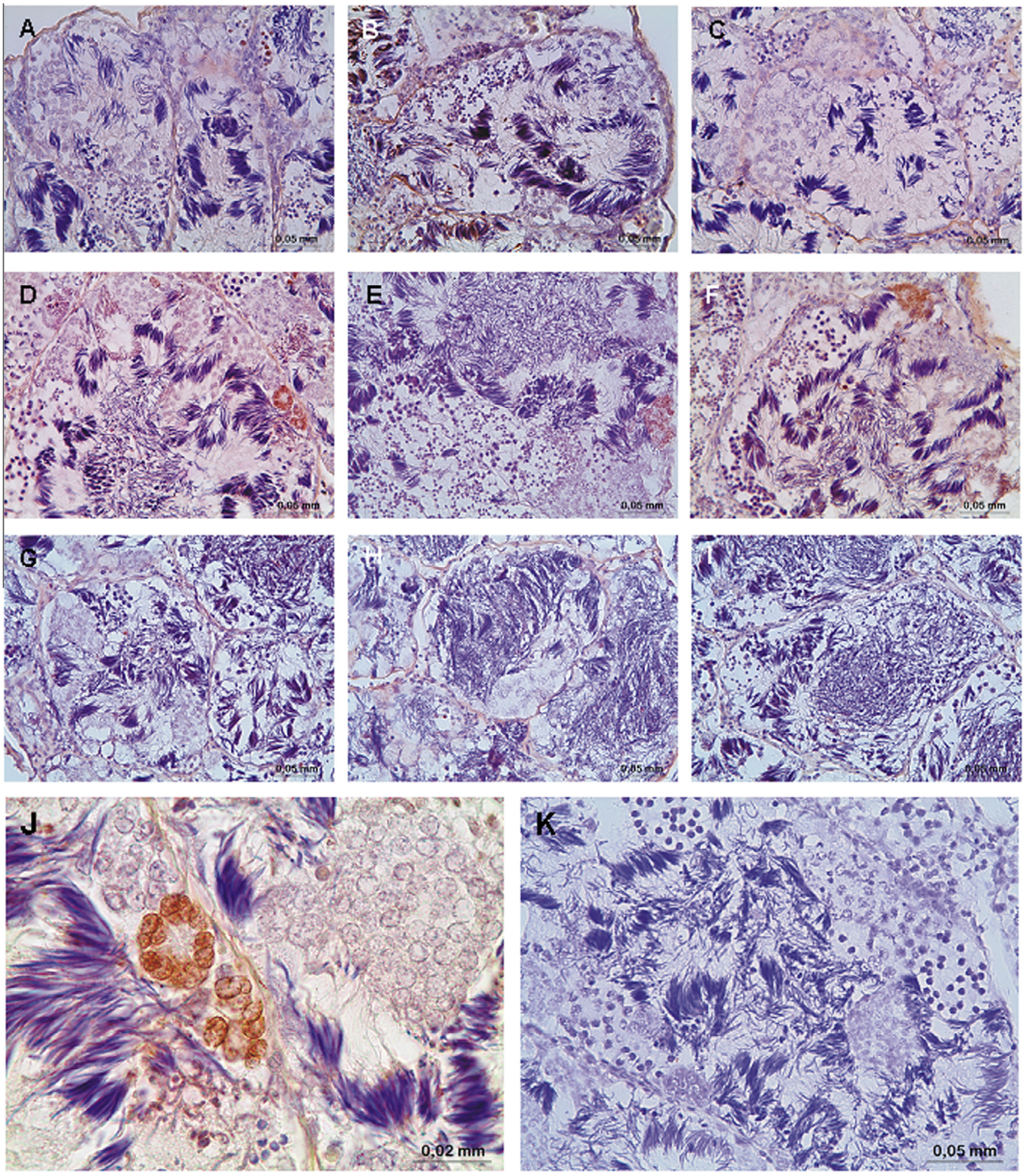

Fig. 8. Effect of E_2 on proliferation. Testes were incubated during 1 h with BrdU and BrdU incorporation was determined by immunohistochemistry in control fragments (A, D, G), and in fragments treated with 2 nM (B, E, H) and 20 nM of E_2 (C, F, I). PreR season (A–C). R season (D–F). PostR season (G–I). Detail of immunopositive spermatogonia from the R season (J). Negative control with no primary antibody (K).

Taking these results into account, one of the main purposes of this paper was to study the effect of E_2 on testicular steroidogenic enzymes in *R. arenarum*, since this hormone reduces androgen production in *X. laevis* and *P. esculentus* (Fasano et al., 1991; Hecker et al., 2005; Pierantoni et al., 1986). Our results indicate that E_2 has no effect on CypP450c17 activity or on the protein level, suggesting that seasonal variations in E_2 are not responsible of the seasonal changes in the activity of this enzyme. In the toad, CypP450c17 is one of the main regulating steps in testicular steroidogenesis, since its reduction leads to a decrease in androgen levels during the R season and the decrease in its activity could be due to, at least in part, the high levels of FSH during the breeding (Canosa and Ceballos, 2002a, 2002b; Fernández Solari et al., 2002). Our results differ from those obtained in *P. esculentus*, in which E_2 inhibits 17-hydroxylase activity of CypP450c17 (Fasano et al., 1991), and this difference is probably due to the diversity of mechanism among the different families of amphibians.

On the other hand, in the toad E_2 reduces the activity of 3β-HSD/I during the PostR season, when plasma E_2 achieves its highest levels (Scaia et al., 2013). These results indicate that E_2 may

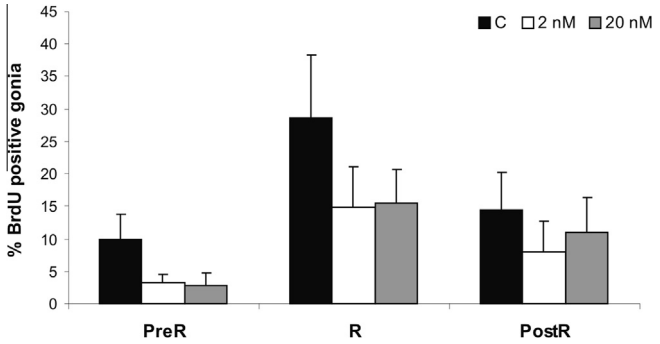

Fig. 9. Effect of E_2 on proliferation. Proliferation rate is expressed as the percentage of immunopositive cysts related to the total number of cysts in gonia stage. Values are expressed as means of 5 lobules from 5 animals in each season ± SE. No significant differences were detected (PreR: $p = 0.095$; R: $p = 0.3418$; PostR: $p = 0.6320$).

regulate testicular steroidogenesis, at least during the PostR season. Previous results in *R. arenarum* showed that 3β-HSD/I is not regulated by gonadotropins or GnRH (Canosa and Ceballos, 2002b; Canosa et al., 2002) and that there are seasonal variations in its activity, being higher during the breeding (Pozzi et al., 1997). The fact that this enzymatic activity decreases after the breeding, when plasma estradiol levels reach the highest concentrations, reinforces the idea that estradiol could be involved in the regulation of 3β-HSD/I also *in vivo*. These results also suggest that in *R. arenarum* estradiol could inhibit the conversion of the 3β-hydroxy-5-ene-steroids into 3-oxo-4-ene-steroids. Similarly to the toad, in fish E_2 reduces the expression of 3β-HSD/I (Govoroun et al., 2001). Unfortunately, there are no other results in amphibians regarding the effect of estradiol on steroidogenic enzymes.

In mammals, the role of estrogens in the regulation of spermatogenesis has been clearly established and, for example, they induce apoptosis in spermatogenic cells (Mishra and Shaha, 2005; Nair and Shaha, 2003). Unfortunately, until now there were no studies regarding the effect of E_2 on amphibian testicular apoptosis. Our results indicate that there is almost no apoptosis in the germ line in none of the *in vitro* experimental conditions during the PreR season, although E_2 induces apoptosis during the breeding. In addition, high apoptosis was detected in all the conditions during the PostR season, which is characterized by high levels of plasma E_2. Seasonal variations in apoptosis have been studied in *Cynops pyrrhogaster* and in *L. catesbeianus*. In the newts apoptosis occurs in winter or in both winter and summer, depending on the species (Ricote et al., 2002; Yazawa et al., 1999) while in *L. catesbeianus* high levels of apoptosis have been detected at the end of the breeding season (Sasso-Cerri et al., 2004).

Apoptosis is an important physiological process because germ cells undergoing this type of cell death during spermatogenesis are phagocytosed by Sertoli cells, playing a key role in testicular involution during the annual cycle (Sasso-Cerri and Miraglia, 2002). In this context, it is important to determine which cell types are undergoing apoptosis. In newts, apoptosis was detected in early spermatids and mature spermatozoa (Wang et al., 2012) and also in spermatogonia during the winter (Yazawa et al., 1999). In the toad, apoptosis induced by E_2 in the breeding was mostly detected in spermatocytes, while in the PostR season apoptosis occurs also in spermatids. It is worth mentioning that almost no apoptosis was detected in spermatogonia. These results suggest that late spermatogenic stages that do not reach spermatozoa maturity may undergo apoptosis via an estradiol-mediated mechanism.

In amphibians, E_2 is involved in the regulation of spermatogenesis, having different actions depending on the spermatogenic stage, the developmental stage and the species. In *X. laevis*, larval exposure to E_2 induces an acceleration of spermatogenesis by increasing the percent of spermatocytes, spermatids and spermatozoa (Hu et al., 2008). Otherwise, in *L. pipiens* estrogens retard the formation of secondary spermatogonia and spermatocytes and accelerate the final stages of spermatogenesis (Tsai et al., 2003). Furthermore, E_2 is associated with high mitogenic activity of spermatogonia in *P. esculentus* (Fasano et al., 1989; Rastogi et al., 1985; Varriale et al., 1986) via Fos and mitogen-activated kinase (Chieffi et al., 1995, 2000; Cobellis et al., 1997, 1999, 2002; Minucci et al., 1997). Our results in *R. arenarum* indicate that in spite of the season, estradiol has no effect on spermatogonial proliferation. Similarly, but using an *in vivo* approach, Tsai et al. (2003) were not able to detect any stimulatory effect of E_2 in *L. pipiens* after 20 days of treatment. However, it is important to take into account that there are differences in the experimental approaches employed in all the species studied. For instance, papers differ not only in the species but also in the dose and duration of E_2 treatment or in the use of an *in vivo* or *in vitro* system. Then, in spite of the lack of effect of estradiol on spermatogonial proliferation in the testes of *R. arenarum*, further studies and an *in vivo* approach would be necessary to confirm our *in vitro* results. In addition, proliferation of spermatogonia in *R. arenarum* could be regulated by hormones other than E_2. For instance, gonadotropins may play an important role, since they induce spermatogonial proliferation in newts (Abé and Ji, 1994; Ito and Abé, 1999; Ji et al., 1992). In *P. esculentus*, besides the role of estradiol on proliferation previously described, spermatogonial proliferation is also associated with seasonal changes in testosterone (Raucci and Di Fiore, 2007) while in *L. catesbeianus* both testosterone and E_2 control proliferation (Caneguim et al., 2013a). Melatonin is another hormone that has also been suggested to regulate proliferation by inhibiting estradiol-stimulated mitotic activity of spermatogonia in *P. esculentus* (ĺIstria et al., 2003). Overall, amphibian spermatogenesis is a highly complex event, and the regulation of proliferation in *R. arenarum* may involve interactions between different factors.

In conclusion, this study shows that E_2 has no effect on the activity and the expression of CypP450c17 but it reduces the activity of 3β-HSD/I during the PostR. Our results also show that E_2 induces apoptosis mainly in spermatocytes in the breeding and that there is no effect on proliferation of the germ line. Altogether, this study suggests that E_2 could regulate androgen production and spermatogenesis in adult males of *R. arenarum*. However, more studies regarding seasonal variations in the testicular estrogen receptor and in apoptosis and proliferation of the germ line may clarify the relationship between E_2 and spermatogenesis.

Acknowledgments

This work was supported by grants from Universidad de Buenos Aires – Argentina (UBACYT 20020100100786), Consejo Nacional de Investigaciones Científicas y Técnicas – Argentina (PIP 292) and Agencia Nacional de Promoción Científica y Tecnológica de la Argentina (PICT 2011-0813) to NRC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2014.12.016.

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