RESEARCH ARTICLE

Estradiol Production by the Bidder's Organ of the Toad *Rhinella arenarum* (Amphibia, Anura). Seasonal Variations in Plasma Estradiol

A Journal of Integrative Biology

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ABSTRACT

In bufonids, the Bidder's organ (BO), located in the anterior pole of the testis, is sometimes referred to as a rudimentary ovary because of the presence of previtellogenic follicles. In males of *Rhinella arenarum* it has been demonstrated that some follicles are vitellogenic and also express several steroidogenic enzymes in follicular cells. The purpose of this study is to describe seasonal variations in plasma estradiol (E_2) and in aromatase activity of the BO, and to determine the capacity of the BO to synthesize E_2 from cholesterol in males of *R. arenarum*. E_2 was determined by radioimmunoassay and aromatase activity was measured by transformation of radioactive substrates into products. Results indicate that plasma E_2 reached the highest concentration in April and the lowest one in animals captured in June, showing a progressive increase to the end of the year. Plasma E_2 and total activity of aromatase in the BO were significantly lower during the pre-reproductive season than during the reproductive and post-reproductive seasons. It was also demonstrated that the BO is able to produce E_2 from endogenous substrates throughout the year. No correlation was found between plasma E_2 and total BO weight, while there was a significant correlation between plasma E_2 and total activity of aromatase, and between plasma E_2 and E_2 produced in vitro. Taken together, these results demonstrate seasonal variations in plasma E_2, in bidderian total activity of aromatase and, that the BO of adult males of *R. arenarum* is able to produce E_2 from endogenous substrates. *J. Exp. Zool. 9999A: XX–XX, 2013.* © 2013 Wiley Periodicals, Inc.

J. Exp. Zool. 9999A:1–10, 2013

How to cite this article: Scaia MF, Regueira E, Volonteri MC, Ceballos NR. 2013. Estradiol production by the Bidder's organ of the toad *Rhinella arenarum* (Amphibia, Anura). Seasonal variations in plasma estradiol. J. Exp. Zool. 9999:1–10.

Among anurans, bufonids are characterized for the presence of a structure called the Bidder's organ (BO). The BO is located in the cephalic portion of the testis of males of the Bufonidae family (Davis, '36; Dubois, '47), and it is sometimes referred to as a rudimentary ovary because of the presence of previtellogenic follicles. Ponse ('27) demonstrated in *Bufo vulgaris* that the complete removal of both testes induced the development of functional ovaries with vitellogenic follicles. Years later, Pancak-Roessler and Norris ('91) also showed that in *Bufo woodhousii* bilateral

Grant sponsor: The University of Buenos Aires; grant number: X042; grant sponsor: The National Research Council of Argentina (CONICET); grant number: PIP2433.
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Received 1 February 2013; Accepted 12 March 2013
DOI: 10.1002/jez.1799
Published online XX Month Year in Wiley Online Library (wileyonlinelibrary.com).

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orchidectomy provokes a shift towards the later stages of oogenesis and an increase in the BO weight. Moreover, in *Bufo marinus* orchidectomy induced a greater vascularization of the BO and the development of vitellogenic follicles (Brown et al., 2002). This evidence suggests that the development of the BO is inhibited by the differentiation of the corresponding gonad. Furthermore, in *Bufo melanostictus* the administration of testosterone caused atrophy of the BO, suggesting that the inhibition of oogenesis could be due to testicular products (Deb and Chatterjee, ’63).

The BO develops in both sexes early during the larval life and before the differentiation of the gonads (Beccari, ’25; Vitale-Calpe, ’69; Petrini and Zaccanti, ’98). In *Bufo bufo*, *Bufo ictericus*, and *Bufo vulgaris*, BOs are conserved in adults of both sexes (Ponse, ’27; Farias et al., 2004; Falconi et al., 2007) while in other species such as *B. marinus*, *Bufo lentiginosus*, and *Rhinella arenarum* the BO disappears in adult females (King, ’08; Echeverría, ’90; Brown et al., 2002).

R. arenarum, formerly known as *Bufo arenarum* (Frost, 2011), is a species from the Bufonidae family with wide geographic distribution throughout the south of South America. The breeding behavior of this species correlates with the heavy rains of spring and summer and, consequently, it has been classified as an opportunistic or explosive breeder. For those populations surrounding Buenos Aires City, the reproductive season is restricted to the period between September and December (Gallardo, ’74). In adult males, BOs show a cyclic pattern of growth and involution during the year (Echeverría, ’90), and it has been recently demonstrated that the weight of BOs is significantly lower during the pre-reproductive season (PreR, from May to August), reaching the highest values during the reproductive season (R, from September to December). Moreover, histological observations have indicated that although most bidderian follicles are pre-vitellogenic, there are others in early or late vitellogenesis (Scaia et al., 2011).

In amphibians, testosterone and 5α-dihydrotestosterone (5αDHT) are the major androgens secreted by the testis (Wada et al., ’76; Müller, ’77; Kime and Hews, ’78; Canosa et al., ’98). In males of several species, including *R. arenarum*, androgen secretion exhibits seasonal variations throughout the year (Garnier, ’85; Itoh et al., ’90; Canosa and Ceballos, 2002). In some species, plasma androgens reach the highest levels during the breeding season in spring (Siboulet, ’81; Rastogi et al., ’86). However, other species like *R. arenarum* have a dissociated reproductive pattern, since plasma androgens peak before the beginning of the breeding and decline during spring, when reproduction takes place (Canosa and Ceballos, 2002; Fernández Solari et al., 2002; Canosa et al., 2003; Denari and Ceballos, 2005). In this species the ratio testosterone/5αDHT is around 1.0–1.5 all year long (Canosa et al., 2003).

The presence of estradiol (E_2) in plasma has been detected, and seasonal changes have been described in several amphibian species (Polzonetti-Magni et al., ’84; Cayrol et al., ’85; Lecouteux et al., ’85; Varriale et al., ’86; Fasano et al., ’89; Gobbetti et al., ’91a, b). In females of *R. arenarum*, plasma E_2 concentrations are low during winter, in the preovulatory period, and increase during spring, reaching the highest value in summer, during the early postovulatory season (Medina et al., 2004). However, no information about seasonal changes in plasma E_2 has been reported for males of *R. arenarum*. Furthermore, even if in other anuran males it has been demonstrated that plasma E_2 is produced by the testis (Varriale et al., ’86; Fasano et al., ’89), in *R. arenarum*, similarly to *B. marinus* (Kime and Hews, ’78), no estrogen biosynthesis could be detected in the testis. The lack of E_2 production by testicular tissue has been determined by different approaches including biosynthetic studies using different precursors (Canosa et al., ’98; Canosa and Ceballos, 2002). This difference between bufonids and ranids is probably due to the presence of BOs in adult males of the former group, which could produce E_2. The steroidogenic capacity of the BO has been studied in other bufonid species such as *B. bufo*, *B. melanostictus*, and *B. woodhousii* (Colombo and Colombo Belvedere, ’80; Ghosh et al., ’82; Pancak-Roessler and Norris, ’91). In the BO of *R. arenarum*, the presence of cytochrome P450 side-chain-cleavage (CYP11A) and aromatase has been demonstrated by immunohistochemistry, while activities of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD), cytochrome P450 17-hydroxylase, C17,20-lyase (CYP17) and aromatase were detected by the transformation of radioactive substrates into products (Scaia et al., 2011). However, despite the evidence showing the steroidogenic potentiality of the BO, there is no information indicating that the BO is able to produce E_2 from cholesterol.

The main purpose of this study is to determine seasonal variations in plasma E_2 and in aromatase activity of the BOs in males of *R. arenarum* as well as to analyze the capacity of the BO to synthesize E_2 from endogenous substrates.

MATERIALS AND METHODS

Animals

Adult male toads of *R. arenarum* were monthly collected during 2010 and 2011 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 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.

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 3,500 rpm. Aliquots of plasma (100 μL) were treated overnight with 10 volumes of methanol, evaporated, and re-dissolved in radioimmunoassay (RIA) buffer for E_2 determinations (10 mM phosphate buffer, 0.1% w/v gelatin, 140 mM NaCl, 0.1% w/v sodium azide, pH 7.4) or in RIA buffer for testosterone determinations (10 mM phosphate buffer, 1% w/v gelatin, 140 mM NaCl, 0.1% w/v sodium azide, 20 mM EDTA, pH 7.4).

Tissue Collection and Incubations

BOs were rapidly dissected, excised and weighed after fat bodies, testes and mesorchia were removed. BOs were obtained no more than 5 min after animals were deeply anesthetized, a deep plane of anesthesia being indicated when the withdrawal reflex to deep pain is lost (Gentz, 2007). Organs from 47 animals (16: PreR season, 17: R season; 14: PostR season) were individually incubated in multiwell plates for 2 h at 28°C in 1 mL Krebs-Ringer-Glucose medium containing 0.026 M Hepes buffer (KRGH, pH 7.4) with continuous shaking. These conditions were determined in preliminary experiments. Aliquots of incubation media without extraction (200 μL) were used to measure E_2 production by RIA.

Radioimmunoassay

E_2 was measured by RIA using [2,4,6,7-^{3}H]E_2 (70.0 Ci/mmol; Perkin-Elmer Life Science, MA, USA) and sheep anti-estradiol (Colorado State University, CO). This E_2 antibody has very low (<1%) cross-reactivity with estrone, progesterone, 17-hydroxyprogesterone and 5α-pregnane-3,20 dione, and negligible ones (<0.1%) with testosterone and 5α-dihydrotestosterone. The antiserum was used in a final dilution of 1:250,000. Plasma testosterone was also measured by RIA using [1,2,6,7-^{3}H] testosterone (70.0 Ci/mmol; Perkin-Elmer Life Science) and sheep anti-testosterone (Colorado State University). The cross-reactivity of the antibody with 5α-dihydrotestosterone was 6.8% and the antibody was employed in a dilution of 1:125,000. Dilutions of both antibodies were calculated as the amount of antibody producing between 40% and 50% of specific maximum binding. Standard curves were performed with radioinert E_2 or testosterone (Sigma Chemical Co., St. Louis, MO, USA) in concentrations ranging from 6.25 to 800 pg/mL and 10.0 to 1,000 pg/mL for E_2 and testosterone, respectively. The intra and inter-assay coefficients of variation for E_2 assay were 6.7% and 11.5%, respectively. The intra- and inter-assay coefficients of variation for testosterone assay were 8.2% and 9.5%, respectively. Charcoal-dextran method was used 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 or medium.

Aromatase Activity

Freshly isolated BOs obtained during the PreR ($n = 12$), R ($n = 8$), and PostR ($n = 4$) seasons were used to determine aromatase activity. Both BOs from each animal were homogenized together in 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA and 3 mM $MgCl_2$. Protein concentration was estimated by the method of Bradford using bovine serum albumin as standard (Bradford, '76). Aromatase activity was determined by incubating 200 μg proteins for 30 min at 28°C in 1 mL buffer with 5 μM [^{3}H] testosterone, and a NADPH regenerating system containing 5 mM glucose-6-phosphate, 0.25 mM NADPH, 0.25 mM NADP and 1 IU/mL glucose-6-phosphate dehydrogenase. The reaction was stopped with 2 mL cold methylene chloride and media were extracted three times with the same solvent. Substrate and product were separated by thin layer chromatography (TLC) using methylene chloride:acetone (75:5, v/v) as solvent system. Positions of radioactive steroids on TLC were determined by using radioinert standards. Testosterone was detected by UV absorption at 254 nm and E_2 at 365 nm after spraying with primuline (Wright, '71). After determining the position of standard E_2 and testosterone each spot was scraped and counted. Steroids other than E_2 and testosterone were not detected in TLC. The mass of the products was calculated from the known specific activities of the substrate. The specific activity of the enzyme was expressed as pmole of product/min/mg of protein and the total activity of the enzyme was expressed as pmole of product/min.

Statistical Analysis

Steroid concentrations and aromatase activity were expressed as means ± SE. Data from seasonal variations in plasma E_2 and in the production of E_2 were log10 transformed to correct for heteroscedasticity. Values were analyzed and compared by using a one-way ANOVA and a posteriori contrast by Tukey's test (Steel and Torrie, '80). Moreover, taking into account that androgens reach the lowest values during the PreR season, and that androgens may affect BO development and consequently steroidogenic capacity (Ponse, '27; Deb and Chatterjee, '63; Pancak-Roessler and Norris, '91; Brown et al., 2002), variations in aromatase activity were analyzed using a priori orthogonal contrasts. In this model, contrast 1 compares aromatase activity in PreR with R and PostR, while contrast two only compares the activity in R with PostR. Pearson Correlation was used to examine the relationship between total aromatase activity and plasma E_2, total BO weight and plasma E_2, total aromatase activity and total BO weight, and finally plasma E_2 and E_2 produced in media. For

this analysis, data were log10 transformed to normalize values. Pearson correlation analysis was chosen because it allows measuring the strength of the linear relationship between two continuous random variables to determine the covariance of the two variables. To analyze the strength of the correlations, P-value ≤ 0.05 was used as the cut off in all cases.

RESULTS

Plasma concentrations of E_2 were determined in toads collected during the PreR, R, and PostR seasons. Figures 1 and 2 show, respectively, the annual and seasonal patterns of E_2 level. Figure 1 indicates that the lowest concentration of E_2 was found in animals captured in June (205 ± 41 pg/mL), showing a progressive increase during the subsequent months of the year, and reaching the highest value in April (1,291 ± 188 pg/mL). Figure 2 shows that E_2 concentrations are significantly lower during the PreR (May–August) season than during the R (September–December) and PostR (January–April) seasons (PreR: 414 ± 43; R: 727 ± 77; PostR: 1,039 ± 117 pg/mL, $P < 0.0001$). As regards androgens, Figure 1 shows that the lowest concentration of testosterone was found during the R season, particularly in November (2.9 ± 0.6 ng/mL), while the highest values occurred during the PreR season in August (44.1 ± 3.8 ng/mL). This figure also shows an opposite pattern between E_2 and testosterone concentrations.

In order to study the capacity of the BO to synthesize E_2 from endogenous substrates, organs collected from animals during different seasons were incubated in media for E_2 determination. To analyze a possible correlation between plasma levels of E_2 and E_2 production by the BO, the most relevant variable for the physiology of the animal is total amount of E_2 produced by both BOs. Then, the emphasis of this correlation has been made on total E_2 secretion and not on E_2 secretion per mg of BO. Figure 3 shows that the sum of the in vitro production of E_2 by both organs from each animal was significantly lower in animals collected during the R season, and the highest production of E_2 was found in the PostR season (PreR: 2,539 ± 240; R: 956 ± 152; PostR: 5,306 ± 734 pg/mL, $P < 0.0001$). These results indicate that the BO is able to produce E_2 from endogenous substrates throughout the year. To study if there was a parallelism between the in vitro

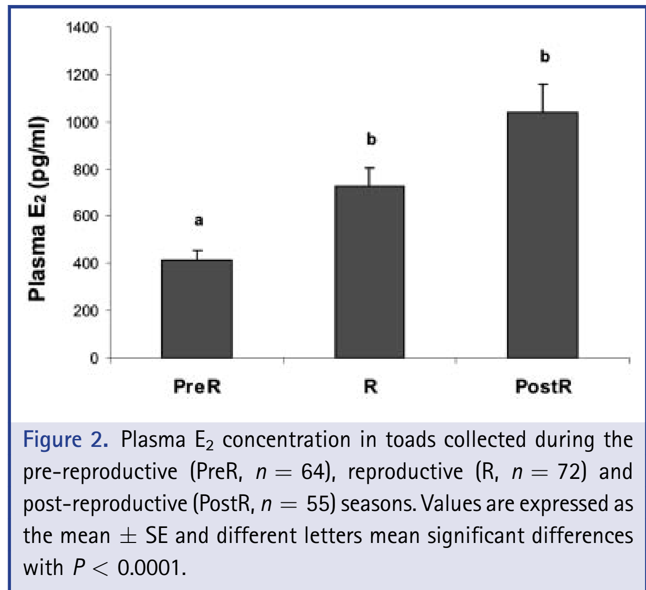

Figure 2. Plasma E_2 concentration in toads collected during the pre-reproductive (PreR, $n = 64$), reproductive (R, $n = 72$) and post-reproductive (PostR, $n = 55$) seasons. Values are expressed as the mean ± SE and different letters mean significant differences with $P < 0.0001$.

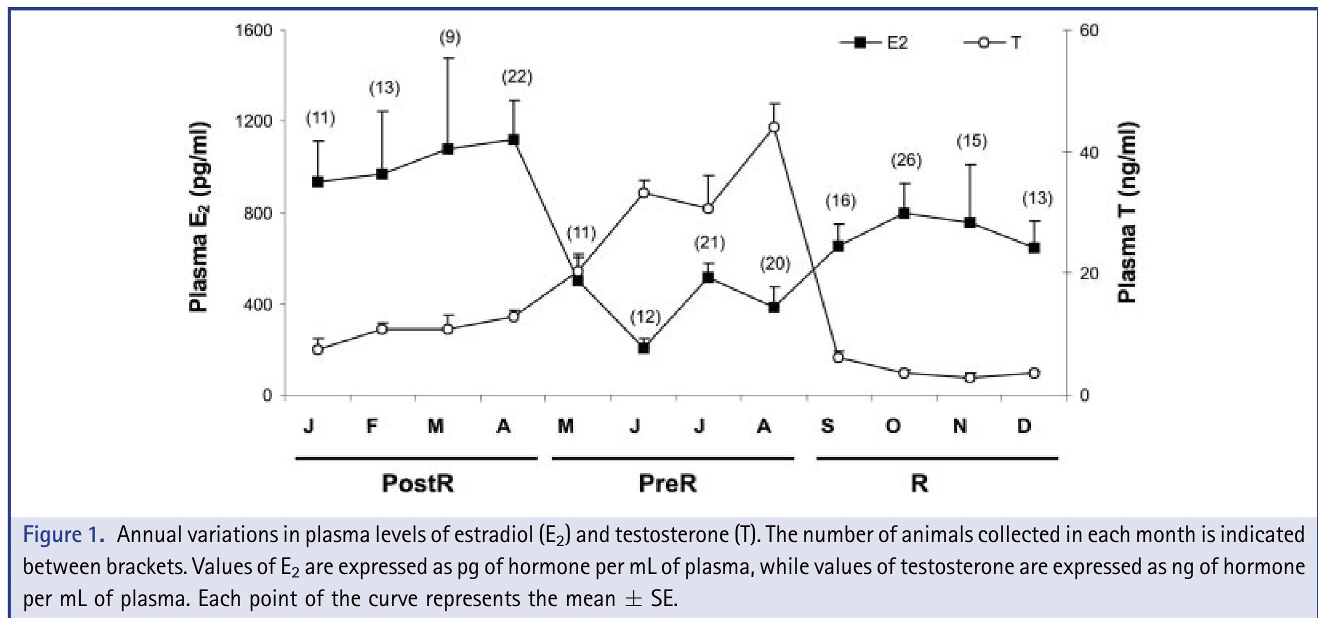

Figure 1. Annual variations in plasma levels of estradiol (E_2) and testosterone (T). The number of animals collected in each month is indicated between brackets. Values of E_2 are expressed as pg of hormone per mL of plasma, while values of testosterone are expressed as ng of hormone per mL of plasma. Each point of the curve represents the mean ± SE.

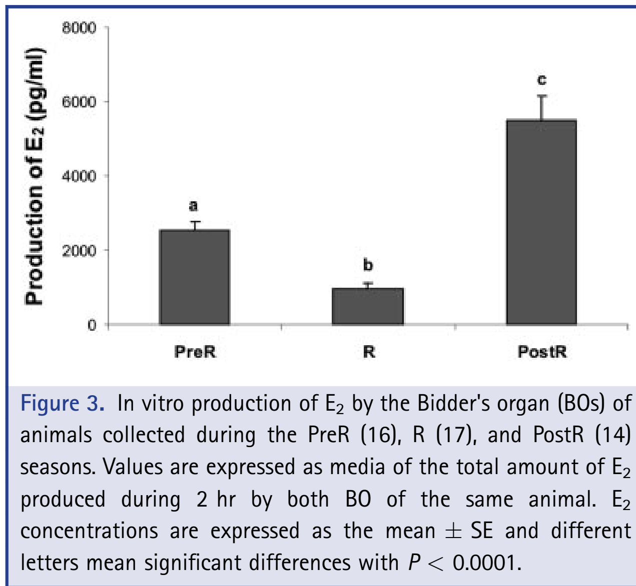

Figure 3. In vitro production of E_2 by the Bidder's organ (BOs) of animals collected during the PreR (16), R (17), and PostR (14) seasons. Values are expressed as media of the total amount of E_2 produced during 2 hr by both BO of the same animal. E_2 concentrations are expressed as the mean $\pm$ SE and different letters mean significant differences with $P < 0.0001$.

production of E_2 and plasma E_2, data from each animal were analyzed using Pearson Correlation. Statistical analysis indicates that there was a significant correlation between the levels of E_2 produced by in vitro incubations of BOs, and plasma concentrations of E_2 (Fig. 4, $r = 0.54$, $P = 0.0016$). Moreover, to analyze if both organs from one given animal have the same steroidogenic capacity, the amount of E_2 produced by each BO of the same animal was expressed as pg of E_2 produced per BO. Data from this normalization show that in some cases BOs of similar weights produce similar amounts of E_2 but in others the production of E_2 differs. Furthermore, in all the animals having BOs with different weights, the production of E_2 per organ is also different. This variability is shown in Table 1 that indicates values of nine representative animals from the PreR, R, and PostR seasons. Three categories were emphasized in this table, animals with both BO of similar weight and similar production of E_2, similar BO weight but different production of E_2, and different BO weight and different production of E_2. For each category, animals were randomly chosen.

Table 1. In vitro production of E_2 by Bidder's organs (BOs) from representative toads.*

Toad no.	Period	BO	BO weight	BO/testes index	E_2
1	PreR	r	15	0.07	1,581
		l	18	0.08	1855.8
2	PreR	r	2	0.01	1,175
		l	6	0.02	2201.4
3	PreR	r	17	0.12	2191.3
		l	6	0.03	1,233
4	R	r	9	0.09	360.9
		l	8	0.06	342.4
5	R	r	12	0.05	512.4
		l	14	0.05	218.4
6	R	r	28	0.16	585.2
		l	5	0.04	291
7	PostR	r	6	0.04	2782.2
		l	6	0.04	2734.8
8	PostR	r	3	0.03	4874.4
		l	6	0.04	1954.8
9	PostR	r	8	0.04	3587.2
		l	14	0.06	1542.8

*Animals were collected during the PreR, R and PostR seasons. r: corresponds to the right organ. l: corresponds to the left organ. In toads no. 1, 4, and 7 both BO have similar weight and produce similar amounts of E_2, while in toads no. 2, 5, and 8 both BOs are similar but they have different production of E_2. In toads no. 3, 6, and 9 both BOs have different weight and E_2 production. BOs weight is expressed in mg and hormone production is expressed as pg of E_2 per mL of media per BO.

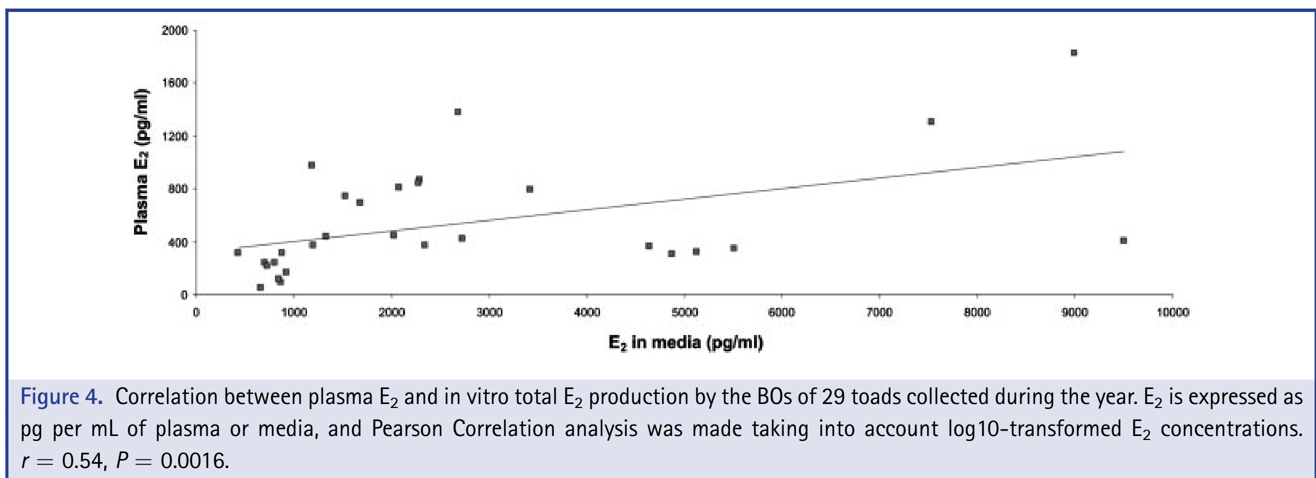

Figure 4. Correlation between plasma E_2 and in vitro total E_2 production by the BOs of 29 toads collected during the year. E_2 is expressed as pg per mL of plasma or media, and Pearson Correlation analysis was made taking into account log10-transformed E_2 concentrations. $r = 0.54$, $P = 0.0016$.

The capacity of BO to synthesize E_2 depends, at least in part, on the activity of aromatase. To study seasonal changes in the synthesis of E_2 from androgens, aromatase activity was measured in BOs from animals collected during the PreR, R, and PostR seasons. As shown in Figure 5A, total activity of aromatase was significantly lower during the PreR than in both R and PostR seasons ($P = 0.0178$ for contrast 1; $P = 0.0562$ for contrast 2). However, the analysis of the specific activity shows no significant differences among periods (Fig. 5B, $P = 0.0597$ for contrast 1; $P = 0.6415$ for contrast 2). Since the BO is one of the sources of plasma E_2 and it has been reported that BO weight is lower during the PreR season, the correlation between plasma E_2 and the weight of both BOs obtained from 137 animals was analyzed by using Pearson correlation. Statistical analysis indicates that there was no significant correlation between plasma levels of E_2 and the total weight of BOs (Table 2A, $r = -0.02$, $P = 0.8$). Moreover, there was no correlation between total aromatase activity and total weight of BOs (Table 2B, $r = 0.09$, $P = 0.73$).

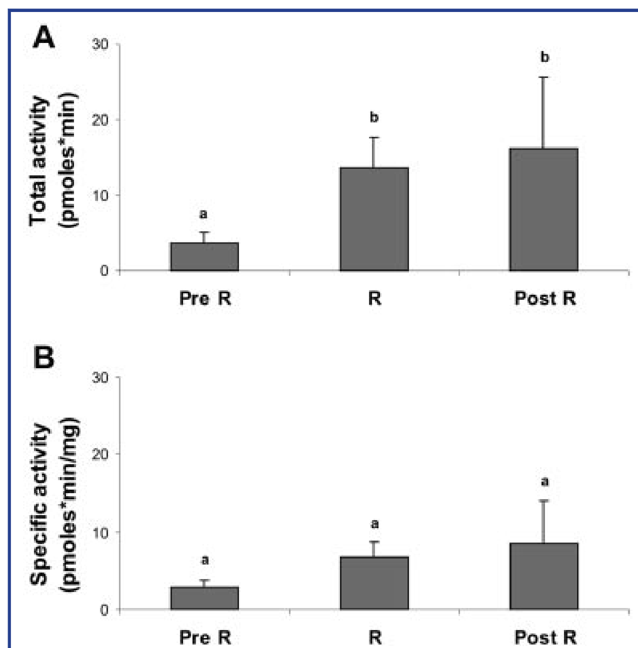

Figure 5. Variation in aromatase activity in BO from animals collected during the PreR ($n = 12$), R ($n = 8$), and PostR ($n = 4$) seasons. Aromatase activity was determined by the transformation of $[^3H]$testosterone to $[^3H]E_2$ in the presence of NADPH. Values are expressed as the mean ± SE and different letters mean significant differences. (A) Total activity of aromatase is expressed as pmoles of E_2 produced per minute ($P = 0.0178$ for contrast 1; $P = 0.0562$ for contrast 2). (B) Specific activity of aromatase is expressed as pmoles of E_2 produced per minute per mg of protein ($P = 0.0597$ for contrast 1; $P = 0.6415$ for contrast 2).

Table 2. Pearson correlation analysis.*

	log 10 plasma E2	log 10 total BO weight
(A)		
log 10 plasma E2	1.00	0.8
log 10 total BO weight	−0.02	1.00
	log 10 total activity of aromatase	log 10 total BO weight
(B)		
log 10 total activity of aromatase	1.00	0.73
log 10 total BO weight	0.09	1.00

*Analysis was made taking into account log 10 transformed values to normalize data, and total weights were calculated as the sum of right and left organs. (A) Analysis of correlation between plasma E_2 levels and total BO weight of 137 toads collected during the year. E_2 is expressed as pg per mL of plasma, while total BO weight is expressed in mg. $r = -0.02$, $P = 0.8$. (B) Correlation between total activity of aromatase and total BO weight. Total activity of aromatase is expressed as pmoles of E_2 produced per minute. $r = 0.09$, $P = 0.73$.

Furthermore it was finally analyzed if there was a correlation between the total activity of aromatase and plasma levels of E_2. Statistical analysis shows that there was a significant positive correlation between plasma levels of E_2 and the total activity of aromatase (Fig. 6, $r = 0.5$, $P = 0.04$).

DISCUSSION

Androgens are known to play an important role in male amphibian reproduction and seasonal variations in plasma testosterone levels have been determined in several anuran species such as *Rana catesbeiana* (Licht et al., '83), *Rana esculenta* (Varriale et al., '86), *Pachymedusa dacnicolor* (Rastogi et al., '86), *Rana perezi* (Delgado et al., '89), *Bufo japonicus* (Itoh et al., '90), *Rana italica* (Guarino et al., '93) *Rana rugulosa* (Kao et al., '93), and *R. arenarum* (Canosa et al., '98; Denari and Ceballos, 2005). However, there is little information regarding seasonal changes in plasma E_2. In *R. esculenta*, high levels of plasma E_2 have been measured in PostR males associated with a low level of androgens (Polzonetti-Magni et al., '84). In addition, the complete cycle of plasma E_2 studied in the same species shows that E_2 peaks when androgens start to decrease (Varriale et al., '86). However, there was no information regarding the presence of E_2 in males of the Bufonidae family, like *R. arenarum*. In this paper, seasonal fluctuations in plasma E_2 in adult male toads, and its relationship with plasma testosterone were analyzed. Statistical analysis indicated that plasma levels of this hormone were significantly lower in the PreR season than in the R and PostR seasons, the PreR

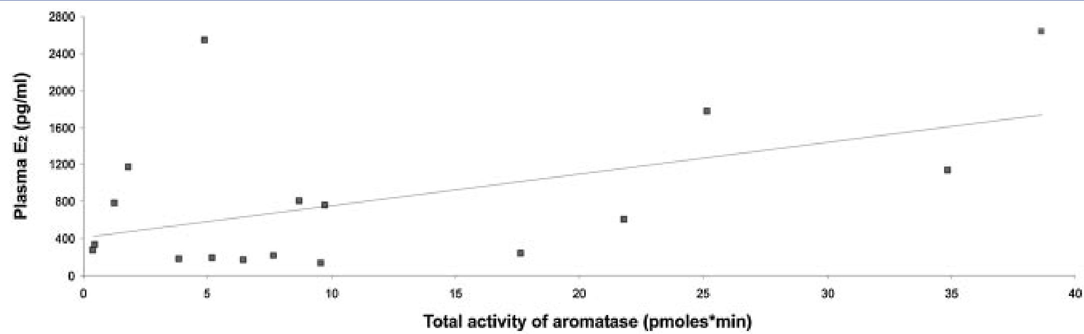

Figure 6. Correlation between plasma concentration of E_2 and total activity of aromatase. Pearson Correlation analysis was made taking into account data from 17 toads. $r = 0.5$, $P = 0.04$. E_2 is expressed as pg per mL of plasma, while total activity of aromatase is expressed as pmoles of E_2 produced per minute.

being a season characterized by high concentrations of androgens, as it was demonstrated in this paper and by Denari and Ceballos (2005). However, even if testosterone is a substrate for E_2 production, the decrease in testosterone levels during the R season could not be explained in terms of E_2 increase because in males of *R. arenarum* plasma androgen levels are much higher than E_2 levels. Similarly to the results described in *R. esculenta*, the highest concentration in plasma E_2 was measured during the PostR season. In addition, E_2 variations in males of *R. arenarum* show a similar pattern to that described in adult females of the same species (Medina et al., 2004), since in both cases E_2 reaches its lowest level during the PreR season, and shows a progressive increase during the subsequent months. However, there is a difference between sexes: in males the highest value was found at the end of the PostR season, while in females the highest ones occur at the end of the breeding and the beginning of the PostR season.

Regarding the origin of E_2, in *R. esculenta* it has been proposed that plasma E_2 is synthesized by the testis (Varriale et al., '86; Fasano et al., '89). In *R. arenarum* steroidogenic studies employing different substrates such as pregnenolone, progesterone and testosterone indicated that E_2 is not produced by testicular tissue (Canosa et al., '98; Canosa and Ceballos, 2001) not even during the PostR season (Canosa and Ceballos, 2002). In another bufonid, *B. melanostictus*, it has been suggested that E_2 could have bidderian origin, since administration of this estrogen to BO ectomized toads normalizes the testicular activity, which was impaired after the removal of the BO (Ghosh, '91). In *R. arenarum*, the BO is one of the candidates for being the source of E_2, since the presence of steroidogenic enzymes has been recently demonstrated by using different approaches (Scaia et al., 2011). However, in that paper there was no evidence showing bidderian production of E_2 from endogenous substrates. In the present study, in vitro incubations were used to demonstrate that the BOs from toads collected throughout the year produce E_2 from endogenous substrates. The significant positive correlation found throughout the year between plasma E_2 and E_2 produced in vitro by the BOs, supports the hypothesis that this organ could be, at least in part, a source of plasma estrogens.

On the other hand, it has been proposed that oogenesis in the BO is inhibited by testicular androgens (Deb and Chatterjee, '63; Calisi, 2005). Moreover, in *B. woodhousii*, the failure of hCG to stimulate the BO suggested that the inhibition of oogenesis is not due to the lack of circulating gonadotropins, but rather due to testicular inhibition (Pancak-Roessler and Norris, '91). In *R. arenarum* there is a significant reduction in BO weight during the PreR season and an increase during the R season (Scaia et al., 2011), which are characterized by high and low androgen levels, respectively (Fig. 1 of this paper; Denari and Ceballos, 2005). The fact that the BO is able to produce E_2 from endogenous substrates poses the question whether there are seasonal variations in aromatase activity that could explain seasonal fluctuations in plasma E_2 levels. Our results indicate that total activity of aromatase is significantly lower during the PreR than in both the R and PostR seasons. In conclusion, and taking into account that these are in vitro and in vivo determinations, there seems to be an opposite relationship between the lower total activity of aromatase and the higher concentration of plasma androgens determined during the PreR season. However, since there were no significant variations in the specific activity of aromatase throughout the year, lower values of total aromatase activity during the PreR season could be explained by a reduction in BO size (Scaia et al., 2011). The decrease in total enzymatic activity during the PreR season is associated with an increase in plasma androgen concentration during this season, suggesting that androgens could inhibit E_2 production in adult males of *R. arenarum*, probably by regulating the growth of BO. However, this hypothesis needs to be confirmed.

A marked parallelism emerges from seasonal variations in plasma E_2 and total activity of aromatase since both parameters

reach their lowest values during the PreR season. Moreover, significant positive correlation between both variables suggests that in male toads the BO could be the main source of plasma E_2. However, the lack of correlation between total activity of aromatase and total BO weight indicates that the bidderian contribution to plasma E_2 is not related to the weight but probably to the amount of follicular cells or aromatase activity per follicular cell. Despite the fact that the BO could be the main source of plasma E_2 and that bidderian total activity of aromatase plays a central role in E_2 production, it is worth mentioning that the regulation of other steroidogenic enzymes, such as CYP11A, 3β-HSD, and CYP17 are clearly involved and organs other than the BO could also contribute to plasma E_2. Preliminary results from our laboratory indicate that fat tissue, one of the strongest candidates to produce estrogens, does not produce E_2.

Several papers have reported that in anurans from temperate zones plasma testosterone levels were highest during the winter in association with spermatid formation while the lowest concentrations were detected in the summer during early spermatogenesis (spermatogonia I to spermatocytes II) (Rastogi et al., '76; Rastogi and Iela, '80; Pierantoni et al., 2002; Delgado et al., '89; Guarino et al., '93). In *R. arenarum*, the highest concentration of testosterone was determined also during the winter associated with the late spermatogenesis (Burgos and Mancini, '48) but the lowest one was measured during the breeding (Denari and Ceballos, 2005). In anurans, several factors such as gonadotropins and E_2 act on spermatogonial proliferation (Minucci et al., '86; Di Matteo et al., '88; Cobellis et al., '99). In mammals, several papers have also suggested that estrogens could regulate spermatogenesis (Ebling et al., 2000; Mahatto et al., 2000). In the toad, during the PostR season, there is an active proliferation of spermatogonia (Burgos and Mancini, '48), and results of this paper indicate that this proliferation could be associated with the highest concentration of plasma E_2, suggesting that, as in *R. esculenta*, E_2 could promote spermatogonial proliferation (Minucci et al., '97). However, this hypothesis needs to be confirmed.

In conclusion, this study shows that the BO of male toads collected during different seasons produces E_2 from endogenous substrates, and that it could be one of the main sources of plasma E_2. Our results also show that plasma E_2 and bidderian total activity of aromatase are significantly lower during the PreR season, a season characterized by high concentration of plasma androgens. However, more studies regarding the effect of androgens on aromatase activity and the action of E_2 on testicular parameters are necessary to clarify the relationship between the BOs and the testes in males of the toad *R. arenarum*.

ACKNOWLEDGMENTS

This work was supported by grants from The University of Buenos Aires (X042) and The National Research Council of Argentina (CONICET, PIP2433).

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