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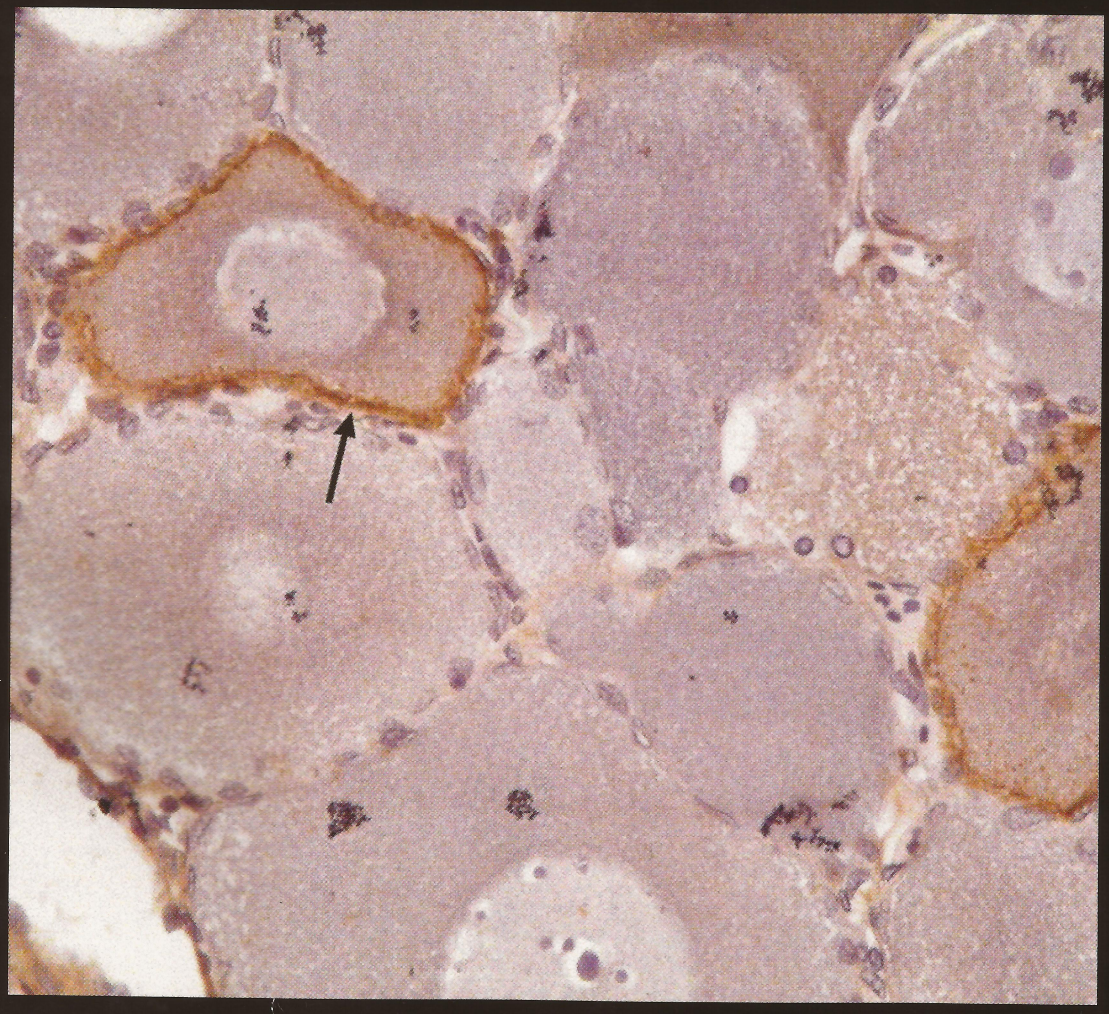

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ON THE COVER: The Bidder's organ of male true toads of Bufonidae family, located in the anterior pole of the testis, has been compared to a rudimentary ovary due to the presence of previtellogenic follicles. Analysis of the presence of steroidogenic enzymes via immunohistochemistry showed that all follicles were immunoreactive with the antibody against aromatase, yet only a few were positive for the cytochrome P450 side-chain cleavage. See related article by Scaia et al., pages 439–446.

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RESEARCH ARTICLE

The Bidder's Organ of the Toad *Rhinella arenarum* (Amphibia, Anura). Presence of Steroidogenic Enzymes

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART A
ECOLOGICAL GENETICS AND PHYSIOLOGY
A Journal of Integrative Biology

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ABSTRACT

The Bidder's organ (BO) of male true toads of Bufonidae family is located in the anterior pole of the testis and it has been compared to a rudimentary ovary because of the presence of previtellogenic follicles. In some species, BO remains in both sexes, while in others only adult males preserve the structure. Several studies suggest that the development of BO is inhibited by the differentiation of the corresponding gonad. The purpose of this study is to describe morphological and histological variability of the BO of *Rhinella arenarum* and also analyze its steroidogenic capacity. Observations indicate that although most bidderian follicles are in pre vitellogenesis, there are others in early or late vitellogenesis. Moreover, we found that BOs weight was significantly lower in males during the pre-reproductive period and that there is no significant correlation between the weights of BO and the adjacent testis. We also analyzed the presence of steroidogenic enzymes using immunohistochemistry. Results indicate that all the follicles were immunoreactive with the antibody against aromatase, while only few of them were positive for the cytochrome P450 side-chain cleavage. Furthermore, activities of 3β-hydroxysteroid dehydrogenase/isomerase, cytochrome P450 17-hydroxylase, C17,20-lyase and aromatase were detected by the transformation of radioactive substrates into products. Taken together, these results confirm the steroidogenic capacity of the BO in adult males of *R. arenarum*. *J. Exp. Zool. 315:439–446, 2011.* © 2011 Wiley-Liss, Inc.

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The Bidder's organ (BO) of male true toads of Bufonidae family is located in the anterior pole of the testis and it has been compared to a rudimentary ovary because of the presence of previtellogenic follicles (Echeverría, '90). In *Bufo ictericus*, the structure of this organ is similar to a typical ovary, composed by a cortex with follicular cells and follicles in different stages of development and, also, a medulla containing blood vessels and cells pigmented with melanin (Farias et al., 2002). BO differentiation has been previously described by several authors (Beccari, '25; Ponse, '25, Ponse and Dovaz, '43; Vitale-Calpe, '69; Petrini and Zaccanti, '98). BO develops in both sexes very early during the larval stage and prior to the first appearance of ovarian and testicular differentiation. In some species such as *Bufo bufo*, *B. ictericus* and *Bufo vulgaris*, BO is present in adults of both sexes (Ponse, '27; Farias et al., 2004; Falconi et al., 2007).

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However, in *Bufo marinus* and *Bufo lentiginosus*, this organ remains only in adult males (King, 1908; Brown et al., 2002).

Rhinella arenarum (formerly known as *Bufo arenarum*) is a species with wide geographic distribution throughout South America. It has been classified as an opportunistic breeder, since its breeding behavior correlates with the heavy rains of spring and summer (Gallardo, '74). Moreover, this author has characterized populations surrounding Buenos Aires City in which the breeding season extends from the end of September to the beginning of December. This is a species with a dissociated reproductive pattern, since the reproductive behavior in males is associated with low levels of plasma androgens (Canosa and Ceballos, 2002; Fernández Solari et al., 2002; Canosa et al., 2003). Regarding BO, in females of *R. arenarum* atresia of bidderian follicles increases markedly during the third winter after metamorphosis, followed by a reduction in size and a final reabsorption of BO during the fourth summer. On the contrary, adult males retain one or two BO with a cyclic pattern of growth and involution during the year (Echeverría, '90).

Several studies suggest that the development of BO is inhibited by the differentiation of the corresponding gonad. Following gonadectomy of males and females of *B. vulgaris*, BOs develop into functional ovaries with vitellogenic follicles (Ponse, '27). Bilateral orchidectomy of males of *Bufo woodhousii* also caused a shift toward the later stages of oogenesis and an increase in the organ weight, suggesting that oogenesis is inhibited by the presence of functional testes (Pancak-Roessler and Norris, '91). Furthermore, as a result of orchidectomy in males of *B. marinus*, BO became more vascularized and oocytes became vitellogenic (Brown et al., 2002). Moreover, administration of testosterone in *Bufo melanostictus* caused atrophy of BO (Deb and Chatterjee, '63). These authors propose that the inhibition of oogenesis could be due to some testicular product.

Few studies have studied the steroidogenic capacity of the BO in different bufonid species. The presence of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/I) and 17β-hydroxysteroid dehydrogenase (17β-HSD) has been described in follicles of BO of adult males of *B. melanostictus* by using histochemistry and biochemical methods (Ghosh et al., '82). These enzymatic activities have been localized in ooplasm and follicular cells in the BO of *B. woodhousii* (Pancak-Roessler and Norris, '91). Furthermore, in the case of *B. bufo* it has been demonstrated that this organ is capable of converting pregnenolone or progesterone into several steroid hormones such as 17-hydroxyprogesterone, androstenedione, testosterone, estrone and 17β-estradiol (Colombo and Colombo Belvedere, '80). Taken together, these results confirm the presence of steroidogenic activity in the BO of at least three species of the Bufonidae family.

The main purpose of this study is to describe the structure and variability of BO of *R. arenarum* collected throughout the year as well as to determine the steroidogenic capacity of this organ by immunohistochemistry and enzymatic assays.

MATERIALS AND METHODS

Animals

A total of 101 male toads of *R. arenarum* were collected during the pre-reproductive (PreR), reproductive (R) and post-reproductive (PostR) seasons (Canosa et al., 2003) in a nonagricultural area near Buenos Aires City during 2008 and 2009. Animals were maintained with free access to water and fed with crickets, diet being supplemented with liver and zophobas. Animals were anaesthetized with 1% MS222, 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.

Tissue Collection, Histology and Immunohistochemistry

Testes and BOs were rapidly excised and weighted after fat bodies and mesorchia were removed. Organs were analyzed with a stereoscopic microscope Leica EZ4D and macroscopic images were captured with an incorporated digital camera. Testes and BOs 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 stained with hematoxylin–eosin or Masson's trichromic stain. Immunohistochemical techniques were carried out after blocking biotin with the avidin–biotin blocking kit (Vector Laboratories Inc., Burlingame, CA). To assess the presence of steroidogenic enzymes, immunohistochemical staining was performed in BOs from five animals collected during the R season, employing a polyclonal anti-aromatase antibody (1:300; rabbit anti-human, USBiological, Swampscott, MA) and a polyclonal anti-cytochrome P450 side-chain cleavage ($CypP450_{scc}$) antibody (1:200; rabbit anti-rat, Chemicon International, Billerica, MA). Later, sections were incubated with goat anti-rabbit secondary polyclonal antibody conjugated with biotin (1:500; DAKOCytomation, Denmark), 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. Sections were examined using a Leica DM2000 microscope and images were captured with an incorporated digital camera.

Enzymatic Assays

Freshly isolated organs from animals collected during the R season were used to determine the activity of three steroidogenic enzymes. For the determination of these activities both BOs from each animal were homogenized together.

For determining the activity of 3βHSD/I, BOs 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. ('97). Enzymatic activity was measured in homogenate preparations containing 200 µg proteins in 1 ml buffer, with 0.5 mM NAD^+ and 25 µM [^{3}H]pregnenolone and samples were incubated for 30 min at 28°C. For this assay, five animals were employed.

For the determination of cytochrome P450 17-hydroxylase, C17,20-lyase ($CypP450_{c17}$) and aromatase activity, BOs were homogenized in 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA and 3 mM $MgCl_2$. $CypP450_{c17}$ activity was determined by incubating 200 µg proteins for 20 min at 28°C in 1 ml buffer containing 10 µM [^{3}H]pregnenolone, 5 mM glucose-6-phosphate, 0.25 mM NADPH, 0.25 mM NADP and 1 IU/ml glucose-6-phosphate dehydrogenase (Fernández Solari et al., 2002). Aromatase activity was determined in the same way but employing 5 µM [^{3}H]testosterone as substrate and incubating for 30 min at 28°C. This activity was determined in both BO and fat bodies. Three animals were employed for each enzymatic assay.

For all the activities, substrates and products were separated by thin layer chromatography (TLC) using methylene chloride: acetone (75:5, v/v) as solvent system. The specific activities of the enzymes were expressed as pmole of product/min/mg of protein. Positions of radioactive steroids on TLC were determined by using radioinert standards. Standard 3-oxo-4-ene steroids were detected by UV absorption. Nonabsorbing standard steroids were revealed after spraying with primuline (Wright, '71).

Statistical Analysis

BO weights and the ratios of total testes weight/total BO weight were expressed as mean ± SE. Values from PreR, R and PostR seasons were analyzed and compared by using a one-way ANOVA test and a posteriori contrast by using Tukey's test (Steel and Torrie, '80). For ANOVA test, a Log10 transformation was used and for Pearson Correlation analysis, a square root-transformation of BO and testes weights was employed to normalize values. The correlation analysis excluded animals with only one BO, reducing the number of animals from 101 to 93.

RESULTS

Macroscopic observations demonstrated that the size of BOs and testes differs markedly throughout the year, and in some animals both testes also differ in size. Most of the toads have well-developed testes with reduced BOs (Fig. 1A). However, other animals possess well-developed BOs with testes and BOs with similar sizes (Fig. 1B). In other toads testes are reduced, while BOs

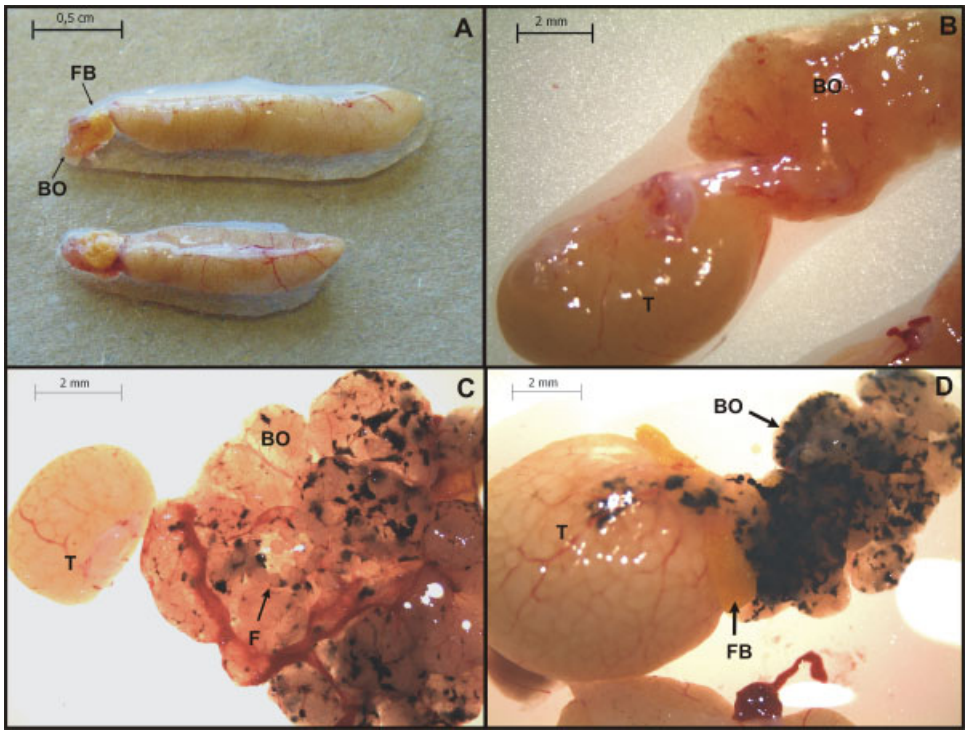

Figure 1. (A) Well-developed testes (T) with small Bidder's Organs (BO) and fat bodies (FB). Both testes of different sizes correspond to the same animal collected during the PreR period. (B) BO with similar size than the corresponding T. (C) Reduced T and highly developed and vascularized BO. Bidderian follicles (F) of several sizes can be seen. (D) Pigmented BO belonging to animals collected during PreR period.

are bigger than testes (Fig. 1C) and are also highly pigmented and vascularized (Fig. 1C and D).

Histological studies show bidderian follicles near to the seminiferous lobules with spermatocysts in different stages (Fig. 2A and B). Most bidderian follicles are previtellogenic, with a nucleus in central position and homogeneous ooplasm (Fig. 2C and D). In addition, it is possible to identify follicles in early vitellogenesis with a heterogeneous cytoplasm, and a central germinal vesicle (Fig. 2C–F). Figure 2F shows a detail of an early vitellogenic follicle having a germinal vesicle with irregular nuclear membrane and numerous nucleoli. In addition, other follicles show yolk in their ooplasm, which is characteristic of late vitellogenesis (Fig. 2C). Atresic follicles are also present (Fig. 2D).

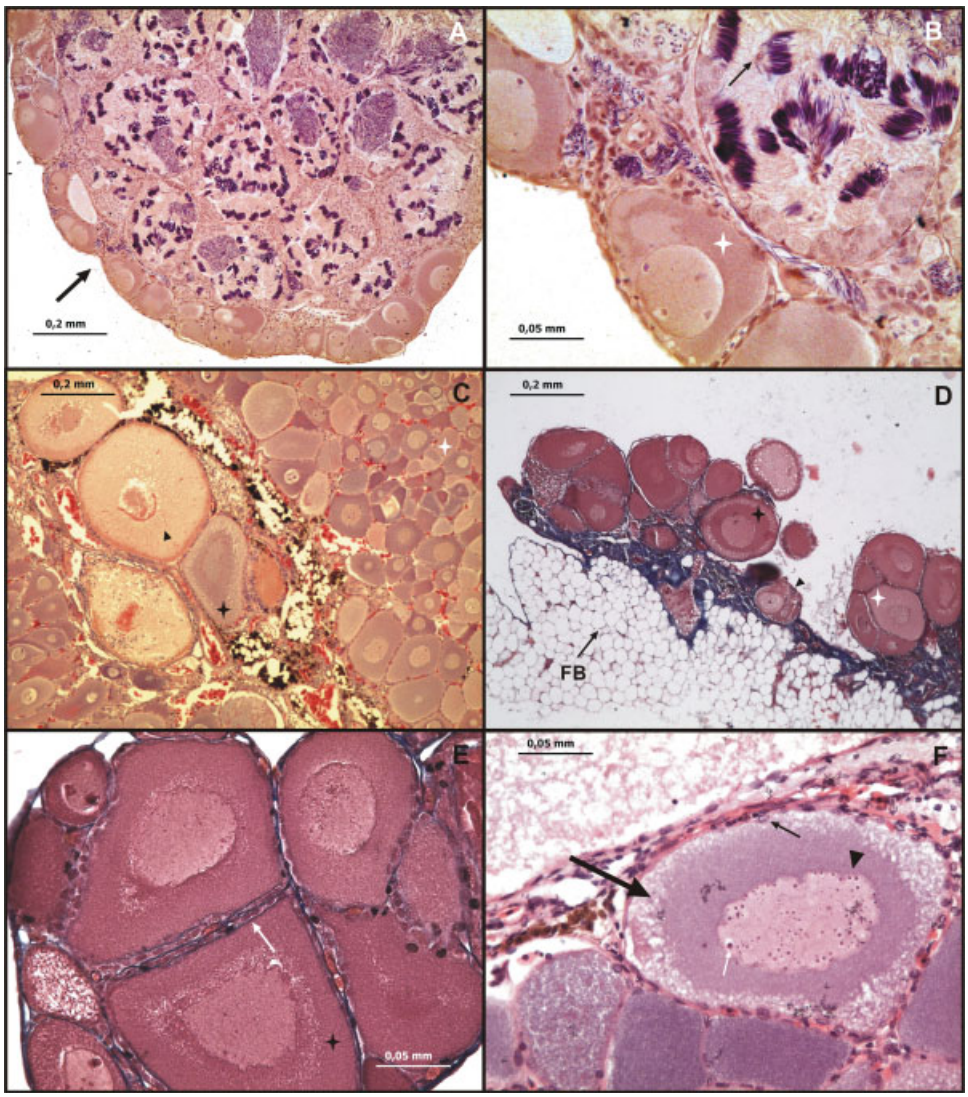

Figure 2. (A) Cross section of testes and BO. Arrow indicates the region amplified in (B). (B) Previtellogenic bidderian follicles (white star) close to spermatocysts in different stages. Arrow indicates spermatozoa attached to Sertoli cells. (C) Bidderian follicles in several stages. Previtellogenic follicles (white star), early vitellogenic follicle (black star) and late vitellogenic follicle (head of arrow). (D) Cross section of BO and FB. Bidderian follicles in different stages of development: previtellogenic (white star), early vitellogenic (black star) and atresic (head of arrow). (E) Follicle in early vitellogenesis (black star). White arrow indicates follicular cells surrounding bidderian oocytes. (F) Detail of bidderian follicle in early vitellogenesis. Long arrow shows the heterogeneous cytoplasm with peripheric yolk and the head of arrow indicates the central germinal vesicle with irregular nuclear membrane. Numerous nucleoli (white arrow) and follicular cells (short arrow) can be observed. A, B, C, F: stained with hematoxylin–eosin. D, E: stained with modified Masson's trichromic. FB, fat bodies.

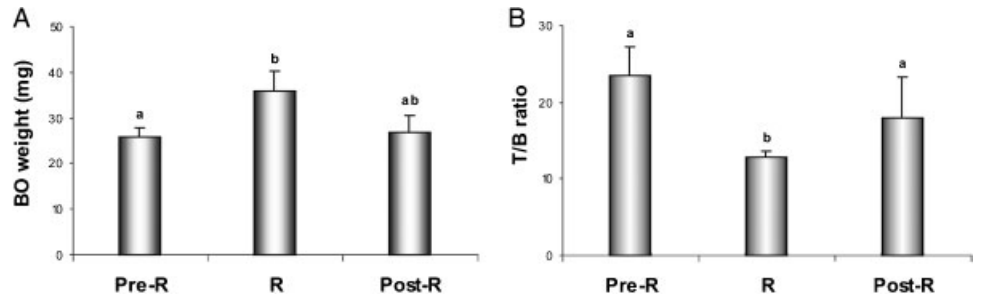

Figure 3. Variation in BO weight and ratios of total testes weight/total BO weight (T/B ratio) in PreR ($n = 52$), R ($n = 33$) and PostR ($n = 16$) period. (**A**) BO weight. (**B**) Ratios of total testes weight/total BO weight (T/B ratio). Values are expressed as the mean ± SE. Different letters mean significant differences ($P = 0.0227$ for BO weight, $P = 0.0279$ for T/B ratio).

Table 1. Weight of testes in toads with only one BO.

BO (mg)		Testes (mg)		
R	L	R	L	Month
6	0	207	238	May
0	15	182	113	June
0	3	254	272	June
5	0	86	90	July
25	0	105	11	August
61	0	127	156	October
73	0	146	120	October
14	0	203	224	January

R, corresponds to the right organ; L, corresponds to the left organ.

Table 2. Pearson coefficients of correlation.[1]

		Square root total BO weights	Square root total testes weights
a.	Square root total BO weights	1.00	0.69
	Square root total testes weights	−0.04	1.00
b.[2]		Square root BO weights	Square root testes weights
	Square root BO weights	1.00	0.60
	Square root testes weights	−0.04	1.00

[1]Total weights were calculated as the sum of right and left organs. Analysis was made taking the square root of total BO weights in each animal as *X* variable, and the square root of total testis weights in each animal as *Y* variable. $r = -0.04$, $P = 0.69$.
[2]In this case, the weight of each organ was taken as an independent value. $r = -0.04$, $P = 0.60$. A total of 93 animals was analyzed.

Annual variation in BO weight was analyzed in animals collected during PreR, R and PostR seasons. As shown in Figure 3A, BO weights were significantly lower in PreR than in R males ($P = 0.0227$), but no significant differences were found with PostR males. In addition, the ratio total testes weight/total BO weight was significantly lower in R animals (Fig. 3B; $P = 0.0279$) even if testicular weight was slightly higher during the R season than in PreR and PostR seasons (PreR: 344 ± 18; R: 392 ± 12; PostR: 359 ± 39, mg).

When the presence and weight of BO was analyzed in relationship with the testicular weight, it was observed that while most animals possess two BOs and two testes ($n = 93$), others have only one BO and both testes with similar or different weights ($n = 8$). In animals with only one BO, sometimes this organ is associated to the smallest testis but in others to the biggest one. Besides, the presence of only one BO seems not to be related to a particular period. Table 1 shows the values of the eight toads with only one BO.

In order to analyze whether there is an inverse correlation between BO and testis, total and individual weights of each organ were analyzed by using Pearson correlation. Table 2 shows that there is no significant correlation neither between the weight of total BOs and testes ($r = -0.04$, $P = 0.69$) nor between the weight of each BO and the adjacent testis ($r = -0.04$, $P = 0.60$).

To study the capacity of BO to synthesize steroids, immunohistochemistry was used to study the presence and localization of steroidogenic enzymes such as $CypP450_{scc}$ and aromatase. In the first case, immunoreactivity was observed only in follicular cells of a few follicles, indicating that at least some of them are able to transform cholesterol into pregnenolone (Fig. 4A). On the other hand, when aromatase was determined, immunoreactivity was located in follicular cells of all bidderian follicles, suggesting that BO is able to produce estradiol from androgens (Fig. 4C). In both cases, no immunoreactivity was observed when primary antibody was omitted (Fig. 4B and D).

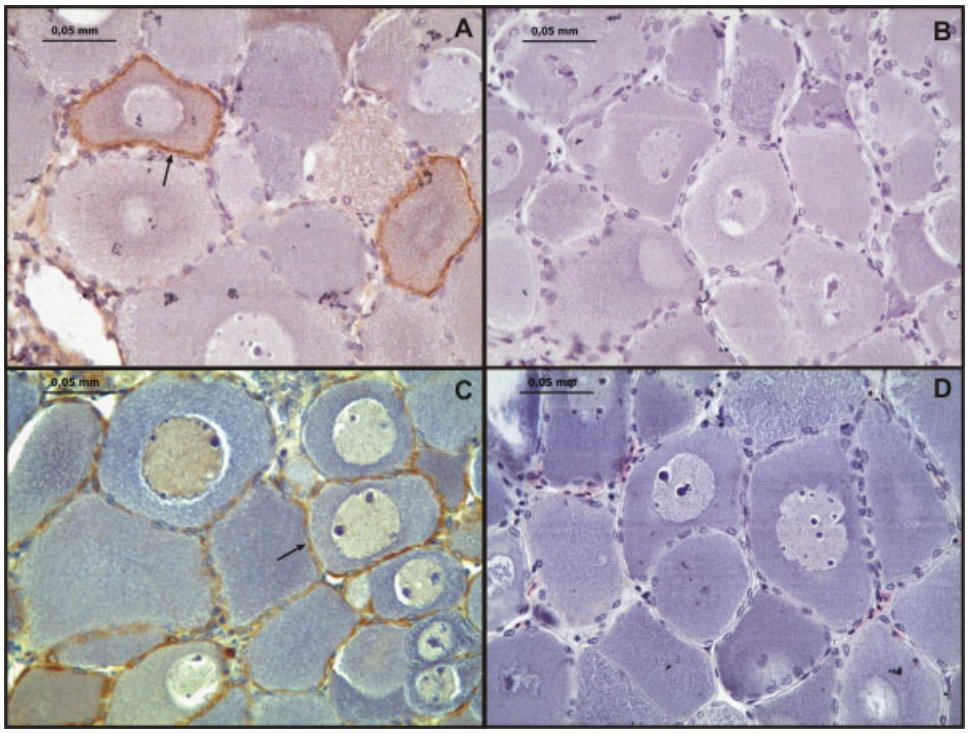

Figure 4. Immunohistochemistry of Cyp450$_{scc}$ (**A**, **B**) and aromatase (**C**, **D**) in BO. (A, C) Sections incubated with the primary antibody. Immunoreactivity is indicated with an arrow. (B, D) Negative controls without the primary antibody.

Table 3. Enzymatic activities in the BO.[1]

Enzymatic activity	Mean (pmol/min/mg)	SE
3βHSD/I	3.04	0.83
Aromatase	4.86	2.56
CypP450c17	31.85	9.90

[1]3βHSD/I activity was determined by the transformation of [^{3}H]pregnenolone to [^{3}H]progesterone in the presence of NAD$^+$. CypP450$_{c17}$ activity was assayed employing [^{3}H]pregnenolone as substrate and registering production of [^{3}H]dehydroepiandrosterone and [^{3}H]17-hydroxypregnenolone in the presence of NADPH. Aromatase activity was determined by the transformation of [^{3}H]testosterone to [^{3}H]estradiol in the presence of NADPH. 3βHSD/I ($n = 5$), CypP450$_{c17}$ ($n = 3$) and aromatase activities ($n = 3$) are expressed as pmoles of product per minute per mg of protein.

The presence of steroidogenic enzymes in BO was confirmed by assaying the activity of CypP450$_{c17}$, 3βHSD/I and aromatase in homogenates. All enzymatic activities were detected, confirming the steroidogenic capacity of the BO in males of *R. arenarum* (Table 3). The determination of aromatase activity in fat bodies—another possible source of estradiol—rendered negative results (data not shown).

DISCUSSION

Macroscopic observations of BO of *B. marinus* indicated that some bidderian follicles are in early or late vitellogenesis (Mc Coy et al., 2008). In *R. arenarum*, both macroscopic and histological observations show that although most follicles are previtellogenic, others are in early or late vitellogenesis. In previous publications, the presence of vitellogenic follicles in BO has only been supported by histological studies in orchidectomized males of *B. woodhousii* but not in control animals (Pancak-Roessler and Norris, '91). In this study, we described that in *R. arenarum* late vitellogenic follicles were also found in animals with well-developed testes.

As mentioned in the Introduction, it has been suggested that oogenesis in BO is inhibited by testicular androgens since the administration of testosterone induced BO atrophy (Deb and Chatterjee, '63). The study of annual variations in Bidder volume in *B. woodhousii* has suggested that this organ is reduced during the R season (Calisi, 2005). On the contrary, previous studies proposed that BO in *R. arenarum* proliferates during the R season, followed by degeneration during the nonreproductive period (Echeverría, '90). Furthermore, in this study we reported a significant reduction in BO weight during the PreR period, while there is an increase during the R season. The differences between both species could be due to the fact that in *B. woodhousii* the

reproductive behavior seems to be associated with high levels of plasmatic androgens. In the case of *R. arenarum* high concentrations of these hormones are characteristic of the nonreproductive season (Canosa and Ceballos, 2002; Fernández Solari et al., 2002; Canosa et al., 2003). Therefore, in both species degeneration of BO occurs when plasma androgens are high, suggesting that androgens could inhibit the development of this organ in adult males.

In this paper we also demonstrated that there is no statistical correlation between BO and testicular weight in *R. arenarum*. These results differ from those obtained in *B. woodhousii*, where BO volume was inversely related to testicular volume (Calisi, 2005). This difference could be due to the fact that in *B. woodhousii* the volume of BO was analyzed, this being estimated taking into account the length and width of the organs.

In amphibians, high plasmatic and testicular levels of estradiol in males of *Rana esculenta* have been described (Polzonetti-Magni et al., '84; Varriale et al., '86; Fasano et al., '89). However, in *R. arenarum*, testes do not synthesize estrogens, even during the PostR season (Canosa et al., '98). In that study, authors proposed that the difference with ranids could be due to the presence of steroidogenic enzymes in BOs. In this study, we used several approaches to corroborate the proposal that the BO from *R. arenarum* produces estradiol from androgens. For instance, we employed immunohistochemistry to demonstrate the expression of aromatase in follicular cells of all bidderian follicles. In addition, we determined the activity of aromatase for the conversion of testosterone into estradiol. These results suggest that BO is able to use androgens to produce estradiol and that it could be one of the sources of circulating estradiol. In other species such as *B. bufo* the production of estradiol by BOs was demonstrated by biosynthetic studies (Colombo and Colombo Belvedere, '80). In *B. woodhousii*, it was suggested that testes may not be the main source of estradiol since orchidectomy produced a reduction in androgen levels but estradiol remained unaffected. BO, fat bodies and liver were proposed as organs for aromatizing activity (Pancak-Roessler and Norris, '91). In *R. arenarum* no aromatization of androgens was detected in fat bodies (data not shown).

The source of androgens employed by BO for estradiol synthesis has not been determined. One possibility is that this organ uses circulating androgens as the main source of aromatase substrate. Another one is that BO is able to synthesize androgens. To determine the capacity of de novo synthesis of androgens from cholesterol of the BO, we employed immunohistochemistry and demonstrated that CypP450$_{scc}$ is expressed in some bidderian follicles. These results suggest that only some follicles are able to start the steroidogenic pathway from cholesterol. The presence of the activities of CypP450$_{c17}$ and 3βHSD/I—determined by the transformation of radioactive substrates into products—also suggests that follicles expressing CypP450$_{scc}$ could convert cholesterol into androgens, BO being a source of aromatase substrate. The expression of CypP450$_{scc}$ in only few follicles is intriguing and clearly more observations are needed to clarify whether the expression of this enzyme varies in different stages of oogenesis, as it was documented in other models like the fish *Ictalurus punctatus* (Kumar et al., 2000).

In amphibians, several studies have reported that some herbicides of worldwide application have an endocrine-disrupting effect and induce gonadal abnormalities, a decrease in plasma testosterone and fertility rates, and even feminization (Hayes et al., 2002, 2003, 2010). Particularly, *B. marinus* collected from completely suburban to completely agricultural areas show gonadal and Bidder's organ abnormalities increasing with agriculture in a dose-dependent fashion (McCoy et al., 2008). However, there is discrepancy about whether reproductive abnormalities are caused by endocrine-disrupting chemicals or by natural reasons (Coady et al., 2005; Hecker et al., 2005; Murphy et al., 2006; Kloas et al., 2008). The discussion on whether hermaphroditism in amphibians is related to agricultural contaminants enhances the interest of studying gonadal morphology and endocrine regulation in *R. arenarum*, a species in which adult males have functional testis and also BO.

Results in this study demonstrate that the BO of the toad has both previtellogenic and vitellogenic follicles and that this organ is able to produce estradiol from androgens and expresses several steroidogenic enzymes. However, more studies regarding the localization of some enzymes such as CypP450$_{c17}$ and 3βHSD/I as well as seasonal changes in the expression of CypP450$_{scc}$ could clarify the function of BOs.

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