RESEARCH ARTICLE

Development and Steroidogenic Properties of the Bidder's Organ of the Tadpole of *Rhinella arenarum* (Amphibia, Anura)

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ABSTRACT

Several studies suggested that in anuran amphibians steroidogenic enzymes are critical for gonadal differentiation, proposing that the amount of sex steroids would adjust this differentiation. Among anurans, bufonids are important for the study of sex differentiation due to the presence of Bidder's organ (BO) that differentiates as a rudimentary ovary in the cephalic portion of the genital ridge. Considering that in adult males of *Rhinella arenarum*, the BO synthesizes estradiol, the main purpose of this work is to examine, in this species, the morphogenesis of BO and the steroidogenic capacity of this organ during larval development. BO and the proper gonads are distinguished from Gosner stage 26. During metamorphosis, BO primary oogonia develop in oogonia in nests, early previtellogenic oocytes and late previtellogenic oocytes in follicles while proper gonads remain undifferentiated. Aromatase was detected by immunohistochemistry in almost all the largest follicles of the BOs while the cytochrome P450 side-chain cleavage was observed in only few oocytes. The proper gonad was not immunoreactive in any stage. The determination of aromatase and 5α-reductase activities showed that the population of tadpoles between stages 36–41 is not homogeneous in terms of aromatase activity. In addition, from stage 26 to the end of metamorphosis, all the stages were able to produce estradiol from endogenous substrate but stages 40–41, corresponding to the end of pro-metamorphosis, produced the highest values. In conclusion, BO is able to synthesize estradiol from endogenous precursors and proper gonad remains undifferentiated at least until the end of the metamorphosis. *J. Exp. Zool. 323A:137–145, 2015.* © 2014 Wiley Periodicals, Inc.

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In vertebrates, gonadal differentiation is a sequential and ordered process that begins during early stages of embryonic development. In females, the differentiation of the ovary is directed by germ cells while in males, somatic cells induce germ cells toward spermatogenesis (Barske and Capel, 2008). In mammals, sex differentiation depends on the expression of several genes such as *Sry*, *Sox9,* and *Dax1*. In amphibians, despite many studies have shown that sex is genetically determined (Hayes, '98), no sex-determining genes have been identified (Kelley, '96; Uno et al., 2008) and in many species sex chromosomes are morphologically indistinguishable from autosomal ones (Schmid and Steinlein, '91). In last years, a candidate for an ovary-determining gene (*DM-W*) was found in *Xenopus laevis* (Yoshimoto et al., 2008) but this gene was not further investigated in other species. Several papers have suggested that steroidogenic enzymes might be critical for gonadal differentiation, proposing that the amount of sex steroids synthesized by these enzymes could finely adjust this differentiation (Yu et al., '93; Zaccanti et al., '94; Hayes, '98; Petrini and Zaccanti, '98). In *Rugosa rugosa*, steroidogenic enzymes such as CYP11A (cytochrome P450 side-chain cleavage), 3βHSD/I (3β-hydroxysteroid dehydrogenase-isomerase), CYP17 (cytochrome P450 17α-hydroxylase/C17–20 lyase), 17β-HSD (17β-hydroxysteroid dehydrogenase), aromatase and 5α-reductase, as well as the steroidogenic acute regulator protein (StAR) are expressed in the undifferentiated gonad of tadpoles (Maruo et al., 2008). Particularly, the expression of aromatase is higher in the undifferentiated gonad of female tadpoles than in male ones, while CYP17 is enhanced in the undifferentiated male gonads prior to sex determination and continues at high levels thereafter (Sakurai et al., 2008; Nakamura, 2012). This same pattern was found in *X. laevis* and *Lithobates sylvaticus* (Okada et al., 2009; Navarro-Martín et al., 2012).

Anurans are characterized by having different rates of ovarian development, which could affect their susceptibility to estrogenic contaminants. Taking into account somatic development, Ogielska and Kotusz (2004) described three rates of ovarian development: basic, retarded, and accelerated. In general, most of the studied species undergo the basic rate, while several toads follow the retarded rate (Ogielska and Kotusz, 2004). These authors have also described that some members of the genus *Rana* appear to follow the accelerated rate (*Pelophylax lessonae*, *Pelophylax ridibundus*, *Lithobates catesbeianus*, *Lithobates pipiens*) while others follow the basic rate (*Rana temporaria*, *Rana arvalis*).

Among anurans, bufonids are particularly important for the study of sex differentiation due to the presence of a structure called Bidder's organ (BO) that differentiates as a rudimentary ovary in the cephalic portion of the genital ridge. BO develops in both sexes very early during the larval stage and prior to the first appearance of ovarian and testicular differentiation. The morphogenesis of this structure has been described by several authors (Ponse, '49; Vitale-Calpe, '69). In *Bufo bufo* and *Rhinella icterica*, the BO is conserved in adults of both sexes (Farias et al., 2004; Falconi et al., 2007) while in other species, such as *Rhinella marina* and *Rhinella arenarum,* BO disappears in adult females (Echeverria, '90b; Brown et al., 2002). In males of all the bufonids species analyzed, the BO is located in the anterior pole of the testis.

Many studies refer to the BO as a vestigial ovary and it has been an increase of evidence concerning to the steroidogenic activity of this structure. In the BO of at least three species of bufonids (*Duttaphrynus melanostictus*, *B. bufo,* and *Anaxyrus woodhousii*), the presence of steroidogenic enzymes such as 3β-HSD/I and 17β-HSD has been determined (Colombo and Colombo-Belvedere, '80; Ghosh et al., '82; Pancak-Roessler and Norris, '91). As well, it has been described that in adult males of *R. arenarum,* the BO expresses measurable activity of aromatase, CYP17, and 3β-HSD/I (Scaia et al., 2011) and synthesizes steroids from endogenous cholesterol (Scaia et al., 2013). Besides, it has been established that the enzyme aromatase as well as genes involved in gonadal differentiation are expressed in the BO of recently metamorphosed and early juveniles of *R. marina* (Abramyan et al., 2009, 2010).

During larval stages, but in a greater extension at the end of the metamorphosis, it takes place the differentiation and development of organs and systems characteristic of post larval life, including gonads. The length of larval life determines the period animals that are exposed to aquatic endocrine disrupting contaminants (EDCs), ubiquitous substances present in the environment due to the extensive use of pesticides and industrial chemicals. Storrs and Semlitsch (2008) observed that the effects of "estrogenic contaminants" in the differentiation of the ovary are more evident in species having an ovarian accelerated rate of differentiation. Species with slower ovarian rates may be less vulnerable to contaminants exposure during sexual differentiation. The development of a tool to predict phenotypic sex in "retarded" ovarian development amphibian species would help to identify the possible effects of EDC on gonadal differentiation and sex ratio balance.

Taking these antecedents into account, and considering that the BO of adult males of *R. arenarum* synthesizes sexual steroids like estradiol, the main purpose of this work is to examine the morphogenesis of BO in *R. arenarum* as well as the steroidogenic capacity of this organ during larval development. If BO actively contributes to the hormonal pathway directing gonadal differentiation, then it represents a possible target for EDCs.

MATERIALS AND METHODS

Materials

[1,2,6,7(n)-^{3}H]testosterone (114.5 Ci/mmol) was acquired from NEN (Boston, MA, USA). Reference steroids, tricaine methanesulfonate (MS-222), paraplast, and cofactors were purchased

from Sigma Chemical (St. Louis, MO, USA). Human chorionic gonadotropin (hCG) was obtained from ELEA Laboratory (Buenos Aires, Argentina). Silica gel plates 60–GF 254 on aluminum, and Gill's hematoxylin were purchased from Merck (Darmstadt, Germany). All chemicals and solvents were of analytical grade. Acid fuchsine, ponceau xylidine, acid orange 10, blue aniline, and phosphomolibdic acid were obtained from Anedra (Buenos Aires, Argentina).

Animals

Tadpoles of *R. arenarum* in different stages were obtained by in vitro fertilization. Female ovulation was hormonally induced by injecting intraperitoneally 2,500 IU hCG. In vitro fertilization was performed with sperm suspension prepared in Holtfreter solution (60 mM NaCl, 0.6 mM KCl, 0.9 mM $CaCl_2$, 0.2 mM $NaHCO_3$). Embryos were maintained in Holtfreter solution until experimental stages were achieved. Embryos and tadpoles were staged according to Gosner ('60). In addition, metamorphosis process was divided into three stages according to Echeverria ('90a) and Gilbert (2003): Pre-metamorphosis from stage 25 to stage 34, pro-metamorphosis from stage 35 to stage 41, and climax from stage 42 to stage 46. Reproductive male and females of *R. arenarum* were collected near Buenos Aires City (Argentina), maintained with free access to water and fed with crickets and zophobas. Adults and tadpoles were over-anesthetized by immersion in 1% (w/v) and 0.1% (w/v) neutralized MS-222, respectively (Gentz, 2007). Experiments were performed 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

Proper gonads and BO were rapidly excised and weighted. Due to their small sizes, both structures were processed together and named as "genital ridge". Organs were analyzed with a stereoscopic microscope Leica EZ4D and macroscopic images were captured with an incorporated digital camera. Tissue was fixed in Bouin's solution, dehydrated, and embedded in paraplast. Serial sections were cut at 7 μm, deparaffinized, hydrated, and stained with hematoxylin or Masson's modified trichromic. Immunohistochemical techniques were carried out after blocking endogenous biotin with an avidin-biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA). To assess the presence of steroidogenic enzymes, immunohistochemical staining was performed according to Scaia et al. (2011) by employing a polyclonal anti-aromatase antibody (1:300; rabbit anti-human, USBiological, Swampscott, MA, USA, catalogue number C9095–14A) and a polyclonal anti-CYP11A antibody (1:200; rabbit anti-rat, Chemicon International, Billerica, MA, USA, catalogue number AB1244). Afterward, 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, United Kingdom). Immunohistochemical staining was visualized with 3,3′-diaminobenzidine solution (DAKO North America, Inc., Carpinteria, CA, USA) and counterstained with hematoxylin. Sections were examined by using a Leica DM2000 microscope and images were captured with an incorporated digital camera. Western blot analysis was performed for both primary antibodies and a single band with expected molecular weight was obtained for each one (Supplementary material).

Enzymatic Assays

Both aromatase and 5α-reductase use testosterone as substrate and NADPH as cofactor for the synthesis of estradiol and 5α-dihydrotestosterone (DHT), respectively. In consequence, it is possible to determine and compare both activities in a single homogenate of the genital ridge of each tadpole. Both activities were determined in some intervals of the metamorphosis, intervals corresponding to gross morphological changes. Genital ridges were individually homogenized in 50 mM sodium phosphate buffer (PBS), 0.1 mM EDTA and 3 mM $MgCl_2$, pH 7.4. Aromatase and 5α-reductase activities were assayed by the incubation of homogenates for 120 min at 28°C with 5 μM [^{3}H] testosterone, 5 mM glucose-6-phosphate, 0.25 mM NADPH, 0.25 mM NADP and 1 IU/mL glucose-6-phosphate dehydrogenase (Fernandez Solari et al., 2002). After incubations, steroids were extracted twice with 3 mL of cold methylene chloride. Substrates and products were separated by thin layer chromatography (TLC) by using methylene chloride:acetone (75:5, v/v) as a solvent system. Standard radioinert 3-oxo-4-ene steroids were detected by UV absorption. Non-absorbing standard steroids were revealed after spraying with primuline (Wright, '71). The amounts of tritiated products were quantified after chromatography, the mass of the products being calculated from the known specific activities of the substrates. Specific activity of each enzyme was expressed as pmol of product per minute and per milligram of tissue.

Estradiol Production

Genital ridges from each stage were incubated individually in 500 μl of Krebs–Ringer-glucose solution containing 10 mM Hepes, pH 7.4 (KRGH) during 3 hr at 28°C. After the incubation, media were collected and stored at −20°C until estradiol determination.

Radioimmunoassay (RIA)

Production of estradiol (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-17β-6-BSA (Colorado State University, CO, USA). This E_2 antibody has very low (<1%)

cross-reactivity with estrone, progesterone, 17-hydroxyprogesterone and 5α-pregnane-3, 20 dione, and negligible (<0.1%) with testosterone and 5α-dihydrotestosterone. The antiserum was used in a final dilution of 1:600,000. Standard curves were 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 used to separate bound and free hormones (Dufau et al., '72). 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 medium per mg genital ridge.

Statistical Analyzes

Specific activity of the enzymes and production of estradiol were expressed as means ± standard error (SE). Enzymatic activities were analyzed by a Median test followed by multiple contrasts (Conover, '99). Aromatase activity of stages 36–39 and 40–41 and production of estradiol were analyzed and compared by using a one factor ANOVA test and a posteriori contrast by using Tukey's test (Steel and Torrie, '80). Data were transformed every time they did not satisfy the ANOVA assumptions. Shapiro–Wilk test was used to verify normality in the distribution of the variables and Levene test to verify homogeneity of variances.

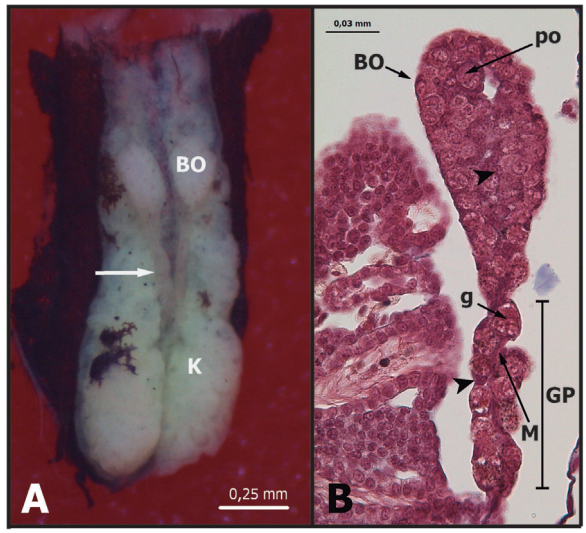

Figure 1. **A.** Genital ridges of a distinctive tadpole in stage 26 situated ventral to the kidneys (K). Bidder's organs (BO) and proper gonads (white arrow) are located in the cephalic and caudal portion of the genital ridge, respectively. **B.** Longitudinal section of BO and proper gonad (GP) of a tadpole in stage 26 stained with modified Masson's trichromic method. Arrowhead: somatic cell; po: primary oogonia; g: germ cell; M: medullar layer.

RESULTS

Morphogenesis of Bidder's Organ During Larval Development

Genital ridges are distinguished from stage 26 as a pair of structures located ventrally to each kidney, with a conspicuous spherical organ in the cephalic portion that corresponds to the BO (Fig. 1A). The histological analysis of the genital ridge reveals the presence of somatic cells and primary oogonia in the BO while the proper gonad shows two compartments, the cortical, and the medullar layers. In the cortical layer there are germ cells surrounded by somatic cells as described in other bufonids (Viertel and Richter, '99; Falconi et al., 2004), whereas the medullar compartment is made up of connective tissue (Fig. 1B).

As the metamorphosis proceeds, most of the germ cells of the BO develop in oocytes. At the end of the metamorphosis (stage 46), in the BO it can be distinguished (Fig. 2A): Nests of oogonia (mitotic germ cells enveloped by somatic cells), early previtellogenic single oocytes (larger than the germ cells, with a well-defined conspicuous germinal vesicle), and late previtellogenic single oocytes (larger than early previtellogenic oocytes, with nucleoli) in follicles (Dumont, '72; Echeverria, '90b). Despite the proper gonads remain undifferentiated throughout all the metamorphosis, some morphological changes can be observed. They become larger, accompanying body growth, and its metamerism is better differentiated. The development of the basal laminae makes the division between the cortex and medulla more distinguishable. The germ cells are located within the peripheral cortex while the central medulla is devoid of them (Fig. 2B).

Immunodetection of Steroidogenic Enzymes

The first approach used to elucidate the steroidogenic capacity of the genital ridge was the detection by immunohistochemistry of two steroidogenic enzymes: Aromatase, responsible of estradiol production, and CYP11A, first enzyme in steroid biosynthetic pathway. In the case of aromatase, immunoreactivity was observed in follicular cells of almost all the largest follicles of the BOs obtained from tadpoles in stages 35 to 46. Figure 3 shows an example of BO of a typical tadpole in stage 40 expressing aromatase in follicular cells. On the other hand, only few cells were stained with the antibody against CYP11A and the location of immunoreactivity changed during metamorphosis (Fig. 4). While at stages 35 to 39, CYP11A was detected in oocytes cytoplasm (Fig. 4A,B), in tadpoles from stages 40 to 46 CYP11A immunoreactivity was lost in oocytes and gained in follicular cells (Fig. 4C,D). These results indicate that at least some of the follicles of the BO would be able to transform cholesterol into pregnenolone whereas most of them would have the capability of producing estradiol from androgens. On the other hand, the

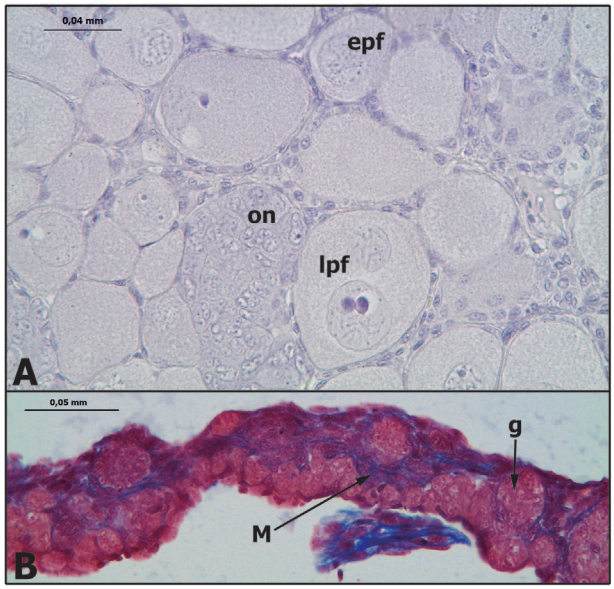

Figure 2. **A.** Cross section showing the Bidder's organ of a typical tadpole in stage 46. Slices were stained with hematoxylin. on: oogonial nest; epf: early previtellogenic oocyte in follicle; lpf: late previtellogenic oocyte in follicle. **B.** The longitudinal section corresponds to the proper gonad of a tadpole in stage 46. Section was stained with modified Masson's trichromic method. g: germ cell; M: medullar layer.

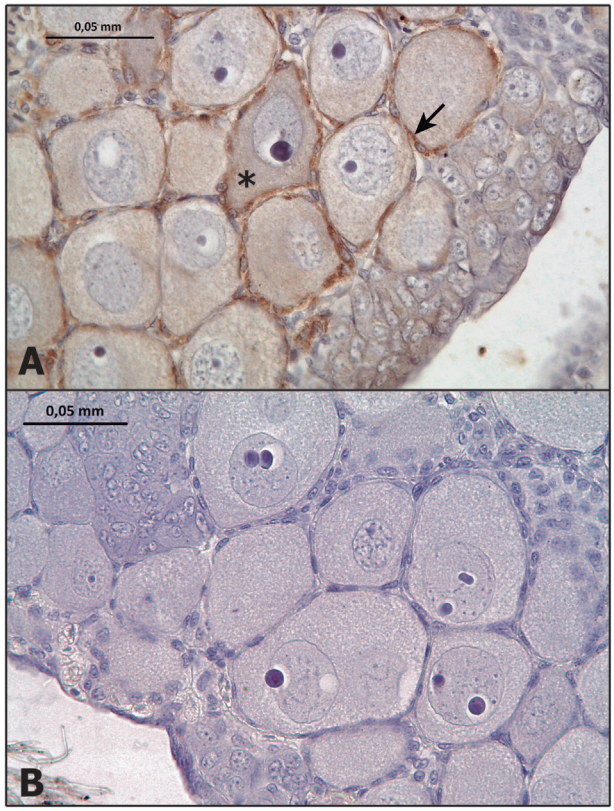

Figure 3. Immunohistochemistry of aromatase in Bidder's organ of a characteristic tadpole in stage 40 incubated with (**A**) or without (**B**) the primary antibody in a dilution of 1:300. The arrow indicates the immunoreactivity in follicular cells. With an asterisk is indicated a large previtellogenic oocyte in follicle.

proper gonad was not immunoreactive with either anti-aromatase or anti-CYP11A in any stage of the metamorphosis (data not shown).

Steroidogenic Activity in the Genital Ridge During Metamorphosis

Aromatase and 5α-Reductase Activities. The final enzymes in the synthesis of estrogens and androgens, sexual development related hormones, are aromatase and 5α-reductase. The characterization of these enzymes activity profiles started at stage 35, corresponding to the beginning of the pro-metamorphosis (Echeverria, '90a). At stage 35, the ratio 5α-reductase/aromatase is around two while from stage 36 to the end of the metamorphosis, this ratio is lower than one (Table 1). The activity of aromatase reaches the highest level between stage 36 and the beginning of the metamorphic climax (stage 42), while 5α-reductase activity reaches the lowest one in the same stages (Fig. 5A). Figure 5A also shows that at stage 42, the activity of 5α-reductase increases five times over its minimum value, being constant until the end of the climax while aromatase reaches its minimum value at stage 46.

Since values of standard error for aromatase activity during the pro-metamorphosis (from stage 36 to stage 41) were very high, approximately two times higher than for the rest of the values, in each stage data were divided into two groups taking into account the appropriate median value (Fig. 5B). Statistical analysis of these groups revealed that there are significant differences among groups ($F_{3,26} = 26.60$; $P < 0.0001$), suggesting that the population of individuals between stages 36 and 41 is not homogeneous in terms of aromatase activity.

Estradiol Synthesis in the Genital Ridge During Metamorphosis

The capability of the genital ridge to synthesize estradiol from endogenous substrates was evaluated after 3 hr of incubation. All the stages were able to produce estradiol from endogenous substrate but stages 40–41, corresponding to the end of pro-metamorphosis, produced the highest values (Fig. 6, $F_{6,43} = 5.64$; $P = 0.0002$).

DISCUSSION

As previously described (Echeverria, '90b), tadpoles of *R. arenarum* have a BO located in the cephalic portion of the

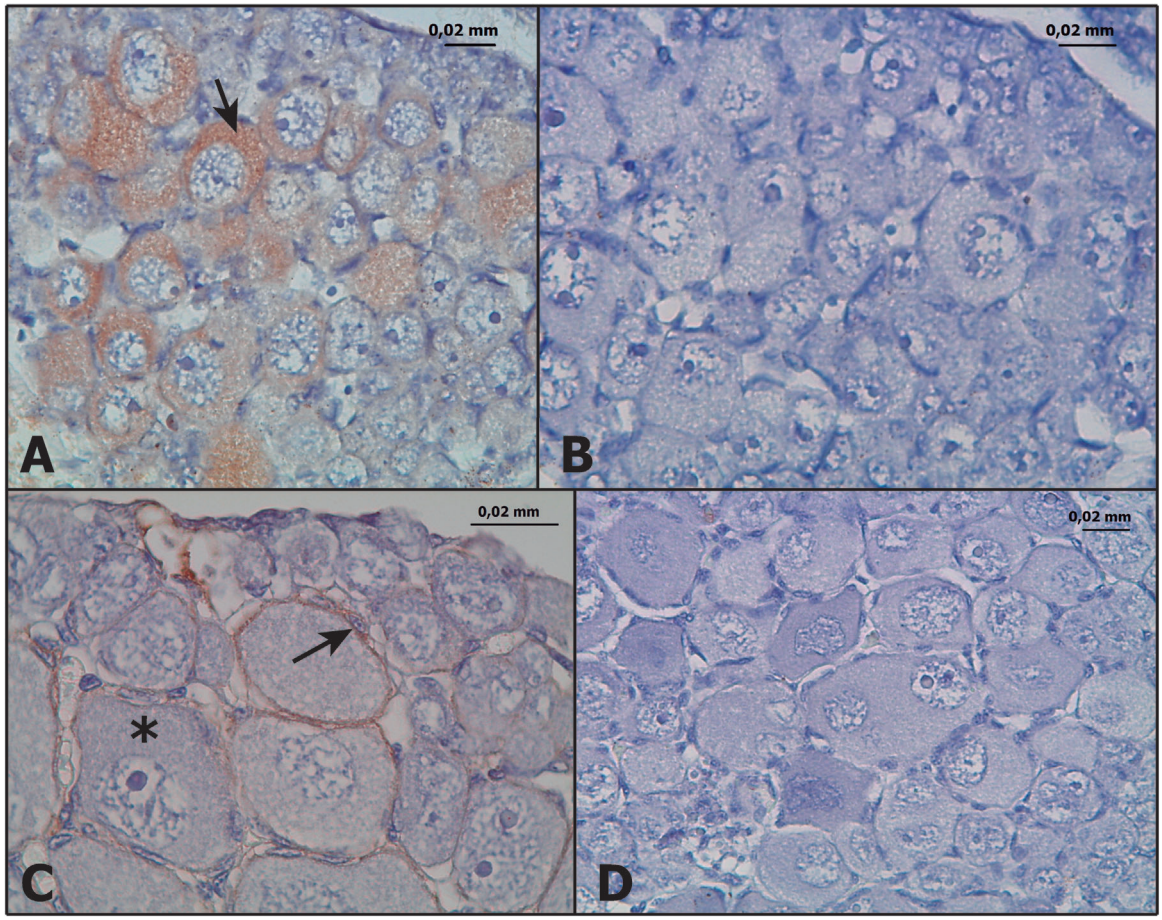

Figure 4. Immunohistochemistry of CYP11A of typical tadpoles in stages 35 and 46. Sections were incubated with (**A, C**) or without (**B, D**) the primary antibody in a dilution of 1:200. **A.** Bidder's organ of stage 35 tadpole. The arrow indicates immunoreactivity in the cytoplasm of an oocyte. Note that only few oocytes are immunoreactive. **B.** Negative control. **C.** Bidder's organ of stage 46 tadpole. Immunoreactivity is indicated with an arrow in the follicular cells. Asterisk indicates large previtellogenic oocyte. **D.** Negative control.

Table 1. Enzymatic activities in the genital ridge of tadpoles in different stages.

Stage	N	Aromatase activity	5α-reductase activity	Ratio 5α-reductase/aromatase
35	7	0.022 ± 0.006	0.050 ± 0.004	2.3
36–39	16	0.075 ± 0.012	0.034 ± 0.002	0.5
40–41	17	0.062 ± 0.008	0.006 ± 0.002	0.1
42	17	0.029 ± 0.005	0.018 ± 0.003	0.6
43–44	10	0.033 ± 0.007	0.020 ± 0.003	0.6
45	5	0.032 ± 0.005	0.026 ± 0.006	0.8
46	14	0.027 ± 0.004	0.015 ± 0.001	0.6

Aromatase activity is expressed as pmol estradiol per minute per milligram of tissue whereas 5α-reductase activity is expressed as pmol DHT per minute per milligram of tissue. Data are expressed as means ± standard error.

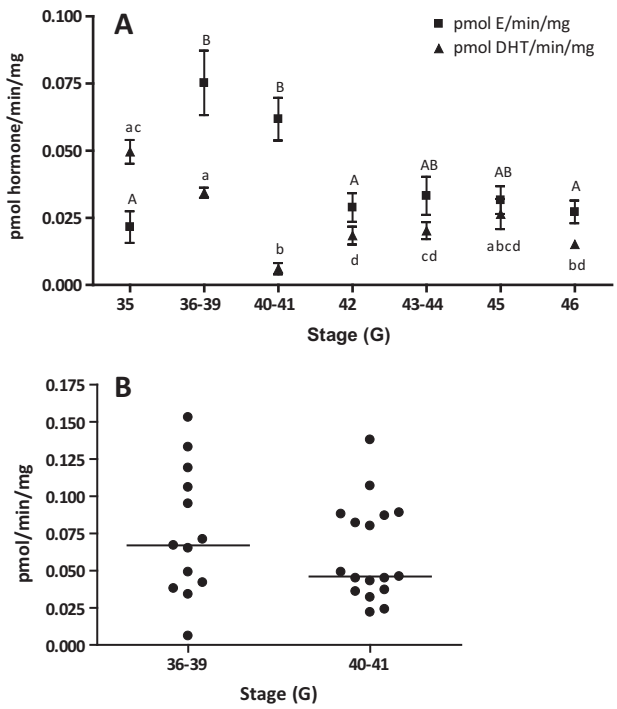

Figure 5. Enzymatic activities. **A.** Aromatase and 5α-reductase activities in the genital ridge of tadpoles in different stages (35 to 46). Statistical differences among aromatase activities are indicated with different uppercase letters ($P = 0.0002$) while statistical differences among 5α-reductase activities are indicated with different lowercase letters ($P < 0.0001$). **B.** Aromatase activity distribution among tadpoles of stages 36–39 and 40–41. The medians are represented by the horizontal lines that cross each distribution, E: estradiol, DHT: 5α-dihydrotestosterone, G: Gosner.

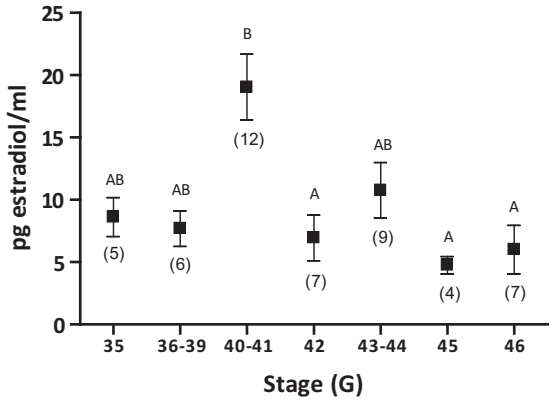

Figure 6. Estradiol synthesis in the genital ridge of tadpoles in different stages (35–46). The production of estradiol was determined by radioimmunoassay. Genital ridges were obtained and incubated as described in materials and methods. Different letters indicates statistical differences with $P \leq 0.0002$, number of replicates are between brackets. G: Gosner.

genital ridge. This structure develops very early in the larval life (stage 26) and has ovarian-like previtellogenic oocytes in different stages of development. The predominance of a particular oogenetic stage depends on the progress of the metamorphic process. The development of the proper gonad is clearly delayed since at the end of the metamorphosis, it remains still undifferentiated. These observations suggest that *R. arenarum* expresses a "retarded" rate of ovarian development (Ogielska and Kotusz, 2004). This fact, together with the lack in this species of a sex-specific DNA marker, disables us to sex the individuals during the larval period.

As mentioned in the introduction, in some species, sex steroids induce sex reversal although the molecular mechanism of this reversal is largely unknown. In *L. sylvaticus* (Navarro-Martín et al., 2012), *R. rugosa* (Nakamura, 2010) and *X. laevis* (Bögi et al., 2002), it was proposed that sex steroids play a major role in sexual differentiation during metamorphosis. Therefore, the main focus of this paper is made on the events related to the steroid synthesis through metamorphosis. In *R. arenarum*, larval BOs are able to synthesize estradiol from endogenous or exogenous substrates because they express aromatase in almost all the follicular somatic cells from the pro-metamorphosis until the end of metamorphosis. However, only few follicles express CYP11A. Between stages 35 and 39, the expression of this enzyme is located in the cytoplasm of few oocytes while from stage 40, the expression switches from oocytes cytoplasm to the cytoplasm of follicular somatic cells. These results suggest that only a small amount of bidderian follicles are able to synthesize estradiol from endogenous cholesterol, as previously described in adult males of the same species (Scaia et al., 2011). In both cases, one possibility that cannot be excluded is that follicles able to produce steroids from cholesterol constitute a source of precursors for aromatase and/or 5α-reductase in almost all the follicles. In *R. rugosa*, Isomura et al. (2011) described the presence of aromatase in the gonad of undifferentiated females by using immunohistochemistry but in *R. arenarum,* no aromatase was detected in the proper gonad. The lack of expression of aromatase in the toad proper gonad is probably due to the fact that in bufonids sexual differentiation occurs later than in other anurans. On the other hand, in another species of the genus *Rhinella*, *R. marina*, aromatase expression is only detected in BOs of recently metamorphosed undifferentiated juveniles by using reverse transcriptase-polymerase chain reaction and in situ hybridization of tissue sections (Abramyan et al., 2010).

The results of the immunohistochemistry against aromatase enabled us to conclude that estradiol synthesized in the incubation of the genital ridge with testosterone was mainly produced by the activity of bidderian aromatase in previtellogenic follicles.

Moreover, BOs of sexually undifferentiated tadpoles not only produce estradiol from exogenous testosterone but also from endogenous substrates, suggesting that these organs could have an important role in steroid production during metamorphosis, hence being an important target for disrupting agents during the long period tadpoles live in the water. As mentioned before, the proper gonad does not express detectable amounts of aromatase or CYP11A during the metamorphic process.

Activities of aromatase and 5α-reductase in the BO during the pro-metamorphosis have different profiles. In fact, both enzymes have an opposite pattern of activity with aromatase maximum coincident with 5α-reductase minimum. Since both enzymes are able to use the same substrate and cofactor, and both reactions are irreversible: in non-saturating conditions, 5α-reductase and aromatase would compete for the same substrate, making the ratio between both enzymes an important aspect to take into account when the ratio DHT/estradiol is analyzed. Bögi et al. (2002) proposed that in *X. laevis*, the relationship between DHT and estradiol could determine the course of gonadal differentiation. In *R. arenarum*, the profile of aromatase activity from stage 36 to stage 41 is not homogeneous allowing the separation of each group into two statistically different subgroups. These results would suggest that the pro-metamorphosis constitutes a key period in the synthesis of sexual steroids, steroids that could play an important role in sexual differentiation, as suggested by Bögi et al. (2002).

In *R. rugosa*, undifferentiated females are able to produce more estradiol from testosterone than undifferentiated male. Indeed, the profile of aromatase activity in the population of those tadpoles would be heterogeneous (Isomura et al., 2011). Furthermore, Navarro-Martín et al. (2012) determined in *L. sylvaticus* that at early developmental stages, aromatase expression shows a heterogeneous profile. This profile allows them to identify two groups of tadpoles named as "presumptive females" and "presumptive males".

The expression of both aromatase and 5α-reductase in the BO is an important issue to take into account since the ratio between both enzymes could determine the predominance in the production of estrogens or androgens. However, it is also important to evaluate the capability of the BO to produce steroids from endogenous substrates. Subsequently, we studied the synthesis of estradiol from endogenous precursors in the genital ridge of tadpoles in different stages. Our results show that the maximal capacity of estradiol production is achieved in stages 40–41. The higher biosynthesis of estradiol detected in stages 40–41 suggests that in stages 36–39, where maximal aromatase activity was determined, the expression of other/others steroidogenic enzyme/s, which is/are involved in the pathway of testosterone synthesis, could be a limiting factor for the synthesis of estradiol.

In conclusion, we have demonstrated the presence of steroidogenic enzymes in the BO during the metamorphic process of individual genital ridges. None of the steroidogenic enzymes detected in the BO could be observed in the proper gonad until the end of the metamorphic process. In addition, we have characterized the variation of aromatase and 5α-reductase activity during metamorphosis. During the pro-metamorphosis, the activity of aromatase could lead to the division of tadpoles in two populations in terms of estradiol synthesis capability. This difference could be related to the beginning of the sexual differentiation process.

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