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Author(s): Javier Goldberg
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Gonadal Differentiation and Development in the Snouted Treefrog, *Scinax fuscovarius* (Amphibia, Anura, Hylidae)

JAVIER GOLDBERG

Instituto de Bio y Geociencias (IBIGEO), Centro Científico Tecnológico-CONICET y Universidad Nacional de Salta. 9 de Julio 14, 4405, Rosario de Lerma, Salta, República Argentina; E-mail: jgoldberg@conicet.gov.ar

ABSTRACT.—**Accurate descriptions of gonadogenesis are necessary to have a baseline to detect abnormalities during development and to assess the time a species may be more vulnerable to the action of pesticides, endocrine disruptors, and pharmaceuticals to which abnormal conditions have been attributed. Here, I describe the histomorphological changes during gonadal differentiation in the Snouted Treefrog *Scinax fuscovarius*. I sexed and measured a total of 302 tadpoles between Gosner stages 26–46 and 21 juvenile and adults. The results show that 1) the species possesses the undifferentiated type of gonadal differentiation—ovaries differentiate much earlier (at stage 26) than testes (stage 36); 2) oogenesis begins almost simultaneously with ovarian differentiation; and 3) ovaries and testes exhibit different rates of gonadal differentiation with respect to somatic development—the ovaries have an accelerated rate (the ovarian cavity differentiates early during larval development and previtellogenic oocytes develop at early premetamorphic stages), whereas the testes have a decelerated rate (seminiferous tubules differentiate at the juvenile stage). The comparison of these characteristics with those of other co-occurring anuran species reveals important interspecific variation in the relative timing of gonadogenesis. Finally, and as a byproduct of the extensive sampling, I describe several cases of gonadal malformations.**

Abnormal patterns of gonadogenesis in amphibians can result in abnormal development of the individual and impaired reproduction after maturity. Exogenous factors that can lead to abnormal gonadogenesis include various environmental toxins, including agricultural pesticides (Hayes et al., 2003), which also represent an important contributor to global amphibian population declines (Mann et al., 2009; Brühl et al., 2013). Because amphibians spend much of their life in aquatic habitats, they are susceptible to toxins in agricultural and industrial runoff (Haughton and Terns, 1999; Hayes et al., 2003).

In the last 15 years, several studies have used the development of the anuran reproductive system as an indicator of the toxicity of herbicides, pesticides, endocrine disruptors, and their accumulated effects (reviewed in Mann et al., 2009). These studies demonstrated a variety of malformations, such as skewed sex ratios, intersexes, feminization, masculinization, and altered gonadal development in response to the exposure to these chemicals (Hayes et al., 2002, 2003; Mann et al., 2009; Rohr and McCoy, 2010; Papoulias et al., 2013; among others). Yet there is a need for studies in different environments to broaden our knowledge of the natural occurrence of gonadal malformations, which can serve as a reference for the assessment of the effects of chemical exposure (Hecker et al., 2006).

To recognize abnormal patterns of gonadogenesis, however, biologists need clear descriptions of normal gonadogenesis to use as baselines as well as to assess critical periods in which a species may be more vulnerable to the action of pesticides, endocrine disruptors, or pharmaceuticals. Unfortunately, accurate descriptions of gonadogenesis exist for only ~20 taxa among >6,200 known anuran species (e.g., Iwasawa et al., 1987; Lopez, 1989; Ogielska and Bartmanska, 1999; Chavadej et al., 2000; Gramapurohit et al., 2000; Falconi et al., 2001, 2004; Ogielska and Kotusz, 2004; El Jamil et al., 2008; Downie et al., 2009; Fabrezi et al., 2010; Piprek et al., 2010; Sandoval and Gomez, 2010; Flament et al., 2011; Haczkiewicz and Ogielska, 2013; Phuge and Gramapurohit, 2013); therefore, accurate descriptions are needed.

Over the last 12 years in South America, the Chacoan forests of southern Bolivia, northwestern and central Argentina, eastern Paraguay, and southern Brazil have been lost at an alarming rate (Fig. 1). Approximately 85% of the area that was originally undisturbed forest is now occupied by crops, pastures, and secondary scrub (Hansen et al., 2013). The main cause of deforestation has been agricultural expansion, particularly corn and soybean cultivation, with the consequent use of pesticides, herbicides, and fertilizers (Zak et al., 2008). In this context, and considering the previous reports of the effect of these chemicals on the anuran development, the diverse anurans inhabiting this ecosystem have much to offer as indicators of Chacoan ecosystem health.

The Snouted Treefrog *Scinax fuscovarius* is a member of the family Hylidae, currently placed in the *Scinax ruber* group (Faivovich et al., 2005). The species occurs throughout urban, periurban, and open nonforested lands of southeastern Brazil, northern Argentina, Paraguay, and Bolivia (Frost, 2014), in close association with the Gran Chaco extension (Fig. 1). Because of its wide distribution, great abundance in disturbed and undisturbed areas (Vaira et al., 2012), and reproductive season and larval development concurrent with the fumigation and seeding times (pers. obs.), *S. fuscovarius* can be used as a good indicator of ecosystem health. The complete histomorphological characterization of its gonadogenesis under the current conditions will allow us to recognize, in the future, possible changes related to land-use change and the accumulated effects of contaminants.

The aim of this study is to describe the sequence of events related to gonadal differentiation and development in *S. fuscovarius*. As a byproduct of the large data set, resulting from fieldwork in different Chacoan localities over many years, I also describe several naturally occurring gonadal malformations. Finally, I compare gonadal development in *S. fuscovarius* with data recorded for sympatric species that develop in the same breeding sites.

MATERIALS AND METHODS

I studied larval ($N = 302$) and post-metamorphic ($N = 21$) specimens of *S. fuscovarius* collected in ephemeral ponds that fill only during the rainy season with unpredictable hydroperiods during March 2006 in "El Tunal" (25°15′17.97″S; 64°23′33.31″W);

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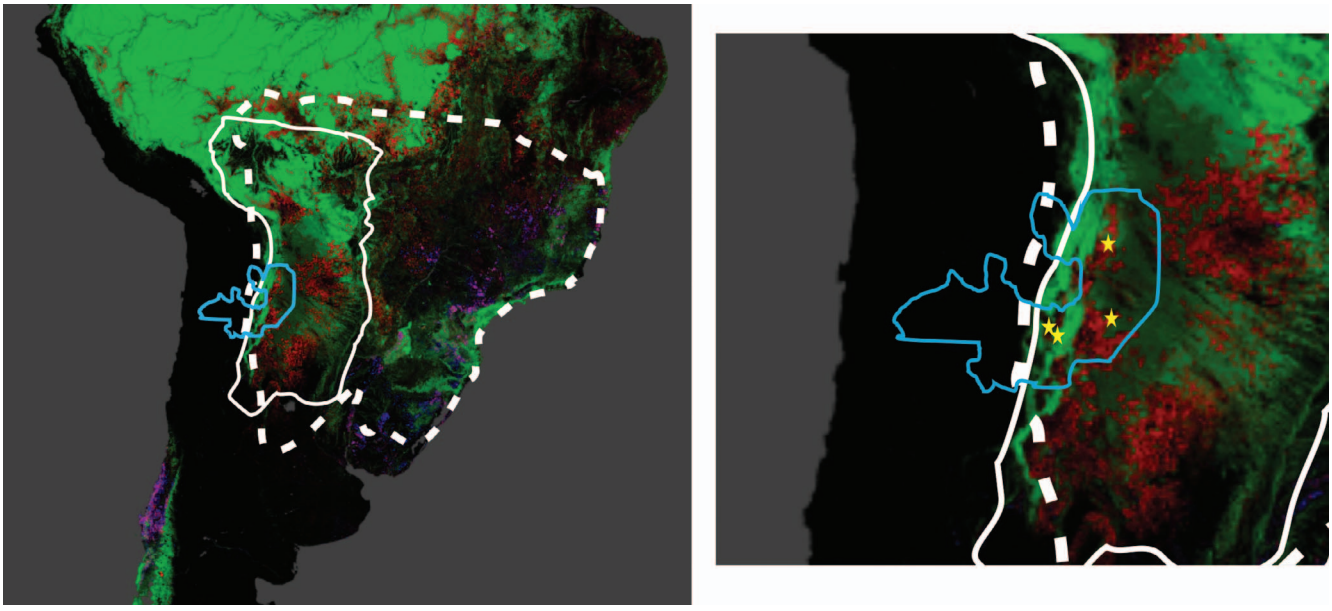

FIG. 1. Superposition of the geographic range of *Scinax fuscovarius* (modified from BerkeleyMapper) depicted with a white dashed line, with the Gran Chaco extension (continuous white line) on the map of South America forest change in the last 12 years. Red areas show high rates of forest loss attributable to deforestation; green indicates forest extent; and purple indicates forest loss and gain (source: Hansen/UMD/Google/USGS/NASA). The right picture depicts a detail of Salta Province (turquoise line), with yellow stars indicating the main locations were tadpoles were collected.

Metán Department, Salta (Argentina); January 2012 along National Route 81 (23°10′14″S, 63°31′39″W) in San Martin Department, Salta (Argentina); January and February 2013 and February 2014 in ephemeral ponds beyond the western border of Salta, capital city (24°45′22.82″S, 65°27′42.41″W); February 1997 in Lesser, Vaqueros Department, Salta, and March 2013 in ephemeral ponds beyond the northern border of Salta, capital city (24°44′40.28″S, 65°23′18.57″W). Specimens were euthanized in an aqueous solution of chloretone, fixed in neutral-buffered formalin (10%), and adults were preserved in 70% ethanol. Specimens accessioned as lots are deposited in the Herpetological Collection of the Museo de Ciencias Naturales (MCN), Universidad Nacional de Salta (Argentina) with the following numbers: MCN 106, MCN 319, MCN 325, MCN 407, MCN 447, MCN 472, MCN 473, MCN 1108, MCN 1573, MCN 1574, and MCN 1575. Larval development was staged according to Gosner (1960). Data were obtained from manual dissection and histological sections of larval and post-metamorphic specimens to describe changes in gonads. The age of juveniles and sexually mature specimens was estimated by counting the number of lines of arrested growth (LAGs) in transverse section of phalangeal bones of toe IV. Gonads along with the kidneys ($N = 40$) and Toe IV ($N = 18$) were separated from preserved specimens (Toe IV was decalcified), dehydrated, cleared in xylene, embedded in paraffin, sectioned at 6 μm, and stained with haematoxylin and eosin. Oocytes in adult specimens were staged according to Dumont (1972). The analysis of LAGs was performed according to the technique detailed in Hemelaar (1986). Descriptions and photographs were made with a Nikon (Nikon, Inc., Tokyo, Japan) microscope (either a SMZ1000 stereo dissection or an E200 compound microscope), and equipped with a digital camera. Measures of oocyte diameters and gonads are expressed as mean ± SD. Significance in mean differences between populations ($\alpha < 0.05$) was determined by single factor analysis of variance (ANOVA).

RESULTS

No differences were observed in the sequence of gonadogenesis and gametogenesis among the different populations, or in the size of ovaries (Table 1), oocytes (Table 2), and testis (Table 3). Therefore, I describe the general pattern of ovarian and testicular differentiation for the species.

Ovarian Differentiation.—The first sign of ovarian differentiation was discernible at Gosner stage 26 ($N = 21$ female frogs) with the shortening and incipient lobulation of the cords, which reflects the arrangement of the future ovarian sacs (Fig. 2A). Histologically, the beginning of ovarian differentiation was evident by the

TABLE 1. Comparison of ovary length (mean ± SD) during development of *Scinax fuscovarius* at different Gosner stages from each population sampled. (A: "El Tunal" modified environment; B: non-anthropogenic environment at the western border of Salta city; C: slightly modified environment at the northern border of Salta city). *N*: number of measured ovaries

	Ovary length (in mm)				ANOVA	
Stage	A	B	C	Average	*F*	*P*
30	0.981 ± 0.11 ($N = 10$)	0.965 ± 0.09 ($N = 10$)	0.993 ± 0.08 ($N = 6$)	0.979 ± 0.02	0.19	0.82
33	1.297 ± 0.07 ($N = 10$)	1.313 ± 0.07 ($N = 6$)	1.294 ± 0.09 ($N = 10$)	1.302 ± 0.06	0.41	0.66
35–36	1.592 ± 0.12 ($N = 10$)	1.616 ± 0.10 ($N = 10$)	1.571 ± 0.18 ($N = 10$)	1.597 ± 0.10	0.11	0.89
38	1.864 ± 0.11 ($N = 10$)	1.830 ± 0.18 ($N = 10$)	1.899 ± 0.21 ($N = 10$)	1.857 ± 0.11	2.69	0.08
42–46	2.419 ± 0.22 ($N = 10$)	2.546 ± 0.26 ($N = 10$)	2.511 ± 0.11 ($N = 10$)	2.514 ± 0.19	0.57	0.57

TABLE 2. Comparison of oocyte diameter (mean ± SD) during development of *Scinax fuscovarius* at different Gosner stages from each population sampled. (A: "El Tunal" modified environment; B: non-anthropogenic environment at the western border of Salta city; C: slightly modified environment at the northern border of Salta city). *N*: number of measured oocytes. juv: juvenile stage.

	Oocyte diameter (in μm)			ANOVA	
Stage	A	B	C	*F*	*P*
31	23.21 ± 4.32 ($N = 10$)	27.25 ± 3.44 ($N = 10$)	22.94 ± 8.65 ($N = 10$)	2.63	0.10
33	53.81 ± 7.21 ($N = 10$)	45.22 ± 7.21 ($N = 10$)	49.34 ± 4.66 ($N = 10$)	1.67	0.21
35–36	66.97 ± 5.33 ($N = 10$)	67.70 ± 5.98 ($N = 10$)	62.54 ± 6.28 ($N = 10$)	2.22	0.13
38–juv	75.21 ± 4.30 ($N = 10$)	78.33 ± 6.45 ($N = 10$)	72.69 ± 8.57 ($N = 10$)	1.54	0.24

formation of the ovarian cavity (Fig. 2B). A few small spaces first formed within the medulla and then fused to form a larger space, surrounded by an irregular, developing epithelium (Fig. 2B). Thus, a cortico-medullary structure was clearly defined in which primordial germ cells and darkly stained somatic cells were located.

As development progressed, through stages 27–30 ($N = 17$), gonad size increased with an increasing number of germ cells (Table 1). Externally, by stage 31, the ovaries were morphologically distinct as they became divided into lobules (Fig. 2C). The cortex was composed of primary and secondary oogonia, and the first oocytes appeared, indicating the entry of some oogonia into meiosis (Fig. 2D). The mean diameter of these oocytes was 24.46 ± 2.41 μm (Table 2). The nucleus was relatively large compared with the total cellular diameter and contained few nucleoli.

At stage 33 ($N = 16$ frogs), the ovaries continued to grow in size (Table 1), and lobulation was evident, resulting in a multilobed organ (Fig. 2E). The anlage of the fat bodies developed as fingerlike projections from the median anterior end of the left ovary with a whitish-translucent coloring (Fig. 2E). In the right ovary, there was no sign of differentiation of fat bodies. In light microscopy, the epithelium surrounding the ovarian cavity appeared well defined (Fig. 2F). The size of the ovaries increased both because of the enlarged ovarian cavity and because of the growing thickness of the cortex; the number of oocytes in diplotene notably increased (Fig. 2F). Mean diameter of oocytes was 49.45 ± 4.29 μm (Table 2). These previtellogenetic primary oocytes had a highly basophilic cytoplasm and a discernible (acidophilic) nucleus with a smooth nuclear membrane and several, darkly stained, peripheral nucleoli of different sizes (Fig. 2F). Scattered dense inclusions of lipids appeared unstained in the cytoplasm of each oocyte. Prefollicular cells proliferated, surrounding and individualizing each oocyte. In the cortex, there were also primary and secondary oogonia (Fig. 2F).

At stages 35–36 ($N = 24$ frogs), the ovary continued to grow (Table 1). Fat bodies were distinct, became separated from the gonad proper, and appeared as a uniformly granular-looking mass (Fig. 2G). Histologically, the majority of the diplotene oocytes in the ovary were wrapped within a single layer of follicle cells (Fig. 2H). The oocytes became larger with a mean diameter of 65.73 ± 2.59 μm (Table 2), with a highly basophilic cytoplasm and a larger nucleus with numerous nucleoli. The cortex was composed of diplotene cells, secondary oogonia, and the outermost layer of single primary oogonia (Fig. 2H). Blood vessels began to invade the cortex.

At stage 38 ($N = 22$ frogs), the ovaries continued to grow in size (Table 1). Fat bodies became larger and displayed a yellowish color indicating the accumulation of lipids (Fig. 2I).

TABLE 3. Comparison of testis sizes (mean ± SD) during development of *Scinax fuscovarius* from each population sampled. (A: "El Tunal" modified environment; B: non-anthropogenic environment at the western border of Salta city; C: slightly modified environment at the northern border of Salta city). *N*: number of measured testis.

				ANOVA	
Measure (in mm)	A	B	C	*F*	*P*
			Left testis		
Stage 38					
length	1.604 ± 0.18 ($N = 9$)	1.623 ± 0.25 ($N = 15$)	1.554 ± 0.13 ($N = 14$)	0.21	0.81
width	0.110 ± 0.03 ($N = 9$)	0.091 ± 0.04 ($N = 15$)	0.103 ± 0.02 ($N = 14$)	1.22	0.31
Stage 41					
length	1.457 ± 0.09 ($N = 2$)	1.442 ± 0.11 ($N = 2$)	1.425 ± 0.10 ($N = 2$)	0.32	0.75
width	0.130 ± 0.04 ($N = 2$)	0.150 ± 0.04 ($N = 2$)	0.149 ± 0.02 ($N = 2$)	0.03	0.97
Stage 43					
length	1.371 ± 0.19 ($N = 7$)	1.431 ± 0.18 ($N = 5$)	1.429 ± 0.15 ($N = 4$)	0.26	0.77
width	0.190 ± 0.03 ($N = 7$)	0.220 ± 0.02 ($N = 5$)	0.181 ± 0.04 ($N = 4$)	1.69	0.22
			Right testis		
Stage 38					
length	1.387 ± 0.26 ($N = 9$)	1.415 ± 0.08 ($N = 15$)	1.397 ± 0.28 ($N = 14$)	0.14	0.86
width	0.130 ± 0.04 ($N = 9$)	0.112 ± 0.08 ($N = 15$)	0.119 ± 0.03 ($N = 14$)	1.67	0.21
Stage 41					
length	1.182 ± 0.14 ($N = 2$)	1.203 ± 0.15 ($N = 2$)	1.164 ± 0.18 ($N = 2$)	0.41	0.66
width	0.148 ± 0.02 ($N = 2$)	0.151 ± 0.02 ($N = 2$)	0.150 ± 0.04 ($N = 2$)	0.05	0.95
Stage 43					
length	1.311 ± 0.18 ($N = 7$)	1.346 ± 0.12 ($N = 5$)	1.323 ± 0.11 ($N = 4$)	0.45	0.50
width	0.211 ± 0.09 ($N = 7$)	0.231 ± 0.09 ($N = 5$)	0.194 ± 0.06 ($N = 4$)	0.17	0.34

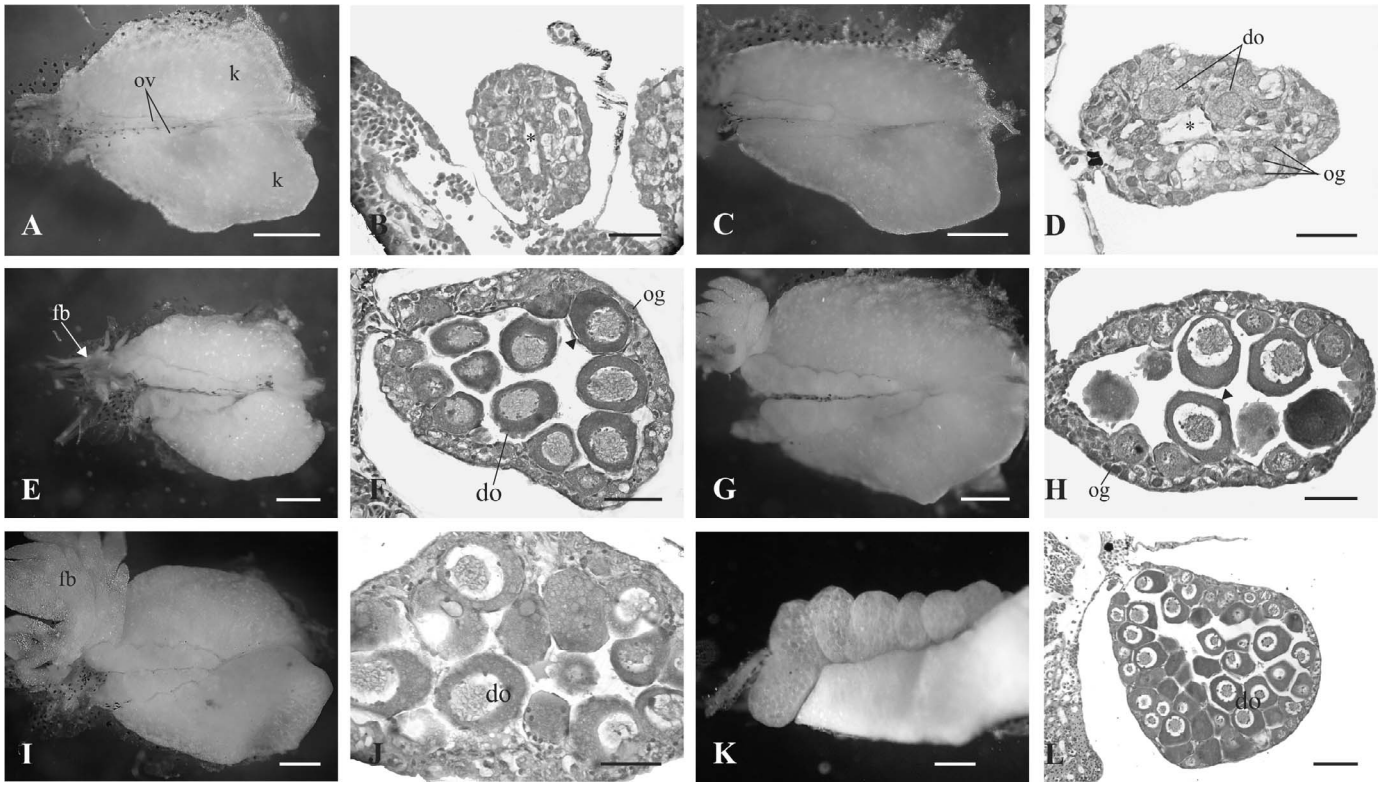

FIG. 2 Sequence of events during ovarian differentiation in *Scinax fuscovarius*. (A–B) Stage 26: (A) external morphology; (B) differentiated ovary with a distinct central lumen (asterisk), and primordial germ cells in mitotic division placed in the cortex. (C–D) Stage 31: (C) external lobulation becomes evident; (D) the ovarian cavity enlarges; the cortex is formed by several oogonia and few oocytes (prediplotenic oocytes sensu Falconi et al., 2001). (E–F) Stage 33: (E) lobulation of the ovary is evident and the first signs of fat body differentiation occur; (F) the ovary contains numerous diplotene oocytes surrounded by follicular cells (arrowhead), and the location of primary and secondary oogonia is reduced to the outer part of the cortex. Nucleoli in oocytes locate at the periphery of the nuclei. The cortex appeared lined by a clearly discernible basal lamina. (G–H) Stage 36: (G) ovarian size and lobulation increases. Fat bodies grow; (H) diplotene oocytes are larger. Arrowhead indicates follicular cells. (I–J) Stage 38: ovarian size increases attributable to an increase in the number and size of diplotene oocytes. (K–L) Stages 42–43: (K) numerous oocytes can be identified in the ovary; (L) the ovarian sacs have numerous diplotene oocytes reducing the lumen. do = diplotene oocytes, fb = fat bodies, k = kidneys, og = oogonia, ov = ovary. Scale bars = 0.5 mm in A, C, E, G, I, K; 50 μm in B, D, F, H, J; and 100 μm in L.

The enlargement in the size of the ovaries was mainly attributable to an increase in the number and size of diplotene oocytes, with a mean diameter of 75.41 ± 2.82 μm (Table 2), which also shrank the ovarian cavity (Fig. 2J).

In female tadpoles from stage 39–46 (prometamorphic stages to the end of metamorphosis; $N = 40$ frogs), ovaries reached larger sizes than in previous stages (Table 1), and their maximum number of lobules reached between 9 and 10; the left ovary always had 1 or 2 more lobules than the right one. On average the left ovary had 9 lobules per ovary (from 8–10 lobules), whereas the right ovary had 8 lobules (7–9 lobules). Ovarian lobules were bigger than in previous stages and appeared composed mainly of diplotene oocytes (Fig. 2K, L). These oocytes were easily seen as globular cells with a translucent cytoplasm (Fig. 2K). Histologically, the ovary was almost fully occupied by diplotene oocytes with the largest ones adjoining the ovarian cavity (Fig. 2L). Other germ cells in earlier stages of development appeared exclusively located in the most external part of the cortex, which was very thin.

The condition observed during metamorphosis (Fig. 2L), in which most cells were suspended in the diplotene stage of meiosis, continued during juvenile stages (Fig. 3). There was no increase in the diameter of diplotene oocytes ($N = 10$; 76.70 ± 8.25 μm). Fat bodies of the right ovary began to differentiate after metamorphosis. In adults, oogenesis persisted in the multilobed ovaries, with oogonia constantly differentiating into oocytes. Thus, individual lobules contained developing oocytes in different stages that ranged from about 90–270 μm ($N = 10$; 178.83 ± 55.37 μm) in stage I oocytes to 1,066.23 ± 63.10 μm ($N = 10$) in stage V and VI oocytes.

Testicular Differentiation.—During those stages when the presumptive ovaries underwent differentiation and development (beginning at stage 26), and external changes occurred, testes remained similar in appearance to the undifferentiated gonad ($N = 23$ frogs). Even so, the development of fat bodies from the anterior part of the left cord began by stage 28 ($N = 44$ frogs).

At Gosner stage 36–37 ($N = 35$ frogs), both testes remained as long, thin cords, and there was no external change in their morphology indicating testis differentiation (Fig. 4A). However, given the early differentiation of ovaries with a clear discernible lobulated morphology by Gosner stage 31, those cords without ovarian morphology can be inferred to be presumptive testis.

By stage 37, the left cord was now longer, and fat bodies associated with it were well developed. Histologically, each testis appeared as a massive structure with numerous medullary cells and germ cells in mitotic division. In the medulla, primary spermatogonia exhibited prominent nuclei with a single nucleolus and appeared surrounded by flattened, crescent-shaped, darkly stained, somatic cells (Fig. 4B). By stage 38 ($N = 34$ frogs), the shortening and thickening of the testes began, and the differences in size between the left and the right testis became conspicuous (Table 3).

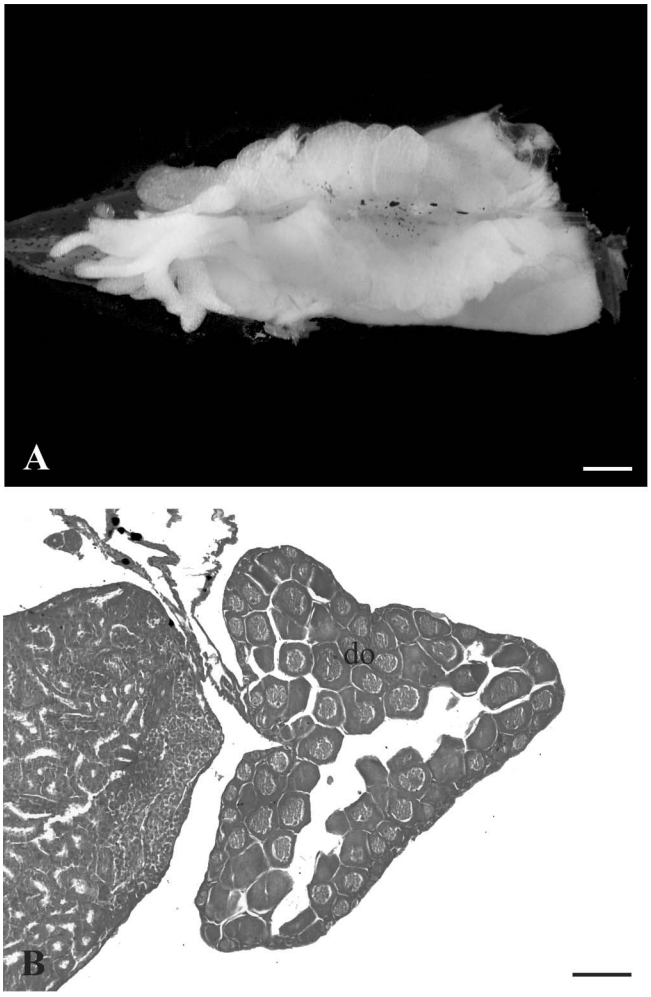

FIG. 3. Juvenile ovary of *Scinax fuscovarius*. (A) External morphology. (B) Histological section of the ovary showing a similar structure and size to that of metamorphosing individuals. The ovary contained numerous diplotene oocytes. Scale bar = 0.5 mm in A, and 100 μm in B.

By stage 41 ($N = 6$ frogs), a proximal and a distal part of the developing testes were discernible, with a thinner, spindle-shaped, distal part (Fig. 4C, D, F). Before the beginning of metamorphosis, primary spermatogonia divided mitotically and produced clusters of smaller cells, the secondary spermatogonia, which remained together within a membranous cyst (Fig. 4E).

During metamorphic stages ($N = 20$ frogs), the distinction between the distal (posterior) and the proximal (anterior) part of the testes was remarkable. The anterior part became thicker, whereas the distal portion began to degenerate (Fig. 4G, I). Histologically, there was no change in morphological organization, but an increased number of primary and secondary spermatogonia were present (Fig. 4H). Fat bodies related to the right testis began to differentiate by stage 42. Juvenile specimens still exhibited vestiges of the distal part of the testes (Fig. 4J, L), and the proximal part showed sign of seminiferous cord organization (Fig. 4K).

Adult testes were paired, ovoid structures, with a complete absence of pigmented cells on their surface (Fig. 4M), and appeared surrounded by a thin layer of connective tissue, the tunica albuginea. Seminiferous tubules were discernible. Each seminiferous tubule was composed of several cysts of germ cells at different stages of differentiation but within each cyst, germ cells appeared at approximately the similar stage (Fig. 4N). Seminiferous tubules exhibited an average size ($N = 10$) of 373.10 ± 23.38 μm. The intertubular or interstitial compartment contained steroidogenic Leydig cells, connective tissue, and blood vessels.

Aging.—The analysis of gonadal differentiation and maturation together with skeletochronological analysis on juvenile (immature gonads and absence of secondary sexual characters) and adult specimens (mature gonads and presence of secondary sexual characters such as the vocal sacs) provided indications of the species' life span and age when sexual maturity is attained. The phalangeal cross-sections in four juveniles of both sexes (two males with SVL = 22.16 mm and 23.06 mm, and two females with SVL = 23.76 mm and 31.40 mm) were composed of a single layer of avascular and parallel-fibered bone encircling a wide medullary cavity (Fig. 5A), whereas three juvenile specimens presented one LAG (one male with SVL = 24.46 mm and two females with SVL = 23.46 mm and 29.34 mm). In adults of both sexes, the results revealed: 2 LAGs (female SVL = 46.22 mm); 3 LAGs (female SVL = 32.22 mm; 2 males with 44.82 mm and 46.12 mm); 4 LAGs (female SVL 38.22 mm; male SVL = 45.42 mm); 5 LAGs (female SVL = 51.28 mm); 6 LAGs (two males with SVL = 41.28 mm and 47.06 mm); and 7 LAGs (female SVL = 46.32 mm; male SVL = 49.02 mm; Fig. 5B) . These data suggest the juvenile stage lasts up to the second year of life (in both sexes) when sexual maturity is attained.

Gonadal Abnormalities.—The observation and description of gonadal development in *S. fuscovarius* allowed the recognition of several malformations in the developing testes and ovaries, although with a very low incidence (0.3%). In some cases, there were encysted metacercariae in the urogenital system, but it was not possible to show a direct relationship between infestation and malformations (Fig. 6A). Malformations included the following:

Unpaired gonad ($N = 5$): This condition was observed in both sexes, mainly at stages 41 and 42, and it was the right gonad that was always absent (Fig. 6A, B). The morphology of the developing left gonad was similar to that observed in normal, female or male, tadpoles at the same stage.

Unpaired and segmented gonad ($N = 2$): The left gonad was absent, and the right one appeared abnormally segmented, without the characteristic morphology of testes or ovaries (Fig. 6C). However, fat bodies corresponding to the left gonad appeared normally developed. Histologically, the gonad was an ovary, but it was poorly developed with respect to normal ovaries at the same developmental stage (stage 40). The abnormal gonad showed a well-developed central lumen, corresponding to the ovarian cavity, which was surrounded by a regular epithelium and a cortex formed only by somatic cells and primary oogonia (Fig. 6D).

Asymmetric ovaries (Fig. 6E): In a specimen at stage 42, ovaries presented large size discrepancies, with the right one

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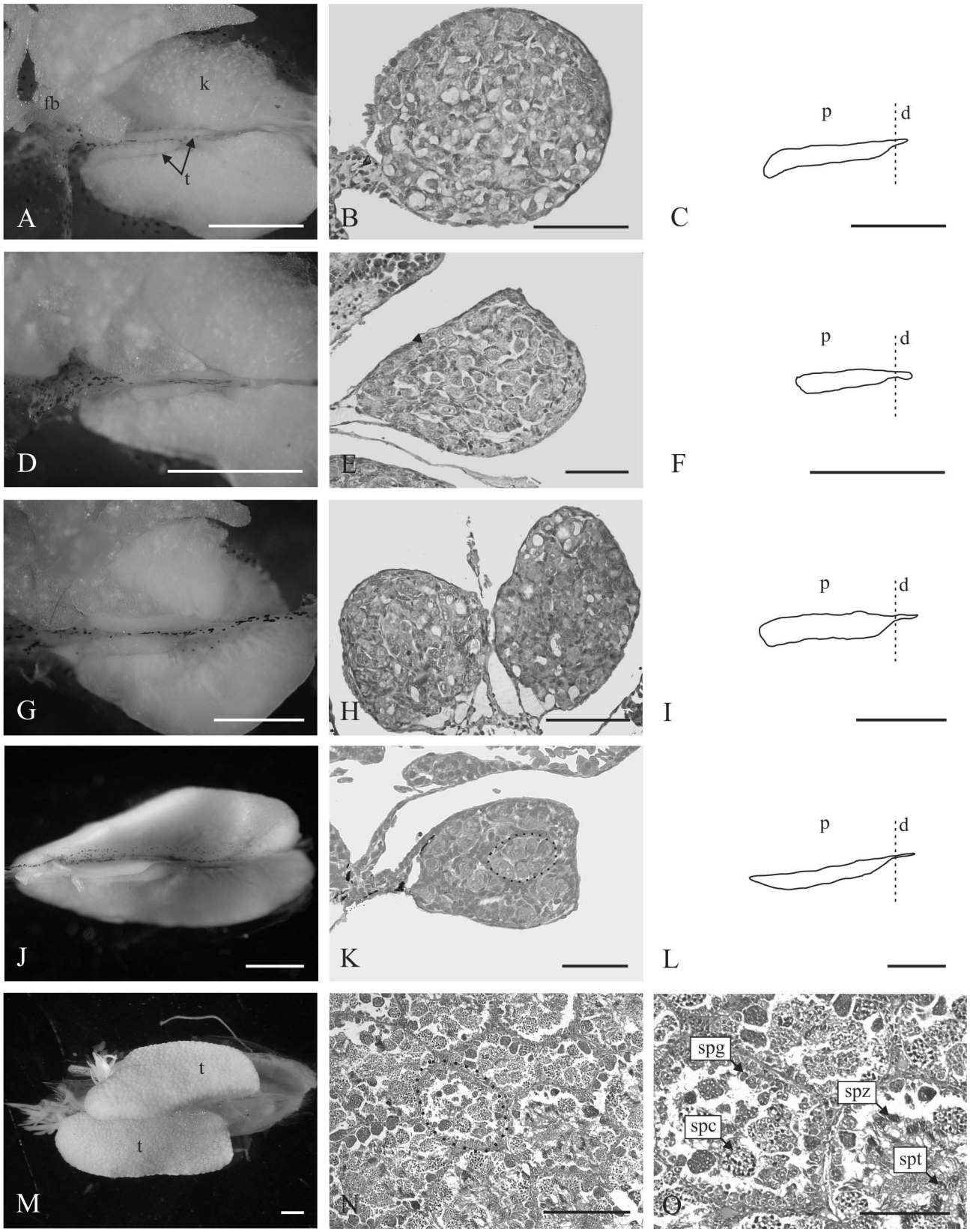
FIG. 4. Sequence of events during testes differentiation in *Scinax fuscovarius*. (A–C) Stage 37: (A) externally, testes show no sign of differentiation (fat bodies are well-developed); (B) few primary spermatogonia are distributed throughout the testis; (C) scheme of testis shape with a proximal and a distal part (divided by the dashed line). (D–F) Stage 41: (D) testes are shorter than in previous stages and the proximal and distal parts become recognizable; (E) secondary spermatogonia (arrowhead) appear; (F) the constriction between the proximal and the distal part becomes conspicuous.



(G–I) Stage 43: (G, I) testes get thicker, and the distal part becomes acuminate; (H) primary and secondary spermatogonia increase in number; (J–L) juvenile testes: (J, L) the distal part degenerates. (K) Formation of seminiferous tubules begins. For recognition, one seminiferous tubule is outlined with a dashed line. (M–O) Adult testes: (M) adult testes have grown much more than in the previous stages and the numerous seminiferous tubules can be identified as small, rounded structures distributed all along the testes; (N) seminiferous tubules (outlined with a dashed line) with spermatogonia, spermatocytes, spermatids, and spermatozoids; (O) detail of fully developed tubules. d = distal part of testis, fb = fat bodies, k = kidney, p = proximal part of testis, t = testis, spc = spermatocytes, spg = spermatogonia, spt = spermatids, spz = spermatozoids. Scale bar = 1 mm in A, C, D, F, G, I, J, L; 50μm in B, E, H, K; 360 μm in N; and 200 μm in O.

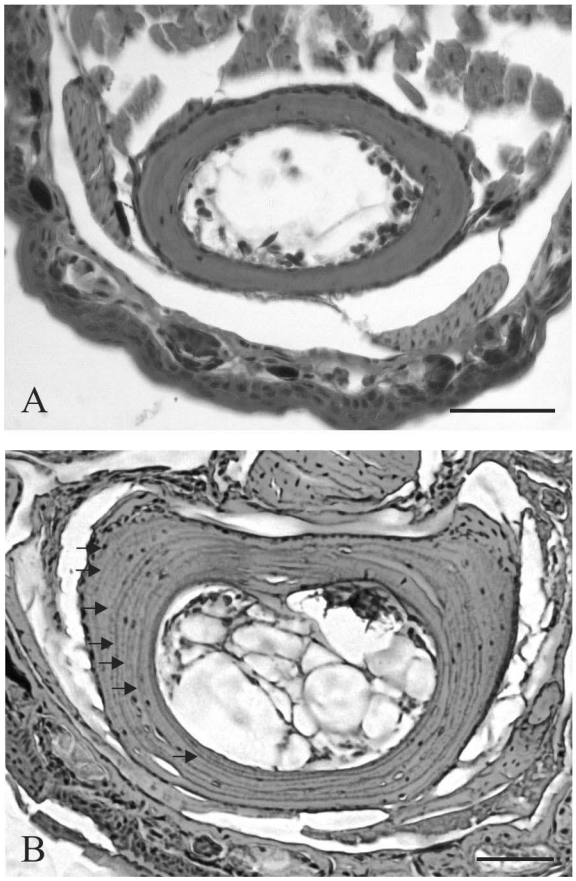


FIG. 5. Phalangeal cross sections in *Scinax fuscovarius*. A) A female juvenile with no LAGs, 23.76 mm SVL. B) A 7-yr old male, 47.60 mm SVL. Scale bar = 50 μm.

smaller and poorly lobulated. In light microscopy, both gonads presented numerous diplotene oocytes and primary and secondary oogonia, but in the right ovary, these cells were stacked and the ovarian cavity was not discernible (Fig. 6F).

Fusion of both ovaries: In a specimen at stage 42, both gonads were separated at their proximal end but merged into a single ovary in the distal portion (Fig. 6G). Histologically, both gonads appeared well separated at the proximal end (each suspended from the peritoneal wall by a short mesogonium), and diplotene oocytes were the most numerous germ cells (Fig. 6H). In a distal direction both ovaries got closer; then they merged but retained both mesogonia (Fig. 6I) and toward the distal end a single ovary appeared suspended by both mesogonia (Fig. 6J).

True hermaphroditism: This condition in a specimen at stage 41 from "El Tunal" pond has already been reported and described (Goldberg, 2013). The left gonad corresponded to an ovary, whereas the right one was a testis. Both gonads were similar to those normal gonads at the same developmental stage.

DISCUSSION

Events of gonadal differentiation in *S. fuscovarius* occur in a similar manner to that of most other anurans described (e.g., Lopez, 1989; Gramapurohit et al., 2000; Ogielska and Kotusz, 2004; Fabrezi et al., 2010; Piprek et al., 2010; Flament et al., 2011; Haczkiewicz and Ogielska, 2013), and all mature cellular types are likewise similar to those described so far (Oliveira and Vicentini, 1998; Oliveira et al., 2003; Oliveira and Souza Santos, 2004).

Scinax fuscovarius exhibits a type of gonadal sex differentiation classified as "undifferentiated" (Gramapurohit et al., 2000); that is, gonads differentiate early into ovaries or remain in indifferent condition and then differentiate into testes. The same type has been reported for the Common Toad *Bufo bufo*, the Marsh Frog, *Pelophylax ridibundus*, the Common Frog *Rana temporaria,* and the Montane Brown Frog *Rana ornativentris* (Gramapurohit et al., 2000). Among anurans, two other types of gonadal sex differentiation have been described (Gramapurohit et al., 2000; Fabrezi et al., 2010; Haczkiewicz and Ogielska, 2013): "differentiated" (indifferent gonads directly differentiate directly either into ovary or testis); and "semi-differentiated" (gonads differentiate initially into ovaries regardless of the genetic sex; then the gonad in a genetic male transforms into testis).

Ogielska and Kotusz (2004) distinguished three types of developmental rates of ovaries and germ cells relative to somatic development: basic, retarded, and accelerated. In the basic and retarded types, at the end of metamorphosis, the ovary reaches the differentiation of an ovarian cavity or the differentiation of diplotene oocytes, respectively (Ogielska and Kotusz, 2004). *Scinax fuscovarius*, by contrast, displays the accelerated type: the ovarian cavity differentiates in earlier tadpoles (during limb bud appearance), and ovarian development progresses at a rapid rate, with previtellogenic oocytes present long before metamorphosis. Folliculogenesis also occurs quite early during ovarian differentiation (stage 33). The accelerated pattern has also been recorded for the Bicolored Frog *Clinotarsus curtipes* (Gramapurohit et al., 2000), the Common Skittering Frog *Euphlyctis cyanophlyctis* (Phuge and Gramapurohit, 2013), the Pool Frog *Pelophylax lessonae* and *P. ridibundus* (Ogielska and Kotusz, 2004), Black-spotted Frog *Pelophylax nigromaculatus* (Iwasawa et al., 1987), *Pseudis platensis* (Fabrezi et al., 2010), and the Paradoxical Frog *Pseudis paradoxa* (Downie et al., 2009). Interestingly, another species of the same genus, the Mato Grosso Snouted Treefrog *Scinax acuminatus*, presents the retarded type, in which ovarian differentiation occurs after metamorphosis (Fabrezi et al., 2010).

The histology of ovarian germinative cell types in adults of *S. fuscovarius* has previously been described by Oliveira and Souza Santos (2004) and presents similar characteristics to those of other hylid species. However, morphometric data are scarce. In *S. fuscovarius*, oocytes increase greatly in size during oogenesis (1,200%) and become full grown oocytes at approximately 1.1 mm in diameter, slightly less than the average for anuran species (Uribe Aranzabal, 2011).

With respect to testis development, the sequence of morphological events related to testicular development is similar in all anurans studied so far (Witschi, 1929; Hsu and Liang, 1970; Tanimura and Iwasawa, 1987; López, 1989; Duellman and Trueb, 1994; Ogielska and Bartmanska, 1999; Chavadej et al., 2000; Falconi et al., 2004; El Jamil et al., 2008; Downie et al, 2009; Fabrezi et al., 2010; Piprek et al., 2010; Haczewicsz and Ogielska, 2013; Phuge and Gramapurohit, 2013). In contrast, the onset of these events has been reported from stage 24 in *P. nigromaculatus* to juvenile stages as in the Argentine Toad *Rhinella arenarum* (Duellman and Trueb, 1994) such that the range of temporal variation is considerably wide. In *S. fuscovarius*, testis differentiation begins at prometamorphic stages and is retarded (i.e., seminiferous tubules develop at juvenile stages).

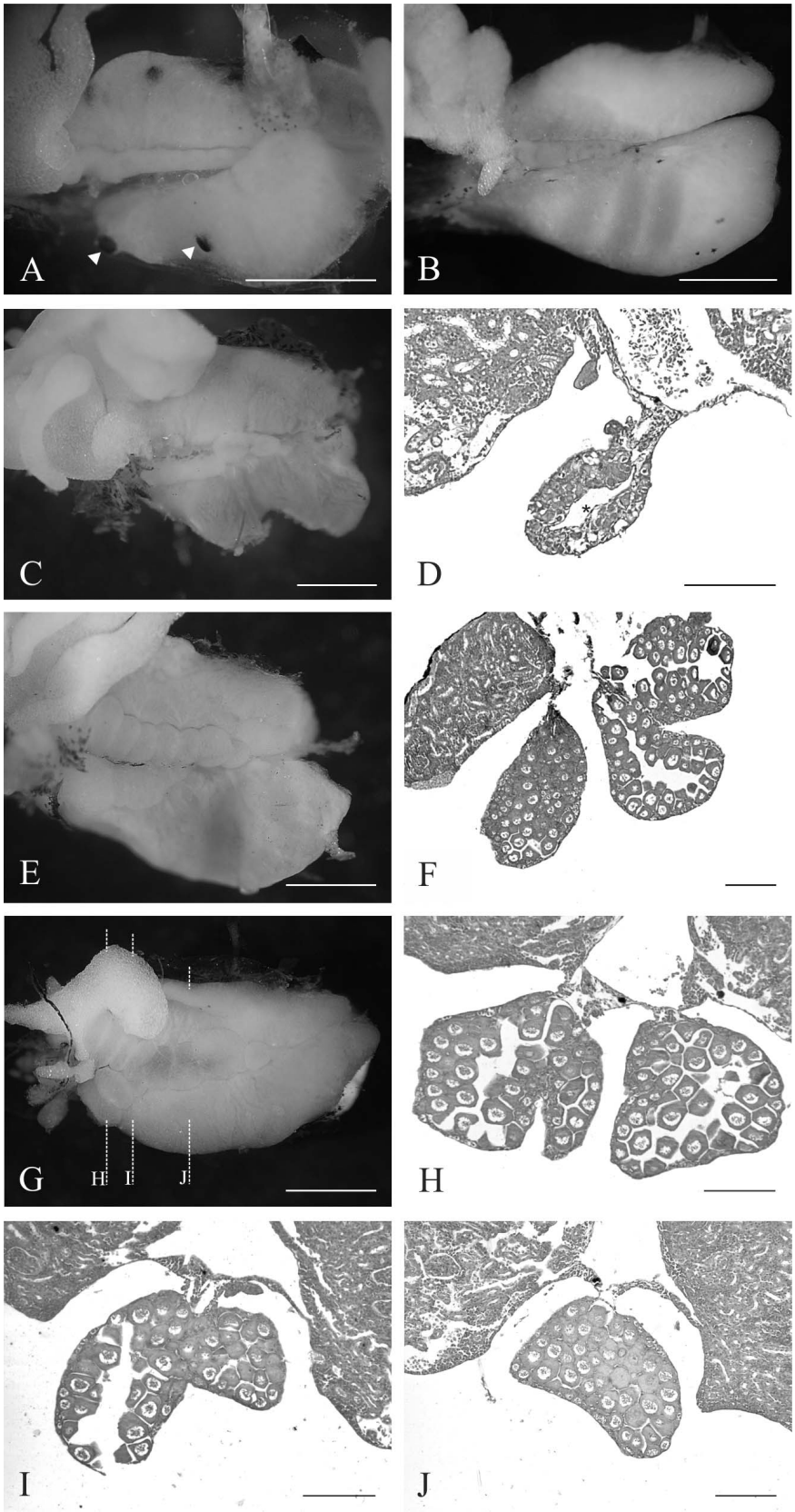


FIG. 6. Gonadal abnormalities in *Scinax fuscovarius*. (A) Unpaired ovary in a tadpole at Gosner stage 41. Arrowheads show encysted metacercariae. (B) Unpaired testis in a tadpole at Gosner stage 42. (C–D) Unpaired and segmented gonad in a tadpole at Gosner stage 40. (C) In gross morphology, sex differentiation is not evident and the single gonad appeared with an odd morphology. (D) In transverse sections, the ovarian cavity (asterisk) was detected, and evidenced of a poorly developed ovary. (E–F) Asymmetric ovaries in a tadpole at Gosner stage 42. The right ovary is underdeveloped but has numerous oocytes in diplotene, as does the left one. (G–J) Posterior fusion of both ovaries in a tadpole at stage 42. Anteriorly, both ovaries are separated but become fused in a posterior direction. Dashed lines in G indicate the location of serial cross-sections in H, I, and J. Scale bar = 1 mm in A, B, C, E, G; and 200 μm in D, F, H–J.

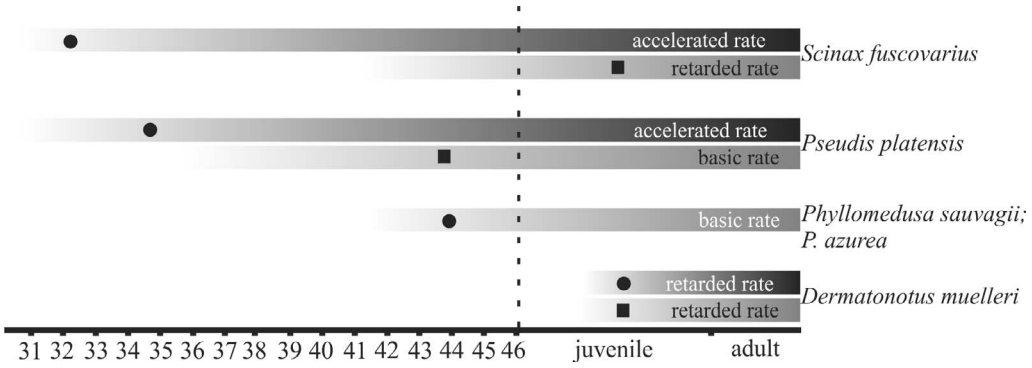


FIG. 7. Comparison of relative timing of gonadal differentiation in anuran species from semiarid environments of Argentina. Gonadal differentiation and development progress in a general pattern but with heterochronic changes that characterize each species. Dark and light bars indicate ovarian and testicular differentiation, respectively. Black circles indicate differentiation of diplotene oocytes. Black squares indicate differentiation of seminiferous tubules. Differentiation rates are indicated, next to species names, for each gonad. Data for *Pseudis platensis*, *Phyllomedusa azurea*, and *Phyllomedusa sauvagii* was taken from Fabrezi et al., (2010), and data for *Dermatonotus muelleri* was taken from Fabrezi et al. (2012).

Testicular development up to maturation has been classified in a variable number of stages based mainly on germinal cell characteristics (Kalt, 1976; Chavadej et al., 2000; El Jamil et al., 2008; Haczkiewicz and Ogielska, 2013). Most studies, however, describe only the morphology of the germinal cells, with special emphasis on the ultrastructure, with little attention paid to other features (such as the onset of seminiferous tubule formation or Sertoli cell differentiation), that would allow an interspecific comparison. Nevertheless, comparing testes differentiation in *S. fuscovarius* with data from the literature allows the distinction of three types of testis developmental rate (following Ogielska and Kotusz, 2004, for ovaries) considering the differentiation of seminiferous tubules with reference to metamorphosis: 1) basic: during metamorphosis as in the Red-bellied Toad *Bombina orientalis* (López, 1989), and *P. platensis* (Fabrezi et al., 2010); 2) retarded: after metamorphosis as in *S. fuscovarius* (this study), the Yellow-bellied Toad *Bombina variegata* (Piprek et al., 2010), the Muller's Termite Frog *Dermatonotus muelleri* (Fabrezi et al., 2012), *E. cyanophlyctis* (Phuge and Gramapurohit, 2013), the Bull Frog *Lithobates catesbeianus* (Chavadej et al., 2000), and the Painted-belly Leaf Frog *Phyllomedusa sauvagii* and *S. acuminatus* (Fabrezi et al., 2010); and 3) accelerated: before metamorphosis as in *C. curtipes* (Gramapurohit et al., 2000), the Wood Frog *Lithobates sylvaticus* (Hacziewicz and Ogielska, 2013), and *P. paradoxa* (Downie et al., 2009).

All these parameters that involve patterns of differentiation, type of differentiation rate, and the ontogenetic trajectory of gonadal differentiation and development are useful to 1) characterize each species, 2) analyze the variation in a phylogenetic context and interpret evolutionary patterns, and 3) develop hypotheses about the evolution of life cycles. Figure 7 depicts the comparison of gonadal differentiation and development between *S. fuscovarius* and four other species (the microhylid *D. muelleri*, and the hylids *Phyllomeusa azurea*, *P. sauvagii*, and *P. platensis*) living in similar geographic and ecological ranges. Among the selected species, gonadal development progresses with different onsets, quite early in *S. fuscovarius* for ovarian differentiation but with a retarded rate for testicular differentiation. This implies that 1) ovarian and testicular developments have their own independent pathways in each species, and 2) gonadogenesis is not entirely dependent on environmental factors.

The different rates of gonadal development, between sexes and among species, may be related to the susceptibility of each species to develop malformations attributable to chemical exposure. *Scinax fuscovarius* would be expected to run an increased risk of ovarian malformations with respect to testicular, and to other species with a basic or retarded rate of differentiation, because of an earlier and a greater exposure of differentiating gonads to aquatic contaminants. In fact, most abnormalities in *S. fuscovarius* were seen on ovaries and, interestingly, at prometamorphic stages. If gonadogenesis proceeds mainly during or after metamorphosis, as in *D. muelleri* (Fabrezi et al., 2012), the risk would be diminished.

Gonadal malformations were observed in tadpoles inhabiting both 1) ponds directly related to crop and soybean fields (cases of hermaphroditism and asymmetry) and 2) periurban ponds, located in undisturbed military fields with occasional livestock (impaired, segmented, and fused gonads). These cases, even with a low frequency, represent interesting documented cases of malformations in tadpoles living in natural conditions, because most malformations described in the literature have been in juveniles and adults from experimental treatments (Reeder et al., 1998; Hayes et al., 2002, 2003; Carr et al., 2003; Coady et al., 2004; Jooste et al., 2005; Skelly et al., 2010; Papoulias et al., 2013; Goldberg, 2013).

Hermaphroditism has been previously reported mainly in experimental conditions that examined the response of larvae to estrogenic and antiestrogenic compounds in juveniles and adults of the Cricket Frog *Acris crepitans* (Reeder et al., 1998), the Leopard Frog *Lithobates pipiens* (Mackenzie et al., 2003), and the Clawed Frog *Xenopus laevis* (Carr et al., 2003) and to atrazine in *X. laevis* (Hayes et al., 2002), whereas the first case on the occurrence of histologically confirmed true hermaphroditism in anuran tadpoles living in natural conditions was recently reported in *S. fuscovarius* (Goldberg, 2013). Because I have now shown that this species has an undifferentiated pattern of gonadal differentiation, this anomaly (the observation of germinal cells of both sexes) cannot be assigned to an intermediate step in a semidifferentiated type but is indeed a real case of hermaphroditism. The other described anomalies have been rarely mentioned in the literature. Size discrepancies between gonads have been reported in tadpoles of *X. laevis* both in laboratory conditions in response to atrazine (Jooste et al., 2005) and in natural conditions (Smith et al., 2005); no correlation was found between the incidence of this malformation and concentration of the herbicide. One case of unpaired

testis also has been reported in *L. catesbeianus* (Murphy et al., 2006).

In Argentina, as in the entire Gran Chaco, there is a scarcity of precise information about the levels of different chemicals in soil and water (Agostini et al., 2013; Gimenez et al., 2013), with the exception perhaps of natural arsenic and fluoride (Bundschuh et al., 2009). The analyses in progress are generally related to specific human activities with the objective to show they are contaminating (Rosenberg et al., 2001; Gonzalez Alonso et al., 2010). Studies evaluating the evolution (changes or constancy) of physico-chemical characteristics of sediment and water, and their relation to gonadal anomalies in anurans, represent one of the main objectives in present/future work in the area.

The description of gonadogenesis in *S. fuscovarius* presented here, from different disturbed and undisturbed sites, allowed the recognition of natural occurrence of gonadal malformations that can serve as a baseline for the assessment of the effect of chemical exposure. The present study, which showed no apparent differences among different populations of *S. fuscovarius* (Tables 1–3), can serve as a useful starting point to evaluate any short-term changes and effects that may occur because of land-use changes.

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