

# Testicular histology of Anurans that deposit eggs out of the water

## Histología testicular de Anuros que depositan huevos fuera del agua

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### ABSTRACT

Anuran amphibians show the largest diversity of amphibian reproductive modes, many of which imply egg deposition out of the water. This kind of egg deposition requires specialisations to avoid egg desiccation. Physiological, anatomical and ethological traits integrate to define these modes. In particular, morphological features of the urogenital system correlate with these reproductive modes and the environmental conditions where egg-laying occurs. In this study, we describe the testicular histology and spermatogenesis of the nest-building frog *Leptodactylus latinasus*, and we compare it with other species that breed out of water. We found variations in testis size, the thickness of interstitial tissue, tunica albuginea, and peritubular tunics, flagellum length, and in the shape and size of the spermatozoal nucleus. Certain specifics' characters differed at the species level, but not between families. Such variation could be an indicator of spermatozoal performance and environmental constraints under which fertilisation takes place.

**Keywords** — Anurans, terrestrial reproduction, testicle, histology.

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## RESUMEN

Los anfibios anuros muestran la mayor diversidad de modos reproductivos, muchos de los cuales implican la deposición de huevos fuera del agua. Este tipo de puesta requiere de especializaciones para evitar la desecación de la puesta. Los rasgos fisiológicos, anatómicos y etológicos se han integrado para definir estos modos. En particular, las características morfológicas del sistema urogenital se correlacionan con estos modos reproductivos y las condiciones ambientales donde tiene lugar la puesta. En este estudio describimos la histología de los testículos y la espermatogénesis de la rana excavadora *Leptodactylus latinasus* y se las compara con otras especies que se reproducen fuera del agua. Encontramos variaciones en el tamaño de los testículos, el grosor del tejido intersticial, la túnica albugínea y peritubular, la longitud del flagelo, forma y tamaño del núcleo del espermatozoide. Ciertos caracteres específicos difieren a nivel de especie y no entre familias. Tal variación podría ser un indicador del desempeño de los espermatozoides y de las limitaciones ambientales bajo las cuales tiene lugar la fertilización.

**Palabras clave** — Anuros, reproducción terrestre, testículo, histología.

## INTRODUCTION

Among vertebrates, anuran amphibians have the highest diversity of reproductive modes (Salthe and Duellman, 1973; Taylor and Gutman, 1977; Duellman and Trueb, 1986; Haddad and Prado, 2005). These modes have been characterised based on features describing the different stages of the reproductive function, such as amplexus type, number and size of eggs, oviposition frequency, deposition place, breeding sites, parental care. (Duellman, 1985; Lavilla and Rougés, 1992; Lavilla, 2004; Haddad and Prado, 2005). Physiological, anatomical, and ethological traits have been integrated to define these modes. In particular, morphological features of the urogenital system are correlated with these reproductive modes (Oliveira, Zanettoni and Zieri, 2002; Alcaide, Lavilla, and Pucci Alcaide, 2009; Leite, Franco-Belluci, Provete and Oliveira, 2015).

In anurans, the most generalised and phylogenetically prevalent oviposition site is water (Duellman and Trueb, 1986); nevertheless, among the reproductive modes described by Duellman and Trueb (1986), 18 of 29 categories exhibit terrestrial or arboreal egg depositions. Such deposition out of the water has been proposed as an adaptation against aquatic predators (Magnusson and Hero, 1991; Martins, 1993). This behaviour would imply an extra cost since the eggs and hatchlings would be under the pressure of terrestrial predators (Downie, Disney, Collins, and Hancock, 1995; Prado, Toledo, Zina, and Haddad, 2005; Lingnau and Di-Bernardo, 2006; Ponssa and Barrionuevo, 2008).

When eggs are laid outside of water (e.g. in mosses, wet ground land, moist soil, holes, leaves hanging from trees, mud chambers, natural burrows, or trees), larval development continues with a passive fall or active displacement to water,

where free-living larval development is completed (Noble, 1927; Salthe and Meham, 1974; Duellman and Trueb, 1986; Wells, 2007). This type of egg deposition requires specialisations to avoid egg desiccation. For example, the vesicles found in nests of *Phyllomedusa sauvagii* frogs are filled with metabolic water, which plays a key role in maintaining an appropriate humid environment for eggs and their embryonic development (Pyburn, 1980; Pucci Alcaide, Alcaide, Pucci Alcaide, and Lavilla, 2011). These vesicles originate from glycoconjugates, proteins, and lipids secreted in the preconvolute and convolute parts, and organise in the oviductal lumen; at least four types can be recognised according to their content (Pucci Alcaide *et al.*, 2011). Another example is the foam generated by tadpoles of certain *Leptodactylus* species, which prevents from desiccation when the dry season lasts longer (Downie, 1984; Caldwell and Lopez, 1989; Downie, 1990; Downie and Smith, 2003; Ponssa and Barrionuevo, 2008).

In regard to the male reproductive system, testes have been studied in certain anuran species (Oliveira *et al.*, 2002; Oliveira, Sant'Anna, Munhoz de Omena, Souza Santos, Zieri, 2003; Oliveira and Zieri, 2005; Asenjo, Siu Ting, and Pino, 2011; Leite *et al.*, 2015; among many other). Particularly, sperm morphology and ultrastructure have been used to infer taxonomic and phylogenetic relationships in *Ascaphus* (Jamieson, Lee, and Long, 1993), *Colostethus* (Veiga-Menoncello, Lima, and Recco-Pimentel, 2006), certain *Hylidae* species, (Lee and Jamieson, 1993), *Leiopelma* (Scheltinga, Jamieson, Eggers, and Green, 2001), *Leptodactylinae* (Salles, Zara, and Prado, 2017), *Myobatrachids* (Lee and Jamieson, 1993), *Pleurodema* (Cruz, Ferraro, Farías, Santos, Recco-Pimentel, Faivovich, and Hermida, 2016); *Pseudopaludicola* (dos Santos, Orlandi Introíni, Prado Veiga-Menoncello, and Recco-Pimentel, 2015), and *Pseudinae* (Garda, Costa, Colli, and Báó, 2004). Spermiogenesis has also been studied in association with different types of fertilisation since spermatozoon morphology is thought to be the result of evolutionary pressure from the fertilisation environment (Jamieson *et al.*, 1993; Lee and Jamieson, 1993). In anurans, fertilisation environments can be diverse; consequently, since spermatozoa are the most diverse cell type, they are expected to exhibit wide evolutionary divergence in their form (Pitnick, Hosken, and Birkhead, 2009). The relationship between spermatozoal ultrastructure and spawning location has been discussed for certain anurans (Garda *et al.*, 2004; Muto and Kubota, 2013).

The morphology of the male reproductive organs can present anatomical variants in shape and size, according to the reproductive period (Asenjo *et al.*, 2011). Many studies have focused on cell and morphology changes in the testes and seminiferous tubules throughout the anuran reproductive cycle (e.g. Alves Santos, Santana, and Pacheco, 2017; Asenjo *et al.*, 2011; Carezzano and Cabrera, 2010; Curi, Olea, Álvarez, Céspedes, and Lombardo, 2014). However, the associations between testicular morphology and egg-laying mode out of the water, have been notoriously less explored (Leite *et al.*, 2015).

The anuran genus *Leptodactylus* (74 species) is divided into four species groups: *Leptodactylus latrans*, *Leptodactylus melanonotus*, *Leptodactylus pentandactylus*, and *Leptodactylus fuscus* (Heyer, 1969; de Sá, Grant, Camargo, Heyer, Ponssa, and Stanley, 2014). There are four reproductive modes reported for *Leptodactylus* (Haddad and

Prado, 2005): 1) eggs in aquatic foam nests and exotrophic tadpoles in ponds (mode 11); 2) foam nests floating on water accumulated in constructed basins and exotrophic tadpoles in ponds (mode 13); 3) foam nests with eggs and early larval stages in subterranean nests built after flooding; and 4) exotrophic tadpoles in ponds (mode 30), or streams (mode 31). These two latter modes are typical of species of the group *L. fuscus*, and of species of the related genus *Adenomera*. The testicular histomorphology of species of the genus *Leptodactylus* that lay eggs out of water (such as species of the group *L. fuscus*) has not been described yet.

It is the aim of the present study to describe the testicular histology and structural characteristics of the germ cells during development and maturation of male gametes, and their cystic arrangement in *Leptodactylus latinasus*, a member of the group *L. fuscus* with terrestrial egg deposition. The characteristics observed in *L. latinasus* are compared with those of other species with egg deposition out of the water *Leptodactylus bufonius*, *Leptodactylus fuscus*, *Phyllomedusa boliviana*, *Phyllomedusa sauvagii*, *Pithecopus azureus*, and *Oreobates discoidalis*, in order to propose particularities of testicular morphology that contribute to the reproductive success of these species of anurans with egg-laying out of water.

## MATERIALS AND METHODS

Seven species with egg deposition outside the water were selected: *Leptodactylus latinasus*, *L. bufonius* and *L. fuscus* (Leptodactylidae) lay their eggs in foam placed in underground terrestrial incubation chambers, where tadpoles hatch and complete their development in water bodies (Heyer, 1969; Solano, 1987) (Mode 12, Duellman and Trueb, 1986; Mode 30, Haddad and Prado, 2005). *Phyllomedusa boliviana*, *Ph. sauvagii* and *Pithecopus azureus* (Phyllomedusidae) lay their eggs in nests made of leaves hanging over water bodies, and larvae complete their development in the water (Ihering, 1886; Vaira, 2001; Pyburn and Glidewell, 1971) (Mode 18, Duellman and Trueb, 1986; Mode 24, Haddad and Prado, 2005). *Oreobates discoidalis* (Craugastoridae) eggs are laid directly on the ground, in moist and protected places, and their development is direct (Köhler, 2000; Vaira, 2002) (Mode 17, Duellman and Trueb, 1986; Mode 27, Haddad and Prado, 2005).

*Leptodactylus latinasus* was used as a comparative model among the studied species since it is known that this species is well adapted to anthropogenic pressure (Ponssa and Barrionuevo, 2008). That allowed us to have a greater sample availability and, therefore, to determine all stages of cystic spermatogenesis during the development and maturation of male gametes.

Specimens were collected during the reproductive period (October 2008 to March 2017) in Northern Argentina localities: San Pablo, Lules, San Javier, Yerba Buena, Tafi Viejo, San Miguel de Tucumán (Tucumán), and Salta Capital (Salta). (Table 2).

Males were anaesthetised with 10% Xylocaine spray solution. The testes were removed through an abdominal incision and photographed by using a Leica EZ4 simple stereoscopic microscope. They were fixed in 10% buffered formalin (0,2 M,

pH 7) and were preserved in 70% ethanol, dehydrated in a series of graded ethanol-solutions, cleared in xylene, and embedded in paraffin. Serial sections (4 to 5  $\mu\text{m}$  thick) were cut in a transverse plane with a microtome Zeiss HYRAX S30. The remaining testes were embedded with Cryoplast (freezing mounting medium), at a temperature between  $-20^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$ , thin cuts (3 to 4  $\mu\text{m}$  thick) were made on these samples with Cryostat Zeiss HYRAX C25, which allowed the identification of the gametes throughout the different stages of spermatogenesis.

Sections were stained with hematoxylin-eosin (H&E), for the detection of the different cells and tissues as components according to their acidic and basic groups (nucleus and cytoplasm). Although this coloration was used for histomorphological demonstrations, it has histochemical value, since Hematoxylin, due to its basophilia, has an affinity for the acidic groups of glucides and proteins. Eosin, on the other hand, reacts in a specific way in the basic proteins (Martoja and Martoja Pierson, 1970). To identify histones, protamines, and nucleic acids we used the nuclear basic dye toluidine blue (TB) at pH 4,4 and 7, which stains tissues based on the principle of metachromasia (Sridharan and Shankar, 2012). Tissues rich in histones are stained violet-blue; while chromatin rich in protamines is stained blue (Pearse, 1960; Barka and Anderson, 1965; Humason, 1979).

The present work is an exhaustive study carried out on numerous histological sections of testicles belonging to each of the different specimens of the species studied, which were observed under an optical microscope ZEISS Lab.A1, with a ZEISS AxioCam ERc5s camera, and Leica DM2000 with Leica ICC50HD camera. Microscopic measurements were performed with a Leica LAZ V4.9 Software.

The histological preparations were added to the Anatomico-Histological Collection belonging to the Institute of Animal Morphology of Foundation Miguel Lillo. (Table 2).

## RESULTS

### Testicular morphology of *Leptodactylus latinasus*

Macroscopically, the male gonads of *Leptodactylus latinasus* are asymmetrical, paired, smooth, and oval organs. They are located in the abdominal cavity and linked to the kidney through a thin, vascularised, innervated, connective tissue covered with mesothelial cells. The testicles are  $2154.77 \pm 74.29 \mu\text{m}$  long and  $1199.28 \pm 47.34 \mu\text{m}$  wide (N= 14); fat bodies are associated with the cephalic area of the gonads. The testicles are surrounded by a thin capsule of connective tissue: the tunic albuginea, which is  $4.667 \pm 0.726 \mu\text{m}$  thick and confers them a light-yellow colour (unpigmented) (Figs 1, 2) (Table 1). This connective tissue has blood vessels, collagen fibres, fibroblasts, muscle cells, and nerves (Figs 3, 4, 26). Within the testes, there are several seminiferous tubules forming the testicular parenchyma, which is surrounded by a stroma of connective tissue: the peritubular tunic. This tunic originates from the albuginea tunic; and is part of the connective tissue that forms the interstice, where blood vessels, collagen fibres, fibroblasts, macrophages, and Leydig

cells can be observed (Figs. 5, 6). In the testicular interstitium, there are efferent ducts made up of a simple cuboidal epithelium in the lumen of which spermatozoa can be distinguished (Fig. 3).

### Spermatogenesis of *Leptodactylus latinasus*

**Seminiferous tubules.**— Inside the seminiferous tubules, two cell groups can be identified: germ cells and Sertoli cells. The latter are responsible for the maturation of the spermatozoa lineage (Figs. 7, 8). The germ cells are undergoing different stages of spermatogenesis. Spermatogenesis occurs in groups called cysts, which are found inside the seminiferous tubules. Each cyst consists of a single type of germ cells in the same differentiation stage. *L. latinasus* showed a large number of cysts, containing primary and secondary spermatogonia (Figs. 7, 8), primary and secondary spermatocytes (Figs. 9, 10), spermatids (Figs. 9, 10, 11), and spermatozoa (Figs. 7, 9, 12). The Sertoli cells are cylindrical with a large euchromatic nucleus in a basal position, prominent nucleolus, and acidophilic cytoplasm. Up to three Sertoli cells were identified in each cyst, probably due to the large cyst size (Figs. 6, 8, 11, 12).

The different developmental stages of the germ cells throughout spermatogenesis are classified by the cell size, cytoplasmic and nuclear characteristics (chromatin size and degree of compaction). In *L. latinasus*, we observed all the stages of the spermatic cycle (spermatogonia, spermatocyte and spermiogenesis).

**Spermatogonia.**— Primary spermatogonia of  $5.96 \pm 0.21 \mu\text{m}$  of diameter, which are diploid cells characterised as being much larger than the other germ cells. They present a clear nucleus and chromatin with a thin, granular appearance and are divided by mitosis, giving rise to secondary spermatogonia of  $4.76 \pm 0.18 \mu\text{m}$  of diameter. The secondary spermatogonia are discretely smaller, and their chromatin is slightly more condensed than in primary spermatogonia. That is because their nuclei have several small sectors of heterochromatin, which gives them greater basophilia (Figs. 7, 8). Both primary and secondary spermatogonia stain with TB and show a  $\beta$ -metachromasia, with blue-violet nuclei due to the presence of histones (Figs. 9, 10).

**Spermatocytes.**— These cells originate from the secondary spermatogonia. Their presence indicates the beginning of the first meiotic division (Figs. 9, 10). The spermatocytes show more condensed chromatin concentration, and they are smaller than the spermatogonia. The primary spermatocytes in different phases of the first meiotic division are discernible as diplotene, metaphase and anaphase, (Fig. 9). The chromosomes in diplotene are observed as large pieces. In the metaphase, the chromosomes are arranged throughout the equatorial region. During anaphase, the chromatids are separated and located at the poles of the cells. As a result of the first meiotic division, secondary spermatocytes are observed, which are characteristically smaller ( $3.16 \pm 0.11 \mu\text{m}$  of diameter) than the primary spermatocytes. They exhibit more nuclear compaction, show a strong basophilia with H&E (Figs. 4, 6), and orthochromasia with TB at pH 7 and 4,4 (Figs. 9, 10).

**Spermatids.**— The secondary spermatocytes division generates primary spermatids, which are small and spherical approximately  $1.62 \pm 0.165 \mu\text{m}$  diameter. Throughout spermiogenesis, spermatid differentiation and nuclear elongation can be observed in ‘step’ stages. The spermatids are differentiated and give rise to spermatozoa, which allows the observation of spermatids in the first (spermatid 1) and second (spermatid 2) stage of spermiogenesis. During the spermiogenesis, chromatin condensation, cell size modification, and changes in the shape of the nuclei that goes from oval to cylindrical are observed. Spermatids in the first stage of spermiogenesis are spherical and disposed in groups in the cyst (Fig. 9) while spermatids in the second stage of spermiogenesis have an oval nucleus and are organised in bundles attached to the apical zone of the Sertoli cells (Fig. 8, 10, 11, 12). Both primary and secondary spermatids have a nucleus with strong basophilia due to chromatin condensation.

**Spermatozoa.**— Spermatids in the second stage of spermiogenesis generate spermatozoa, which are elongated cells with totally compacted chromatin (Figs. 9-12). Mature spermatozoa present elongated cylindrical nuclei (total length =  $9.37 \mu\text{m}$  diameter =  $1.05 \pm 0.07 \mu\text{m}$ ), with their anterior end slightly pointed and arrow-shaped. Posterior to the nucleus is the acidophilic flagellum. The spermatozoa are arranged in groups resembling eyelashes in the apical region of the Sertoli cell; while free spermatozoa are already in the lumen of the tubule (Fig. 12).

In the testicle samples of *Leptodactylus latinasus*, three stages of the reproductive cycle are distinguished: (1) Testicles with cysts in early spermatogenesis stages with a greater number of spermatogonia and spermatocytes, few spermatids, and empty tubule lumen (Fig. 11); (2) Testicles with a large number of cysts, with cells in advanced stages of spermatogenesis (secondary spermatogonia and secondary spermatocytes), spermatids in the first and second stages of spermiogenesis, spermatozoa anchored to the apical region of the Sertoli cell, and free ones in the lumen of the tubule (Fig. 3, 10); (3) Testicles in regression with many cysts with germ-line cells in apoptosis, observed through morphological and biochemical characteristics of the process: DNA fragmentation, reduction of cell volume, cytoplasmic vacuolisation, and formation of apoptotic bodies (Fig. 6).

### Comparative study of the testes

When comparing *Leptodactylus latinasus* to the other studied species (*Leptodactylus bufonius*, *L. fuscus*, *Phitecopus azurea*, *Phyllomedusa boliviana*, *Phyllomedusa sauravagii*, *Oreobates discoidalis*), variations in both, testicular morphology and spermatogenesis are observed.

Testicular pigmentation, thickness and/or abundance of the albuginea tunic, peritubular tunic, interstitial tissue, and spermatozoon shape stand out. In all the studied species, testes are unpigmented due to the absence of chromatophores (Figs. 13, 16, 19), and show size variation (Figs. 14, 17, 20). The albuginea tunic, constituted by dense irregular connective tissue varies from two to three layers in *Leptodactylus latinasus* (Fig. 4), *L. fuscus*, *Oreobates discoidalis* (Fig. 21) and *L. bufonius* (Fig.

15); from four to eight layers in *Pithecopus azureus*, *Phyllomedusa sauvagii* (Fig. 18) and *P. boliviana*.

The peritubular tunic constitutes a thick layer of connective tissue with peritubular myoid cells, fibroblasts and collagen fibres (Figs. 22, 25, 28). The connective tissue that forms the interstitium is dense, but it is characterised not only by an abundance of collagen fibres but also by an abundance of cells. The interstitial tissue, in turn, shows variations between species (Figs. 23, 26, 29); in *Phyllomedusa boliviana* the abundance of interstitial tissue and the presence of smooth muscle cells stand out (Fig. 26).

Three morphologies are observed among the spermatozoa: (a) Arrow-shaped spermatozoa in Family Leptodactylidae (Fig. 24); (b) curved nail-shaped spermatozoa in Family Phyllomedusidae; in the spermatozoa head region of *Phyllomedusa boliviana*, two very acidophilous sectors are observed: the anterior acrosome, and the midpiece of the flagellum in the posterior region of the nucleus (Fig. 27); (c) in Family Craugastoridae, long thin sperm of filamentous aspect, *Oreobates discoidalis* (Fig. 30).

The observed variations in the selected characters among species are synthesised in Table 1.

Regarding spermatogenesis, differences in the development and maturation stages of the germ cells are observed. *Leptodactylus bufonius* presents cysts with secondary spermatogonia, a few cysts with secondary spermatocytes and spermatids in the second stage of spermiogenesis. This species shows abundant spermatozoa attached to the Sertoli cells, in the tubular lumen and the efferent duct (Fig. 31). *Leptodactylus fuscus* shows abundant cysts with primary and secondary spermatogonia, secondary spermatocytes, and few spermatozoa attached to the Sertoli cells. These features would indicate that most of the germ cells are in the initial stage of spermatogenesis (Fig. 32). *Pithecopus azurea* shows an abundance of cysts with secondary spermatogonia, secondary spermatocytes, and spermatozoa attached to the Sertoli cells. These features correspond to advanced spermatogenesis, but not to the final stage since there are no free spermatozoa observed in the lumen of the seminiferous tubule (Fig. 33). In *Phyllomedusa boliviana*, we found few cysts with primary spermatogonia, abundant cysts with secondary spermatogonia, and secondary spermatocytes and spermatozoa attached to the apical pole of the Sertoli cells. The lumen of the seminiferous tubule and those of the efferent duct are empty. Spermatogenesis is at a similar stage as in *P. azurea* (Fig. 34). *Phyllomedusa sauvagii* shows abundant cysts with secondary spermatogonia and secondary spermatocytes, spermatids in the second stage of spermiogenesis, and spermatozoa attached to the Sertoli cells, both in the tubular lumen. These features would indicate complete spermatogenesis and abundant release of mature sperm (Fig. 35). Cysts with secondary spermatogonia abound in *Oreobates discoidalis*, spermatids in the second stages of spermiogenesis and spermatozoa anchored in the apical region of Sertoli cells, as well as spermatozoa in the process of being released into the light of the seminiferous tubule, indicating a final stage in the spermatogenesis (Fig. 36).



**Table 1.** Variable characters analyzed in species with deposition mode outside of water. N: specimen number; TL: testicle length; TW: testicle width; P: pigmentation; AT: albuginea tunic; PT: peritubular tunic; IT: interstitial tissue; S: spermatozoa length; SNS: spermatozoa nucleus shape; FL: flagellum length; SNL: spermatozoa nucleus length (calculated as the difference between total length and flagellum length).

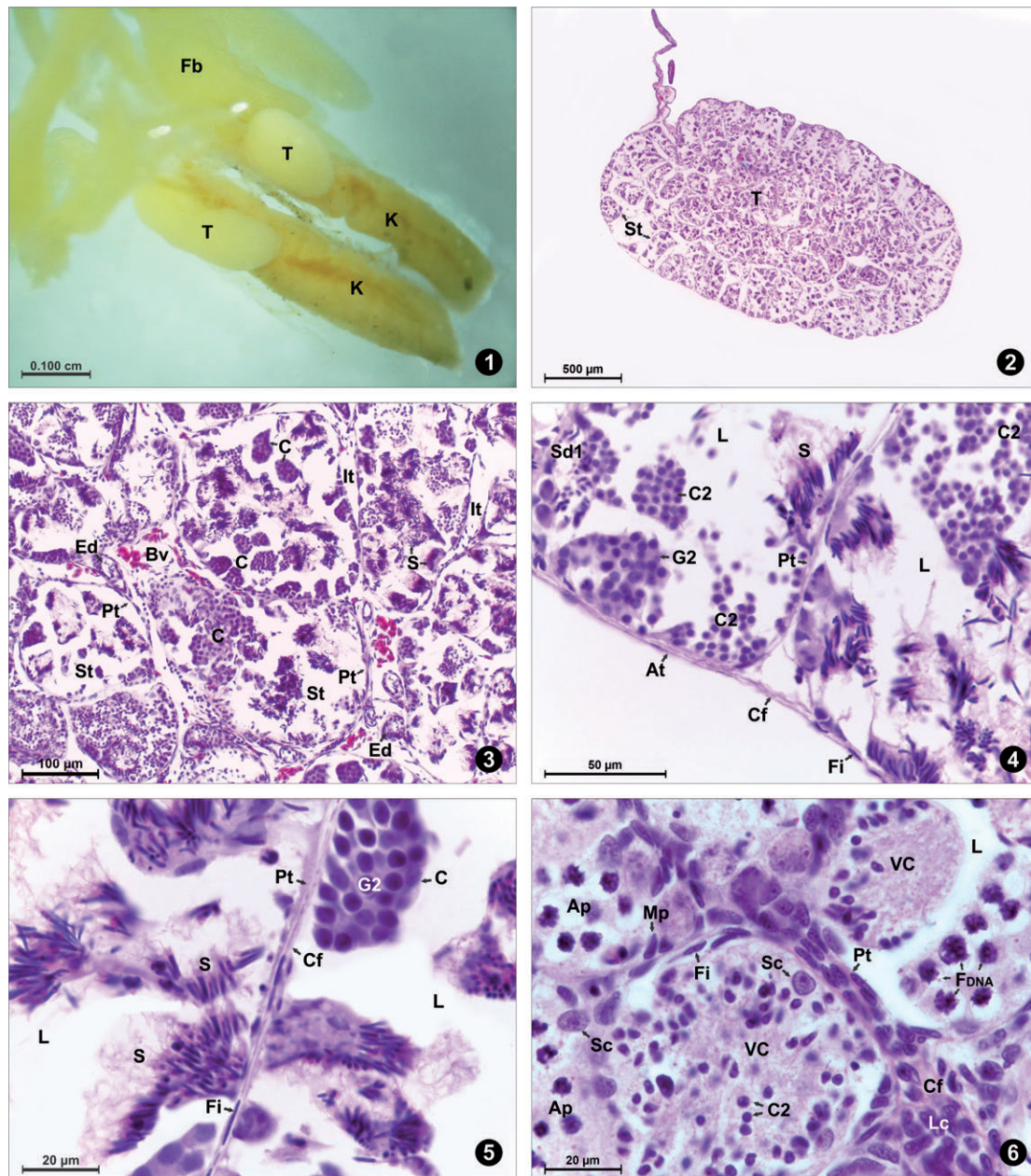
**Tabla 1.** Caracteres variables analizados en especies con modo de deposición fuera del agua. N: número de muestra; TL: longitud del testículo; TW: ancho de testículo; P: pigmentación; AT: túnica de albuginea; PT: túnica peritubular; IT: tejido intersticial; S: longitud de los espermatozoides; SNS: forma del núcleo de los espermatozoides; FL: longitud del flagelo; SNL: longitud del núcleo de los espermatozoides (calculada como la diferencia entre la longitud total y la longitud del flagelo).

Family	Species	N	TL ( $\mu\text{m}$ )	TW ( $\mu\text{m}$ )	P (present/ absent)	AT ( $\mu\text{m}$ )	AT (number of layers)	PT (number of layers)	IT	S ( $\mu\text{m}$ )	SNS	FL ( $\mu\text{m}$ )	SNL ( $\mu\text{m}$ )
Leptodactylidae	<i>Leptodactylus latinasus</i>	14	2154.77 $\pm$ 74.29	1199.28 $\pm$ 47.34	A	4.667 $\pm$ 0.726	2	1	Scarce	28.13 $\pm$ 1.89	Arrow	15.71 $\pm$ 1.70	9.3713
Leptodactylidae	<i>Leptodactylus bufonius</i>	3	1668.47 $\pm$ 95.39	1032.86 $\pm$ 55.83	A	7.22 $\pm$ 1.08	3	1	Abundant	32.24 $\pm$ 2.699	Arrow	21.24 $\pm$ 1.43	10.99
Leptodactylidae	<i>Leptodactylus fuscus</i>	2	2333.09 $\pm$ 75.70	1179.80 $\pm$ 71.8358	A	4.616 $\pm$ 0.411	2	1	Scarce	21.7155 $\pm$ 1.925	Arrow	14.60 $\pm$ 1.88	7.11
Phyllomedusidae	<i>Pithecopus azurea</i>	3	4125.909 $\pm$ 140.971	1622.32 $\pm$ 45.98	A	8.98 $\pm$ 1.51	4 to 5	1	Scarce	58.53 $\pm$ 5.20	Claw	39.45 $\pm$ 3.82	19.08
Phyllomedusidae	<i>Phyllomedusa boliviana</i>	3	5593.571 $\pm$ 172.118	34.056 $\pm$ 4.806	A	34.056 $\pm$ 4.806	7 to 8	1	Abundant	60.855 $\pm$ 3.771	Claw	40.38 $\pm$ 3.41	20.48
Phyllomedusidae	<i>Phyllomedusa sauvagii</i>	2	9528.182 $\pm$ 239.297	17.10 $\pm$ 2.20	A	17.10 $\pm$ 2.20	5 to 6	1	Scarce	54.22 $\pm$ 2.85	Claw	38.48 $\pm$ 3.99	19.73
Craugastoridae	<i>Oreobates discoidalis</i>	3	2818.8 $\pm$ 80.0356	1403.79 $\pm$ 56.7908	A	4.577 $\pm$ 0.894	2	1	Scarce	61.364 $\pm$ 7.240	Filamentous	46.16 $\pm$ 3.42	15.20

**Table 2.** List of species examined. Colección Anatómo-Histológica, Instituto de Morfología Animal (Dirección de Zoología, Fundación Miguel Lillo).

**Tabla 2.** Lista de especies examinadas. Colección Anatómo-Histológica, Instituto de Morfología Animal (Dirección de Zoología, Fundación Miguel Lillo).

Species	Number of collections (histological samples)	Collection dates in brakes (dd/mm/yy)	Collection location
<i>Leptodactylus latinasus</i>	APLL0002	06/III/2007	Lules (Tucumán)
	APLL0025	04/XII/2008	Lules (Tucumán)
	APLL0030	04/XII/2008	Tafí Viejo (Tucumán)
	APLL0033	XII/2011	Tafí Viejo (Tucumán)
	APLL0035	XII/2011	San Javier (Tucumán)
	APLL0036	XII/2013	San Javier (Tucumán)
	APLL0037	XII/2015	San Pablo (Tucumán)
	APLL0038	XI/2015	San Pablo (Tucumán)
	APLL0039	XI/2015	S. M. de Tucumán (Tucumán)
	APLL0040	XI/2015	San Pablo (Tucumán)
	APLL0041	XI/2015	San Pablo (Tucumán)
	APLL0042	XI/2015	Yerba Buena (Tucumán)
	APLL0043	XI/2015	Yerba Buena (Tucumán)
	APLL0044	XI/2015	Yerba Buena (Tucumán)
<i>Leptodactylus bufonius</i>	APLB0001	25/II/2006	Salta Capital (Salta)
	APLB0002	20-23/II/2010	Salta Capital (Salta)
	APLB0003	08/II/2005	Salta Capital (Salta)
<i>Leptodactylus fuscus</i>	APLF0003	03/I/2018	Salta Capital (Salta)
	APLF0004	09/I/2018	Salta Capital (Salta)
<i>Pitherocopus azureus</i>	APPA0007	13/I/2017	Salta Capital (Salta)
	APPA0009	13/I/2017	Salta Capital (Salta)
	APPA0011	13/I/2017	Salta Capital (Salta)
<i>Phyllomedusa boliviana</i>	APPB0001	15/I/2017	Salta Capital (Salta)
	APPB0002	15/I/2017	Salta Capital (Salta)
	APPB0003	15/I/2017	Salta Capital (Salta)
<i>Phyllomedusa sauvagii</i>	APPS0004-1	24/XI/2016	San Javier (Tucumán)
	APPS0009	12/XII/2002	Salta Capital (Salta)
<i>Oreobates discoidalis</i>	APOD0007	XI/2013	Lules (Tucumán)
	APOD0008	XI/2013	Lules (Tucumán)
	APOD0009	XI/2013	Lules (Tucumán)



**Figure 1.** Macroscopic views showing oval unpigmented Testes associated with Fat Bodies and Kidneys in *Leptodactylus latinasus*. Fb: Fat Body; T: Testis; K: Kidney.

**Figura 1.** Vistas macroscópicas que muestran testículos ovalados no pigmentados asociados con cuerpos grasos y riñones en *Leptodactylus latinasus*. Cg: Cuerpo Graso; T: Testículo; R: Riñón.

**Figure 2.** Histological section of the Testis in *L. latinasus*. T: Testis; St: Seminiferous tubules. Staining: Hematoxylin-Eosin (H-E).

**Figura 2.** Corte histológico del testículo en *L. latinasus*. T: Testículo; Ts: Túbulos seminíferos. Tinción: Hematoxilina-eosina (H-E).

**Figure 3.** Arrangement of the Seminiferous Tubules in *L. latinasus*. Bv: Blood vessel; C: Cysts; Ed: Efferent duct; It: Interstitial tissue; Pt: Peritubular tunic, S: Spermatozoa; St: Seminiferous tubules. Staining: H-E.

**Figura 3.** Disposición de los Túbulos Seminíferos en *L. latinasus*. Vs: Vaso sanguíneo; C: Cistos; Ce: Conducto eferente; Ti: Tejido intersticial; Tp: Túnica peritubular; E: Espermatozoides; Ts: Túbulos seminíferos. Tinción: H-E.

**Figure 4.** Albuginea Tunic in *L. latinasus*. At: Albuginea tunic; C2: Secondary Spermatocyte; Cf: Collagen fibres; Fi: Fibroblasts; G2: Secondary Spermatogonia; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa; Sd1: Primary Spermatid. Staining: H-E.

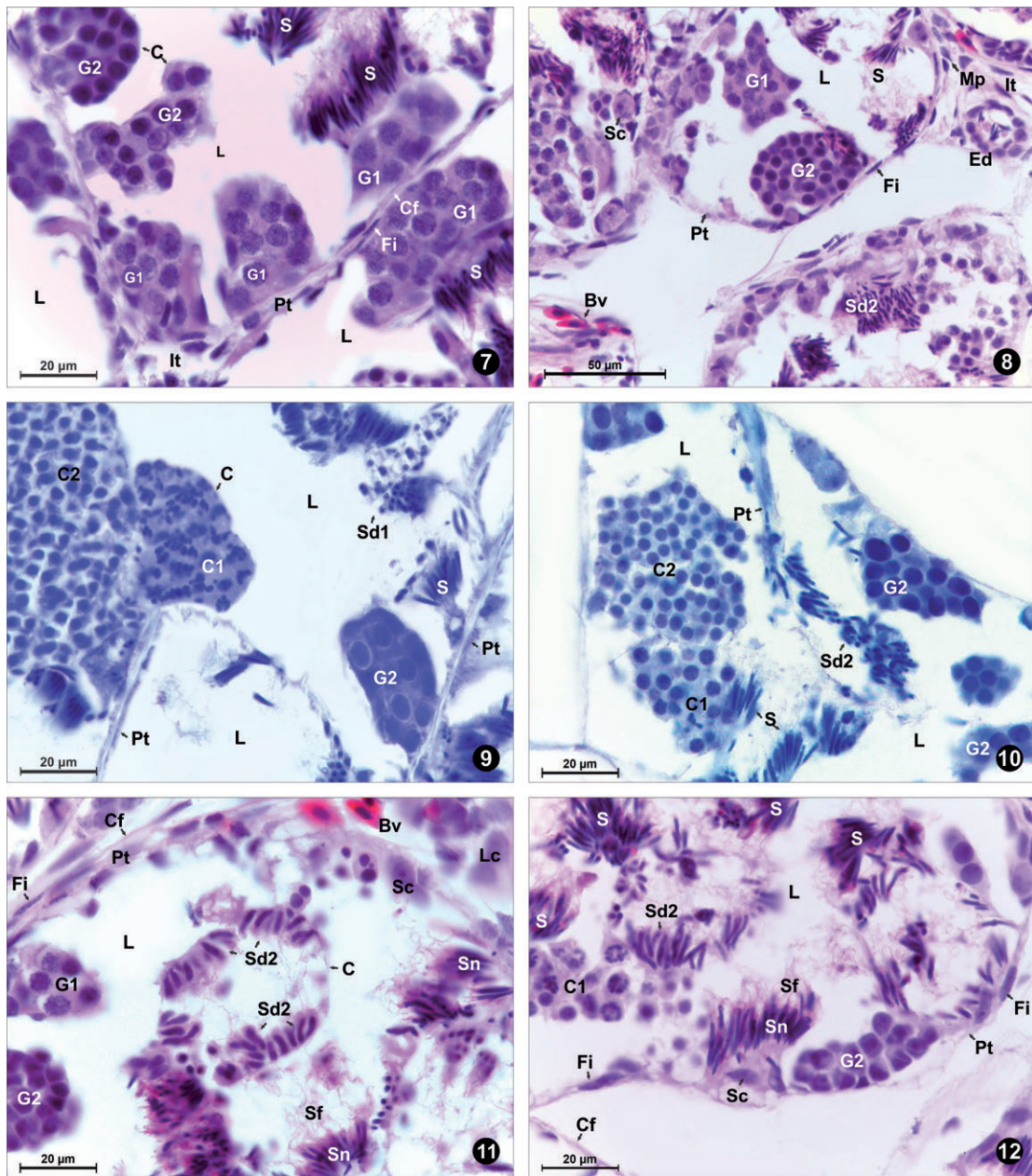
**Figura 4.** Túnica Albugínea en *L. latinasus*. Ta: Túnica Albugínea; Ec2: Espermatocito Secundario; Fc: Fibras de colágeno; Fi: Fibroblastos; Eg2: Espermatogonias Secundarias; L: Lumen Tubular; Tp: Túnica peritubular; E: Espermatozoides; Ed1: Espermátida Primaria. Tinción: H-E.

**Figure 5.** Peritubular Tunic in *L. latinasus*. C: Cysts; Cf: Collagen fibres; Fi: Fibroblasts; G2: Secondary Spermatogonia; L: Tubule Lumen; S: Spermatozoa. Staining: H-E.

**Figura 5.** Túnica Peritubular en *L. latinasus*. C: Cistos; Fc: Fibras de colágeno; Fi: Fibroblastos; Eg2: Espermatogonias Secundarias; L: Lumen Tubular; E: Espermatozoides. Tinción: H-E.

**Figure 6.** Interstitial Tissue in *L. latinasus*. Ap: Apoptosis; C2: Secondary Spermatocyte; Cf: Collagen fibres; Fi: Fibroblasts; F<sub>DNA</sub>: DNA fragmentation; L: Tubule Lumen; Lc: Leydig cell; Mp: Macrophage, Pt: Peritubular tunic, Sc: Sertoli cell; CV: Cytoplasmic Vacuolization. Staining: H-E.

**Figura 6.** Tejido Intersticial en *L. latinasus*. Ap: Apoptosis; Ec2: Espermatocito Secundario; Fc: Fibras de colágeno; Fi: Fibroblastos; FADN: fragmentación de ADN; L: Lumen Tubular; CL: Célula de Leydig; Ma: Macrófago; Tp: Túnica peritubular; CS: Célula de Sertoli; VC: Vacuolización Citoplasmática. Tinción: H-E.



**Figure 7.** Testicular histology and spermatogenesis of *L. latinasus*. Cysts with Primary Spermatogonia. C: Cysts; Cf: Collagen fibres; Fi: Fibroblast; G1: Primary Spermatogonia; G2: Secondary Spermatogonia; It: Interstitial tissue; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa. Staining: Hematoxylin–Eosin (H-E).

**Figura 7.** Histología testicular y espermatogénesis de *L. latinasus*. Cistos con Espermatogonias primarias. C: Cistos; Fc: Fibras de colágeno; Fi: Fibroblasto; Eg1: Espermatogonias Primarias; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; L: Lumen Tubular; Tp: Túnica peritubular; E: Espermatozoides. Tinción: Hematoxilina-eosina (H-E).

**Figure 8.** Testicular histology and spermatogenesis of *L. latinasus*. Cysts with Secondary Spermatogonia. Bv: Blood vessel; Ed: Efferent duct; Fi: Fibroblast; G1: Primary Spermatogonia; G2: Secondary Spermatogonia; It: Interstitial tissue; L: Tubule Lumen; Mp: Macrophages; Pt: Peritubular tunic; S: Spermatozoa; Sc: Sertoli cell; Sd2: Secondary Spermatid. Staining: H-E.

**Figura 8.** Histología testicular y espermatogénesis de *L. latinasus*. Cistos con Espermatogonia Secundaria. Vs: Vaso sanguíneo; Ce: Conducto eferente; Fi: Fibroblasto; Eg1: Espermatogonias Primarias; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; L: Lumen Tubular; Ma: Macrófagos;

Tp: Túnica Peritubular; E: Espermatozoides; CS: Células de Sertoli; Ed2: Espermátida Secundaria. Tinción: H-E.

**Figure 9.** Testicular histology and spermatogenesis of *L. latinasus*. Cysts with Primary Spermatocytes. C: Cysts; C1: Primary Spermatocyte; C2: Secondary Spermatocyte; G2: Secondary Spermatogonia; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa; Sd1: Primary Spermatid. Staining: Toluidine Blue pH 7 (TB).

**Figura 9.** Histología testicular y espermatogénesis de *L. latinasus*. Cistos con Espermatocitos Primarios. C: Cistos; Ec1: Espermatocito Primario; Ec2: Espermatocito Secundario; Eg2: Espermatogonias Secundarias; L: Lumen Tubular; Tp: Túnica peritubular; E: Espermatozoides; Ed1: Espermátida Primaria. Tinción: Azul de Toluidina pH 7 (AT).

**Figure 10.** Testicular histology and spermatogenesis of *L. latinasus*. Cysts with Secondary Spermatocytes. C1: Primary Spermatocyte; C2: Secondary Spermatocyte; G2: Secondary Spermatogonia; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa; Sd2: Secondary Spermatid. Staining: TB.

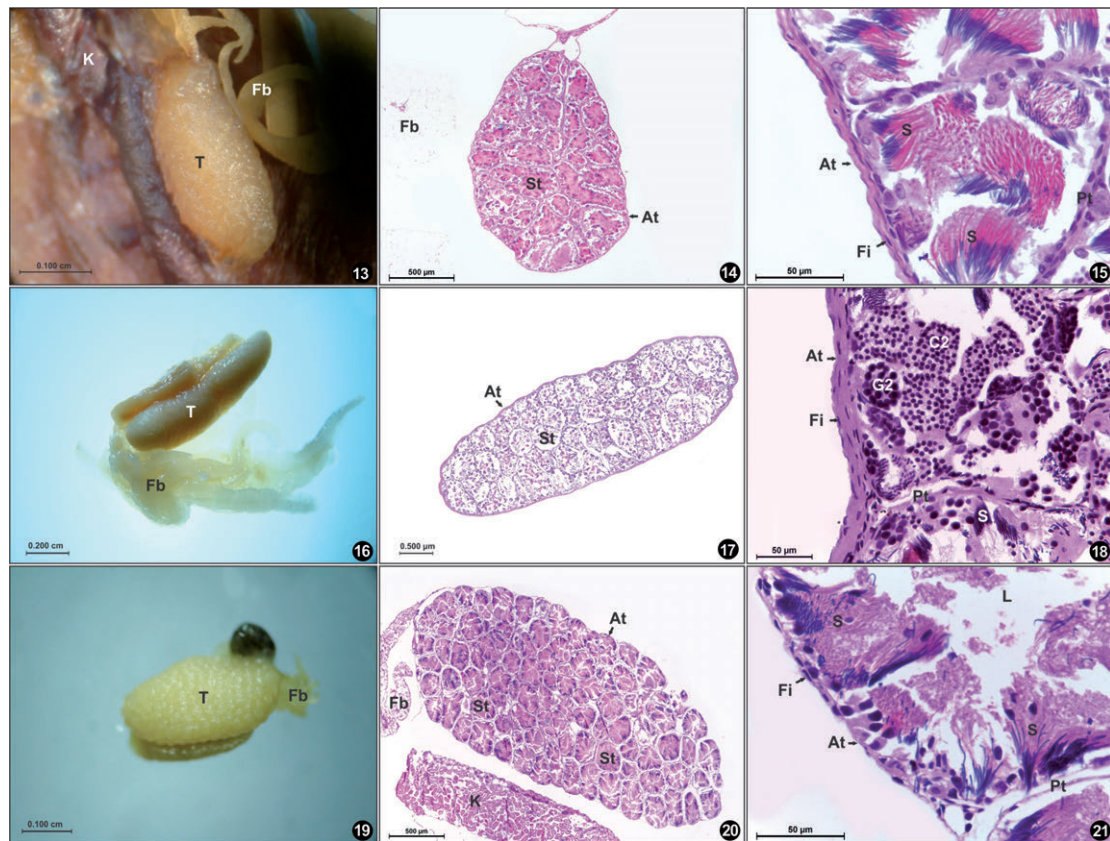
**Figura 10.** Histología testicular y espermatogénesis de *L. latinasus*. Cistos con Espermatocitos Secundarios. Ec1: Espermatocito Primario; Ec2: Espermatocito Secundario; Eg2: Espermatogonias Secundarias; L: Lumen Tubular; Tp: Túnica peritubular; E: Espermatozoides; Ed2: Espermátida Secundaria. Tinción: AT.

**Figure 11.** Testicular histology and spermatogenesis of *L. latinasus*. Cysts with Spermatids. Bv: Blood vessel; C: Cysts; Cf: Collagen fibres; Fi: Fibroblast; G1: Primary Spermatogonia; L: Tubule Lumen; Lc: Leydig cell; Pt: Peritubular tunic; Sc: Sertoli cell; Sd2: Secondary Spermatid; Sf: Spermatozoa flagellum, Sn: Spermatozoa nucleus. Staining: H-E.

**Figura 11.** Histología testicular y espermatogénesis de *L. latinasus*. Cistos con Espermátidas. Vs: Vaso sanguíneo; C: Cistos; Fc: Fibras de colágeno; Fi: Fibroblasto; Eg1: Espermatogonias Primarias; L: Lumen Tubular; CL: Célula de Leydig; Tp: Túnica peritubular; CS: Célula de Sertoli; Ed2: Espermátida Secundaria; Fe: Flagelo del espermatozoide; Ne: Núcleo del espermatozoide. Tinción: H-E.

**Figure 12.** Testicular histology and spermatogenesis of *L. latinasus*. C1: Primary Spermatocyte; Cf: Collagen fibres; Fi: Fibroblast; G2: Secondary Spermatogonia; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa; Sd2: Secondary Spermatid; Sf: Spermatozoa flagellum, Sn: Spermatozoa nucleus. Staining: H-E.

**Figura 12.** Histología testicular y espermatogénesis de *L. latinasus*. Ec1: Espermatocito Primario; Fc: Fibras de colágeno; Fi: Fibroblasto; Eg2: Espermatogonias Secundarias; L: Lumen Tubular; Tp: Túnica peritubular; E: Espermatozoides; Ed2: Espermátida Secundaria; Fe: Flagelo del espermatozoide; Ne: Núcleo del espermatozoide. Tinción: H-E.



**Figure 13.** Macroscopy of the testicles showing the unpigmented surface of the organ and its association with fat bodies in *Leptodactylus fuscus*. Fb: Fat body; T: Testicle.

**Figura 13.** Macroscopia de los testículos que muestra la superficie no pigmentada del órgano y su asociación con cuerpos grasos en *Leptodactylus fuscus*. Cg: Cuerpo grasso; T: testículo.

**Figure 14.** Histological characterisation of the testes in *Leptodactylus bufonius*. At: Albuginea tunic; Fb: Fat body; St: Seminiferous tubules. Staining: Hematoxylin–Eosin (H-E).

**Figura 14.** Caracterización histológica de los testículos en *Leptodactylus bufonius*. Ta: Túnica Albugínea; Cg: Cuerpo grasso; Ts: Túbulos seminíferos. Tinción: Hematoxilina-Eosina (H-E).

**Figure 15.** Histological characterisation of the testes in *Leptodactylus bufonius*. At: Albuginea tunic; Fi: Fibroblasts; S: Spermatozoa. Staining: H-E.

**Figura 15.** Caracterización histológica de los testículos en *Leptodactylus bufonius*. Ta: Túnica albugínea; Fi: Fibroblastos; E: Espermatozoides. Tinción: H-E.

**Figure 16.** Macroscopy of the testicles showing the unpigmented surface of the organ and its association with fat bodies in *Phyllomedusa sauvagii*. Fb: Fat body; T: Testicle.

**Figura 16.** Macroscopia de los testículos que muestra superficie no pigmentada del órgano y su asociación con cuerpos grasos en *Phyllomedusa sauvagii*. Cg: Cuerpo grasso; T: Testículo.

**Figure 17.** Histological characterisation of the testes in *Phyllomedusa boliviana*. At: Albuginea tunic; St: Seminiferous tubules. Staining: H-E.

**Figura 17.** Caracterización histológica de los testículos en *Phyllomedusa boliviana*. Ta: Túnica albugínea; Ts: Túbulos seminíferos. Tinción: H-E.

**Figure 18.** Histological characterisation of the testes in *Phyllomedusa sauvagii*. At: Albuginea tunic; C2: Secondary Spermatocyte; Fi: Fibroblasts; G2: Secondary Spermatogonia; Pt: Peritubular tunic; S: Spermatozoa. Staining: H-E.

**Figura 18.** Caracterización histológica de los testículos en *Phyllomedusa sauvagii*. Ta: Túnica albugínea; Ec2: Espermatocito Secundario; Fi: Fibroblastos; Eg2: Espermatogonias Secundarias; Tp: Túnica peritubular; E: Espermatozoides. Tinción: H-E.

**Figure 19.** Macroscopy of the testicles showing the unpigmented surface of the organ and its association with fat bodies in *Oreobates discoidalis*. Fb: Fat body; T: Testicle.

**Figura 19.** Macroscopia de los testículos que muestra la superficie no pigmentada del órgano y su asociación con cuerpos grasos en *Oreobates discoidalis*. Cg: Cuerpo graso; T: Testículo.

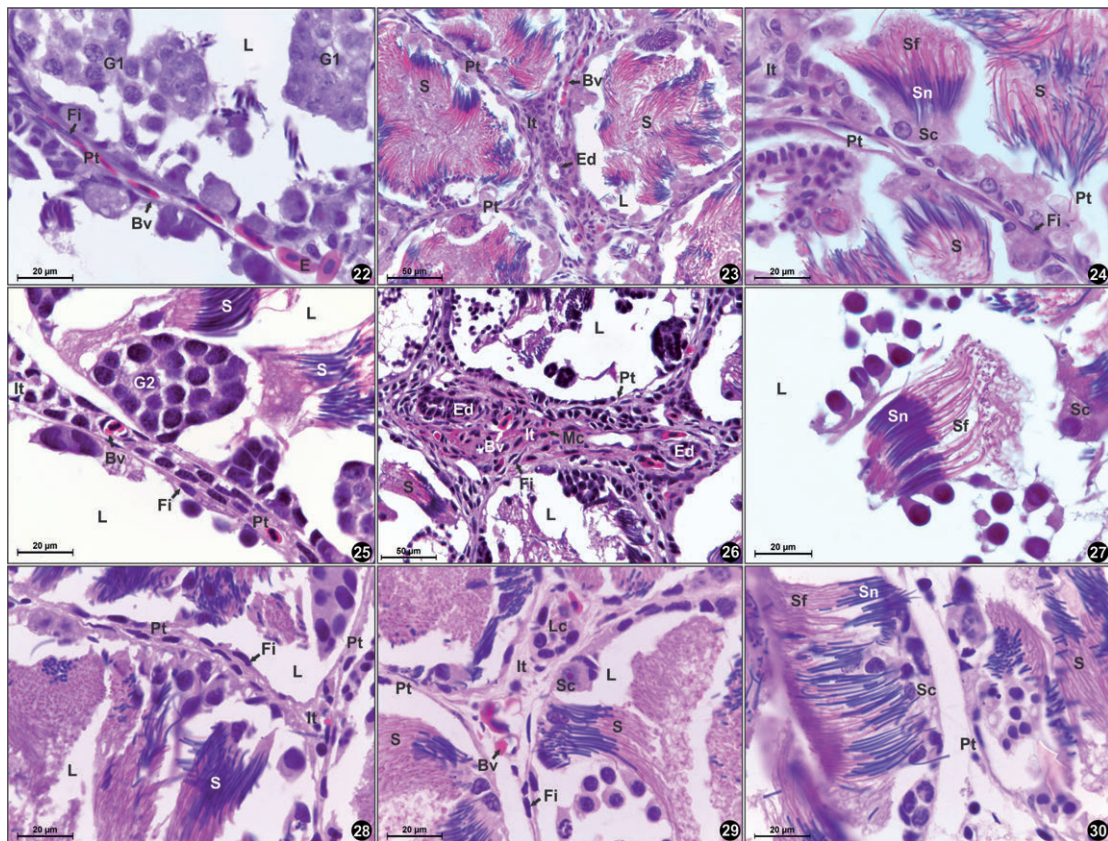
**Figure 20.** Histological characterisation of the testes in *Oreobates discoidalis*. At: Albuginea tunic; C2: secondary spermatocyte; Fb: Fat body; K: Kidney; St: Seminiferous tubules. Staining: H-E.

**Figura 20.** Caracterización histológica de los testículos en *Oreobates discoidalis*. Ta: Túnica albugínea; Ec2: Espermatocito Secundario; Cg: Cuerpo graso; R: riñón; Ts: Túbulo seminífero. Tinción: H-E.

**Figure 21.** Histological characterisation of the testes in *Oreobates discoidalis*. At: Albuginea tunic; Fi: Fibroblasts; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa. Staining: H-E.

**Figura 21.** Caracterización histológica de los testículos en *Oreobates discoidalis*. Ta: Túnica albugínea; Fi: Fibroblastos; L: Lumen Tubular; Tp: Túnica peritubular; E: Espermatozoides. Tinción: H-E.





**Figure 22.** Histological characterisation and spermatogenesis of the testes in *Leptodactylus fuscus*. Bv: Blood vessel; E: Erythrocyte; Fi: Fibroblast; G1: Primary Spermatogonia; L: Tubule Lumen; Pt: Peritubular Tunic. Staining: Hematoxylin–Eosin (H-E).

**Figura 22.** Caracterización histológica y espermatogénesis de los testículos en *Leptodactylus fuscus*. Vs: Vaso sanguíneo; Er: Eritrocito; Fi: Fibroblasto; Eg1: Espermatogonias Primarias; L: Lumen Tubular; Tp: Túnica peritubular. Tinción: Hematoxilina-Eosina (H-E).

**Figure 23.** Testicular histology and spermatogenesis of *Leptodactylus bufonius*. Bv: Blood vessel; Ed: Efferent conduct; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa. Staining: H-E.

**Figura 23.** Histología testicular y espermatogénesis de *Leptodactylus bufonius*. Vs: Vaso sanguíneo; Ce: Conducto eferente; L: Lumen Tubular; Tp: Túnica Peritubular; E: Espermatozoides. Tinción: H-E.

**Figure 24.** Testicular histology and spermatogenesis of *Leptodactylus bufonius*. Fi: Fibroblast; It: interstitial tissue; Pt: Peritubular tunic; S: Spermatozoa; Sc: Sertoli cell; Sf: Spermatozoa flagellum; Sn: Spermatozoa nucleus. Staining: H-E.

**Figura 24.** Histología testicular y espermatogénesis de *Leptodactylus bufonius*. Fi: Fibroblasto; Ti: Tejido intersticial; Tp: Túnica peritubular; E: Espermatozoides; CS: Célula de Sertoli; Fe: Flagelo del espermatozoide; Ne: Núcleo del espermatozoide. Tinción: H-E.

**Figure 25.** Testicular histology and spermatogenesis of *Pithecopus azureus*. Bv: Blood vessel; Fi: Fibroblast; G2: Secondary Spermatogonia; It: Interstitial tissue; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa. Staining: H-E.

**Figura 25.** Histología testicular y espermatogénesis de *Pithecopus azureus*. Vs: Vaso sanguíneo; Fi: Fibroblasto; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; L: Lumen Tubular; Tp: Túnica peritubular; E: Espermatozoides. Tinción: H-E.

**Figure 26.** Testicular morphology of *Phyllomedusa boliviana*. Bv: Blood vessel; Ed: Efferent conduct; Fi: Fibroblast; Mc: muscle cells; L: Tubule Lumen; Pt: Peritubular tunic. It: interstitial tissue. Staining: H-E.

**Figura 26.** Morfología testicular de *Phyllomedusa boliviana*. Vs: Vaso sanguíneo; Ce: Conducto eferente; Fibroblasto; Cm: células musculares; L: Lumen Tubular; Tp: Túnica peritubular. Ti: tejido intersticial. Tinción: H-E.

**Figure 27.** Testicular histology and spermatogenesis of *Phyllomedusa boliviana*. L: Tubule Lumen; Sc: Sertoli cell; Sf: Spermatozoa flagellum; Sn: Spermatozoa nucleus. Staining: H-E.

**Figura 27.** Histología testicular y espermatogénesis de *Phyllomedusa boliviana*. L: Lumen Tubular; CS: Célula de Sertoli; Fe: Flagelo del espermatozoide; Ne: Núcleo del espermatozoide. Tinción: H-E.

**Figure 28.** Histological characterisation and spermatogenesis of Craugastoridae species. Photomicrographs showing the peritubular tunic in detail in *Oreobates discoidalis*. Fi: Fibroblast; It: interstitial tissue; L: Tubule Lumen; Pt: Peritubular tunic; S: Spermatozoa. Staining: H-E.

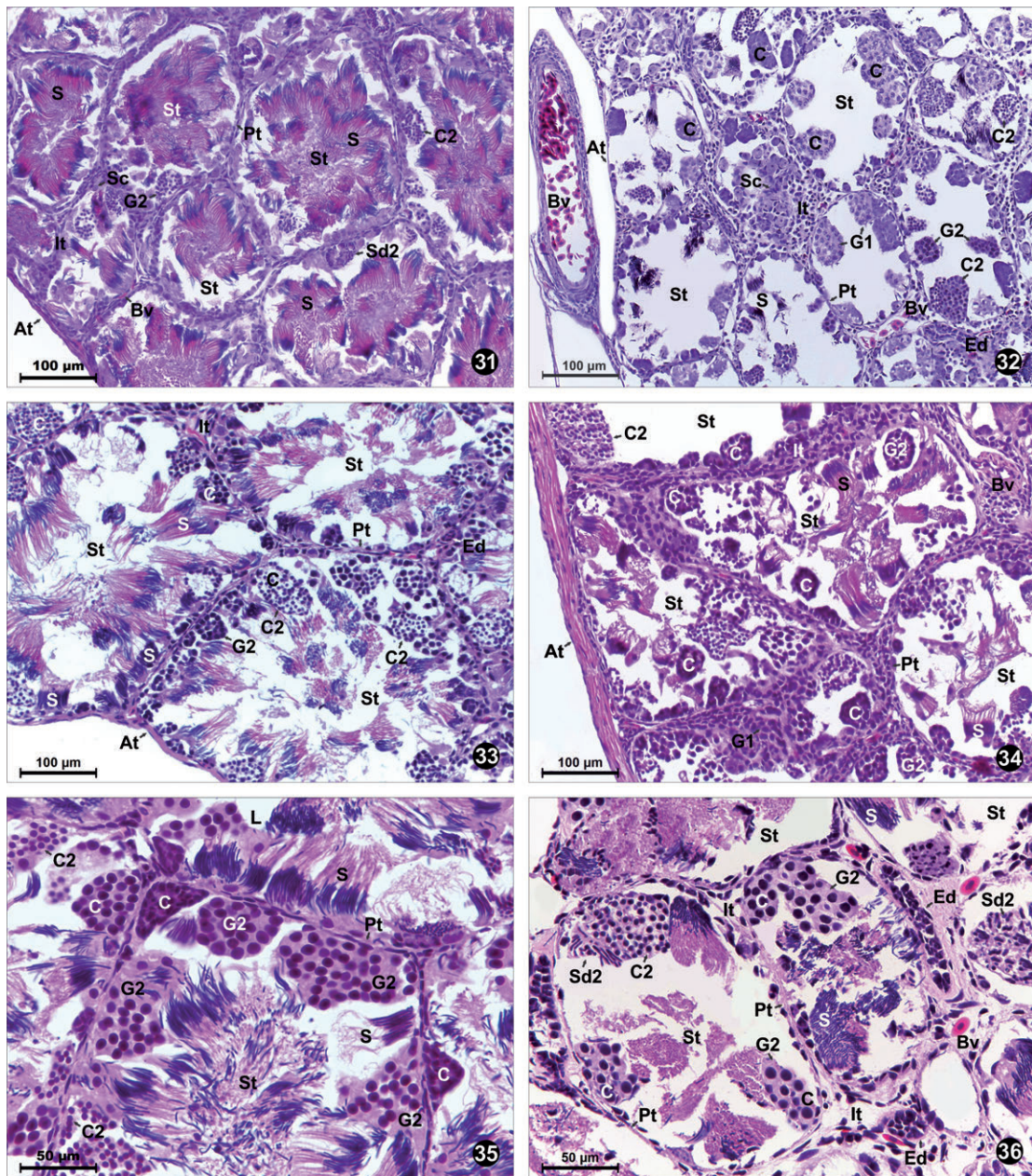
**Figura 28.** Caracterización histológica y espermatogénesis de especies de Craugastoridae. Microfotografías que muestran en detalle la túnica peritubular en *Oreobates discoidalis*. Fi: fibroblasto; It: tejido intersticial; L: lumen tubular; Pt: túnica peritubular; S: espermatozoides. Tinción: H-E.

**Figure 29.** Testicular histology and spermatogenesis of *Oreobates discoidalis*. Bv: Blood vessel; E: erythrocyte; Fi: Fibroblast; It: interstitial tissue; L: Tubule Lumen; Lc: Leydig cell; Pt: Peritubular tunic; S: Spermatozoa. Staining: H-E.

**Figura 29.** Histología testicular y espermatogénesis de *Oreobates discoidalis*. Vs: Vaso sanguíneo; Er: Eritrocito; Fi: Fibroblasto; Ti: Tejido intersticial; L: Lumen Tubular; CL: Célula de Leydig; Tp: Túnica peritubular; E: Espermatozoides. Tinción: H-E.

**Figure 30.** Testicular histology and spermatogenesis of *Oreobates discoidalis*. Photomicrographs showing the spermatozoa in detail. Pt: Peritubular tunic; S: Spermatozoa; Sc: Sertoli cell; Sf: Spermatozoa flagellum; Sn: Spermatozoa nucleus. Staining: H-E.

**Figura 30.** Histología testicular y espermatogénesis de *Oreobates discoidalis*. Microfotografías que muestran los espermatozoides en detalle. Tp: Túnica peritubular; E: Espermatozoides; CS: Célula de Sertoli; Fe: Flagelo del espermatozoide; Ne: Núcleo del espermatozoide. Tinción: H-E.



**Figure 31.** Testicular histology and spermatogenesis of *Leptodactylus bufonius*. At: Albuginea tunic; Bv: Blood vessel; C2: Secondary Spermatocyte; G2: Secondary Spermatogonia; It: Interstitial tissue; Pt: Peritubular tunic, S: Spermatozoa; Sc: Sertoli cell; St: Seminiferous tubules; Sd2: Secondary Spermatid. Staining: Hematoxylin–Eosin (H-E).

**Figura 31.** Histología testicular y espermatogénesis de *Leptodactylus bufonius*. Ta: Túnica albugínea; Vs: Vaso sanguíneo; Ec2: Espermatocito Secundario; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; Tp: Túnica peritubular; CS: Célula de Sertoli; Ts: Túbulos seminíferos; Ed2: Espermátida Secundaria. Tinción: Hematoxilina-eosina (H-E).

**Figure 32.** Testicular histology and spermatogenesis of *Leptodactylus fuscus*. At: Albuginea tunic; Bv: Blood vessel; C: Cysts; C2: Secondary Spermatocyte; Ed: Efferent duct; G1: Primary Spermatogonia; G2: Secondary Spermatogonia; It: Interstitial tissue; Pt: Peritubular tunic, S: Spermatozoa; Sc: Sertoli cell; St: Seminiferous tubules. Staining: H-E.

**Figura 32.** Histología testicular y espermatogénesis de *Leptodactylus fuscus*. Ta: Túnica albugínea; Vs: Vaso sanguíneo; C: Cistos; Ec2: Espermatocito Secundario; Ce: Conducto eferente; Eg1: Espermatogonias Primarias; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; Tp: Túnica peritubular; E: Espermatozoide; CS: Célula de Sertoli; Ts: Túbulos seminíferos. Tinción: H-E.

**Figure 33.** Testicular histology and spermatogenesis of *Pithecopus azureus*. At: Albuginea tunic; C: Cysts; C2: Secondary Spermatocyte; Ed: Efferent duct; G2: Secondary Spermatogonia; It: Interstitial tissue; Pt: Peritubular tunic, S: Spermatozoa; St: Seminiferous tubules. Staining: H-E.

**Figura 33.** Histología testicular y espermatogénesis de *Pithecopus azureus*. Ta: Túnica albugínea; C: Cistos; Ec2: Espermatocito Secundario; Ce: Conducto eferente; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; Tp: Túnica peritubular; E: Espermatozoides; Ts: Túbulos seminíferos; Tinción: H-E.

**Figure 34.** Testicular histology and spermatogenesis of *Phyllomedusa boliviana*. At: Albuginea tunic; It: interstitial tissue; Bv: Blood vessel; C: Cysts; C2: Secondary Spermatocyte; G1: Primary Spermatogonia; G2: Secondary Spermatogonia; It: Interstitial tissue; Pt: Peritubular tunic, S: Spermatozoa; St: Seminiferous tubules. Staining: H-E.

**Figura 34.** Histología testicular y espermatogénesis de *Phyllomedusa boliviana*. Ta: Túnica albugínea; Ti: Tejido intersticial; Vs: Vaso sanguíneo; C: Cistos; Ec2: Espermatocito Secundario; Eg1: Espermatogonias Primarias; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; Tp: Túnica peritubular; E: Espermatozoides; Ts: Túbulos seminíferos. Tinción: H-E.

**Figure 35.** Testicular histology and spermatogenesis of *Phyllomedusa sauvagii*. C: Cysts; C2: Secondary Spermatocyte; G2: Secondary Spermatogonia; L: Tubule Lumen, Pt: Peritubular tunic, S: Spermatozoa; St: Seminiferous tubules. Staining: H-E.

**Figura 35.** Histología testicular y espermatogénesis de *Phyllomedusa sauvagii*. C: Cistos; Ec2: Espermatocito Secundario; Eg2: Espermatogonias Secundarias; L: Lumen Tubular; Tp: Túnica peritubular; E: espermatozoides; Ts: Túbulos seminíferos. Tinción: H-E.

**Figure 36.** Testicular histology and spermatogenesis of *Oreobates discoidalis*. Bv: Blood vessel; C: Cysts; C2: Secondary Spermatocyte; Ed: Efferent duct, G2: Secondary Spermatogonia; It: Interstitial tissue; Pt: Peritubular tunic, S: Spermatozoa; Sd2: Secondary Spermatid, St: Seminiferous tubules. Staining: H-E.

**Figura 36.** Histología testicular y espermatogénesis de *Oreobates discoidalis*. Vs: Vaso sanguíneo; C: Cistos; Ec2: Espermatocito Secundario; Ce: Conducto eferente; Eg2: Espermatogonias Secundarias; Ti: Tejido intersticial; Tp: Túnica peritubular; E: Espermatozoides; Ed2: Espermátida Secundaria; Ts: Túbulos seminíferos. Tinción: H-E.

## DISCUSSION

In the present study, we describe the histomorphology of the testes of *Leptodactylus latinasus*. We also identify morphological traits of the testes that vary among species of different families with egg deposition out of the water, and with three different reproductive modes. Certain specific characters do not differ at the family level, but they do at the species level. However, the observed variations suggest that the hypothesis of a relationship between these features and phylogeny needs to be confirmed by future phylogenetic comparative analyses.

Despite the wide diversity of the genus *Leptodactylus* (75 spp., Frost, 2020), only the histological aspects of the spermatozoa of *Leptodactylus ocellatus* (Rosembliit, Pozzi, and Ceballos, 2006), *Leptodactylus labyrinthicus* (Prado, Abdala, Silva, and Zina, 2004), *Leptodactylus podicipinus* (Ferreira, dos Santos Rosa, and Mehanna, 2009), and *Leptodactylus chaquensis* (Ferreira et al., 2009; Iruzubieta Villagra et al., 2012) have been analysed. In *Leptodactylus latinasus*, males build chambers with their snouts, where amplexus takes place, the construction of the foam nest occurs, and the earlier larval stages develop, even before the first rains (Gallardo, 1958, 1964). The larvae complete their development in temporal puddles. This behaviour would be an advantage for this species over others that develop in the same water body since at the beginning of the rainy season *L. latinasus* larvae are already in more advanced stages of development (Heyer, 1969). This particular breeding behaviour and the capacity of the tadpoles to generate their own foam allow the species to survive in the case of prolonged drought periods (Downie, 1984, 1989, 1990, 1994; Caldwell and Lopez, 1989; Downie and Smith, 2003; Ponssa and Barrionuevo, 2008).

The presence of testicles with many cysts in apoptosis found in *L. latinasus* specimens could be related to the climatic conditions of the sampled year, which was especially dry (4/12/2008, Lules, Tucumán). Thus, the temporary water bodies might have been absent, or soil moisture might not have been optimal for the construction of the nuptial chamber, where the amplexus and the first stage of larval development occur. Female specimens of *L. latinasus* collected during the drought months of the same year (2008) showed ovaries in the process of atresia (Pucci Alcaide et al., 2012). Alterations in the periods of activity and quiescence are related to climatic conditions (Rastogi, Lela, Saxena, Chieffi, 1976).

A remarkable characteristic was the large size of the testis in species with arboreal egg deposition. For example, the testis of the smallest Phyllomedusidae species studied (*Pithecopus azureus* SVL =  $38.64 \pm 0.96$  cm), were larger than those of the largest Leptodactylidae species (*Leptodactylus fuscus* SVL =  $99.99 \pm 2.58$ ). This feature does not seem to be related to terrestrial breeding, but it is rather considered as an excellent example of adaptation to spermatozoa competition (Emerson, 1997; Ramm and Scharer, 2014). Many species with multiple breeding have relatively larger testes compared to other species without this behaviour (Kusano, Toda, and Fukuyama, 1991; Jennions and Passmore, 1993; Emerson, 1997; Prado and Haddad, 2003). To be more precise, this behaviour was reported in *P. Azureus* (Dias, Maragno, Prado, and Cechin, 2012). This relationship was also observed in other *Rhacophoridae* arboreal species, e.g. *Rhacophorus arboreus* (Kusano et al., 1991)

and *Chiromantis xerampelina* (Jennions and Passmoren, 1993), but also in species of the genus *Leptodactylus*, *L. chaquensis* and *L. podicipinus* (Prado and Haddad, 2003). Other ways in which testicles can regulate spermatozoa production are independent of testicle size, such as increases in the proportion of spermatogenic tissue (Ramm and Scharer, 2014), or testis morphology (Leite *et al.*, 2015).

The thickness and number of layers of the albuginea tunic revealed a significant variation among the studied species, from a thin tunic composed by one layer in *Oreobates discoidalis*, to being composed of six or more layers in *Phyllomedusa boliviana*. The more noticeable number of layers corresponded to *Phyllomedusa boliviana* and *P. sawagii*, which exhibited approximately five and two times the maximum value of that of other studied species, respectively. Differences in the thickness of the albuginea tunic have been reported in Hylodids and Leiuperines (Leite *et al.*, 2015), and in *Eleutherodactylus* species (Rodríguez Gómez, Sanz Ochotorena, Segura Valdés, Martínez, and Jiménez García, 2012). The latter authors proposed that these variations might indicate that species with more terrestrial habits tend to be more protective of their seminiferous tissue from solar radiation and other physical aggressions, compared to those inhabiting areas closer to water. In *Phyllomedusa boliviana* and *P. sawagii*, the positive relationship between the size of the testicle and the increase of the albuginea tunic could be associated with the need for protecting and supporting larger testes and a greater number of seminiferous tubules. That relationship could also be related to an increase in the smooth muscle cells, which would be necessary for the compression of the testicle, and the movement of the spermatozoa from the seminiferous tubules to the efferent ducts. Moreover, there is evidence supporting the fact that these parameters can be adjusted according to environmental conditions (Rammand Scharer, 2014).

A previously analysed characteristic in the tissues here studied is the presence of pigmentation. A common state of character among the species studied here is the lack of testicular pigmentation (e.g., the presence of milky white testes), which is a usual trait in anurans (Oliveira and Zieri, 2005, Asenjo *et al.*, 2011; Duellman and Trueb, 1986). The absence of pigmented cells has been reported in several leptodactylid species (Franco-Belucci, Zieri, de Souza Santos, Moresco, Oliveira, 2009), and a weak pigmentation has only been observed in *Leptodactylus furnarius* and *Adenomera bokermanni* (as *Leptodactylus bokermanni*) (Franco-Belussi *et al.*, 2009). Testicular pigmentation has also been described in Leiuperidae (Aoki, Vitale-Calpe, and Pisano, 1969; Carezzano and Cabrera, 2010; Oliveira and Zieri, 2005; Oliveira *et al.*, 2002, 2003; Franco-Belussi *et al.*, 2009), and in species of *Colestethus* (Grant, 2004). This extracutaneous pigmentary system still exerts an unknown function (Zuasti, Jimenez-Cervantes, García-Barrón and Ferrer, 1998; Franco-Belussi *et al.*, 2009), although it has been suggested to be linked to spermatogenesis thermoregulation (Guillette, Weigeland Flater, 1983), to the protection of sperm from oxidative stress (Almbro, Dowling and Simmons, 2011), or to the amount of pre-mating sexual investment (Rammand Scharer, 2014). Our data do not support this last hypothesis, since the investment of *Leptodactylus* species of the *fuscus* group and *Phyllomedusa* species in the construction of nests of mud and leaves, respectively, occurs before mating and they do not present testicular pigmentation.

The cells of the spermatic lineage of the studied species have similar characteristics as those observed in other species (e.g. Oliveira *et al.*, 2002, Jamieson *et al.*, 1993, Oliveira *et al.*, 2005, Carrezano *et al.*, 2010, Iruzubieta Villagra, Ramos, Cisint, Crespo, and Fernández, 2017). Among the species studied, differences in the abundance of cysts during the spermatogenesis process would be related to the reproductive moment in which the studied specimens are found. The early onset of thyroid development appears to be related to direct developmental evolution in anuran amphibians (Goldberg J., Taucce Pedro P.G., Quinzio Silvia Ines, Haddad Celio F.B. and Vera Candiotti Florencia, 2020). The shape of sperm nuclei distinguishes the samples of the studied families. The spermatozoal nucleus contains DNA and nuclear proteins, and its shape might be determined by the DNA-protein association patterns (Báo, Dalton, and Oliveira, 1991). The nucleus occupies a significant part of the head, and its shape ultimately determines the shape of the spermatozoa head. In order to guarantee fertilisation, several factors are involved, including environmental conditions, cell-cell interactions, gene expression, and phenotypic spermatozoa traits (Zeng, Ling Lou, Bo Liauo, and Jehle, 2014; García Vázquez, Gadea, Matás, and Holt, 2016); thus, head morphology and tail size would influence the selection of the spermatozoa that reach the fertilisation place. In anurans, the nuclear morphology is quite variable (Zeng *et al.*, 2014), and, in this study, we recorded three morphological patterns: arrow (*Leptodactylidae*); claw (*Phyllomedusidae*), and filamentous (*Craugastoridae*). Other described shapes include a pointed head in *Ranidae*, and a spindle-shaped head in *Bombina bombina* (as *Bombina torigneus*) (Blüm, 2012). In mammals, the influence of the shape of the spermatozoa heads over hydrodynamics was found to be considerable (Gomendio and Roldan, 2008; Firman and Simmons, 2010). Further, several studies have evaluated the relationship between spermatozoa morphology and fertility, which varies intraspecifically; thus, it is not possible to propose a single cause of the observed differences between mammal species (García Vazquez *et al.*, 2016). The site where spermatozoa compete is apparently related to interspecific differences (Gomendio and Roldan, 2008). In animals with internal fertilisation, spermatozoa face different physical barriers and undergo complex interactions (García Vazquez *et al.*, 2016). Within the female reproductive tract, mammalian spermatozoa are exposed to more complex influencing factors than fish spermatozoa in water, since the spermatozoa must swim along epithelial surfaces (Nosrati, Driouchi, Yip, and Sinton, 2015). Anurans select environmental conditions that maximise mating success (Anssi, 2006; Indermaur, Schaub, Jokela, Tockner, and Schmidt, 2010).

The three reproductive modes exhibited by the studied species are related to different fertilisation environments: open-air in *Phyllomedusidae* (Ihering, 1886); foam nests produced by female oviductal secretions and deposits in incubation chambers of mud on land in *Leptodactylus* of the *L. fuscus* group (Heyer, 1978); and direct development in *Oreobates discoidalis* (Akmentis, 2011; Goldberg, J., Vera Candiotti, F. and Akmentins, M. S., 2012; Vitt and Caldwell, 2013). These three reproductive modes showed three different sperm head morphologies, which is probably an adaptive advantage that favours fertilisation.

The ratio between head and tail length might be a good predictor of the swimming speed of the spermatozoa (Gomendio and Roldan, 2008). The variation in spermatozoa length has been related to spermatozoa swimming speed since it has been hypothesised that spermatozoa competition among species can favour longer spermatozoa with higher swimming speed (Zeng *et al.*, 2014). This hypothesis is both supported by many authors (Blengini, Naretto, Cardozo, Giojalas, and Chiaraviglio, 2014; Ross-Santaella, Pintus, and Garde, 2015; Simpson, Humphries, Evans, Simmons, and Fitzpatrick, 2013), and rejected by empirical evidence (Hosken, 1997; Anderson and Dixon, 2002; Firman and Simmons, 2010).

In our study, we found differences in this ratio: species with arboreal spawning locations (Phyllomedusidae) showed longer spermatozoa tails than those with terrestrial (*Leptodactylus* of the group *L. fuscus*), or direct development (*Oreobates discoidalis*) (Goldberg *et al.*, 2020). These results partially agree with those found in Chinese frogs, with terrestrial-arboreal spawning species showing longer spermatozoa than species with aquatic spawning (Zeng *et al.*, 2014). The relationship between spermatozoa morphology (spermatozoa head and flagellum length ratio) and swimming speed would be influenced by the fertilisation mode (Simpson *et al.*, 2013).

In the female reproductive tract, the physics of the viscoelastic fluid motion would act on the performance, since the elasticity of the medium implies forces that affect spermatozoa movement and that are not present in water (Lauga, 2007).

In *Oreobates discoidalis*, the spermatozoa exhibited the longest flagella; however, the flagellum length to head length ratio in this species was similar to that found in species with terrestrial-external fertilisation, and it was smaller than that of species with aerial-external fertilisation. These characters could have a relationship with putting mode out of the water.

In species with external fertilisation, more subtle aspects should be taken into account as responsible for the variations in the relationships between spermatozoa size and speed, e.g. anuran oocytes are covered by an extracellular matrix of oviductal secretions forming a variable number of layers of gelatinous aspect (Alcaide *et al.*, 2009) through which spermatozoa must swim and penetrate (Reinhart, Ridgway, and Chandler, 1998). Consequently, in anurans, the external environment might be different from that of fishes or molluscs (Simpson *et al.*, 2013), and even among species, since the egg glandular aggregations differ in species with different oviposition modes (Alcaide *et al.*, 2009). That highlights the importance of analysing the anuran urogenital system in a context of spermatozoa morphological coevolution and female reproductive tract morphology.

The most outstanding characters were found in species with egg deposition in trees (family Phyllomedusidae). Some of the described features can be related to species deposition modes which imply different environmental conditions, e.g. the thickness of albuginea tunic with arboreal breeding, alterations in spermatogenesis development in adverse environmental conditions, variations in spermatozoa shape within fertilisation environments, the ratio between head and tail length with terrestrial or external fertilisation.

Anurans are an excellent group to test specialisations of the urogenital tract in relation to phylogeny or adaptation since they present species with both internal



and external fertilisation and the greatest variation of reproductive modes among vertebrates. The histomorphological study of the testicles of the species studied in this work allowed us to establish particularities in gonadal morphology and morphological-physiological characteristics of male gametes related to the ways that deposit eggs out of water. In turn, it provides information to understand the success of such a diversity of reproductive modes in the evolutionary history of the group.

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### PARTICIPATION

The authors A. Pucci Alcaide and F. Pucci Alcaide planned, designed, and executed the experimental work and A. Pucci Alcaide, F. Pucci Alcaide, A.A. Michel, M.L. Ponssa conducted data analyses and wrote the manuscript.

### CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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