Zoologischer Anzeiger 257 (2015) 29–38

Contents lists available at ScienceDirect

Zoologischer Anzeiger

journal homepage: www.elsevier.com/locate/jcz

Zoologischer Anzeiger

Comparative testis morphology of Neotropical anurans

Gabriela Baroni Leite[a,*], Lilian Franco-Belussi[b], Diogo B. Provete[c,1], Classius de Oliveira[b]

[a] Graduate Program in Animal Biology, Department of Biology, São Paulo State University (UNESP), 15054-000, São José do Rio Preto, São Paulo, Brazil
[b] Department of Biology, São Paulo State University (UNESP), 15054-000, São José do Rio Preto, São Paulo, Brazil
[c] Graduate Program in Ecology and Evolution, Department of Ecology, Institute of Biological Sciences, Federal University of Goiás, Goiânia 74001-970, Goiás, Brazil

ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 16 April 2015
Accepted 24 April 2015
Available online 25 April 2015

Keywords:
Reproductive biology
Evolutionary morphology
Hylodidae
Leptodactylidae
Leiuperinae
Urogenital morphology

ABSTRACT

Anuran testes have generally similar germinative and somatic anatomical components, but their morphology varies among species groups. However, little is known about how environmental and evolutionary factors influence testis shape and sperm quantity. Here, we tested the hypothesis that the arrangement of both germinal compartment (seminiferous locules containing germ cells) and somatical compartment (interstitial fibers and somatic cells) differ between species with distinct reproductive modes and breeding activities. We compared the testes of eight species from two families of Neobatrachian anurans: *Physalaemus centralis*, *Physalaemus cuvieri*, *Physalaemus marmoratus*, *Physalaemus olfersii*, *Pseudopaludicola* sp. (aff. *murundu*) (Leptodactylidae: Leiuperinae), *Crossodactylus caramaschii*, *Hylodes cardosoi*, and *Hylodes sazimai* (Hylodidae). We built a 3D model of the testis to visualize the morphology and arrangement of seminiferous locules. We measured locular diameter and area, thickness of reticular fiber bundles, and area of both collagen fibers and sperm bundles. Hylodids had the highest values for most characters, except for sperm and reticular fiber bundles. For example, their locular diameter was 50%, and locular area 20% larger than leiuperines, which usually had thicker reticular fibers bundles. These fibers provide greater stability to the large quantity of small, spherical seminiferous locules found in leiuperines. Conversely, hylodids had larger area of collagen fibers, which provides greater flexibility and resistance to a few large and flattened seminiferous loci. Our results can shed light on how testicular morphology is influenced by evolutionary processes and ecological strategies, such as breeding seasonality and reproductive modes in anurans.

1. Introduction

Amphibians have the greatest diversity of reproductive modes among tetrapods (Duellman and Trueb, 1994; Wells, 2007). Morphological characteristics of the urogenital system in amphibians are related to these reproductive modes, including internal fertilization and viviparity. These characteristics make them excellent models for comparative studies on the evolution of vertebrate reproduction (Kühnel et al., 2010). Testes architecture is one of the most important features in the evolution of male reproductive characters, since their reproductive success is correlated to the number, morphology, and maturation time of sperm (reviewed by Ramm and Scharer, 2014). As a result, testes are subjected to natural selection pressures, which may influence the development of testicular characteristics (Ramm and Scharer, 2014).

Anuran testes are paired, smooth, rounded, and compact structures, usually yellowish, and smaller than the ovaries of the same species. However, they are usually asymmetric in size, independently of age and health condition (Duellman and Trueb, 1994; Liu et al., 2011; Zhou et al., 2011; Mi et al., 2012) and position in abdominal cavity (Oliveira and Vicentini, 1998). Additionally, melanocytes have been reported on the tunica albuginea of testes in some species (reviewed in Oliveira and Franco-Belussi, 2012), and their presence seems to exhibit phylogenetic signal in Neotropical anuran lineages (Provete et al., 2012). In contrast to anurans, the testes of caudates and caecilians have lobular segmentation, whose dimensions vary strongly among families (Wake, 1968; Ingersol et al., 1991).

The anuran testis is covered by a thin tunica albuginea that is rich in collagen fibers, but lack septa dividing the parenchyma (Oliveira and Vicentini, 1998). They include seminiferous locules surrounded by an elastic fibrous layer that delimits the interlocular

* Corresponding author at: Departamento de Biologia, Instituto de Biociências, Letras, e Ciências Exatas, Universidade Estadual Paulista, Rua Cristóvão Colombo, 2265, São José do Rio Preto, 15054-000, São Paulo, Brazil. Tel.: +55 1732212387.
E-mail addresses: gabriela.bleite@gmail.com (G.B. Leite), lilian.belussi@gmail.com (L. Franco-Belussi), dbprovete@gmail.com (D.B. Provete), classius@ibilce.unesp.br (C. de Oliveira).
[1] Present address: CNPq post-doctoral fellow, Department of Biological and Environmental Sciences, University of Gothenburg, SE 405 30, Göteborg, Sweden.

http://dx.doi.org/10.1016/j.jcz.2015.04.005
0044-5231/

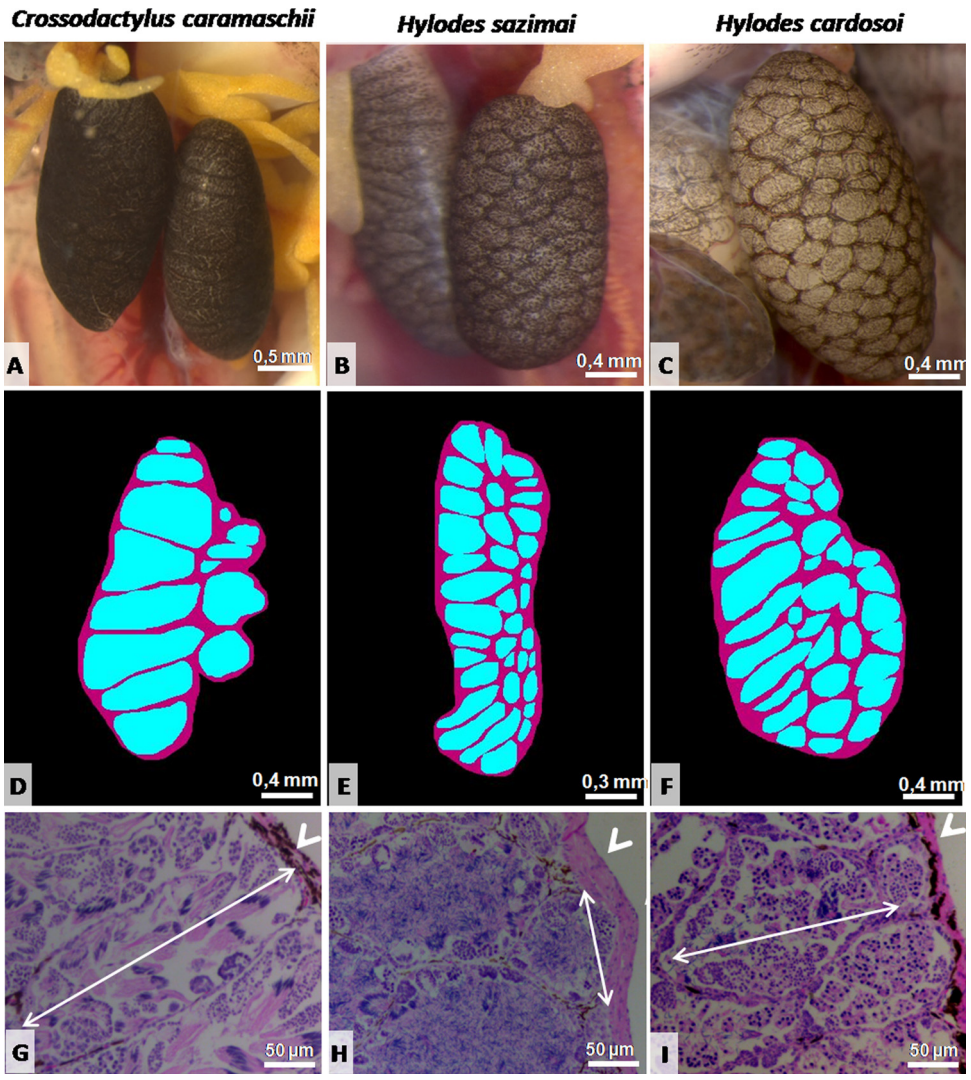

Fig. 1. Testicular morphology of *Crossodactylus caramaschii*, *Hylodes sazimai*, and *H. cardosoi*, showing the organ's surface pigmented (A, B, C) and location of seminiferous locules (D, E, F). The photomicrograph is showing differences in the thickness of the tunica albuginea (arrowhead) and in the locular diameters (double arrow) between species (G, H, I). Staining: Hematoxylin/Eosin (G, H, I).

area (interstitial region), which is formed by connective tissue (Lofts, 1976; Oliveira and Vicentini, 1998). This interstitial region contains fibroblasts, blood vessels, efferent ductules, and testicular melanocytes, giving a dark coloration to the testis of some species (Oliveira et al., 2002, 2003; Oliveira and Franco-Belussi, 2012).

Germ cells in the seminiferous locules are supported by Sertoli cells, forming spermatocysts. Each cyst contains cells at the same spermatogenetic stage, establishing a synchrony in their development, a common characteristic of amphibians (Cavicchia and Moviglia, 1983; Rastogi et al., 1988; Báo et al., 1991; Santos and Oliveira, 2008). However, internal characteristics of testicular components greatly vary interspecifically, e.g., sperm production increases with testis size, improving reproductive success (Emerson, 1997). Also, there is a strong relationship between sperm morphology and testis size, suggesting that sperm competition has influenced not only sperm (Zeng et al., 2014), but also testis morphology. Previous studies have focused on the causes and consequences of variation in testis size in amphibians (reviewed in Ram and Scharer, 2014). However, little is known about how differences in the histology of germinative (e.g., seminiferous locules) and somatic components (e.g., arrangement of fibers that support the organ) are influenced by evolutionary and environmental factors. Thus, a comparative analysis could help to understand not only how testis morphology evolved, but also the mechanisms responsible for changes in some of its structures. Some aspects of testicular pigmentation have been analysed from the perspective of phylogenetic systematics (e.g., Grant et al., 2006) and phenotypic evolution (e.g., Provete et al., 2012). Thus, comparative data on locule and fiber arrangement may also provide new insights into how testis anatomy has evolved in anurans.

Leptodactylidae (sensu Frost, 2014) is an exclusive Neotropical family of anurans. Most leptodactylids build foam nests into which they deposit eggs and where the initial tadpole development takes place (Haddad and Prado, 2005). Conversely, hylodids usually lay eggs inside constructed subaquatic chambers in montane streams in the Brazilian Atlantic Forest (Haddad and Prado, 2005; Wells, 2007). It seems that foam nests is a plesiomorphic character, while eggs in subaquatic chambers are more derived (Gomez-Maestre et al., 2012). It is thought that placing eggs in foam nests is a strategy to decrease water dependency, while species that lay eggs directly in the water depend on high humidity (Gomez-Mestre et al., 2012). Also, hylodids usually have continuous reproduction, whereas most of leptodactylids are explosive breeders. Therefore, these groups have contrasting traits that make them useful to understand how testicular morphology is correlated with reproductive modes, breeding activity, and environmental characteristics. Specifically,

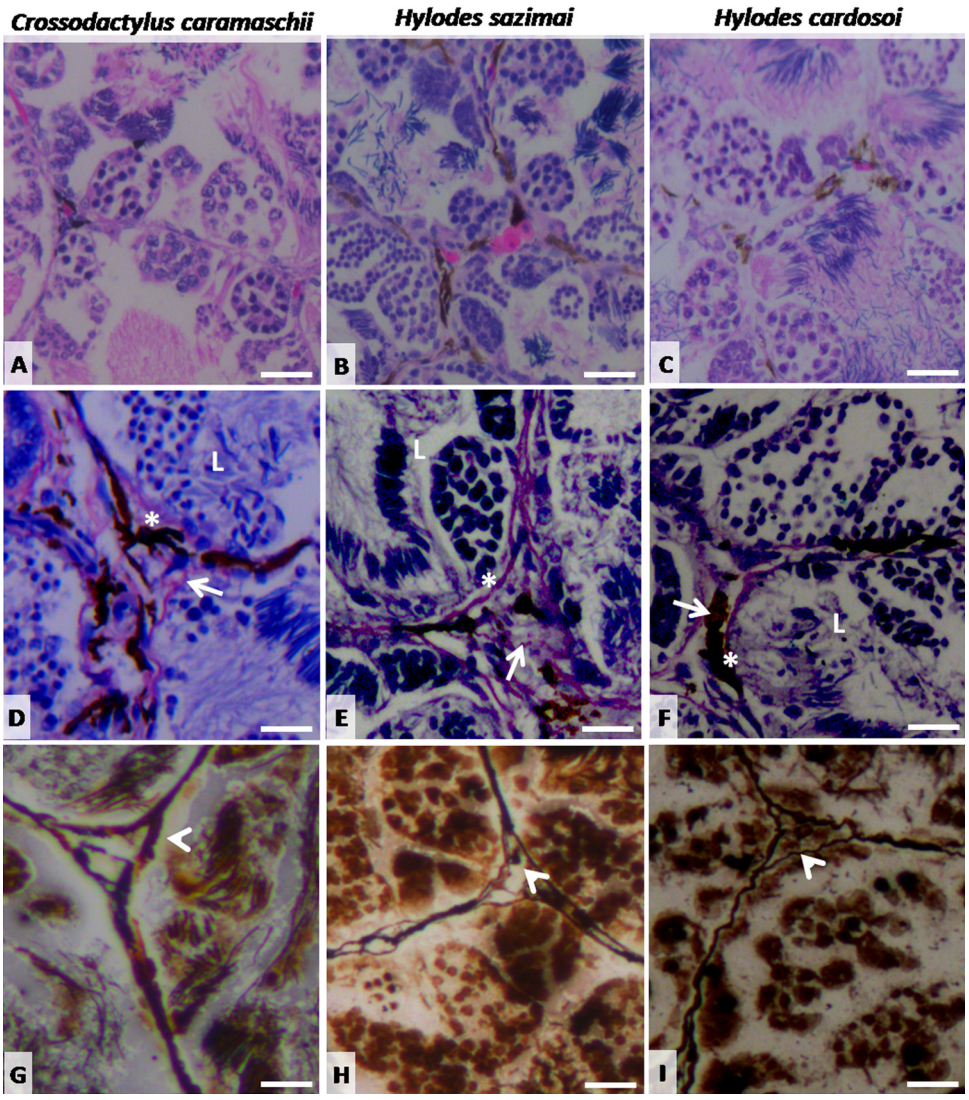

Fig. 2. Histological characterization of the testes of *Crossodactylus caramaschii*, *Hylodes sazimai*, and *H. cardosoi*, showing the locular area (lines A, B, C), arrangement of collagen fibers (arrow), testicular melonocytes (*) (D, E, F), and reticular fibers beam (arrowhead) in the interstitium (G, H, I). L: seminiferous locule. Staining: Hematoxylin/Eosin (A, B, C), Picrossirius (D, E, F), and Gömöri's reticulin (G, H, I). Bars = 25 μm.

we expect that the arrangement of both testicular compartments will differ between species of these families.

Here, we compared the testes of eight species: *Physalaemus centralis* Bokermann, 1962, *Physalaemus cuvieri* Fitzinger, 1826, *Physalaemus marmoratus* (Reinhardt and Lütken, 1862), *Physalaemus olfersii* (Lichtenstein and Martens, 1856), *Pseudopaludicola* sp. (aff. *murundu*) (Leptodactylidae: Leiuperinae), *Crossodactylus caramaschii* Bastos and Pombal, 1995, *Hylodes cardosoi* Lingnau et al., 2008, and *Hylodes sazimai* Haddad and Pombal, 1995 (Hylodidae). We used histochemical analysis to characterize the network of testicular fibers. In addition, we built three-dimensional models of the testes to compare the shape of seminiferous locules and the morphometry of sperm bundles and locule diameters.

2. Materials and methods

2.1. Specimen sampling

We examined five male specimens of each species collected during the reproductive period (November 2006–January 2007) and deposited in the herpetological collection of our department. The choice to only examine specimens during this period was based on Santos et al. (2011), who showed that the size of seminiferous locules of a hylid did not vary throughout the annual cycle. Voucher specimens are housed in the collection of the Laboratório de Anatomia Comparada, Departamento de Biologia, State University of São Paulo, São José do Rio Preto, Brazil (see Appendix S1 of Supplementary material).

2.2. Histological analyses

One testis from each male was submitted to routine histological procedures and embedded in Histosec (Merk, Darmstadt, Germany®) to quantify the area of collagen fibers. The other testis was embedded in historesin (Leica®) to quantify the area of sperm bundle, build three-dimensional models, and measure the thickness of reticular fibers bundles, locular area and diameter. Only sperm arranged in a bundle were quantified, that is, the gamete in the final maturation phase inside a cyst. Sections of 2 μm for historesin and 4 μm for paraffin were mounted on slides and stained with Hematoxilyn/Eosin, Gömöri's Reticulin, and Picrossirius. Images were captured with an optical microscope (Leica DM4000) coupled with an image capture system (Leica

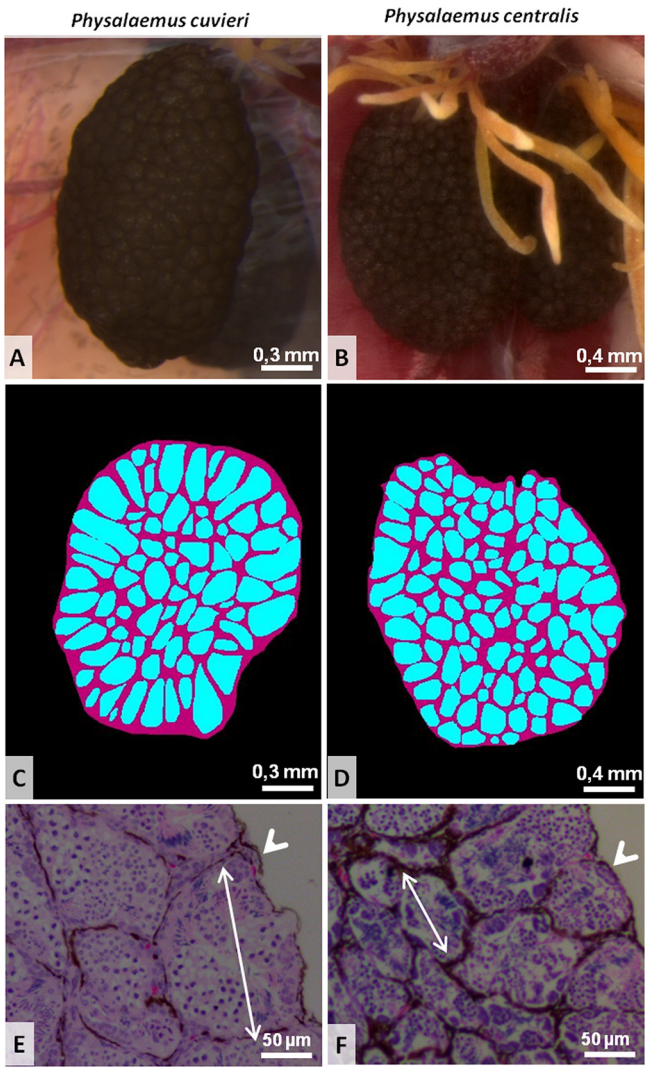

Fig. 3. Testicular morphology of *Physalaemus centralis* and *P. cuvieri* showing the surface of the organ pigmented (A, B) and arrangement of seminiferous locules (C, D). The photomicrograph of testis is showing the thickness of the tunica albuginea (arrowhead) and differences in locular diameter (double arrow) between species (E, F). Staining: Hematoxylin/Eosin (E, F).

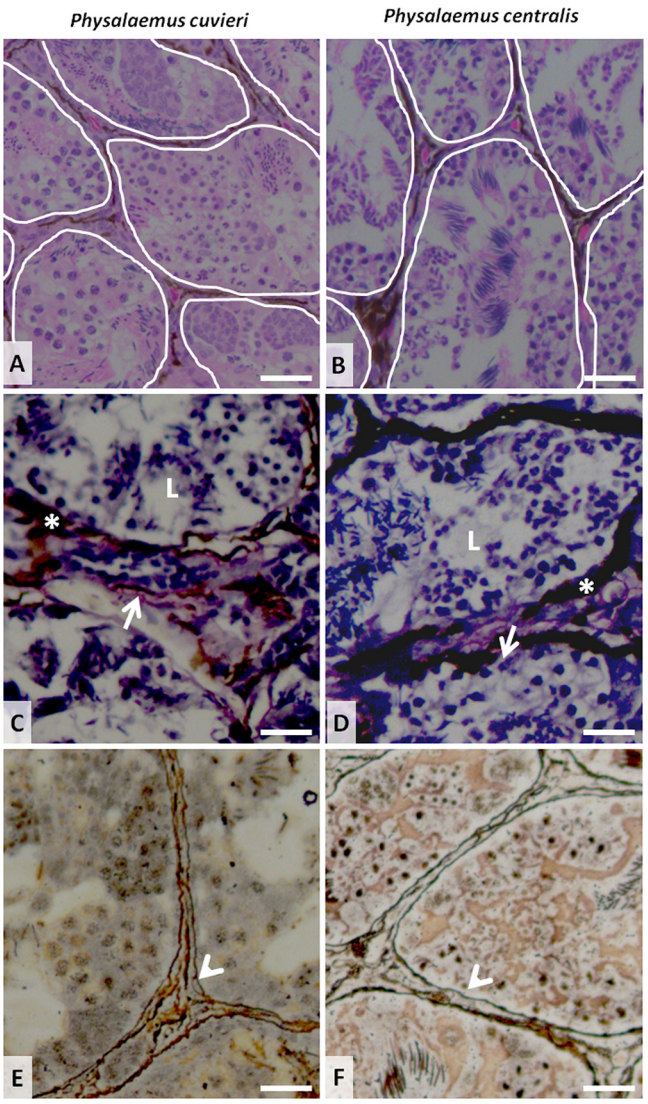

Fig. 4. Histological characterization of the testes of *Physalaemus cuvieri* and *P. centralis*, showing the locular area (lines A, B), arrangement of collagen fibers (arrow), testicular melonocytes (*, C, D), and reticular fibers bundle (arrowhead) in the interstitium (E, F). L: seminiferous locule. Staining: Hematoxylin/Eosin (A, B), Picrossirius (C, D), and Gömöri's reticulin (E, F). Bars = 25 µm.

DFC295), and analyzed in the Image-Pro Plus v.6.0 software (Media Cybernetics).

2.3. Morphometric analyses

For morphometric analysis, 25 histological fields of 25 sections chosen at random were analysed. Locular diameter was measured at 100× magnification and the other variables (collagen and reticular fibers, locular area, and sperm bundle) at 200× magnification. Interstitial area was obtained by calculating the difference between the total and locular areas.

To calculate the locular diameter, 100 measurements of the largest diameter of the seminiferous locules of each specimen for each species were made. The areas of sperm bundles, collagen fibers, and locules were measured in 25 fields randomly chosen. The thickness of reticular fibers was measured in 100 fields per animal.

For three-dimensional reconstruction, 100 sections for each animal (e.g., equivalent to half of the testicles) were stained with Hematoxilyn-Eosin and photographed (Leica MZ16) at either 25 or 50× depending on the size of the testis. Then, images were analysed in the program Reconstruct® (Fiala, 2005) to obtain a 360° view of the testis.

2.4. Statistical analyses

To test for differences in morphometric parameters between species of the two families, we used a phylo-MANOVA (Garland et al., 1993). Since this is a cross-species analysis, the phylogenetic relationship among the sampled species must be accounted for in order to correctly estimate the degrees of freedom and consequently *P*-value (Felsenstein, 1985; Nunn 2011). Then, a phylogeny with branch lengths for the eight species was pruned from the chronogram of Pyron and Wiens (2013). The best evolutionary model that described all traits was the Brownian motion (data not shown), thus there was no need to transform branch lengths for the phylogeny to reflect the evolutionary history of traits (Nunn, 2011). This analysis tested for a difference in all means of morphological variables between species of the two families (Text S1). Analysis was conducted in the Geiger package (Pennell et al., 2014) of the R software v.3.0.2 (R Core Team, 2014). Additionally, we tested for

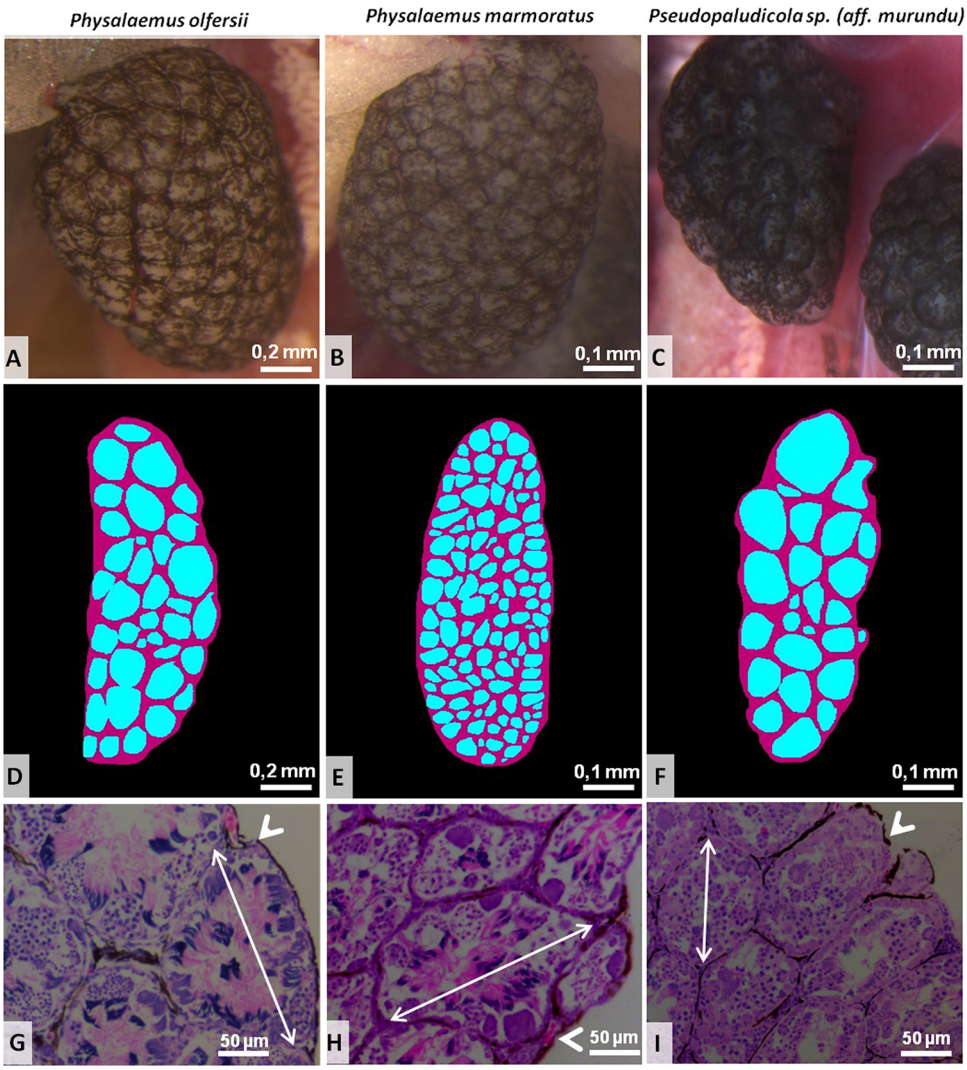

Fig. 5. Testicular morphology of *Physalaemus olfersii*, *P. marmoratus*, and *Pseudopaludicola* sp. (aff. *murundu*), showing the surface of the organ pigmented (A, B, C) and arrangement of the seminiferous loci (D, E, F). The photomicrograph is showing the thickness of the tunica albuginea (arrowhead) and differences in the locular diameters (double arrow) between species (G, H, I). Staining: Hematoxylin/Eosin (G, H, I).

differences between species of the two families in each morphological variable using a phylo-ANOVA (Garland et al., 1993), followed by post-hoc test with adjusted *P*-values (Benjamini and Yekutieli, 2001). This analysis used the same pruned tree from the previous one and was conducted in R package phytools (Revell, 2012).

3. Results

3.1. Gross anatomy

The testes of the species analysed were paired structures, asymmetric in size and located at the anterior portion of the kidneys, similarly to all anurans (Figs. 1A–C; 3A and B; 5A–C). Species of both families shared different degrees of pigmentation in the testes, due to testicular melanocytes in the cortex and in between seminiferous locules (Figs. 2D–F; 4C and D; 6D–F).

The shape, size, and number of seminiferous locules differed between families. Seminiferous locules were flat in hylodids, but rounded in leuperines (Fig. S1–8). For example, *C. caramaschii*, *H. sazimai*, and *H. cardosoi* have a few large locules (Fig. 1D–F), whereas leiuperines had many small locules (Figs. 3C and D; 5D–F). Furthermore, the shapes of seminiferous locules varied between species, being flattened in hylodids (Fig. 1D–F), but rounded in leiuperines (Figs. 3C and D; 5D–F).

3.2. Histology

The thickness of the tunica albuginea differed between species of both families, being thicker in hylodids (Fig. 1G–I) than in leiuperines (Figs. 3E and F; 5G–I). Furthermore, *C. caramaschii* (Fig. 1G) and *H. cardosoi* (Fig. 1I) had melanocytes in the tunica, while they occurred only between seminiferous locules in the interstitium in *H. sazimai* (Fig. 1H).

Testes were composed of seminiferous locules and germ cells grouped in cysts in all species. The size and morphology of seminiferous locules (Figs. 1D and F; 3C and D; 5D–F), and the largest diameter of the testes also differed between species (Figs. 1G–I; 3E and F; 5G–I). The interstitium comprised the tissue between locules and had a large amount of somatic and immune cells (Figs. 1–7). Collagenous (Figs. 2D–F; 4C and D; 6D–F) and reticular fibers (Figs. 2G–I; 4E and F; 6G–I) also occurred in this region, along with melanocytes, which rendered the organ black.

The quantity of sperm in seminiferous locules was different between species (Fig. 7). However, this character did not show a clear phylogenetic pattern, differing at the species level instead of

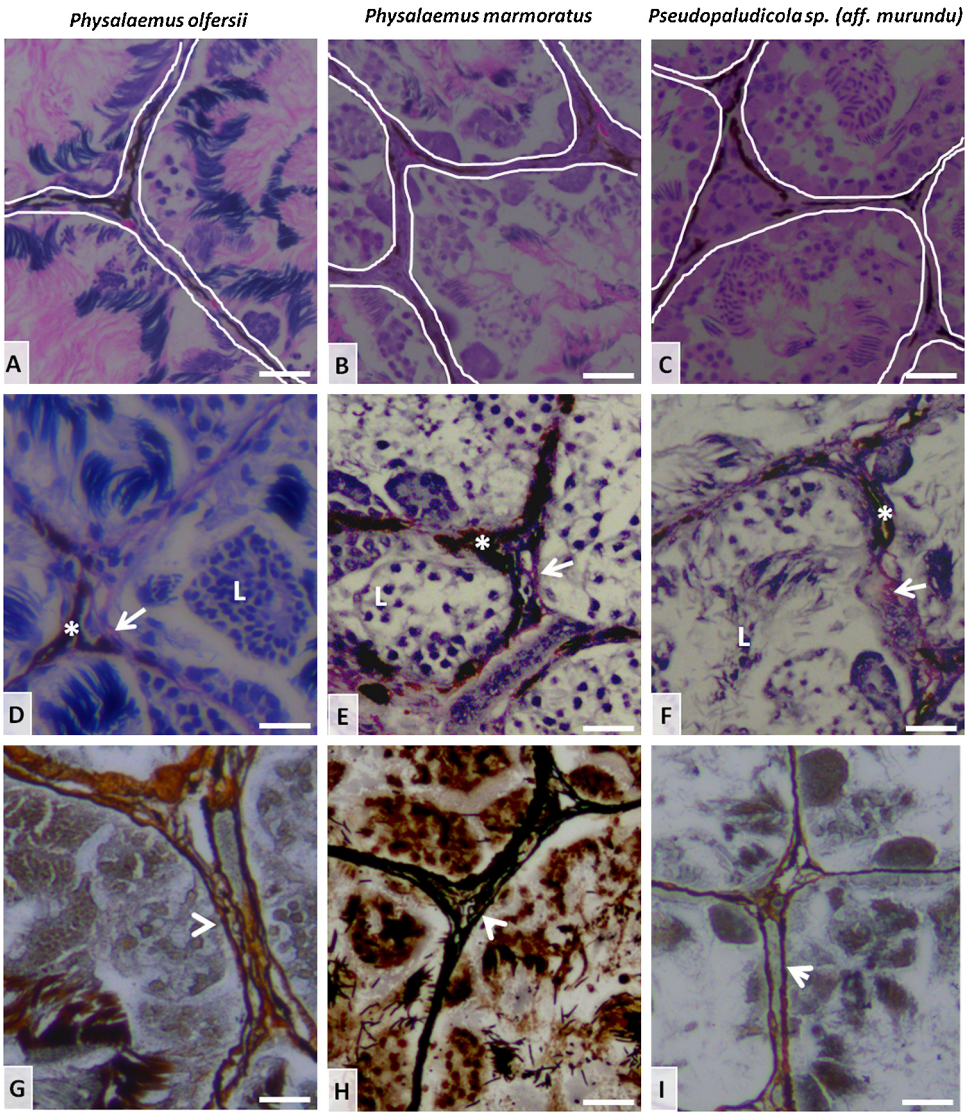

Fig. 6. Histological characterization of the testes of *Physalaemus olfersii*, *P. marmoratus,* and *Pseudopaludicola* sp. (aff. *murundu*), showing the locular area (lines A, B, and C), arrangement of collagen fibers (arrow), testicular melonocytes (*; D, E, F), and reticular fibers bundle in the interstitium (arrowhead; G, H, I). L: seminiferous locule. Staining: Hematoxylin/Eosin (A, B, C), Picrossirius (D, E, F), and Gömöri's reticulin (G, H, I). Bars = 25 μm.

between families, since *P. olfersii*, *P. marmoratus*, and *C. caramaschii* had the highest values (Fig. 8).

3.3. Quantitative analyses

There were differences between species of the two families when we consider all characters together (Wilks' $\lambda_{1,6} = 0.0027$; $P < 0.01$ Fig. 8). Also, hylodids had the highest values for most characters, except for sperm bundle and length of reticular fiber (Table S1).

There was a great difference between families in characters of germinal compartmet, such as locular diameter. For example, the locular diameters of *C. caramaschii* and *H. cardosoi* were about 50% larger than those of leiuperines. The locular area was greater in hylodids, those of *H. sazimai* and *H. cardosoi* being approximately 20% higher than that in leiuperines. However, this difference was not significant (Fig. 8; $F_{1,6} = 0.436$; $P = 0.667$).

Conversely, the interstitial area was larger in leiuperines. Collagen and reticular fibers occurred in the somatic cells around locules. The area and length of these fibers, respectively, was different between leuperines and hylodids (Fig. 8). The area of collagen fibers in hylodids was about 50% greater than in leuperines (Fig. 8; $F_{1,6} = 55{,}007$; $P < 0.05$). Reticular fibers were thicker in leuperines than hylodids, except for *H. cardosoi*, which resembled leiuperines. However, this difference was not significant (Fig. 8; $F_{1,6} = 7.612$; $P = 0.10$).

P. olfersii had the highest areas occupied by sperm bundles, followed by *P. marmoratus*. However, this variable did not show a phylogenetic pattern, differing among species regardless of the family (Fig. 8; $F_{1,6} = 1.119$; $P = 0.498$).

4. Discussion

Testicular morphology observed in histology and 3D reconstruction varied interspecifically, mainly in the shape of seminiferous locules and thickness of the tunica albuginea. For example, hylodids had a few large and flattened locules, and a thick tunica albuginea, whereas leuperines had many small, spherical locules, and a thin tunica albuginea.

Anurans have non-lobate, paired testes similarly to those of birds, squamate reptiles, and mammals, whereas the testes of caecilians and caudates have segmented lobes, whose number and

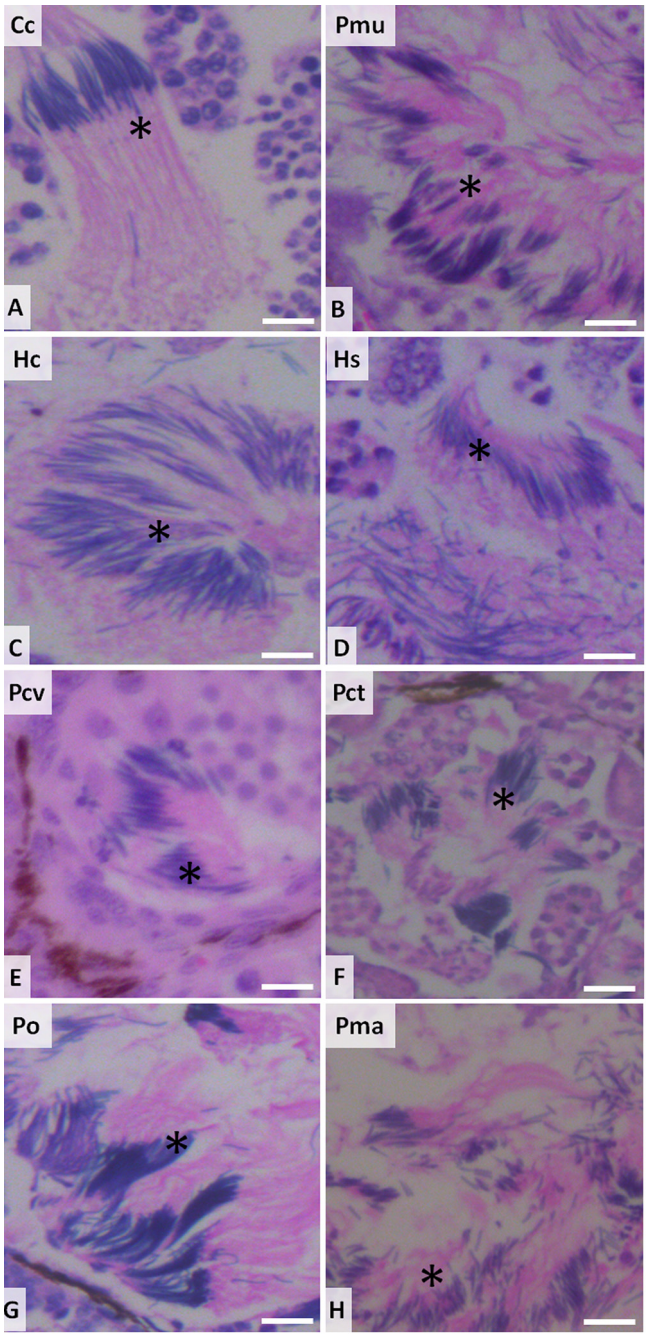

Fig. 7. Sperm bundle inside seminiferous locules in *Crossodactylus caramaschii* (Cc), *Pseudopaludicola* sp. (aff. *murundu*) (Pmu), *Hylodes cardosoi* (Hc), *H. sazimai* (Hs), *Physalaemus cuvieri* (Pcv), *P. centralis* (Pct), *P. olfersii* (Po), and *P. marmoratus* (Pma). Differences in area occupied by sperm (*). Staining: Hematoxylin/Eosin. Bars = 5 μm.

volume vary among families (Wake, 1968; Ingersol et al., 1991; Oliveira and Vicentini, 1998). Lobate testes are typically found in caecilians and salamanders, in which the number of lobes appear to increase with age in some species. However, having multiple lobes seem to be a plesiomorphic state of this character, since they tend to be merged in more derived groups (Wake, 1968). Lobes are not distinct in anurans and their testes are delimited externally by a sheath of fibrous tissue (Wake, 1979). Differences in testis morphology between these two orders and anurans may be due to the retention of plesiomorphic states of the urogenital system in caecilians and caudates. Conversely, anurans have a well-developed testis cell mass surrounded by the tunica albuginea, which connects to a transverse duct originating in the kidney and interconnected by central ducts, similar to that of fish (Wake, 1979). The tunica albuginea supports seminiferous locules and its thickness varies depending on both the microhabitat used by a species (Guillama et al., 2014) and among families, since the tunica is thicker and thinner in hylodids than in leuperines.

The amount of collagen fibers was different between the two families. Collagen fibers have flexible proteoglycans and high resistence (Ushiki, 2002). The undulating arrangement of these fibers also provides resiliency against direct tensions (Ushiki, 2002). Hylodids have greater amount of collagen fibers and locular diameter than leiuperines. The amount of collagen fibers and locular size may be correlated, since larger locules are required for physiological adjustments to cope with environmental variation.

Most leiuperines had thicker reticular fibers. Reticular fibers form a dark fiber framework, very thin and continuous with

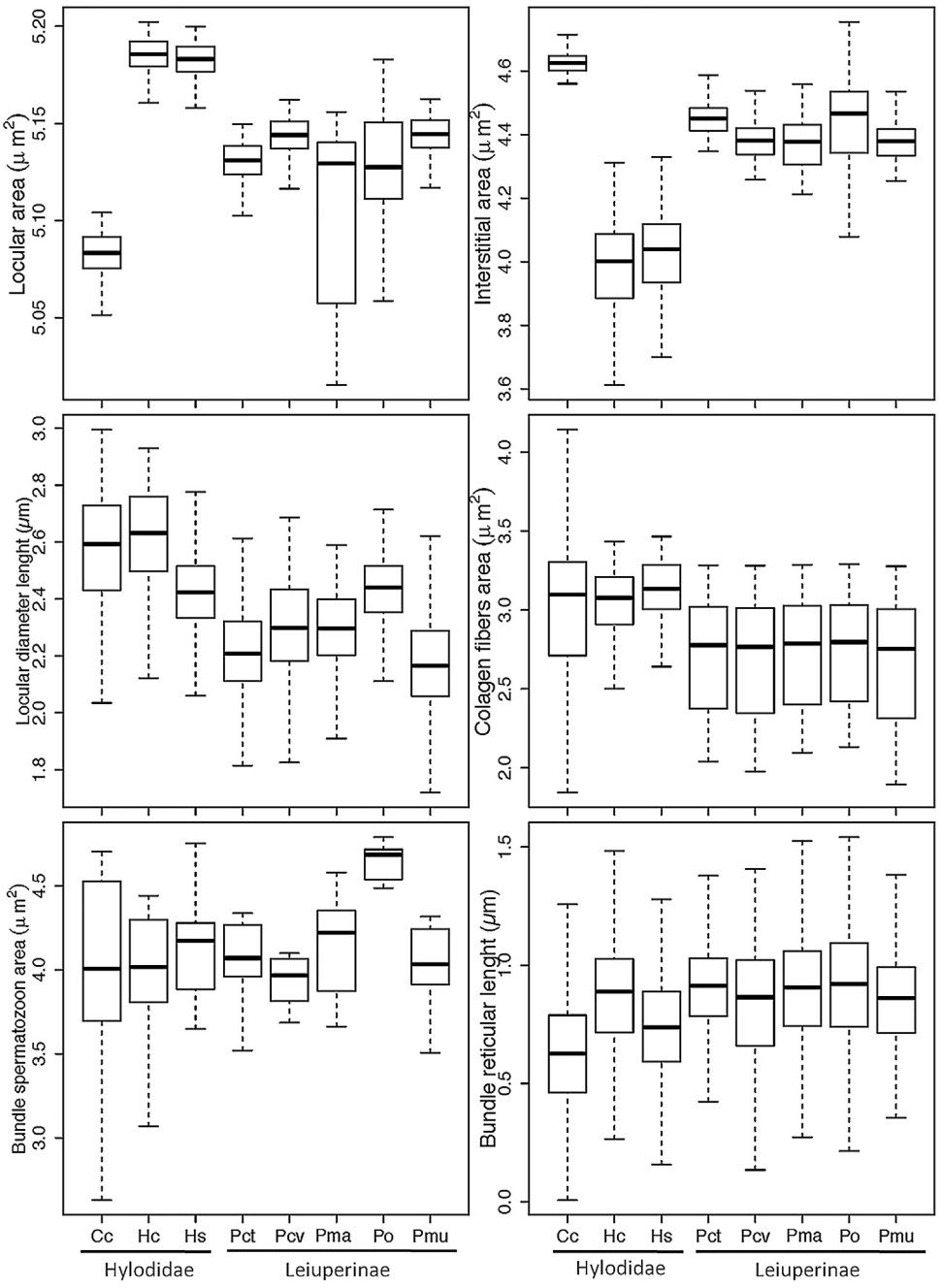

Fig. 8. Boxplot showing measurements for each species of testicular area, locular diameter, sperm bundle, interstitial area, area of collagen fiber bundles, and reticular fibers length. The line represents the median value, box are the first and third quantile. Dashed lines correspond to higher and lower values. Notice the log scale in the *y*-axis. Abbreviations: *Crossodactylus caramaschii* (Cc), *Hylodes cardosoi* (Hc), *H. sazimai* (Hs), *P. centralis* (Pct), *Physalaemus cuvieri* (Pcv), *P. marmoratus* (Pma), *P. olfersii* (Po), and *Pseudopaludicola* sp. (aff. *murundu*) (Pmu).

collagen fibers and abundant glycoproteins, providing great stability (Ushiki, 2002). Therefore, reticular fibers have a different biochemical structure, function, and arrangement of fiber bundles from collagen, but both help to support the testis (Ushiki and Fujita, 1992). The variation in fiber thickness is probably due to the smaller locules of leiuperines, which have higher mobility and resistence. This characteristic may be associated with explosive breeding, since leiuperines produce large amounts of sperm in a short period of time (Haddad and Prado, 2005; Wells, 2007), which results in a wide variation in fiber framework during the reproductive cycle.

The framework of fibers might also be related to sperm transport from the seminiferous locules, through the kidneys to the cloaca. Amphibians have mesonephric kidneys, whose anterior portion carries sperm to outside through the Wolff's ducts (Hiragond et al., 2000; Sandoval and Gómez, 2010). However, the connections between seminiferous locules and the cloaca may vary among species. For example, species of the genus *Bombina* have an additional duct to the renal lateral channel, which is not found in other anurans, or even a direct association between the ductus deferens and the Wolffian duct in discoglossids (Hiragond et al., 2000). Thus, the variation of somatics cells between species may be related to sperm transport out of the body and ultimately external fertilisation.

The compartment fulfilled by sperm bundles varies independently of phylogenetic relatedness. These differences are likely due to different reproductive strategies of the two families. Leiuperines

are usually explosive breeders, reproducing only for a few days per year and lay eggs in foam nests in ponds in open areas (Haddad and Prado, 2005). On the other hand, hylodids are associated with fast-flowing, montane streams along the Brazilian Atlantic Forest (Frost, 2014) and usually have continuous reproduction. Explosive breeders must produce a large amount of sperm in a short period of time, while prolonged breeders generally distribute energy investment over several months. Moreover, testis sizes differ between leptodactylids with different reproductive modes, whose testis weight:body weight ratio is higher than in other congeneric species. Those species are unique within the family by having multi-male spawning, which favors sperm competition and alter testes size (Prado and Haddad, 2003; Liao et al., 2011). Sperm can also influence testicular morphology, such as in *Discoglossus pictus* (Piprek et al., 2013). Therefore, the area occupied by sperm appears to be a plastic character, probably influenced by local environmental characteristics.

5. Conclusion

In conclusion, testicular histological parameters differ interspecifically in anurans, varying both in locular shape and the testicular framework. Hylodids have larger seminiferous locules supported by a larger area of collagen and tunica albuginea, while leiuperines have a smaller reticular fiber bundle. These characteristics are likely related to the distinct reproductive modes of species investigated. Our results may help understanding the morphological evolution of reproductive traits in anuran amphibains.

Acknowledgements

We would like to thank Marvalee Wake for constructive comments on an earlier version of this manuscript. Luiz R. Falleiros Jr. helped with histological processing. IBAMA provided collecting permits (#18573-1). GBL was supported by a FAPESP IC fellowhsip (#2013/07284-4), and a master's fellowship from FAPESP during the final preparation of this manuscript (#2014/16900-3). LF-B received a post-doc grant from FAPESP (#2014/00946-4). DBP was supported by a PhD fellowship from CAPES-DS and by a post-doc fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (#203734/2014-9) during the final preparation of this manuscript. This project was supported by a FAPESP grant (#2013/02067-5).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2004.08.011.

References

Báo, S.N., Dalton, G.C., Oliveira, S.F., 1991. Spermiogenesis in *Odontophrynus cultripes* (Amphibia, Anura, Leptodactylidae): ultrastrutural and cytochemical studies of proteins using E-PTA. J. Morphol. 207, 303–314.
Bastos, R.P., Pombal Jr., J.P., 1995. New species of *Crossodactylus* (Anura: Leptodactylidae) from the Atlantic Rain Forest of southeastern Brazil. Copeia 1995, 436–439.
Benjamini, Y., Yekutieli, D., 2001. The control of the false discovery rate in multiple testing under dependency. Ann. Stat. 29, 165–1188.
Bokermann, W.C.A., 1962. Sôbre uma pequena coleção de anfíbios do Brasil Central com descrição de uma espécie nova de *Physalaemus* (Amphibia Salientia). Braz. J. Biol. 22, 213–219.
Cavicchia, J.C., Moviglia, G.A., 1983. The blood-testis barrier in the toad (*Bufo arenarum* Hensel): a freeze-fracture and lanthanum tracer study. Anat. Rec. 205, 387–396.
Duellman, W.E., Trueb, L., 1994. Biology of Amphibians. Johns Hopkins University Press, Baltimore, pp. 670.
Emerson, S.B., 1997. Testis size variation in frogs: testing the alternatives. Behav. Ecol. Sociobiol. 41, 227–235.
Fiala, J.C., 2005. Reconstruct: a free editor for serial section microscopy. J. Microsc. 218, 52–61.
Fitzinger, L.J.F.J., 1826. Neue Classification der Reptilien nach ihren Natürlichen Verwandtschaften nebst einer Verwandtschafts-Tafel und einem Verzeichnisse der Reptilien-Sammlung des K. K. Zoologisch Museum's zu Wien. Wien: J. G. Heubner.
Felsenstein, J., 1985. Phylogenies and the comparative method. Am. Nat. 125, 1–15.
Frost, D.R., 2014. Amphibian Species of the World: an Online Reference. American Museum of Natural History, New York, USA, Version 6.0 (13 September 2014). Accessible athttp://research.amnh.org/herpetology/amphibia/index.html
Garland, J.T., Dickerman, A.W., Janis, C.M., Jones, J.A., 1993. Phylogenetic analysis of covariance by computer simulation. Syst. Biol. 42, 265–292.
Gomez-Mestre, I., Pyron, R.A., Wiens, J.J., 2012. Phylogenetic analyses reveal unexpected patterns in the evolution of reproductive modes in frogs. Evolution 66, 3687–3700.
Guillama, L.C., Rodríguez, Y., Sanz, A., Lara, R., Segura, M.L., Jiménez, L.F., 2014. Descripiín morfolígica de gínadas y células sexuales de dos especies de ranas cubanas del género Eleutherodactylus (Anura: Eleutherodactylidae). Rev Cub Cienc Biol. 3, 38–47.
Grant, T., Frost, D.R., Caldwell, J.P., Gagliardo, R., Haddad, C.F.B., Kok, P.J.R., Means, D.B., Noonan, B.P., Schargel, W.E., Wheeler, W.C., 2006. Phylogenetic systematics of Dart-Poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae). Bull. Am. Mus. Nat. Hist. 299, 1–262.
Haddad, C.F.B., Pombal Jr., J.P., 1995. A new species of *Hylodes* from southeastern Brazil (Amphibia: Leptodactylidae). Herpetologica 51, 279–286.
Haddad, C.F.B., Prado, C.P.A., 2005. Reproductive modes in frogs and their unexpected diversity in the Atlantic Forest of Brazil. BioScience 55, 207–217.
Hiragond, A.N.C., Saidapur, S.K., Hiragond, N.C., 2000. The excurrent duct system of sperm transport in *Rana cyanophlyctis*, *Rana limnocharis*, *Polypedates maculatus*, *Microhyla rubra*, *Bufo melanostictus*, and *Bufo fergusonii*. Zool. Sci. 17, 453–458.
Ingersol, C.A., Wilkinson, R.F., Peterson, C.L., Ingersol, R.H., Naturalist, T.S., Mar, N., 1991. Histology of the reproductive organs of *Cryptobranchus alleganiensis* (Caudata: Cryptobranchidae) in Missouri. Southwest. Nat. 36, 60–66.
Kühnel, S., Reinhard, S., Kupfer, A., 2010. Evolutionary reproductive morphology of amphibians: an overview. Bonn Zool. Bull. 57, 119–126.
Liao, W.B., Mi, Z.P., Zhou, C.Q., Jin, L., Lou, S.L., Han, X., Ma, J., 2011. Relative testis size and mating systems in anurans: large testis in multiple-male mating in foam-nesting frogs. Anim. Biol. 61, 225–238.
Lichtenstein, H., Martens E.V, 1856. Nomenclator Reptilium et Amphibiorum Musei Zoologici Berolinensis. Namenverziechniss der in der zoologischen Sammlung der Königlichen Universität zu Berlin aufgestellten Arten von Reptilien und Amphibien nach ihren Ordnungen, Familien und Gattungen. Berlin.
Lingnau, R., Canedo, C., Pombal Jr., J.P., 2008. A new species of *Hylodes* (Anura: Hylodidae) from the Brazilian Atlantic Forest. Copeia 2008, 595–602.
Liu, Y.H., Liao, W.B., Zhou, C.Q., Mi, Z.P., Mao, M., 2011. Asymmetry of testes in Guenther's Frog, *Hylarana guentheri* (Anuar: Ranidae). Asian Herpetol. Res. 2, 234–239.
Lofts, B., 1976. Reproduction. In: Lofts, B. (Ed.), Physiology of the Amphibia. Academic Press, New York, pp. 107–218.
Mi, Z.P., Liao, W.B., Jin, L., Lou, S.L., Cheng, J., Wu, H., 2012. Testis asymmetry and sperm length in *Rhacophorus omeimontis*. Zool. Sci. 29, 368–372.
Nunn, C.L., 2011. The Comparative Approach in Evolutionary Anthropology and Biology. University of Chicago Press, Chicago, pp. 392.
Oliveira, C., Vicentini, C.A., 1998. Descrição anatômica dos testículos ecorpos adiposos de *Scinax fuscovarius* (Anura). Biociências 6, 79–88.
Oliveira, C., Zanetoni, C., Zieri, R., 2002. Morphological observations on the testes of *Physalaemus cuvieri* (Amphibia, Anura). Rev. Chil. Anat. 20, 263–268.
Oliveira, C., SantíAnna, A.C., Omena, P.M., Santos, L.R.S., Zieri, R., 2003. Morphological considerations on the seminiferous structures and testes of anuran amphibians: *Bufo crucifer Physalaemus cuvieri* and *Scinax fuscovarius*. Biociências 11, 39–46.
Oliveira, C., Franco-Belussi, L., 2012. Comparative morphology of anuran testes. In: Nemoto, Y., Inaba, N. (Eds.), Testis: Anatomy, Physiology and Pathology. Hauppauge: Nova Science Publishers, pp. 111–126.
Pennell, M.W., Eastman, J.M., Slater, G.J., Brown, J.W., Uyeda, J.C., FitzJohn, R.G., Alfaro, M.E., Harmon, L.J., 2014. geiger v2: 0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. Bioinformatics 30, 2216–2218.
Piprek, R.P., Pecio, A., Szymura, J.M., 2013. Modifications of the testis in response to sperm bundle formation in the Mediterranean painted frog *Discoglossus pictus* Otth, 1837 (Amphibia: Anura: Discoglossidae). J. Herpetol. 47, 331–336.
Prado, C.P.A., Haddad, C.F.B., 2003. Testes size in leptodactylid frogs and occurrence of multimale spawning in the genus *Leptodactylus* in Brazil. J. Herpetol. 37, 354–362.
Provete, D.B., Franco-Belussi, L., Santos, L.R.S., Zieri, R., Moresco, R.M., Martins, I.A., Almeida, S.C., Oliveira, C., 2012. Phylogenetic signal and variation of visceral pigmentation in eight anuran families. Zool. Scr. 41, 547–556.
Pyron, R.A., Wiens, J.J., 2013. Large-scale phylogenetic analyses reveal the causes of high tropical amphibian diversity. Proc. Royal Soc. B 280, 1622.
R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing. URL http://www.R-project.org/
Ramm, S.A., Scharer, L., 2014. The evolutionary ecology of testicular function: size isn't everything. Biol. Rev. 89, 874–888.
Rastogi, R.K., Bagnara, J.T., Iela, L., Krasovich, M.A., 1988. Reproduction in the Mexican leaf frog, *Pachymedusa dacnicolor*. IV. Spermatogenesis a light and ultrasonic study. J. Morphol. 197, 277–302.

Reinhardt, J. T., and Lütken, C. F. 1862 "1861"', Bidrag til Kundskab om Brasiliens Padder og Krybdyr. Förste Afdeling: Padderne og Öglerne. Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening i Kjøbenhavn, Serie 2, 3, 143-242.
Revell, L.J., 2012. Phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3, 217–223.
Sandoval, M.T., Gómez, M.L., 2010. Desarrollo y morfología del sistema urogenital de *Phylsaemus biligonigerus*. Facena 26, 29–41.
Santos, L.R.S., Franco-Belussi, L., Oliveira, C., 2011. Germ cell dynamics during the annual reproductive cycle of *Dendropsophus minutus* (Anura: Hylidae). Zool. Sci. 28 (11), 840–844.
Santos, L.R.S., Oliveira, C., 2008. Histological aspects and structural characteristics of the testes of *Dendropsophus minutus* (Anura, Hylidae). Micron 39, 1266–1270.
Ushiki, T., 2002. Collagen fibers, reticular fibers and elastic fibers: a comprehensive understanding from a morphological viewpoint. Arch. Histol. Cytol. 65, 109–126.
Ushiki, T., Fujita, T., 1992. The three-dimensional ultrastructure of the collagen fibres: reticular fibers and elastic fibers: a review. Acta. Anat. Nippon. 67, 186–199.
Wake, M.H., 1968. Evolutionary morphology of the caecilian urogenital system. I. the gonads and the fat bodies. J. Morphol. 126, 291–331.
Wake, M.H., 1979. The comparative anatomy of the urogenital system. In: Wake, M.H. (Ed.), Hyman's Comparative Vertebrate Anatomy. , 3rd ed. University of Chicago Press, Chicago, pp. 555–614.
Wells, K.D., 2007. The Ecology and Behavior of Amphibians. University of Chicago Press, Chicago.
Zeng, Y., Lou, S.L., Liao, W.B., Jehle, R., 2014. Evolution of sperm morphology in anurans: insights into the roles of mating system and spawning location. BMC Evol. Biol. 14, 104.
Zhou, C.Q., Mao, M., Liao, W.B., Mi, Z.P., Liu, Y.H., 2011. Testis asymmetry in the dark-spotted frog *Rana nigromaculata*. Herpetol. J. 21, 181–185.