Revista Nordestina de Zoologia
ISSN 1808-7663

Boana albomarginata (SPIX, 1824) (ANURA: HYLIDAE) SPERMATOGENESIS IN A HIGHLAND ATLANTIC FOREST REMNANT IN PERNAMBUCO STATE, NORTHEASTERN BRAZIL

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ABSTRACT

Knowledge on anurans reproductive biology is still incipient in Brazil, and almost totally unknown in northeastern Brazil. Understanding the various aspects of reproductive patterns is essential for determining the population dynamics of this group. Spermatogenesis is an important animal reproduction phase and strongly regulated by environmental conditions in anurans, mainly by the presence of rainfall, that significantly alters temperature and humidity, two extrinsic parameters that control sex hormone release. Here we describe some aspects of the spermatogenesis of *Boana albomarginata* from a population located in a highland Atlantic Forest remnant in the semiarid northeastern region of Pernambuco state. We sampled 23 individuals bimonthly between October 2017 and October 2019 from a population located in the Brejo dos Cavalos. The results indicate that spermatogenesis in this population is regulated by rainfall, with consistently high level of sperm production before the onset of rainfall. Thus, this population presents rainfall-regulated spermatogenesis.

Key-words: Amphibia; Brejo de Altitude; Gametogenesis; Histology; Reproductive biology

RESUMO

O conhecimento sobre a biologia reprodutiva de anuros ainda é incipiente no Brasil, e quase totalmente desconhecido no nordeste brasileiro. Compreender os diversos aspectos dos padrões reprodutivos é essencial para determinar a dinâmica populacional desse grupo. A espermatogênese é uma importante fase da reprodução animal e fortemente regulada pelas condições ambientais nos anuros, principalmente pela presença de chuvas, que alteram significativamente a temperatura e a umidade, dois parâmetros extrínsecos que controlam a liberação de hormônios sexuais. Aqui, descrevemos alguns aspectos da espermatogênese de *Boana albomarginata* de uma população localizada em um remanescente de Mata Atlântica de altitude no semiárido nordestino pernambucano. Amostramos 23 indivíduos bimestralmente entre outubro de 2017 e outubro de 2019 de uma população localizada no Brejo dos Cavalos. Os resultados indicam que a espermatogênese nesta população é regulada pela chuva, com nível consistentemente alto de produção de espermatozoides antes do início das chuvas.

Palavras-chave: Amphibia; Brejo de Altitude; Gametogênese; Histologia; Biologia Reprodutiva

INTRODUCTION

One of the key factors determining the success of vertebrate dispersal is their ability to adapt reproductive cycles to the environment in which they live (Haddad & Prado, 2005; Moore *et al.,* 2005). According to Callard *et al.* (1978), the plasticity in reproductive cycle control allows the organisms to adjust of physiological parameters according to the necessary conditions to the changes in the environment.

For most anuran species, the reproduction process is closely related to rainfall, which alters temperature and humidity (Duellman & Trueb 1994; Sasso-Cerri *et al.,* 2004; Haddad & Prado, 2005; Moore *et al.,* 2005). These environmental changes lead anurans to release hormones and initiate gametogenesis process (Lofts, 1974). A few weeks after this process begins, rain fills the natural water bodies that will serve as breeding sites and areas of larval development (Haddad & Prado, 2005). Many species prefer hot and humid periods for reproduction and demonstrate a preference for temporary ponds as fewer predators are present (Altig & McDiarmid, 1999). Thus, the maintenance of these water bodies is intrinsically related to rainfall, which in turn influences the entire reproductive process (Bertoluci & Rodrigues, 1997; 2002a; b).

Reproductive cycle control in anurans is mediated by the interaction of environmental influence rhythms that affect endogenous animal factors (Sasso-Cerri *et al.,* 2004; Wilczynski *et al.*, 2005). Thus, for anurans, temperature and rainfall are the main factors influencing reproductive patterns in areas that are strongly affected by these physical environmental conditions (Cardoso & Martins, 1987). As such, anurans are noteworthy for being excellent experimental models (Ferreira et al., 2008; Chaves *et al.,* 2017).

Anuran reproductive cycles were explored and classified by Lofts (1974), according to the sum of behavioral patterns and histological gonad characteristics, as: continuous, potentially continuous and discontinuous. The discontinuous cycle is typical of temperate climate zones and is characterized by a discreet breeding period with pronounced changes in gonad size, gamete production and accessory sexual structures. Continuous and potentially continuous cycles are typical of tropical regions and are characterized by constant gamete production. On the other hand, partial gametogenesis interruption is observed during certain periods in potentially continuous cycles (Santos & Oliveira, 2007). Therefore, knowledge on the reproductive aspects of anurans in different ecoregions, and even in different areas of ecoregions that present high environmental and climatic heterogeneity, is essential.

In the Neotropical region, the Atlantic Forest is characterized by the occupation of an extensive latitudinal and longitudinal range and is associated with climatic variation. This variation includes tropical and subtropical regions and precipitation and humidity gradients from the coast to the mainland (Ribeiro *et al.,* 2009; Haddad *et al.,* 2013), as well as variation in elevation and vegetation (0–2,700 meters; Pinto & Brito, 2003). This habitat heterogeneity allows for the emergence and maintenance of high anuran species richness and endemism (719 species; Rossa-Feres *et al.,* 2017; Figueiredo *et al*., 2021). Many of these species present high micro-environmental specificities and a poor ability to adapt to human interference, making them extremely sensitive to environmental changes (Shlaepfer & Gavin, 2001; Vitt & Caldwell, 2001; Caorsi *et al*., 2019; Estrela *et al*., 2020; Getelina *et al*., 2022).

Singular areas known as “brejos de altitude” (highland humid forest remnants scattered throughout the semiarid lowlands) are found in the northernmost Atlantic Forest. These areas occur at a minimum of 600 m above sea level, in mountain ranges and plateaus in northeastern Brazil (Tabarelli & Santos, 2004; Freitas *et al.,* 2019).

Boana albomarginata (Spix, 1824) is one of the several anuran species that inhabits these regions. This medium-sized species presents nocturnal and arboreal habits and deposit its eggs in lentic water bodies (Pombal-jr & Gordo, 2004). This species presents a marked sexual dimorphism in size (snout-vent length [SVL] = 55.0 mm in males and 60 mm in females) and is distributed throughout northeastern Brazil, from the Atlantic Forest of Paraíba state to southern Brazil in Santa Catarina state (Frost, 2021).

Here, we describe some aspects of the spermatogenesis of *Boana albomarginata* in a highland Atlantic Forest remnant, strongly influenced by climate due to its insertion in the Brazilian semiarid region.

MATERIAL AND METHODS

Study area

The study was carried out at Brejo dos Cavalos, located in the municipality of Caruaru, state of Pernambuco, northeastern Brazil (8.376944ºS, 36.059167ºW; WGS84; Fig. 1A). It is a rainforest area located on the Borborema

Plateau, surrounded by a semiarid region in the Caatinga biome. The altitude varies from 750 to 1,040 meters above sea level, with characteristic vegetation of the Atlantic Forest biome (Fig. 1B-C).

Data sampling

Bimonthly sampling excursions were conducted during a period of two years (October 2017 to October 2019), with the aim of increasing the chances of specimen capture at different spermatogenesis stages. We searched for at least three males during each field trip. Samplings were carried out at night, between 6:00 to 9:00 PM, totaling 39 hours of active searches.

Captured specimens were euthanized with 5% lidocaine (samplings and procedures permissions: ICMBio/SISBio 57963-1 and CEUA-UFRPE 040/2018). After euthanizing the individuals, we removed their gonads, and they were then fixed in a 10% formaldehyde solution, kept in alcohol 70% and later incorporated into the collection of 'Laboratório de Estudos Herpetológicos e Paleoherpetológicos' of 'Universidade Federal Rural de Pernambuco' (LEPH-UFRPE), under registration numbers LEPH 5643–67.

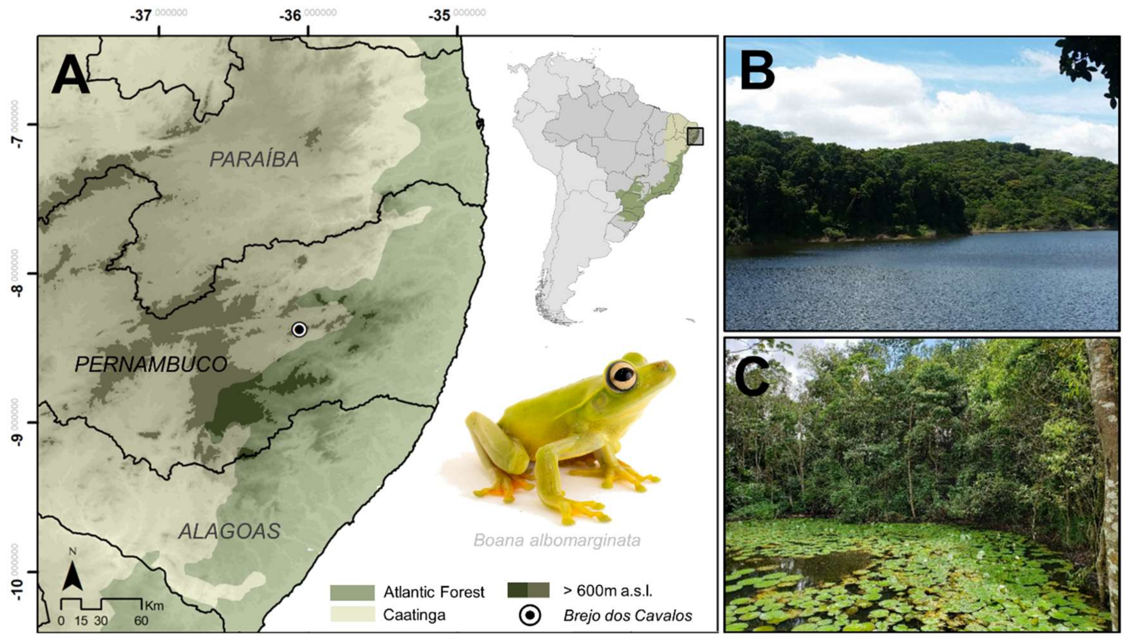

Figure 1. Brejo dos Cavalos, municipality of Caruaru, state of Pernambuco, northeastern Brazil. A = Map indicating the location of the study area; B = Overview of the Atlantic Forest mosaic in the area; C = Pond within the sampling area investigated. Inset map: South America.

Laboratory procedures

The testicles were taken to the 'Laboratório de Biologia de Anfíbios' of 'Universidade Federal de Campina Grande' (LABAN-UFCG), where they were fixed in a 10% formaldehyde solution for 24 hours, followed by the protocol proposed by Hopwood (1990), consisting of the following steps: (i) Dehydration through a series of increasing alcohol baths (70% - 30 min, 80% - 30 min, 90% - 30 min, 95% - 30 min, 100% - 30 min and 100% - 30 min), (ii) diaphanization (two 30 min Xylol baths), (iii) inclusion (50% paraffin + Xylol - 1 hour), (iv) pure paraffin exposure for 24 hours, (v) paraffin-embedding and, finally, (vi) sectioning at 5.0 µm using a microtome.

Slides were stained following the methodology of Robinson and Gray (1990): Xylol I - 6min; 100% alcohol - 1 min; Alcohol 90% - 1 min; Alcohol 70% - 1 min; Distilled water - 1 min; Hematoxylin - 30 sec; Tap water - 10 min; Eosin - 20 sec; Alcohol 70% - 1 min; Alcohol 90% - 1 min; 100% alcohol I - 1 min; 100% Alcohol II - 1 min; Xylol - 6 min. Cell types were identified according to Hermosilla *et al.* (1983), Oliveira *et al.* (2003a; b) and Chaves *et al.* (2017).

A qualitative analysis was performed using a conventional trinocular and bench microscope, coupled with a 4x and 40x digital objective imaging system, where 10 different focal fields were acquired from each slide, per specimen. Stereological cell analyses were carried out according to Weibel (1979) and Mandarin-de-Lacerda (1991), where the volume density (Vv) of spermatids I (St1), spermatids II (St2), and sperm (Z) were calculated, as they are excellent cellular models and extremely useful for bioindicating sexual maturation stage. As spermatogonia and spermatocytes consist of initial cell stages, they do not provide the necessary information concerning the reproductive period near the sampling period (Weibel, 1979). The sample calculations were performed using Hally's formula (Hally, 1964) and corrected for the result that represents stereological precepts (Mandarin-de-Lacerda, 1991). To quantify profile density (Q_A), fields were counted in a 0.044 mm² Test Area (TA) for each animal. The final result (mm²) was then calculated after using the mean for each profile and applying the following formula (Mandarin-de-Lacerda, 1991): $Q_A = \Sigma \text{profiles}/TA$.

The density values of the reproductive lineage cells were analyzed between sampling months by the Kruskal-Wallis test. We tested the relationship between rainfall and the population density of the reproductive lineage cells using a Linear Regression test. The climatic data encompass the time of expeditions and was provided by the state climatic agency (APAC 2020). Statistical significance was set at p <0.05, with all analyses based on Zar (1999).

RESULTS AND DISCUSSION

A total of 23 male specimens of *Boana albomarginata* were collected (one to three per expedition). During the months of August 2018 and June 2019, no males were found in the area.

All cell types evaluated were identified throughout the study period. Each cyst was presented as a cluster of germ cells in the same differentiation state. The albuginous tunic, delimiting the locules (Fig. 2B, TA), is observed at the center of the testicular structure, in the mediastinum region (Fig. 2B, Med), both formed by connective tissue.

Primary spermatogonia were easily recorded, as they are located in the locular periphery, bordering the albuginous tunic and presenting granular chromatin. Secondary spermatogonia appear more intensely stained than other cysts, located immediately after the primary lumen of the seminiferous locus, both presenting similar sizes.

Primary spermatocytes are large cells, although smaller than primary spermatogones, with looser chromatin. Secondary spermatocytes are slightly smaller than primary spermatocytes, with a much denser nucleus and a large surrounding eosinophilic cytoplasmic area. Primary spermatids (Fig. 2A-B, St1) are rounded cells, smaller than spermatophytes (Sp), with a slightly compacted nucleus and slightly fusiform shape.

Secondary spermatids (Fig. 2A-C, St2) undergo nucleus elongation alongside progressive nuclear compaction, disrupting the cystic organization to become arranged in bundles supported by Sertoli cells, although they are still considered germinal cysts. Sperm (Fig. 2C, Z) presented nuclear compaction and often free and disordered cytoplasmic reduction in the center of the locules.

The spermatogenesis in anurans is cystic, with differences in frequency of each cell stage (Santos & Oliveira, 2007; Leite *et al.,* 2015; Chaves *et al.,* 2017), as found in this study for *B. albomarginata*. However, cyst organization may vary between different families (e. g. Ferreira *et al.,* 2008) and according to the environment in which these animals occur (e. g. Ferreira *et al.,* 2008; Chaves *et al.,* 2017). Anurans of the family Hylidae generally have well-defined cysts, in which the spermatogonia are located on the periphery of the tubule (for exemple in *Dendropsophus nanus*, *Pseudis limellum*, *P. paradoxa* and *Scinax acuminatus*; Ferreira *et al.,* 2008), as found in *B. albomarginata*.

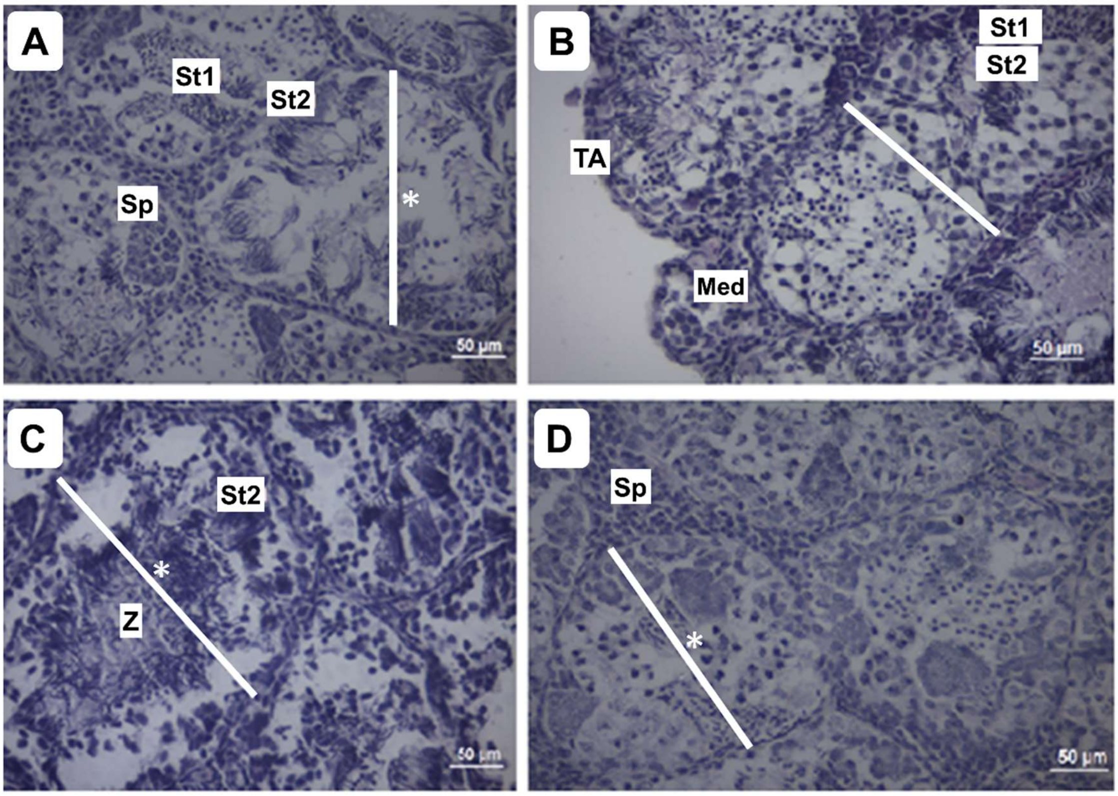

Figure 2. Histologic sections of *Boana albomarginata* collected in Brejo dos Cavalos, municipality of Caruaru, state of Pernambuco, northeastern Brazil. A = section at 40x: St1 [Primary spermatid], St2 [Secondary spermatid], Sp [Spermatocyte]; B = section at 40x: TA [albuginous tunic], Med [Mediastinum]; C = section at 40x: Z [sperm], * [locular area]; D = section at 40x: Sg [Spermatogonia].

The individuals sampled in this study presented oval and compact testicle structures, with anatomical variations in shape and weight (0.001 – 0.04g; Table 1) according to reproductive period, as well as other morphofunctional changes associated with reproduction seasonality, corroborating Duellman & Trueb (1994) and Lofts (1974).

Table 1. Monthly values (mean ± standard deviation) for density of snout-vent length (SVL), total weight, gonada weight, and primary spermatids (St1), secondary spermatids (St2) and sperm (Z)(mm^2) of the *Boana albomarginata* individuals collected in the Brejo dos Cavalos, municipality of Caruaru, state of Pernambuco, Brazil.

	N	SVL (cm)	Total weight (g)	Gonada weight (g)	St1	St2	Z
Oct/2017	2	7.85 ± 0.35	53.5 ±2.12	0.005 ± 0	448.09 ± 21.99	144.19 ±41.42	483.98 ± 40.51
Dec/2017	1	6.3	52	0.02	422.98	118.43	476.26
Feb/2018	2	6.2 ± 0	52.5 ±3.54	0.022 ± 0.03	401.57 ± 88.13	67.42 ±95.35	587.5 ± 99.1
Apr/2018	3	8 ± 0.17	50 ±1	0.01 ± 0	424.71 ± 153.35	439.39 ±130.19	199.58 ± 39.15
Jul/2018	2	6.92 ± 0.49	51 ±2.83	0.007 ± 0	557.2 ± 97.32	166.16 ±135.71	70.71 ± 20
Oct/2018	1	8.9	46	0.001	138.01	263.64	602.02
Dec/2018	2	10.62 ± 3.09	49.5 ±0.71	0.001 ± 0	179.17 ± 41.25	140.28 ±8.03	574.37 ± 302.31
Feb/2019	3	9.49 ± 0.84	54 ±3.21	0.001 ± 0.01	174.67 ± 43.56	250.29 ±52.77	377.69 ± 228.31
Apr/2019	3	8.15 ± 1.11	53 ±4.58	0.008 ± 0.01	184.43 ± 27.05	234.54 ±77.73	494.25 ± 144.63
Aug/2019	2	10 ± 1.27	55.5 ±2.12	0.016 ± 0	245.37 ± 12.73	354.9 ±45.93	199.67 ± 19.53
Oct/2019	2	11.8 ± 1.41	54 ±0	0.016 ± 0	317.93 ± 101.42	252.15 ±173.03	201.64 ± 74.46

Regarding histological characterization, *B. albomarginata* testicles presented varied cellular characteristics, with seminiferous locule size displaying different forms and presenting different germ cell patterns. The relationship values between cell types density values and rainfall indicate a significant relationship only for Secondary spermatids (St2; Linear Regression p = 0.002; Table 1). With regard to the relationship between secondary Spermatids (St2) and sperm (Z), no significant influence of rainfall was noted (Linear Regression p = 0.890 and 0.251, respectively; Table 1), despite that high values preceding the rainy season and low values appearing during the rainy season, suggesting that the specimens had indeed reproduced.

The monthly densities of three cell types evaluated varied significantly (Kruskal-Wallis = p <0.05; Table 1). Sperm values were high from October 2017 to February 2018, as well as from October 2018 to February 2019. This period characterizes water stress that precedes the highest rainfall periods which began, respectively, from February to June 2018 and February to August 2019. Sperm values fell sharply during the rainy season, corroborating the occurrence of copulations during this wetter period (MAF personal observation). However, for April 2019, although elevated rainfall values were observed, high sperm values were also recorded. Primary spermatids were high from October 2017 to June 2018, corroborating high sperm production, and from October 2018 to April 2019, indicating low reproductive activity (Fig. 3).

Secondary spermatids (St2) displayed significant variations, with low values from October 2017 to February 2018 and high values from October to December 2018, and in February and April 2019. Taking into account monthly average rainfall values, the data indicate that the higher the number of rainfall events, the lower the sperm density, thus demonstrating a direct relationship between these variables.

Boana albomarginata presented constant spermatogenic production and rapid germ cell maturation. During the period when adult males were not found (August 2018 and June 2019), the sampling area was very dry, with almost no visible water bodies. These two dry periods did not provide an adequate breeding environment for this species, corroborating the importance of rainfall for the study population, reducing the observed richness and diversity rates of species that inhabit this community as a whole and affecting reproduction rates. Therefore, due to the characteristics of this investigated species, it likely acts as a great biological model that can be used to test the hypothesis that rainfall is one of the most important spermatogenesis regulators in the brejos de altitude of Atlantic Forest. These results can serve as a parameter for future studies carried out in coastal Atlantic Rainforest areas that display constant environmental factors, such as rainfall and humidity, throughout the year.

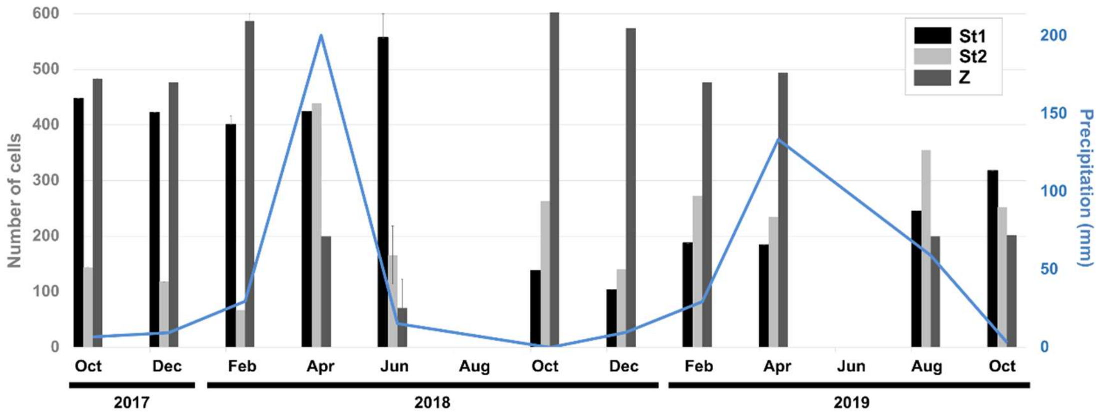

Figure 3. Correlation between the average number of Primary spermatid (St1; Black bar), Secondary spermatid (St2; light gray bar) and sperm (Z; dark gray bar) of *Boana albomarginata* and monthly rainfall (blue line) between October 2017 to October 2019 in Brejo dos Cavalos, municipality of Caruaru, state of Pernambuco, northeastern Brazil.

ACKNOWLEDGEMENTS

The authors thank the staff and managers at the Parque Natural Municipal Professor João Vasconcelos Sobrinho, especially João Domingos, João Freitas, Adriano, Alexandre and Ari, who have always supported research activities in this conservation unit; to Ms. Rebecca Umeed and Dra. Rachel Davis for English editing. MJMD thanks Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco – FACEPE (IBPG-1117-2.04/19).

REFERENCES

Altig, R. & McDiarmid, R.W. 1999. Body plan: Development and morphology. In Tadpoles: The Biology of Anuran Larvae (R.W. McDiarmid & R. Altig, eds). University of Chicago Press, Chicago, p.24–50.

APAC. 2019. Boletim Pluviométrico. http://www.apac.pe.gov.br/ (last access on 13/dez/2019)

Bertoluci, J.A. & Rodrigues M.T.U. 2002a. Seasonal patterns of breeding activity of Atlantic Rainforest anurans at Boracéia, Southeastern Brazil. Amphibia-Reptilia, 23(2): 161–167.

Bertoluci, J.A. & Rodrigues M.T.U. 1997. Fenologia e seleção de hábitat em girinos da Mata Atlântica em Boracéia, São Paulo (Amphibia, Anura). Universidade de São Paulo, São Paulo.

Bertoluci, J.A. & Rodrigues M.T.U. 2002b. Utilização de hábitats reprodutivos e micro-hábitats de vocalização em uma taxocenose de anuros (Amphibia) da Mata Atlântica do sudeste do Brasil. Papéis Avulsos de Zoologia, 42(11): 287–297.

Callard, L.P.; Callard, G.V.; Lance, V.; Bolafft, J.L. & Rosset, J.S. 1978. Testicular regulation in nonmammalian vertebrates. Biology of Reproduction, 18(1): 1–11.

Caorsi, V., Guerra, V., Furtado, R., Llusia, D., Miron, L.R., Borges-Martins, M., Both, C., Naris, P.M., Meenderink, S.W.F. & Márquez, R. 2019. Anthropogenic substrate-borne vibrations impact anuran calling. Scientific reports, 9(1): 1–10.

Cardoso, A.J. & Martins, J.E. 1987. Diversidade de anuros durante o turno de vocalização em comunidades Neotropicais. Papeis Avulsos de Zoologia 36: 279–285.

Chaves, M.F.; Moura, G.J.; Tenório, F.D.C.; Baptista, J.D.S.; Lapa, C.J.; Texeira, V.W. & Texeira, Á.A. 2017. Influence of rainfall and temperature on the spermatogenesis of *Leptodactylus macrosternum* (Anura: Leptodactylidae). Zoologia, 34: 1–7.

Duellman, W.E. & Trueb, L. 1994. Biology of Amphibians. Johns Hopkins University press, Baltimore.

Estrela, M.D.N., Simões, C.R., Vieira, G.H., & de Araújo, C.B. 2020. Predicting the effects of noise on Anuran spatial distribution: the case of *Scinax nebulosus*. Bioacoustics, 29(4): 481–497.

Ferreira, A., Rosa, A.B.S., & Mehanna, M.M. 2009. Organização celular dos Testículos em Hylidae e Leptodactylidae, no Pantanal (Estado do Mato Grosso do Sul, Brasil). Acta Scientiarum, 31: 447–452.

Ferreira, A.; Mehanna, M. & Prado, C. 2008. Morphologic and morphometric analysis of testis of *Pseudis limellum* (Cope, 1862) (Anura, Hylidae) during the reproductive cycle in the Pantanal, Brazil. Biocell, 32(2): 185–194.

Figueiredo, M.D.S.L., Weber, M.M., Brasileiro, C.A., Cerqueira, R., Grelle, C.E., Jenkins, C.N., Solidade, C.V., Thomé, M.T.C., Vale, M.M. & Lorini, M.L. 2021. Tetrapod diversity in the Atlantic Forest: maps and gaps. In The Atlantic Forest (pp. 185-204). Springer, Cham.

Freitas, M.A.; Abbeg, A.D.; Araujo, D.; Coelho, H.E.A.; Azevedo, W.S.; Chaves, M.F.; Rosa, C.M. & Moura, G.J.B. 2019. Herpetofauna of three Brejos de Altitude in the interior of the state of Pernambuco, northeastern Brazil. Herpetology Notes 12: 591–602.

Frost, DR. 2021. Amphibian Species of the World 6.0. Online Reference. http://research.amnh.org/vz/herpetology/amphibia. Accessed on 30 June 2021.

Getelina, M.A., Schwantes, J.B., Graichen, D.A.S., & Schuch, A.P. 2022. Influence of anthropogenic pressure on the genetic diversity and chromosomal instability of an endangered forest-specialist anuran. Hydrobiologia, 1–13.

Haddad, C.B.F. & Prado, C.P.A. 2005. Reproductive modes in frogs and their unexpected diversity in the Atlantic Forest of Brazil. Bioscience 55: 207–217.

Haddad, C.F.B; Toledo, L.F; Prado, C.P.A; Loebmann, D.; Gasparini, J.L. & Sazima, I. 2013. Guia dos Anfíbios da Mata Atlântica: Diversidade e Biologia. Anolis Books, São Paulo.

Hally, A.D. 1964. A counting method for measuring the volumes of tissue components in microscopical sections. Quarterly Journal of Microscopical Science, 105: 503–17.

Hermosilla, I.B.; Urbina, A.P. & Cabrera, J.C.P. 1983. Espermatogénesis en la rana chilena *Caudiverbera caudiverbera* (Linne, 1758) (Anura, Leptodactylidae). Boletin de la Sociedad de Biología de Concepción, 54: 103–115.

Hopwood, D. 1990. Fixation e fixatives: 21-42. *In*: Bancroft, JD; Stevens, A. (eds). Theory and practice of histological techniques. Churchill Livingstone, New York.

Leite, GB, Franco-Belussi, L, Provete, DB, de Oliveira, C. 2015. Comparative testis morphology of Neotropical anurans. Zoologischer Anzeiger, 257: 29–38.

Lofts, B. 1974. Seasonal changes in the functional activity of the interstitial and spermatogenetic tissues of the green frog, *Rana esculenta*. General and Comparative Endocrinology, 4: 550–562.

Mandarim-de-Lacerda, C.A. 1991. Métodos quantitativos em morfologia. Eduerj, Rio de Janeiro.

Moore, F.L., Boydb, S.K. & Kelleyc, D.B. 2005. Historical perspective: Hormonal regulation of behaviors in amphibians. Hormone Behavior, 48: 373–383.

Oliveira, C.; Sant'Anna, 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(1): 39–46.

Oliveira, C.; Vicentini, C.A. & Taboga, S.R. 2003. Structural characterization of nuclear phenotypes during *Scinax fuscovarius* spermatogenesis (Anura, Hylidae). Caryologya, 56: 75–83.

Pinto, L.P. & Brito, M.C.W. 2003. Dynamics of biodiversity loss in the Brazilian Atlantic Forest: an introduction. The Atlantic Forest of South America: biodiversity status, trends, and outlook, 27–30.

Pombal-jr, J.P. & Gordo, M. 2004. Anfíbios anuros da Juréia. Estação Ecológica Juréia–Itatins. Ambiente Físico, Flora e Fauna, pp.243–56.

Ribeiro, M.C.; Metzger, J.P.; Martensen, A.C.; Ponzoni, F.J. & Hirota, M.M. 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. Biologia e Conservação, 142(6): 1141–1153.

Robinson, G. & Gray, T. 1990. Electron microscopy 2: Tissue preparation, sectioning and staining. In: Bancroft JD, Stevens A (Eds) Theory and practice of histological techniques. Edinburgh, 3rd ed., 525–562.

Rossa-Feres, D.C.; Garey, M.V.; Caramaschi, U.; Napoli, M.F.; Nomura, F.; Bispo, A.A.; Brasileiro, C.R.A.; Thomé, M.T.C.; Sawaya, R.J.; Conte, C.E.; Cruz, C.A.G.; Nascimento, L.B.; Gasparini, J.L.; Almeida, A.P. & Haddad, C.F.B. 2017. Anfíbios da Mata Atlântica: lista de espécies, histórico dos estudos, biologia e conservação: 237-314. *In*: Monteiro-Filho E.L.A. & Conte C.E. (org.). Revisões em Zoologia: Mata Atlântica. Editora UFPR, Curitiba.

Santos, L.S. & Oliveira, C. 2007. Morfometria testicular durante o ciclo reprodutivo de *Dendropsophus minutus* (Peters) (Anura, Hylidae). Revista Brasileira de Zoologia 24: 64–70.

Sasso-Cerri, E.; Faria, F.; Freymuller, E. & Miraglia, S.M. 2004 Testicular morphological changes during the seasonal reproductive cycle in the Bulfrog *Rana catesbiana*. Journal of Experimental Zoology, 301: 249–260.

Tabarelli, M. & Santos, A.M.M. 2004. Uma breve descrição sobre a história natural dos Brejos Nordestinos: 17-24. *In*: Porto, KC; Cabral, JJP & Tabarelli, M. (eds.). Brejos de Altitude em Pernambuco e Paraíba, História Natural, Ecologia e Conservação. Ministério do Meio Ambiente, Distrito Federal.

Weibel, E.R. 1979. Fleischener Lecture: looking into the lung: what can tell us? American Journal of Roentgenology, 6: 1021–1031.

Wilczynski, W., Lynch, K.S. & O'Bryant, E.L. 2005. Current research in amphibians: Studies integrating endocrinology, behavior, and neurobiology. Hormone Behavior, 48: 440–450.

Zar, J.H. 1999. Biostatistical Analysis. Prentice Hall, New Jersey.