Environmental and abiotic factors interfere in *Leptodactylus macrosternum* (Anura, Leptodactylidae) reproduction despite no changes in sexual hormones levels

O ambiente e os fatores abióticos interferem na reprodução de *Leptodactylus macrosternum* (Anura, Leptodactylidae) apesar de não haver mudanças nos hormônios sexuais

El ambiente y los factores abióticos interfieren en la reproducción de *Leptodactylus macrosternum* (Anura, Leptodactylidae) aunque no hay cambios en las hormonas sexuales

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Abstract

In tropical regions presenting severe seasonality, the reproductive activity of many species occurs during the most appropriate periods of the year. Therefore, several physiological and behavioral adjustments are needed during their reproductive cycle. This study aims to evaluate possible changes in histologic and morphometric parameters of the ovaries as well in estrogen and progesterone plasma levels of *Leptodactylus macrosternum* females and their relationship with temperature and precipitation. Specimens were collected in the Horto Florestal Olho d'Água da Bica - HFOB (06°49´20''S/36°15´85''W) area in the municipality of Cuité, brazilian semiarid, every 15 days, only between the months of May to August and November to December. The quantification of population density of oocyte types and dosages of progesterone and estrogen levels were used to determine the reproductive activity of the species. Population densities of oogonia and oocyte II did not vary significantly during the months in which the animals were sampled. Population densities of oocyte I was significantly higher during the months of June ($p= 0.04$) and August ($p= 0.03$). The highest values for the population density of oocyte III and IV were found in the months of May ($p=0.04$) and June ($p=0.04$). The amounts of estrogen concentrations (pg / ml) and progesterone (pg / ml) did not change significantly over the sampling periods. The population density of the types of oocytes III and IV showed dependence on the variation in rainfall.

Keywords: Frogs; Oocyte; Rainfall; Semiarid; Sex hormones.

Resumo

Em regiões tropicais com forte sazonalidade, a atividade reprodutiva de muitas espécies ocorre nos períodos mais adequados do ano. Portanto, vários ajustes fisiológicos e comportamentais são necessários durante seu ciclo reprodutivo. Este estudo tem como objetivo avaliar possíveis alterações nos parâmetros histológicos e morfométricos dos ovários, bem como nos níveis plasmáticos de estrogênio e progesterona de fêmeas de *Leptodactylus macrosternum* e sua relação com a temperatura e precipitação. Os espécimes foram coletados na área do Horto

Florestal Olho d'Água da Bica - HFOB (06 ° 49´20"S / 36 ° 15´85"W) no município de Cuité, semiárido brasileiro, a cada 15 dias, apenas entre os meses de maio a agosto e de novembro a dezembro. A quantificação da densidade populacional dos tipos de ovócitos e dosagens de progesterona e níveis de estrogênio foram utilizados para determinar a atividade reprodutiva das espécies. As densidades populacionais de oogônios e oócitos II não variaram significativamente durante os meses em que os animais foram amostrados. As densidades populacionais do oócito I foram significativamente maiores durante os meses de junho (p = 0,04) e agosto (p = 0,03). Os maiores valores para a densidade populacional do oócito III e IV foram encontrados nos meses de maio (p = 0,04) e junho (p = 0,04). As quantidades de concentrações de estrogênio (pg / ml) e progesterona (pg / ml) não se alteraram significativamente ao longo dos períodos de amostragem. A densidade populacional dos tipos de ovócitos III e IV mostrou dependência da variação da precipitação.
Palavras-chave: Sapos; Oócito; Chuva; Semiárido; Hormônios sexuais.

Resumen

En las regiones tropicales que presentan una estacionalidad severa, la actividad reproductiva de muchas especies ocurre durante los períodos más apropiados del año. Por lo tanto, se necesitan varios ajustes fisiológicos y de comportamiento durante su ciclo reproductivo. Este estudio tiene como objetivo evaluar los posibles cambios en los parámetros histológicos y morfométricos de los ovarios, así como en los niveles plasmáticos de estrógeno y progesterona de las hembras de *Leptodactylus macrosternum* y su relación con la temperatura y la precipitación. Los especímenes fueron recolectados en el área Horto Florestal Olho d'Água da Bica - HFOB (06 ° 49´20"S / 36 ° 15´85"W) en el municipio de Cuité, semiárido brasileño, cada 15 días, ocurrieron entre los meses de mayo a agosto y de noviembre a diciembre. Se utilizó la cuantificación de la densidad poblacional de los tipos de ovocitos y las dosis de progesterona y niveles de estrógenos para determinar la actividad reproductiva de la espécie. Las densidades de población de ovogonias y ovocitos II no variaron significativamente durante el experimento. La densidad poblacional de ovocito I fue significativamente mayor durante los meses de junio (p = 0.04) y agosto (p = 0.03). Los valores más altos para la densidad poblacional de ovocito III y IV se encontraron en los meses de mayo (p = 0.04) y junio (p = 0,04). Las cantidades de concentraciones de estrógeno (pg / ml) y progesterona (pg / ml) no cambiaron significativamente durante los períodos de muestreo. La densidad de población de los tipos de ovocitos III y IV mostró dependencia de la variación de las precipitaciones.
Palabras clave: Ranas; Ovocito; Lluvia; Semiárido; Hormonas sexuales.

1. Introduction

Factors which determine the reproductive success of anurans involve the adaptation of their reproductive cycles to the environment in which they live (Haddad & Prado, 2005). Tropical regions with seasonal pattern, as in the Brazilian semiarid, adaptation to these changes requires Species that habitat tropical regions with accentuate seasonality concentrate their reproductive activity during the period that their offspring has higher changes to survive (Anthes et al., 2010; Oliveira et al., 2008). Thus, organisms perceive environmental changes and promote physiological adjustments to determine their reproductive cycles according to the necessary environmental conditions (W. Duellman, 1985).

With specific reference to amphibians, the abiotic environmental conditions, which most influence the occurrence of reproductive activity, are the air temperature and relative humidity: both highly related to rainfall (Sasso-Cerri et al., 2004). However, the reproductive period can also occur during the dry season, when there is availability of appropriate environments such as permanent water bodies (Bastos et al., 2003). These factors associated with endogenous rhythm are responsible for the control of gametogenesis in various frog species (Sasso-Cerri et al., 2004).

In semi-arid regions, the occurrence and reproduction of most species are associated with the rainy season (Chaves, Moura, et al., 2017, p. 7; Prado & Uetanabaro, 2000; Rodrigues & Bertoluci, 2002). Therefore, these variables determine the favorable time of year when frogs remain active, causing their seasonal activity pattern (W. E. Duellman & Trueb, 1994).

In these areas, the production peaks of the major sex hormones coincide with physiological preparations for the breeding period (Wilczynski et al., 2005), where in males highest hormone production rates result in the development of secondary sexual characteristics, maintaining sexual behavior and starting the gametogenesis of anurans (Chaves, Moura, et al., 2017; Moore et al., 2005).

To test the hypothesis of the influence of seasonal variations on the reproductive dynamics of frogs in the caatinga areas, we used the *Leptodactylus macrosternum* is a biological role model (Arzabe, 1999; Leal et al., 2003; Velloso et al., 2002). Given its wide distribution in the semiarid region, it presents generalist behavior and is well adapted to the disturbed areas (De La Riva & Maldonado, 1999). In the Northeast region, this species is a hunting target for human subsistence (Alves et al., 2009) and is sometimes used for husbandry purposes as an alternative for alien species in frog farms (eg .: *L. catesbiana*, Shaw, 1802) (Cunha & Delariva, 2009). Therefore, this study aims to evaluate possible changes in the histology of the ovaries and the levels of estrogen and progesterone in females of the target species (*L. macrosternum*), and its relations with rainfall and temperature variations in the study area.

2. Methodology

This is a descriptive study delineated in a specific semi-arid region at Paraíba, which used a qualitative and a morphoquantitative analysis of specimens, and a quantitative analysis of female sex hormones.

Study and sampling area

Night expeditions were undertaken every 15 days during the period between January and December 2013 in order to verify the reproductive activity of *L. macrosternum* females. The females were collected manually in the Horto Florestal Olho d'Água da Bica - HFOB (06 ° 49'20 "S / 36 ° 15'85"W) (Figure 1) area in the municipality of Cuité, state of Paraíba, Brazil with prior authorization from IBAMA (44134-1). The specimens collected were placed in the Herpetological Collection of the Federal Rural University of Pernambuco- UFRPE (municipality Recife, state of Pernambuco, Brazil).

The area is situated in the Brazilian caatinga region and is characterized by its hot, dry climate with temperatures ranging between 17° and 28 ° C, average monthly rainfall of 76.35 mm and relative humidity of approximately 70%. It presents a peculiar hydrography, with ephemeral rivers, ponds, streams, reservoirs and wetlands as well as sloped areas from which the water sources flow. The region is made up of 70 hectares of bush and arboreal caatinga (Abrantes et al., 2011).

The monthly weather temperature (° C) and rainfall (mm) data were obtained from the Executive Agency Centre of Water Management of the state of Paraíba (AESA) database, at the weather station neighboring the sampling location.

Figure 1 - Location of *Leptodactylus macrosternum* sampling points in the Horto Florestal Olho d'Água da Bica (HFODB) area in the municipality of Cuité - state of Paraíba – Brazil between January and December 2013.

Source: Authors.

Histological analysis

The animals were taken to the Biosystematics Amphibian Laboratory (LABAN) of the Federal University of Campina Grande-UFCG, Cuité Campus- state of Paraiba, Brazil, and were euthanized by anesthetic overdose (lidocaine 5%). With the use of ophthalmic surgical materials, we proceeded with the dissection and removal of the ovaries. The ovaries were fixed in 10% formalin solution for 24 hours and then dehydrated in an ascending series of alcohol. The embedding was performed with paraffin and 5μm transverse sections in order to obtain slides, which were subsequently stained with hematoxylin and eosin (Stevens & Bancroft, 1990). Qualitative analysis was obtained in a conventional, trinocular, bench microscope, (Olympus AX70, Tokyo, Japan - UFES), coupled to a digital image acquisition system (camera ERC 5s and Axiovision 6.3, Carl Zeiss, Jena, Germany - UFES). The quantification of the types of oocytes was performed by the density method in a test area of 88 mm^2 (Axiovision 6.3, Carl Zeiss, Jena, Germany - UFES). The cell types were identified according previous works (J. S. Baptista et al., 2020; V. I. de A. Baptista et al., 2019).

Hormone dosage

In laboratory, blood samples were collected in previously heparinized syringes (Liquemin, Roche 0.1% solution). After collection, the samples were transferred to 1.5 mL micro centrifuge tubes then submitted to cooled centrifugation for 10 minutes at a rotation of 300 rpm. After centrifugation, plasma was separated into new tubes and stored at -20 ° C. Dosages of progesterone and estrogen levels were performed using the ELISA method.

Data analysis

The values of the density of cells in the reproductive lineage and in progesterone and estrogen levels were assessed monthly by the Kruskal-Wallis test, with Dunn's post-hoc tests. The covariation between climate variables (rainfall and temperature) and the population density of oogonia and oocytes was verified by the simple linear regression test. Statistical significance was considered to be $p < 0.05$(Zar, 1999).

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3. Results

At the end of the collection period, 24 *L. macrosternum* females were sampled, distributed between the months of May (4 individuals), June (4 individuals), July (5 individuals), August (4 individuals), November (3 individuals) and December (4 individuals) of 2013, coinciding with the rainy months in the region. Between the months of January to April and September to October 2013, no *L. macrosternum* individuals were found during field trips.

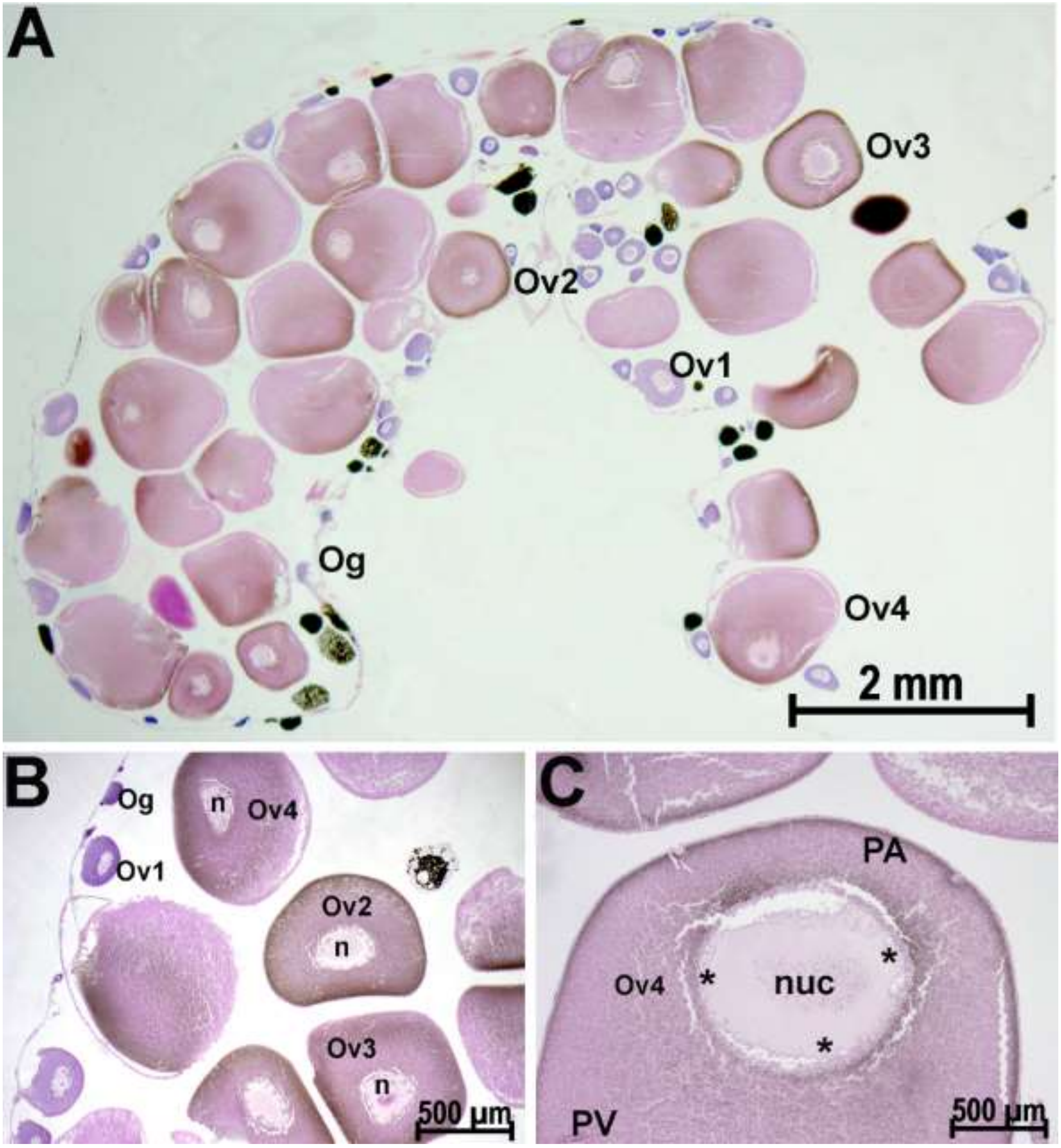
It was possible to verify all cell types constituting the *L. macrosternum* ovary (Figure 2). The oogonia (og) (Figure 2 A) were confirmed as the smaller cells of the oogenic line; located in the periphery and organized in clusters. The oocytes I (Ov1) (Figure 2 B) have a slightly larger cell volume than the oogonia, with a bulky cytoplasm of rough, grainy appearance and basophils with a large nucleus, showing a clear and well-defined outline.

The oocytes II (Ov2) (Figure 2B) had abundant cytoplasm, a grainier appearance and a less basophil aspect compared to the oocytes I, also having a large nucleus and evident nucleolus with dispersed chromatin.

The oocytes III (Ov3) (Figure 2B) is characterized by its intense process of vitellogenesis, with clear appearance in colorations with H-E and growing accumulation of eosinophils granules (yolk granules), occupying much of its length. The core is large, granular, centered and slightly basophil.

The oocytes IV (Ov4) (Figure 2C) are the major cell types present in *L. macrosternum* ovaries. In this development phase, the cell polarization process (animal and vegetal poles) was clear. The core is large, shifting towards the animal pole, presenting in its large nucleolus interior, interspersed chromatin which in turn, display a slightly basophil aspect. The cytoplasm presents an intense vitellogenic process (vegetal pole) and remains full of calf, with acidophilus aspect.

Figure 2 - Histologic sections of Leptodactylus *macrosternum* tests, stained in HE, collected between the months of May to August and November to December in the Horto Florestal Olho d'Água da Bica (HFOB) area in the municipality of Cuité - state of Paraíba – Brazil. [Cross-section of the *Leptodactylus macrosternum* ovary (Miranda-Ribeiro, 1926) (H/E); Oogonia (Og), oocytes I (Ov1), oocytes II (Ov2), oocytes III (Ov3) and oocytes IV (Ov4); B- Detail of oocyte I (Ov1), *- nucleus; Detail of oocyte II (Ov2), *- nucleus, - chromosomes dispersed in the nuclear membrane periphery; Detail of oocyte III(Ov3), *- nucleus, nuc – nucleolus; C- Detail of oocyte IV(Ov4), PA – animal pole, PV – vegetal pole, n – nucleus, nuc – nucleolus.]



Source: Authors.

Densities of oocyte cell populations showed significant differences (p <0.05, Kruskal-Wallis) over the months of collection (Table 1). Population densities of oogonia and oocytes II did not vary significantly during the months in which the animals were sampled. Population densities of oocyte I was significantly higher during the months of June (p= 0.04) and August (p= 0.03). The highest values for the oocyte III and oocyte IV population densities were observed during the months of May (p=0.04) and June (p=0.04) 2013.

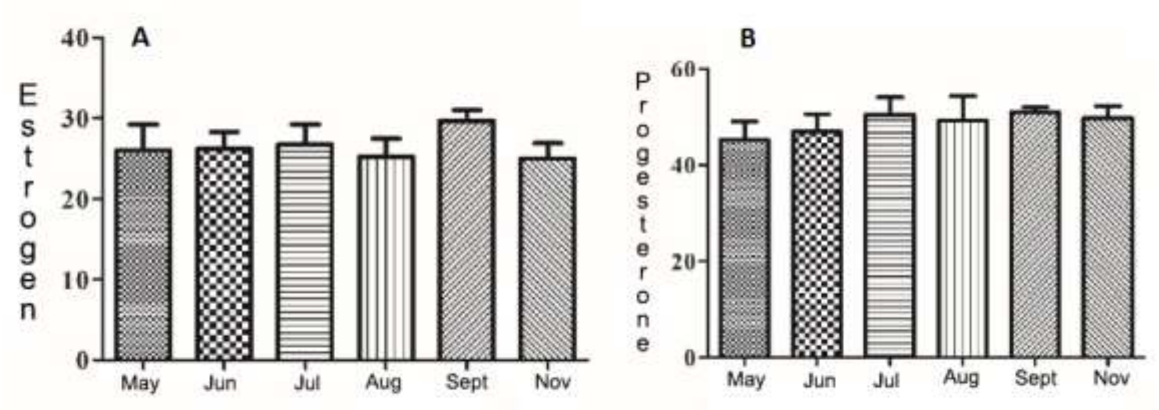
Table 1 - Monthly population density values (average and standard error) (mm^2) in cells present in ovaries of *Leptodactylus macrosternum* (Miranda- Ribeiro, 1926) species collected from May to August and November to December in the Horto Florestal Olho d'Água da Bica (HFOB) area in the municipality of Cuité - state of Paraíba – Brazil. N – monthly number of females; Og – oogonia; Ov1 – oocyte I; Ov2 – oocyte II; Ov3 – oocyte III, Ov4 – oocyte IV. * Represents significant differences (p < 0.05, Kruskal- Wallis).

	N	Og	Ov I	Ov II	Ov III	Ov IV
May	4	0.03± 0.01	0.09± 0.02	0.03± 0.01	0.21± 0.03*	0.14± 0.02*
Jun	4	0.02± 0.01	0.18± 0.02*	0.06± 0.01	0.17± 0.01*	0.15±0.01*
Jul	5	0.01± 0.01	0.03± 0.01	0.02± 0.01	0.05± 0.01	0.08± 0.05
Aug	4	0.01± 0.01	0.14± 0.01*	0.05± 0.01	0.09± 0.01	0.06 ± 0.01
Nov	3	0.02± 0.01	0.12± 0.01	0.05± 0.01	0.08 ± 0.01	0.07± 0.02
Dec	4	0.01± 0.01	0.06 ± 0.02	0.02± 0.01	0.05 ± 0.02	0.07± 0.02

Source: Authors.

Estrogen (pg/ml) and progesterone (pg/ml) concentrations did not change significantly over the sampling periods (p <0.05, Kruskal-Wallis). Estrogen levels (pg/ml) ranged from 25 to 27 pg / ml between the months of May and December 2013 (Figure 3A). Progesterone levels ranged from 43 to 45 pg/ml between the months of May and December 2013 (Figure 3 B).

Figure 3 - A – Monthly values (average and standard error) for estrogen (pg/ml) and; B – progesterone (pg/ml) in *Leptodactylus macrosternum* (Miranda- Ribeiro, 1926) females collected between the months of May to August and November to December in the Horto Florestal Olho d'Água da Bica (HFOB) area in the municipality of Cuité - state of Paraíba – Brazil.



Source: Authors.

As shown in Table 2, only the oocyte III (r^2 = 0.11; p = 0.04) and oocyte IV (r^2 = 0.16; p = 0.04) population densities showed a significant relationship with precipitation. Similarly, significant relationships were observed between the variation of temperature and oocytes III (r^2 = 0.27; p = 0.01) and oocytes IV (r^2 = 0.19; p = 0.03) population densities.

Table 2 - Relations between the density of Og – oogonia; Ov1 – oocyte I; Ov2 – oocyte II; Ov3 – oocyte III, Ov4 – oocyte IV in *Leptodactylus macrosternum* females in relation to rainfall and temperature in the Horto Florestal Olho d'Água da Bica (HFOB) area in the municipality of Cuité- state of Paraíba, in the periods from May to August and November to December 2013. * = Represents significant differences.

	Rainfall		**Temperature**	
	r^2	**p**	r^2	**p**
Og	0.04	0.4	0.04	0.77
Ov1	0.02	0.7	0.01	0.2
Ov2	0.21	0.5	0.05	0.5
Ov3	0.11	0.04*	0.27	0.01*
Ov4	0.16	0.04*	0.19	0.03*

Source: Authors.

4. Discussion

Identification and characterization of these cells in the ovaries of *L. macrosternum* species were similar to those observed in the literature: Hermosilla et al. (1983) evaluating the ovaries of *Caudiverbera caudiverbera* (Linnaeus, 1758); Oliveira and Andrade (1997) checking the ovaries of *Scinax fuscovarius* (Peters, 1871); Prado et al. (2004) in working with the ovaries of *Leptodactylus labyrinthicus* (Spix, 1824); Oliveira and Santos (2004) analyzing the ovaries of *Scinax fuscovarius*; and Azzouzi and Tekaya (2007) in work undertaken with the *Dicoglossus* genus. Therefore, it can be observed that these characteristics are quite conservative in frogs since similarities occur in different and phylogenetically distant species.

In this study, the low precipitation levels observed associated with the high temperatures recorded in 2013 (Agência executiva de águas do estado da Paraíba, 2014), are factors that explain the absence and the low sampling of female *L. macrosternum*. The occurrence and reproduction of most frog species are associated with the presence of rain, thus determining this pattern of frog active (Araújo et al., 2020; Chaves, Moura, et al., 2017; Chaves, Tenório, et al., 2017; Prado & Uetanabaro, 2000; Rodrigues & Bertoluci, 2002).

Low precipitation volumes and high temperatures are factors, which influences the frog activy in their environments (Prado & Uetanabaro, 2000; Rodrigues & Bertoluci, 2002). In caatinga areas, unpredictability and temporal and spatial distribution of rainfall, often compounded by high annual temperatures (Ab'Sáber, 2012; Velloso et al., 2002), directly affect the onset and duration of the breeding season of anurans (Araújo et al., 2020; Chaves, Moura, et al., 2017; Chaves, Tenório, et al., 2017; Gottsberger & Gruber, 2004). Adaptations in the areas are expected as part of anuran populations such as the reproductive activity concentrated during the rainy season, with short reproduction periods (Arzabe, 1999; Chaves, Moura, et al., 2017).

As expected., the significant presence of oocytes in an advanced stage of differentiation (oocytes III and IV) during the months of May and June (the months with the highest precipitation levels and the lowest average temperature in the HFOB area) confirm that *L. macrosternum* has a short breeding period, with ovogenesis peaks concentrated in May and June with the month of July having the highest reproductive potential. During the rainy season, frogs may present an increased gonadal development, a larger body size, a higher growth rate as well as greater accumulation of energy reserves (Brown et al., 2011; Chaves, Moura, et al., 2017).

In tropical regions of seasonal climate, the reproductive activity of anurans is sincronized to the rainy season (Santos & Oliveira, 2008) supporting the absence of Register of activityin the dry period between the months of January and April, with lower humidity and rainfall rates in the Curimatau macroregion in the state of Paraíba.

However, the occurrence of oocytes III and IV s in the months with lower precipitation volumes and higher average temperature (August, November and December 2013) in the study area, we can affirm that in this period, different degrees of fertility were observed. This did not completely halt reproductive activity in comparison to the other observed in this study, however no specimens were captured during the months of September and October 2013. Thus, it is probable that in seasonal environments, these animals have a constant production of gametogenesis, with partial interruption of this activity however, when the environments where these animals occur do not present ideal weather conditions for reproduction. Therefore, this information corroborates the research by Loft (1974) when classifying this type of reproductive cycle as "potentially continuous" and typical of seasonal, tropical environments (Lofts, 1974).

Although the concentrations of estrogen and progesterone do not show significant variations in the sampled months, it is supposed that these hormones are constantly related to the maturation process, regardless of other factors. In fact, it is already postulated that in amphibians, sex steroids play an important role in gonad development and oocyte maturation (Madelaire & Gomes, 2016; Moore et al., 2005; Scherer, 1999).

Linear regression analysis was effective to confirm the relationship between ovarian follicle densities at an advanced stage of development (oocyte III and IV) and precipitation levels and monthly temperature averages. This shows that *L. macrosternum* presents short reproductive cycles. The variation of monthly temperature averages showed a significant relationship with the density of both oocytes III and oocytes IV, showing that in caatinga areas, temperature variation is determinant in the reproductive activity of these animals (Chaves, Moura, et al., 2017).

5. Conclusion

Our results confirm the initial research hypothesis, that environmental and abiotic conditions (air temperature and precipitation) can interfere with frog reproductive activity in highly seasonal environments (Sasso-Cerri et al., 2004), despite no changes in hormones levels. This indicates L. macrosternum females reproduce during the rainy season, and temperature and rainfall affects their reproductive cycle as well as their activity pattern (W. E. Duellman & Trueb, 1994; Moore et al., 2005). Specific to these animals, and for the region and the period studied, the oocyte maturation processes are correlated with weather patterns, with the reproductive activity of the species classified as “potentially continuous," “or opportunistic’’ however, with a defined ovogenesis peak between 30-60 days in May and June. Future work should focus on the analysis of other hormonal and growth factors that may be involved with seasonality and abiotic factors that influence the reproduction of these animals.

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