Comparative leukocyte morphometric analysis between endemic Anurans from Brazil and the invasive species Lithobates catesbeianus

Análise morfométrica comparada entre Anuros endêmicos do Brasil e a espécie invasora Lithobates catesbeianus

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Abstract

Amphibians are potentially reliable and efficient bioindicators. Existing anuran white blood cell morphology studies are limited, with only a few morphometric studies available. We employed morphometric techniques to characterize leukocytes of selected Neotropical anurans from Brazil and compare our findings with the exotic American Bullfrog (*Lithobates catesbeianus*), genus *Ranidae*. We compared blood smears of 28 specimens from six different genera (*Hyla*, *Phyllomedusa*, *Hypsiboas*, *Scinax*, *Physalaemus,* and *Proceratophrys*) with samples from *L. catesbeianus*. Leukocyte average diameter was calculated by an image analysis software. One-way analyses of variance and Bonferroni tests were used on statistical analyses. Lymphocytes, neutrophils, eosinophils, and basophils were significantly smaller than the reference ranges reported for other amphibian genera, including *Lithobathes*, whereas monocyte diameters did not differ significantly between genera. This is the first study to evaluate leukocyte morphometrics of Brazilian anuran species. Our findings suggest that geographical separation could possibly influence leukocyte morphometry.

Keywords: Anura. Morphometry. South America. White blood cells.

Resumo

Anfíbios são indicadores ambientais potencialmente confiáveis e eficientes. Estudos referentes à morfologia de leucócitos de anuros são limitados, com poucos estudos morfométricos disponíveis em literatura. O presente estudo empregou técnicas morfométricas para caracterizar leucócitos de anuros Neotropicais brasileiros selecionados e compará-los com a espécie exótica rã-touro (*Lithobates catesbeianus*), família *Ranidae*. Esfregaços sanguíneos de 28 espécimes pertencentes a seis gêneros diferentes (*Hyla*, *Phyllomedusa*, *Hypsiboas*, *Scinax*, *Physalaemus* e *Proceratophrys*) foram comparados com amostras de esfregaços de *L. catesbeianus*. A média do diâmetro dos leucócitos foi calculada por um software de análise de imagens. One-way e teste de Bonferroni foram utilizados para avaliação estatística. Linfócitos, neutrófilos, eosinófilos e basófilos mostraram-se significativamente menores que os valores de referência reportados em outros gêneros de anfíbios, incluindo *Lithobathes*; por outro lado, a média do diâmetro dos monócitos não demonstrou variação significativa entre os gêneros. Esse é o primeiro estudo de avaliação morfométrica de leucócitos em espécies de anuros brasileiros. Nossos resultados sugerem que a separação geográfica possivelmente influencia a morfometria leucocitária.

Palavras-chave: Anura. Morfometria. América do Sul. Leucócitos.

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Introduction

Amphibians often correspond to a significant proportion of the vertebrate biomass in forest and wetland ecosystems (HAMER;MCDONNELL, 2008), therefore being potentially reliable and efficient bioindicators (GÜL; TOSUNOĞLU; ERDOĞAN, 2011; MAHAPATRA et al., 2012; ZHELEV et al., 2013), very sensitive to the slightest fluctuations in their habitat (GÜL; TOSUNOĞLU; ERDOĞAN, 2011; MAHAPATRA et al., 2012).

The hematopoietic tissue is particularly sensitive to environmental changes and is an important indicator of physiological response and stress agents, which are quickly reflected in hematologic parameters (ALLENDER; FRY, 2008; DAVIS; MANEY; MAERZ , 2008; SHUTLER; MARCOGLIESE, 2011). Hematologic and biochemical evaluation of amphibians is of great clinical importance, and provides valuable diagnostic information and understanding of the pathogenesis of diseases, their progression, and response to therapy (SCHMID-SCHÖNBEIN et al., 1980; ALLENDER; FRY, 2008; DAVIS; DURSO, 2009; YOUNG et al., 2012). Nevertheless, there are few studies available on Neotropical amphibian hematology (CABAGNA-ZENKLUSEN et al., 2005; CABAGNA-ZENKLUSEN et al., 2011; LAJMANOVÍCH et al., 2012), and scarce studies on anuran white blood cell morphology (ARIKAN; ÇIÇEK, 2010; DAS; MAHAPATRA, 2012). Few morphometric studies of red blood cells (BARNI et al., 1992; ATATÜR et al., 1999; WOJTASZEK; ADAMOWICZ, 2003; GRENAT et al., 2009) and white blood cells (FOXON 1964; NANO et al., 1991; TOZETTI et al., 2014) are available. To the authors' knowledge, this is the first study on white blood cell morphology in Neotropical anurans from South America.

We employed morphometric techniques to characterize leukocytes of selected Neotropical anurans from Brazil. We also compared our findings with an exotic species of the same order: the American Bullfrog (*Rana catesbeiana*), genus *Ranidae,* recently named *Lithobates catesbeianus* (FROST et al., 2006).

Materials and Methods

Sample Collection and Processing

Because of technical restrictions, we evaluated 28 out of 55 free-ranging animals collected at Chapada Diamantina National Park, in the state of Bahia, Brazil. All animals were individually placed in labeled plastic bags that allowed free air exchange, over a substrate of leaves and a small amount of water to mimic their natural habitat. Bags were placed in a quiet and temperature-controlled environment in order to minimize stress, until the administration of topical lidocaine (Xylocaína 5%, AstraZeneca do Brasil. Cotia, SP, Brazil). Sample collection and humane euthanasia were performed only after the animals were anesthetized and unconscious.

Blood sampling through cardiocentesis avoided sampling limitations related to the small size of the individuals and reduced the amount of circulating blood. Each animal was placed on dorsal recumbence while the cardiac pulse could still be clearly discerned. Blood samples were immediately collected with a disposable 25 G x 5/8 in. needle, inserted in the direction of the ventricular apex. Insulin needles were used in the smaller species. A single drop of blood was placed on the edge of a slide, and equally distributed by a spreader slide angled at 45°. The coelomic cavity was incised immediately after anesthetic absorption and cessation of cardiac contractions in those species considered too small for cardiocentesis. The whole heart was excised, sectioned and slid on the slide surface, so the blood cells present in the tissue section could be visualized. Slides were stained with Rosenfeld stain using an adapted protocol version based on Rosenfeld (1947).

We analyzed blood smears from 28 Neotropical specimens, from six different genera: *Hyla* ($n = 7$), *Phyllomedusa* ($n = 5$), *Hypsiboas* ($n = 5$), *Scinax* ($n = 4$), *Physalaemus* ($n = 4$), and *Proceratophrys* ($n = 3$). To enable the comparison between the Brazilian and North American species, we included blood smears of *Lithobates catesbeianus* ($n = 6$).

All animals used in this study were collected in full compliance with specific federal permits issued by the Brazilian Ministry of Environment and approved by the Biodiversity Information and Authorization System – SISBIO, License nº 14555-2, to be incorporated into the collection of the Museum of Zoology, University of São Paulo (MZUSP). All procedures were performed according to the Ethical Committee in Animal Research of the College of Veterinary Medicine and Animal Sciences, University of São Paulo (Proccess number 1686/2009).

Slide Interpretation

Leukocytes were analyzed and counted under 100x magnification and immersion oil. We identified and documented the following blood cell types: neutrophils, eosinophils, basophils, monocytes, and lymphocytes. Leukocytes were identified and evaluated according to previous authors (WRIGHT 1996; CATHERS et al., 1997; DAVIS; DURSO, 2009; HEATLEY; JOHNSON, 2009). Thrombocytes and erythrocytes were not included in this study.

Cell Measurement and Data Analysis

Blood smears were observed under a microscope equipped with a digital camera and connected to a computer with image analysis software (Image ProPlus, v 5.1.2.59 Media Cybernetics, Inc., Bethesda, MD). The whole slide was examined and all visualized leukocytes were individually analyzed and manually outlined to determine the cell average diameter (in µm, equivalent to the mean measurement of the diameters measured at 5 intervals around the center of the cell). We chose this technique because of the small size of some of the evaluated specimens and consequently small blood volume available for analysis.

Statistical analysis was performed by One-way analysis of variance followed by Bonferroni tests, with the significance level set at 5%. Data were analyzed with GraphPad Prism (Prism 4.02 for Windows, GraphPad Software Inc.).

Results

Lymphocytes were basophilic, showed round nuclei with dense chromatin aggregates and scarce cytoplasm with frequent cytoplasmic "pseudopods". Lymphocytes of the *Scinax* genus were significantly smaller than the ones from *Hyla*, *Phyllomedusa* ($P < 0.05$), and *Lithobathes* ($P < 0.01$) genera. *Hypsiboas* lymphocytes were also smaller than *Lithobathes* ($P < 0.05$), but no differences were found in relation to *Scinax* (Figure 1a; Table 1).

The majority of amphibian neutrophils had lobulated nuclei. Neutrophils of the *Scinax* genus were significantly smaller than the ones from the *Hyla* ($P < 0.01$), *Phyllomedusa* ($P < 0.05$) and *Lithobathes* ($P < 0.05$) genera. (Figure 1b; Table 1). *Physalaemus* neutrophils were also smaller than *Hyla*, *Phyllomedusa* ($P < 0.01$), and *Lithobathes* ($P < 0.05$), but no differences were found between *Scinax* and *Physalaemus*.

Eosinophils were similar to neutrophils, but presented big, refringent, and eosinophilic intracytoplasmic granules. Eosinophils of the *Scinax* ($P < 0.01$) and *Physalaemus* ($P < 0.05$) genera were significantly smaller than the ones from *Lithobathes* genus. *Proceratophrys* neutrophils were smaller than *Hyla* ($P < 0.01$) and *Lithobathes* ($P < 0.001$) (Figure 1c; Table 1).

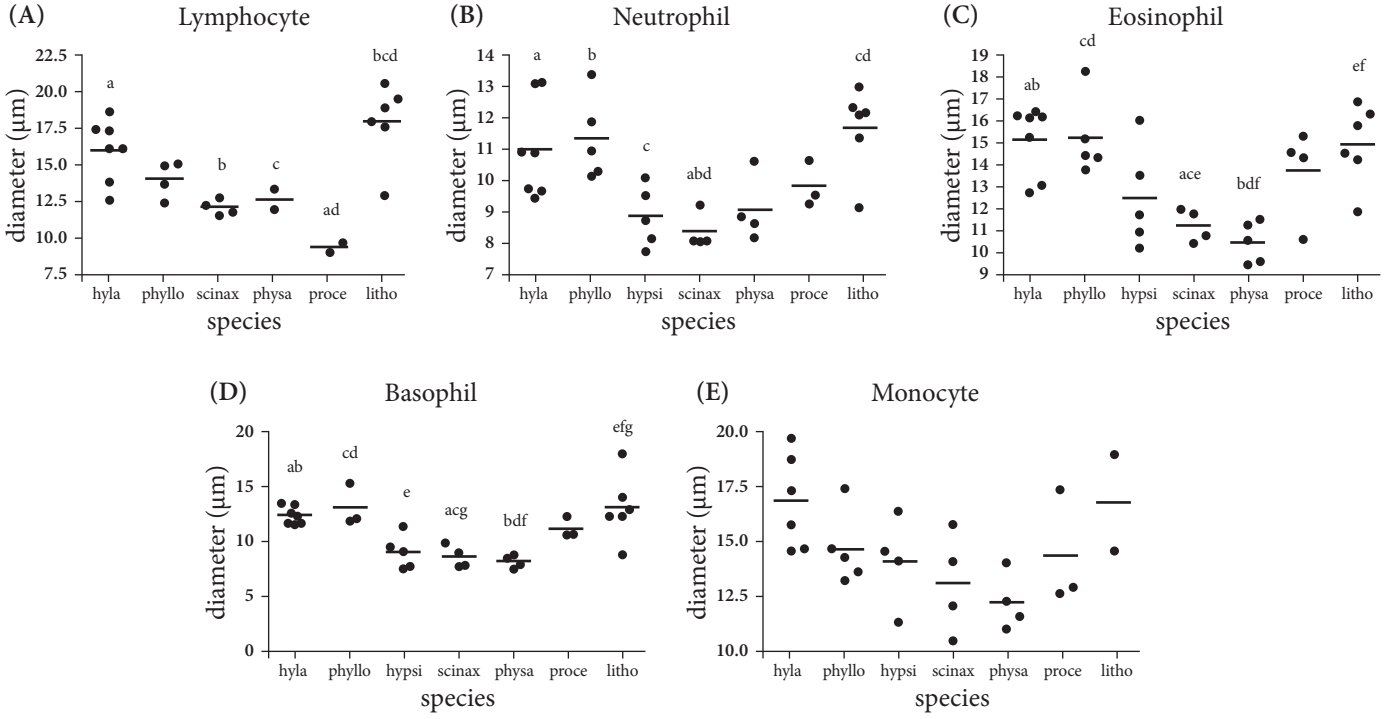

Figure 1 - Leukocyte diameter values for the analyzed species: (a) Lymphocytes, (b) Neutrophils, (c) Eosinophils, (d) Basophils, and (e) Monocytes

Table 1 - Leukocyte diameter values for the analyzed genera and species – São Paulo – October 11, 2016

Genus	Parameters	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes
Hyla	Mean ± SD	10.98 ± 1.60^{a}	15.16 ± 1.58^{ab}	16.05 ± 2.13^{a}	12.37 ± 0.80^{ab}	16.78 ± 2.16
	Max – Min	(9.39–13.16)	(12.73 – 16.43)	(12.66 – 18.65)	(11.59 – 13.44)	(14.55 – 19.70)
Phyllomedusa	Mean ± SD	11.33 ± 1.35^{b}	15.20 ± 1.78^{cd}	14.05 ± 1.25	13.09 ± 2.00^{cd}	14.60 ± 1.65
	Max – Min	(10.14–13.4)	(13.78 – 18.23)	(12.44 – 15.11)	(11.83 – 15.40)	(13.18 – 17.37)
Hypsiboas	Mean ± SD	8.82 ± 0.99^{c}	12.50 ± 2.33	–	9.00 ± 1.60^{e}	14.05 ± 2.08
	Mean ± SD	(7.69 – 10.08)	(10.23 – 16.04)	–	(7.42–11.39)	(11.29 – 16.32)
Scinax	Max – Min	8.33 ± 0.59^{abd}	11.25 ± 0.74^{ace}	12.12 ± 0.52^{b}	8.56 ± 1.06^{acf}	13.04 ± 2.31
	Mean ± SD	(8.02 – 9.20)	(10.46 – 11.97)	(11.60 – 12.79)	(7.65 – 9.85)	(10.42 – 15.71)
Physalaemus	Max – Min	9.04 ± 1.07	10.51 ± 0.92^{bdf}	12.68 ± 1.00^{c}	8.14 ± 0.61^{bdg}	12.19 ± 1.31
	Mean ± SD	(8.16 – 10.59)	(9.49 – 11.53)	(11.97 – 13.39)	(7.44 – 8.77)	(10.99 – 14.00)
Proceratophrys	Max – Min	9.79 ± 0.76	13.72 ± 2.09	9.40 ± 0.44^{ad}	11.18 ± 0.98	14.27 ± 2.67
	Mean ± SD	(9.23 – 10.64)	(10.64 – 15.30)	(9.08 – 9.71)	(10.57 –12.30)	(12.60 – 17.35)
L. catesbeianus	Mean ± SD	11.69 ± 1.37^{cd}	14.95 ± 1.81^{ef}	17.94 ± 2.68^{bcd}	13.03 ± 3.01^{efg}	16.73 ± 3.14
	Max – Min	(9.13 – 13.02)	(11.90 – 16.88)	(12.96 – 20.62)	(8.78 – 18.03)	(14.51 – 18.95)

Similar letters indicate significant differences. No eosinophils were observed in the *Hypsiboas* genus

Basophils were round-shaped and in some cases elliptical, presenting well delimitated and numerous intense basophilic granules that prevented the observation of the nucleus. The outlining of the cell followed the granules adhered to the cytoplasmic membrane. Basophils of the *Scinax* genus were significantly smaller than the ones from the *Hyla* ($P < 0.05$), *Phyllomedusa* ($P < 0.05$), and *Lithobathes* ($P < 0.01$) genera. Basophils of the *Physalaemus* genus were significantly smaller than the ones from the *Hyla* ($P < 0.05$), *Phyllomedusa* ($P < 0.05$), and *Lithobathes* ($P < 0.01$) genera. Moreover, basophils of the *Hypsiboas* genus were significantly smaller than those from the *Lithobathes* genus ($P < 0.05$). No differences were found between *Proceratophrys* and any of the other evaluated genera (Figure 1d; Table 1).

In this study, the monocytes studied were horseshoe- or kidney-shaped, presenting eccentric nuclei. Monocyte diameters varied from 10.42 to 19.70 μm, but no significant differences were found between genera (Figure 1e; Table 1).

Discussion

Amphibians have suffered massive, widespread, often unexplained, and probably irreversible decline of population worldwide over the last several decades. Among the world's vertebrates, amphibians present the highest proportion of species on the verge of extinction (HAMER; MCDONNELL, 2008), with thousands of species with diminished population and hundreds on the brink of extinction or already vanished (STUART et al., 2004; POUNDS et al., 2006). Since 1980, between 9 and 122 species have been considered "possibly extinct" (STUART et al., 2004).

The amphibian population decline phenomenon is complex in origin and multifactorial in etiology, caused by some of the following identified causes: habitat loss and overutilization, fragmentation, degradation, pathogens, pollution, introduced exotic species, climate change and associated atmospheric processes, and overexploitation, including collection for pet and food industries (STUART et al., 2004; POUNDS et al., 2006; HAMER; MCDONNELL, 2008; BLAUSTEIN et al., 2011). Declines in population are nonrandom in terms of species' ecological preferences, geographic ranges, and taxonomic associations, probably varying between species, populations, and life stages within a population, and most prevalent in Neotropical mountainous stream-associated species (STUART et al., 2004; BLAUSTEIN et al., 2011).

According to the latest update of the List of Brazilian Amphibians (July 2014) there are 1026 recognized amphibian species in Brazil: 988 Anurans, 5 Caudatas, and 33 Gymnophionas (SEGALLA et al., 2014). However, the information about these species is scarce, and the accelerated global amphibian decline indicates the need to rapidly expand conservation research programs and emergency strategies (SILVANO; SEGALLA, 2014).

Blood analyses are useful in the diagnosis and monitoring of animal health and disease and in the differentiation of physiological processes (ARIKAN; ÇIÇEK, 2010). In environmentally sensitive animals such as anurans, variation in white blood cell parameters is also an indicator of environmental stress (DAS; MAHAPATRA, 2012). However, amphibian hematology is specially challenging because of a combination of several factors:

lack of analytical methods, challenges to sample collection (small size, few venipuncture sites, sample volume restrictions, and contamination of blood with lymph), and the variability in amphibian red blood cell (RBC) and white blood cell (WBC) counts (ALLENDER; FRY, 2008; ARIKAN et al., 2010; GÜL; Tosunoğlu; Erdoğan, 2011; MAHAPATRA et al., 2012).

Native to the eastern United States and Canada (GIOVANELLI et al., 2008; BOTH et al., 2011), *L. catesbeianus* was introduced in Brazil in the 1930s, in association with aquaculture (GIOVANELLI et al., 2008; ROCHA et al., 2010). *L. catesbeianus* was included in this study because of its economical importance and the variety of hematologic studies of this species available (CATHERS et al., 1997; COPPO et al., 2005; DAVIS, 2009; ROCHA et al., 2010).

Lymphocytes were by far the most frequently observed leukocyte in the evaluated species and genera, as previously reported in other amphibian species (ARSERIM; MERMER, 2008; ARIKAN et al., 2010; ARIKAN; ÇIÇEK, 2010; DAS; MAHAPATRA, 2012). The lymphocyte morphology observed in this study is consistent with descriptions by other authors (ARSERIM; MERMER, 2008; ARIKAN et al., 2010; CAMPBELL 2012; DAS; MAHAPATRA, 2012). We also observed different lymphocyte sizes (large and small), as previously described (WRIGHT, 1996; ARSERIM; MERMER, 2008; ARIKAN et al., 2010; ARIKAN; ÇIÇEK, 2010), but in order to simplify our correlations, we decided to classify these cells only as "lymphocytes", instead of identifying them according to size. In amphibians, the lymphocyte diameter varies between 10 and 25 µm in most species (WRIGHT, 1996; HEATLEY; JOHNSON, 2009; ARIKAN et al., 2010; CAMPBELL, 2012). The mean lymphocyte diameters of *Scinax* and *Hypsiboas* genera (8.33 ± 0.59 and 8.82 ± 0.99 µm, respectively) were similar to those of balloon frogs (*Glyphogloossus molossus,* 8.56 ± 2.74 µm, range 7.74 – 11.30 µm) (SINSCH, 1990).

Monocyte morphology reported in this study is in agreement with previous reports (ARSERIM; MERMER, 2008; ARIKAN; ÇIÇEK, 2010; OMONONA; EKPENKO, 2011; CAMPBELL, 2012). The mean monocyte diameters found for *Hypsiboas* (14.06 ± 2.09 µm), *Phyllomedusa* (14.60 ± 1.66 µm), and especially for *Proceratophrys* (14.27 ± 2.67 µm), were comparable to those found for Long–legged wood frog (*Rana macrocnemis*, 14.30 ± 0.19 µm, range 10.00 – 18.50 µm) (ARSERIM; MERMER, 2008), and the ones found for *Scinax* (13.05 ± 2.32 µm) were comparable to those of *G. molossus* (13.89 ± 3.34 µm) (PONSEN et al., 2008). As with other vertebrates, monocytes are phagocytic and can migrate to tissues to turn into macrophages. Apart from directly eliminating pathogens, monocytes and macrophages can process certain antigens and stimulate lymphocyte production (DAVIS; DURSO, 2009; CAMPBELL, 2012). Monocytes showed eccentric, horseshoe- or kidney-shaped nuclei, as reported by previous authors (ARSERIM; MERMER, 2008; ARIKAN; ÇIÇEK, 2010; OMONONA; EKPENKO, 2011; CAMPBELL, 2012).

Granulocytes include neutrophils, eosinophils, and basophils. Neutrophils are usually round-shaped, and may be defined as "small eosinophilic granulated lymphocytes", with a typical elongated shape (WRIGHT, 1996). The majority of amphibian neutrophils presented lobulated nuclei, but Allender and Fry (2008) have reported clinically normal amphibians with hyposegmented nuclei. Neutrophil morphology is in agreement with the literature (ARIKAN et al., 2010; ARIKAN; ÇIÇEK, 2010; DAS; MAHAPATRA, 2012; MAHAPATRA et al., 2012). Neutrophil diameters found for *Hyla* (15.24 ± 1.65 µm) and *Phyllomedusa* (15.19 ± 1.77 µm) were similar to those found for *L. catesbeianus* by Coppo et al. (2005) (15.2 ± 2.1 µm, range 11.3 – 20.5 µm), but not to the values for *L. catesbeianus* (17.93 ± 2.67 µm) found in the present study.

Eosinophil and basophil descriptions are in agreement with previous authors (ARSERIM; MERMER, 2008; ARIKAN et al., 2010; ARIKAN; ÇIÇEK, 2010). The eosinophil diameter found for *Hyla* (16.06 ± 2.13 µm) was comparable to those found for *R. macrocnemis* (16.30 ± 0.21 µm, range 11.75 – 19.75 µm) (ARSERIM: MERMER, 2008), fire-bellied toad (*Bombina bombina*, 16.40 ± 1.026 µm, range 15 – 18.5 µm) (ARIKAN et al., 2010), and *L. catesbeianus* (16.2 ± 2.5 µm, range 11.3 – 21.5 µm) (COPPO et al., 2005). However, *L. catesbeianus*' eosinophils were smaller in our study (14.94 ± 1.80 µm). Regarding basophils, *Phyllomedusa* (13.10 ± 2.00 µm) and *Lithobathes* (13.03 ± 3.02 µm) mean diameter values were comparable to those found in *R. macrocnemis* (13.69 ± 0.15 µm, range 10.50 – 16.25 µm) (ARSERIM; MERMER, 2008). As in other species, basophils may fulfill a role in surveillance and eosinophil recruiting on the event of a helminthic infection (WRIGHT, 1996; YOUNG et al., 2012). Degranulated basophils can also be identified in the bloodstream (ALLENDER; FRY, 2008), being relatively common in some species (YOUNG et al., 2012).

Conclusion

This study compared the mean diameters of leukocytes (neutrophils, basophils, eosinophils, lymphocytes, and monocytes) of six different amphibian genera within the anuran order native of Chapada Diamantina (South America), and the exotic North American species *L. catesbeianus,* showing significant differences in leukocyte morphometry. We initially expected some similarities in blood cell morphometry amongst the South American genera and significant differences with the North American genus. However, the South American genera presented few morphometric similarities, as shown in table 1. Our results suggest that the geographical separation between the North American genus and the six South American genera could possibly have influenced a certain differentiation in leukocyte morphometry, once the migratory behavior of anuran amphibians consists of short-distance or microgeographic dislocations, generally not longer than 1500 m, and site fidelity to these spatial units (SINSCH, 1990; RUSSELL et al., 2005). Further research in the field of amphibian hematology is needed to better characterize the Brazilian species. To the authors' knowledge, this is the first study to evaluate leukocyte morphology of Brazilian anuran species. We hope our results may contribute as a general reference and stimulate future leukocyte morphology and morphometric investigations involving anuran species from the Brazilian caatinga and *Lithobathes*.

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