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Article

Hematological parameters of a Neotropical wild frog population, with a phylogenetic perspective on blood cell composition in Anura

Lilian Franco-Belussi [a,*,†], Diogo B. Provete [a,b,†], Taynara R. F. Leão [c], Mayara S. Siqueira [a], Bruno S. L. Valverde [c], Brenda O. Martins [d], Classius De Oliveira [e], and Carlos Eurico Fernandes [a]

[a]Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul 79002970, Brazil, [b]Gothenburg Global Biodiversity Centre, Göteborg, Box 100, S 405 30, Sweden, [c]Graduate Program in Biodiversity. Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista, São José do Rio Preto, São Paulo, Brazil, [d]Graduate Program in Animal Biology. Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil, and [e]Departamento de Biologia. Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto, Universidade Estadual Paulista, Brazil

*Address correspondence to Lilian Franco-Belussi. E-mail: lilian.belussi@gmail.com†These authors contributed equally to this article.

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Abstract

Hematological parameters can provide key information to an animal health status. However, this information is usually hard to obtain. Here, we described the hematological parameters of *Leptodactylus podicipinus* in the Brazilian Pantanal. We measured red blood cell morphometrics, erythrogram, and leukogram. We also tested for phylogenetic signal in the erythrogram and leukogram of 48 frog species from 15 families, testing if body size explains their variation. Lymphocytes were the most abundant leukocytes (>60%) in *L. podicipinus*, followed by neutrophils (10%). Given that *L. podicipinus* is an abundant and widely distributed species in central Brazil, knowing its hematological pattern can help establish a baseline and improve its use as a bioindicator of environmental degradation. Mean corpuscular hemoglobin and value contributed more to the phylomorphospace of erythrogram, in which *Leptodactylus* spp. and *Hypsiboas raniceps* had lower values of these variables, whereas *Bufotes viridis* and *Hyla arborea* had high values. The phylogenetic signal was spread throughout the dimensions of the leukogram phylomorphospace. The variables that most contributed to it were total leukocytes counts, lymphocytes, and neutrophils. We also found a moderate phylogenetic signal for both the erythrogram and leukogram. Accordingly, body size accounted for a low proportion of variation in both the leukogram (4.7%) and erythrogram (0.57%). By applying phylogenetic comparative methods to hematological parameters, our results add a new perspective on the evolution of blood cell physiology in frogs.

Key words: erythrogram, leukogram, conservation physiology, macroevolution, population monitoring programs.

Hematology has long been used as a tool to study the physiology and health of frog species, especially in natural environments (Allender and Fry 2008). The analysis of hematological parameters provides relevant information for the diagnosis of many disorders

(Campbell 2015). Additionally, they can also be used as biomarkers for metabolic processes and physiological stress in response to changes in exogenous and endogenous conditions (Davis et al. 2008; Davis and Maerz 2011; Brodeur et al. 2020). Therefore, establishing baseline patterns is critical to interpret hematological responses to stressful environmental conditions.

Hematological dynamics may reflect early and late effects of environmental stressors to which individuals were exposed at early life stages (Ceccato et al. 2016). For instance, adult frogs whose tadpoles were exposed to UltraViolet radiation have lower leukocyte count, which increases their susceptibility to diseases (Ceccato et al. 2016). However, to assess and contrast hematological patterns, we need baseline values, which are still scarce for many Neotropical frog species.

Cell morphometrics has long been considered as an important indicator of cell plasticity (Rodrigues et al. 2017). Several physiological events cause morphological variations in size, volume, diameter, and relationships among structural components of red blood cells (Allender and Fry 2008; Garcia-Neto et al. 2020; Mahapatra et al. 2012; Silva et al. 2017). This may imply that subsequent adaptations that occur in blood cells involve alterations driven by critical physiological events (Garcia-Neto et al. 2020). However, whereas the hematological and erythrocyte morphometric features of several temperate frog species have been described, these aspects of tropical species are less known. These two sets of variables can help to find efficient biomarkers for testing the effect of environmental change on frogs, which can be useful in environmental monitoring programs.

The baseline leukocyte profile varies considerably among vertebrate taxa. For example, the most abundant white blood cell in mammals is the neutrophil, whereas there is high variation in amphibians, lizards, and fish, but a predominance of lymphocytes (Davis et al. 2008). The frequency of each type of leukocytes expresses the state of the immune response to physiological and continuous adaptation to environmental changes (Newman et al., 1997). While providing basic descriptions, previous studies (e.g., Glomski et al. 1997; Davis 2009; Davis and Durso 2009; Zhelev et al. 2017) have also compiled datasets of leukogram and erythrogram of amphibians to compare the parameters found for a given species with other known species or find general patterns. However, little is known about the evolutionary determinants of leukogram and erythrogram in frogs, and no study to date has explicitly applied phylogenetic comparative methods to provide a phylogenetically informed comparison of leukogram and erythrogram among species.

Our goal in this article is two-fold: first, we describe the hematological parameters and red blood cell morphometric features of the Pointedbelly Frog, *Leptodactylus podicipinus* (Cope 1862). Second, we provide a novel phylogenetic perspective on the evolution of hematological parameters in frogs by assembling the largest dataset to date with leukogram and erythrogram information for 48 anurans species from 15 families. Using this dataset, we tested for phylogenetic signal and also related species body size to erythrogram and leukogram. We expect a strong phylogenetic signal in the erythrogram, but not in the leukogram, because white blood cells tend to respond quickly to exogenous stress sources (Davis et al. 2008; Rollins-Smith 2017), whereas hemoglobin and hematocrit are determined by bone marrow at the embryogenesis (Glomski et al. 1997). Environmental stressors vary at a local scale, whereas our main goal is to evaluate the macroevolutionary pattern of white cell profile. Thus, we acknowledge that there can be variation in blood cell profile in response to several stressors, but we expect that interspecific variations will be larger, making the phylogenetic relationships between species a good representation of this pattern. We also expect body size to be a good predictor of those hematological parameters. Because the main organ involved in white blood cell maturation in anurans is the spleen (Franco-Belussi and De Oliveira 2016), and due to body size–spleen size relationships, we expect larger species to have larger spleens, which could potentially confer distinct white blood cell compositions. Our results can help not only understand the evolutionary pattern of leukogram and erythrogram, but also predict values for hematological parameters for additional species.

Materials and Methods

Animal model

We collected 22 adult females of *L. podicipinus* by active search in breeding sites at night at the UFMS Pantanal Field Station (19°34′ 37″ S; 57°00′42″ W), Corumbá, Central Brazil in February 2019. We used females in this study because *L. podicipinus* is a small species (4.67 ± 0.51 g) and females have more blood volume than males, making it easier to extract the amount needed to perform all analyses. In addition, previous studies found none or negligible differences in hematological and cell morphometric parameters between sexes (Forbes et al. 2006; Cabagna Zenklusen et al. 2011; Das and Mahapatra 2014). All specimens were transported to the laboratory and remained in acclimation for 12 h before blood sampling. *Leptodactylus podicipinus* is an abundant species and well-adapted to several environments, such as open areas, wetlands, lakes, and urban environments (AmphibiaWeb 2021).

For blood collection, we anesthetized and euthanized animals by topical application of 5% xylocaine. Afterward, we measured body mass (g) and snout–vent length (SVL; cm). All procedures for the maintenance and handling of animals were carried out in accordance with theNational Institute of Health Guide for Care and Use of Animals in the Laboratory and are approved by the Ethics Committee of the Federal University of Mato Grosso do Sul - (CEUA #997/2018).

Hematological analysis

Blood was collected by cardiac puncture in syringes and needles with 3% Ethylenediamine tetraacetic acid (Ranzani-Paiva et al. 2013). We calculated the following hematological parameters: hematocrit (%) using the microhematocrit technique (Goldenfarb et al. 1971); total hemoglobin (g dL^{1}) using the cyanomethaemoglobin technique (Collier 1944) with modifications, in which 5 L of blood was used; count of red blood cells and total leukocytes in Neubauer chamber (number of cells 106 L^{1}) after blood dilution (1:200) in Natt and Herrick (1952) solution. We calculated the following hematimetric indices from the values obtained in the hematocrit, total hemoglobin, and the number of erythrocytes: mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), and MCH concentration (MCHC, g dL^{1}), following Wintrobe (1934).

To obtain a differential leukocyte count, a blood smear was made immediately after blood collection, which was stained with May–Grunwald–Giemsa–Wright by the modified Rosenfeld method (Tavares-Dias and Moraes 2003). For this analysis, 100 leukocytes were counted in each smear to establish the percentage of each cell type (Rosenfeld 1947).

Erythrocyte morphometry

We captured 10 images of the smear per animal at 1,000 magnification with a Nikon 3500 camera attached to a light microscope (Zeiss Primo Star). In each image, we measured area (m^2), perimeter (m), and cell and nucleus diameter (m) of 5 erythrocytes, totaling 50 cells per animal. We used only 7 out of the 22 specimens for these measurements. Measurements were performed using Motic Images Plus version 2.0 (Rodrigues et al. 2017). From these data, we calculated second-order morphometric measurements: cell volume (m^3) based on the formula: EV = (4/3) * radius; nucleus: cytoplasm ratio (%) calculated by the formula: (nucleus area/cell area)*100; and nuclear volume (m^3) calculated by the formula NV = (cell volume * nucleus: cytoplasm ratio)/100. The shape factor (Sf) was calculated using the formula: Sf = (4..A)/P^2, where A = total cell area (m^2); P = perimeter (m). It ranges from 0 (elliptical shape) to 1 (circular shape).

Data analysis

To conduct phylogenetic comparative analysis, we first pruned the Maximum Clade Credibility Tree from Jetz and Pyron (2018) to only contain species to which we have trait data. Afterward, we obtained body size data for all species from the amphiBIO version 1 dataset (Oliveira et al., 2017), except for *Rhinella limensis*, whose SVL was taken from Vellard (1959), and *Telmatobius pefauri* and *Telmatobius peruvianus* whose body size were taken from (Veloso and Trueb, 1976) and (Schmidt, 1928), respectively. Then, we searched for leukogram and erythrogram data of frogs in the published literature using Google Scholar and a snowball approach. We only gathered data from wild populations, preferably females (because we had data for only females *L. podicipinus*) and excluded those used in experiments or infected with parasites. We found data for 48 species from 15 families of anuran amphibians (Franco-Belussi et al. 2021).

However, 38% of the data (30 species lack data) for leukogram were missing, whereas erythrogram variables had 27% of missing data (15 species lack data; see Supporting information). To provide a comprehensive analysis of the dataset, we used phylogenetic imputation to estimate missing values in the R package Rphylopars (Goolsby et al. 2016), assuming Brownian Motion (BM) as a model of evolution. This is the most accurate method to estimate missing data for phylogenetic autocorrelated data (see Penone et al. 2014) and performed better under a range of bias types (Johnson et al. 2020). Recently, a study (Jardim et al. 2021) found that measuring and testing for phylogenetic signal in simulated datasets with up to 30% of missing data and assuming BM had better performance. They also found that when traits were correlated (as in our case) and the missing data were phylogenetically correlated, the imputed values were closer to the real data (Jardim et al. 2021). The amount of missing data in our data set is very close to this threshold and given that we based our estimates on BM, we are confident that values are reasonable (see Franco-Belussi et al. 2021 for results with alternative evolutionary models and standard deviation of values). We also run the analysis using only species to which we have data, and the results did not change qualitatively (see Franco-Belussi et al. 2021).

Then, we conducted a phylogenetic Principal Component Analysis (phylo-PCA; Polly et al. 2013 ; Revell 2009; Collyer and Adams 2020) separately for the imputed leukogram and erythrogram data using the correlation matrix. This analysis starts by calculating a $\boldsymbol{C}$ matrix describing the expected variance–covariance structure of species given the phylogeny and BM as a model of trait evolution and a matrix $\boldsymbol{Z}$ that is the GLS mean-centered residuals to obtain an $\boldsymbol{R}$ matrix, onto which eigendecomposition is lastly done to obtain eigenvectors and eigenvalues, along with species scores (Revell 2009; Collyer and Adams 2020). To build a phylomorphospace (Adams and Collyer 2019), we plotted the first two PCs of each data set and then superimposed the phylogeny onto it. Analysis was conducted in the R package phytools (Revell 2012). Complementarily, we also built a phylogenetically aligned component analysis (PaCA; Collyer and Adams 2020) to show in which dimension of the data the phylogenetic signal was concentrated. PaCA is an alternative ordination method that rotates the reduced space to align with phylogenetic signal in species data.

We used all Principal Components (PCs) to test for a phylogenetic signal separately for the leukogram and erythrogram using the Blomberg's K statistic (Blomberg et al. 2003) generalized for multivariate data (K_{mult}; Adams 2014a, 2014b; Adams and Collyer 2019). Phylogenetic signal is the tendency of closely related species to resemble each other more than species taken randomly in the phylogeny (Blomberg and Garland 2002; Münkemüller et al., 2012 ; Kamilar and Cooper 2013). This statistic measures phylogenetic signal of a multivariate dataset by comparing the observed with the expected values given a BM model. Blomberg's K varies from $0 < K > 1$, when $K < 1$ related species resemble each other less than expected under BM, when $K > 1$ means that related species are more similar than expected under BM. Therefore, a strong phylogenetic signal only happens when $K > 1$. Finally, we used a multivariate Phylogenetic Generalized Least Squares (PGLS; Adams 2014b) to test the effect of body size on the erythrogram and leukogram (described by the phylo-PCA). This analysis assumes traits evolved under a BM model of evolution. Analysis was conducted in the R package geomorph (Adams et al. 2021).

Results

Hematological parameters of *L. podicipinus*

The total erythrocytes in *L. podicipinus* are 3.31 ± 0.35 10^6 L^1, whereas hematocrit values are 13.75 ± 1.18% and hemoglobin 4.66 ± 0.42 g/dL (Table 1). Erythrocytes had an elliptical shape, with an average Sf of 0.70 ± 0.04 (Table 1). The cell nucleus is central, with an average volume of 229.75 ± 14.53 m^2, occupying 20% of the cell (Figure 1, Table 1).

Table 1. Hematological parameters and red blood cell morphometrics of *Leptodactylus podicipinus*

	Mean ± standard error	Min–Max
Hematological parameters		
Erytrocytes (×10^6 µL^{-1})	3.31 ± 0.35	1.40–6.50
Hematocrit (%)	13.75 ± 1.18	6.70–19.70
Hemoglobin (g/dL)	4.66 ± 0.42	2.07–7.77
MCV (fL)	52.32 ± 8.46	10.31–100.50
MCH (pg)	15.24 ± 1.52	6.97–29.34
MCHC (g dL^{-1})	36.77 ± 10.32	14.72–115.92
Red blood cell morphometrics		
Cell volume (µm^3)	1191.43 ± 75.35	486.19–2342.07
Nucleus volume (µm^3)	229.75 ± 14.53	29.48–435.79
N:C ratio	20.11 ± 1.27	1.54–34.40
Sf	0.75 ± 0.04	0.62–0.89

We calculated hematological parameters based on 22 specimens, cell morphometrics based on 7, and data are presented as mean ± standard error and minimum and maximum values. = MCH;

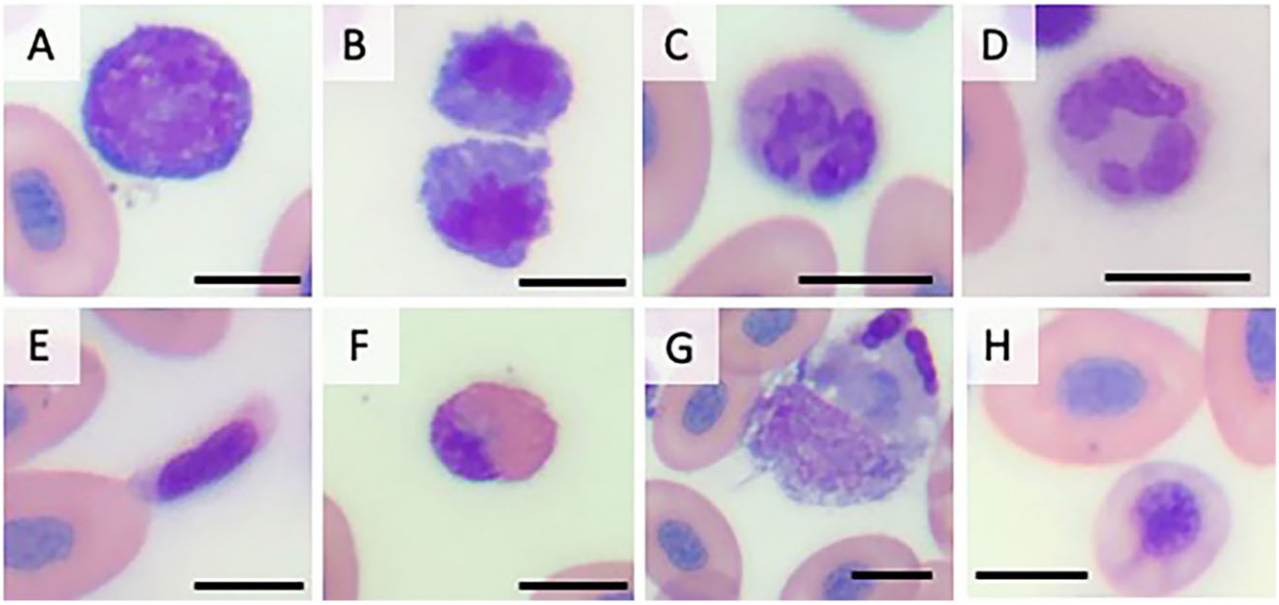

Figure 1. Blood smear showing the white and red blood cells of *L. podicipinus*. Scale bar = 5 μm. (**A**) and (**B**) Lymphocyte; (**C**) and (**D**) Neutrophils; (**E**) Thrombocyte; (**F**) Eosinophil; (**G**) Monocyte; (**H**) Erythrocytes. Coloration = May–Grunwald–Giemsa–Wright.

Table 2. Leukocyte and thrombocyte count of 22 females of *Leptodactylus podicipinus*

	Mean ± standard error	Min–Max
Monocytes (%)	6.89 ± 1.17	0.00–17.12
Lymphocytes (%)	64.72 ± 3.38	39.29–92.08
Neutrophils (%)	10.48 ± 1.69	2.40–30.91
Immature leukocytes (%)	3.92 ± 0.94	0.62–20.83
Eosinophils (%)	2.65 ± 0.48	0.00–8.33
Basophils (%)	0.89 ± 0.22	0.00–2.88
Thrombocytes (%)	10.45 ± 1.85	0.99–38.51
Leukocytes ($\times 10^3$/mm^3)	27.1 ± 2.32	12.98–59.84

Data are presented as mean values ± standard error, and minimum and maximum values.

Lymphocytes were the most abundant leukocytes (>60%), followed by neutrophils (Table 2). Lymphocytes had a proportionally large nucleus with almost no cytoplasm (Figure 1), which often had cytoplasmic projections. Neutrophils had a segmented nucleus with several morphologies. Basophils are smaller than other leukocytes. However, they had a typically granular cytoplasm with a basophilic reaction, sometimes with nuclear overlap. In contrast, monocytes were the largest leukocytes and had an abundant cytoplasm rich in vacuoles. The nucleus is prominent in relation to the cytoplasm, occupying 50% of the cell volume. Eosinophils had many eosinophilic granules, with an eccentric and poorly segmented nucleus (Figure 1). Immature leukocytes (3.92%) were relatively common in the circulating blood of *L. podicipinus*. These cells occur under normal physiological conditions in the circulating blood of this species. They can be distinguished by their basophilic aspect besides large size and an irregular nucleus, varying from reniform to rounded.

Phylogenetic signal of erythrogram and leukogram in anurans

We found that both erythrogram (K_{mult} = 0.632; Effect size = 3.793; P = 0.001) and leukogram (K_{mult} = 0.6099; Effect size = 3.759; P = 0.001) exhibit a moderate phylogenetic signal. These results suggest that in both data sets closely related species resemble each other less than expected under BM. The high effect size of erythrogram, combined with the Phylogenetic aligned Components Analysis (Supplementary Figure S1), showed that the phylogenetic signal was mainly concentrated along the PC1 (Figure 2). Species associated positively with PC1 of the erythrogram (e.g., *Bufotes viridis* and *Hyla arborea*) had high values of MCH and MCV, whereas those negatively associated with PC1 (e.g., *H. raniceps* and *Leptodactylus* spp.) had decreasing values of those variables.

The phylomorphospace of the leukogram was quite distinctive, with phylogenetic signal spread throughout the PCs (Supplementary Figure S2). Species associated positively with PC1 of the leukogram (e.g., *Hoplobatrachus rugulosus*, *Pelobates syriacus*, and *H. arborea*) had high lymphocyte counts, whereas those negatively related to PC1 (e.g., *Anaxyrus americanus* and *Lithobates catesbeianus*) had high leukocytes and neutrophil counts (Figure 3). Neither the leukogram nor the erythrogram was influenced by body size (Table 3), which explained only a small amount of their variance.

Discussion

The total number of erythrocytes in *L. podicipinus* was 5 times higher than those reported for *Polypedates maculatus* and *H. rugulosus* (Mahapatra et al. 2012; Meesawat et al. 2016). However, *L. podicipinus* had lower values of hematocrit, hemoglobin, MCV, and MCH than *P. maculatus* (Mahapatra et al. 2012). This difference can be explained by the fact that *L. podicipinus* had many erythrocytes that were about 1.5 times smaller than that of *P. maculatus*. This relationship is the same for nucleus area, because *P. maculatus* had a larger nucleus than *L. podicipinus* (Mahapatra et al. 2012). These differences in the leukogram and erythrogram may be related to life history traits, such as terrestrial or semi-aquatic habits (Gül et al. 2011), as well as abiotic factors, such as ambient temperature (Allender and Fry 2008).

We found a higher proportion of lymphocytes in *L. podicipinus*, 60% of the total leukocyte count. This seems to be a common

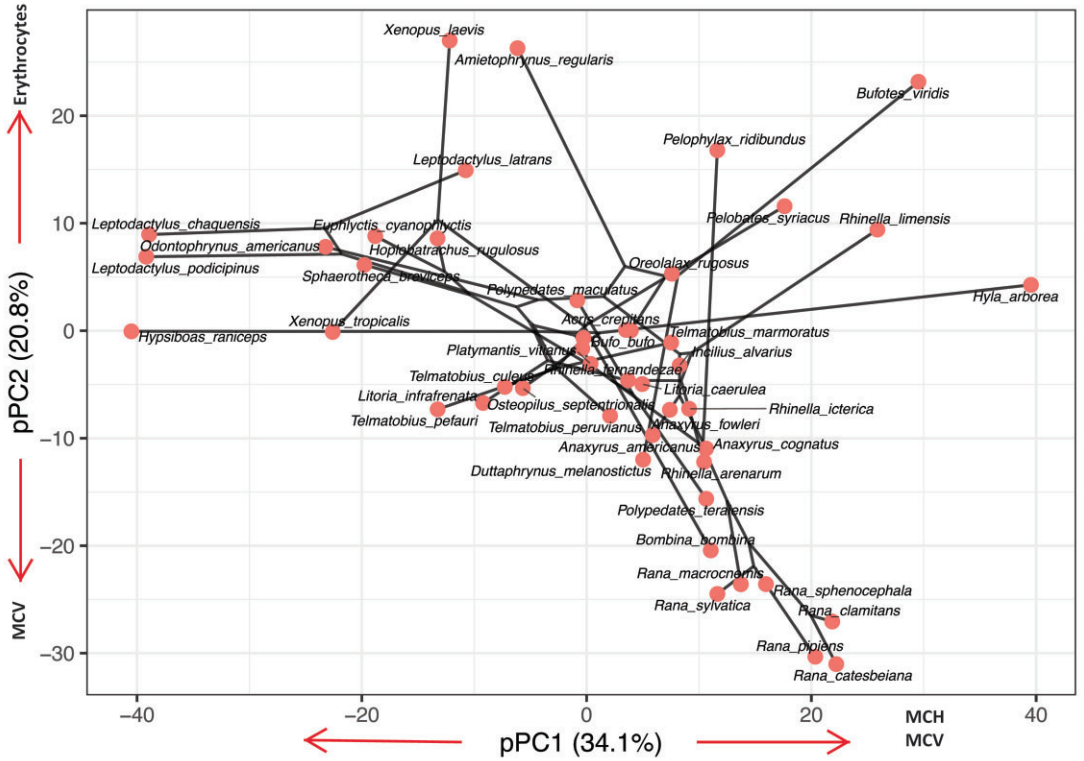

Figure 2. Phylomorphospace for the erythrogram variables built with the first 2 axes of the phylogenetic PCA showing the distribution of species in the reduced space with the phylogeny superimposed. The variables that most contributed (highest loading) to each PC are showed next to them.

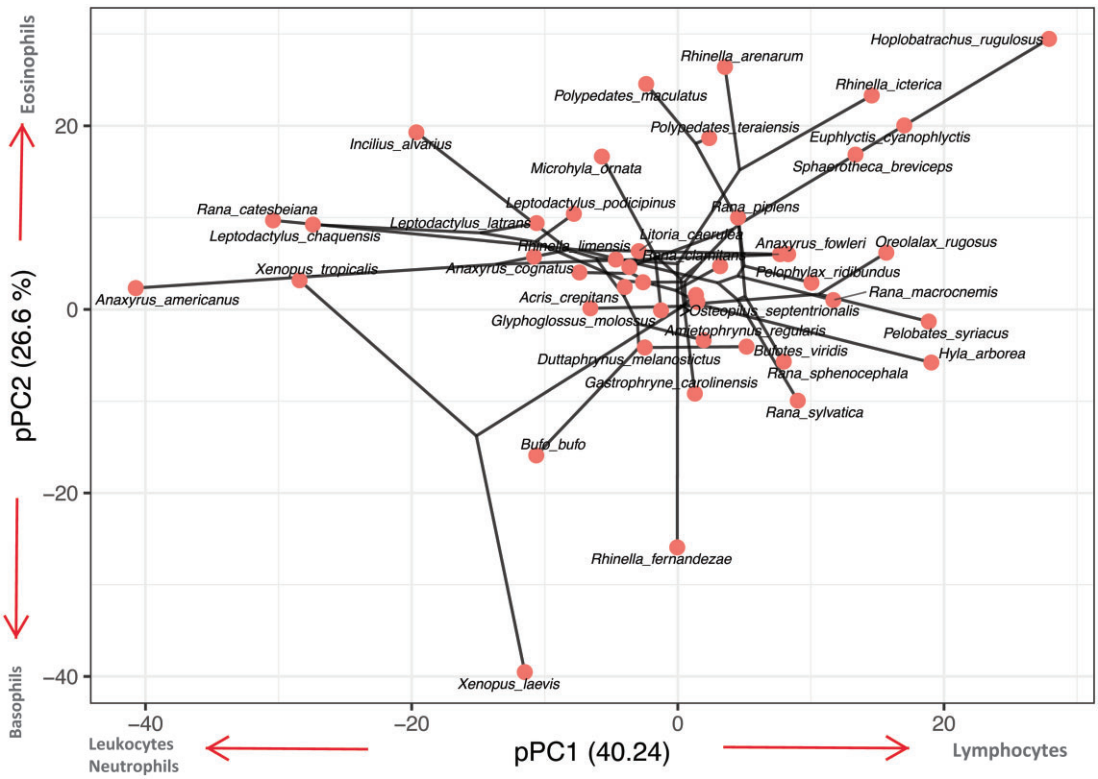

Figure 3. Phylomorphospace for the leukogram variables built with the first 2 axes of the phylogenetic PCA showing the distribution of species in the reduced space with the phylogeny superimposed. The variables that most contributed (highest loading) to each PC are showed next to them.

Table 3. Results of the PGLS s for testing the effect of body size on erythrogram and leukogram variables

Erythrogram	*df*	SS	MS	R^2	*F*	*Z*	*P*-value
Body size	1	1.607	1.6072	0.0057	0.2637	−1.5225	0.934
Residuals	46	280.393	6.0955	0.9943			
Total	47	282.000					
Leukogram							
Body size	1	13.225	13.2254	0.0469	2.2635	1.513	0.062
Residuals	46	268.775	5.8429	0.9531			
Total	47	282.000					

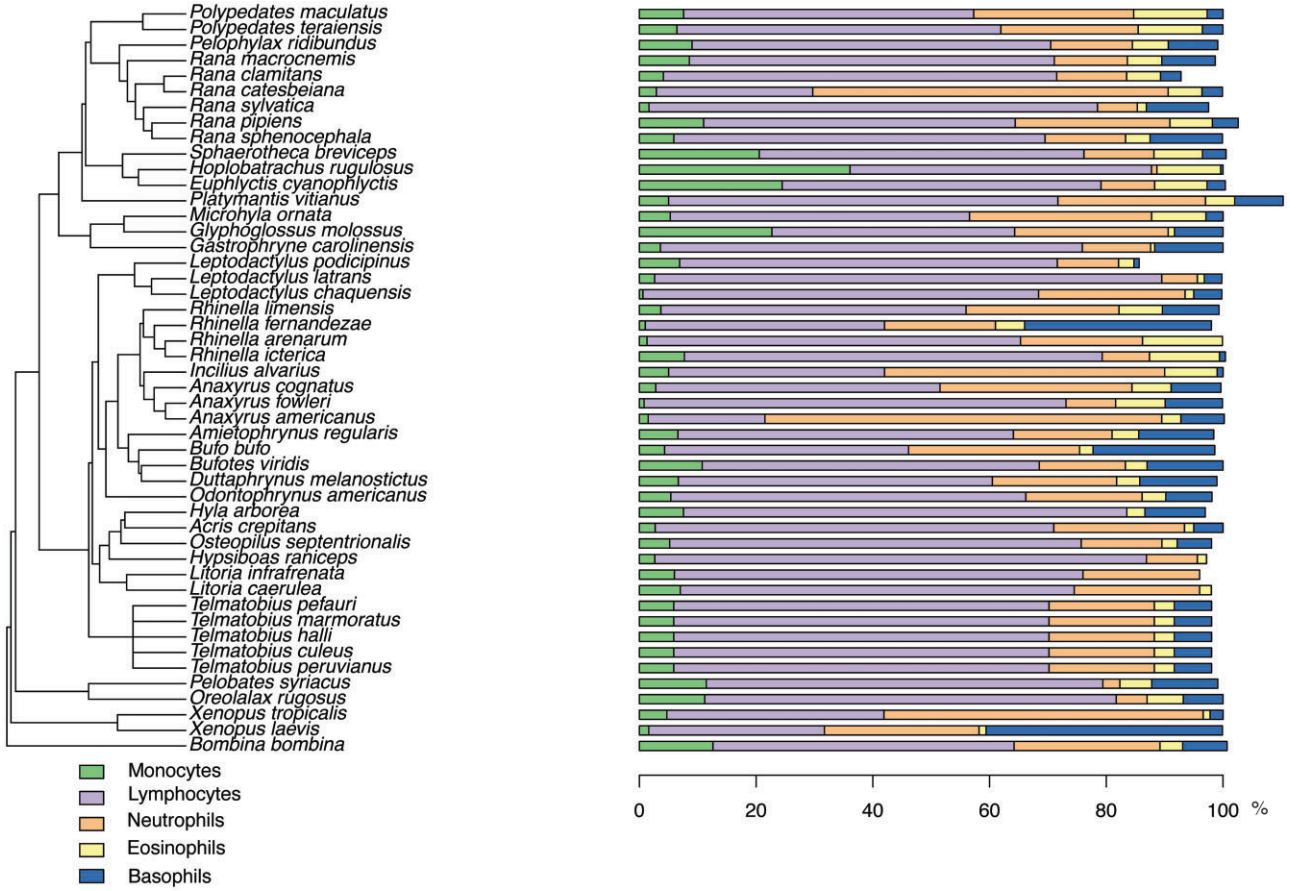

Figure 4. Proportion of each white blood cell in the leukogram of each species. Notice that, with a few exceptions, the cell type with the highest proportion is the lymphocyte, followed by neutrophils. The sum of *L. podicipinus* was >100% because we included immature leukocytes in our counts.

pattern for amphibians (Allender and Fry 2008; Figure 4). The proportions of lymphocytes in anurans can vary from 20% in *L. catesbeianus* (Peng et al. 2016), 50% in *P. maculatus* (Mahapatra et al. 2012) and *Physalaemus cuvieri* (Fanali et al. 2018), to >80% in *Pelophylax ridibundus* (Davis et al. 2008; Zhelev et al. 2018) and *Leptodactylus latrans* (Brodeur et al. 2020). In addition, *L. podicipinus* has many total leukocytes (27.5 10^3/mm^3). The highest leukocytes in *L. podicipinus* were neutrophils, thrombocytes, and monocytes. Species of the genus *Leptodactylus* (*L. podicipinus* and *L. latrans*; Brodeur et al. 2020) apparently share a high count of thrombocytes (>10%). Conversely, neutrophils can vary more widely in anurans, from 0.92% in *H. rugulosus* (Dicroglossidae) to 60% in *L. catesbeianus* (Meesawat et al. 2016; Peng et al. 2016). The white cell with the lowest proportion are eosinophils, varying from 0% in *Litoria infrafrenata* (Pelodryadidae) to 12.59% in *P. maculatus* (Racophoridae). Nonetheless, no study so far investigated the hematological parameters in frogs under a phylogenetic perspective. Our results contribute to understanding the role of species evolutionary history as a non-negligible aspect that can influence both red and white blood cell composition.

We found a moderate, though significant, phylogenetic signal in the leukogram. Also, the most important white cell types that contributed to build the phylomorphospace were Neutrophils, Lymphocytes, total Leukocytes to PC1, and Eosinophils and Basophils to PC2. Interestingly, the pattern in the reduced ordination space for the leukogram was complex, as shown in the PaCA.

This is because we need more dimensions of the data to represent phylogenetic signal. The higher proportion of these cells in anurans (Davis 2009) can explain why they contributed disproportionally to the ordination space. Interestingly, species of *Telmatobius* were clustered in the center of the ordination diagram together with other distantly related species. This is surprising given that species of this genus inhabit high elevations in the Andes and had distinct erythrogram values (Franco-Belussi et al. 2021). Therefore, our result suggests that neutrophils and lymphocytes are the most relevant cell types, in addition to total leukocytes count to characterize leukogram parameters in anurans. Neutrophils and lymphocytes have opposite responses to environmental stressors (Davis et al. 2008). Indeed, the neutrophil:lymphocyte ratio is a useful tool to test the effects of environmental stressors (Davis et al. 2008) on blood parameters. For example, frog species inhabiting highly impacted habitats have a higher neutrophil:lymphocyte ratio (Garcia et al. 2020). As a result, experiments testing the effect of contaminants and UV radiation on immune response of frogs (e.g., Carvalho et al. 2017; Franco-Belussi et al. 2018) only need to estimate these two cell types. Thus, our initial hypothesis about the role of evolutionary history on the leukogram was not entirely supported, because we found a small, though significant, contribution of species phylogeny to the variation in white cell composition. As such, species response to local environmental stressors (e.g., pollutants, temperature, and UV) seems to play a larger role than phylogenetic relationships to determine white cell profile.

We did not find a strong influence of body size on erythrogram or leukogram. Taken together with the high phylogenetic signal for these data, they suggest that body size alone does not account for the variation in white blood cell composition in anurans, and phylogenetic inertia (Blomberg and Garland 2002; Revell et al. 2008) seems to play a strong role. While white blood cell composition can vary quickly in response to exogenous stimuli (Newman et al. 1997), our result shows that apparently there is somewhat a common standing cell composition that is shared by closely related species. The main center of white blood cell maturation in anurans is the spleen (Franco-Belussi and De Oliveira 2016). This organ plays a key role in the immune response of species to exogenous stimuli (Rollins-Smith 2017; Yaparla et al. 2017; Zhelev et al. 2020). However, the activity and size of the spleen are shaped by organogenesis, whose development varies at the level of more inclusive lineages instead of species (Neely and Flajnik, 2016).

We found a moderate phylogenetic signal for erythrogram. However, contrarily to the leukogram, phylogenetic signal in the erythrogram variables was mostly concentrated in the PC1 (RV_1 = 99.87%). It is known that K_{mult} is rarely >1 (Adams and Collyer 2019), because phylogenetic signal is usually concentrated in a few dimensions of multivariate data. The high effect size and the ordination diagram with PaCA confirm that only the first dimension of the erythrogram exhibits phylogenetic signal. This axis is correlated with indices derived from hemoglobin and the hematocrit, such as MCV and MCH. This result suggests that the amount of hemoglobin in red blood cells has a phylogenetic pattern (see also Glomski et al. 1997), whereas the hematocrit and the number of erythrocytes are more plastic variables, varying at shallow phylogenetic scales. The phylogenetic pattern in the erythrogram may be due to the morphometry of red blood cells (see Martins et al., 2021 for an example with fish), because red blood cell volume is negatively correlated with hemoglobin concentration (Lay and Baldwin, 1999). This emphasizes the importance of collecting these data when evaluating hematological parameters in frogs, as we did with *L. podicipinus*. Unfortunately, we do not have cell morphometric data for all the species, but future studies should ideally assemble a large dataset to test this hypothesis.

In conclusion, hematological parameters are related to the phylogeny to some extent, and the erythrogram seems more conserved than the leukogram. Conversely, local environmental changes in habitats where species occur (e.g., stressors) can also play a role in determining white cell profiles. The main contribution of our study was to provide a macroevolutionary perspective to the study of hematological parameters in frogs, pointing which components of the blood are more similar between related species. One limitation of our study was that we were unable to analyze the influence of environmental factors on blood cell composition. Future studies should conduct phylogenetically-informed experiments to disentangle the effect of local environmental stressors from the evolutionary adaptative component of blood parameters in frogs.

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Author's Contributions

L.F.B., T.R.F.L., M.S.S., B.S.L.V., B.O.M., and C.E.S.F. collected data. L.F.B. performed laboratory analyses. B.O.M. collected cell morphometric data. M.S.S. collected cell count data. D.B.P., C.O., L.F.B., and C.E.S.F designed the analyses and wrote the manuscript. D.B.P. and L.F.B. gathered literature data. D.B.P. performed data analysis and curated the data. C.O. and C.E.S.F provided funding.

Conflict of Interest Statement

Authors have no conflicts of interest to declare.

Supplementary Material

"Supplementary material can be found at https:// academic. oup. com/ cz".

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