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Intraspecific variation in erythrocyte sizes among populations of *Hypsiboas cordobae* (Anura: Hylidae)

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Abstract. We studied the morphology and size of erythrocytes of *H. cordobae*, and analysed the geographic variation of this character along the distribution of the species, in relation to the latitudinal and altitudinal distances. Erythrocyte shape of the *H. cordobae* is ellipsoidal and the nuclei are also ellipsoidal and centrally oriented. Erythrocyte and nuclear size showed significant differences among populations, with the highest mean size corresponding to the population of Achiras (low altitude site) and the lowest mean size to Los Linderos (high altitude site). There was no significant relationship between the latitude of each population and the both erythrocyte and nuclear size. The altitudinal variation in erythrocyte cell size may be attributable to the surface available for gas exchange; a small erythrocyte offers a possibility of greater rate of exchange than a larger one. Our results are consistent with studies of other amphibians, where intraspecific comparisons of populations at different altitudes show that individuals at higher altitudes are characterized by smaller erythrocytes.

Keywords. *Hypsiboas cordobae*, erythrocyte and nuclear size, geographic variation.

The description of the anuran amphibian hematology is insufficient, although this is a diverse group of vertebrates (Cabagna et al., 2011). The majority of the references to hematology in different species of anurans have been limited to blood cell counts (Martínez et al., 1985; Arıkan, 1990; Arserim and Mermer, 2008; Dönmez et al., 2009). However, there are also some studies on erythrocyte sizes of several amphibian species (Hartman and Lessler, 1964; Matson, 1990; Atatür et al., 1998, 1999, 2001; Wojtaszek and Adamowicz, 2003; Zhelev et al., 2006; Gao et al., 2007; Grenat et al., 2009a, b; Arıkan et al., 2010).

Some investigators have stressed that erythrocyte size in amphibians may be used to ploidy determination, because blood cells of amphibians conserve their nucleus and the erythrocyte size is correlated with the DNA content (Stöck and Grosse, 1997; Schröer and Greven, 1998; Atatür et al., 1999; Martino and Sinsch, 2002; Rosset et al., 2006; Gao et al., 2007; Grenat et al., 2009a, b; Valetti et al., 2009). This method is simple, rapid and minimally invasive (Grenat et al., 2009a). In this paper, we studied six populations of a single species, in which ploidy level is the same (Baraquet et al., 2013).

It is well-known that in amphibians there is an extensive range in the erythrocyte size. Morphology and size of erythrocytes have shown great inter-specific and even intra-specific variations (Arıkan and Çiçek, 2010). Furthermore in comparison with other organisms, amphibian red blood cells tend to be larger (Duellman and Trueb 1994; Gregory, 2001; Campbell, 2004). This relationship between erythrocyte size and the level of ploidy has also been discussed on the basis of differences in metabolic rates between different groups of vertebrates (Gregory, 2000), because the size and shape of red blood cells give

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an indication of the surface available for the exchange of gases in respiratory functions (Hartman and Lessler, 1964; Sevinç et al., 2000).

The availability of oxygen limits the metabolic potential and, therefore, the behaviour of animals in a particular environment. Thus, the adaptation to an environment depends on the development of suitable mechanisms to overcome these limitations. So, it is not strange that these adaptations in amphibians influence the properties of blood and parameters that most affect this tissue (Martínez et al., 1985).

Several studies have demonstrated that variations in erythrocyte counts and size are correlated with metabolic activity of the animal, indicating that the more active species have smaller erythrocytes while those with less oxygen consumption have bigger ones (Evans, 1939; Smith, 1925; Szarski, 1970, 1976).

The distribution of the species under study, *Hypsiboas cordobae* (Barrio 1965), is restricted to Córdoba and San Luis provinces, Argentina (Barrio, 1965; Cei, 1980; Gallardo, 1974, 1987; di Tada, et al. 1996; Faivovich, et al. 2004). This restricted distribution and a broad altitudinal range, together with the reported IUCN status (i.e., data deficient), make this species an interesting research model.

Although, various hematological studies were carried out on many anuran species, information is not available for *H. cordobae*. Here, we examine the morphology and size of erythrocyte of *H. cordobae* and report their geographic variation along a latitudinal and altitudinal gradient in Cordoba and San Luis provinces, Argentina.

A total of 66 adult individuals of *H. cordobae* (57 ♂♂ and 9 ♀♀) were collected from six localities of Cordoba and San Luis Provinces (Argentina), between September 2006 and May 2011. The study area covers a latitudinal gradient across an area of approximately 20 000 km^2, with an altitudinal range between 800 m and 2300 m in elevation. The sampled localities were: Achiras (n = 10, 808 m a.s.l., 33º 09'S, 64ºW), Las Guindas (n = 21, 930 m a.s.l., 32º S, 64º W), La Carolina (n = 15,1634 m a.s.l., 32º 48'S, 66º 05'W), Los Tabaquillos (n = 9, 2107 m a.s.l., 32º 23'S, 64º 55'W), Pampa de Achala (n = 6, 2150 m a.s.l., 31º 49'S, 64º 51'W), Los Linderos (n = 5, 2310 m a.s.l., 32º 00'S, 64º 56'W).

The blood samples were obtained by *angularis* vein puncture (Nöller, 1959). Smears of fresh blood were air-dried and stained with a 10% solution of Giemsa for 5 min. Slides were observed by using a microscope Carl Zeiss trinocular Primo Star (Pack 5), photographed with a Canon Power Shot G10 Digital Camera and processed using the image software AxioVision 4.8.

The photographs were used to record the erythrocyte measurement by Adobe® Photoshop® 9.0. On each blood smear, length (L) and width (W) of forty randomly chosen erythrocytes and their respective nuclei were measured. Erythrocyte and nuclear areas were calculated assuming an ellipsoid shape according to formula $L*W*\pi/4$.

We calculated mean, standard deviation and maximum and minimum values for each variable. Since these variables had a normal distribution (Shapiro-Wilks test, $P > 0.05$), differences between males and females were compared by t-tests and inter-population comparisons by analyses of variance (ANOVA). If the ANOVA revealed significant differences among populations, pairwise Tukey's HSD tests were used to determine which groups differed significantly from one another.

Pearson correlation coefficient (r) was used to measure association of erythrocyte and nuclear size with latitude and altitude of the population studied to investigate geographic variation. Mean values of each individual were used and all data were processed using Statgraphics Plus 5.0.

Because no sex differences were found for any of the size variables (t tests, $P > 0.05$ in all cases), data from both sexes were pooled. The mean erythrocyte and nuclear length, width, area and length/width ratio for each population sampled of the *H. cordobae* are given in Table 1.

The characteristic erythrocyte shape of the *H. cordobae* was ellipsoidal (L/W = 1.51). Nuclei were also ellipsoidal (l/w = 1.79) and centrally located.

In the population studied, erythrocyte lengths and sizes varied between 21.14 μm and 23.66 μm and 230.56 μm^2 and 280.72 μm^2, respectively. The longest erythrocytes were observed in the population from Las Guindas. The largest erythrocyte areas were observed in the population from Achiras while the shortest and the smallest erythrocytes were observed in Los Linderos. In terms of L/W ratio, the most ellipsoidal cells were those of La Carolina and Las Guindas populations while the least ellipsoidal ones were observed in Los Tabaquillos (Table 1).

The longest and the largest nuclei were observed in Achiras while the shortest and the smallest nuclei were measured in the population from Los Linderos. The most ellipsoidal nuclei were observed in La Carolina and the least ellipsoidal ones were found in Pampa de Achala (Table 1).

Erythrocyte and nuclear size showed significant differences among populations (ANOVAs: $F = 2.88$, $P = 0.02$; $F = 3.70$, $P \leq 0.01$, respectively). Pairwise test showed that erythrocyte and nuclear sizes of Achiras and Los Linderos populations differed significantly (Tukey's HSD tests, $P < 0.05$, in both cases). In these populations we found the extreme erythrocyte and nuclear sizes: the largest size in Achiras and the smallest in Los Linderos.

Table 1. Erythrocyte data in six populations of *H. cordobae*. n: sample size, L: erythrocyte length, W: erythrocyte width, A: erythrocyte size, L/W: erythrocyte ratios of length/width, l: nuclei length, w: nuclei width, a: nuclei size, l/w: nuclei ratios of length/width (means ± standard deviations).

Population	n	L (μm)	W (μm)	A (μm^2)	L/W	l (μm)	w (μm)	a (μm^2)	l/w
Achiras (808 m a.s.l.)	10	23.42 ± 1.60	15.18 ± 1.55	280.72 ± 45.40	1.55 ± 0.10	9.99 ± 0.85	6.00 ± 1.12	47.72 ± 12.99	1.69 ± 0.18
Las Guindas (930 m a.s.l.)	21	23.66 ± 1.14	15.06 ± 1.05	280.58 ± 28.22	1.58 ± 0.10	9.92 ± 0.83	5.55 ± 0.64	43.57 ± 7.91	1.80 ± 0.14
La Carolina (1634 m a.s.l.)	15	22.93 ± 1.01	14.62 ± 1.23	263.83 ± 30.05	1.58 ± 0.11	9.66 ± 0.60	5.09 ± 0.42	38.63 ± 4.20	1.91 ± 0.17
Los Tabaquillos (2107 m a.s.l.)	9	21.62 ± 0.67	15.35 ± 0.61	260.91 ± 15.99	1.41 ± 0.05	9.44 ± 0.54	5.19 ± 0.40	38.51 ± 4.41	1.83 ± 0.14
Pampa de Achala (2150 m a.s.l.)	6	22.41 ± 0.52	15.04 ± 1.31	265.39 ± 27.32	1.50 ± 0.11	9.12 ± 0.84	5.44 ± 0.26	39.02 ± 4.11	1.68 ± 0.17
Los Linderos (2310 m a.s.l.)	5	21.14 ± 0.80	13.85 ± 0.94	230.56 ± 20.44	1.53 ± 0.10	9.01 ± 0.30	4.77 ± 0.07	33.69 ± 1.37	1.89 ± 0.06
H. cordobae (range)	66	22.54 ± 0.99 (21.14-23.62)	14.94 ± 0.62 (13.85-15.59)	265.40 ± 19.00 (230.56-280.72)	1.51 ± 0.07 (1.41-1.57)	9.54 ± 0.41 (9.01-9.99)	5.33 ± 0.42 (4.77-6.00)	40.86 ± 5.60 (33.69-47.72)	1.79 ± 0.10 (1.66-1.90)

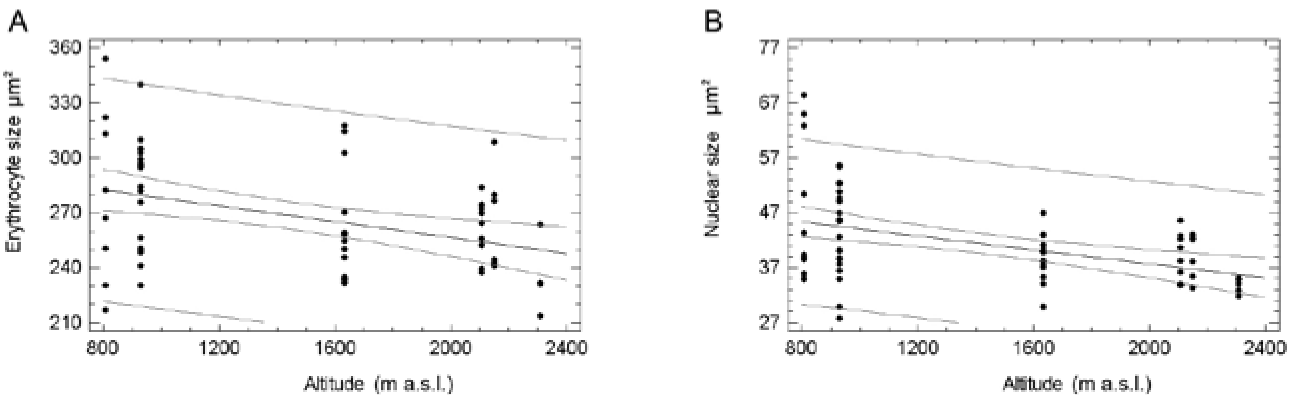

Fig. 1. Correlation of erythrocyte (A) and nuclear (B) size with altitude of *H. cordobae*.

Pearson correlation tests indicated there was not a significant relationship between latitude of each population and size of both erythrocyte and nuclei (r = 0.12, P = 0.81; r = 0.40, P = 0.43). Conversely, correlations showed a negative significant relationship between altitude and size of both erythrocyte and nuclei (r = -0.82, P = 0.04; r = -0.91, P = 0.01, respectively). Erythrocyte and nuclear size decreased significantly with increasing altitude of *H. cordobae* populations (Figure 1).

In the present paper, we have analysed the spatial pattern of erythrocyte size variation along the distribution of *H. cordobae*. The univariate analyses revealed significant differences among populations. There are many ways in which erythrocyte size is of relevance to organism biology; larger erythrocytes contain more hemoglobin (Gregory, 2001). One of the most important functions of erythrocytes is to carry oxygen and carbon dioxide. The erythrocyte size and shape are indicators of the area available for gas exchange in respiratory function. Therefore, small erythrocyte offers a possibility of greater rate of exchange than a larger one (Hartman and Leesler, 1964; Martinez *et al.*, 1985; Sevinç *et al.*, 2000; Wojtaszek and Adamowicz, 2003). Consequently, at altitude where there are lower levels of oxygen available smaller erythrocytes should be selected. Indeed, our results showed a negative relationship among the altitude and size of both erythrocyte and nuclei in the six populations studied. Moreover, Achiras and Los Linderos populations showed the erythrocyte and nuclear sizes values extremes.

Besides, the study of erythrocytes in different species provides an interesting comparison of the erythrocyte size in relation to activity and habitat (Hartman and Lessler, 1964). In amphibians, erythrocyte size has long been known to correlate negatively with metabolic rates (Smith, 1925; Vernberg, 1955; Monnickendam and Balls, 1973). Small erythrocytes improve the uptake of oxygen joined to a high number of red blood cells; this allows the organism to adapt to environments with low oxygen pressures (Hutchison et al., 1976). This relationship stems from the fact that larger surface-area-to volume ratios in smaller cells allow for more efficient exchange of oxygen. This idea is exemplified in intraspecific comparisons of amphibians at different altitudes, where animals at higher latitudes have smaller erythrocytes (Ruiz et al. 1983; Arıkan, 1989; Weber, 2007), presumably to maximize cellular efficiency of oxygen transport and exchange in a low oxygen environment. Our results are in agreement with this, strongly suggesting a negative correlation between altitude and erythrocyte size. However, further studies about metabolic rate and oxygen consumption would be required to analyse the causes of erythrocyte size variation in populations of *H. cordobae* living at different altitudes.

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