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Variation in the erythrocyte size among larvae, juveniles and adults of *Hypsiboas cordobae* (Anura, Hylidae)

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We aimed at determining sizes and shape of erythrocytes and evaluating the differences among larva, juvenile and adult *Hypsiboas cordobae*. Length and width of 40 randomly chosen erythrocytes and their respective nuclei from individuals of different ages collected in the experimental field "Las Guindas" (Córdoba, Argentina) were measured. Erythrocyte and nuclear areas were estimated assuming an ellipsoidal shape, and the aspect ratio, which reflects the shape of the cell, was calculated. The erythrocytes were oval, and their nuclei were also oval and centrally located. Erythrocyte size increased with age, whereas nuclei were larger in tadpoles than in juveniles. The cell and nucleus shapes also changed with age from the spherical shape in larvae to the more ellipsoidal one in adults. Discriminant analysis confirmed the existence of highly significant ($P < 0.0001$) differences in erythrocyte and nuclear areas among larvae, juveniles and adults, with a classification rate of 93.33%.

Key words: age-related differences; erythrocyte; haematology; *Hypsiboas cordobae*.

Variación en el tamaño de los eritrocitos entre larvas, juveniles y adultos de *Hypsiboas cordobae* (Anura, Hylidae). Determinamos el tamaño y forma de los eritrocitos de *Hypsiboas cordobae*, evaluando las diferencias entre larvas, juveniles y adultos. Medimos la longitud y anchura de 40 eritrocitos seleccionados aleatoriamente en individuos de diferentes edades colectados en el campo experimental "Las Guindas" (Córdoba, Argentina). Estimamos las áreas de los eritrocitos y de sus núcleos asumiendo formas elipsoidales, y calculamos la razón de aspecto, indicativa de la forma de la célula. Tanto los eritrocitos como los núcleos mostraron forma ovalada, apareciendo los últimos en posición central. El tamaño de los eritrocitos aumentó con la edad, mientras que los núcleos fueron mayores en larvas que en juveniles. Las formas tanto de la célula como del núcleo también cambiaron con la edad, siendo esféricas en larvas y más ovaladas en adultos. Mediante un análisis discriminante confirmamos la existencia de diferencias altamente significativas ($P < 0.0001$) entre larvas, juveniles y adultos en las área de eritrocitos y núcleos, con una tasa de clasificación del 93.33%.

Key words: diferencias relacionadas con la edad; eritrocitos; hematología; *Hypsiboas cordobae*.

The majority of studies in haematology of anurans are limited to blood cell counts (Atatür *et al.*, 1998, 1999; Dönmez *et al.*, 2009) and erythrocyte size determination (Hartman & Lessler, 1964; Matson, 1990; Atatür *et al.*, 1998, 1999, 2001; Zhelev *et al.*, 2006; Gao *et al.*, 2007; Grenat *et al.*, 2009a,b), while studies on cell size variation among larvae, juveniles and adults are very scarce. One of the most important functions of erythrocytes is to carry oxygen and carbon dioxide, and their size and shape are indicators of the area available for gas exchange. For instance, a small

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erythrocyte possesses a comparatively greater rate of exchange than a large one. The study of erythrocytes in different species provides an interesting comparison of the erythrocyte size in relation to activity and habitat (HARTMAN & LESSLER, 1964; MARTÍNEZ *et al.*, 1985; SEVINÇ *et al.*, 2000; WOJTASZEK & ADAMOWICZ, 2003). Amphibians show an extensive range in erythrocyte sizes, being these relatively large in comparison with other vertebrates (DUELLMAN & TRUEB, 1994; GREGORY, 2001; CAMPBELL, 2004, 2012).

Little is known about the relationship between ontogenetic growth and erythrocyte size, which could be relevant to organism biology especially in animals such as amphibians that have indeterminate growth (DAVIS, 2008; ARIKAN & ÇIÇEK, 2011). Amphibian larval growth and metamorphosis have effects on blood cells such as erythrocytes (DAVIS, 2009; DAS & MAHAPATRA, 2012). As part of the changes happening during metamorphosis involving the substitution of specific larval organs or cells by adult ones (OHMURA & WAKAHARA, 1998), larval erythrocytes are replaced by adult ones (DORN & BROYLES, 1982; YAMAGUCHI & WAKAHARA, 1997; TAMORI & WAKAHARA, 2000; WAKAHARA & YAMAGUCHI, 2001). Consequently, larval and adult erythrocytes differ in size and morphology, being large and elongated in larvae and smaller and rounder in adults (BENBASSAT, 1974; DAVIS, 2008).

In the present paper we examine and evaluate differences in morphology and size of erythrocytes of larvae, juveniles and adults *Hypsiboas cordobae* (Barrio, 1965). *Hypsiboas cordobae* is restricted to highlands of Córdoba and San Luis provinces, in central Argentina (BARRIO, 1965; FAIVOVICH *et al.*, 2004). This restricted distribution with a broad altitudinal range, its consideration by the IUCN Red List of Threatened Species as a taxon of Data Deficient, and the lack of information about its haematology make it an interesting species for study.

A total of 46 individuals of *H. cordobae* were collected from the experimental field "Las Guindas" (Alpa Corral, province of Córdoba, Argentina, 32º35'35.22" S, 64º42'38.92"W; 930 m above sea level) in October and November months between 2007 and 2009. These included 15 tadpoles in stages 35 to 39 according to GOSNER (1960), 10 recently metamorphosed individuals (hereafter juveniles) with the tail completely reabsorbed obtained from captive-raised larvae, and 21 adults. Blood samples were obtained by angularis vein puncture in juveniles and adults (NÖLLER, 1959), and directly from the heart after anaesthesia via immersion in 1% tricaine methanesulfonate in larvae. Smears of fresh blood were air-dried and stained with a 10% solution of Giemsa for 5 min. Slides were observed under a trinocular microscope Primo Star (Pack 5) and the image processing software AxioVision 4.8 (Carl Zeiss, Oberkochen, Germany). The photographs were used to measure erythrocytes with Adobe Photoshop 9.0 (Adobe Systems, San Jose, California, USA). On each blood smear, length (L) and width (W) of 40 randomly chosen erythrocytes and their respective nuclei were measured using a scale of 20 microns as a reference. Erythrocyte and nuclear areas were calculated assuming an ellipsoid shape according to the formula $L \times W \times \pi / 4$. We compared each variable among larvae, juveniles and adults using analyses of the variance (ANOVAs) followed by Tukey's

HSD post hoc comparisons. Then, we used a discriminant function analysis to know the variables that better defined the variation among these groups.

Adult individuals showed the largest erythrocyte and nucleus areas (Table 1; Erythrocyte area: $F_{2,43}$ = 35.69, P < 0.0001. Nucleus area: $F_{2,43}$ = 9.13, P = 0.0005). Larval nuclei were larger than juvenile ones, whereas the opposite was true for erythrocyte size (Table 1), although in the latter case differences among age groups were not significant. The L / W ratio results revealed significant differences among age groups in shape of both erythrocytes ($F_{2,43}$ = 5.48, P = 0.0077) and nucleus ($F_{2,43}$ = 43.05, P < 0.0001). For both parameters, adults showed oval or elliptical shapes while in larvae they were rounder or more spherical (L / W closer to one, Table 1). In juveniles, erythrocyte shape was similar as in larvae while nucleus shape resembled that of adults.

Discriminant analysis based on length and width of erythrocytes and nuclei yielded two highly significant functions (P < 0.0001). The first function, with an eigenvalue of 6.12, explained 89.26 % of the observed variation. The high canonical correlation (0.92715) indicated a high weight of the function, while the low Wilks' Lambda (0.08084) indicated that the two selected variables (length and width of nuclei) were appropriate for discriminating age groups (Fig. 1). A total of 93.33% of the cases was correctly classified (100% of larvae, 90% of juveniles and 90.48% of adults).

Our results showing differences in erythrocyte and nuclear size and shape among larvae, juveniles and adults agree with previously reported differences between larval and adult erythrocytes in amphibians. Many studies report that during metamorphosis, larger and elongated erythrocytes of larvae are replaced by smaller and rounder cells in adults (BENBASSAT, 1974; BROYLES, 1981; DORN & BROYLES, 1982; DUELLMAN & TRUEB, 1994; YAMAGUCHI & WAKAHARA, 1997, 2001; HASEBE *et al.*, 1999; DAVIS, 2008; ARIKAN & ÇIÇEK, 2011; CAMPBELL, 2012). However, we observed that the erythrocyte and nuclear sizes in larvae were smaller than in adults, and that both cells and nuclei were more elongated in adults than in larvae. Our results are in agreement with those by YAMAGUCHI & WAKAHARA (1997) in the salamander *Hynobius retardatus*, who found

Table 1: Mean ± standard deviation of erythrocyte and nuclear measurements of larvae (N = 15), juveniles (N = 10) and adults (N = 21) of Hypsiboas cordobae. Lower case letters indicate different groups defined by post hoc tests (P < 0.05). Lar = Larvae; Juv = Juveniles; Adu = Adults.

	Erythrocytes				Nuclei			
	L (µm)	W (µm)	A (µm²)	L / W	L (µm)	W (µm)	A (µm²)	L / W
Lar	20.47 ± 1.00	13.90 ± 0.45	223.98 ± 16.71[a]	1.48 ± 0.05	8.22 ± 0.33	6.07 ± 0.19	39.26 ± 2.51[b]	1.39 ± 0.11
Juv	20.87 ± 0.84	13.69 ± 0.58	224.73 ± 12.13[a]	1.53 ± 0.10	8.40 ± 0.63	5.03 ± 0.59	33.38 ± 5.88[a]	1.69 ± 0.13
Adu	23.66 ± 1.14	15.06 ± 1.05	280.58 ± 28.22[b]	1.58 ± 0.10	9.92 ± 0.83	5.55 ± 0.64	43.57 ± 7.91[c]	1.80 ± 0.14

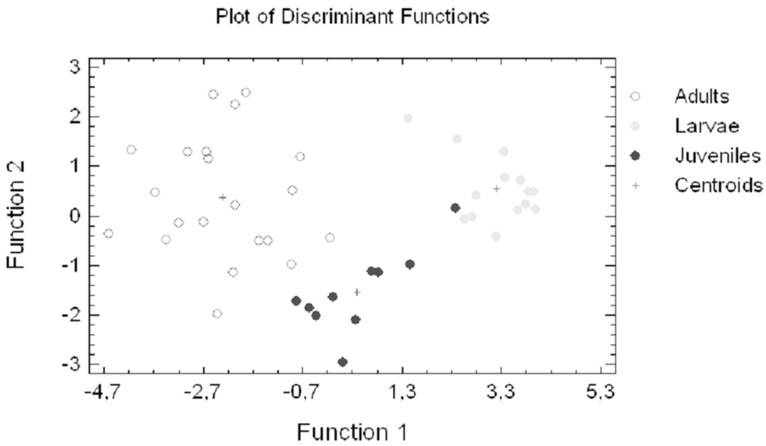

Figure 1: Representation of the first two canonical functions from the discriminant analysis performed on erythrocyte and nucleus variables of larvae, juveniles and adults of *Hypsiboas cordobae*.

larger erythrocytes in adults than in larvae. Furthermore, these authors also showed that in larvae, erythrocyte shape is round or spherical while in adults is oval or elliptical, and indicated that during the transition, erythrocytes showed an intermediate form between typical larval and adult cells, which also coincides with what we have observed in *H. cordobae*.

Because erythrocytes are responsible for storing and transporting oxygen (HARTMAN & LESSLER, 1964) their characteristics determine in part the efficiency of this transport from respiratory systems to tissues (HOLLAND & FORSTER, 1966). Thus, amphibian erythrocyte size relates to the respiratory needs of the individuals (ARSERIM & MERMER, 2008), and therefore increasing the amount of hemoglobin per cell, as it would happen in larger erythrocytes, would be one way to meet the body's increasing demands for oxygen as individuals grow in size. However, DAVIS *et al.* (2009) argued that the increased size of erythrocytes with growth might merely reflect the allometric scaling of body and cells (i.e. as capillary size grows, cell sizes increase).

The age-related variation in erythrocyte morphology clearly warrants further studies to clarify the physiological mechanism involved and the implications of these changes. Our results may be helpful as reference values for future investigations and could also be used in combination with other hematological parameters for the study about the changes that occur in blood cells during metamorphosis and identifying hematopoietic organs in *H. cordobae*.

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