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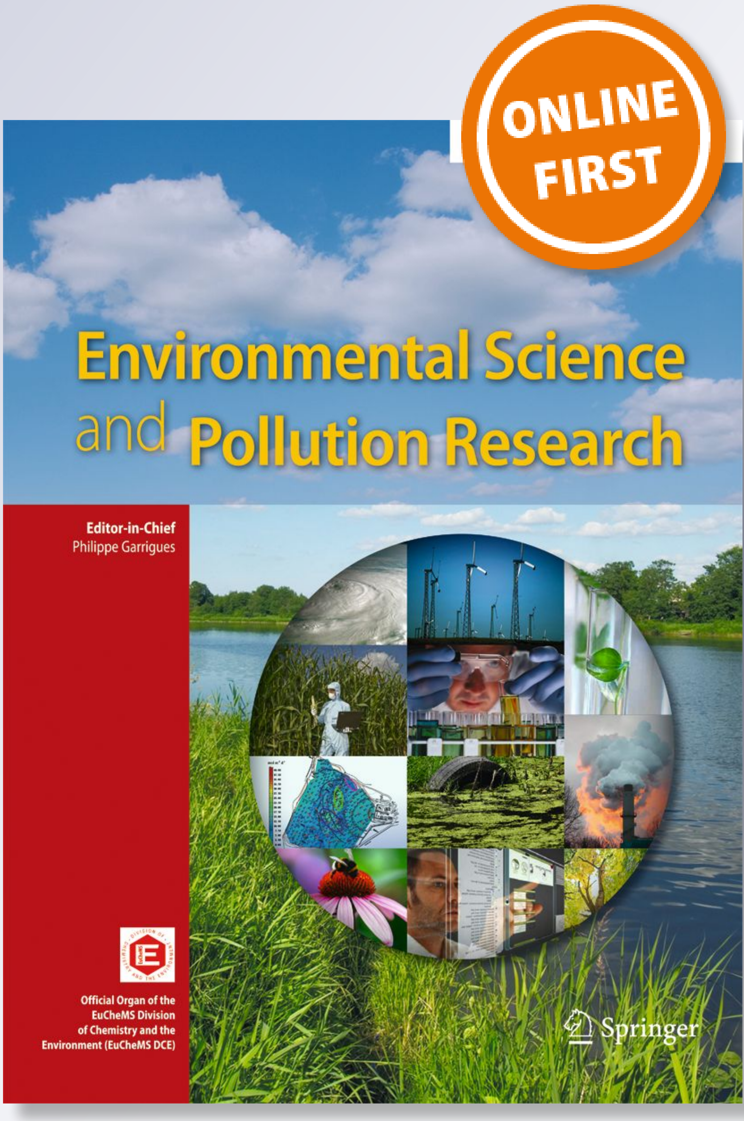

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RESEARCH ARTICLE

Morphology and size of blood cells of *Rhinella arenarum* (Hensel, 1867) as environmental health assessment in disturbed aquatic ecosystem from central Argentina

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Abstract Four populations of *Rhinella arenarum* from aquatic environments with different degrees of disturbance in central Argentina were compared to assess the ability of cytomorphology and cytomorphometry of blood cells as a hematological biomarker. A total of 93 specimens of *R. arenarum* (adults sexually mature) were captured during the spring. From the analysis of cell, no variations were found in terms of morphology, whereas in nuclear and cell areas and Price-Jones curves, we observed a smaller size in erythrocytes of individuals inhabiting the site most altered, "Villa Dalcar," as well as for leukocytes, lymphocytes, neutrophils, and eosinophils for the same site. This could be caused by presence of different pollutants in the lake. Furthermore, this was confirmed by the high levels of environmental variables (conductivity, total dissolved solids, and salinity) show that Villa Dalcar is the site most affected by human activities.

Keywords *Rhinella arenarum* · Biomarkers · Cytomorphology · Price-Jones curves · Aquatic environments · Pollutants

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Introduction

In recent years, conservation biologists have intensified studies to monitor the impact caused by pollution from human activities on the ecosystem (Cabagna et al. 2005; Zhelev 2007; Pollo et al. 2012). The expansion of agricultural systems and extreme urbanization has simplified the landscape (McLaughlin and Mineau 1995) affecting biodiversity (Blaustein and Bancroft 2007). Evaluation of quality of the environment, particularly in aquatic ecosystems, has traditionally been based on physicochemical measurements of water. Organisms in aquatic ecosystems are usually exposed to mixtures of xenobiotics in low concentrations, and it is difficult to predict adverse effects when information is obtained exclusively from chemical analyses (Pollo et al. 2015), because amphibians are particularly sensitive to environmental pollutants because they have a biphasic life cycle (aquatic and terrestrial) and a highly permeable skin that exchanges materials with the environment (Cabagna et al. 2005; Blaunstein et al. 2011), making them good bioindicators of environmental health (Young et al. 2004). This has led several scientists to work with different parameters related to amphibian populations, such as use of hematological biomarkers (Attademo et al. 2005; Peltzer et al. 2008; Bionda et al. 2011). This biomarker is very important because it represents the first level in which the initial interaction of pollutants occurs with the organism and can be used as early warning signals for higher levels (Cajaraville et al. 2000). In this context, hematological investigations become important because through these studies we can infer health and immune status of different species (Lajmanovich and Peltzer 2008). While, morphological analysis of blood cells are performed to evaluate the presence of alterations in size, color, and shape of erythrocytes and leukocytes and their frequencies, which can increase in cases of anemia, chronic diseases, malnutrition (Campbell 2004),

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poisoning (Chiesa et al. 2006), and environmental pollution (Barni et al. 2007), is fundamental to determine the state of metric parameters of blood cell of anurans that inhabit sites anthropogenically polluted, which has been poorly investigated (Zhelev et al. 2016).

The aim of this study is to determine the ability of blood cytomorphology and cytomorphometry as hematologic biomarker on individuals of *Rhinella arenarum* inhabiting aquatic environments with different degrees of disturbance from central Argentina.

Materials and methods

Study areas

We gathered samples from four sites: both urban and suburban aquatic environments with different disturbance types in the south of the province of Córdoba. The importance of selection of such environments for this study is based on previous results showing changes in population parameters in different amphibian and fish species, as well as changes in the properties of communities, possibly by the degree of environmental alteration (Pollo et al. 2012; Bionda et al. 2013). The first site, "Alpa Corral" (32° 42′ S, 64° 42′ W; 880 m a.s.l.) is a mountainous area known as Sierra de Comechingones, and is dominated by vegetation characteristic of highland forests and is not affected by crops or livestock. Given the characteristics of this site, it is considered as less disturbed of all.

The second site is a pond called "Charca de las Brujas" (33° 06′ S, 64° 25′W; 465 m a.s.l.), which is also not affected by crops or livestock and is near to a protected natural area the native forest El Espinal, located in Universidad Nacional de Río Cuarto Campus, considered a semimodified environment. This is an area with permanent and temporary ponds and a small strip of forest surrounding the ponds with grasslands and forest formations of native and non-native trees (Doffo 1989).

These are the sites which are considered to have the lowest degree of human disturbance.

The third site "Cultivo" is on agricultural land (9 ha: 33° 05′ S, 64° 26′ W; 465 m a.s.l.). This site is sparsely vegetated with low growing plants (heights <0.5 m), permanent and temporary ponds used intensively for cattle grazing. During the time the study took place, these ponds were surrounded by soybean crops.

The fourth site, "Villa Dalcar" Lake (33° 05′ S, 64° 26′ W; 467 m a.s.l.), is an urban aquatic system located in the city of Río Cuarto; it serves as an important location for fishing and recreation (Mancini et al. 2012). Several studies have been conducted in this sampling site due to multiple incidents of mass mortality of fish recorded in this lake (Mancini et al. 2000), as well as unusual algal growth (Novoa et al. 2006).

The latter two sites are considered to be areas with high levels of degradation due to urbanization, grazing, and cropland.

Data collection

A total of 93 specimens of *R. arenarum* (adults sexually mature) were captured during the spring, corresponding to the beginning of the reproductive period of this species, to avoid the effect of the seasonal variation (Table 1) (Varela and Sellares 1973b; Zhelev et al. 2016). All specimens were in a healthy condition. A set of environmental parameters of water were recorded in situ (temperature, pH, conductivity, total dissolved solids, and salinity) using a digital equipment Tests™ Multiparameter 35-Series 35425-10. A blood sample was taken from each specimen by puncturing the angularis vain (Nöller 1959) and extended prepared in field to avoid causing stress to the animals. The smears were kept cold until transportation to the laboratory, where they were dried at temperature before being stained with May Grunwald-Giemsa differential staining (Dacie and Lewis 1984). Blood smears were observed with Zeiss Primo Star iLED, and five types of leukocytes were distinguished following Hadji-Azimi et al. (1987) and Coppo (2003): lymphocytes, neutrophils, eosinophils, basophils, and monocytes. Only mature forms were considered. To obtain the leukocyte relation, the total number of leukocytes was first calculated relative to 10,000 erythrocytes (Davis et al. 2004). Then, knowing the standard percentage of the leucocyte formula for the species *B. arenarum* (Salinas 2012), the relation for each type of cell was calculated. A total of 40 erythrocytes, 24 lymphocytes, 11 neutrophils, 2 eosinophils, 2 basophils, and 1 monocyte per individual were photographed as Tiff image using the Canon digital camera Power Shot G10 for measurement of morphometric variables. Cellular (CA) and nuclear areas (NA) of erythrocytes and leukocytes were measured (μm^2) using the measurement area tool of the software Axio Vision 40V4.8.2.0. The nuclear/cell area ratio (N/C) was calculated. Additionally, Price-Jones curves using cell areas were performed to determine the variation in erythrocyte sizes (anisocytosis) between sites (Bessman et al. 1979). Mean and standard deviations of each variable recorded were calculated.

Data analysis

We tested the normal distribution of data by mean Shapiro-Wilks test. Then, ANOVAs (with those variables where normality was proved) or Kruskal-Wallis (for variables not normally distributed) were performed to test statistically significant differences between sexes and between sites. If the ANOVA revealed significant differences among sites, it performed a post hoc test (LSD de Fisher) to determine which

Table 1 Number of individual females and males collected at each sampling site

Sample site	Females	Males	Total
Alpa Corral	8	21	29
Charca de las Brujas	9	13	22
Villa Dalcar	11	13	24
Cultivo	5	13	18
Total			93

sites differed significantly from one another. For comparison of less disturbed site (Alpa Corral) and all sites, we conducted a Mann-Whitney test. InfoStat software was used for data analysis (Di Rienzo et al. 2012). The criterion for significance was $p < 0.05$.

Results

Salinity, conductivity, and total dissolved solids of water presented the lowest values in Alpa Corral, while Villa Dalcar showed the highest values of these parameters (Table 2). The pH values were also high for Villa Dalcar (9, 8) relative to other sites (Cultivo = 9, Alpa Corral = 8, 8, and Charca de las Brujas = 7, 9).

Cytomorphology

Erythrocytes are the most abundant nucleated cells in blood tissue. In the preparations, appearance of regular oval in shape was observed for these cells, with a blue-gray cytoplasm. As for nuclei, it is violet, with a central position and distinguishable. Like the cell, the nucleus also has a regular aspect (Fig. 1a).

In the blood preparations, lymphocytes were generally rounded and much less frequent oval with regular contour but may exhibit some cytoplasmic blebs. The cytoplasm presented a grayish coloration, and, in general, was a very narrow zone in the form of halo. This is because the nuclei occupied most of the cell and it is round to board in color (Fig. 1a).

In the case of neutrophils, the blue-gray cytoplasm evidenced a slight eosinophilia, and in some cases the presence of slight spots. The nucleus rarely centered was violet and with less granulation than the cytoplasm. Different types of lobularity were observed for its nuclei attached through a plasma bridge (Fig. 1c).

Eosinophils, in general, were observed to be irregularly oval shape, and in some cases circular. The cytoplasm presented large amounts of pink-orange granules of different sizes; not much cytoplasm can be seen. The purple nucleus, however, was observed smooth. The most common type of lobularity observed was bilobed, and in smaller, unilobed cases. On the other hand, the nucleus is located at one pole of the cell (Fig. 1e).

The basophils have a round or oval shape. They are presented a large number of granulocytes without being able to distinguish, in most cases, their round nuclei. It presents a colorless cytoplasm, whereas granulocytes and the nuclei of dark blue (Fig. 1b).

In this study, the monocytes had a round shape and grayish-blue cytoplasm. While its nuclei turned out of rounded aspect with horseshoe or kidney shape, and never in central position, and coloration was violet (Fig. 1d).

Cytomorphometry

The values of CA and NA for *R. arenarum* species are shown in Table 3. Because no sex differences were found for any of the size variables (ANOVA, $p > 0.05$ in all cases), data from both sexes were pooled. All variables showed significant differences among sites when compared using ANOVA or Kruskal-Wallis ($p < 0.05$), except basophils (CA $H = 1.2$, $p = 0.75$; NA $H = 0.4$, $p = 0.76$) and monocytes (CA $F = 1.05$, $p = 0.37$; NA $F = 0.26$, $p = 0.85$). The post hoc test indicated that in most variables, Alpa Corral differed with other sites. Individuals from Alpa Corral had the highest CA and NA in most of cell types analyzed (Table 3).

According to these results, and knowing that Alpa Corral correspond to the less disturbed site, we used Mann-Whitney test to compare this site with the others. The analysis showed that in the comparison of Alpa Corral and each disturbed sites in CA and NA, individuals from Villa Dalcar and Alpa Corral showed significant differences in CA and NA in alls variables, except in basophils (CA $U = 243$, $p = 0.5$; NA $U = 266$, $p = 0.208$) and monocytes (CA $U = 219$, $p = 0.949$; NA $U = 215$, $p = 0.989$). With regard to the other sites, individuals of Charca de las Brujas and Cultivo also showed statistically significant differences with individuals of Alpa Corral in relation to the CA of eosinophils ($p < 0.05$). For neutrophils only, individuals of Charca de las Brujas showed statistically significant differences with individuals of Alpa Corral ($p < 0.05$), but not for Cultivo ($U = 136$, $p = 0.458$). Regarding the NA, only erythrocytes of individuals to Cultivo differ statistically ($p < 0.05$) with erythrocytes of individuals in Alpa Corral, but not for lymphocytes ($U = 145$, $p = 0.270$), neutrophils ($U = 122$, $p = 0.857$), eosinophils ($U = 147$, $p = 0.237$), basophils ($U = 134$, $p = 0.508$), and monocytes ($U = 144$, $p = 0.288$).

Individuals from Villa Dalcar showed erythrocyte sizes ranging between 161 and 195 μm^2, while in the other sites, erythrocytes had values between 229 and 263 μm^2 (Fig. 2). Individuals from Alpa Corral and Charca de las Brujas showed very similar cell areas.

The analysis of N/C ratio for each cell type showed similar values (erythrocytes 0.15, neutrophils 0.37,

Table 2 Average salinity, conductivity, and total dissolved solids for each site studied

	Salinity (ppm)	Conductivity (μS)	Total dissolved solids (ppm)
Alpa Corral	62.05	127.70	91.70
Charca de las Brujas	114.50	243.50	172.50
Villa Dalcar	*446.50*	*923.50*	*655.50*
Cultivo	354.00	735.00	522.00

eosinophils 0.37, basophils 0.54, monocytes 0.59) in all sites except in lymphocytes of Alpa Corral individuals (0.9), the nuclei of which had about the same area as cells (Fig. 3).

Discussion

Hematic morphology of the species under study coincided with those of other amphibians (Varela and Sellares 1973a;

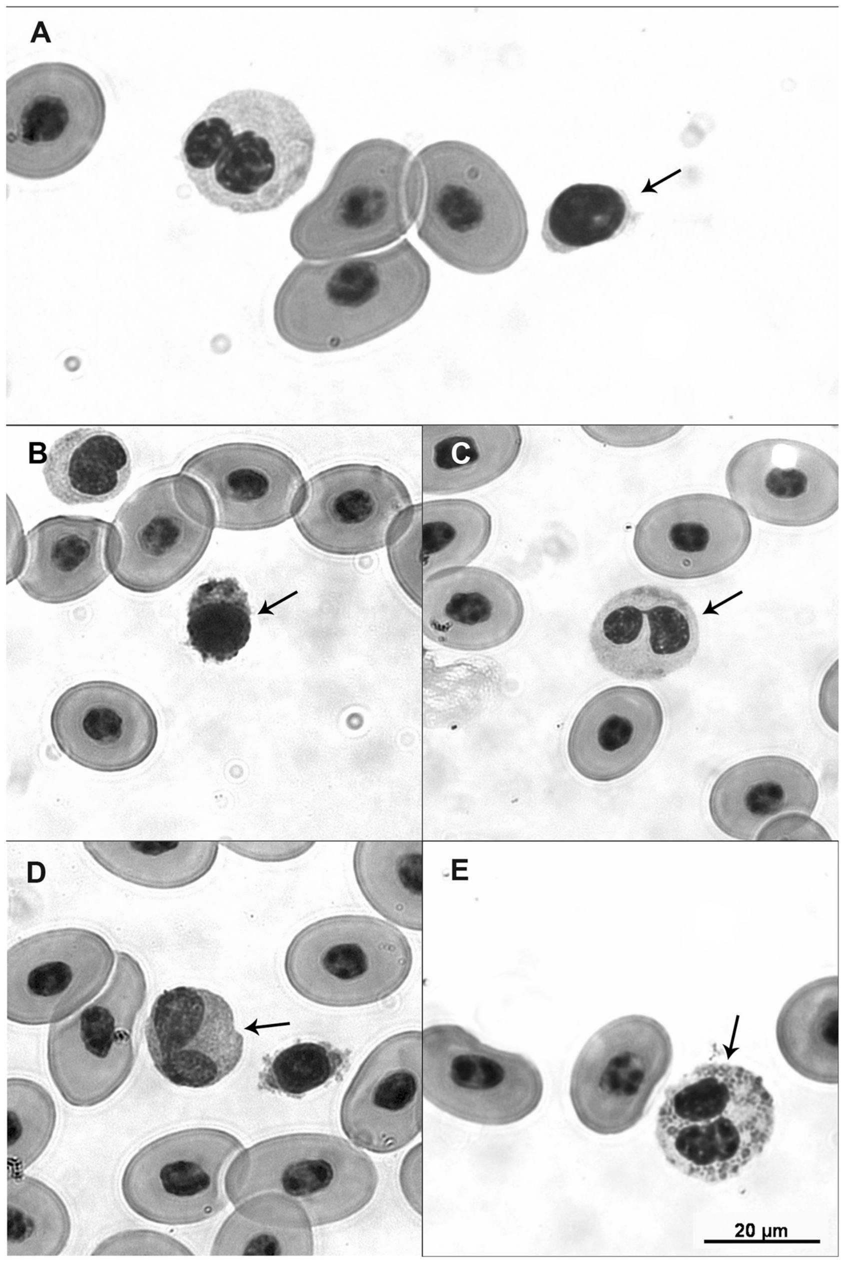

Fig. 1 **a** Left lymphocyte is observed. **b** Basophils. **c** The arrow indicates a neutrophil surrounded by red blood cells. **d** Monocyte. **e** Eosinophil

Table 3 Averages and standard deviations of nuclear (NA) and cell area (CA) of each cell type for species *R. arenarum* and each site

	Alpa Corral		Charca de las Brujas		Villa Dalcar		Cultivo		*R. arenarum*	
	CA (μm^2)	NA (μm^2)	CA (μm^2)	NA (μm^2)	CA (μm^2)	NA (μm^2)	CA (μm^2)	NA (μm^2)	CA (μm^2)	NA (μm^2)
Erythrocytes	232.9 ± 21.7	36.1 ± 4.7	226.8 ± 70.5	33.4 ± 10.4	193.1 ± 58.9	28.3 ± 7.6	231.9 ± 23.4	32.5 ± 2.5	*218.5 ± 53.8*	*32.3 ± 7.8*
Lymphocytes	75.9 ± 16.0	70.3 ± 78.3	63.2 ± 20.1	45.3 ± 14.4	58.8 ± 17.0	42.5 ± 12.2	65.8 ± 16.5	48.0 ± 13.1	*65.3 ± 18.4*	*50.8 ± 40.5*
Neutrophils	232.5 ± 46.5	81.1 ± 12.2	180.4 ± 71.8	70.0 ± 24.4	171.7 ± 53.5	61.6 ± 16.0	218.9 ± 57.5	79.4 ± 17.3	*196.6 ± 62.7*	*71.6 ± 62.7*
Eosinophils	271.5 ± 57.7	93.9 ± 20.5	213.7 ± 118.3	80.3 ± 34.9	170.5 ± 60.1	59.8 ± 14.5	215.3 ± 48.4	84.5 ± 21.0	*214.0 ± 85.8*	*77.8 ± 27.0*
Basophils	127.7 ± 55.6	68.5 ± 22.2	125.5 ± 66.1	65.5 ± 40.9	111.6 ± 46.4	59.0 ± 25.3	108.9 ± 49.4	63.3 ± 20.0	*118.8 ± 54.6*	*63.8 ± 28.8*
Monocytes	166.5 ± 49.7	93.7 ± 22.6	156.4 ± 71.0	90.9 ± 37.1	172.1 ± 88.3	96.4 ± 41.7	131.6 ± 40.6	86.2 ± 32.9	*159.5 ± 69.0*	*92.5 ± 34.7*

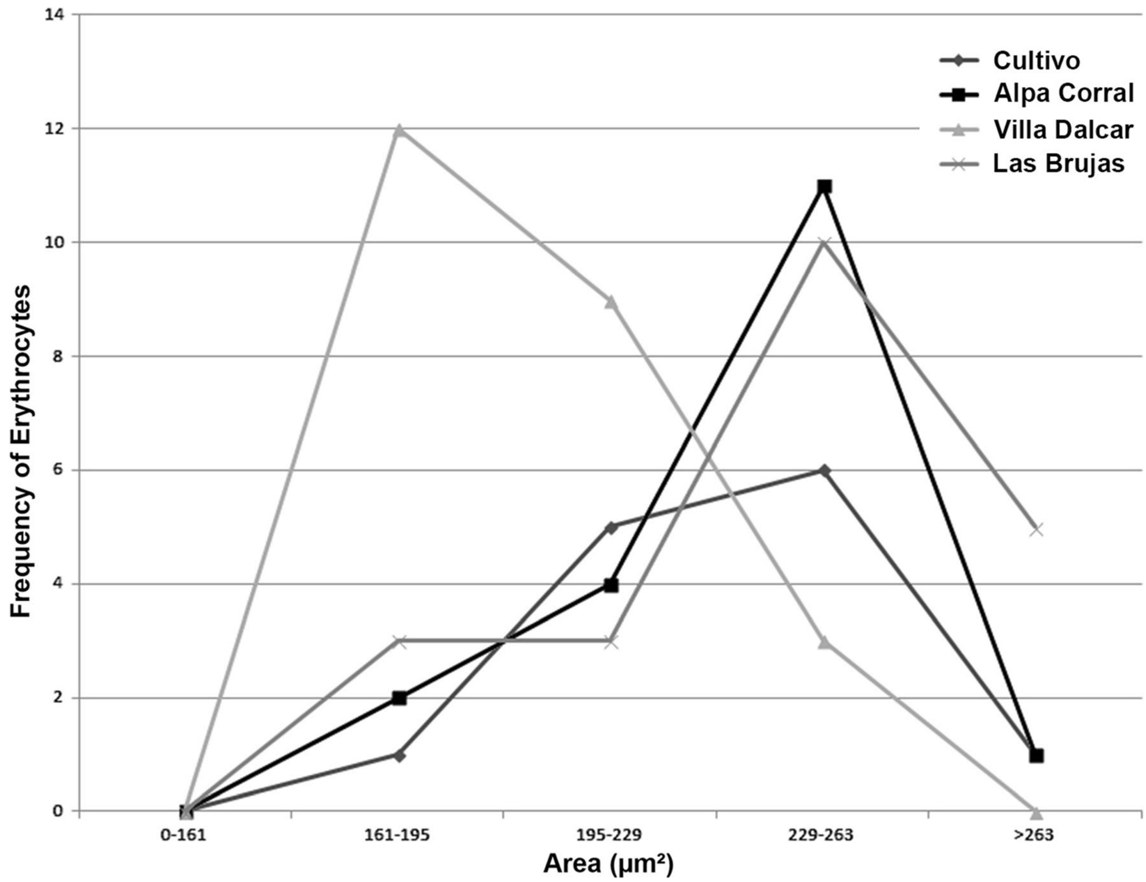

Fig. 2 Price-Jones curves using erythrocyte cell areas for comparison among sites (ref. Las Brujas = Charca de las Brujas)

Hadji-Azimi et al. 1987; Arserim and Mermer 2008; Arıkan 2010; Cabagna et al. 2011). However, through evaluating various parameter morphometrics within the same species (*R. arenarum*) and environmental characteristics in several sites with different degrees of disturbance, we observed that contrasting differences exist between the sites of major human disturbance compared to sites lesser degree of disturbance in terms of morphometry of blood cells.

Since there are no studies on the metric measurements of erythrocytes and leukocyte in *R. arenarum*, parameter morphometrics was compared with a species of the same genus, *Rhinella fernandezae* (Cabagna et al. 2011).

The N/C ratio obtained for *R. arenarum* was similar to that of *R. fernandezae* erythrocytes. However, cell and nuclear areas of erythrocytes *R. arenarum* (218.5 μm^2) were higher compared with *R. fernandezae* (150.79 μm^2) (Cabagna et al. 2011). According to Wintrobe (1933) and Campbell (2004), the size of erythrocytes reflects the position of a species in the scale of evolution. In vertebrates such as fish and frogs, erythrocytes are large, unlike mammals in which this cells are smaller and do not contain nuclei (Tok et al. 2009). From this, it is noted that within the class Amphibia, anurans have both cellular and nuclear areas significantly lower than in the order Caudata (Arıkan et al. 2001). However, it is known that the amount of erythrocytes is smaller (Arıkan et al. 2001). Furthermore, erythrocytes of terrestrial amphibians are smaller than those of aquatic species (Arserim and Mermer 2008).

As for the white series, the morphologic description of the five leukocyte types coincided with the description of Varela and Sellares (1973a) for the same species, and other studies in several anuran species (Hadji-Azimi et al. 1987; Arserim and Mermer 2008; Arıkan 2010; Cabagna et al. 2011). The mean lymphocyte area of *R. arenarum* (65.3 μm^2) was lower than in *R. fernandeze* (70.85 μm^2). This relationship is reversed for neutrophils and basophils. Eosinophils of *R. arenarum* (214.0 μm^2) were greater than those of *R. fernandezae* (147.01 μm^2). Finally, monocyte areas were similar in all two species. Nuclear areas of lymphocytes, neutrophils, eosinophils, and basophils were larger than nuclear areas reported by Cabagna et al. (2011) for *R. fernandezae* while monocytes had similar areas. The N/C ratio in lymphocytes and basophils of *R. arenarum* was higher than that recorded by these authors, while for neutrophils, basophils, and monocytes were observed similar values. Finally, our results are in agreement with other studies (Atatür et al. 1998; Arserim and Mermer 2008; Arıkan 2010; Cabagna et al. 2011; Baraquet et al. 2013); no significant differences between sexes were found in the morphometry of each cell type.

We found statistically significant differences in the size (cell and nuclear area) of erythrocytes, lymphocytes,

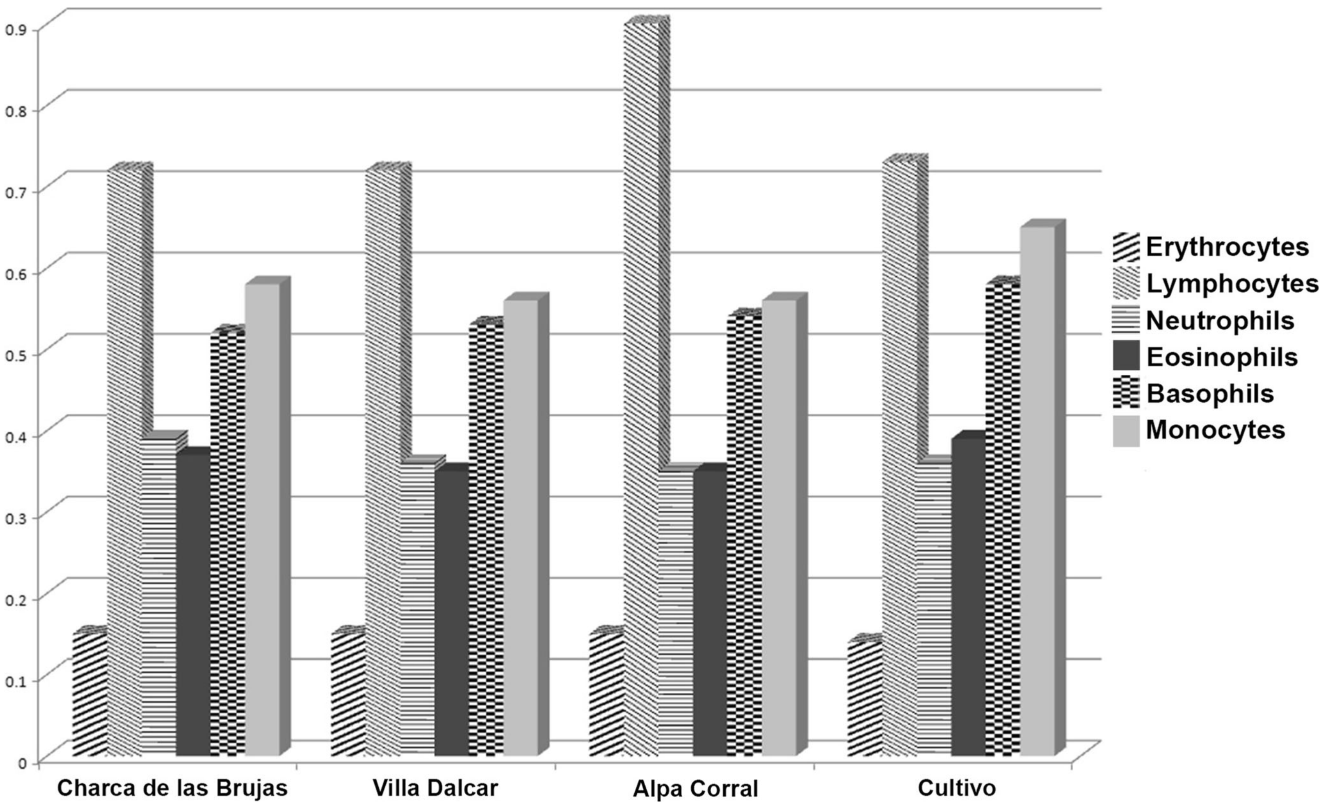

Fig. 3 N/C ratio for each cell

neutrophils, and eosinophils between individuals from Alpa Corral and from Villa Dalcar. Numerous studies indicate that hematological variation in erythrocyte size of amphibians could be associated with the metabolic activity (Wojtaszek and Adamowicz 2003; Arserim and Mermer 2008; Arıkan 2010). Several authors suggested that this variation is probably due not only to different geographical adaptations to high-altitude regions but also to a recent reduction in habitat as a result of human activities and the presence of different pollutants (Goniakowska 1973; Wojtaszek and Adamowicz 2003). Evidence for this is given by changes in hematological parameters as well as other genetic and morphological patterns (Arıkan 2010).

For the case of erythrocytes, several authors indicate that there are a number of environmental factors and activity levels (Atatür et al. 1998), as well as variations in habitat (Atatür et al. 1998) that affect the erythrocyte sizes (Tok et al. 2009). So, the dimensions of erythrocytes are used as biomonitoring of contaminated areas (Zhelev et al. 2006; Zhelev et al. 2017).

In a study carried out at the same seasonal (spring) by Zhelev et al. 2016 and Zhelev et al. 2017, a variation in the size of erythrocytes was observed based on different hematological parameters of individuals located in an area affected by heavy metals. The erythrocytes and their nucleus of the individuals at this site were smaller in size compared to erythrocytes at other sites sampled. In the case of Villa Dalcar, something similar is observed with respect to the sizes of erythrocytes and could be associated with the presence of the same type of substances. The presence of several car salvage yards near the lake represents potential revenue of toxic substances as heavy metals. Furthermore, its watershed sump has historically supported varied agricultural activities. Studies in the last decade indicate a high degree of environmental degradation of this urban lake, raising serious questions about management and use as a recreational area and fishing site (Pollo et al. 2015).

The values in the environmental variables can give evidence of this. According to Zhelev et al. (2017), existing synergism of the effects of toxic substances and their interaction with other environmental factors (pH, temperature, electroconductivity, oxygenation, etc.) arise an additional difficulty. In particular, the pH is important parameter because biological and chemical processes can only take place at a given pH (Addy et al. 2004). The values recorded in Villa Dalcar were the most basic compared to the other sites, with values close to 10. Both acid and alkaline stress cause mainly in amphibians a genetic disorder in the egg stage of some species. In freshwater, an optimum pH range ranges from 6.5 to 8, which would be optimal for survival and physiology in most aquatic organisms; outside of this range, direct toxic effects may occur and stress levels will be high. However, specifically in amphibians, the limit values of pH considered for normal development are a little more limited; in some species, a lower limit of 6.3 and higher has been mentioned (7.7) (Salinas et al. 2015).

On the other hand, the toxicity levels of certain environmental contaminants depend largely on pH, in addition to the temperature factor. For example, there has been a substantial change in the natural pattern of location and deposition, magnifying the distribution of heavy metals in the ecosystems which, in turn, has provoked a sustained increase in the exposure of the biota to their toxicity (Chiesa et al. 2006). Heavy metals strongly contribute to environmental pollution, and it is in function of the present pH that generates greater toxicity (Prieto Méndez et al. 2009). According to Zhelev et al. (2016), pH correlates heavy with hematological parameters associated with erythrocyte size, such as erythrocyte size (ES), nucleus size (NS), and nucleus cytoplasm ratio (NS/ES).

Added to this, the toxic effects of heavy metals destroy the immune system of aquatic animals (for example, fish) and cause hematological disturbances: erythrocyte destruction (haemolysis) and leukocytosis (Javed and Usmani 2012). In anurans, heavy metals attack the liver and kidney and cause changes in basic hematological parameters (Priyadarshani et al. 2015; Carvalho et al. 2016; Medina et al. 2016; Zhelev et al. 2017).

Although it is somewhat complex to perform this type of studies due to the influence of the stations on the hematological values (Varela and Sellares 1973b; Zhelev et al. 2016), besides, the dose, degree of exposure, environmental conditions, and genotype may influence toxicity effects, future studies are recommended to complement the present. Nevertheless, according to the results of our study, the hematologic evaluation is one of the simplest and least invasive methods, allowing early detection of physiological changes related to processes of environmental pollution. The changes in their values, especially of those in the populations that live in condition pollution, can be successfully used for preliminary general assessments of the ecological status of a water body without any need of expensive and labor-intensive physicochemical analyses; such could subsequently be applied to provide a detailed assessment of environmental pollution (Zhelev et al. 2016). In this regard, the immediate development of management plans and monitoring for potential generators of toxic substances in urban aquatic environments is needed to prevent long-term consequences in these ecosystems, as well as to the health of society itself.

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