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Blood differential count and effect of haemoparasites in wild populations of Pyrenean lizard *Iberolacerta aurelioi* (Arribas, 1994)

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Differential cell count in four wild populations of *Iberolacerta aurelioi* (the smallest and most endangered lizard species found in the Pyrenees) has been studied. *Iberolacerta aurelioi* has cytological values similar to those described for other lacertid species. Moreover, the survey has detected the presence of haemoparasites genus *Haemogregarina* in this species. Haemoparasites are very abundant in the population of Mont-Roig, being almost insignificant in Pica d'Estats. The presence of haemoparasites is significantly related to erythroblast occurrence. Results suggest that haemoparasitism in this species stimulates high rates of blood cell regeneration.

Key words: *Haemogregarina*; haemoparasites; *Iberolacerta aurelioi*; leukocyte differential count.

Recuento diferencial y efecto de los hemoparásitos en la sangre de poblaciones silvestres de lagartija pirenaica *Iberolacerta aurelioi* (Arribas, 1994). Se ha estudiado el recuento sanguíneo diferencial en cuatro poblaciones de *Iberolacerta aurelioi* (el lacértido endémico más amenazado y de menor tamaño de los Pirineos). *Iberolacerta aurelioi* tiene valores citológicos similares a los descritos para otras especies de lacértidos. Asimismo, el análisis de frotis sanguíneos ha permitido detectar la presencia de hemoparásitos del género *Haemogregarina*. Estos hemoparásitos son abundantes en una de las poblaciones estudiadas (Mont-Roig) y son minoritarios en otra (Pica d'Estats). La presencia de hemoparásitos está significativamente relacionada con la aparición de eritroblastos. Los resultados sugieren que el hemoparasitismo en esta especie estimula unas altas tasas de regeneración celular sanguínea.

Key words: *Haemogregarina*; hemoparásitos; *Iberolacerta aurelioi*; recuento diferencial leucocitario.

The Pyrenean's Rock lizard *Iberolacerta aurelioi* (Arribas, 1994) is endemic to the easternmost part of Central Pyrenees, where it only inhabits three mountain massifs: Mont-Roig, Pica d'Estats and the nucleus of Coma Pedrosa, distributed across Catalonia (north-east Spain), Andorra and the Ariège (south France). This species, discovered in the field in 1991 and described in 1994, is a small lizard (males up to 57.6 mm with average 52.05 mm, females up to 62.21 mm with average 54.32 mm) with a body mass between 6 and 8 g. It inhabits alpine belts, usually between 2100 and 2940 m, being more abundant from 2300 to 2500 m. It lives on rocks, boulders and screes, usually in glacial cirques, in sheltered, well insolated and moderately sloped localities (ARRIBAS, 2007). Due to the harsh climate of the areas where it is present, the species has a short annual

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period of activity (four or five months, from mid-May to late September or early October; ARRIBAS, 2009).

Iberolacerta aurelioi is an endangered species, nominally protected in Spain, France and Andorra. Apart from climatic change, which is forcing this species higher up the mountain beyond its survival range, conservation problems involve any activity that implies change in the habitat of separated micropopulations. In the short term, any action involving the modification and disappearance of fragile high alpine habitats should be avoided: ski resorts, mountain refuges that attract mass tourism to inhabited areas or flooding due to hydroelectric dams, among others (ARRIBAS, 2004, 2007).

Some of the most important tools to learn about the physiological adaptations of reptiles to special environmental conditions (e.g. high mountain or insularity, among others) are haematology and blood cytology (MARTÍNEZ SILVESTRE, 2011). Both are also very important aspects of the diagnostic evaluation of endangered saurians of the Lacertidae family (HERNANDEZ-DIVERS *et al.*, 2003; MARTÍNEZ SILVESTRE *et al.*, 2007; SACCHI *et al.*, 2011). The objective of this study is to describe basic haematological values for *I. aurelioi*. Reference intervals are useful for physiological evaluation and detection of pathological alterations in wild lizards, which might be ultimately relevant for conservation purposes. Although some studies exist on haematological and biochemical values in other lacertid lizards such as the giant Canary lizards (*Gallotia simonyi*, *G. bravoana* and *G. intermedia*; MARTÍNEZ SILVESTRE *et al.*, 2004, MARTÍNEZ-SILVESTRE *et al.*, 2005), or Mediterranean lizards (SEVINÇ & UGURTAS, 2008), there are not, as far as we know, comparative haematological and cytological reference values for Pyrenean lacertid lizards. In addition, we will investigate the presence of haemoparasites. This study constitutes the first description of these parameters in the rare, endangered and high-mountain dwelling lizard *I. aurelioi*.

MATERIALS AND METHODS

A total of 17 specimens were captured during the reproductive period (from mid-June to mid-July) of 2006 in four localities (see Table 1 for more details) and sexed using described morphological characteristics (ARRIBAS, 2009).

Table 1: Localities, number of specimens, coordinates and altitude of the *Iberolacerta aurelioi* samples analysed. N, number of samples analysed (M: males, F: females, Sub: subadults).

Locality	Country	Locality Code	N (M / F / Sub)	Coordinates	Altitude (m)
Port de Rat (Coma Pedrosa Massif)	Andorra	AND	5 (2 / 3 / 0)	42º 37' 23.46" N 1º 28' 34.84" E	2387
Estany d'Estats (Pica d'Estats Massif, Lleida)	Spain	P	5 (0 / 5 / 0)	43º 39' 11.50" N 1º 22' 55.6" E	2400
Salòria Massif (Lleida)	Spain	CAP	4 (2 / 1 / 1)	42º 30' 52.27" N 1º 23' 5.75" E	2450
Calberante (Mont-Roig Massif, Lleida)	Spain	MR	3 (2 / 1 / 0)	42º 41' 33.64" N 1º 10' 18.01" E	2361

All animals were apparently clinically healthy at the time of sampling. Blood samples were collected in the field, with blood extraction in situ and immediate release of the specimens.

Blood smears were prepared using blood obtained from the ventral coccygeal vein, using disposable syringes and 23 gauge needles. Due to the little size of the lizards, only 0.1 ml was taken for cytological assessment.

Differential leucocyte counts were made from blood smears using 1000x magnification lens. A total of 100 leucocytes per slide were examined. The air-dried smears were studied by conventional light microscopy and stained with a commercial Wright's stain (Quick Panoptic®, Química Clínica Aplicada S.A., Amposta, Spain) for differential leucocyte counts and description of erythrocytes, thrombocytes, leucocytes and hemoparasites. Leucocytes were categorized as: heterophils, eosinophils, basophils, azurophils, lymphocytes and monocytes. Presence of cytological disorders (mitosis and parasitologic assessment) was also performed. Blood parasites were detected, and 20 of them were measured using an optical microscope.

Multivariate statistical analysis (correspondence analysis) was performed with the software NCSS 2001 (NCCS LLC, UTAH, USA; HINTZE, 2001). Correspondence analysis (CA) is a very useful exploratory technique for graphically displaying a two-way table by calculating coordinates representing both its rows and columns using Chi-square (thus, nonparametric) distances (GREENACRE, 1993; HINTZE, 2001). Rows in the original matrix were the different samples, and columns were the number (counts) of heterophils, eosinophils, basophils, azurophils, monocytes, lymphocytes, erythrocytes, erythroblasts, mitoses and haemoparasites observed. Comparison between cell counts by sex and localities and correlations between cell types were done using nonparametric approaches (Mann-Whitney significance test and Spearman correlation test, respectively; SOKAL & ROHLF, 1969).

RESULTS

According to our data, sex had no relation to special blood parameters (Mann-Whitney U-test, $P > 0.05$ in all variables). The haematological differential reference values for the 17 specimens of *I. aurelioi* analysed are presented in Table 2. Presence of immature forms of erythrocytes (erythroblasts) was detected in 45% of the animals sampled, and mitoses were observed in 11.8% of counted erythrocytes. Parasites measured between 2.9 to 3.6 µm of

Table 2: Descriptive statistics of the cyto-hematologic reference values and erythrocytes parasitized per microscopic field of the 17 wild-caught *I. aurelioi* included in this study.

	Heterophils	Eosinophils	Basophils	Azurophils	Monocytes	Lymphocytes	Parasitized erythrocytes (%)
Mean	40	6	0	9	6	35	5
Std. Dev(sd)	11,24	7,84	1,33	9,91	6,35	13,48	15,8
Max	56	24	4	28	20	70	7
Min	15	0	0	0	0	15	1

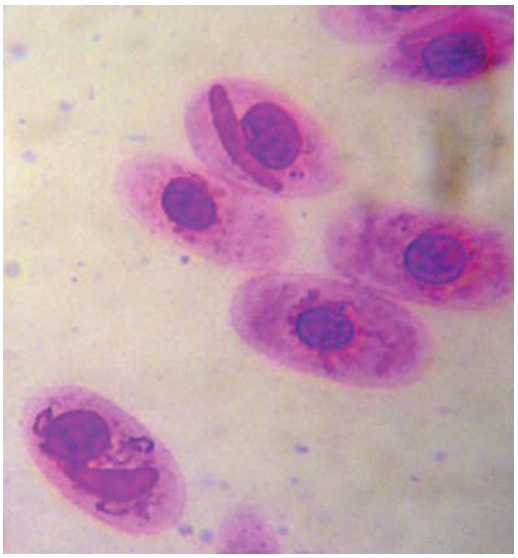

Figure 1: *Haemogregarina* spp. parasitizing erythrocytes in the lizard *I. aurelioi*, inducing a displacement in nucleus position and a change in cell shape.

width and 15.3 to 14.6 µm of length (Fig. 1). These protozoa affected 5% (average) of the cells and had an intracellular location. Affected erythrocytes had the nucleus marginalized and increased cell size, being the parasites identified as belonging to the group of haemogregarines. Cytological identification of the species was concordant with genus *Haemogregarina*, family Haemogregarinidae (TELFORD, 2009) according to their disposition, aspect, shape and morphology. However, definitive identification via molecular testing or other methods was not performed. Haemoparasites were detected in 50% of the animals analysed, but percentages varied depending on the micropopulation of origin of the host as follows: Pica d'Estats Massif (20%), Andorra (40%), Saloria (50%), and Mont-Roig (100%).

Correspondence analysis was run to explore the relationships among the most determinant factors in the observed inter-sample variability (Fig. 2). In our study the first two axes represented graphically accounted for 51.23% of the total variability. Relationships among the haematological parameters can be extracted from the axis structure. In detail; the first axis (eigenvalue, E = 0.12) explains 26.8% of total variability, with a high contribution (loadings with higher absolute value) of basophils (-1.65) and mitosis (-1.037), both in the negative part of the bivariate space. The second axis (E = 0.11) explains 24.43% of the total variability, and the most contributing variables were basophils (-1.800) in the negative part and mitosis (1.059) plus haemoparasites (1.009) in the positive part of the bivariate space (Fig. 2). Several blood parameters appear in the analysis, which are more or less grouped and seem to be correlated among them (monocytes,

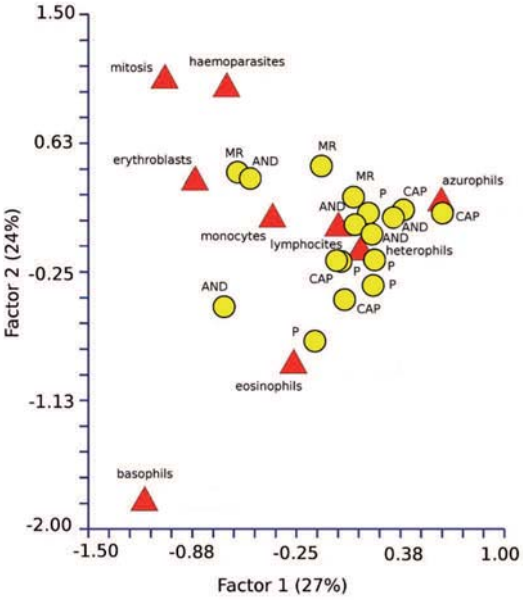

Figure 2: Representation of the haematological parameters (triangles) of the *I. aurelioi* samples studied (circles). Locality codes are provided for each sample (see Table 1 for more details). The oval shows the location of the number of mitoses and the presence of haemoparasites in the CA multivariate space.

lymphocytes, heterophils, azurophils and erythroblasts) with no specific clinical application, but two parameters appear surprisingly related and individualized in the graph: the number of mitoses and the presence of haemoparasites. Although the relationship of these two parameters seems to be accidental, as their pairwise relationship is not significant (Spearman Rank correlation, rs = 0.18, $P > 0.05$), we find a significant correlation (one tailed test) between the presence of haemoparasites and erythroblasts (rs = 0.44, $P < 0.05$) and also between the number of mitoses and the number of erythroblasts (rs = 0.6, $P < 0.01$), both reflected in Fig. 2.

DISCUSSION

Our study reveals a significant correlation between the presence of haemoparasites and erythroblasts. Such correlation may be due to the effect of parasitism in the stimulation of blood regeneration, as described in other reptiles (MARTÍNEZ-SILVESTRE *et al.*, 2011). Moreover, correlation between the number of mitoses and the number of erythroblasts might be related to the high mountain environments. In fact, high blood regeneration to compensate lower oxygen pressure is a physiological adaptation to altitude described in several animal groups (MANI, 1968; RIVOLIER *et al.*, 1985). On the other side, the correspondence analysis showed the existence of two cell types that are independent from the others and between them: eosinophils and basophils. This is a usual situation, since there is great variability in these cell types, as seen in other lacertilian reptiles such as *Gallotia* lizards (MARTÍNEZ SILVESTRE *et al.*, 2004). Although eosinophils and basophils increase is associated with parasitism, this is not the case here. This fact is observed in other studies where the presence of haemoparasites is not related to variations of these kinds of cells, either in tortoises (LAWRENCE & HAWKEY, 1985) or crocodilians (GLASSMAN *et al.*, 1979). As consequence, it should be inferred that haemoprotozoa may not trigger the same immune reaction as other kinds of parasites do.

In the representation of samples based on their haematological parameters, Mont-Roig samples are fairly similar among them, whereas Andorra, Pica d'Estats and Capifonts are more heterogeneous. It is possible that the high degree of parasitization and other possibly linked phenomena such as the large number of mitoses, made the three Mont-Roig samples appear so close in the analysis.

The joint representation of samples and haematological parameters showed that the Mont-Roig samples are notable particularly in terms of number of mitoses, haemoparasites and erythroblasts. The remaining samples are fairly well correlated with the abovementioned parameters that appear more or less grouped together (monocytes, lymphocytes, heterophils, azurophils and erythroblasts) with values that we can consider as the usual for the species, at the light of our data. These results are not coincident with the ones observed in other Lacertidae studies such as the ones carried out on the giant lizards from Canary Islands (Spain) (genus *Gallotia*) (MARTÍNEZ SILVESTRE *et al.*, 2004). In this genus, higher values of heterophils (33%), basophils (10%) and azurophils (12%) were observed, whereas eosinophils (1.5%), monocytes (1.5%) and lymphocytes (14%) had higher values in *I. aurelioi* (Table 1). These differences may be indicative of

physiological adaptations to different environmental conditions (SACCHI *et al.*, 2011). Interestingly, mitoses in this species show a relatively higher occurrence in *I. aurelioi* when compared to other species (SALAKIJ *et al.*, 2002; MARTÍNEZ SILVESTRE *et al.*, 2004; STRIK *et al.*, 2007).

Mitosis and regeneration resulting from haematological parasitosis could be related to heterophilia or eosinophilia, since they are also usually associated with parasitosis in reptiles (JACOBSON, 2007). However, in our case, the presence of haemoparasites is not related with eosinophilia in any of our sampled populations. This is in agreement with other studies carried out with haemoparasites in crocodilians (GLASMANN *et al.*, 1979) and tortoises (LAWRENCE *et al.*, 1985). In our study, monocytosis was neither associated with haemoparasitism, contrary to the results obtained in the lizard *Ameiva ameiva* parasitized with the protozoan *Plasmodium* (BONADIMAN *et al.*, 2010). Moreover, animals with higher number of erythroblasts also show erythrocytes in mitosis, which although not rare, is neither very common in reptiles.

Finally, the presence of haemoparasites seems to be an important factor in the observed results. The biological cycle of the parasite causes the destruction of the infected erythrocytes, leading to blood regeneration. This relationship has been shown in several studies (MARTÍNEZ-SILVESTRE *et al.*, 2011). Erythrocyte regeneration is characterized by a high frequency of young cells in peripheral blood (also confirmed in this study) and an activation of cell regeneration processes in a frequency higher than usual. The presence of mitoses is not rare in peripheral blood, but, although not significant, such a high number of mitoses as observed in these lizards is infrequent. In our analysis, the correlation is almost significative and probably it would be with a larger sample size.

Populations of this lizard are not as dense as in other known lacertid species, which may be important from an epidemiological viewpoint. Maximum densities for the species of 175 and 145 individuals per hectare (ind / ha) were estimated in two successive years in Andorra (Ordino-Arcalís) in particularly favourable sites (ARRIBAS, 2009). During the collection of samples for this study (end of June and early July 2006), densities of 20.8 ind / ha in Mont-Roig massif, 25.8 ind / ha in Pica d'Estats and 10.27 ind / ha in Salòria (Capifonts) were observed (ARRIBAS, 2006). Theoretically, the more crowded lizards live, the more possibilities have parasites to infest new hosts. In our case, low lizard densities combined with the isolation of the populations in different mountain massifs might have helped to avoid high infestation levels and explain differences among micropopulations. In other words, orography of the habitat of *I. aurelioi* could represent a true barrier for parasitaemia levels become homogeneously epidemic.

We can conclude that parasitized animals have higher erythrocyte regeneration, and hence parasites are probably directly involved in this situation. The most important question to answer is whether the lizards are able to co-evolve and reach equilibrium with the parasite or whether these parasites represent a threat to the conservation of *I. aurelioi* in the affected mountain micropopulations.

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