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ANIMAL SCIENCE

Hematology of *Liolaemus pacha* (Iguania: Liolaemidae) and its relationship with mite infestation, reproductive period and body condition

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Abstract: Variations in hematological profile in reptiles can be caused by multiple factors, including parasites presence. Our goals were to identify and morphologically describe blood cells of *Liolaemus pacha* and analyze their relationship with sex, body condition, individual reproductive/post-reproductive period and mite infestation. Blood smear analyses do not indicate the presence of hemoparasites, suggesting that the mites *Neopterygosoma* do not serve as vectors for these organisms, as has been proposed for other genera of ectoparasitic mites. In post-reproductive period, there was a reduction in specimens' body condition and a higher leukocyte count in uninfected lizards. This could be a consequence of the testosterone effects, in higher concentration during the reproductive season, which can increase the metabolic rate, decreasing feeding rate. Infested and non-infested lizards showed no differences in body condition, as well as in leukocyte count, hence the host's immune system could be developing infestation tolerance. Infested specimens had a higher count of monocytes, thrombocytes, heterophils and lymphocytes. Based on cells function, mites' effect could be associated with inflammatory processes, allergic reactions or infectious diseases. These results suggested a complex interaction between lizards' hematological parameters and factors associated to ectoparasites or body conditions. We consider this work as a diagnostic tool for genus *Liolaemus*, to evaluate health quality, with relevance to the conservation or management of this lizard's genus.

Key words: Argentina, blood cells, ectoparasites, *Liolaemus*, lizards, mites.

INTRODUCTION

Blood constitutes an effective approach of evaluating a large amount of information about individuals (Lochmiller et al. 1985, Hannon & Grant 1988). In this context, hematology constitutes a main physiological parameter that allows measuring health condition and nonspecific immune response, contributing to diagnosis and management of a wide variety of diseases (Kolmer 1981, Vassart et al. 1994). Variations in cytomorphology, as well as changes in hematological values, have important implications for animal health (Chamut & Arce 2018).

In reptiles, variations in the hematological profile may have their origin in factors such as: species, age and sex (Duguy 1963), seasonal factors (Frye 1991, Chamut & Arce 2018), nutritional status (Bolten & Bjorndal 1992, Alleman et al. 1999, LeBlanc 2001, Dickinson et al. 2002, Simpson 2006), stress (Aguirre et al. 1995), parasitism (Campbell 2004, Innis et al. 2007, Martínez Silvestre 2011, Selleri & Hernandez Divers 2006), environmental pollution and immune responses

(Duguy 1970, Frye 1991, Campbell 1998, 2004, Pal et al. 2008, Olayemi 2011, Parida et al. 2011, Stacy et al. 2011). These variations allow both diagnosis of diseases and characterization of physiological and pathological conditions of animals in captivity or in the wild (Harder 1994). Consequently, those parameters can be used as habitat quality indices (Franzmann & LeResche 1978, Seal & Hoskinson 1978, Lochmiller et al. 1985) and to evaluate population's health status. Knowledge of hematological parameters in free-living individuals is important to evaluate and manage their populations, because blood counts provide a useful tool to easily detect pathological processes within them (Martínez Silvestre et al. 2005). To generate the aforementioned inferences, reference values must be generated "a priori", that is, knowing the normal blood parameters in a population, allowing "a posteriori" comparisons with the same or other populations (Franzmann & LeResche 1978, Weaver & Johnson 1995).

Currently, the scientific community resumed activity in the field of hematology, noticing increased knowledge in individual management techniques. This includes, improvements in blood extraction techniques (point of sample collection, maintenance, optimal volume and condition of the individual), as well as staining techniques (Wilmorth 1994, Parpiñan et al. 2006, Sykes & Klaphake 2008, Padilla et al. 2009).

The classification proposed by Hawkey & Dennett (1994) is commonly used in hematological studies carried out in reptiles (Davis et al. 2008, Troiano et al. 2008, Rojas Moreno & Varillas 2013, Kindlovits et al. 2017). It is based on the staining morphological analysis of blood: erythrocytes, mononuclear leukocytes (lymphocytes, monocytes, and azurophils), and granulocytic leukocytes (heterophiles, eosinophils, and basophils). Heterophils are equivalent to mammalian neutrophils, while reptile monocytes and lymphocytes have the same function and morphology as those of mammals, birds and fish. Azurophiles are a specific cell type present in Squamata (lizards and snakes) and Crocodilia (caimans, crocodiles and gharials) (Raskin 2000, Martínez Silvestre 2011, Martínez Silvestre et al. 2005), while they are sometimes identified in chelonians as neutrophils or neutrophilic azurophiles (Alleman et al. 1992, Anderson et al. 1997, Wilkinson 2004, Zhang et al. 2011).

Among parasites, mites can cause various health problems and diseases in reptiles. Some studies showed that high loads of mite have a high metabolic cost, lower weight gain (Klukowski & Nelson 2001), lower resistance and therefore, reduced activity; and local skin inflammations in the attack areas (Reardon & Norbury 2004) among other effects. In addition to the likely transmission of blood parasites (Newell & Ryckman 1964, Fajfer 2012). Although, there are five mite families that have been reported to carry pathogens: Amblyommidae, Argasidae, Ixodidae, Macronyssidae and Pterygosomatidae (Fajfer 2012), with a large amount of research; the direct impact of ectoparasitic mites on the health of reptiles is still unclear (Reardon & Norbury 2004).

Liolaemus pacha (Juárez Heredia et al. 2013) mites belong to *Neopterygosoma* genus, (Pterygosomatidae Fajfer 2019). In a previous study, males lizards presented a greater intensity of infestation of *Neopterygosoma* mites, and this difference was not modified by mass or size of the host (Juárez Heredia et al. 2014). *Neopterygosoma* mites, are concentrated in the ventral region (specifically on belly' sides and in the gular region of the host), located below their host' scales (Juárez Heredia et al. 2014, 2020). Histological analysis of *Neopterygosoma* insertion point into the host's skin showed that there is no epidermal damage or inflammatory

reactions (Juárez Heredia et al. 2020). The type of feeding of *Neopterygosoma* mites is not defined (Fajfer 2019), but according to histological observations by Juárez Heredia et al. (2020) in mite's insertion point , they cast doubt on the type of hematophagous feeding. Therefore, it is relevant to investigate host hematology, and evaluate the effect produced by the presence of ectoparasites (e.g. lesions due to allergic reaction Arnold 1986). Thus being able to explore the feeding type of *Neopterygosoma*.

Hematological studies in the *Liolaemus* genus are scarce (eg. Engbretson & Hutchison 1976, Ceballos de Bruno 1995, Peña Roche 1939, Ruiz et al. 1993), considering the large number of species reported for the genus (283 spp, Abdala et al. 2021). Although *L. pacha* is categorized as Least Concern in the IUCN (Abdala & Ávila 2016), this study could be relevant to the conservation or management of other lizards of the genus *Liolaemus* that are endangered. Due to the above, this study aims to: 1) analyze blood smears from the lizard *L. pacha*, 2) identify and morphologically describe the blood cells found, 3) perform the differential count of leukocytes; and finally, 4) assess the existence of differences related to the individual's sex, its body condition, the reproductive/post-reproductive period and the infestation by *Neopterygosoma* mites.

MATERIALS AND METHODS

Liolaemus pacha is a lizard' species which inhabits Los Cardones' site (26° 40' 1.5" S, 65° 49' 5.1" W, 2700 masl), located 20 km east of the Amaicha del Valle commune, Tafí del Valle department, Tucumán, Argentina. Monthly visits were carried out from October 2013 to February 2015, during lizards' activity period (from 9 a.m. to 5 p.m.). Seventy-two adult lizards of both sexes were captured (fifty-three males and nineteen females) were captured with the noose technique (Scrocchi & Kretzschmar 1996), which ensures that the specimen will not suffer any damage. Two or three blood smears were taken in field from each lizard individual, then all smears were analyzed in laboratory. After blood collection, the lizards were released in the exact location of collection.

Data on weight (P), snout-vent length (LHC), and mite intensity (*Neopterygosoma* (Pterygosomatidae) Juárez Heredia et al. 2014, Fajfer 2019) were recorded for each lizard specimen.

Animal maintenance and experimental procedures were in accordance with Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research (Herpetological Animal Care and Use Committee of the American Society of Ichthyologists and Herpetologists 2004), and the corresponding approval permit from the Dirección de Flora, Fauna silvestre y suelo de Tucumán, Argentina (Resol. #169-13).

Sampling

Samples for blood smears were collected by puncture of the coccygeal vein, present along the ventral midline of the tail. It is a recommended technique due to its advantages related to sample volume, a minimally invasive procedure, with low risk for the individual of feeling pain (compared to cardiac puncture, tail tip cutting and retro-orbital venous sinus) (Sykes & Klaphake 2008, Troiano 2013), and the low probability of sample contamination (Frye 1991, Hernandez Divers 2006, Campbell & Ellis 2007, Strik et al. 2007, Martínez Silvestre et al. 2011). The extraction began by holding the lizard in a supine position and disinfecting the area with 70% ethyl alcohol. Subsequently, the needle (0.5 x 15 mm) was carefully introduced into the coccygeal vein at a 90° angle. The blood drop was placed on a slide (25.4 x 76.2 mm - 1mm x 1.2 mm wide) and the smears were

made following routine techniques (De Rodak et al. 2012, Carr & De Rodak 2014). Once the spreading was completed, it was left to dry at room temperature. The blood smear was fixed with 70% methyl alcohol and labeled (date, individual identity, sex, total number of mites).

Smear staining

Staining process was carried out using the May-Grünwald Giemsa technique (Solís 1996, Troiano et al. 2008, Martínez Silvestre et al. 2011, Troiano & Gould 2011). This differential staining technique is based on the use of two dyes: the May-Grünwald solution containing the anionic dye eosin, which upon binding to acidophilic cellular structures stains them in shades of orange to red; and the cationic dye methylene blue that stains in different shades of blue by selectively binding to basophilic components. The second dye used is Giemsa, which contains eosin, methylene blue and certain oxidation compounds of these, the azures. This staining highlights the chromatin and the azurophilic granules, especially highlighting the leukocyte granulations and also improving the staining of the erythrocytes.

As a first step, the smears were covered with May-Grünwald solution for three minutes. Then an equal volume of distilled water was added for one minute. After this time, the dye was removed and rinsed with distilled water. Finally, it was covered for fifteen minutes with a Giemsa solution diluted ten times in distilled water. Finally, it was rinsed with distilled water and allowed to air dry.

For erythrocytes and leukocytes identification, the criteria of Hawkey & Dennett (1994) and Stacy et al. (2011) were followed, based on tintorial and morphological terminology.

Blood smear analysis

Red series or erythrocytes

The erythrocyte series was morphologically evaluated and the presence of senile erythrocytes was recognized (Ceballos De Bruno 1995, Stacy et al. 2011). They were counted (with a 40x objective), to identify possible differences between non-infested and mite-infested specimens. The count was performed by dividing the smear into two areas (area b and area c, see Fig. 1c, each made up of five fields (total of 10 fields). In each field, a total of 100 erythrocytes were counted, differentiating senile and normal erythrocytes number. To obtain the senile erythrocytes percentage in each sample, the average of the 10 fields was calculated, divided by 10 (total fields) and multiplied by 100 (Martínez Silvestre et al. 2011).

Measurements of erythrocytes were taken and three levels of poikilocytosis (type of variation of erythrocytes which take irregular

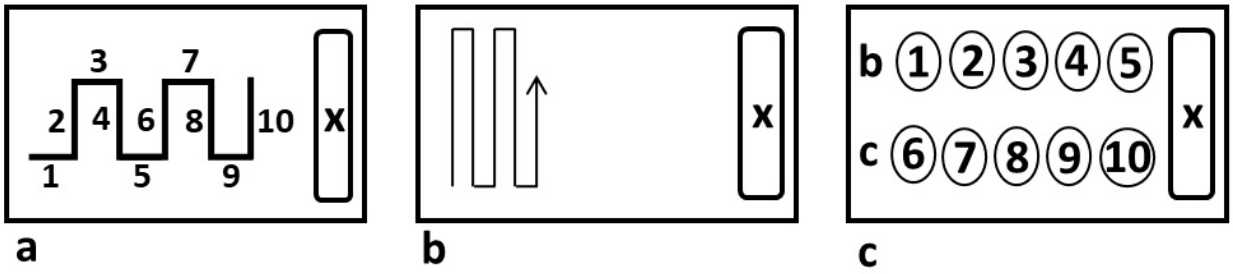

Figure 1. Sheme counting technique. a: leukocyte counting technique in 10 fields, following a Greek guard (40x). b: differential leukocyte count technique following serpentine pattern (100x). c: senile erythrocyte, areas b and areas c: smear areas up of five fields (40x). x: label with sample identification data.

shapes Campbell 1998, Stacy et al. 2011) were qualitatively defined: null, mild and high (Fig. 2, chamber digital AxioCam ERC5S, images processed with ZEN 2012-Blue edition® software).

White series or leukocytes

A descriptive and quantitative analysis of white series was carried out. The technique of manual counting of total leukocytes was used (Martínez Silvestre et al. 2001, 2004, 2011, Martínez Silvestre & Arribas 2014, Rojas Moreno & Varillas 2013). For this, the number of leukocytes was counted in 10 fields with a 40x objective, following the direction of a Greek guard (De Rodak et al. 2012, Carr & De Rodak 2014) (Fig. 1a). The average of 10 fields was calculated, divided by 10 (total fields) and multiplied by 1000, the total being expressed in the number of leukocytes/µl.

For differential leukocyte count or leukocyte formula, the classification criteria of the white series were followed based on the staining and morphological terminology proposed by Hawkey & Dennett (1994). The classification of blood cells was as follows: erythrocytes, granulocyte leukocytes (heterophils, eosinophils and basophils), mononuclear leukocytes (lymphocytes, monocytes and azurophils) and thrombocytes.

Counting was performed with a 100x objective, using immersion oil. Counting and classification of 100 consecutive leukocytes was performed (De Rodak et al. 2012, Carr & De Rodak 2014), reporting the leukocyte types percentage. Differential counting was performed systematically following the serpentine pattern, which minimizes leukocyte distribution errors (Fig. 1b). Linear regression was performed between mite intensity and each type of leukocytes to analyze the relationship between both variables.

Statistical analysis

Descriptive statistics were used to report estimated hematological parameters, expressed as mean (x) ± standard deviation (SD). Due to non-compliance with data normality and homoscedasticity assumptions, comparisons between infested and non-infested groups were analyzed with non-parametric statistics with Infostat software (Di Rienzo et al. 2015). Linear regression was performed between mite intensity (total number of ectoparasites in a sample, divided by the infested hosts number) and each type of leukocytes to analyze the relationship between both variables.

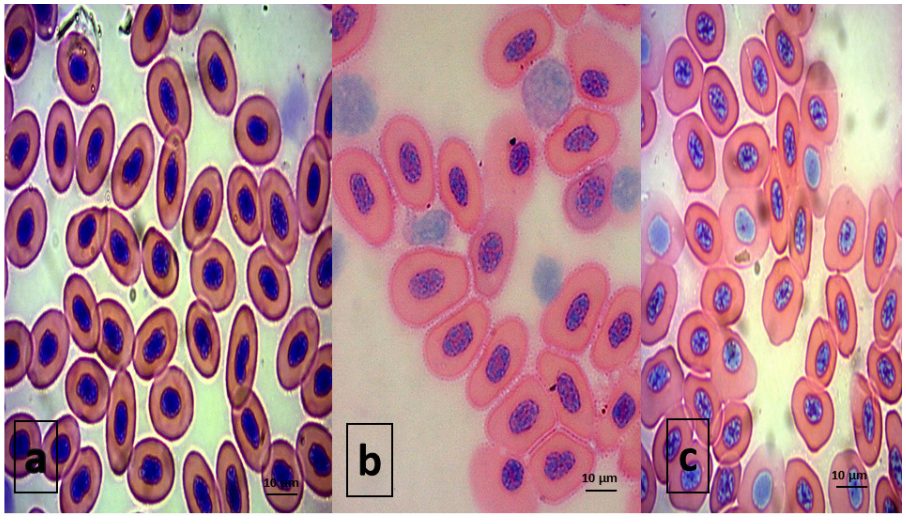

Figure 2. Qualitative analysis of Poikilocytosis level in *L. pacha* blood sample. a: without poikilocytosis. b: mild poikilocytosis. c: high poikilocytosis (100x). An AxioCam ERC5S digital camera was used and images were processed with the ZEN 2012-Blue edition® software.

Body Condition (BC) of *L. pacha* was calculated, assessing the residuals of the general linear regression between weight (P) (non-transformed variable) and snout-vent length (LHC) (Madsen & Shine 2001, Bertona & Chiaraviglio 2003).

Wilcoxon-Mann Whitney test was used to analyze the percentage of senile erythrocytes, the leukocyte count in infested and non-infested specimens and BC in the reproductive and post-reproductive period. Linear Regression analysis was used in the relationship between the percentage of senile erythrocytes and the differential leukocyte count with the intensity of mites, in addition between BC and the leukocyte count. To verify whether BC and the differential leukocyte count varies in infested and non-infested specimens, the T-Test and Friedman statistics were used, respectively.

RESULTS

Seventy-two blood smears of *L. pacha* adults were analyzed: 31 from specimens without mites (10 females and 21 males) and 41 with mites (9 females and 32 males), which presented a range between 1 to 212 (SD 49.7) mites per individual.

Morphological description

Red series or erythrocytes

Mature erythrocytes of *L. pacha* are elliptical, with abundant eosinophilic cytoplasm, centrally positioned, elliptical nucleus and basophilic condensed chromatin (Fig. 3). Their average measurements are: 16 x 9.6 µm (SD 1.9 x 0.7). Occasionally, immature erythrocytes were observed (Fig. 3), which are characterized by being rounded cells, with central, rounded nuclei and basophilic cytoplasm. Senile erythrocytes were identified by presenting a cell membrane with irregular and diffuse borders, as well as its nucleus. Average measurements of this cells type are not reported because their margins are diffuse (Fig. 4). No significant differences were found between the senile erythrocytes percentage of infested and non-infested lizards, so their presence does not depend on

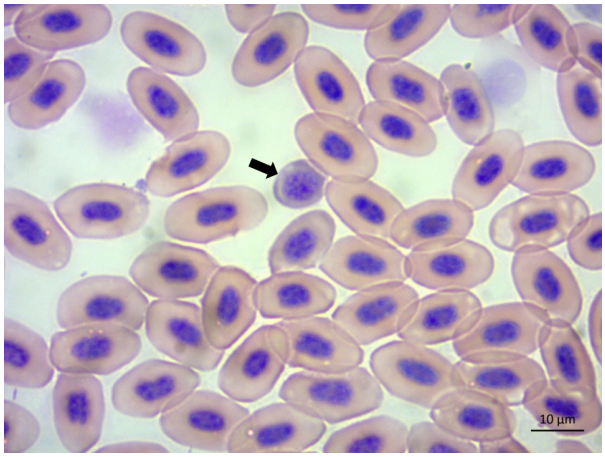

Figure 3. ***Liolaemus pacha*** **mature erythrocytes. Immature erythrocyte is indicated with a black arrow (100x). An AxioCam ERC5S digital camera was used and images were processed with the ZEN 2012-Blue edition® software.**

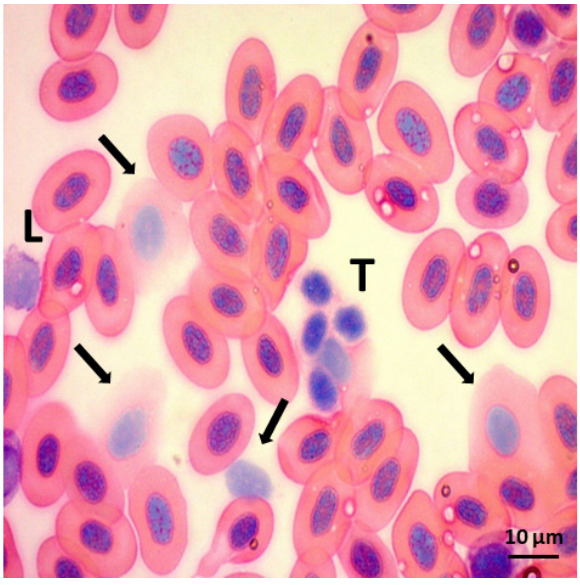

Figure 4. Senile erythrocytes (100x) identified by their membrane, cytoplasm and diffuse nucleus. In the right image, senile erythrocytes are indicated with black arrows; thrombocytes (T); lymphocytes (L). An AxioCam ERC5S digital camera was used and images were processed with the ZEN 2012-Blue edition® software.

mites presence (W = 1162.5; p = 0.7; Maximum senile erythrocytes percentage: non-infested: 29.7%, infested = 32.2%). In infested specimens, no relationship was found between mite load and senile erythrocytes percentage (R^2 = 0.06, p = 0.1). Qualitative analysis of poikilocytosis degree showed 44% without poikilocytosis, 44% had mild poikilocytosis and 12% had high poikilocytosis (Infested samples n = 41). In 31 samples of non-infested specimens, 52% did not register poikilocytosis, 32% presented mild poikilocytosis and 16% a high level of poikilocytosis (Fig. 2).

Thrombocytes

Cells similar in appearance to lymphocytes were differentiated by their particular grouping (Fig. 5). These small, elliptical or oval cells, with little clear cytoplasm and absence of granules, are sometimes difficult to identify. Its nucleus is oval and central, with dense dark chromatin (n = 72).

Mononuclear leukocytes

Lymphocytes

Rounded cells, with centrally located spherical nucleus. They have little homogeneous and slightly basophilic cytoplasm. They may present cellular extensions or pseudopodia on cell periphery (Fig. 6). Its average size is 5.4 - 14.5 µm (SD 2.5).

Monocytes

Considered the largest leukocytes in blood periphery, whose average measurement is 8.1 - 19.2 µm (SD 3.5). They are round or oval cells and the nucleus has variable shapes between round, oval or lobed. The cytoplasm stains grayish blue in which small vacuoles or fine granules may appear (Fig. 6).

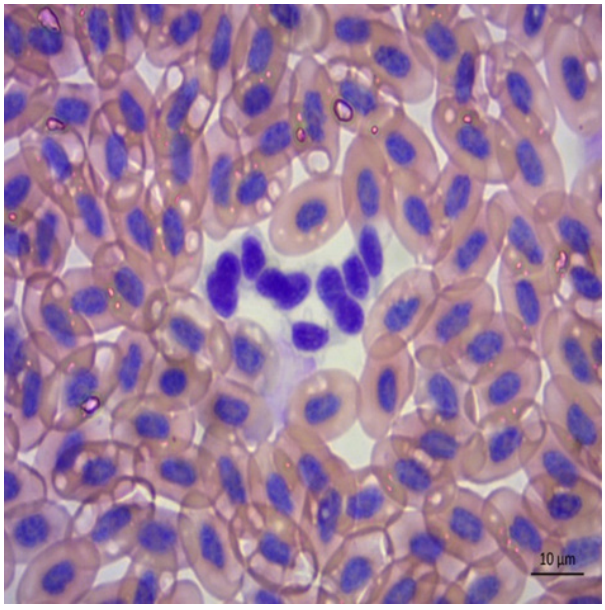

Figure 5. Central thrombocytes aggregates (central group of cells with bluish nuclei) from *L. pacha* blood sample (100x). An AxioCam ERC5S digital camera was used and images were processed with the ZEN 2012-Blue edition® software.

Granulocytic leukocytes

Heterophiles

Large, rounded cells, whose cytoplasm has refractive and/or opaque fusiform granules, which stain bright orange with May-Grünwald Giemsa. Nucleus is eccentric and rounded oval in shape. Heterophiles with bilobed and trilobed nuclei were also observed; as well as in the degranulation process, so its average measurement depends on its state (10.6 - 20 µm) (SD 2.5, Fig. 7).

Eosinophils

Rounded cells similar in size to heterophils (9.8 - 18 µm, SD 3.3), their cytoplasm presents abundant eosinophilic granules. Eccentric nucleus rounded or oval (Fig. 6).

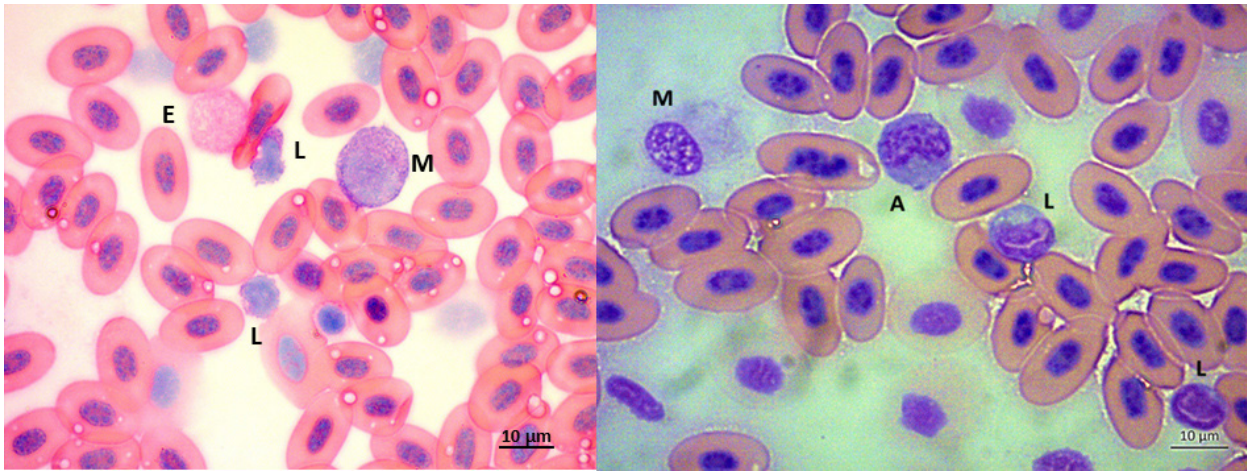

Figure 6. Leukocytes identification: Lymphocytes (L), eosinophils (E), monocytes (M) and azurophils (A) of *L. pacha* (100x). For the identification of the white series based on the tintorial and morphological terminology, the criterion of Hawkey & Dennett 1994. An AxioCam ERC5S digital camera was used and images were processed with the ZEN 2012-Blue edition® software.

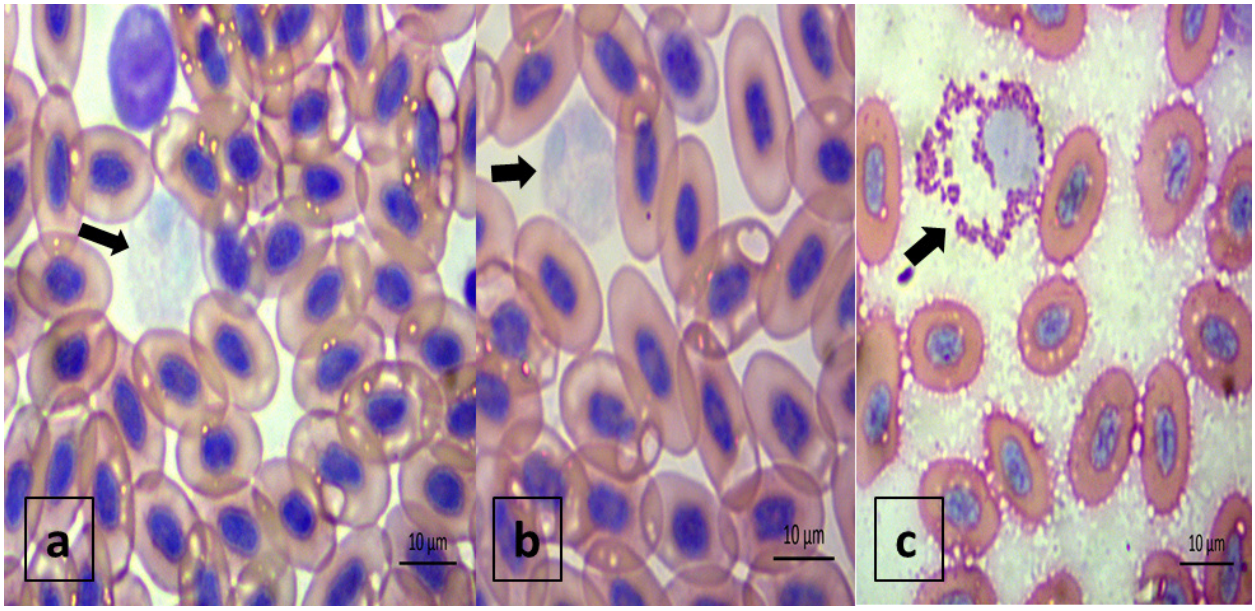

Figure 7. Heterophiles types of *L. pacha* (indicated with black arrow). a: Heterophile with bilobed nucleus. b: trilobed heterophile. c: degranulated heterophile (100x). An AxioCam ERC5S digital camera was used and images were processed with the ZEN 2012-Blue edition® software.

Basophils

Rounded cells with basophilic granules in the cytoplasm which almost completely cover the central or eccentric rounded nucleus. Its average size is 12.3 - 15.2 µm (SD 1.3) (Figs. 6 and 8).

Azurophils

Cells with an irregular size (13 µm), whose cytoplasm presents basophilic granules and small vacuoles. Its nucleus is eccentric and has an irregular, rounded or oval shape (Figs. 6 and 8).

Leukocyte count

Summary measures of leukocyte number for *L. pacha* in infested and non-infested specimens are showed in Table I. Leukocyte count was significantly higher for those non-infested lizards (W = 1313.5; p = 0.03). No significant differences were found in leukocyte count between individuals sexes (W = 781.5; p = 0.2) (Table I). The same was found between infested and non-infested males and females (Males infested and non-infested, W = 638, p = 0.2; Females infested and non-infested, W = 67, p = 0.06) (Table I). In differential leukocyte count, infested specimens had a significantly higher number of monocytes, thrombocytes, heterophils and lymphocytes (T^2 = 54.28; p = 0.0001) (Table II). Samples from non-infested lizards had a higher differential lymphocyte count (Nº lymphocytes= 1932, T^2 = 49.25; p = 0.0001) than in infested individuals (Nº lymphocytes= 1128, W = 1605, p = 0.0001) (Table II). To verify whether monocytes, thrombocytes, heterophils and lymphocytes counts of infested individuals depends on mite load, a linear regression was applied. Only lymphocytes showed a positive and significant relationship with mite load (R^2 = 0.12, p = 0.02) (Fig. 9a). Although there was no significant difference in eosinophil count, considering cell function, a regression analysis was performed, finding a positive and significant relationship with mite load (R^2 = 0.25, p = 0.0008) (Fig. 9b). In a qualitative analysis of regression graphs of each leukocyte type related to mite infection intensity, activity peaks of each cell type were observed in a range between 1 to 50 mites (see Fig. 9a, b). Body condition (BC) did not show significant differences between infested and non-infested individuals (T = 0.001, p = 0.9), as well as between infested/non-infested males and females (non-infested and infested males, T = 0.006, p = 0.09; non-infested and infested females, T = 0.007, p = 0.09). Based on obtained results, the BC values of infested/non-infested males and females were unified and a regression was applied with leukocyte count, which did not show any relationship between leukocytes count and BC (R^2 = 0.0002, p = 0.9). BC in post-reproductive period was significantly lower (reproductive and post-reproductive period, W = 882.5, p = 0.004). During reproductive period, non-infested specimens had a significantly higher leukocyte count (W = 420.5, p = 0.02). Conversely, post-reproductive period did not show differences between infested and non-infested specimens (W = 253, p = 0.6).

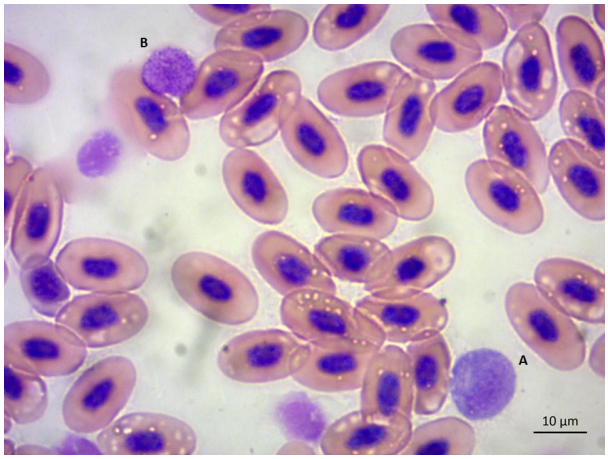

Figure 8. Identification of Azurophils (A) and basophils (B) in blood smear of *L. pacha* (100x). Technique used according to the criteria of Hawkey & Dennett (1994) and Stacy et al. (2011). An AxioCam ERC5S digital camera was used and images were processed with the ZEN 2012-Blue edition® software.

DISCUSSION

Most mite genera of the Pterygosomatidae family are considered vectors of hemoparasites, except for the *Neopterygosoma* mites, for which there is no information (Newell & Ryckman 1964, Fajfer 2012). In our study, blood smear analyzes do not reflect the presence of hemoparasites, providing new information for the genus *Neopterygosoma*. This result coincides with what was reported by Juárez Heredia et al. (2020), where they observed,

Table I. Leukocyte count (Leuc./µl) for males and females infested and non-infested of *L. pacha* specimens. Range: indicates the minimum and maximum number of leukocytes. n: number of samples analyzed.

	Non-infested (n=31)	Infested (n=41)	Females (n=19)	Males (n=53)	Females infested	Females non-infested	Males non-infested	Males infested
Range	5.600-78.800	3.000-74.200	4.400-66.700	3.000-78.800	4.400-31.200	7.100-66.700	5.600-78.800	3.000-74.200
Mean ± SD	23.120 ± 17.033	16.290± 16.480	21.150 ± 15.010	18.540± 16.480	14.910 ± 9.600	26.770 ± 17.130	21.380± 17.120	16.680± 16.040

Table II. Differential leukocyte count (Leuc./µl) of infested and non-infested specimens of *L. pacha*. (*)Values with significant differences between infested and non-infested specimens (p≤0.05). n: number of samples analyzed.

	Non- infested				Infested			
Leucocytes formula	**Total number of cells (n=31)**	**Range**	**Mean**	**SD**	**Total number of cells (n=41)**	**Range**	**Mean**	**SD**
Heterophiles	379	1-44	22.23	10.46	904*	2-50	22	13.1
Eosinophils	59	0-9	1.9	2.6	151	0-24	4.6	5
Lymphocytes	1932**	23-94	62.32	21.74	1128*	1-73	27.5	20.3
Basophils	120	0-18	3.87	4.9	219	0-32	5.3	6.9
Thrombocytes	402	0-49	12.97	13.2	1101*	0-51	26.8	20.1
Monocytes	201	0-24	6.48	6.03	593*	0-33	14.4	10,4
Azurophils	2	0-1	0.06	0.25	3	0-1	0.06	0.2

due to the type of insertion of the mite into the skin of its host, it would not be in contact with blood vessels, therefore no hemoparasites are found.

Poikilocytosis levels do not differ between parasitized and non-parasitized individuals. Therefore, the presence of these types of abnormal erythrocytes could be attributed to several factors, as stress processes, inflammatory diseases (spleen, kidneys, liver), malnutrition, post hibernation, neoplasia (Hawkey & Dennett 1989, Canfield 1998, Campbell 2004, Saggese 2009) or it is simply due to a common process, typical of the rest of reptiles (Campbell 2004). During sampled years, amputated fingers and/or autotomized or regenerated tails with malformations were observed in some specimens of *L. pacha*, which could be some of the reasons for the presence of poikilocytosis in this species (V.I. Juárez Heredia, personal communication).

Erythrocytes morphological measurements of *L. pacha* were higher than those obtained in *L. weigmannii* (X- 12 µm Ceballos De Bruno 1995) and slightly lower than those of *L. pictus* and *L. nigromaculatus* (X- 18.7 µm Peña Roche 1939). Senile erythrocytes identification in *L. pacha* would be the second record for the genus *Liolaemus* (*Liolaemus wiegmanni* Ceballos De Bruno 1995). The absence of a relationship between these types of erythrocytes with individual sex and degree of mite infestation forces us to consider their presence would only be a final part of red series life cycle (Saint Girons 1970, Stacy et al. 2011).

Leukocyte count did not show differences between sexes, which agrees with results obtained for *L. wiegmanni* (Ceballos De Bruno 1995). *Liolaemus pacha* lymphocytes represented 60% of the leukocyte count and their highest number was in non-infested individuals, results also reported in other studies (Divers et al. 1996, Troiano et al. 1997, Work et al. 1998, Strik

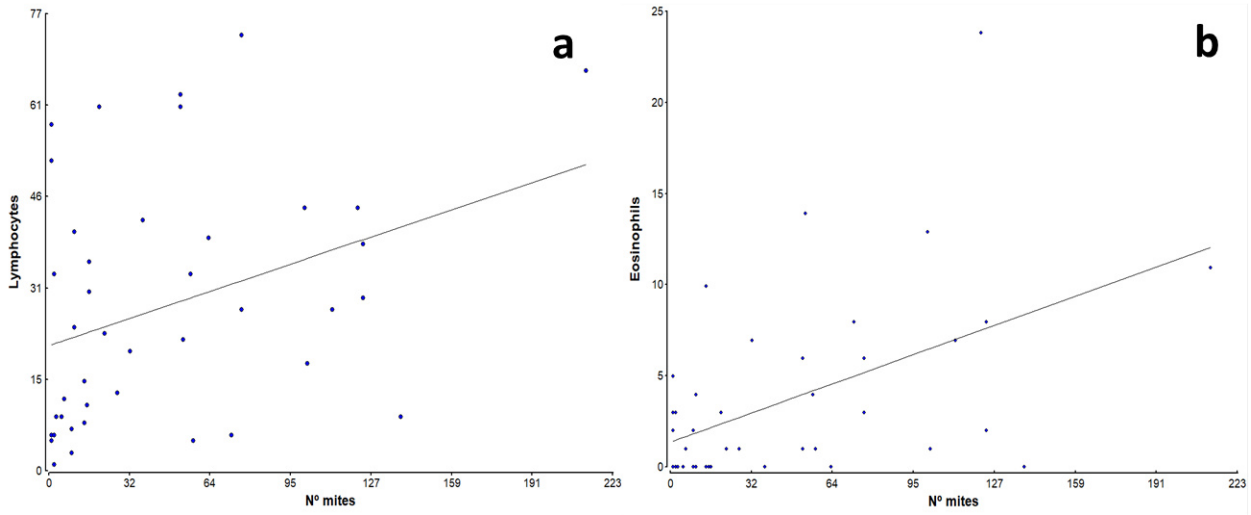

Figure 9. Regression graphs between number of lymphocytes (a) and eosinophils (b) and mite intensity. On a sample of n = 41 infested individuals.

et al. 2007). Mites presence did not influence lymphocytes number, so lymphocytosis in those individuals without mites may be associated with other factors, e.g. inflammatory processes, viral infections or wound healing parasitism (e.g. anisakiasis, espirorquidiasis, hematozoos Campbell 2004, Martínez Silvestre 2011). There are also studies indicating that during the summer season there is an increase in lymphocytes (Campbell 2004, Deem et al. 2009, Machado et al. 2006, Muñoz & Fuente 2004). This would be related to the poikilothermy of reptiles. That is to say, as the environmental temperature increases, the animal begins to activate, intake increases, resulting in the time necessary to form different antigens being shortened and the total number of leukocytes increasing. Said increase in white blood cells during the summer would be due to a faster immune response related to environmental temperature. Conversely, the opposite occurs during the winter (Haggag et al. 1966, Ultsch 1988). Infested lizards had a higher count of monocytes, thrombocytes, heterophils and a positive relationship between mite intensity and the number of lymphocytes and eosinophils. Furthermore, a peak of activity of certain leukocytes was observed in a range of mite numbers (1-50 mites), considering this ectoparasites intensity would be sufficient to activate an immune response. Therefore, since the general function of these leukocytes, the action of mites could be associated with inflammatory processes, allergic reactions or infectious diseases (Frye 1991, Bolten & Bjorndal 1992, Campbell 1996, 2004, Raskin 2000, Selleri & Hernandez Divers 2006, Simpson 2006, Zhang et al. 2009). However, in a histological analysis, Juárez Heredia et al. (2020) reported that *Neopterygosoma* mites do not generate epidermal damage or inflammatory reactions in *L. pacha* skin, just as their type of insertion calls into question the type of hematophagous feeding. Based on this background and our results, we consider it necessary to continue with studies to elucidate how mites activate the leukocyte response.

It should be noted that mites from the genera Neopterygosoma are associated with lizards of *Liolaemus* genus (Sauria: Liolaemidae) from South America (Chile and Argentina), highly specific parasites living mainly under the hosts' scales (monoxenous) and permanent

ectoparasites (Juárez Heredia et al. 2014, Fajfer 2019). Certain adaptations have evolved such as their specific ventral distribution in regions such as the neck, eyelids, flanks, neck, groin or the presence of packages/pockets (*Agama caudospinosa* Bertrand & Modrý 2004, *Liolaemus pictus* Espinoza Carniglia et al. 2016, *Mabuya agilis* Cunha Barros & Rocha 1995, *Pseudtrapelus sinaitus* Bochkov et al. 2009). These protects them from solar radiation, high temperatures or desiccation and gives them protection against scratching or friction of the host's body with vegetation (Cunha Barros & Rocha 1995). Mites of *L. pacha* are distributed in specific regions of the host's body (Juárez Heredia et al. 2014) and do not cause epidermal or inflammatory damage at the insertion points (Juárez Heredia et al. 2020). The feeding behavior of *Neopterygosoma* genus is still unconfirmed, with its potential hematophagous feeding considered doubtful based on histological results (Juárez Heredia et al. 2020). Based on these findings, the higher leukocyte count in infested lizards may be a response to mitigate epidermal damage.

Some authors describe azurophiles and monocytes as the same type of cell (Alleman et al. 1999) and others consider them as a different type within leukocytes (Ellman 1997, Wilkinson 2004). In this work, based on its morphological characteristics, it was considered a different type of leukocyte. An increase in azurophiles is associated with infections and inflammatory processes generated by parasites (Martínez Silvestre et al. 2001, Salakij et al. 2002), but their low count in infested and non-infested specimens of *L. pacha* rules out this relationship. The record of these cells in *L. pacha* is the first in the *Liolaemus* genus from Argentina since it was not reported in *L. wiegmanii* (Ceballos de Bruno 1995).

Immunocompetence Handicap Hypothesis (Folstad & Karter 1992) proposes that testosterone, in addition to stimulating the development of characteristics used in sexual selection (e.g. vocalizations, color production, visual displays), has an immunosuppressive effect. Therefore, signals that depend on testosterone are considered honest signals since they would reflect the male health state, because only healthy individuals can express their elaborate sexual signals and resist parasitic infections (Folstad & Karter 1992). In this context and given our results, the testosterone of *L. pacha* males would not be affecting the immune response since we did not find differences in the leukocyte count between sexes.

Body condition usually reflects an animal's overall health, energy status, and survival capabilities (Beldomenico et al. 2008, Budischak et al. 2018, Schulte Hostedde et al. 2005). Besides, parasites take resources from their hosts (Schmid Hempel 2011), reducing their energy reserves and damaging their body condition (Sánchez et al. 2018, Mougeot et al. 2009). *Liolaemus pacha* individuals infested and not infested did not show differences in their BC or leukocyte count, so we could interpret that the host's immune system would be developing a tolerance to infestation (ability of the host to resist a certain load of parasites and maintain fitness in the presence of infestation) (Ayres & Schneider 2008, Medzhitov et al. 2012). *L. pacha* mating occurs at the end of October and beginning of November (Ramírez Pinilla 1992), recording a higher rate of chemical and visual signals in the reproductive season (Vicente & Halloy 2016, 2017), correlated with an increase in testosterone (Martín et al. 2007). Therefore, the decrease in BC during post-reproductive period may be a consequence of testosterone effects. This hormone increases metabolic rate (Oppliger et al. 2004) and decreases feeding rate (Marler et al. 1995), causing a reduction in lipids

total amount in liver or in plasma and in body mass (Lacy et al. 2002, Lance et al. 2002).

Blood assessment is important to understand reptile physiology, since its detailed examination can provide important parameters used as markers and indicators of alterations in organisms metabolism and physiology (Campbell & Ellis 2007). Thus, we consider essential this kind of studies reporting reference intervals, describing blood types, morphology and its relationship with environmental, climatic, nutritional conditions, parasitism and possible contaminants. These data allowed us to increase the scarce diagnostic references in reptiles, specifically in *Liolaemus* genus, which is scarce (Engbretson & Hutchison 1976, Ceballos de Bruno 1995, Peña Roche 1939, Ruiz et al. 1993) providing monitoring tools for endangered species.

Finally, we highlight that the present study, in addition to reporting the hematological parameters in *L. pacha*, explores the proximal factors that influence its variability, such as parasitism, behaviors and/or environmental variables. Which allows us to understand the selective pressures that individuals face and the possible evolutionary effects.

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VIVIANA ISABEL JUÁREZ HEREDIA: conducted the field sampling, collected samples, performed data analysis, and drafted the initial version of the manuscript. MARÍA EUGENIA PÉREZ: provided expert advice on blood smear staining and assisted in the identification of blood cells. ANA GABRIELA SALVA: translated the manuscript into English. CECILIA INÉS ROBLES and MARCELA BEATRIZ HERNÁNDEZ: critically reviewed and thoroughly revised the manuscript. MONIQUE HALLOY: provided unwavering academic and personal support. All authors have approved the final version of the manuscript for publication and are collectively responsible for all aspects of the work, ensuring the integrity and accuracy of each component.