# Impact of immunological state on eco-physiological variables in one of the southernmost lizards in the world

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**Abstract:** The immune state is an essential component of survival as it directly influences physiological performance and health status. Variation in the leukocyte profile, a significantly increase in body temperature, and a detriment of the eco-physiological performance are among the possible consequences of an unhealthy state. In this study we analyse and discuss how field body temperature, preferred body temperature, the speed for sprint and long runs, locomotor stamina, and body condition can be affected by the immunological state (i.e. leukocyte profile) in a wild population of *Liolaemus sarmentoi*. Juveniles and adult males with a high percentage of eosinophils, basophils, and a low percentage of monocytes preferred higher body temperatures in a thermal gradient, while pregnant females maintained thermal preferences independently of leukocyte profile. Although juveniles with a high percentage of heterophils showed less locomotor stamina, adult males and pregnant females showed no differences in locomotor performance in relation to leukocyte profile. This study represents a starting point in eco-immunology of a wild lizard population of *Liolaemus* in cold and temperate environments of Patagonia where the southward shift in the geographic ranges of pathogen populations due to global warming represents a threat to resident host populations.

**Key words**: body condition, leukocyte profile, *Liolaemus sarmientoi*, locomotor performance, thermoregulation.

## INTRODUCTION

The interplay between immune system responses and phenotypic plasticity of physiological performance related with fitness defines the resilience of populations (Graham et al. 2011). The immune response depends on several factors such as the type of pathogens, the body condition, and body temperature of the host (Zimmerman et al. 2010). Non-specific defence components, such as leukocyte mobility, lymphocyte transformation, and the effects of interferon, are optimized at high body temperature ($T_b$; Kluger et al. 1975, 1998,

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Zimmerman et al. 2010). A diversity of vertebrates enhance the immune system's capacity by changes in body temperature (Rakus et al. 2017). Even ectotherms, that depend on external heat sources, modify their $T_b$ (Bernheim and Kluger 1976, Kluger 1977, 1979) mitigating or neutralizing an infection (Hallman et al. 1990, Deen and Hutchison 2001, do Amaral et al. 2002, Merchant et al. 2008). Vertebrate ectotherms and mainly Lepidosaurs can use behavioural and physiological thermoregulation mechanisms to modify $T_b$ and optimize physiological performance within a limited range of temperatures ($T_p$; Carothers et al. 1997, Ibargüengoytía et al. 2010, Fernández et al. 2011). The elevation of body temperature via behavioural thermoregulation in response to bacterial infections has been described in turtles (Monagas and Gatten 1983), crocodiles (Merchant et al. 2007), snakes (Burns et al. 1996) and lizards (Muchlinski et al. 1989, Ortega et al. 1991).

Although immune function may benefit from modified body temperature, in ectotherms the energetic costs of immune responses and other physiological demands, such as reproductive output, and ultimately fitness, result in a balance between the benefits accrued from the control of body temperature and the costs of thermoregulation (Hallman et al. 1990, Ortega et al. 1991, Merchant et al. 2008). In many cases, when individuals allocate resources to immune processes, they may suffer a general decrease in body condition (amount of energetic reserves stored as fat; Peig and Green 2009, 2010) affecting overall performance and the individual's interactions with environment. Locomotor performance is one of the most relevant eco-physiological variables since it affects the ability to disperse, to forage, to socialize and to evade predators (Greenwald 1974, Bennett 1980, Christian and Tracy 1981). Thus a decrease in speed for sprint and long runs, and stamina could negatively affect individual fitness, and ultimately, survival (Schall et al. 1982,

Oppliger et al. 1996, Garrido and Pérez-Mellado 2013, Zamora-Camacho et al. 2014). During an infection, the activity of the immune system is costly in terms of the re-allocation of energetic reserves as well as specific resources, such as proteins and amino acids (Schmid-Hempel 2011). The allocation of energetic reserves to the immune response reduces the availability of energy for other functions, such as reproduction, particularly embryonic development in pregnant females (Wang et al. 2014). For example, in *Urosaurus ornatus* wound healing was approximately 30% slower during the energetically taxing period of vitellogenesis (French and Moore 2008). Hence, there is a range of costly consequences impacting behavioural and eco-physiological performance in ectotherms whose avoidance is determined not only by immune system responses, but also by thermoregulatory efficiency (Deen and Hutchison 2001, Zamora-Camacho et al. 2016).

The immune system includes a series of white blood cells (leukocytes) that recognize foreign agents or pathogens and respond by phagocytosis (cellular response), antibodies (humoral response), and oxidizing agents or lysozymes (Wakelin and Apanius 1997, Zimmerman et al. 2010). In this regard, blood tissue can be assayed to provide valuable information and diagnoses on the general health of the animals (leukocyte profile and haematological values such as haematocrit, total counts of erythrocytes and haemoglobin), as well as clear signs of pathology such as blood parasites (Sykes and Klaphake 2008, Stacy et al. 2011). Some of the leukocytes present in blood tissue are important components of the innate or non-specific immune system and are the first line of defence against a foreign agent (Zimmerman et al. 2010).

The immunological function of leukocytes, although varying among taxa, is similar in most vertebrates (Davis et al. 2008). It is known that in most organisms, a high concentration of stress hormones (glucocorticoid hormones, such as

corticosterone) alters the number of leukocytes in circulation, increasing the number of heterophils and decreasing lymphocytes (Davis et al. 2008). In consequence, the heterophil:lymphocyte ratio (H:L) is frequently used as a proxy measure of stress. Additionally, an infection causes an increase in the number of circulating heterophils, which are the first phagocytic cells to attack and absorb particles and foreign organisms (Davis et al. 2004, 2010). In the different avian and non-avian reptiles, the heterophils are considered equivalent to mammalian neutrophils because they exhibit the same immune function (Stacy et al. 2011). The eosinophils are associated with parasitic infections and modulate the immune response by the secretion of chemicals that promote phagocytosis, although the number in circulation is typically low (Rothenberg and Hogan 2006). Basophils are involved in inflammation and, when they are activated by an antigen, they degranulate and release histamine (Campbell 1995). Meanwhile, lymphocytes are related to a variety of immunological functions such as immunoglobulin production and modulation of immune defence (Campbell 1996). Monocytes are also phagocytic and may increase in circulation during infections (Davis et al. 2004, Bonadiman et al. 2010). Finally, azurophils are a type of leukocyte found mainly in squamata that increase during infections and protozoal blood parasitism (Salakij et al. 2002, Stacy et al. 2011).

The genus *Liolaemus* is the most diversified genus of the family Liolaemidae and has broad latitudinal and altitudinal distribution, extending from the Andes of Peru to Tierra del Fuego in Argentina and Chile (from 10°S to 54° 30'S), and from sea level to more than 5000 m above sea level (m asl; Schulte et al. 2000, Aparicio and Ocampo 2010, Aguilar et al. 2013). This genus shows a wide variety of physiological responses that accommodates the diversity of climates and environments they inhabit (Ibargüengoytía et al. 2008, Cruz et al. 2009, Medina et al. 2011, Corbalán

et al. 2013, Moreno-Azócar et al. 2013). However, there are few studies of the immune system in liolaemids (Ceballos de Bruno 1995) and there are not previous studies about eco-immunology in the genus. *Liolaemus sarmientoi* is a medium-sized lizard (mean snout-vent length, $SVL_{females}$: 76.90 ± 1.21 mm; $SVL_{males}$: 76.82 ± 2.02 mm; Ibargüengoytía et al. 2010), viviparous, omnivorous, and saxicolous (Cei 1986, Scolaro and Cei 1997). They inhabit the central and southern regions of Santa Cruz province, Argentina, reaching the Strait of Magellan at the South (Breitman et al. 2014). *Liolaemus sarmientoi*, together with the sympatric *Liolaemus magellanicus,* are the southernmost reptile species of the world. It shows the second lowest mean field body temperature of liolaemids ($T_b$ = 26.2 °C, Ibargüengoytía et al. 2010), which is lower than their optimal body temperature for locomotor performance (Fernández et al. 2011).

The aim of this study is to determine the effects of the immune state on eco-physiological traits impacting individual survival and viability of a free-ranging population of the lizard *Liolaemus sarmientoi* (Donoso-Barros 1973). In particular, we estimate the percentages of heterophils, eosinophils, basophils, lymphocytes, or monocytes based on leukocyte profile (blood smear), and relate them to body temperature in the field, preferred body temperature, speed for sprint and long runs, locomotor stamina, and body condition, all response variables relate directly to fitness. We hypothesize that the immunological state of lizards induces changes in thermoregulation and a reduction in locomotor performance. In particular, individuals with a high percentage of leukocytes are expected to show higher body temperatures and decreased overall physiological performance. Finally, we analyse constraints on thermoregulation imposed by body condition and discuss the vulnerability of a resident lizard population to pathogens.

## MATERIALS AND METHODS

### STUDY AREA AND CAPTURED SPECIMENS

Field work was carried out in Santa Cruz province, Argentina (51 °S, 69 °W; 109 m asl) in November 2011. The climate is cold-temperate, semiarid (Soto and Vázquez 2001), dominated by sub-polar cold and humid air masses with winds increasing toward the south which contribute to aridity, a distinctive feature of the Patagonian climate (Camilloni 2007). Winds are very strong, with a mean speed of 37 km/h during spring and summer, and a maximum speed during summer that reaches 120 km/h, which results in ever-changing weather conditions. The mean annual air temperature is 8.04 ± 1.37 °C (ranging from 1.2 to 14.1 °C), but the mean air temperature during the lizards' activity period from October to March is 12.1 ± 0.81 °C (Meteorological Station in Río Gallegos, Santa Cruz).

We captured 8 juveniles (all males), 14 adult males and 15 adult females (all pregnant) of *L. sarmientoi* ($N = 37$) by hand or loop when they were active between 0900 to 1900 hours. Immediately after capture, the body temperature ($T_b$) was measured (TES 1303, ± 0.03 ºC digital thermometer) using a thermocouple (TES TP-K01, 1.62 mm diameter) inserted approximately 1 cm inside the cloaca. The temperature measurements were taken within 10 seconds of capture to prevent heat transfer from the operator's hands. Every capture micro-site was geo-referenced (GPS data 3 m resolution, GARMIN), allowing us to return lizards precisely after experiments. Capture permit (No. 09/09) was obtained from the Wildlife Delegation of Santa Cruz Province, Argentina. Lizards care followed ASIH/HL/SSAR Guidelines for Use of Live Amphibians and Reptiles, as well as the regulations detailed in Argentinian National Law No. 14346.

### *Laboratory experiments*

We brought the lizards to the laboratory in individual cloth bags. Lizards were kept in a quiet place, handled carefully, and all experiments were performed individually to avoid behavioural interference among individuals and to minimize stress. Experiments were performed during their natural daily activity period (0900 to 1900 hours) and within 48 hs after capture. First, each lizard performed the thermoregulation trial (2:30 hs duration) followed by at least 2 hs rest before the running trial which lasted < 15 min. Each lizard then rested at least 2 hs before the 30 min stamina trial. Immediately after the stamina trial, a blood sample was taken and the SVL and body mass were measured. After the experiments, each lizard was released at their capture site using GPS.

### PREFERRED BODY TEMPERATURE

Lizards were placed individually in open-top terraria with 24 individual tracks (100 x 25 x 15 cm) made of medium density fiberboard wood (MDF; in walls and substrate). A thermal gradient was created in each track using a heating lamp (75-W, full spectrum). Aluminium foil covered the hot end of each track and around the heating lamp so as to concentrate the heat in the track and to minimize heat loss to surroundings during the experiment. In this set up, we achieved a thermal gradient of 17 to 45 °C along each track. The temperature gradients were continuously monitored during the experiment using a thermometer (TES 1303, ± 0.03 ºC digital thermometer). The terraria did not contain any special substrate, shelter, water or food during experiments in order to offer the individuals the possibility to behaviourally choose their preferred temperature without any distractions. The body temperature of each lizard was measured using an ultra-thin (0.08 mm) catheter thermocouple inserted approximately 1 cm inside the cloaca and fastened to the base of the tail, which allowed the normal movement and behaviour of the lizards (Ibargüengoytía et al. 2010). The other end of each probe was connected to a Data Acquisition Module

(USB-TC08, OMEGA) which automatically recorded body temperatures simultaneously at 1 min intervals. The lizards, with thermocouple probes attached, were placed in the thermal gradient and allowed to acclimatize and to recover from manipulation for 20 min after which their $T_b$ was recorded continuously for 2 hs (according with the methodologies of Ibargüengoytía et al. 2010, Paranjpe et al. 2013). The mean temperature ($T_{pref}$), minimum value ($T_{pref\,min}$), and the maximum value ($T_{pref\,max}$) were obtained for each lizard and used to relate them to their leukocyte profile.

LOCOMOTOR PERFORMANCE

Before the sprint and long runs (SR and LR respectively) and locomotor stamina experiments, the lizards were maintained 30 min at the mean $T_{pref}$ for the species ($34.4 \pm 0.28$ °C; Ibargüengoytía et al. 2010) in a terrarium (35 x 20 x 20 cm) conditioned with 75-W incandescent bulbs. The body temperature ($T_b$) was measured using a thermocouple inserted approximately 1 cm inside the cloaca connected to a digital thermometer (TES TP-K01, 1.62 mm diameter and TES 1303, $\pm 0.03$ ºC, respectively). In all experiments (SR, LR and stamina), lizards were encouraged to run by touching them on their hind legs or tail gently in order not to interfere with the locomotor performance.

*Speed for sprint and long runs*

The speed for sprint and long runs was calculated for each lizard as $V_i = d_i / t_i$, where *d* is the distance between the first and the last sensor (1.05 m) and *t* is the elapsed time between sensors. Running trials were conducted in the laboratory maintained at temperatures near the mean $T_{pref}$ ($T_{b\,run\,speed} = 32.81 \pm 0.33$ °C) on a racetrack (0.07 m width, 1.20 m length), with cork as a substrate, and a shelter at one end. The speed of the race was measured using eight infrared photoreceptors positioned at 0.15

m intervals to sense lizard motion. The racetrack was connected to a computer. During analysis, each run was broken into a sprint-run component (SR; first 0.15 m section), indicative of the fright reaction, and secondly, a long-run component (LR; 0.15–1.20 m), indicative of locomotor capacity of the lizard to perform activities such as foraging, territorial defence and courtship. Each lizard ran three consecutive times and only the maximum run speed of the three runs performed for either SR or LR by each lizard was used in analyses. We followed the methodologies used before for *L. sarmientoi* (Fernández et al. 2011) and other *Liolaemus* (Kubisch et al. 2011).

*Locomotor stamina*

We measured the locomotor stamina on a 0.5 km/h treadmill following the methodology of Sinervo et al. (2000). Stamina was defined as the time spent running on the treadmill before exhaustion, indicated by the inability of individuals to right themselves when placed on their back (Sinervo and Huey 1990, Sinervo et al. 2000). Lizards ran at the mean $T_{pref}$ ($T_{b\,locomotor\,stamina} = 34.36 \pm 0.08$ °C), and the temperature was maintained with a 75-W incandescent bulb mounted over the track (Sinervo et al. 2000).

BODY MEASUREMENTS AND SEX DETERMINATION

Snout-vent length (SVL, Vernier calliper $\pm 0.01$ mm) and body mass (BM, 50-g Pesola© spring scale, $\pm 0.3$ g) of each specimen were registered. Sex was determined by the presence of pre-cloacal glands in males. Adults were defined as individuals of SVL > 57.2 mm in females (Fernández et al. 2015) and > 63.2 mm in males (Fernández et al. 2017a).

BODY CONDITION INDEX

For the estimation of body condition, we calculated the scaled mass index (M) of each individual as

an estimator of stored (fat) energy (*sensu* Peig and Green 2009, 2010) as:

$$M = M_i \times [SVL_0/SVL_i]^{\,b\mathrm{SMA}};$$

where $M_i$ and $SVL_i$ are the mass and SVL of the individual, $SVL_0$ is the arithmetic mean SVL of the population, and $b^{\mathrm{SMA}}$ exponent is the standardized major axis slope from the regression of ln mass on ln SVL for the population (Peig and Green 2009, 2010). The $b^{\mathrm{SMA}}$ exponent was calculated using the package 'lmodel2' (Legendre 2015) in R (R Core Team 2015). We calculated the M of each juvenile and adult male; adult females were excluded from the analyses since they were all pregnant.

*Blood smears*

At the end of the experiments and at ambient temperature (20 to 25°C), we prepared a smear on a glass slide from a tail blood sample of each individual. The blood smear was stained with May-Grünwald Giemsa (Biopack®) that highlights granulations and improves staining of erythrocytes (Martínez-Silvestre et al. 2011). Blood smears were used to determine leukocyte profiles (expressed as a percentage of each type of white blood cell), following a manual counting method known as "*greek guard*" in which the observer picks randomly a field and moves the slide from top to bottom and from left to right through different fields until 100 leukocytes are counted. Leukocytes were classified as heterophils, eosinophils, basophils, lymphocytes, monocytes, and azurophils using the categories for reptiles (Stacy et al. 2011). The H:L was also calculated. Azurophils were not included in the subsequent analysis because they were found in only one smear (an adult male). The blood smears were analysed by only one observer (FD) under an optic microscope (Olympus® BX51, America Inc., Melville, NY, USA; 1000X with immersion oil) equipped with a camera (TUCSEN® DigiRetina16; 16mp CMOS sensor). A subsample of 21 smears of juveniles ($N = 6$), males ($N = 7$)

and females ($N = 8$), were randomly chosen to photograph and measure the diameter of each type of leukocyte (μm), using the open-source image-analysis software program ImageJ 1.51n/Fiji (Wayne Rasband, National Institutes of Health, USA). Each leukocyte measurement was the mean of the measurements of each smear obtained for each individual in order to avoid pseudoreplication.

*Statistical analyses*

The differences in the percentages of heterophils, eosinophils, basophils, lymphocytes, monocytes and H:L among juveniles, adult males and pregnant females were analysed using either Analysis of Variance (One-Way ANOVA) or Kruskal-Wallis and Dunn's non-parametric tests (as posteriori; SPSS 17.0® or Sigma Stat 3.5® software). The effect of SVL on all variables was analysed using simple regression. For those variables that showed a significant dependence on SVL, we obtained the residuals using SPSS 17.0® and replaced the original variables (residuals of $T_{\mathrm{pref\ min}}$ in adult males, and the residuals of stamina in pregnant females were obtained).

The effects of the leukocyte profile on $T_b$, $T_{\mathrm{pref}}$, $T_{\mathrm{pref\ min}}$, $T_{\mathrm{pref\ max}}$, speed for sprint and long runs, and stamina of juveniles, adult males, and pregnant females were analysed by Multiple Stepwise Regression (SPSS 17.0®). R (R Core Team 2015) was used to obtain the body condition index. The relationships of body condition of juveniles and adult males to the leukocyte profile were analysed by Multiple Stepwise Regression. The relationship between the physiological variables ($T_b$, $T_{\mathrm{pref}}$, $T_{\mathrm{pref\ min}}$, $T_{\mathrm{pref\ max}}$, speed for sprint and long runs, and stamina), with either body condition or H:L were analysed using Linear Regression (all regressions were performed with SPSS 17.0®). The mean body condition of juveniles and adult males were compared using t-test. The assumptions of normality and homogeneity of variance for parametric

procedures were checked using Kolmogorov-Smirnov and Levene's tests, respectively (Sokal and Rohlf 1969) with SPSS 17.0® and Sigma Stat 3.5®. Figures were created using Sigma Plot 10.0®. Means are given with standard errors (± 1 SE).

### RESULTS

DESCRIPTION OF WHITE BLOOD CELLS AND LEUKOCYTE PROFILE

The most frequent cells were lymphocytes, and the second most frequent were heterophils. The abundances of other cell types (eosinophils, basophils, and monocytes) were much lower (Table I). The heterophils exhibited a medium size (mean $11.59 \pm 0.22$ μm, ranging from 8 to 17 μm, measured on 70 cells from $n = 15$ lizards, measuring 4 or 5 cells per lizard). Heterophils exhibited a round and eccentric bilobed nucleus with clear cytoplasm fill with oval or elongated granules of bright pink-orange coloration (Figure 1a). The eosinophils were more variable in size than heterophils (mean $16.71 \pm 0.41$ μm, ranging from 12 to 22 μm, measured on 30 cells from $n = 17$ lizards, measuring 1 or 2 cells per lizard). Eosinophils exhibited an eccentric nucleus with a clear cytoplasm, and show spherical pink granules (Figure 1b). Basophils usually exhibited a medium size (mean $10.57 \pm 0.49$ μm, ranging from 7 to 17 μm, measured on 30 cells from $n = 14$, measuring 1 or 3 cells per lizard). Basophils were characterized by the presence of a pale purple cytoplasm and by the presence of abundant darkly basophilic granules that cover the nucleus (nucleus not distinguishable; Figure 1c). Lymphocytes were small agranulitic cells (mean $7.23 \pm 0.18$ μm, ranging from 5 to 11 μm, measured on 70 cells from $n = 15$ lizards, measuring 4 or 5 cells per lizard) characterized by the presence of a high nucleus-to-cytoplasm ratio with basophilic cytoplasm (Figure 1d). Only one monocyte was photographed and measured; it exhibited a medium size (11.68 μm),

characterized by a round, oval or bilobed nucleus, and an abundant pale blue-grey cytoplasm (Figure 1e). Finally, azurophils were similar to monocytes, and exhibited a medium size (mean $11.98 \pm 0.38$ μm, measured on 3 cells from $n = 1$ lizard), characterized by having a central nucleus, usually rounded or oval, with high nucleus-cytoplasm ratio and with little azurophil granules dispersed in the cytoplasm (Figure 1f).

*Comparison of leukocyte profile in juveniles, adult males and pregnant females*

Juveniles exhibited more basophils than pregnant females, but were not different than adult males, and there was no difference between adult males and females (Kruskal–Wallis, $H_2 = 8.146$, $p = 0.017$; Dunn's Method, $Q_{juveniles \, vs \, females} = 2.755$, $p < 0.05$; $Q_{males \, vs \, juveniles} = 2.146$, $p > 0.05$; $Q_{males \, vs \, females} = 0.686$, $p > 0.05$; Table I). Juveniles, adult males, and pregnant females did not show differences in the percentage of heterophils, eosinophils, lymphocytes, and monocytes ($p > 0.05$; Table I). The H:L did not differ in the juveniles, adult males, and pregnant females (Kruskal–Wallis, $H_2 = 1.497$, $p = 0.473$; Table I), and in consequence, they were pulled together to determine the relationship of the thermophysiology and locomotor performance with the H:L (see below).

*Relationship of the body condition, thermophysiology, and locomotor performance, with the leukocyte profile*

Means or medians of thermophysiology variables, locomotor performance, and body condition with a descriptive purpose for juveniles, adult males, and pregnant females are shown in Table II. The body condition of juveniles and adult males did not differ (*t*-test, $t_{20} = -0,140$, $p = 0,890$). In consequence, juveniles and adult males were pooled in the subsequent analyses. Body condition did not show a significant relationship with leukocyte profile
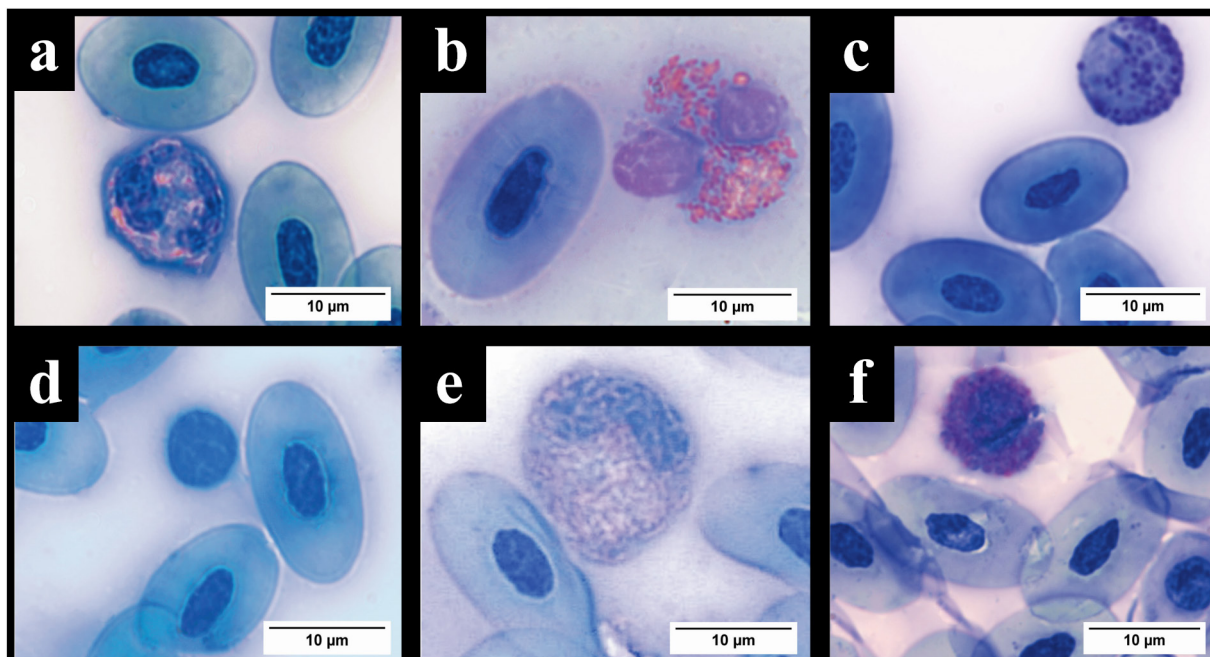
**Figure 1 -** Selected photographs of six types of leukocytes found in *Liolaemus sarmientoi*. Granulocyte cells: heterophil (**a**), eosinophil (**b**), and basophil (**c**); mononuclear cells: lymphocyte (**d**) and monocyte (**e**); and azurophil (**f**) are indicated. May-Grünwald Giemsa stain. Bars = 10 μm.

(Multiple Regression Stepwise, $F_{4, 21} = 0.77$, $r^2 = 0.154$, $p = 0.558$; the lymphocytes were excluded from the model because of collinearity).

In juveniles, the only two physiological variables that showed a relationship with the leukocyte profile were the $T_{pref\ max}$, which increased with the number of basophils (Multiple Regression Stepwise, $F_{1,7} = 7.208$, $r^2 = 0.546$, $p = 0.036$; Figure 2a, Table III), and the stamina, which decreased with the linear combination of the heterophils and eosinophils ($F_{2,7} = 5.859$, $r^2 = 0.701$, $p = 0.049$; Table III). The $T_b$, $T_{pref}$, $T_{pref\ min}$, and speed for sprint and long runs did not exhibit a relationship with the leukocyte profile (Multiple Regression Stepwise, $p > 0.05$; Table III).

In adult males, the $T_{pref\ max}$ increased with the number of basophils ($F_{1,12} = 33.859$, $r^2 = 0.755$, $p = 0.001$; Figure 2b, Table III) and the $T_{pref}$ showed a positive relationship with the number of eosinophils and basophils, and a negative relationship with monocytes (Multiple Regression Stepwise, $F_{3,12} = 17.86$, $r^2 = 0.859$, $p = 0.001$; Table III). The

$T_b$, $T_{pref\ min}$, speed for sprint and long runs, and stamina of adult males did not exhibit a significant relationship with the leukocyte profile (Multiple Regression Stepwise, $p > 0.05$; Table III). Pregnant females did not show a relationship between the physiological variables ($T_b$, $T_{pref}$, $T_{pref\ min}$, $T_{pref\ max}$, speed for sprint and long runs, and stamina) and the leukocyte profile (Multiple Regression Stepwise, $p > 0.05$; Table III).

*Effects of body condition and the relationship H:L on thermophysiology and locomotor performance*

The physiological variables analysed ($T_b$, $T_{pref}$, $T_{pref\ min}$, $T_{pref\ max}$, speed for sprint and long runs, and stamina) did not show a significant relationship with body condition (Linear Regression, $p > 0.05$; Table IV). The physiological variables analysed ($T_b$, $T_{pref}$, $T_{pref\ min}$, $T_{pref\ max}$, speed for sprint and long runs, and stamina) also did not show significant relationships with the H:L (Linear Regression, $p > 0.05$; Table IV).

## TABLE I

Percentage of each type of leukocyte (heterophils, eosinophils, basophils, lymphocytes, and monocytes) and heterophils:lymphocytes ratio (H:L) of the leukocyte profile (count 100 white blood cells), and comparisons among juveniles, adult males, and pregnant females of *Liolaemus sarmientoi*. Coefficients of the variance analyses (ANOVA) or Kruskal-Wallis test, mean (± standard error, SE) or median (when data distribution was not normal), minimum and maximum values, and sample sizes (*N*) are indicated. Significant *p* values are indicated in bold.

| Leukocyte profile | Juveniles N | Juveniles Mean (±SE) or Median | Juveniles min-max | Adult Males N | Adult Males Mean (±SE) or Median | Adult Males min-max | Pregnant females N | Pregnant females Mean (±SE) or Median | Pregnant females min-max | Total Mean (±SE) or Median | Total min-max | Total N | ANOVA or Kruskal-Wallis | p |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Heterophils (%) | 15 | 24.87 (4.04) | 12-43 | 8 | 24.64 (3.58) | 6-55 | 14 | 21.40 (3.49) | 6-53 | 19 | 6-55 | 37 | $H_2 = 1.154$ | 0.561 |
| Eosinophils (%) | 15 | 0.87 (0.39) | 0-3 | 8 | 1.42 (0.27) | 0-3 | 14 | 0 | 0-3 | 1 | 0-3 | 37 | $H_2 = 5.203$ | 0.074 |
| Basophils (%) | 15 | 5.62 (1.80) | 1-17 | 8 | 2.00 (0.57) | 0-7 | 14 | 1.26 (0.30) | 0-3 | 2 | 0-17 | 37 | $H_2 = 8.146$ | **0.017** |
| Lymphocytes (%) | 15 | 68.50 (4.16) | 49-82 | 8 | 71.42 (3.77) | 39-90 | 14 | 76.60 (3.68) | 40-93 | 72.89 (2.26) | 39-93 | 37 | $F_{2,36} = 1.036$ | 0.366 |
| Monocytes (%) | 15 | 0 | 0-1 | 8 | 0 | 0-3 | 14 | 0 | 0-1 | 0 | 0-3 | 37 | $H_2 = 2.767$ | 0.251 |
| H:L | 15 | 0.40 (0.09) | 0.14-0.87 | 8 | 0.40 (0.09) | 0.06-1.41 | 14 | 0.23 | 0.06-1.32 | 0.28 | 0.06-1.41 | 37 | $H_2 = 1.497$ | 0.473 |

**TABLE II**

**Mean (± standard error) or median (when data did not have a normal distribution), and sample size (*N*) of physiological variables measured for *Liolaemus sarmientoi*: snout-vent length (SLV, mm), body mass (BM, g), body temperature ($T_b$, °C), preferred body temperature ($T_{pref}$; °C), minimum preferred body temperature ($T_{pref-min}$; °C), maximum preferred body temperature ($T_{pref-max}$; °C), short run (SR; m/s), long run (LR; m/s), and locomotor stamina (s) of juveniles, adult males, and pregnant females, and body condition index of juveniles and adult males.**

| Physiological variables | Juveniles | | Adult males | | Pregnant females | |
|---|---|---|---|---|---|---|
| | Mean (±SE) | *N* | Mean (±SE) | *N* | Mean (±SE) | *N* |
| SVL | 54.97 (1.47) | 8 | 80.12 (1.69) | 14 | 77.32 (1.64) | 15 |
| BM | 4.61 (0.37) | 8 | 14.64 (0.81) | 14 | 12.33 (0.99) | 15 |
| $T_b$ | 26.40 (2.49) | 6 | 27.44 (1.56) | 13 | 27.99 (1.03) | 15 |
| $T_{pref}$ | 34.09 (0.29) | 7 | 34.29 (0.34) | 13 | 33.54 (0.19) | 15 |
| $T_{pref-min}$ | 29.89 (0.85) | 7 | 28.98 (0.65) | 13 | 29.47 (0.70) | 15 |
| $T_{pref-max}$ | 38.11 (0.31) | 7 | 37.94 (0.49) | 13 | 36.69 (0.40) | 15 |
| SR | 0.98 (0.18) | 8 | 0.85 (0.16) | 14 | 0.99 (0.10) | 15 |
| LR | 1.17 (0.19) | 8 | 1.25 (0.10) | 14 | 1.10 (0.07) | 15 |
| Locomotor stamina | 240.89 (31.62) | 8 | 440.41 (76.20) | 14 | 254.17 (30.35) | 15 |
| Body condition | 9.97 (0.45) | 8 | 10.04 ( 0.27) | 14 | | |

## DISCUSSION

The differences in the percentage of certain leukocytes among genera, species or individuals can be related to both extrinsic and intrinsic variables. For example, differences in abundance of certain leukocytes among seasons (Sandmeier et al. 2016), before and after hibernation (Sykes and Klaphake 2008, Stacy et al. 2011), and also in neonates (Brown and Shine 2016, 2018) have been described. Leukocytes vary widely among different vertebrate groups, and within squamata, in the abundance and morphology of granules, in the cytochemical staining patterns, and in the relative occurrence in the peripheral blood (Stacy et al. 2011). Most studies only report the variation in the proportion of the different leukocyte cells, but in general do not discuss the possible causes or eco-physiological costs. For example, healthy freshwater turtles (*Graptemys gibbonsi*) in

captivity can show a high percentage of basophils (up to 50%) of the total leukocytes in comparison with other reptiles (Perpiñán et al. 2008). In contrast, a predominance of heterophils in the leukocyte profile was reported in crocodiles, like *Crocodylus palustris* (Stacy and Whitaker 2000) and *Caiman crocodilus* (Rossini et al. 2011), in the marine turtle *Caretta caretta* (Casal et al. 2009), and in some lizards like *Uromastyx spp.* (Naldo et al. 2009). The only study in *Liolaemus* provided a description of a high proportion of heterophils in *Liolaemus wiegmannii* (Ceballos de Bruno 1995). However, in the lizards *Pogona vitticeps* (Eliman 1997), *Leiolepis belliana rubritaeniata* (Ponsen et al. 2008), *Ctenosaura melanosterna* (Davis et al. 2011), and in some species of the genus *Podarcis* and *Algyroides* (Sacchi et al. 2011), lymphocytes were the most abundant cells in the leukocyte profile. In *Liolaemus sarmientoi*, the percentage
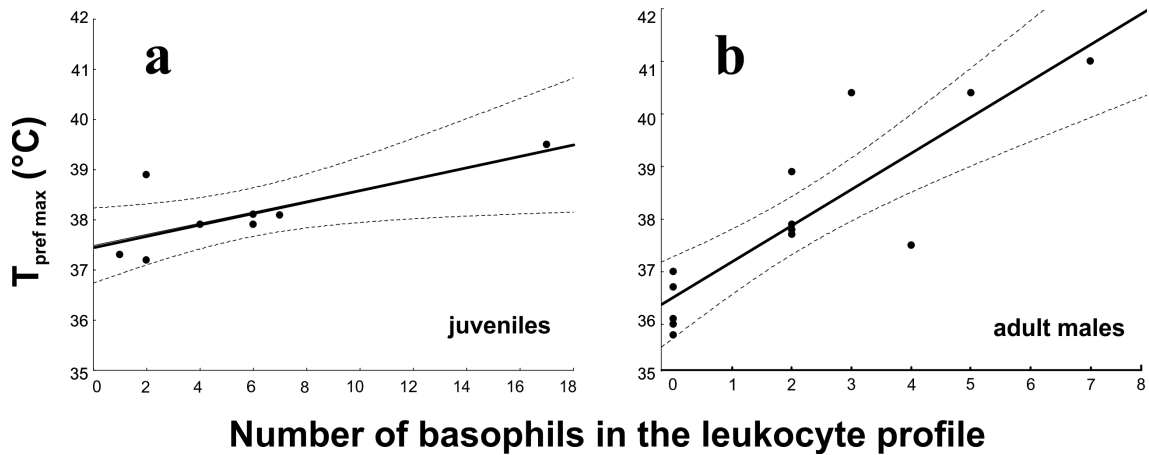
## Number of basophils in the leukocyte profile

**Figure 2** - Significant relationships in the regression analyses among variables related with thermoregulation: mean maximum value ($T_{pref\ max}$) preferred body temperature *versus* percentages of basophils (●) of *Liolaemus sarmientoi*. Linear regression (solid line) and their 95% confidence intervals (dashed lines) of $T_{pref\ max}$ *versus* basophils in juveniles (**a**) and adult males (**b**).

### TABLE III

**Multiple Regression Stepwise of body temperature ($T_b$, ºC), preferred body temperature ($T_{pref}$; ºC), minimum preferred body temperature ($T_{pref-min}$; ºC), maximum preferred body temperature ($T_{pref-max}$; ºC), short run (SR; m/s), long run (LR; m/s), and locomotor stamina (s) versus leukocyte profile of juveniles, adult males, and pregnant females. Regression coefficients, correlation coefficient ($r^2$), and $p$ values are indicated. The asterisk (*) indicates when residuals between the snout-vent length (SVL) and $T_{pref\ min}$ or stamina were used. Significant $p$ values are indicated in bold.**

|  |  | Leukocyte profile | | | | | |
|---|---|---|---|---|---|---|---|
|  |  | **Juveniles** | $p$ | **Adult males** | $p$ | **Pregnant females** | $p$ |
| $T_b$ | *coefficients* | $F_{3,5} = 3.48$ | 0.231 | $F_{4,12} = 0.19$ | 0.933 | $F_{4,14} = 0.60$ | 0.666 |
|  | $r^2$ | 0.839 |  | 0.090 |  | 0.196 |  |
| $T_{pref}$ | *coefficients* | $F_{3,7} = 0.71$ | 0.594 | $F_{3,12} = 17.86$ | **<0.001** | $F_{4,14} = 1.34$ | 0.319 |
|  |  |  |  | $t_{basophils} = 6.84$ | **<0.001** |  |  |
|  |  |  |  | $t_{eosinophils} = 4.63$ | **0.001** |  |  |
|  |  |  |  | $t_{monocytes} = 3.88$ | **0.004** |  |  |
|  | $r^2$ | 0.348 |  | 0.856 |  | 0.350 |  |
| $T_{pref\ min}$ | *coefficients* | $F_{3,7} = 0.57$ | 0.660 | $F_{4,12} = 2.03*$ | 0.183 | $F_{4,14} = 1.34$ | 0.320 |
|  | $r^2$ | 0.302 |  | 0.504 |  | 0.349 |  |
| $T_{pref\ max}$ | *coefficients* | $F_{1,7} = 7.20$ | **0.036** | $F_{1,12} = 33.85$ | **<0.001** | $F_{4,14} = 1.96$ | 0.176 |
|  |  | $t_{basophils} = 2.68$ | **0.036** | $t_{basophils} = 5.81$ | **<0.001** |  |  |
|  | $r^2$ | 0.545 |  | 0.755 |  | 0.440 |  |
| **Sprint run (SR)** | *coefficients* | $F_{3,7} = 1.55$ | 0.332 | $F_{4,13} = 1.83$ | 0.206 | $F_{1,14} = 2.18$ | 0.144 |
|  | $r^2$ | 0.538 |  | 0.449 |  | 0.418 |  |
| **Long runs (LR)** | *coefficients* | $F_{3,7} = 0.69$ | 0.604 | $F_{4,13} = 0.50$ | 0.733 | $F_{4,14} = 0.68$ | 0.621 |
|  | $r^2$ | 0.341 |  | 0.183 |  | 0.214 |  |
| **Stamina** | *coefficients* | $F_{2,7} = 5.85$ | **0.049** | $F_{4,13} = 0.37$ | 0.822 | $F_{4,13} = 1.83*$ | 0.206 |
|  |  | $t_{heterophils} = -2.88$ | **0.034** |  |  |  |  |
|  |  | $t_{eosinophils} = -2.18$ | 0.080 |  |  |  |  |
|  | $r^2$ | 0.701 |  | 0.142 |  | 0.450 |  |

of lymphocytes and heterophils were similar to those recorded for other reptiles, such as lizards and snakes (70-80% of lymphocytes, and 15-40% of heterophils; Martínez-Silvestre et al. 2011, Stacy et al. 2011), while eosinophils, basophils and especially monocytes and azurophils were scarce. In particular, the basophil percentages varied between juveniles and pregnant females.

In cold temperate environments, the attainment of optimal temperatures for physiological performance to fight an infection poses a challenge for species like *L. sarmientoi* because the harsh environment offers few microenvironments to raise body temperatures near their $T_{pref}$ (Ibargüengoytía et al. 2010, Fernández et al. 2011). *Liolaemus sarmientoi* thermoregulation and locomotor performance varied according to the leukocyte profile of individuals, suggesting a physiological adjustment to enhance the immunological response to infection, disease, or stress. Lizards selected high temperatures ($T_{pref}$ and $T_{pref\,max}$) when harbouring high counts of some leukocytes (eosinophils or basophils) related with innate immune responses, suggesting they can improve phagocytic activity by thermoregulation. In particular, juveniles responded by preferring warmer temperatures while showing a high percentage of basophils, as did adult males exhibiting high percentage of eosinophils and basophils, and low percentage of monocytes. In contrast, pregnant females of *L. sarmientoi* did not show a relationship between the leukocyte profile and preferred temperatures in laboratory ($T_{pref}$, $T_{pref\,min}$ or $T_{pref\,max}$).

There is a balance between improving the immune response and the advantages of homeostasis during embryonic development (French and Moore 2008). In nature, *L. sarmientoi* lizards are exposed to a mean air temperature of 12 °C during most of the activity season (spring and summer) but they can reach a mean $T_b$ of 27.5 ± 0.84 °C by active thermoregulation in the field. However, this $T_b$ is well below the $T_{pref}$ selected in

laboratory (33.9 ± 0.16 °C, present result). This constraint is corroborated by previous work on another population of the same species ($T_b$ = 26.2 ± 0.55 °C; $T_{pref}$ = 34.4 ± 0.28 °C; Ibargüengoytía et al. 2010) and on other liolaemids from cold environments (Medina et al. 2011, Moreno Azócar et al. 2013). The low $T_b$ registered in lizards that inhabit cold temperate environments is interpreted as a way to allow pregnant females to maintain stable $T_b$ thereby benefitting offspring fitness (*L. pictus*, Ibargüengoytía and Cussac 2002, *L. sarmientoi*, Fernández et al. 2017b) according to the maternal manipulation hypothesis that explains the evolution of viviparity (Shine 1995, 2004). In particular, pregnant females of *L. sarmientoi* could be constrained from a thermoregulatory response to infection because higher and more variable temperatures have been shown to be detrimental for embryonic development and survivorship (Fernández et al. 2017b). Previous studies on this species show that pregnant females maintain a narrower range of $T_{pref}$ than the rest of the population, providing a stable thermal environment for their offspring during gestation, and ensuring high aptitude after they are born (Fernández et al 2017b). Even though our study is preliminary, the preference for higher temperatures by only juveniles and males of *L. sarmientoi* with increased percentage eosinophils or basophils suggests that they could employ behavioural fever, unlike gravid females.

In addition, there is a physiological trade-off between the benefits of enhanced immune-system performance and consumption of stored energy which could otherwise be used for growth, reproduction, or the maintenance of body condition (Smith and French 2017). Immunity, like all other physiological processes, requires adequate energy to maintain optimal functioning (Demas 2004). Individuals with poor overall physiological and energetic state (low body condition index) are more susceptible to infections or parasites

**TABLE IV**

Linear Regression of body condition index (in pooled data of juveniles and adult males) and heterophil:lymphocyte ratio (H:L; in pooled data of juveniles, adult males, and pregnant females). Coefficients, correlation coefficient ($r^2$), and $p$ values are indicated.

| | Body condition (juveniles and adult males) | | | H:L (juveniles, adult males and pregnant females) | | |
|---|---|---|---|---|---|---|
| | coefficients | $r^2$ | $p$ | coefficients | $r^2$ | $p$ |
| $T_b$ | $F_{1,18} = 0.25$ | 0.015 | 0.622 | $F_{1,33} = 0.84$ | 0.026 | 0.365 |
| $T_{pref}$ | $F_{1,20} = 0.38$ | 0.019 | 0.542 | $F_{1,35} = 1.59$ | 0.045 | 0.216 |
| $T_{pref\ min}$ | $F_{1,20} = 4.24$ | 0.183 | 0.053 | $F_{1,35} = 1.46$ | 0.041 | 0.234 |
| $T_{pref\ max}$ | $F_{1,20} = 0.08$ | 0.004 | 0.779 | $F_{1,35} = 1.29$ | 0.037 | 0.263 |
| Sprint run (SR) | $F_{1,21} = 1.09$ | 0.052 | 0.309 | $F_{1,36} = 2.69$ | 0.071 | 0.110 |
| Long runs (LR) | $F_{1,21} = 1.66$ | 0.077 | 0.212 | $F_{1,36} = 0.95$ | 0.027 | 0.334 |
| Stamina | $F_{1,21} = 0.77$ | 0.037 | 0.389 | $F_{1,36} = 0.38$ | 0.011 | 0.541 |

(Merchant et al. 2008). For example, lizards with low body condition could be limited in their ability to increase their $T_b$ to counteract pathogens because of the energy costs of maintaining high $T_b$ (Romanovsky and Szekely 1998). This is the case of the juvenile green iguanas (*Iguana iguana*) with low energy reserves (poor body condition) which developed hypothermia as a defence strategy when they were infected with LPS (lipopolysaccharide of the cell wall of *Escherichia coli)* to conserve the individual's energy reserves (Deen and Hutchison 2001). The same result was described in green anoles (*Anolis carolinensis*; Merchant et al. 2008). In our observations of free-ranging *L. sarmientoi*, body conditions of juveniles and adult males were similar, and they did not show any relationship with thermal biology, locomotor performance, or leukocyte profile, suggesting that the actual body condition of *L. sarmientoi* would not prevent lizards from modifying their body temperature (i.e., elevating $T_{pref}$) to improve immune system performance if needed.

Infected or unhealthy states not only lead to a change in the blood-cell profile (Schall et al. 1982, Zamora-Camacho et al. 2014), but could also affect much of the general physiological homeorhesis of the individual (*sensu* Balon 1990), as evidenced by the reduction of efficiency of eco-physiological variables such as locomotor performance. For example, the lizards *Podarcis lilfordi* exhibited faster sprint speeds and had a better body condition when they were uninfected or had low blood parasite loads (Garrido and Pérez-Mellado 2013). Also, males of *Psammodromus algirus* lizards inoculated with LPS diminished sprint speed, whereas infected females did not (Zamora-Camacho et al. 2014). In our study, the locomotor performance (speed for sprint and long runs, and stamina), in both adult males and pregnant females were not related to the immune state (measured here as the leukocyte profile). However, in juveniles the capacity to run for long periods (stamina) was reduced in individuals that had a high percentage of heterophils. The reduction of locomotor stamina of juveniles could affect vital activities including predator avoidance and dispersal, pointing out their vulnerability in the population under infection (associated with presence of heterophil cells; Stacy et al. 2011) or stress episodes (which can also decrease lymphocytes; Davis et al. 2008).

The great variety of pathogens exerts strong selection pressures on their hosts, and affects variables intimately linked with biological adaptation and fitness, such as thermoregulation

and physiological performance (Graham et al. 2011, Zamora-Camacho et al. 2014). Thus, lizards can exhibit a great variability in the magnitude and efficiency of the immune response, finding a balance between the activation of the immune defence and the associated costs of thermoregulation and energy expenditure as a consequence of the higher $T_b$. The result of this balance generates differences in the magnitude of the defence deployed and is under strong adaptive pressure (Schmid-Hempel 2011). The cold temperate environments of Patagonia represent a potential thermal refuge for northern lizard populations under a global warming scenario (Piantoni et al. 2016), but infections by new colonizing bacteria, parasites and viruses represent a threat for resident lizard populations (Cahill et al. 2012). Nevertheless, it is expected that host populations in cold-temperate environments could benefit by the reduction in costs of thermoregulation (Piantoni et al. 2016, Fernández et al. 2017b) and may therefore have more opportunities to increase body temperatures to improve immune defence responses. Juveniles are probably the most vulnerable in the population, since a reduction in locomotor stamina could affect their ability to disperse and to evade predators (Main and Bull 2000, Civantos et al. 2010).

Former studies on Liolaemidae from Patagonia discussed the efficiency in thermoregulatory behaviour in relation to the availability of thermal microenvironments (Medina et al. 2011, Duran et al. 2018), refuges (Duran et al. 2018), the condition of pregnancy (Fernández et al. 2017b), and feeding habits (Ibargüengoytía et al. 2008), but this study represents a starting point that provides background information on the leukocyte profile and body condition of a wild lizard population of *L. sarmientoi,* and the first evidence of how immunological state influences thermoregulatory behaviour and locomotor performance in the genus *Liolaemus*.

## AUTHOR CONTRIBUTIONS

Fernando Duran conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft. Jorgelina M. Boretto conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft. Jimena B. Fernández performed the experiments, analyzed the data, approved the final draft. Mora Ibáñez Molina stained the blood smears, analyzed the data, approved the final draft. Marlin S. Medina performed the experiments, analyzed the data, approved the final draft. Nora R. Ibargüengoytía conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.

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