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COMPLEMENTARY/CONTRIBUTED PAPER

Who Rules Over Immunology? Seasonal Variation in Body Temperature, Steroid Hormones, and Immune Variables in a Tegu Lizard

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Synopsis Multiple factors can influence the immune response of ectothermic vertebrates, including body temperature (T_b), gonadal steroids, and seasonality, in ways that are thought to reflect trade-offs between energetic investment in immunity versus reproduction. Hibernating tegu lizards (*Salvator merianae*) are a unique model to investigate how immunocompetence might be influenced by different factors during their annual cycle. We assessed immunological measures (plasma bacterial killing ability, total and differential leukocyte count), plasma hormone levels (testosterone in males, estradiol and progesterone in females, and corticosterone [CORT] in both sexes), T_b, and body condition from adult tegus during each stage of their annual cycle: reproduction, post-reproduction/preparation for hibernation, and hibernation. Our hypothesis that immune traits present higher values during the reproductive phase, and a sharp decrease during hibernation, was partially supported. Immune variables did not change between life history stages, except for total number of leukocytes, which was higher at the beginning of the reproductive season (September) in both males and females. Average T_b of the week prior to sampling was positively correlated with number of eosinophils, basophils, monocytes, and azurophils, corroborating other studies showing that when animals maintain a high T_b, there is an increase in immune activity. Surprisingly, no clear relationship between immune traits and gonadal steroids or CORT levels was observed, even when including life history stage in the model. When gonadal hormones peaked in males and females, heterophil: lymphocyte ratio (which often elevates during physiological stress) also increased. Additionally, we did not observe any trade-off between reproduction and immunity traits, sex differences in immune traits, or a correlation between body condition and immune response. Our results suggest that variation in patterns of immune response and correlations with body condition and hormone secretion across the year can depend upon the specific hormone and immune trait, and that experienced T_b is an important variable determining immune response in ectotherms.

Introduction

Evidence suggests that there is a trade-off between immunity and other fitness-related life history components, such as reproduction and growth (Sheldon and Verhulst 1996; Zuk and Stoehr 2002; Martin et al. 2003, 2008; Alonso-Álvarez et al. 2004; French et al. 2007a). The trade-off between reproduction and immune response relies on the fact that both are energetically demanding (Sheldon and Verhulst 1996; Martin et al. 2003). However, the maintenance of immune surveillance during energetically challenging life history stages is critical to prevent infections and facilitate survival (Martin et al. 2006; Sparkman and Palacios 2009). By studying how immune function interacts with reproductive stage and other physiological traits, we can better understand

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the ecological and evolutionary patterns that determine investment in immune function, and under which conditions individuals are more susceptible to diseases (Carey et al. 1999; Wake and Vrendenburg 2008; Graham et al. 2017; Zimmerman 2020).

Gonadal steroids and glucocorticoids (GCs) broadly affect both physiological and behavioral traits (Whittier and Crews 1987; Sapolsky et al. 2000; Moore and Jessop 2003; Landys et al. 2006), including impacts on different branches of the immune system (Roberts et al. 2004; French et al. 2007b; Kovats 2015; Foo et al. 2017; Madelaire et al. 2017). The immunocompetence handicap hypothesis, for instance, indicates testosterone as the physiological mediator of differential investment in sexual display and immunocompetence in males (Folstad and Karter 1992). Conversely, disparities in the reproductive cost between sexes (Abell 2000; Angilletta and Sears 2000) could result in a more prominent trade-off with immune response in females (Zuk and McKean 1996; Klein 2004; Stahlschmidt et al. 2017). Also, GC release helps individuals respond to environmental changes and regulates the immune response (Williams et al. 2008; Dhabhar 2014; Sheriff et al. 2017; Madelaire et al. 2019). Given the integrative role these hormones play in mediating reproductive activity, energy recruitment, and immunocompetence, they are potential candidates for mediating the trade-off between reproduction and immune response in both males and females (Hahn 1967; Ketterson and Nolan 1994; Piersma et al. 2000; Sapolsky et al. 2000; Moore and Jessop 2003; Klein 2004; Anderson et al. 2014).

Body condition, diet, and body temperature (T_b) are also known to affect trade-offs between reproduction and immunocompetence (Ruiz et al. 2010; Neuman-Lee et al. 2015; Forbes et al. 2016; Husak et al. 2016; Smith et al. 2017). For example, vertebrates that undergo aestivation/hibernation experience drastic changes in access to food, and resulting changes in body condition, which can make them especially susceptible to seasonal alterations in immune response (Cooper et al. 1992; Pratihar and Kundu 2010; Havenstein et al. 2016; Madelaire et al. 2017). In ectotherms, T_b can also directly impact immune activity since immune function follows a classic thermal performance curve, where immunocompetence increases with temperature until achieving optimum performance, while beyond this optimum temperature, immune activity rapidly decreases (Angilletta et al. 2002; Hochachka and Somero 2002; Butler et al. 2013; Moretti et al. 2019). Thus, immunocompetence in thermoregulating ectotherms likely depends on the cost of maintaining an optimum T_b through behavioral thermoregulation (Belliure et al. 2004; Meylan et al. 2013, Goessling et al. 2017; Moretti et al. 2018). Additionally, climatic and temperature variations can drive changes in patterns of disease cycles and increase pathogen abundance and virulence, greatly impacting how ectotherms cope with pathogens (Altizer et al. 2013; Sandmeier et al. 2013; Goessling et al. 2017; Matanza and Osorio, 2018; Hernández-Cabanyero et al. 2020). The study of ectothermic vertebrates that behaviorally thermoregulate offers the potential to better understand the poorly explored interaction between T_b regulation and immunocompetence (Slip and Shine 1988).

Vertebrates that display seasonal reproduction, drastic transitions between life history stages, and behavioral thermoregulation are ideal models to investigate the effects of seasonality, hormone levels, sex, and temperature on immunocompetence. These features are exemplified by the tegu lizard (*Salvator merianae*), thus making it an ideal species in which to explore the interrelationships of immunocompetence, reproduction, and other life history traits (Harvey et al. 2012). In tegus, reproduction initiates early in the austral spring (September and October) following emergence from hibernation, when both metabolic rates and circulating levels of reproductive hormones are elevated and, curiously, sustain T_b above ambient through endogenous heat production (Andrade et al. 2004; Chamut et al. 2012; Tattersall et al. 2016; Zena et al. 2019, 2020). Females can lay in excess of 50 eggs and lose ~40% of their body mass (Lopes and Abe 1999; Andrade et al. 2004). After reproduction, from November to April (summer/fall), reproductive hormone levels decrease and remain low while animals eat and accumulate endogenous energy reserves in preparation for hibernation (Abe 1995; Andrade et al. 2004; Chamut et al. 2012; Zena et al. 2019). During hibernation, which occurs in the months that are cold and dry in the austral hemisphere (May to August; end of fall/beginning of winter), tegus sequester themselves in hibernacula, cease activity, thermoregulation and feeding, reduce metabolic rate, and reproductive hormone levels remain low (Abe 1983, 1995; Andrade et al. 2004; de Souza et al. 2004; Chamut et al. 2012; Sanders et al. 2015; Zena et al. 2019, 2020). Experiments with captive tegus suggest that this annual cycle is probably independent of temperature and food availability (Abe 1995; Milsom et al. 2008; Sanders et al. 2015; Zena et al. 2019, 2020).

For each stage of the annual cycle (reproduction, post-reproduction, and hibernation), we determined

plasma hormone concentrations (testosterone in males, estradiol and progesterone in females, and corticosterone [CORT] in both sexes), T_b, body condition, and three innate immune parameters from adult tegus. The immune measures chosen were plasma bacterial killing ability (BKA), which is mediated by complement, antimicrobial peptides, and natural antibodies (French and Neuman-Lee 2012); total number of leukocytes, which correspond to immune surveillance and response mediated by cell (Zimmerman et al. 2010a; Bílková et al. 2015; Zhang and Zhao 2015); and differential leukocyte count, that is, the relative abundance of different leukocyte types, each of which can change independently due to various factors (e.g., exposure to a specific type of infection or pathogen) (Davis et al. 2008; Cadman and Lawrence 2010; Goessling et al. 2016). We hypothesized that immune traits vary according to the annual cycle, correlating positively with hormone levels, T_b, and body condition. We predicted a sharp decrease in immune performance in both sexes during hibernation, due to lack of thermoregulatory behavior, activity and feeding, as well as reduced exposure to pathogens during this energy-conserving life history stage (Pratihar and Kundu 2010; Refsnider et al. 2015; Madelaire et al. 2017). Additionally, during reproduction, we predicted that females would show lower immune activity than males to offset the higher energetic cost of ovogenesis.

Material and methods

Animals

The animals sampled in this study were captive-bred tegu lizards (*S. merianae*) of both sexes (10 males and 11 females) kept in a communal outdoor enclosure (42 m^2) subject to natural fluctuations in temperature, daylight, humidity, and rainfall, at the campus of Universidade Estadual Paulista in Jaboticabal, São Paulo, Brazil (21°14′05″S and 48°17′09″W). The enclosure contained a shared open area and two common shelters (dimensions: 103 × 72 × 71 cm; lined with wood shavings), which provided options for thermoregulation, ambulation, social behavior, evening retreat, and hibernation which, in southeastern Brazil, occurs from late May or June to mid-August. Animals were treated for parasites before sampling started (Ripercol L Fosfato®, 4 mg/kg, Bayer, Brazil), fed 2–3 times/week with cooked chicken eggs, mice, fruits, and captive-bred cockroaches (*Nauphoeta cinerea*), and supplemented once per week with calcium and vitamin D3 (Zoo Med Reptivite; San Luis Obispo, CA, USA). Even in captivity with *ad libitum* food, tegus voluntarily stop eating at the end of the austral autumn (late May) (Abe 1983; de Souza et al. 2004; Zena et al. 2016, 2019) and begin eating again upon emergence from hibernation ("emergence" hereafter) in early August. Hence, during hibernation, no food was provided. Water was provided *ad libitum* all year long. We measured body mass to the nearest ±1 g on a digital scale (Bel Equipamentos Analíticos, Piracicaba, São Paulo, Brasil), snout–vent length, and tail thickness (±0.01 cm) to calculate body condition traits (body condition index and tail fat index [TFI; the tail is a reservoir for fat in tegu lizards; de Souza et al. 2004]). Samples were obtained during the (1) reproductive stage: September/October; (2) post-reproduction: November to March; and (3) hibernation: July.

Blood sampling

Tegus were individually identified by their size and color pattern, confirmed with a reading from a subcutaneously implanted microchip (transponder ISO FDX-B 134.2 KhZ, Animal TAG, São Carlos, Brazil) as in Zena et al. (2019, 2020). Blood was collected monthly from all animals during reproduction and post-reproduction (September of 2017 through March 2018) and once in the middle of hibernation (July 2018). For blood sampling, animals were individually captured by hand and their heads were covered with a cloth bag. Blood was collected (~3 mL) from the ventral coccygeal vein using a heparinized 5 mL syringe and a 21 G needle; whole blood samples were immediately transferred to plastic tubes (2 mL) on ice. Total handling time was ~3 min to minimize potential effects of handling stress (Romero and Reed 2005; Tylan et al. 2020) and all handling was performed between 8:00 and 10:00 a.m. to eliminate time of day effects on variables of interest (Jessop et al. 2002; Jones and Bell 2004).

Blood samples were maintained on ice for up to 2.5 h and then divided into four aliquots. Two aliquots were used for total and differential leukocyte count, one for quantification of steroid plasma levels, and one to measure plasma BKA. To obtain plasma, blood samples were centrifuged at 9391 *g* for 10 min at 4°C. Plasma samples for hormone quantification were stored at −80°C, and plasma samples for BKA were frozen on dry ice, transferred to the laboratory at the University of São Paulo, and stored at −80°C.

Hormone data

Hormone data from September 2017 to March 2018 have been previously published (Zena et al. 2020)

and are re-analyzed here, along with additional hormone data from the same individual tegus, for comparison to immune data. Hormone data for July 2018 were obtained following the same protocol as in Zena et al. (2020). Briefly, estradiol, testosterone, and progesterone were extracted using solid phase extraction of 500 μL plasma (detailed in Newman et al. 2008). Samples were then eluted in 90% methanol, dried at ambient temperature, and stored at −80°C. One day prior to assay, samples were resuspended in 1 mL assay buffer (Arbor Assays X065 buffer; 1:2 dilution), shaken on a multi-tube vortexer (Glas-Col Large Capacity Mixer, speed set on 50; Glas-Col, Terre Haute, IN, USA) for 1 h and then stored at 4°C overnight. Before assays, tubes were shaken again for 1 h. CORT assays were performed using unextracted plasma, according to the manufacturer's instructions. Commercially available enzyme immunoassay kits (Arbor Assays, Ann Arbor, MI, USA) were used to quantify plasma testosterone (kit # K032), estradiol (kit # KB30), progesterone (kit # K025), and CORT (kit # K014). All assays have previously been validated in our laboratory for tegu plasma (Zena et al. 2019). To improve assay precision for low concentrations of hormone, one additional low-dose standard was added to the testosterone standard curve. Following the manufacturer's protocols, the CORT assays included addition of a dissociation reagent to dissociate hormones from plasma binding proteins. Plasma samples from both male and female tegus were assayed for CORT. Male plasma samples were assayed for testosterone and female plasma samples were assayed for estradiol and progesterone. All assays included a full standard curve. Standards and unknowns were run in duplicate. Any sample that exceeded 10% coefficient of variation between duplicates was re-analyzed. Intra-assay and inter-assay variations were <10%. For antibody cross-reactivities, assay sensitivities, and other methodological details, see Hunt et al. (2017).

T_b data

T_b data have been previously published in Zena et al. (2020) and are presented again here for comparison to immune measures. Briefly, temperature loggers (iButtons, DS1922L, TX, USA) were surgically implanted in each animal's coelomic cavity and sutured to the internal muscle layer. Each logger was programmed to measure and record T_b every 70 min. Details of surgical procedures are provided by Zena et al. (2020). Due to technical issues, T_b data are not available for July 2018. Since little is known about how fast T_b alters immune parameters, we calculated the average T_b experienced for 30 days (monthly average), 1 week (weekly average), 24 h (daily average), and 6 h (morning average) before the blood sampling.

Total and differential leukocyte count

To obtain total leukocyte count, 5 μL of blood from each individual was diluted in 195 μL of toluidine blue saline solution (0.01%) and placed in a Neubauer chamber. Toluidine blue stains cells, facilitating differentiation of leukocytes and erythrocytes (Campbell 2015; Chamut and Arce 2018). The total number of leukocytes was counted under a light microscope (LM2100BL, Lumen; # 40 objective). To obtain a leukocyte profile (differential count), we performed a blood smear on a glass slide for each individual. Slides were fixed using 100% methanol and then stained with Giemsa solution (10%). We counted and classified 100 leukocytes per smear under an optical microscope (# 100 objective; oil immersion; Nikon E200, 104c). The classification of leukocytes (basophils, eosinophils, monocytes, heterophils, and lymphocytes) was based on tegu leukocyte morphology (Campbell 2015; Chamut and Arce 2018). Heterophil: lymphocyte ratio (H:L ratio) was calculated for each blood smear. Due to technical issues, total leukocyte count is not available for October 2017.

Plasma BKA

To assess individual humoral innate immune response based on soluble proteins, plasma samples were tested with a BKA assay following an adapted protocol from Fabrício-Neto et al. (2019). Each plasma sample was diluted in sterile phosphate-buffered saline (PBS) solution (8 μL plasma: 192 μL PBS) and mixed with 10 μL of non-pathogenic *Escherichia coli* (Microbio-Logics, # 24311-ATCC 8739) working solution ($\sim 5 \times 10^4$ microorganisms). The negative control consisted of 210 μL of PBS solution and the positive control was a mixture of 10 μL of *E. coli* working solution diluted in 200 μL of PBS. All samples and controls were incubated for 60 min at 37°C and, after the incubation period, 500 μL of tryptic soy broth was added. Bacterial suspensions were thoroughly mixed and 300 μL of each were transferred in duplicate to a 96-well microplate, which was incubated at 37°C for 2 h. Subsequently, optical densities of samples were measured hourly with a plate spectrophotometer (wavelength 600 nm, Spectra Max 250), for a total of 4 readings. BKA was evaluated at the beginning of the bacterial

exponential growth phase using the formula: [100 − (optical density of sample/optical density of positive control)], which represents the percentage of killed microorganisms in the samples compared to the positive control. All samples were assayed between 2 and 20 days after the blood sampling.

Statistical analysis

To obtain body condition index and TFI, we extracted the residuals of a standard least squares linear regression using snout–vent length as the independent variable and body mass or tail thickness, respectively, as the dependent variable (Madelaire et al. 2017; Sivan et al. 2020). To normalize data, we $\log_{10}$ transformed the following variables: total leukocyte count, BKA, T_b, and plasma hormone levels.

To reduce the number of variables, we performed a Varimax normalized principal component analysis (PCA) with all differential leukocyte variables (number of basophils, eosinophils, monocytes, and azurophils), except heterophils and lymphocytes sampled in each month all together (Supplementary Table S1), but separately for males and females. From the obtained component, we extracted a compound residual by regression and saved it as a variable we designated "differential leukocyte component". To test if the measures of total leukocyte count, differential leukocyte component, H:L ratio, and BKA differed between sexes, we compared a null model with a model that included sex as an explanatory variable using linear mixed modeling (LME) (LMER function package "lme4"; Bates et al. 2015). Since we had repeated measures for the same individual throughout the sampling period, individual identification (ID) was included as a random factor. Once we determined there were no differences between males and females regarding the measured immune variables (Table 1), further analyses (Supplementary Table S2) were conducted with all individuals combined.

To determine which condition index best explained the immune parameters (total leukocyte count, differential leukocyte component, H:L ratio and BKA), we used LME to compare the null model with two models that included either body condition or TFI. Further analyses (Supplementary Table S2) were conducted using only the body condition variable that was the better predictor of variation of the immune variables.

To determine the timeframe of average T_b (monthly, weekly, daily, or morning) that best explains variation of the immune parameters (total leukocyte count, differential leukocyte component, H:L ratio, and BKA), we used LME to compare the null model with four models that included either monthly average, weekly average, daily average, or morning average. Further analyses (Supplementary Table S2) were conducted using the most appropriate T_b for each immune variable.

To test whether total leukocyte count, differential leukocyte component, H:L ratio, and BKA variation were explained by life history stage (categorical variable with three levels: reproduction, post-reproduction, and hibernation), or by plasma hormone levels, TFI, or T_b (continuous variables), we compared a series of proposed models using LME (Models 1–11, Supplementary Table S2). In cases where none of the selected models included T_b, the analysis was re-done excluding this variable (Models 1–3, 7, 10, and 11; Supplementary Table S2). The models were tested separately for each hormone. Thus, for the gonadal steroids (testosterone, progesterone, and estradiol), males and females were tested separately, while for CORT, males and females were tested together. As our experimental design involved repeated measures, all proposed models included ID as a random factor.

All models were submitted to Akaike information criterion (AIC) selection, in which each competitive model receives a ΔAIC and a weight, and the model with the lowest ΔAIC is selected as the most accurate to describe the results; weight can be considered as the power of explanation between selected models (package "bbmle"; Bolker et al. 2009) (Burnham and Anderson 2002). We selected models with $\Delta AIC \leq 2.0$ (Burnham and Anderson 2002). Further, we examined the significance of the fixed effects in the selected models [using the "summary(model)" function in R], considering significant factors with *t*-values higher than 2.0 and smaller than −2.0 (Luke, 2017). Prior to any analyses, data were checked for normality. All analyses were performed using SPSS version 22 (IBM Corporation, Armonk, NY, USA) and R software version 3.5.0 (R Core Team, https://www.R-project.org/) and the script for R analysis is provided in the supplementary material.

Ethical note

All experimental protocols were approved by a local Animal Care and Use Committee of São Paulo State University (CEUA; # 7.434/16). Transport was conducted under IBAMA permit no. 02001-000412/94-28 and SISBIO-ICMBio/n. 26677-1. Transport and maintenance permit: ICMBio # 52085-1.

Results

In males, the differential leukocyte component of the PCA analysis explained 43.8% of variance in leukocyte count data. For females, the differential

Table 1 Selected models (ΔAICc ≤ 2.0) that explain the variation of immune variables (BKA, total and differential leukocyte component, heterophil: lymphocyte ratios) in relation to sex, body condition, and T_b of the tegu lizard (*S. merianae*)

Variable	Model	AICc	ΔAICc	df	Weight
BKA	~ null	196.2	0.0	3	0.66
	~ sex	197.5	1.3	4	0.34
Differential leukocyte component	~ null	435.3	0.0	3	0.73
	~ sex	437.3	2.0	4	0.27
H:L ratios	~ sex	−68.4	0.0	4	0.59
	~ null	−67.6	0.7	3	0.41
Total leukocyte	~ null	−48.8	0.0	3	0.70
	~ sex	−47.1	1.7	4	0.30
BKA	~ TFI	115.3	0.0	4	1.00
Differential leukocyte component	~ TFI	235.4	0.0	4	1.00
H:L ratios	~ TFI	−243.2	0.0	4	1.00
Total leukocyte	~ TFI	−69.8	0.0	4	1.00
BKA	~ monthly T_b	183.5	0.0	4	0.77
Differential leukocyte component	~ weekly T_b	356.1	0.0	4	0.99
H:L ratios	~ null	−67.6	0.0	3	1.00
Total leukocyte	~ null	−48.8	0.0	3	0.88

leukocyte component of the PCA analysis explained 37.8% of variance in leukocyte count data.

There were no differences between males and females in immune variables (Table 1, Fig. 3, and Supplementary Fig. S1), which allowed analyses (Supplementary Table S2) to be conducted with data from all individuals together. TFI was a better predictor of immune variable changes than was body condition index (Table 1). Thus, further analyses (Supplementary Table S2) were conducted using only TFI. The average monthly T_b was a better predictor of BKA variation than were T_b averages that used other timeframes (weekly, daily, and morning) (Table 1). For differential leukocyte components, the weekly T_b was a better predictor than monthly, daily, or morning T_b (Table 1). None of the T_b metrics tested were good predictors of total leukocyte count or H:L ratio variation (Table 1). As a result, BKA analysis (Supplementary Table S2) included average monthly T_b as an explanatory variable; for differential leukocyte count analysis (Supplementary Table S2), we included only the weekly T_b; and for total leukocyte count and H:L ratio, models did not include T_b (Supplementary Table S2).

After running BKA analysis with the complete data set (Supplementary Table S2), the selected models were included as explanatory variables: monthly T_b, TFI, life history stage, testosterone for males, progesterone and estradiol for females, and CORT for both sexes (Table 2). However, none of the explanatory variables were significant as indicated by $|t| < 2.0$ (Supplementary Table 3).

For the differential leukocyte component analysis using the complete data set, the selected models included as explanatory variables: T_b, life history stage, TFI, and plasma CORT levels (Table 2). Of these variables, only weekly T_b had a significant positive effect (Fig. 1A; Supplementary Table S4). Additionally, there was a tendency of females with lower TFI to also have a higher differential leukocyte component score during hibernation (Fig. 1B).

For H:L ratio, none of the T_b metrics were among the selected models (Table 1). Thus, further fitted models only included life history stage, plasma hormone levels, and body condition (Models 1–3, 7, 10, and 11; Supplementary Table S2). Selected models included as explanatory variables: life history stage, TFI, and plasma CORT levels (Table 2). Of these variables, only life history stage showed a significant effect (Supplementary Table S5). Males had higher H:L ratio during the reproductive period (Fig. 2A) and females had higher H:L ratio during the reproductive period and hibernation (Fig. 2B). Peaks of H:L ratio during reproductive period coincided with a peak of testosterone in males and progesterone in females (Fig. 2C–D).

For total leukocyte count, none of the T_b metrics were among the selected models (Table 1). Thus, further fitted models only included life history stage, plasma hormone levels and body condition (Models 1–3, 7, 10, and 11, Supplementary Table S2). Selected models included as explanatory variables: life history stage and plasma CORT levels (Table 2). From these variables, only life history stage showed a significant effect, where males and females had higher total leukocyte count in the beginning of the reproductive phase (Fig. 3; Supplementary Table S6).

Discussion

Our hypothesis that immune traits vary according to the tegus' life history stage, with higher values during the reproductive phase and a sharp decrease during hibernation, was partially supported. Total number of leukocytes was higher at the beginning of the reproductive stage (September) in both males and females, but there was no significant decrease of immune variables during hibernation compared to the post-reproductive stage. The increase in total leukocytes at the beginning of reproduction does not

Table 2 Selected models (ΔAICc $\leq$ 2.0) that explain the variation of immune variables (BKA, heterophil: lymphocyte ratios [H:L ratios], total and differential leukocyte component) in relation to life history stages, hormone levels, TFI, and T_b of the tegu lizard (*S. merianae*)

Variable	Hormone	Model	AICc	ΔAICc	df	Weight
BKA	Testosterone	~ hormone + monthly T_b + TFI	63.4	0.0	6	0.77
	Estradiol	~ hormone + TFI	48.1	0.0	5	0.60
		~ hormone + life history stage + TFI	50.0	1.9	6	0.23
	Progesterone	~ hormone + TFI	50.8	0.0	5	0.56
		~ hormone + life history stage + TFI	52.6	1.8	6	0.23
		~ hormone + TFI + monthly T_b	52.7	2.0	6	0.21
	CORT	~ monthly T_b + TFI	112.1	0.0	5	0.60
		~ hormone + monthly T_b + TFI	113.6	1.5	6	0.28
Differential leukocyte component	Testosterone	~ hormone + weekly T_b + TFI	72.4	0.0	6	0.99
	Estradiol	~ hormone + weekly T_b + TFI	115.4	0.0	6	1.00
	Progesterone	~ hormone + weekly T_b + TFI	115.8	0.0	6	1.00
	CORT	~ weekly T_b + TFI	180.3	0.0	5	0.73
		~ hormone + weekly T_b + TFI	182.2	1.9	6	0.27
H:L ratios	Testosterone	~ life history stage + TFI	−248.6	0.0	5	1.00
	Estradiol	~ life history stage + TFI	−248.6	0.0	5	1.00
	Progesterone	~ life history stage + TFI	−248.6	0.0	5	1.00
	CORT	~ hormone + life history stage + TFI	−249.0	0.0	6	0.48
		~ life history stage + TFI	−248.6	0.4	5	0.39
Total leukocyte count	Testosterone	~ life history stage	−96.3	0.0	5	1.00
	Estradiol	~ life history stage	−96.3	0.0	5	1.00
	Progesterone	~ life history stage	−96.3	0.0	5	1.00
	CORT	~ life history stage	−96.3	0.0	5	0.65
		~ hormone + life history stage	−95.1	1.3	6	0.35

Models were run for each variable and hormone, separately.

support the hypothesis that there is a trade-off between reproduction and immunity. Other reptilian species also show increased total leukocytes during reproduction, for instance, both male and female turtles (*Mauremys capsica* and *Trachemys scripta*) display increased immune activity during spring (reproduction) (Muñoz et al. 2000; Zimmerman et al. 2010a, 2010b). The manifestation and intensity of a trade-off between reproduction and immune function depend on multiple determinants, including hormone concentration and pattern of elevation (acute versus chronic), reproductive stage, and body condition (French and Moore 2008; Dhabhar 2014; Neuman-Lee et al. 2015; Neuman-Lee and French 2017).

Hibernation is a physiological strategy that enables animals to save energy during seasons when energy demands exceed supply (Humphries et al. 2003; Hampton et al. 2010). Thus, we expected that during hibernation, individuals would display lower immune responses. Overall, our results indicate that tegus do not show variation in BKA activity and differential leukocyte count according to phase of the annual cycle. Surprisingly, tegu lizards also maintain high levels of immune traits during hibernation. Classic hibernating mammals downregulate multiple branches of the immune system during hibernation (Bouma et al. 2010), with immune function only restored for brief phases of euthermia (Bouma et al. 2010; Ruf and Geiser 2015). During ectotherm hibernation, T_b and metabolic rate might not correlate as they do in mammals. For instance, individual tegu lizards show relatively high T_b (in July 2017: 20.5 ± 0.3°C; Zena et al. 2020), low metabolic rates, no activity, and no changes in immune traits (Abe 1995; Andrade et al. 2004; this study). The relatively high T_b due to the mild austral winter at the location where animals were kept could be one explanation for the maintenance of BKA and other immune traits in hibernating tegu lizards. Studies including populations that hibernate in colder ambient temperatures (e.g., northern Argentina), or experiments using different temperatures in captivity, could shed light on this question. The lack of seasonal variation in

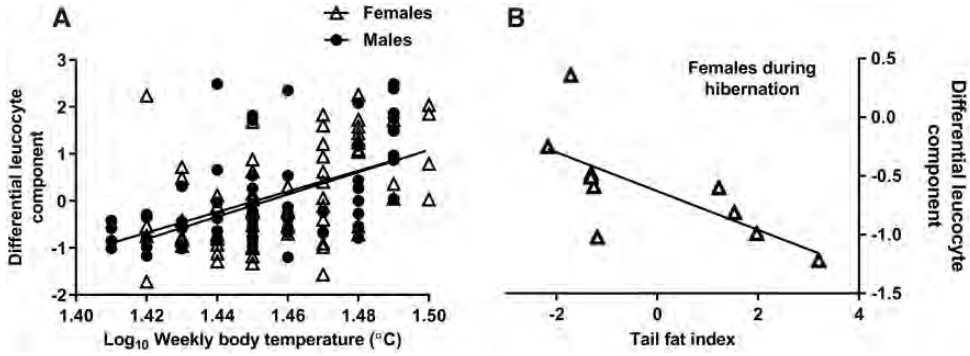

Fig. 1 (**a**) Differential leukocyte component in relation to weekly T_b of male and female tegu lizards (*S. merianae*); (**B**) Differential leukocyte component in relation to tail fat index in hibernating female tegu lizards (*S. merianae*). In (**A**) lines represent best fit regression for males and females, in (**B**) line represents best fit regression.

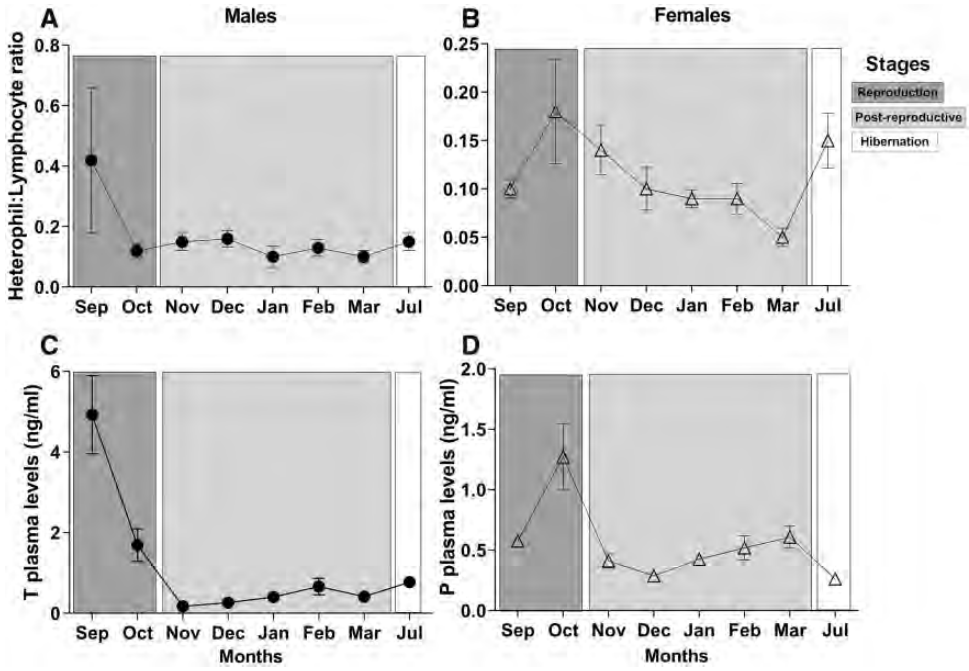

Fig. 2 Heterophil: lymphocyte ratio across life history stages of (**A**) male and (**B**) female tegu lizards (*S. merianae*); (**C**) male testosterone plasma levels and (**D**) female progesterone plasma levels across life history stages of the tegu lizard. Plots display the mean ± standard error of observations.

immune traits could be also attributed to the fact that the tegus can maintain baseline immune surveillance across different life history stages, allowing the ability to detect and fight potential infections, even during hibernation. The fact that these animals did not face nutritional or pathogen challenges in the communal outdoor enclosure might also contribute to the lack of seasonal variation (French et al. 2007c; Husak et al. 2016). Natural populations in the wild may show different relationships between immune response, hormones, and food resource fluctuations (Zapata et al. 1992; Muñoz and de la Fuente 2004; Zimmerman et al. 2010a, 2010b; Schwanz et al. 2011; Spence et al. 2020).

Contrary to our hypothesis, we did not observe a significant correlation of immune variables and body condition features (body condition index and TFI). The trend during the most energetically challenging phase of hibernation was that females with lower TFI showed higher numbers of basophils, eosinophils, monocytes, and azurophils (differential leukocyte component score), but this trend did not persist in other life history stages or in males. Additionally, we also did not observe a clear relationship between BKA and total leukocyte count and either gonadal steroid or CORT levels, even with the additive effect of life history stage. While multiple studies have demonstrated the effects of season, gonadal

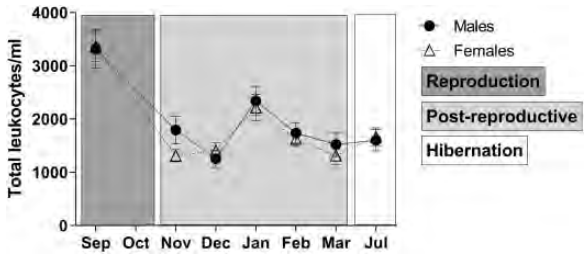

Fig. 3 Total leukocyte count across different life history stages of male and female tegu lizards. Plot displays the mean ± standard error of observations.

hormones, body condition, and CORT in immune response of ectothermic vertebrates (Berger et al. 2005; Meylan et al. 2013; Assis et al. 2015; Madelaire et al. 2017; Titon et al. 2018; Hudson et al. 2020), other studies show no correlation between immune variables and either estradiol levels in females or testosterone levels in males (Mondal and Rai 2002; Foo et al. 2017; Madelaire et al. 2017, 2019; Neuman-Lee and French 2017; Thomas and Woodley 2017). Tegus in this study had an abundant and balanced diet and were vermifugated to eliminate parasites. In natural conditions, individuals face parasites and other pathogens that, combined with a lack of sufficient energy reserves to invest in all physiological activities, might result in a seasonal pattern of variation driven by nutritional constraints (Alonso-Álvarez and Tella 2001; French et al. 2007c; Ruiz et al. 2010; Husak et al. 2016). This supposition emphasizes the importance of investigating the variation and relationship of immune traits with annual cycle, hormones, and body condition in both the field and laboratory (Martin et al. 2021). Future studies might aim to monitor body condition, hormone, and immune levels of tegu lizards in the wild.

Our prediction that T_b and hormone levels would correlate with immune variables was partially supported. The average T_b experienced one week prior to blood sampling was positively correlated with numbers of eosinophils, basophils, monocytes, and azurophils (differential leukocyte component), corroborating other studies showing that when animals maintain a high T_b, there is an increase in immune activity (Butler et al. 2013; Graham et al. 2017; Zimmerman et al. 2017). Interestingly, none of the tested timeframes of T_b variation had a significant effect on BKA or total number of leukocytes, corroborating previous studies showing that immune activity can be viable under a wide range of temperatures even if it is optimized at the species' thermal preference (Butler et al. 2013; Graham et al. 2017; Zimmerman et al. 2017; Moretti et al. 2019). Improved understanding of the relationship between variation in T_b and immune traits and surveillance in ectotherms under field and laboratory conditions is especially important in a climate change scenario. While short-term increase of temperature might increase the amount of circulating immune cells (this study) and possible cell function (Butler et al. 2013), we still do not know how acute heat waves or chronic increases in ambient temperature might affect the overall immune response in ectotherms (Cavieres et al. 2020), patterns of disease cycles (Altizer et al. 2013), or pathogen abundance and virulence (Matanza and Osorio 2018; Hernández-Cabanyero et al. 2020). The exploration of the effects of T_b experienced in both the short- and long-term on different aspects of immunocompetence can be highly relevant to predict how environmental temperature affects and how individuals cope with infections (Rohr and Palmer 2013; Cavieres et al. 2020; Rezende et al. 2020).

We observed that males had their highest H:L ratio during reproduction, whereas females had two peaks in H:L ratio, one occurring during October (oviposition phase) and the other during hibernation. Peaks of H:L ratio during reproduction coincided with a peak of testosterone in males and progesterone in females. The increase of H:L ratio is primarily associated with peaks of circulating GCs, mainly in the context of stress response (Davis et al. 2008). Other steroids can also influence innate immune function, including leukocyte recruitment, and a simultaneous peak of H:L ratio and gonadal hormones is commonly observed across vertebrates (Ots and Hõrak 1996; Davis et al. 2008; Madelaire et al. 2017, Skwarska 2019). For hibernating females, the peak in H:L ratio did not correlate or coincide with peaks of any of the studied hormones, unlike what has been observed in other species (Lance and Elsey 1999a, 1999b; Seddon and Klukowski 2012; Goessling et al. 2015; Madelaire et al. 2017). Increased H:L ratio, even without elevated CORT, can be an indication of stress (Davis et al. 2008). Thus, our results indicate that reproduction for males and females might be considered stressful events for this species.

Contrary to our predictions, no differences were observed between sexes during any of their life history stages. There is no consensus in the literature regarding sex-derived differences in immune investments, while some studies show females have lower immune response (Zuk and McKean 1996; Klein 2004; Stahlschmidt et al. 2017), others indicate no differences between males and females (e.g., Muñoz

et al. 2000; Zuk and Stoehr 2002; Haussmann et al. 2005; Bertrand et al. 2006; Beldomenico et al. 2008; Zimmerman et al. 2010a, 2010b). The lack of differential investment in immune parameters between sexes might be attributed to the abundant energetic resources available in captivity. Food restriction can enhance stress response and decrease reproductive output and immune response (Kitaysky et al. 2001; Titon et al. 2018). In this study, the abundance of resources could allow animals to fully invest in all physiological functions. Future studies could explore whether tegu males and females in the wild that are subject to resource fluctuation might invest differently in immune response.

Conclusions

By examining a variety of physiological and immune variables across the annual cycle of the tegu lizard *S. merianae*, we expected to unveil which of the studied traits were relevant to the modulation of immunity across different life history stages. We observed that the average ambient temperature experienced 1 week prior to blood sampling was positively correlated to the numbers of eosinophils, basophils, monocytes, and azurophils (differential leukocyte component). On the other hand, BKA and total number of leukocytes were not influenced by T_b, indicating that some immune traits are stable across a wide range of temperatures. Additionally, there were coincident peaks of total circulating leukocytes, H:L ratio, and steroids during the reproductive phase, but no significant correlations were found between immune traits and circulating hormone levels/body condition. These results might be attributed to the semi-natural conditions in which the tegus were kept, that is, buffered from nutritional, predation, and other stressors. Our results illustrate the importance of understanding the relationship between these variables in ectotherms, especially in a climate change scenario. Further studies could explore the effects of T_b on immune response to predict how environmental temperature might affect how individuals cope with infections.

Conflict of interest

Authors declare no conflict of interest.

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Data availability statement

The data underlying this article are submitted in Pangea.de repository, at https://doi.pangaea.de/10.1594/PANGAEA.930896.

Supplementary data

Supplementary data available at *ICB* online.

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