Baseline values of immunologic parameters in the lizard *Salvator merianae* (Teiidae, Squamata)

Ana Paula Mestre$^{1,2,3,*}$, Patricia Susana Amavet$^{2,3}$ and Pablo Ariel Siroski$^{1,2,4}$

1Laboratorio de Zoología Aplicada: Anexo Vertebrados, Facultad de Humanidades y Ciencias, Universidad Nacional del Litoral, (FHUC-UNL/MMA), Argentina
2Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina
3Laboratorio de Genética, Departamento de Ciencias Naturales (FHUC-UNL), Santa Fe, Argentina
4Laboratorio de Biología Celular y Molecular Aplicada, Instituto de Ciencias Veterinarias del Litoral (ICiVet-Litoral-UNL-CONICET), Esperanza, Santa Fe, Argentina

**Abstract**

The genus *Salvator* is widely distributed throughout South America. In Argentina, the species most abundant widely distributed is *Salvator merianae*. Particularly in Santa Fe province, the area occupied by populations of these lizards overlaps with areas where agriculture was extended. With the aim of established baseline values for four immunologic biomarkers widely used, 36 tegu lizards were evaluated tacking into account different age classes and both sexes. Total leucocyte counts were not different between age classes. Of the leucocytes count, eosinophils levels were higher in neonates compared with juvenile and adults; nevertheless, the heterophils group was the most prevalent leucocyte in the peripheral blood in all age classes. Lymphocytes, monocytes, heterophils, azurophils and basophils levels did not differ with age. Natural antibodies titres were higher in the adults compared with neonates and juveniles lizards. Lastly, complement system activity was low in neonates compared with juveniles and adults. Statistical analysis within each age group showed that gender was not a factor in the outcomes. Based on the results, we concluded that *S. merianae* demonstrated age (but not gender) related differences in the immune parameters analyzed. Having established baseline values for these four widely-used immunologic biomarkers, ongoing studies will seek to optimize the use of the *S. merianae* model in future research.

**Keywords:** Biomarkers, Immune system, Reptilian, *Salvator merianae*, Sentinel model.

**Introduction**

The genus *Salvator* (previously *Tupinambis*; Harvey et al., 2012) belongs to the order Squamata, and is widely distributed throughout South America. In Argentina, the most abundant and widely distributed species is the "iguana overa (Spanish) or tegu lizard (English)" (*Salvator merianae*) (Ávila-Pires, 1995; Harvey et al., 2012). This lizard is a diet generalist that feeds on a wide range of animals and fruits (de Castro and Galetti, 2004) and whose daily and seasonal activity cycles are strongly related to temperature (Winck et al., 2011). From April-June (colder months), their metabolism decreases until environmental temperature increases. From October-December (warmer months), the annual reproductive cycles takes place. In those times, nests are built in caves in the ground or tree roots that are isolated from the climate changes to ensure a proper incubation temperature and humidity for the development of embryos (Yanosky and Mercolli, 1992). Since 1982 *S. merianae* has been included in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and it is considered of “Least Concern” for conservation status by the IUCN (International Union for Conservation of Nature) (Embert et al., 2009). In addition, this species has been under management on a sustainable use program in Santa Fe province (Argentina), known as Iguana Project (PI - Secretary of State for Environment and Sustainable Development of the Province of Santa Fe. Resolution Number 0031/07). This program is based on the ranching technique, which implies the collection of eggs from natural environment, subsequent artificial incubation, birth and breeding of the animals under controlled conditions until they reach an appropriate size to be released into the wild and to avoid predation or influence of low temperatures (Schaumburg et al., 2012).

In recent years, the habitats of this species have been negatively impacted by expansion of agriculture, especially soybean monocultures. The implementation of a system that combines the use of new technologies includes use/application of chemicals in bulk. The effects of these agents, combined with environmental factors (i.e., habitat fragmentation/degradation, draining of wetlands) have produced a decline in the populations of various wild species, among them...
several types of reptiles (Gibbons et al., 2000; Santos and Llorente, 2009; Weir et al., 2015). In particular, tegu lizard populations have plummeted in areas where agricultural activity has advanced. As with any endangered host species, it is essential to monitor their health status and ability to defend from infections, as a result of changes in their external environments. Immunity to infection is mediated by two general systems, innate (or natural) and acquired (or adaptive). Components of the natural immune system are markedly conserved between insects and mammals, indicating a common ancestral origin for this branch of immunity (Hoffmann et al., 1999). Only vertebrates have the adaptive immune system, which play an important role when innate immunity is not precise enough to a particular challenge or in response to recurrence of a given challenge (Collado et al., 2008). Exposure of native animals to certain pesticides, whether acute or chronic, could affect both their innate and acquired immune responses (Burns et al., 1996). Previous studies have shown some pesticides have effects on immunity; they can alter structures of some components of the immune system (IS), and in some cases also reduce host resistance to antigens and infectious agents (Hernández Coronado, 2007; Modesto and Martinez, 2010; Ray et al., 2015). Studies in broad-snouted caiman (Caiman latirostris), another reptile, demonstrated alterations in the IS of hatchlings and neonates that had been exposed to glyphosate, endosulfan, or cypermethrin formulations (Siroski, 2011; Harvey et al., 2012; Latorre et al., 2013; Siroski et al., 2016).

White blood cells (WBC) are involved in a significant amount of processes in the IS. In certain situations, increases or decreases in values of select blood components can be used as markers to diagnose disease or changes in host nutritional status (González Fernández, 2003). The innate immune system can be monitored through assessments of two humoral components: levels of natural antibodies (NAb) and the functionality of the complement system (CS). Natural antibodies are encoded directly by the germline genome (Avrameas, 1991) and do not require somatic hyper mutation and recombination during ontogenesis as occurs with the adaptive antibody repertoire. The CS is an important part of the innate immune system and can be sequentially activated in a cascade type reaction by numerous routes (Siroski et al., 2016). The aim of this study was to evaluate four widely-used immune biomarkers, e.g., total and differential leukocyte count, NAb levels, and CS activity, in order to determine baseline values for S. merianae of varying ages. This data would, in turn, allow for the potential implementation of this species as a model for use in evaluating exposures of environmentally relevant species to different environmental stressors.

Materials and Methods

Animals
This research was approved by the Ethics Committee and Security (ECAS) of Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (#258/16, Santa Fe, Argentina). All animals were treated in accord with the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals (NSTRC, 2005).

In this study were used 36 specimens of tegu lizards: 12 neonates (NE; 2-days-old), 12 juveniles (JUV; 6-month-old) and 12 adults (AD; > 4-yr-old; 6 males, 6 females). The NE and JUV animals came from eggs collected in the Managed Natural Reserve “El Fisco” located in Santa Fe Province, Argentina; corresponding to a Protected Natural Area (Law 12.930, 2008) situated at least 20 km away from possible pesticide application area or other industrial contaminant activity (Siroski et al., 2016). As part of the ranching program “PI”, the eggs were artificially incubated under controlled conditions of temperature (29-32°C) and relative humidity (< 20%). Adult lizards were taken out from the breeding stock in captivity in the “PI”. All lizards were fed ad libitum three times a week. The NE and JUV could be sexed when they were below 20 cm in snout-vent length (SVL) (Yanosky and Mercolli, 1992). Therefore, gender determination was performed using the mound scale method as well as through observations of hypertrophy of the jaw muscles in males (Hall, 1978; Yanosky and Mercolli, 1992).

Blood samples
Peripheral blood samples were obtained from the caudal vein (Olson et al., 1977) with heparinized syringes (25 G × 5/8” needles for NE lizards and 1” needles (21G) for JUV and AD lizards). Aliquots of the collected blood were used for measures of WBC and for differential leukocyte population counts. The remaining sample was centrifuged at 2500 × g for 15 min and stored at -80°C until used for the determination of NAb levels and CS activity (Siroski et al., 2016).

Total white blood cells and differential leukocyte population counts
WBC counts were performed using a Neubauer chamber. An aliquot of whole blood was diluted 1:200 with a solution of 0.6% NaCl and then examined under microscope at 400X. All results were expressed as total cells/mm³ blood (Lewis et al., 2008). For the differential counts, two smears were prepared/animal, fixed with ethanol, and then stained with May-Grunwald-Giemsa solution. The preparations were then coded to achieve maximum objectivity during the analysis. Amounts of each leukocyte subtype (e.g., heterophil, basophil, eosinophil, lymphocyte, monocyte, azurophil)/100 WBC analyzed were determined manually using an optical microscope.
Each subtype was expressed in relation to WBC count recorded beforehand. Also, lobularity index was calculated (LI = number of counted lobes / number of heterophils counted) from the classification of heterophilic granulocytes according to Arneth (Charipper, 1928) to evaluate the degree of leukocyte maturity (García et al., 1997).

Natural antibodies titres

Determination of agglutinating NAb titres was conducted using a hemagglutination assay described by Matson et al. (2005). This assay is based on agglutination between NAb from lizard plasma samples and rabbit red blood cells (RRBC) obtained from a breeding stock maintained at the Universidad Nacional del Litoral. Here, whole rabbit blood was centrifuged at 2500 x g for 15 min to separate the plasma. A buffer solution was then prepared with phosphate-buffered saline (PBS, pH 7.4) containing rabbit plasma (1%) (Sigma, St. Louis, MO). The pelleted RRBC were then washed with PBS several times until the supernatant was clear, and then a 1% RRBC (v/v) solution in PBS was prepared. For the assay itself, to 96-well round (U)-bottom plates (Corning Costar, Corning, NY), 25 µl (PBS with rabbit plasma) was added into wells in Columns 1-12. Thereafter, 25 µl test plasma was added to wells in the first column. Samples were then serially diluted to a final dilution of 1:2048 (Column 11). As a negative control, no lizard plasma was placed into the Column 12 (i.e., well contained only PBS). Finally, 25 µl RRBC solution was added into wells in all columns (1-12). After incubation at 25°C for 1 hr, NAb titres were determined. Titres were assigned as the inverse of the highest dilution yielding a button; in cases where an individual had negative hemagglutination in all wells, a titre of 0 was assigned. From the average of all titres in a given age group, a mean titre for each group was calculated.

Complement System (CS) activity

Lizard CS activity was determined via assessment of sheep red blood cell (SRBC) hemolysis (Siroski et al., 2010). The SRBC were collected from Merino sheep (Ovis aries) with heparinized syringes. The blood was washed with PBS several times until the supernatant was clear, and then a 2% SRBC (v/v) solution in PBS was prepared. In the assay, lizard plasma was incubated with an equal volume of 2% SRBC for 30 min at 25°C and then centrifuged at 2500 x g for 5 min. Thereafter, 300 µl of the resultant supernatant was transferred to a microplate for measure of optical density [at 540 nm] in a Multiskan RC microplate reader (Multiskan Labsystem, Helsinki, Finland). As a positive control, 2 µl Triton X-100 was added to 1 ml of 2% SRBC and the mixture shaken until complete hemolysis was attained. The level of SRBC hemolysis in each sample was divided by the absorbance of the positive control to obtain the maximum percentage of hemolysis (% MH). All results were expressed as mean % MH [± SE].

Statistical analysis

Data were tested for homogeneity using a Levene test, and for normality using a Kolmogorov-Smirnov test. To determine differences among the groups, data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey test. When the data did not meet the assumptions of normality and / or homogeneity of variances, it were analyzed using a non-parametric Kruskal-Wallis test followed by a Mann-Whitney test. Comparisons of variables analyzed as a function of gender were done using a Student t-test except for eosinophil data that were analyzed using a non-parametric Mann-Whitney test. Differences were considered significant with p < 0.05. All data are reported as means ± SE.

Results

Because the morphological characterization of the leukocytes of this lizard has not yet been reported, we include images of all leucocytes types (Fig. 1).

Fig. 1. Leukocytes of S. merianae. (A, B): Heterophils with cytoplasm full of rod granules (A) and of little round granules (B). (C): Eosinophil with cytoplasm full of round granules. (D): Basophil with big round granules. (E): Monocyte and (F): Big lymphocyte. In the last two, low nucleus/cytoplasm ratio and low concentration of heterochromatin.

The results for the total WBC counts showed there were no differences among the age classes of S. merianae. Nevertheless levels of eosinophils were higher in NE compared with JUV and AD groups (Fig. 2). With respect to the others leucocyte types no differences were observed among age classes. When the mean of all cell types in the three age classes were compared, it was found that the heterophils group was the most prevalent leukocyte in the peripheral blood in all age classes. In this type of leucocyte the nucleus was rounded, with one or two lobes, being infrequent the presence of three or more lobes, determining an IL of 1.76 ± 0.10 for NE, 1.65 ± 0.58 for JUV and 1.61 ± 0.92 for AD. The comparison among age classes was not different. Natural antibody titres differed among the groups. Values associated with AD were higher compared with those for NE and JUV (Fig. 3).
This clearly indicated that the levels of NAb in younger lizards were very low (in many cases, there were titres of 0). With regard to CS activity, the data indicated there were also differences among the age classes. Neonate blood imparted a low value of maximum hemolysis compared with the blood from JUV or AD (Fig. 4). Lastly, comparisons performed for each parameter examined here showed there were no gender-related differences.

**Fig. 2.** Eosinophils level in relation with total number of WBC in each age class. NE (neonate), JUV (juvenile), AD (adult). Values shown are means (± SE) from 12 lizards/group. *Value significantly different from other groups (p < 0.05).

**Fig. 3.** NAb titres in each age class. NE (neonate), JUV (juvenile), AD (adult). Values shown are mean (±SE) from 12 lizards/group. *Value significantly different from other groups (p < 0.05).

**Fig. 4.** Maximum percentage hemolysis (%MH) in each age class. NE (neonate), JUV (juvenile), AD (adult). Values shown are means (± SE) from 12 lizards/group. *Value significantly different from other groups (p < 0.05).

**Discussion**

Reptile immunology involves cell-mediated and humoral components; however, knowledge about immune function in reptiles remains limited. Several characteristics of *S. merianae* suggest it could potentially be useful as a sentinel organism for monitoring of environmental agent impact on their habitat (Schaumburg *et al*., 2016).

The present study sought to establish some baseline parameters for such use; in particular, information on the IS of these hosts. Similar results were obtained for other authors who did not find differences in levels of WBC among different age classes in reptiles. Studies on *Chelonoidis chilensis chilensis* (Troiano and Silva, 1998), from captive *Caiman latirostris* and *C. yacare* (Mussart *et al*., 2006) and *Iguana iguana* (Novoa-Fajardo *et al*., 2008) have reported an absence of influence from age on this hematologic parameter. However, Barboza *et al.* (2008) reported values significantly lower in adult than in both juvenile and sub-adult of *C. latirostris*. Further, the present study showed lower WBC counts compared to Novoa-Fajardo *et al.* (2008) results, and higher values in relation to that seen in other studies (Mussart *et al*., 2006; Barboza *et al*., 2008; Silvestre, 2014).

Regarding the endpoint of differential leukocyte counts, here we have considered azurophils as a variation of normal monocytes, as well as some authors (Montali, 1988; Huber and, 1998; Claver and Quaglia, 2009). In contrast, others researches consider them as a distinct cell type (Troiano *et al*., 1996; Kanchanapangka *et al*., 1999; Salakij *et al*., 2002; Maceda-Veiga *et al*., 2015).

Similar to the one reported by Rios *et al.* (2003) for *Lama guanicoe*, in this study a higher level of eosinophils was seen in neonates compared to juveniles and adult lizards. This could be a consequence of some exposures to allergens present in the nest material in contact with neonates. This result contrast with findings by Barboza *et al.* (2008) who observed an age-related increase in eosinophil levels in *C. latirostris*. Overall, the means levels of eosinophils in adult *S. merianae* were generally higher than those reported by Silvestre (2014).

Similar to what was seen by Troiano *et al.* (1996), Troiano and Silva (1998) and and Rios *et al.* (2003), this study did not reveal any differences among age classes in regard to other leukocyte types, while Stacy *et al.* (2011) described age related changes in lymphocyte and heterophil percentages in *Caretta caretta* turtles. The lobularity index here observed was similar to the ones reported by Cabagna Zenklusen *et al.* (2011) in *Rhinella fernandezae* and Salinas *et al.* (2015) in *Rhinella arenarum*. With regard to NAb levels, our analysis of titres showed the highest values in the adults compared with juveniles and neonate.
lizards. This increase in NAb with age has been also reported in mammals, birds, and other reptiles (Parmentier et al., 2004; Benatuil et al., 2008; Sparkman and Palacios, 2009; Ujvari and Madsen, 2011; Zimmerman et al., 2013). In reptiles of all ages, the specific antibody response is slower and less robust than in mammalian counterparts (Zimmerman et al., 2010). This thus implies that the increase in NAb tires with age might be viewed as a “positive” change in immunity with age in these reptiles (Ujvari and Madsen, 2011). Studies about complement-system activity have been reported with a variety of reptiles. In Alligator mississippiensis (Merchant et al., 2005), Crocodylus porosus and C. johnstoni (Merchant and Britton, 2006), and Caiman latirostris (Siroski, 2011), potent CS were reported; this suggested a strong physiological importance for this system in terms of protection against various pathogens (Siroski, 2011). In the present study, a low percentage of maximum hemolysis was detected in S. merianae compared to the above-mentioned species. This could be a trait of the S. merianae itself. On the other hand, differences in CS activity were noted among the S. merianae age classes, indicating activity of this system increased with age. Strasser et al. (2000) reported similar results in relation to age in mammals. Thus, future studies must consider that results of this technique are likely to be variable with age. According with findings in other studies on sentinel species, the comparisons between sexes revealed no differences in the endpoints assessed here. Khan et al. (2016) found no gender-related differences in WBC levels, differential leukocyte counts, or other biochemical/hematological parameters in dogs in Bangladesh. Latorre et al. (2015) noted no differences between males and female Phrynops hilarii (a side-necked turtle) in any of the variables that were analyzed, including WBC levels and differential leukocyte counts. In contrast, Barboza et al. (2008) found that male C. latirostris had total leukocyte, eosinophil, heterophil, and monocytes levels greater than those of females. Similarly, Stacy and Whitaker (2000) found mean WBC count and mean heterophil counts were significantly greater in adult male C. palustris than in adult females. Troiano et al. (1996) found inter-sex differences too, both in total count and differential leukocyte in other caiman species. Considering the results obtained in this study, sex, unlike age, should not be considered a key variable in studies that may use S. merianae as a model. In conclusion, the baseline values obtained in this study contributed to knowledge of IS of the Salvator merianae. Thus, of these four immunologic biomarkers studied, three showed differences among age classes, unlike sexes, in the analyzed species. These variations among age classes should be considered in future studies that may use S. merianae as sentinel model.

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Conflict of interest

The authors declare that there is no conflict of interest.

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