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Effects of cypermethrin (pyrethroid), glyphosate and chlorpyrifos (organophosphorus) on the endocrine and immune system of *Salvator merianae* (Argentine tegu)

Ana P. Mestre[a,b,*], Patricia S. Amavet[b], Agustín I. Vanzetti[b], Ma. Soledad Moleón[a,c], Ma. Virginia Parachú Marcó[a,c], Gisela L. Poletta[a,d], Pablo A. Siroski[a,c]

[a] Laboratorio de Zoología Aplicada: Anexo Vertebrados, Facultad de Humanidades y Ciencias, Universidad Nacional del Litoral, (FHUC - UNL /MMA) Santa Fe, Argentina
[b] Laboratorio de Genética, Departamento de Ciencias Naturales (FHUC - UNL), CONICET, Santa Fe, Argentina
[c] Laboratorio de Ecología Molecular Aplicada, Instituto de Ciencias Veterinarias del Litoral (ICiVet Litoral) UNL - CONICET, Esperanza, Argentina
[d] Cátedra de Toxicología, Farmacología y Bioquímica Legal (FBCB-UNL), CONICET, Santa Fe, Argentina

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ABSTRACT

Several geographical areas where *Salvator merianae* is distributed in Argentina are included in regions with agricultural activity and exposed to pesticide formulations. Some pesticides could affect defense mechanisms being able alter structures of some components of immune and endocrine systems. To assess the potential effects of pesticides in this reptile under seminatural conditions, on the immune system and endocrine responses in *S. merianae* we analyzed several blood parameters. Total (TWBCC), differential (DWBCC) white blood cells count, heterophils/lymphocytes index (H/L), lobularity index (LI), natural antibodies (NAbs) titres, complement system (CS), and corticosterone concentration were analyzed in animals exposed to a mixture of cypermethrin (25%), glyphosate (66.2%) and chlorpyrifos (48%) formulations. In addition, body size was considered in these analyzes. TWBCC and NAbs revealed lower values in organisms exposed to pesticides respect to a control indicating a possible immunosuppression effect. Besides, the LI showed a greater number of lobes in organism exposed demonstrating symptoms of chronic infection. In addition, we observed a reduced growth in these animals possibly related to a less energy investment in body mass to maintain an active defense against pesticides. Finally, we found high levels of plasma corticosterone in animals exposed to mix formulation that could demonstrate neuroendocrine axis activation. Other parameters like DWBCC, H/L index and activity of CS showed no differences in treated animals respect to control group, which could indicate low sensibility of these parameters to the concentration of pesticides used. Our results provide evidence of the toxic effects of pesticides on different immune system parameters, but also a trade-off among these parameters, corticosterone levels and growth. In this way, we can conclude that the formulated pesticides applied widely and constantly in the areas occupied by *S. merianae*, would be affecting its immune and endocrine systems and therefore its ability to defend against external agents. This kind of studies is of great interest to know the possible responses of wild species to anthropogenic disturbances such as pesticide contamination.

1. Introduction

Agriculture expansion produces a constant increase of habitat fragmentation due to deforestation and a great degradation of ecologic patches with deep consequences for biodiversity that occupy these habitats (Poletta et al., 2011). Pesticide use increased in the last

Abbreviations: IS, Immune system; TWBCC, Total White Blood Cells Count; DWBCC, Differential White Blood Cells Count; TTC, Total Thrombocytes Count; H/L, Heterophils/Lymphocytes index; IL, Lobularity index; NAbs titres, Natural Antibodies; CS, Complement System; MH, Maximum percentage of Hemolysis; RRBC, Rabbit Red Blood Cells; SRBC, Sheep Red Blood Cells; PBS, Phosphate-buffered saline; RIA, Radioimmunoassay; SVL, Snout-vent length; IP, Iguana project; CYP, Cypermethrin; GLY, Glyphosate; CPF, Chlorpyrifos

* Correspondence to: LicLaboratorio de Zoología Aplicada: Anexo Vertebrados, Facultad de Humanidades y Ciencias, Universidad Nacional del Litoral (FHUC - UNL /MMA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina.

E-mail addresses: anapaulamestre@yahoo.com (A.P. Mestre), pamavet@fhuc.unl.edu.ar (P.S. Amavet), aguvanzetti@gmail.com (A.I. Vanzetti), soledadmoleon@yahoo.com.ar (M.S. Moleón), virparachu@gmail.com (M.V. Parachú Marcó), gisepoletta@hotmail.com (G.L. Poletta), cocokaima@hotmail.com (P.A. Siroski).

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decades as agriculture became gradually transformed into a system of high technology, in order to satisfy growing demands (Heinemann et al., 2013). The area planted with soybean in Argentina reached more than 20 million hectares during the 2016–2017 season (Bolsa de Cereales de Buenos Aires, 2017), expanding over native habitat in many areas of the country. This process with the constant application of formulated pesticides (which are complex and variable chemical mixtures), could have been generating a decline in size populations of several wild species, including reptiles (Gibbons et al., 2000; Poletta et al., 2009; Weir et al., 2015). One of the most used pesticide mixture include the herbicide glyphosate (GLY) and the insecticides chlorpyrifos (CPF) and cypermethrin (CYP). The province of Santa Fe is not an exception in Argentina, and the *Salvator merianae* populations occur in areas where agricultural activity has advanced excessively, which imply a continuous exposure to pesticide discharges (Mestre et al., 2017).

As in other countries, currently in Argentina different types of exploitation are developed to *S. merianae*: direct extraction of adults, farming and ranching, giving it great value as an economic and social resource. Particularity, this specie is subject to a management and sustainable use Program in Santa Fe province, known as "Iguana Project" (IP - Secretaría de Estado de Medio Ambiente y Desarrollo Sustentable de la Provincia de Santa Fe, Resolution Number 0031/07), based on the ranching technique (Schaumburg et al., 2012). The high plasticity to live in natural and anthropic habitats, its fidelity to these environments, and its omnivore diet, makes *S. merianae* appropriate as a biological model for the monitoring of pesticide effects (Schaumburg et al., 2014). There are a few studies that have examined the toxic effects of pesticide mixtures on different vertebrates groups (Hayes et al., 2006; Poletta et al., 2011), but no studies have been reported yet in *S. merianae*.

As in any species, the immune system (IS) of *S. merianae* is essential to monitor its ability to defend against infections, health status, and different changes in the external environment. The Immune System is mediated by two general systems, innate and acquired, which have been evolved and diversified in response to many factors including environments in which the organisms live, body complexity, distinct physiology, and lifespan (Zarkadis et al., 2001). The field of immunotoxicology in wildlife is relatively new, and there is scarce knowledge about this subject in reptiles (Siroski et al., 2016). Nevertheless, it has been reported that some pesticides affects defense mechanisms and can alter structures of some components of the IS (Ray et al., 2015). Thus, for example, some authors have reported effects of Roundup (RU, glyphosate-based formulation) on some parameters of the IS and growth of *Caiman latirostris* (Latorre et al., 2013; Siroski et al., 2016). Similarly, have been also reported effects on the IS in humans exposed to chlorpyrifos (Thrasher et al., 1993) and on the heterophils population in caimans exposed in vivo to cypermethrin (Latorre et al., 2016).

As previously reported, the increase or decrease in selected blood components values can be used as markers to diagnose disease (González Fernández, 2003). Thus, the IS could be evaluated through some of the cellular components as total and differential white blood cells counts (TWBCC and DWBCC). In addition, the H/L index was proposed as a measure of the organism's response to stress (Davis et al., 2008) and the lobularity index (LI = number of counted lobes/number of heterophils counted) to assess the degree of leukocyte maturity (García et al., 1997). Another IS parameter can be also monitored through assessments of two humoral components: natural antibodies levels (NAbs) and complement system (CS) (Matson et al., 2005). NAbs are encoded directly by the germ line genome (Avrameas, 1991) and not depend on exogenous antigen stimulation. Natural antibodies mainly belong to the immunoglobulins M class (IgM) and are major humoral components of innate immunity (Palacios et al., 2009). These have a pattern of broad reactivity that is required for the rapid and immediate recognition and protection against invading pathogens (Binder et al., 2005). Nevertheless, different stressors could affect this component of the innate immunity, directly or through alterations in the reaction cascade of the production of natural antibodies. The CS consists of a group of plasma proteins that play critical roles in host defense by interacting with components of both the innate and adaptive immune systems, and can be sequentially activated in a reaction cascade by numerous routes (Siroski et al., 2016). Alterations in any of the steps of this reaction cascade could affects the essential functionality of this immunological parameter.

On the other hand, some authors have reported effects of different pesticides on the endocrine system, i.e., on hormones (Hayes et al., 2002a, 2002b, 2006; Du Preez et al., 2005). In this sense, particularity for analyzed formulates in this study, some authors have reported toxic effects of cypermethrin on endocrine disruption in rats (Elbetieha et al., 2001) and in rabbits (Yousef et al., 2003), of chlorpyrifos in *Oreochromis niloticus* (Oruç, 2010) and of glyphosate in *Rana pipiens* (Howe et al., 2004). Corticosterone is the stress hormone in lizards and reallocates energy from non-essential functions to affect morphological, physiological and behavioral traits that help organisms to deal with acute or chronic stressors (Meylan et al., 2010). This suggests that corticosterone plasma concentration could be a good indicator to evaluate stress in lizards.

Based on the ecologic and economic importance of this species, the identification of new markers of endocrine and immunotoxic effects, related to the intensive use of pesticides on *S. merianae*, is highly relevant. In this way, the aim of this study is to evaluate effects of pesticides on the immune and endocrine responses, of *S. merianae* exposed to sublethal concentrations of a mixture of GLY, CPF and CYP formulations, under controlled conditions.

2. Materials and methods

2.1. Chemicals

Roundup Full II (66.2% glyphosate, GLY), Chlorpyrifos Nufarm (48% chlorpyrifos, CPF) and Cypermethrin Atanor (25% cypermethrin, CYP). Roundup Full II is a liquid water-soluble (12.000 mg/l) herbicide, containing glyphosate potassium salt [N-(phosphonomethyl) glycine monopotassium salt, $C_3H_7KNO_5P$] as its active ingredient (a.i.) (CAS No. 70901-12-1). CPF Nufarm is a liquid water-insoluble (2 mg/l) insecticide (O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS N°: 2921-88-2). CPT Atanor is a liquid water-insoluble (0.01 mg/l) mixture of different cypermethrin isomers ($C_{22}H_{19}Cl_2NO_3$, CAS N°. 52315-07-8).

2.2. 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 handled according to the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals (NSTRC 2005).

A total of 72 tegu lizard eggs from three nests were harvested as part of the routine activities of the IP, in the managed Natural Reserve called El Fisco (30° 11′ 26″ S, 61° 0′ 27″W), an area without agricultural activities or any other source of contaminants, located in Santa Fe province, Argentina. After collection, eggs were immediately transported to the IP facilities (Lab. Zool. Aplicada: Anexo Vert., FHUC-UNL/MMA) for artificial incubation under controlled conditions of temperature (29–31.5 °C) and humidity (< 20%). After hatching, lizards were individually identified by ventral spots (Fig. 1), and maintained under controlled conditions until the beginning of the study. We utilized animals from both sexes and the same age (juveniles) to avoid possible variability associated to those parameters.

Fig. 1. Illustration of pattern of unique and unrepeatable ventral spots (Bar = 1 cm).

2.3. Experimental design and treatments

Four enclosures were built in the IP facilities, with approximately 2 m in diameter and 1.2 m in height, completely closed at the top to prevent the entry of predators and the escape of the animals. Each enclosure had several shelters, a feeder, a recipient with water and one place covered to generate shade. Two of them were exposed to the mixture of GLY, CPF and CYP formulations, and the other two were used as control and were sprayed with potable water, separated from the treatment groups by a distance of 25 m. Each enclosure contained 18 animals belonging four different nest to evaluate the nests effect; (9 animals per 4 nest: 36 animals per experimental group, N total = 72), and they were put into the enclosures one week before spraying for acclimatization. Animals were fed daily ad libitum.

The concentrations of pesticides used in the mixture were equivalent to those recommended for application in soybean crops: GLY formulation 2% (a.p. 66.2%); CPF at 0.8% (a.p. 48%) and CYP at 0.12% (a.p. 25%), and following the schedule used in agricultural practices in the region, which is based on direct sowing following the concentration recommended by the manufacturers. Therefore, treatment groups received first an application of GLY formulation alone, and one month later, the mixture of the three agrochemicals (spraying with GLY pre and post-emergence of plants is generally carried out in agricultural practices). The solutions (potable water or pesticides) were applied through a backpack sprayer, covering the whole surface of the enclosure from a height of 0.5 m. Prior of the application, the animals were removed from the enclosure and placed again 24 h later, to avoid direct spraying over them. The study was conducted for 3 months, and was done during the warm season, coinciding with the time of maximum spraying in soybean crops.

Lizards were weighed (Electronic Compact Scale, TH 5000, precision 0.1–1 g) and measured in snout-vent length (SVL, precision 0.1 cm) before and after exposure.

2.4. Blood samples

Peripheral blood samples were obtained from the caudal vein (Olson et al., 1977) with heparinized syringes (21 G needles). Aliquots of the blood were used for measures of TWBCC, DWBCC and TTC. The remaining sample was centrifuged at 2500 × g for 15 min and stored at −80 °C until used for the determination of NAbs levels, CS activity (Siroski et al., 2016) and corticosterone levels (Parachú-Marcó et al., 2014).

2.4.1. Total and differential white blood cells count

The TWBC count was performed using a Neubauer chamber. An aliquot of whole blood was diluted 1:200 with 0.6% NaCl solution and then examined under microscope at 400X. All results are expressed as total cells counted/mm^3 blood (Lewis et al., 2008). For the DWBCC, two smears were prepared/animal, fixed and 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 TWBC analyzed, were determined in an optical microscope following Mestre et al. (2017). Each subtype is expressed in relation to TWBCC recorded previously. In addition, the H/L index and LI were calculated, the last according to the number of lobes counted by each heterophil (Fig. 2).

2.4.2. Antibodies titres

Determination of agglutinating was conducted using a hemagglutination assay adapted by Mestre et al. (2017). This assay is based on agglutination between NAbs from lizard plasma samples and rabbit red blood cells (RRBC), obtained from a breeding stock maintained at the Laboratory Animal Center from Veterinary Faculty. Here, whole rabbit blood was centrifuged at 2500 × 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, 25 µl (PBS with rabbit plasma) was added in columns 1–12 into 96-well round (U)-bottom plates (Corning Costar, Corning, NY). 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 h, NAb titres were determined. They 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. A mean for each group was calculated.

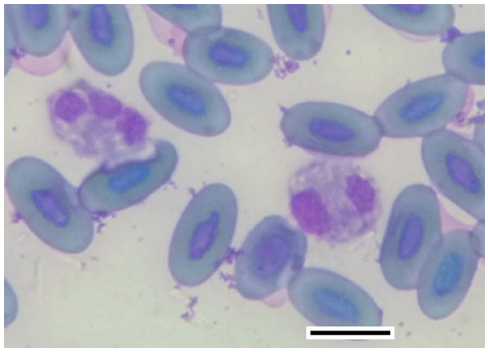

Fig. 2. Heterophils whit two and three lobes (Bar = 10 µm).

2.4.3. Complement system 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 later centrifuged at 2500 × 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].

2.4.4. Corticosterone assay

Plasma corticosterone concentrations were measured by radioimmunoassay (RIA) after extraction with diethyl ether as previously modified for other parented reptile specie by Parachú-Marcó et al. (2014). Samples were run in triplicate.

2.5. Statistical analysis

Data were tested for homogeneity using a Levene test, and for normality using a Kolmogorov-Smirnov test. We determine differences between replicas of each experimental group and between control and treated groups we used the Test T. For those variables that did not meet the assumptions of normality and/or homogeneity of variances (TWBCC, lymphocytes and monocytes population), we used the non-parametric Mann-Whitney test. The correlation of different parameters with the corticosterone levels were analyzed using the Pearson Test. For those variables that did not meet the assumptions of normality and/or homogeneity of variances (Nabs titres, DWBCC), we used the non-parametric Spearman test.

3. Results

No differences were found between replicas for any variable ($p^{>}$ 0.05 in all cases) then all results are reported for each experimental group. Total white blood cell count showed no differences between the control and treated groups but revealed a slight negative trend in organisms exposed to pesticides (Fig. 3). Similarly, no differences were found in different types of leucocytes that were counted. Also, the H/L index revealed higher values in exposed animals, but this difference was not statistically significant. Nevertheless, the comparison between the two groups regarding the number of lobes of the heterophils showed

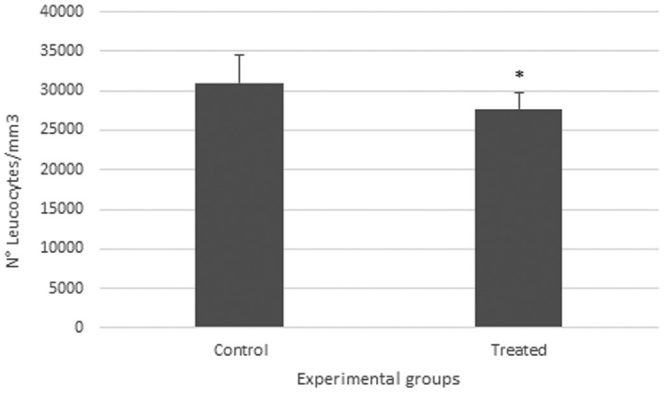

Fig. 3. Comparison the numbers of leucocytes/mm^3 between control vs. treated groups. *Value significantly different from other group ($p < 0.05$).

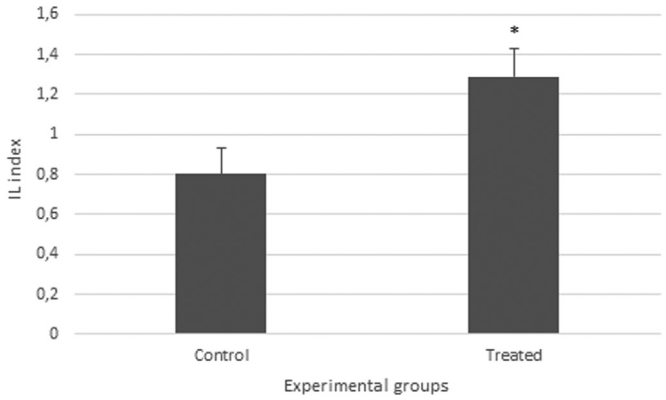

Fig. 4. Comparison the IL indexes between control vs. treated groups. *Value significantly different from other group ($p < 0.05$).

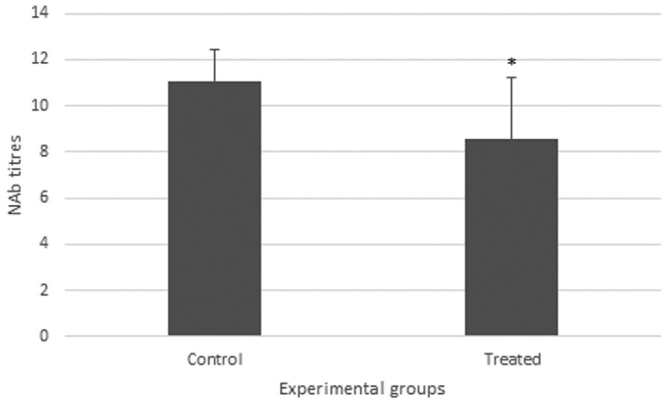

Fig. 5. Comparison of natural antibodies titres between control vs. treated groups. *Value significantly different from other group ($p < 0.05$).

significant differences ($p = 0.009$). The heterophils were rounded, with one or two lobes generally, being more frequent the presence of three or more lobes in animals exposed (IL = 1.29 ± 0.14) than controls (IL = 0.8 ± 0.13) as shown in Fig. 4. Regarding natural antibodies, in the Fig. 5 can be seen that control animals showed higher titres (8.55 ± 2.71%) than the exposed ones (11.09 ± 1.37%, $p = 0.013$). On the other hand, no significant differences were found in the CS activity determined through the SRBC hemolysis evaluation. The body size, analyzed by weight and SVL measurements, increased in all animals, being slightly higher in control animals, although the difference between both groups was not significant (Table 1). The results of RIA assay revealed higher levels of corticosterone (ng/ml) in lizards exposed to pesticides (171.2 ± 14.77%) in relation to the control group (117.68 ± 7. 97%), as shown in Fig. 6. When the corticosterone levels were correlated with the other parameters, a negative correlation was observed with respect to TWBCC (Rho = 0.433; $p < 0.001$), NAbs titres (Rho = 0.544; $p < 0.001$) and SVL (Rho = 0.752; $p < 0.001$).

Table 1
Comparison of growth in weight and snout-vent length between exposed and control animals.

	Experimental group	
	Control	Treated
SVL (cm)		
Pre-experimental	13.37 ± 0.39	13.58 ± 0.35
Post-experimental	20.92 ± 0.66	20.45 ± 0.65
Weight (g)		
Pre-experimental	78.03 ± 8.02	82.26 ± 8.24
Post-experimental	455.14 ± 57.64	391.51 ± 48.12

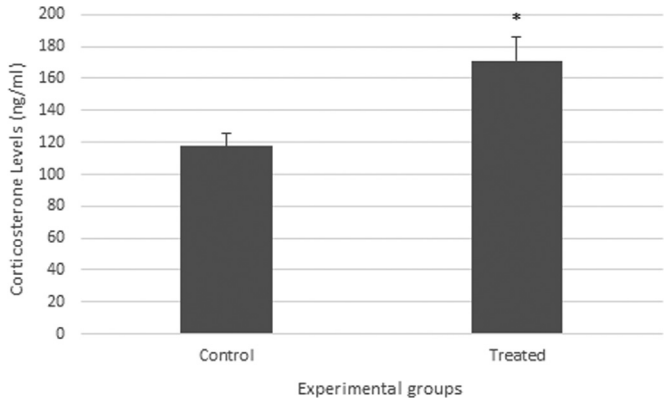

Fig. 6. Comparison of the corticosterone levels between control and treated groups. *Value significantly different from other group ($p < 0.05$).

However, a positive correlation was observed when it was compared to other analyzed parameters. Thus, the eosinophils and heterophils group as well as the H/L index, increased when the corticosterone levels increased (Rho = 0.767; Rho = 0.919 and Rho= 0.565, respectively; $p < 0.001$).

4. Discussion

Low levels of contaminants can cause immunotoxicity, and concentrations of these chemicals much lower than those needed to achieve an effect on a target organ in the short term, could serve as high sensitive indicator of toxicity. Little is known about the immune system of reptiles, and scarce studies have been done in reptilian immunological disorders (Cooper et al., 1985; Munoz and de la Fuente, 2001). In the same way, very few analyzes have been performed about the effects of pesticides on reptiles (Latorre et al., 2013, 2016; Siroski et al., 2016). In this study we found that the mixture of pesticides formulation used could alter some immunological parameters.

The hematological profile of blood can provide the information about the body internal condition of an animal earlier than any noticeable indication of diseases, so peripheral blood smear continues to be the "gold standard" of hematologic diagnosis (Campuzano-Maya, 2014). In our study, TWBCC revealed a slight negative trend in organisms exposed to mixture of pesticides, indicating a possible toxic effect. Some authors that have studied single compounds or mixed formulations have reported similar results. Thus, in *Caiman latirostris* exposed to a commercial glyphosate-based formulation (Siroski et al., 2016); in *Bufo melanostictus* exposed to malathion (Mahananda and Mohanty, 2012), and in *Ambystoma tigrinum* exposed to atrazine (Forson and Storfer, 2006) were observed a significant decrease of peripheral leukocyte levels respect to the controls. The decrease in number of WBC found in the present study, like other reports, in response to different toxicants may be related to compensatory response to circulating leucocytes. Nevertheless, Latorre et al. (2016) found no significant differences in *C. latirostris* specimens exposed to endosulfan and cypermethrin formulations compared to controls. This could suggest that a mixture of formulates would not necessarily affect this immunologic parameter in the same manner.

In vertebrates, DWBC are related to the defense mechanism and consist of lymphocytes, monocytes and granulocytes. Monocytes and granulocytes play function in the removal of injured cell debris, while lymphocytes related to the production of antibodies (Wedemeyer and McLeay, 1981). In contrast to Latorre et al., (2013, 2016) and Siroski et al. (2016) who reported significant differences in DWBCC and H/L index values between treated groups and controls of *Caiman latirostris*, in the present research these parameters showed no significant differences. This could be consequence of a lower sensibility of these variables in *S. merianae* to toxic agents. However, it could also be expected that successive exposures can generate a significant difference. Nevertheless, the LI was significantly different between groups, with higher number of lobes in heterophils of organisms exposed. This result could indicate an unfavorable cellular environment or symptoms of chronic infection (Boxer and Dale, 2002), because a greater number of mature heterophils (more segmented) are in blood circulation. A relatively large number of younger heterophiles (unilobed) would give us data from an acute rather than chronic exposure.

Studies on *Trachemys scripta* (Zimmerman et al., 2013) support the idea that non-specific, natural antibody responses are an important line of defense in reptiles. In this study, the NAbs titres showed significant differences between groups, where the titres values of animals controls were higher than the exposed to pesticides mixture, revealing pesticides effects on the natural antibody responses. Toxic molecules could suppress natural antibodies production altering any-phase of the immune response. Nevertheless, the levels of NAbs never reached null values, suggesting that a basal immunity is maintained even in these cases. Similar results were reported in *Ictalurus punctatus* where the NAbs titres in control animals were higher than the exposed to organophosphorus Malathion (Areechon and Plumb, 1990). Instead, other authors found non-significant positive tendency in *Parus major* exposed to lead, indicating an increase in values of NAbs titres as the concentration of lead increases (Vermeulen et al., 2015). The present study, as well as those above cited, indicated not only toxic effects of different compounds (or mixtures thereof) on the NAbs in different species, but also the importance and usefulness of evaluate this parameter as immunotoxicity marker. It should be noted, that the NAbs titres in the present research were generally low in relation to those reported for other reptiles such as *Buteo galapagoensis* (Whiteman et al., 2006) and *Trachemys scripta* (Zimmerman et al., 2013); but were similar to those reported by our workgroup in lizards of the same age class (Mestre et al., 2017).

The serum complement system is an important element of the innate immune defense of animals against infectious agents (Siroski et al., 2010); so that certain toxic effects could alter the immune response through modifications in this system. Regarding to this parameter CS, some authors reported that toxins could affect its activity, such as pentachlorophenol in mouse (White and Anderson, 1985), and GLY formulation in *C. latirostris* (Siroski et al., 2016). However, in this study no significant differences were found between groups, as it was reported for the fish *Cyprinus carpio* exposed to chlorpyrifos (Li et al., 2013). This result could be consequence of a low sensibility of this parameter to show differences between groups. In addition, it is interesting to note that the results found here were similar to that reported by us in a previous study analyzing juvenile specimens (Mestre et al., 2017).

In relation to body size, similar results were reported in *Xenopus laevis* exposed to pesticides mixture where the growth was lower in exposed animals compared with controls (Hayes et al., 2006). This could be related to a lower energy investment in body mass as result of high energy consumption for individual defense due to toxic exposure. In addition, this could imply an immunosuppression resulting from body mass loss, generating a higher vulnerability to external agents (Flint and Franson, 2009). Barraco (2015) reported the contrary effect on wild *S. merianae*, due to he found higher body size in animals from perturbed areas respect to non-perturbed, due to the preys available for the specie contain a high caloric content (because some fat-soluble pesticides can be accumulated in body fat). According to the explained above, differences in the observed results between different types of studies (semicontrolled experiment vs. environmental tests) could indicate changes in physiological strategies of the species, according to the conditions in which the animals are.

Corticosterone may decrease or increase in presence of a stressor. We found high levels of plasma corticosterone in animals exposed to mix formulation that could demonstrate neuroendocrine axis activation. The increase in corticosterone levels results in mobilization of

energy stores and the appropriate adjustment of behavior and physiology to the conditions encountered (McEwen and Wingfield, 2003). This fact motivated us to make a correlation between the levels of this hormone and other analyzed parameters. Thus, values of TWBCC decreased when corticosterone levels increased (slightly negative correlation). These results coincide with those reported by Hayes et al. (2006) in *X. laevis* exposed to a pesticides mixture, which indicated an increase on plasma corticosterone levels in animals exposed. Likewise, these authors suggested that these increase could be producing alterations on other parameters, including slower growth and development and potential immunosuppression. When the different types of leucocytes were correlated with the corticosterone concentrations, we observed that the eosinophils and heterophils group as well as the H/L index, increased together with corticosterone levels. The effects on the H/L ratio could be explained because this hormone act to increase the number and percentage of heterophils, while decreasing the number and percentage of lymphocytes (Davis et al., 2008). In addition, previous studies showed that high levels of corticosterone lead to an increase in heterophilia and H/L ratios in green sea turtles (Aguirre et al., 1995), in alligators (Morici et al., 1997) and in other vertebrates, in neutrophilia (Davis et al., 2008). These authors highlight the importance of studying the close relationship between stress hormones and H/L ratios in hematological assessments of stress.

Nevertheless, increased levels of corticosterone may cause the opposite effect on other components of the immune system. Thus, as previously mentioned, in non-mammalian tetrapods, glucocorticoids could also establish a close endocrine link between immunocompetence and stress (Apanius, 1998). According to this, the increase of corticosterone in our exposure lizards could be the cause of NAbs decrease, just like in TBWCC. Beside, chronic exposure to sublethal concentrations of toxins leading to structural and functional changes to immune system can result in immunosuppression that reduces resistance of the host (Browne, 2004; Crawshaw and Weinkle, 2000).

In summary, pollutants can cause immunostimulation (sometimes with subsequent hypersensitivity), but more commonly lead to immunosuppression, which increases susceptibility to infectious diseases (Bigazzi, 1996; Gardner and Zelikoff, 1996). Berger et al. (2005) suggest that temporarily elevated plasma glucocorticoids may be immune enhancing, whereas chronically elevated concentrations may be immunosuppressive. In this study, where lizards were exposed to pesticides in sub-chronic condition, we have possibly found two opposite effects according to the kind of parameter analyzed. Considering the high complexity of the immune system, more conclusive results can be obtained when the number of parameters assessed is high.

Our results also showed that the plasma corticosterone level was negatively correlated with body mass (weight and snout-vent length). Morici et al. (1997) reported a similar negative correlation in *A. mississippiensis*. Based on these results, it is reasonable to conclude that any external stressor which produces an increase in corticosterone (such as pesticides exposure), may lead to a suppression of growth.

Overall, these results provide evidence of the potential immunotoxic effects of pesticides, but also about a trade-off between corticosterone levels and immune parameters as well as between fluctuations in this hormone and growth. This supports the hypothesis of an energetic cost associated with the maintenance of the immune system (Viney et al., 2005). In this way, when stressors such as pesticides affect corticosterone concentration, some other parameters could be being affected too. The latter shows the importance of measuring levels of this hormone as a complement to the immunological parameters to assess toxicity, stress, and immunosuppression caused by the formulated products used in crops. Considering our results, we can conclude that pesticide formulated products applied in the areas occupied by *S. merianae*, could be affecting their immune and endocrine systems and its ability to defend himself against external agents. In addition, it constitutes an estimation of what would be happening in the tegu lizard populations continuously exposed in natural environments contaminated with agricultural pesticides.

Studies like this allow broadening the knowledge about immune and endocrine effects of pesticides on *S. merianae* and need to be continued. In this way, use of ecotoxicology and biomarker tests and their application in ecological risk assessments have some way to go to provide a higher level of cost-effective environmental protection (Eason and O'Halloran, 2002).

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Declarations of interest

None.

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