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Immunotoxicity of commercial-mixed glyphosate in broad snouted caiman (*Caiman latirostris*)

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ABSTRACT

The expansion and intensification of agriculture during the past 50 years is unprecedented, and thus environmental problems have been triggered at different scales. These transformations have caused the loss of habitat and biodiversity, and disruption of the structure and functioning of ecosystems. As a result of the expansion of the agricultural frontier in the recent past, many areas of the natural geographic distribution of the local wildlife, among them crocodilians and particularly the broad snouted caiman (*Caiman latirostris*), are being exposed to contaminants.

The present study was designed to evaluate the effect of commercially-mixed glyphosate (RU) on some parameters of the immune system of *C. latirostris*. Two groups of caimans were exposed for two months to different concentrations of RU recommended for its application in the field, while one group was maintained as an unexposed control. The RU concentration was progressively decreased through the exposure period to simulate glyphosate degradation in water. After exposure, total and differential white blood cell (WBC), and complement system activity (CS) were determined. In addition, the animals were injected with a solution of lipopolysaccharide (LPS) from *Escherichia coli* to trigger an immune response and evaluate the parameters associated with it.

The results showed that an effect of the herbicide on CS was observed, as animals exposed to RU showed a lower CS activity than animals from the negative control (NC) but not in total WBC. In the case of leukocyte population counts, differences were only found for heterophils and lymphocytes.

1. Introduction

All organisms, from protozoa to humans, probably resolved extinction risks involving immune defense strategies that ensure the ability to react against foreign molecules and microorganisms, or any attempt to change the homeostatic balance [3]. The immune system (IS) in vertebrates is a complex network of organs, tissues, cells, and circulating molecules. All multicellular organisms have some kind of innate immunity, ranging from small antimicrobial peptides to phagocytic cells [4]. The complement system (CS) is an important part of the innate immune system in both invertebrates and vertebrates, and can be sequentially activated in a cascade reaction by numerous steps and by different routes. The CS in fish and other poikilotherms appears to provide a quick, strong and diverse innate immune response. It is structurally and functionally more diverse than that of higher vertebrates because some of its components have multiple isoforms and this feature is critical for the survival of these species. Some studies have reported the presence

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Abbreviations

IS	Immune system
CS	Complement system
NC	Negative control
RU	Round up®
SS	Saline solution
LPS	Lipopolysaccharide
WBC	White blood cells
SRBC	Sheep red blood cells
MH	Maximum hemolysis
SE	Standard error
TLC	Total leukocyte count
DLC	Differential leukocyte count
GFT	Glyphosate

of CS in a variety of reptiles [5]. Merchant et al. [6] detected CS in the blood of the American alligator (*Alligator mississippiensis*), Merchant and Britton [7] in freshwater (*Crocodylus johnstoni*) and saltwater crocodiles (*Crocodylus porosus*) and Siroski et al. [8] reported similar results in broad snouted caiman (*Caiman latirostris*).

Increased knowledge concerning the crocodilian immune system may allow future consideration as an alternative for the production of drugs, the generation of biological models, the production of nutritional supplements and application of resources to detect and/or disolve environmental problems.

Many substances introduced into the environment by human activities can disturb metabolic systems in animals and humans. Among them, there are persistent, bioaccumulative organohalogens that include some pesticides (fungicides, herbicides and insecticides) and industrial chemicals wastes. The consequences of the alterations produced can be transcendent due to the key role they can play in different biological processes. Agro-industrial activities and urban centers generate waste materials that pollute wetlands and rivers, affecting the health of wildlife and ecosystems [9].

The expansion and intensification of agriculture during the past 50 years is unprecedented, and thus, environmental problems have been triggered at different scales, constituting the most obvious manifestation of human activity. Such transformations of have caused the loss of habitat and biodiversity, disruption of the structure and functioning of ecosystems and the decrease of their abilities to supply vital resources. One of the most prominent examples is the result of the explosion in the use of transgenic soy and implementation of new technologies in tropical and subtropical regions [9,10]. The causes of this expansion can be found in the growing prices of soybean in the highly profitable international market, and the high yields of the genetically modified varieties adapted to less favorable soil and climatic conditions, short rotation times and lower costs by the implementation of non-tillage systems [11].

In Argentina, major threats to biodiversity include deforestation and the draining of marshes in order to allocate more land to agriculture, particularly to soybean crops [12]. This current agricultural model is directly associated with a high usage of pesticide formulations. Generally, pesticides are marketed as formulated complex, and not simple substances. Commercially-mixed varieties include the active substance (a substance that has the property of killing the plague) with other ingredients called adjuvants or surfactants, which function to facilitate the application of the product and increase the effectiveness of the active ingredient. Surfactants and/or adjuvants are present in high percentages in some formulations and are considered inert ingredients; although in many cases exceed the toxicity of the active ingredient [1,2].

The most widely used herbicide in the world is glyphosate (N-phosphonomethyl glycine). This herbicide is a systemic, broad-spectrum herbicide widely used in agriculture and forestry, aquatic environments and gardens in the timber industry, for non-selective control of annual and perennial weeds, grasses and broadleaf plants [13,14]. Its mechanism of action is mediated *via* the alteration of aromatic amino acid biosynthesis. It is an inhibitor of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase, which catalyzes the formation of a precursor for the biosynthesis of three essential amino acids: tryptophan, phenylalanine and tyrosine. This pathway is present in higher plants and some microorganisms but not in animals [15,16].

A significant portion of the pesticides applied in agriculture dissipates into the environment by drift, runoff and leaching, and thus affects wild flora and fauna populations of the surrounding natural areas and causes serious problems to human health [17,18]. Chronic exposure of organisms to pesticides at low concentrations can have cumulative deleterious effects, interfere with development and growth, alter physiological and hematological parameters, suppress immune function and cause genetic instability of organisms that live in environments surrounding crops [19,20].

As a result of the expansion of the agricultural frontier in the recent past, many areas of the natural geographic distribution of the local wildlife, among them crocodilians and particularly the broad snouted caiman (*C. latirostris*), are being exposed to contaminants. Juveniles and adults may be exposed through food, water and sediments presented in the natural environment where they live. In addition, pollutants accumulated in the mother can reach the embryo through the yolk, also affecting embryonic development *in ovo* [21]. Frequently, female caiman build nests near bodies of water adjacent to crops. For this reason, embryos are exposed to pesticides used on these crops. Embryos and hatchlings may be exposed to such compounds spanning eggshell from the atmosphere during incubation or after hatching. The period of maximum pesticide application coincides with the breeding season of this species (November to March), posing a serious contamination risk for developing embryos and neonates [22].

The field of immunotoxicology in wildlife is relatively new, and in reptiles it is very limited. Some studies with wild birds and mammals have provided a basis for the development of this area of study. Results from these studies have demonstrated the implications that depressive effects on the immune system caused by toxic releases to the environment, either voluntarily or involuntarily, have at the population level [23–25]. Other reports confirm suspicions that exposure to major environmental pollutant concentrations can suppress immune function in wildlife and may lead to decreased host resistance, increased susceptibility to disease, increased mortality and therefore, a reduction in the size of the populations [26]. Most of the immunotoxic effects reported at different levels are primarily oriented on adaptive immunity; however, few studies have been conducted to identify the effects of these substances on innate immunity, and even fewer have focused on the CS.

Some authors report the direct activation of the CS cascade after *in vitro* exposure to hexachlorobenzene (compound organochlorine) and malathion (organophosphorus compound) in human serum. With both compounds the activation of the CS cascade was detected although the mechanisms by which this activation occurred could not be elucidated [27]. Other preliminary study revealed similar findings against DDT and endosulfan [28] which

concluded that the CS is a focus of interaction between insecticides and the IS. Considering the extremely important role of innate immunity in reptiles and its possible alteration as a cause of increasing release of toxic substances to the environment, this issue has not been analyzed yet with the concern it deserves.

To assess the potential effects of herbicide on the immune response, we conducted an *in vivo* study during which *C. latirostris* hatchlings were exposed to subchronic concentrations of 66,2% Glyphosate [GFT] commercial formulation under laboratory conditions. After the exposure, the animals were injected with a solution of lipopolysaccharide (LPS) from *Escherichia coli* to trigger an immune response and evaluate the parameters associated with it.

2. Materials and methods

2.1. Animals

All animals in this study were treated in accordance with the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals, using non-invasive techniques of blood collection and minimizing stress and suffering by suitable management methods. The study was evaluated and approved by the Institutional Committee of Animal Use and Care (Faculty of Veterinary Sciences, National University of Litoral, 069/2010).

For this study, 72 *C. latirostris* of approximately 20 days old were used. They were hatched from eggs harvested from three different nests (24 caiman per nest) in the Natural Managed Reserve "El Fisco" (30°11′ 26″S, 61° 0′27″O; San Cristobal, Santa Fe, Argentina), under the 'Proyecto Yacaré' ranching program. This area was selected because it is a Protected Natural Area (Regional Law 12,930/2008), situated at least 20 km away from any pesticide application area or other industrial contaminant activity.

2.2. Experimental protocol

Animals were randomly distributed into three experimental groups (n = 24) divided in 2 replicates each (n = 12). Treatments were: negative control (NC, without exposure), and two treatments exposed to different concentrations of Roundup® (RU). The concentrations of RU chosen were based on the concentration recommended for application in crops (i.e. 2%/ha) and considering the surface of the plastic container base (0.2622 m^2) as the reference area for calculation of the volume of RU to be added to each pen (in a fixed water volume of 5 L), and then doubling this value (RU1 and RU2, respectively). A subchronic exposure (70 days) was performed by immersion in the plastic containers (75 cm long, 35 cm wide, and 37 cm high), tilted to provide 60% dry and 40% tap water surface areas, with a maximum water depth of approximately 15 cm.

Temperature in the containers was maintained at 30 ± 2 °C and monitored with Hobo™ data logger (Onset Computer Corp., Pocasset, MA, USA). All animals were individually marked with foot webbing tags (Monel Natl Band and Tag CO., Newport, Kentucky). Animals were feed with minced chicken head offered *ad libitum* three times a week. Water was renewed every 2 days and the concentration of RU was progressively decreased in each replacement over the exposure period, considering the normal degradation of glyphosate determined previously under the same conditions and concentrations used in the animal experiment. Glyphosate determination was conducted by high-performance liquid chromatography (HPLC) with pre-column derivatization using 9-fluorenylmethyl chloroformate (FMOC-Cl), as thoroughly described in Poletta et al. [29] (Fig. 1).

Through these studies we determined the duration of exposure needed to span the period over which the compound was completely degraded (approximately 2 months), as well as the progressive decreasing concentrations to be used for experimental groups in order to simulate glyphosate degradation in water at 30 ± 2 °C. In RU1, the concentration was decreased from 11 mg/L (initial concentration) to 2.5 mg/L (final concentration), and in RU2 from 21 mg/L (initial) to 5 mg/L (final).

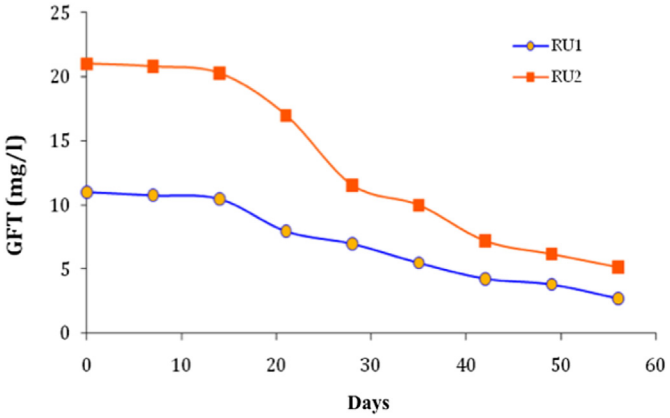

Fig. 1. Glyphosate (GFT) decrease in water analyzed by High Performance Liquid Chromatography (HPLC). Concentration was measured by triplicate and shows progressive GFT (mean ± standard deviation) decrease trough time.

Two days after the end of the exposure period, half of the animals of each experimental group were injected intraperitoneally with a solution of LPS derived from *E. coli* K-235 (Sigma) at a dose of 1 mg/kg [30], while the other half with saline solution (SS) as a control (Table 1). The animals were then maintained for 72 h under the same conditions in which the experiment was conducted, until blood collection. Blood samples were collected from the spinal vein of all hosts as previously described [31] using heparinized sterile syringes fitted with a 25-Gauge needle. This was not done at the beginning of the experiment to avoid any risk of death of the caimans due to their small initial size.

Samples of whole blood were used to measure white blood cells counts (WBC) and differential leukocyte population counts, and the rest were centrifuged and stored at −80 °C until determination of CS activity.

The determination of the total number of leukocytes was performed using a Neubauer chamber. An aliquot of whole blood was diluted 1:200 with a solution of 0.6% NaCl [32]. The NaCl solution acts as a red cell lysing agent without interfering with the integrity of the leukocytes. For the differential leukocyte count, 2 smears were made per animal, fixed with ethanol, and then stained with May Grunwald–Giemsa solution. The preparations were coded to achieve maximum objectivity in the analysis. They were observed under an optical microscope at 1000× and the amount of each leukocytes subtype was determined manually: heterophils, basophils, eosinophils, lymphocytes and monocytes; 100 leukocytes were counted and each subtype was expressed as a percentage.

Table 1
Amount of animals injected with Saline and LPS solutions post-RU *in vivo* exposure per nest and injection treatment.

Experimental group	Injection	Caimans/nest	Caimans/treatm.
NC	Saline solution	4	12
NC	LPS	4	12
RU1	Saline solution	4	12
RU1	LPS	4	12
RU2	Saline solution	4	12
RU2	LPS	4	12

Caiman CS activity was determined by the method of Sheep Red Blood Cells (SRBC) hemolysis following the protocol described by Siroski et al. [8]. The SRBC were obtained from heparinized whole blood collected from Merino sheep (*Ovis aries*). Caiman plasma was incubated with once volume of 2% SRBC (v/v) for 30 min at ambient temperature (25 °C ± 2 °C) and then centrifuged at 2500× *g* for 5 min. Subsequently, 300 uL of supernatant were taken and transferred to a microplate for measuring the optical density in a microplate reader at 540 nm (Multiskan RC Labsystem, Helsinki, Finland).

For a positive control, 2 uL of Triton X-100 was added to 1 mL of 1% SRBC and repeatedly homogenized with a tuberculin syringe, until complete hemolysis was achieved. The result obtained was considered as the maximum hemolysis (MH). All experiments were performed in quadruplicate with different plasma samples and the results of hemolysis of SRBC in each experiment were divided by the absorbance of the positive control to obtain the maximum percentage of hemolysis (% MH). The results are expressed as the mean % MH ± standard error (SE).

2.3. Statistical analyses

Data analyses were performed using the software SPSS 14.5 for Windows. Data were tested in normality by Kolmogorov–Smirnov test and homogeneity of variance by Levene test. Analysis of data from MH%, total leukocyte count (TLC) and differential leukocyte count (DLC) was performed through a two-way ANOVA considering RU exposure and LPS injection as grouping variables. When necessary a post-hoc Tukey test was used to identify differences between treatments. The relationship between MH%, TLC and DLC with growth of the animals was established by linear regressions. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

Unsensitized SRBC assays were conducted to characterize the CS of *C. latirostris* exposed to different RU concentrations and injected with LPS or SS. No statistical differences were found in %MH either between replicates of the treatments nor between nests ($p > 0.05$). In contrast, an effect of the herbicide on CS was observed, the group of animals exposed to RU2 showed a lower CS activity than animals from the NC ($p < 0.05$, Fig. 2). Activity of the caiman CS obtained from animals injected with SS was higher than those animals injected with LPS in all treatments, being statistically significant different in the case of RU2 group ($p < 0.05$). Moreover, %MH obtained from animals injected with LPS or SS had been not influenced by the previous exposure of the animals to the different concentrations of RU, that is, there was no interaction between the variables ($p > 0.05$).

The total leukocyte analyses showed that NC treatment had higher values (38,377 ± 8520 leuk/mm^3) than treatments exposed to pesticides (RU1: 34,163 ± 9862 leuk/mm^3; RU2: 36,283 ± 7321 leuk/mm^3), but these differences were not statistically significant. After injection, the number of WBC in the animals injected with LPS in NC group was higher than in animals injected with SS ($p < 0.05$). In RU1 and RU2 treatments, no differences were found between animals injected with LPS and those injected with SS (Fig. 3).

The results obtained from the differential leukocytes count were:

Heterophils: the percentage of this leukocyte population was affected by the treatment of herbicide exposure and the subsequent injection of LPS or SS ($p < 0.001$) but no interaction was detected between both variables. The groups exposed to RU2 showed that the percentage of heterophils was significantly lower to that of the

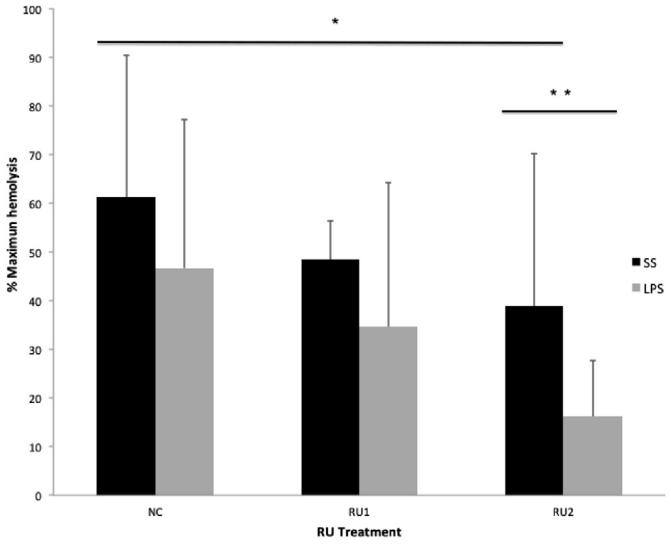

Fig. 2. Percentage of maximal hemolysis (% MH) obtained from the exposure to different concentrations of Roundup® (RU1 and RU2) and negative control (NC). Animals were subsequently injected with saline (SS) and lipopolysaccharide (LPS). The group of animals exposed to RU2 showed a lower CS activity than animals from the NC (*$p < 0.05$), and RU2 showed that LPS-injected group has a significantly lower % MH (**$p < 0.05$) compared to the group injected with SS.

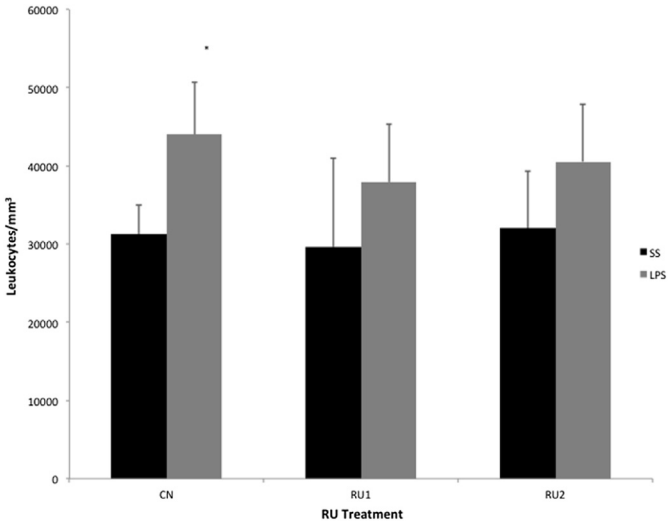

Fig. 3. Total count of leukocytes from animals exposed to different treatments with glyphosate (RU1 and RU2) and subsequently injected with lipopolysaccharide (LPS) or saline (SS). The total white cell count of animals belonging to the NC injected with LPS treatments were higher than those injected with SS (*$p < 0.05$).

NC ($p < 0.001$) and RU1 ($p < 0.05$). In the NC group, the percentage of heterophils in animals injected with LPS was significantly higher than those injected with SS ($p < 0.001$). The same result was obtained in treatments with RU1 and RU2 ($p < 0.001$; Fig. 4A).

Lymphocytes were influenced by exposure to herbicide and LPS injection, but interaction between the variables was not detected ($p > 0.05$). Contrary to that observed in heterophils, the RU2 group (61.83 ± 10.55%) showed a significantly higher number of cells than NC (47.33 ± 10.31%, $p < 0.001$) and RU1 (51.50 ± 7.77, $p < 0.01$). In turn, the number of lymphocytes showed a statistical difference ($p < 0.01$) between animals injected with LPS and SS, in the NC and RU2 treatments but not in RU1 (Fig. 4B).

Unlike heterophils and lymphocytes, herbicide exposure did not influence the percentage of monocytes ($p > 0.05$) but the effect of

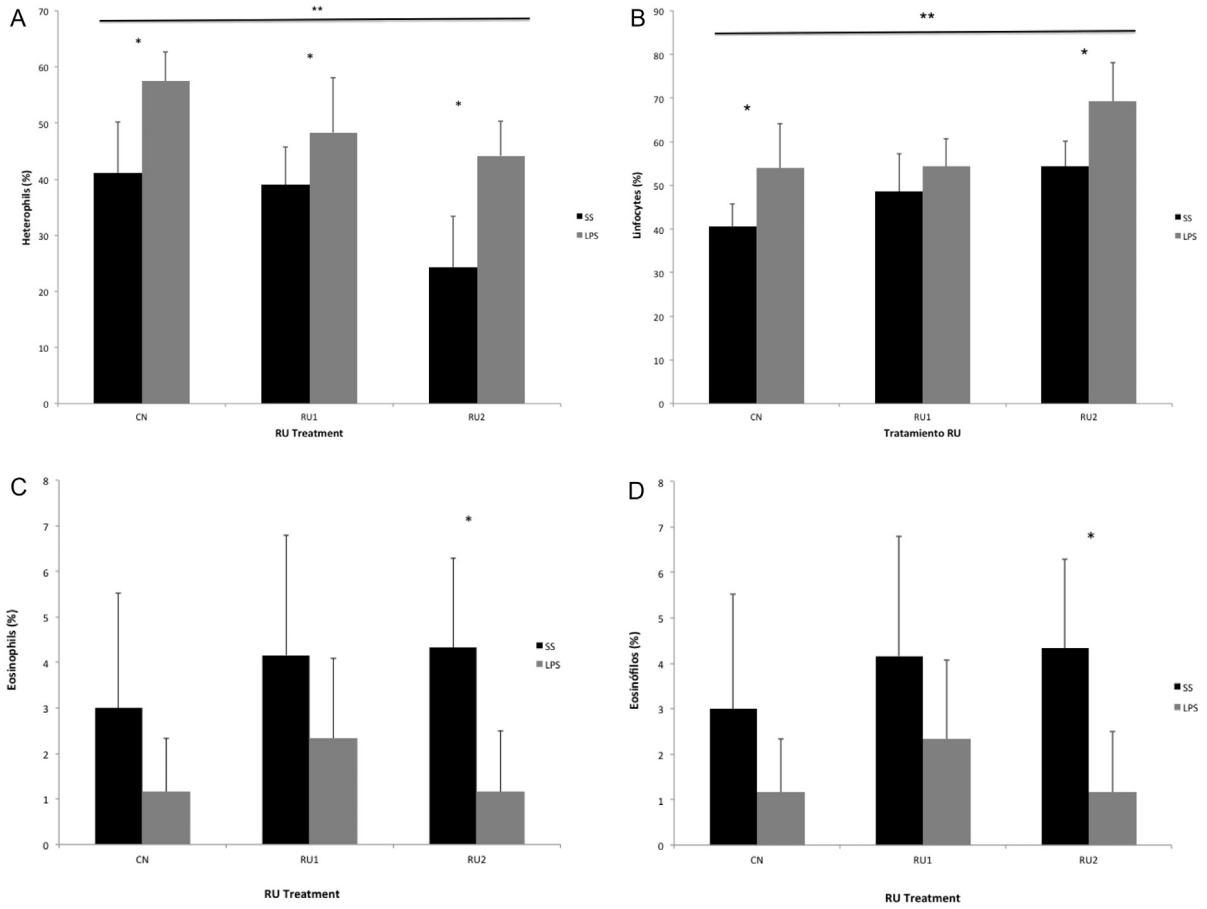

Fig. 4. The percentage of leukocytes populations under different glyphosate concentrations treatments. (A) Heterophils in different herbicide treatments was statistically higher in animals injected with lipopolysaccharide (LPS) than those with saline solution (SS) within the RU1 (*p < 0.05), RU2 (*p < 0.001) and NC (*p < 0.001) treatments. (B) The percentage of lymphocytes among the groups injected with LPS was lower than those injected with SF within treatments RU2 and NC (*p < 0.01). (C) It was found that the percentage of monocytes was lower in animals injected with LPS that SS in both groups of animals exposed to glyphosate (*p < 0.05). (D) The percentage of eosinophils in RU2 was higher in LPS-injected animals than in those injected with SS (*p < 0.01).

LPS injection was detected. This difference was evident only on RU1 ($p < 0.05$) and RU2 treatments ($p < 0.05$) but not on the NC (Fig. 4C).

As with the previous group, there was no effect of herbicide exposure or interaction between the variables on the percentage of eosinophils ($p > 0.05$); only an influence of LPS injection was detected. This difference was evident in RU2 ($p < 0.01$) but not in RU1 ($p > 0.05$) or NC ($p > 0.05$) (Fig. 4D).

Basophils count could not be included in the analysis because the percentage observed in most cases was 0.

4. Discussion

Immune function is an excellent indicator of the health of an organism. Recent studies have shown that some pesticides are immunotoxic as they alter the normal structure and reduce the immune system's response to antigens and infectious agents, increasing disease in exposed organisms [33–35]. Crocodilians are daily exposed to infection by potential pathogens. Despite the constant threat posed by microorganisms in the environment in which they live, these animals do not often show signs of infection [36–38]. This could be possibly because they have evolved and diversified immune responses to many factors that are directly associated with their environment.

Thus, caiman exposure to certain pesticides, whether acute or chronic, could affect both innate and acquired immunity. In addition, low levels of contaminants can cause immunotoxicity and concentrations 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 [39].

In the present study, we determined that different RU concentrations of herbicide negatively affect the activity of the CS in *C. latirostris*. The RU concentrations included in our experimental design aim to detect dose–response relationship on caimans *in vivo*, thus reflecting GFT concentrations in water bodies reported in previous environmental studies. Temporal variations of GFT levels in the environment depend on the time of application and the immediate precipitation and may reach higher concentrations in small water bodies in nature [18,40]. The maximum GFT concentration expected in small bodies of shallow water can reach values between 2.9 and 7.6 mg acid equivalent (a.e.)/L [41–44]. The two ranges of concentrations employed, from the beginning to end of our experimental design, cover the whole range mentioned. The highest concentration of herbicide (5–21 mg/L) showed a strong inhibition of SRBC hemolysis. White and Anderson [45] report similar results when mice exposed orally and subchronically to pentachlorophenol (antimicrobial used for wood preservation) found to exhibited a deleterious dose-dependent effect on CS activation.

In general, studies examining the effect of pollutant exposure on the immune system parameters are carried out in humans and

under different conditions. One of them is a study conducted in workers exposed to chlorinated contaminants, which reported an increase in IgG and reduced IgM and complement C3 protein concentration compared with controls [46]. Similarly, another study with workers exposed to pyrethroid pesticides reported abnormalities in the complement C4 protein but not IgG, IgA, IgM and C3 [47].

In our study, all animals were injected with LPS and SS after the exposure to pesticides. Merchant et al. [48] reported that *A. mississippiensis* responded immunologically to bacterial LPS injection with an increase in the total leukocytes. These results were consistent with those shown in the turtle *Terrapene carolina* [49] and in the cururu toad, *Bufo paracnemis* [50]. Our results showed that after RU exposure, the hemolytic activity exhibited by the animals injected with LPS were lower than in those injected with SS. In this case, we might assume that exposure to pesticides would exert a suppressive effect on the immune response to CS level at high concentrations of RU. This effect was only observed when LPS challenged the system. On the other hand, control animals injected with SS presented a low %MH compared to similar studies conducted in this species [8], so the stress caused in the animals by experimental conditions and injection could be a negative influence on the activity of the CS.

Quantitative and morphological leukocytes determinations of the leukocytes in peripheral blood were included in most immunotoxicity evaluations [51]. These traditional hematological tests can provide information about the general stress of an individual, where an increased heterophil/lymphocyte ratio is used as an index of stress in reptiles [52,53] and it was associated with different diseases [54–56]. Total leukocyte counts from animals exposed and not exposed to pesticides were similar to those found in *A. mississippiensis* [48] and *Cr. porosus* [57] but higher than those reported for the same species and *Caiman yacare* in captivity [58]. It is possible that animals being under captivity for long periods of time could be suffering an immunosuppressed state caused by chronic stress. Within the control group, as was expected after the injection, an increase was observed in the total leukocyte counts in the animals that received the dose of LPS. In contrast, animals exposed to pesticides showed no differences in the response to injection of LPS or SS. The suppression in response to CS stimulation was also observed in turtles exposed to organochlorine contaminants [59].

Each leukocyte population showed a different behavior. Heterophils displayed a low response in RU2 treatment compared to the control, but in all groups greater values were detected in response to stimulation with LPS. These results demonstrated the suppressive effect of pesticides on heterophils, this being one of the most affected populations with a significant intervention in the specific immune response. The high percentages of heterophils in controls are consistent with those reported by Merchant et al. [30].

Conversely, the percentage of lymphocytes of animals exposed to the highest concentration of RU was higher than in the other treatments. The values were similar or lower than that reported in the same species without stimulation [58]. This is coincident with that observed by Merchant et al. [48], who reported that the total lymphocyte population was one of the least affected by LPS stimulation.

Both monocytes and eosinophils were influenced by exposure to LPS injection. In this case, the exposed animals in all groups had lower values than those injected with SF, suggesting that the stress caused by the handling of the animals could be thus decreasing resistance to bacterial component.

Based on the demonstrated importance for innate immunity in reptiles, and the outstanding features of the species as a bioindicator, it is striking this has not been used as a frequent tool in studies of immunotoxicity. This study extends to the few ones carried out in relation to the function of the CS after exposure to chemicals considered to be contaminants. As of yet, no evaluation has been made on the *in vivo* effect of chemicals, particularly pesticides, on the CS or other components of innate or acquired immunity in reptiles.

The immune system is sensitive to changes caused by environmental pollutants and the suppression of immune function can lead to increased risk of diseases in reptiles [60]. There is a great need to understand the effects of environmental contaminants on the health immunity and, ultimately, survival of reptiles. An integrative approach to combine the analysis of markers of exposure and effect will contribute to a better understanding of the mechanisms associated with the observed damage, thereby providing better prediction for environmental risks. Therefore, conducting studies on the effects of pesticide exposure in *C. latirostris* is of particular interest not only to assess the impact on caiman populations, but also to promote the characterization of this species as a sentinel of ecosystem health, which could allow the detection of regions with high pollution.

Crocodilians represent an extremely successful group of organisms that have changed little for millions of years. They are excellent sources of important information for those who investigate breakthroughs in immunology from a phylogenetic viewpoint. Their capacity to resist the attack of microorganisms could be one of the reasons of their longevity but the non-rational use of pesticides and other anthropic activities could put on risk their survival, compromising their abilities to avoid some infections.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cbi.2015.11.031.

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