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RESEARCH ARTICLE

Effects of *in vivo* exposure to Roundup® on immune system of *Caiman latirostris*

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Abstract

The present study aimed to evaluate the effect of Roundup® (RU, glyphosate-based formulation) on some parameters of the immune system and growth of *Caiman latirostris*. Seventy-two caimans (20-day-old) from *Proyecto Yacaré* (Gob. Santa Fe/MUPCN) were used. Two groups were exposed for 2 months to different concentrations of RU (11 or 21 mg/L; taking into account the concentration recommended for its application in the field), while one group was maintained as control. The RU concentration was progressively decreased through the exposure period to simulate glyphosate degradation in water. Animals were measured and weighed at the beginning and end of the experiment, and blood samples taken after exposure to determine total and differential white blood cell (WBC) counts as well as total protein concentration (TPC), and for performing protein electrophoresis. The results showed that, compared against control hosts, there was a decrease in WBC counts, a higher percentage of heterophils, a higher TPC (with a low percentage of F2 protein fraction), and a negative effect on growth in the young caimans exposed to RU. These results demonstrate that *in vivo* exposure to RU induced alterations in the selected immune parameters, plasma proteins, and growth of caimans, thereby providing relevant information about the effects of this type of pesticide in this important species in the Argentinian wetlands.

Keywords

Broad snouted caiman, glyphosate, immune system, pesticides

History

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Introduction

During the last two decades in Argentina, there has been a constant increase in soybean production, mainly based upon economic interests as a consequence of ever-growing international demand. The area planted with soybean in this country reached 19 million hectares during the 2012–2013 season (Bolsa de Cereales de Buenos Aires, 2012). Total replacement of traditional soybean varieties by transgenic soybeans (RR) resistant to glyphosate (*N*-phosphono-methyl glycine [a non-selective herbicide against annual/perennial weeds, grasses, and broad-leaves]) resulted in widespread use of this agro-chemical to allow for weed control at any time in the crop cycle (Penna & Lema, 2002).

Soybean planting in Argentina usually begins in October, with a pre-sowing period in late September when chemical fallow is done using glyphosate/other herbicides. During the culture period, farmers apply insecticides, fungicides, and herbicides (mainly glyphosate) two or three times as necessary. Harvest is done during March–May of the following year. Recommended concentrations for glyphosate application on soybean RR are 1.5–2.5 L/ha (CASAFE, 2010). Because of its wide usage, concerns about contamination of the environment by this agent are well founded. Recent studies made in the Pampas of Argentina revealed glyphosate residues in surface water after spraying, mainly as consequence of drift and run-off (Peruzzo et al., 2008). Further, there has also been great concern about the potential persistence of glyphosate in the environment. Initially, it was reported that the herbicide had a short half-life due to rapid metabolism by micro-organisms; however, other studies claimed it could persist up to 170 days in the environment, with an estimated average of 47 days in soil and from 12 days to 10 weeks in water (EXTOXNET, 1996).

The constant expansion of agricultural frontiers onto natural ecosystems resulted in many areas of the natural domain of the broad-snouted caiman (*Caiman latirostris*) to become closer to areas with high agricultural activity. As a result, wild populations of this species have become continuously exposed to pesticides discharge, particularly glyphosate formulations such as Roundup® (RU). Among the possible effects that could result from repeated *in vivo* exposure of *C. latirostris* to pesticides are alterations on the immune system (IS), considering that this function is particularly vulnerable to numerous xenobiotics in many species of animals.

All organisms, from those very simple, even unicellular, to those with higher levels of complexity, have inherent mechanisms to defend against attack by pathogens. The first defense

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mechanisms work by preventing entry of pathogens; these utilize both physical and chemical barriers. All animals also have a second level of complex mechanisms that form the innate or non-specific immune system (IIS). Only vertebrates have a third level of complexity, i.e. adaptive or specific immune system (AIS), which comes into play when innate immunity is not precise enough to a particular challenge or in response to recurrences (Collado et al., 2008).

One of the cellular components that form part of IS are white blood cells (WBC). WBC are involved in a significant amount of processes in both systems. In certain situations, these processes lead to increases or decreases in the values of select blood components; in turn, these are used for interpretation of physiological phenomena and for diagnosis of diseases/nutritional status (González Fernández, 2003).

Different studies have shown that many pesticides are immunotoxic as they can alter the structure of some components of the immune system and, in turn, generate a lower host resistance to antigens/infectious agents (Christin et al., 2003; Dorucu & Girgin, 2001; Mansour, 2004). A previous study demonstrated that newborn *Caiman latirostris* exposed to glyphosate (Roundup®) showed a decrease in the response of their complement system (CS) and altered leukocyte counts. Therefore, the activity of the caiman CS can be used as an indicator of toxicities induced by pesticides and, potentially, by other environmental factors (Siroski, 2011).

As noted above, *C. latirostris* (broad-snouted caiman) is one (of two) species of crocodilians living in Argentina, distributed in the provinces of Chaco, Corrientes, Formosa, Salta, Santa Fe, Entre Rios, Misiones, Santiago del Estero, and Jujuy (Larriera et al., 2008). Studies on physiological processes in crocodilian blood are scarce, especially those evaluating the influence of external factors on the IS. The objectives of this study were to evaluate the potential effect of RU on select IS parameters in order to generate information about the possible immunotoxic impact of this pesticide on a representative species of wetlands.

Materials and methods

Animals

All animals in these studies were treated in accordance with the *Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals* (National Scientific and Technical Research Council, 2005), using non-invasive techniques of blood collection and minimizing stress and suffering by suitable management methods.

Seventy-two caimans (20-days-old), coming from three clutches collected by *Proyecto Yacaré* (Gob. Santa Fe/MUPCN) ranching activities were used. *Caiman latirostris* eggs were collected from Fisco field (30°11′26″ S; 61°0′27″ W), in Santa Fe Province, Argentina, an area free of farming and urban activities and which falls within the natural distribution range of this species. This area was selected in order to ensure that eggs had not been environmentally exposed to any xenobiotic, as no activity with a potential associated contamination risk is carried out there (Poletta et al., 2009). The experiment lasted 2 months and was conducted in a temperature-controlled environment (30 ± 1°C). Food was offered *ad libitum* three times a week with a mixture of 50% minced chicken head and 50% dry pellets (Larriera et al., 2008).

Experimental protocol

The animals of each clutch ($n = 24$) were randomly divided into three different groups: a negative control group (NC) and two treatments exposed to different concentrations of RU, each one with two replicates of 12 animals (Table 1). Replicas were used to obtain a higher number of animals in each experimental group, as crowding of the caimans can cause high level of stress that leads to negative effects on growth and other physiological parameters (Poletta et al., 2008). Moreover, replicas allow us to accommodate a situation wherein, if any problem occurs during the experiment, not all data for an experimental group is lost. As we found no differences between the two replicas in any of the experimental groups for any of the variables tested, all results are reported as means (±SE) per experimental group.

Animals were measured in snout-vent length (SVL) and weighed at the beginning and end of the experiment. They were exposed during 2 months in plastic pens inclined to offer a dry area and one containing water or RU solution. Pen cleaning was done every 2 days to ensure sanitary conditions.

The RU concentrations used in these studies were chosen to take into account the concentration recommended for product application in crops (i.e. 2%/ha) as well as the area of the pen base (as the reference area for calculation of the volume of RU to be added to each pen in a fixed water volume [5 L]), and then doubling this value (Treatments 1 and 2, respectively). Water was renewed every 2 days and the concentration of RU was progressively decreased in each replacement over the exposure period to take into account the normal degradation of glyphosate; precise values used were based on pilot studies and measures of glyphosate by HPLC, under the same conditions and concentrations used in the animal experiment. Through that pilot study, both the duration of exposure needed to span the period over which the compound was completely degraded (2 months), as well as the progressive decreasing concentrations to be used for experimental groups, were determined. Ultimately, the ranges of exposure concentrations employed herein were as follows: Treatment 1 (RU1) = 11.0 mg/L (initial) down to 2.6 mg/L (final concentration) and Treatment 2 (RU2) = 21 mg/L (initial) down to 5 mg/L (final) (Table 1).

At the end of the experiment (i.e. on Day 60 of regimen), all caimans were placed in pens with only clean water. During the next day (i.e. Day 61), blood samples were taken from the spinal vein of all hosts according to the method of Olson et al. (1977) 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 initial small size.

A WBC count on the drawn blood was performed using a Neubauer chamber. Another aliquot of the blood was diluted with 0.6% NaCl at a ratio of 1:200 and then analyzed using a light microscope at 400× and results expressed as total cells/mm^3 blood (Lewis et al., 2008). To perform differentials, two blood smears were prepared per animal, fixed with ethanol, and then stained with May Grünwald–Giemsa solution. Amounts of each

Table 1. Distribution of *C. latirostris* hatchlings, experimental groups and treatments applied in the *in vivo* exposure assay to Roundup® (RU).

Experimental group	Component	Initial conc.	Final conc.	Hatchlings/nests	*n*
Negative control	Tap water	—	—	8	24
RU1	RU	11 mg/L	2.6 mg/L	8	24
RU2	RU	21 mg/L	5.0 mg/L	8	24

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immune cell sub-type (e.g. heterophils, basophils, eosinophils, lymphocytes, monocytes) per 100 WBC analyzed was determined using a light microscope at 1000× magnification. The heterophil/lymphocyte index (H/L) was subsequently calculated and used as a marker of any stress related to the exposures.

From the remaining blood sample collected, plasma was isolated using standard protocols. Thereafter, plasma total protein (TP) and the proportions of each plasma protein fraction (Díaz Portillo et al., 1996) were estimated using a Genio S automatic electrophoresis system (Interlab Srl, Roma, Italy) and cellulose acetate templates to perform the high resolution process.

Statistical analysis

Data were tested for normality using the Kolmogorov–Smirnov test and homogeneity of variances using the Levene test. Differences among nests for all variables were analyzed using ANOVA. To determine differences among experimental groups, data for total WBC count, differential count of monocytes and eosinophils, protein fractions, and increase in weight (final − initial values) were also analyzed by ANOVA. Heterophils and lymphocytes counts, H/L ratios, total protein values, and increase in SVL (final − initial values) were analyzed using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney. In these cases, a Bonferroni correction was applied according to the number of analysis by pairs carried out; here, a p-value < 0.016 was considered statistically significant.

Results

The results presented in Figure 1 show that total WBC count was lower in the caimans exposed to RU. Those exposed to RU2 had the lowest WBC count (20 282 [±2302] WBC/mm^3) compared with the values for NC (29 142 [±1882] WBC/mm^3, $p = 0.011$) and RU1 animals (28 937 [±1949] WBC/mm^3, $p = 0.01$). The results also showed that the relative population of heterophils in animals exposed to RU2 (28.24 [±1.54]%) was higher compared to values in the NC animals (19.46 [±1.68]%, $p < 0.001$). No significant differences were observed between RU1 (24.77 [±2.35]%) caimans and those in either of the other experimental groups ($p > 0.016$; Figure 2). In the case of lymphocytes, monocytes and eosinophils, no significant differences were observed among the experimental groups. Calculated H/L indices were higher in animals that were in the RU1 (0.47 ± 0.05) and RU2 (0.45 ± 0.03) treatment group as compared to indices for the NC animals (0.30 ± 0.03, $p < 0.016$); however, no significant differences were observed between the RU1 and RU2 caimans (Figure 3).

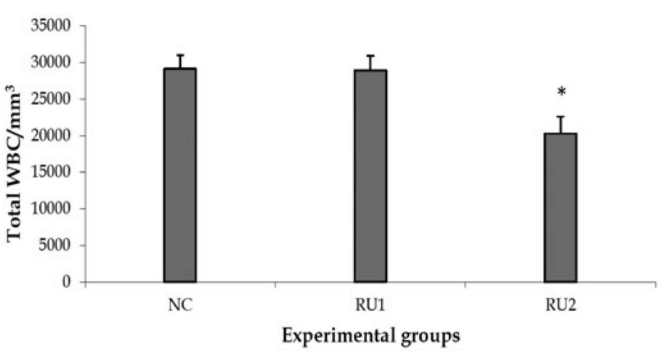

Figure 1. White blood cell count. NC (negative control); RU1 (treatment 1: 11.0 mg/L down to 2.6 mg/L) and RU2 (treatment 2: 21 mg/L down to 5 mg/L): groups exposed to different levels of Roundup®. Values shown are mean (±SE) total cells/mm^3 from 24 caiman/group. *Value significantly different from NC (ANOVA-Tukey).

The total protein concentration was significantly higher in RU1 caimans compared to those in the NC hosts ($p = 0.009$); in contrast, there were no differences noted in this parameter between the RU2 caimans and those in either the NC or RU1 groups ($p > 0.016$). The analysis of protein fractions revealed there was a significant difference in composition between the samples from the caimans in the RU2 and the NC groups, but only in the case of the F2 fraction ($p = 0.026$; Table 2).

Caimans exposed to the highest concentration of RU (i.e. RU2) showed less growth (reflected in SVL) than their NC counterparts (2.80 [±0.36] versus 4.17 [±0.29] cm, $p = 0.006$). There were no significant differences in this end-point noted among the RU1 caimans (4.06 [±0.19] cm) when compared with values in the other experimental groups ($p > 0.016$ in both cases; Figure 4). In the case of body weight, animals in the RU2 group grew less (54.13 [±6.80] g) than did the NC (69.44 [±6.02] g) and RU1 (82.16 [±5.11] g) hosts, although the differences were not significant.

Discussion

Crocodilians, particularly due to their occurrence in a wide variety of habitats, their position at a higher level of the food chain, permanence in aquatic bodies, wide geographical distribution, longevity, and fairly restricted home ranges, are especially vulnerable to pesticide exposure (Campbell, 2003; Beldoménico et al., 2007). In comparison with other vertebrate groups,

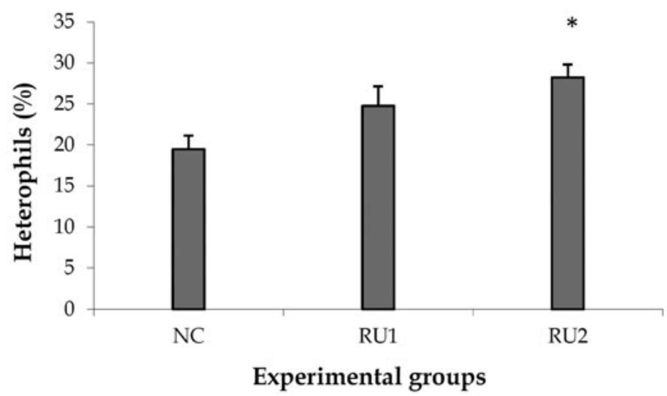

Figure 2. Percentage of heterophils. NC (negative control); RU1 (treatment 1: 11.0 mg/L down to 2.6 mg/L) and RU2 (treatment 2: 21 mg/L down to 5 mg/L): groups exposed to different levels of Roundup®. Values shown are mean (±SE) percentages from 24 caiman/group. *Value significantly different from NC (Kruskal Wallis – Mann Whitney).

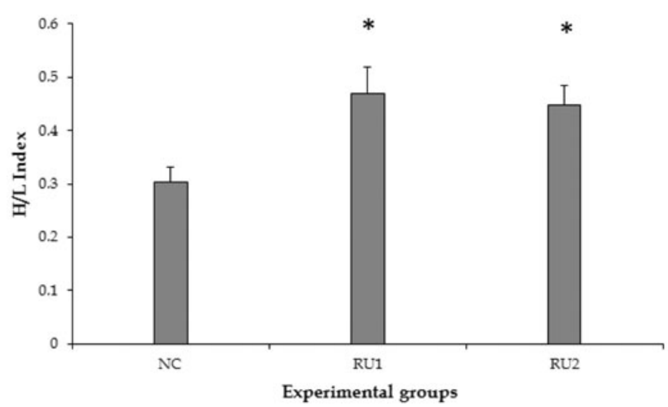

Figure 3. Heterophil/lymphocyte index of caimans in the different exposure regimens. Values shown are mean (±SE) from 24 caiman/group. *Value significantly different from NC (Kruskal Wallis – Mann Whitney).

Table 2. Total protein (TP) and protein fractions (F1–F6) levels in the serum of caimans in the different experimental groups.

	NC	RU1	RU2
TP	3.89 ± 0.05	4.17 ± 0.10*	4.04 ± 0.13
F1	28.33 ± 0.60	28.73 ± 0.61	28.5 ± 0.76
F2	3.67 ± 0.46	2.69 ± 0.42	2.13 ± 0.24*
F3	18.12 ± 0.90	18.82 ± 0.56	18.07 ± 0.40
F4	21.22 ± 0.42	20.95 ± 0.31	20.65 ± 0.35
F5	18.54 ± 0.42	18.4 ± 0.44	18.98 ± 0.39
F6	10.11 ± 0.67	10.41 ± 0.61	10.88 ± 0.92

TP values shown are means (±SE; $n = 24$/group) in units of g/dl. F1–F6 protein fractions are presented as percentages of total protein (TP). *Value significantly different from that of the NC hosts at $p < 0.05$.

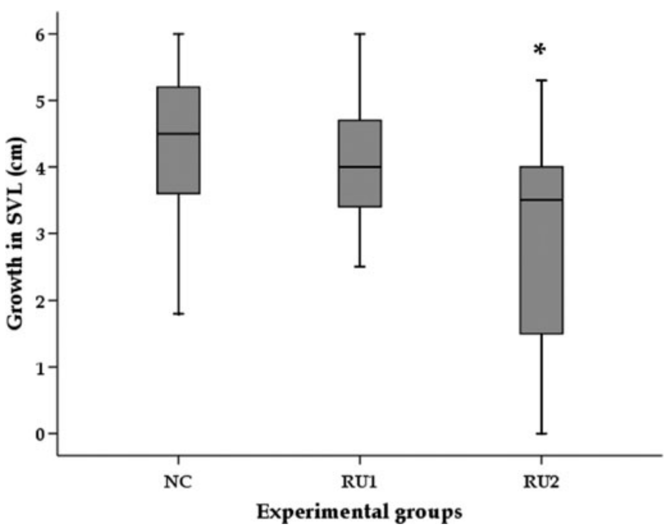

Figure 4. Growth in snout-vent length (SVL, final – initial length) observed among the caimans in the different experimental groups. NC (negative control); RU1 (Treatment 1: 11.0 mg Roundup®/L down to 2.6 mg Roundup®/L); RU2 (Treatment 2: 21 mg Roundup®/L down to 5 mg Roundup®/L). Values are presented in a box-plot showing median, quartiles, and lowest and highest values (range-error bars) from 24 caiman/group. *Value significantly different from NC (Kruskal Wallis – Mann Whitney).

information available on the toxicity of glyphosate and its formulations on reptiles is extremely scarce.

Hematologic investigations are important to wildlife because they can infer the health state of the populations, giving valuable information in relation to the immune system (Gilbertson et al., 2003). The reptilian immune response is profoundly affected by ecological factors, including population dynamics, stress, nutritional state, environmental temperature, seasonal variations, age, and infectious pathogens. The dramatic effects of seasonal changes and related steroid fluctuations have been the topic of many studies on the reptilian immune system (Zapata et al., 1992).

Leukocytes (or white blood cells) are important mediators of the immune system, carrying out the tasks of sensing and ridding the body of pathogens. Heterophils are the functional equivalent of mammalian neutrophils and, as such, act as phagocytes. However, unlike mammalian neutrophils, crocodilian heterophils stain eosinophilic, possessing fusiform-shaped eosinophilic cytoplasmic granules (Jacobson, 2007; Maxwell & Robertson 1998). Heterophils comprise >50% of the circulating leukocytes in *Alligator mississippiensis*, although quantitative differences may arise due to other factors, such as infection, seasonality, and age, among others. Similar to mammalian neutrophils, heterophils are among the first cells to arrive at the site of infection, followed by mononuclear phagocytes (Jacobson, 2007).

Different studies have shown the detrimental effect of glyphosate on different organisms (Glusczak et al., 2006; Modesto & Martínez 2010; Salbergo et al., 2010). In our study, exposure to RU induced a decrease in the total WBC counts in caimans, with the lowest WBC count observed at the highest RU concentration (RU2). These values were higher than those previously found in captive sub-adults of *C. latirostris* (13 700 [±2500] WBC/mm^3 [Mussart et al., 2006] and 13 240 [±3800] WBC/mm^3 [Barboza et al., 2008]), and could be due to chronic stress produced by long-term captivity in those animals or could be associated with different age of the caimans, as the only data reported is in sub-adults while in our study we used yearlings. In the same way, the percentage of heterophils found in caimans exposed to RU2 (28.24 [±1.54]%) was higher than those reported by others for sub-adults of this species (17.5 [±3.4]% [Mussart et al., 2006] and 10.2 [±5.3]% [Barboza et al., 2008]). The values of WBC counts for our study were lower than those found in *C. latirostris* (Siroski, 2011) and *A. mississippiensis* (Merchant et al., 2006); however, in both of those studies, the hosts were previously injected with lipopolysaccharide before being assessed for an immune response to infection.

Traditional hematological parameters may provide information about the general state of an individual, where an increase in the heterophil/lymphocyte (H/L) index is a common response to stress caused by different factors, especially in birds and reptiles (Gross & Siegel, 1983; Lance & Elsey, 1999; Morici et al., 1997). In the current study, the H/L index was significantly higher in the animals from the groups exposed to RU compared to those from the NC, suggesting that pesticide exposure induced a state of stress. Lance and Elsey (1999) showed that handling and sequential bleeding of juvenile alligators (*Alligator mississippiensis*) generate a lot of stress for animals, causing an increase in the H/L index. Morici et al. (1997) studied the effects of corticosterone implants (hormone related to stress levels) in juvenile alligators, observing a higher H/L index in treated animals compared to the control hosts. Merchant et al. (2006) reported that *A. mississippiensis* injected with bacterial lipopolysaccharides had an increase in H/L index. Green turtles (*Chelonia mydas*) with fibropapillomas showed a significant increase in H/L ratios that positively correlated with increases in corticosterone levels, providing further evidence of the impact of chronic stress on reptilian immune parameters (Aguirre et al., 1995).

Plasma proteins help maintain circulating fluid volume and assist in the inactivation of toxic compounds and defense against pathogens. Alterations in total protein levels occur during pathological conditions, including exposure to xenobiotic agents. As such, determination of total protein (levels and profiles) is commonly used as an end-point of overall health of an organism. The results of the protein electrophoresis analyses performed here revealed that the total protein concentration was significantly higher in the RU1 caimans compared to the values for the NC hosts ($p < 0.016$). However, no differences were noted among other groups in this study ($p > 0.016$). The total protein levels were similar to those reported by other studies in wild *C. latirostris* and *C. yacare* (Uhart et al., 2001) and in those in captivity (Coppo et al., 2006; Ferreyra & Uhart 2001; Troiano & Althaus 1994; Uhart et al., 2001). Complementary analysis of the plasma protein fractions indicated a significant difference among the treatment groups, but only with respect to the F2 fraction. This could possibly indicate there was some impact from the exposure on the presence of proteins included in this fraction, i.e. α1-antitrypsin, a protein that acts as an acute phase reactant (taking part in inflammatory processes or trauma), as well as in stressful

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situations in humans (Brandán et al., 2008; Vidala et al, 2006; Zulet et al., 2007). At present, we cannot confirm this potential alteration, as we made no measures of α1-anti-trypsin *per se* in the F2 protein fraction of the *C. latirostris*. To our knowledge, no information exists about what specific components are included in each protein fraction in this species. We consider this an interesting end-point for further investigations in ongoing and future studies, i.e. to determine the particular components affected and potential consequences.

Growth is an integrated response to numerous physiological processes, resulting in positive and negative factors that influence the production of a body balance. Thus, growth rates are often used as an index of overall individual health. Chronic sub-lethal exposure to contaminants has been shown to result in elevated Standard Metabolic Rate (SMR) in reptiles. Given no compensatory increase in feeding or assimilation, individuals having SMR levels elevated above normal would experience fitness costs associated with reduced growth as a result of decreased energetic contributions to the production budget. Environmental factors that lead to a period of reduced growth can be particularly important during the first months of life when animals grow at a maximum rate to attain a size at which certain predators can be avoided. Thus, reductions in growth rates can serve as a biomarker of stressful environmental conditions, although mechanisms responsible for the reductions may not be immediately evident (Mitchelmore et al., 2005).

Existing data in our region related to *C. latirostris* has shown that Roundup® formulation and the mixture of Roundup®-Endosulfan-Cypermethrin formulations lead to lower growth of caimans at birth and during the first months of life, after *in ovo* exposure under similar conditions to those which may happen in natural environments near crops (Poletta et al., 2011). Our results regarding SVL and body weights show that exposure to RU has a negative effect on growth of these animals, in agreement with previous studies made under *in ovo* exposure (Poletta et al., 2011). The lack of significant difference in weight can be explained, in part, by the fact that length is a more conservative measure that commonly reflects long-term effects more clearly than weight, as the latter can fluctuate easily and with great variation among different individuals (Siroski, Personal Communication). This was also observed in previous studies on the effect of rearing density on growth (Poletta et al., 2008).

Considerable scientific evidence demonstrates that early life stages of oviparous organisms often exhibit a greater toxicological sensitivity to chemical contaminants than adult life stages (Russel et al., 1999). Growth and survival are important indicators of normal function. From the point of view of screening, size gain, and deviations from a normal range of body size at a given time in development, the results here suggest that ongoing exposure to this particular pesticide may cause significant developmental toxicity (refer to comments noted in IPCS, 2001).

Conclusion

This is the first study to report the effect of a pesticide formulation on the immune system of *C. latirostris*. Specifically, this study indicated that exposure to Roundup® caused alterations in the immune system and growth of young caimans. The observed effects on the former may be indicative of an overall reduced ability by caimans (especially hatchlings whose immune system is still immature) exposed in natural environments, to respond to infectious agents. Furthermore, any environmental factor that leads to slower growth is of particular importance during first months of life in the wild, when these animals grow at a maximum rate to reach a size that could mitigate the risk of attack by some predators. Thus, apart from the Roundup®-induced changes that could impact each young animal's normal ability to resist pathogens, the co-induced reduction in growth at this stage of life could decrease their risk for survival against ''macro'' organisms in their environment (Mitchelmore et al., 2005).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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