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# Oxidative damage and antioxidant defense in *Caiman latirostris* (Broad-snouted caiman) exposed *in ovo* to pesticide formulations

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ABSTRACT

The surface used for agricultural production in Argentina significantly increased in recent years, mainly due to the expansion of soybean crops. As a result, the use of agrochemicals increased too. Many natural populations of *Caiman latirostris* (broad-snouted caiman) are affected by habitat fragmentation and the constant exposure to pesticides. This exposure could produce Reactive Oxygen Species. The negative imbalance between ROS generation and the capacity of the biological systems to eliminate the reactive intermediaries or avoid the damage is called Oxidative Stress. The aim of this study was to evaluate oxidative damage and antioxidant defense in *C. latirostris* hatchlings after *in ovo* exposure to widely used pesticide formulations. Embryos were exposed by topical exposure on the eggshell, from the beginning of incubation period, to sub-lethal concentrations of two glyphosate formulations: PanzerGold® (PANZ) and Roundup® Full II (RU): 500, 750, 1000 μg/egg; to the endosulfan (END) formulation Galgofan® and the cypermethrin (CYP) formulation Atanor®: 1, 10, 100, and 1000 μg/ egg. Blood samples were taken to all animals immediately after hatching for the application and comparison of the following oxidative stress biomarkers between the exposed groups and their respective controls: lipoperoxidation through thiobarbituric acid reactive substances (TBARS), DNA base oxidation through the modified comet assay, and the activities of Catalase (CAT) and Superoxide dismutase (SOD) in erythrocytes. Our results showed lipoperoxidation in caiman exposed to END (10, 100, 1000 μg/egg), CYP (1, 10, 1000 μg/egg), RU (500, 1000 μg/egg) and PANZ (500, 1000 μg/egg), DNA base oxidation in those exposed to END (10, 100, 1000 μg/egg), CYP (1, 10 μg/egg) and PANZ (500, 750 μg/egg) as well as alteration in the activity of SOD in END 1 μg/egg and CYP (10, 1000 μg/egg). This study demonstrated the incidence of oxidative stress in animals exposed to pesticide formulations widely used in agricultural activity associated mainly with soybean crops, and add further information to that previously reported about pesticide effects in this native reptile species.

## 1. Introduction

Organisms produce Reactive Oxygen Species (ROS) and other free radicals constantly. ROS may be internally produced as subproducts of the mitochondrial respiratory chain, but organisms can also receive ROS from exogenous sources such as smoke, radiation, UV light and contamination (Södergren, 2000). In order to keep ROS in healthy levels to the cells, organisms have antioxidants defenses that work together: antioxidant molecules as Glutation (GSH), C and E vitamins, carotenoids, among others; and antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), GSH reductase, GSH peroxidase (GPx) and glutathione-S-tranferase. The negative imbalance between ROS generation and the capacity of the biological systems to eliminate the reactive intermediaries or avoid the damage is called Oxidative Stress (OS) (Limón-Pacheco and Gonsebatt, 2009).

Pesticides are known to be very reactive substances that cause oxidative damage to biomolecules, such as proteins, lipids, and nucleic acids, due to the production of ROS. They can act as pro-oxidant in a variety of tissues; they produce ROS accumulation, DNA damage, alteration of the antioxidants defenses and lipid peroxidation, causing a great perturbation at intra- and intercellular homeostasis (Halliwell, 2012).

Over the last ten years in Argentina, the agrochemical consumption has raised from 73 to 236 million kg per year (De Gerónimo et al., 2014). Transgenic crops are associated with different pesticide formulations, the most used worldwide are glyphosate-based formulations, followed by different insecticides including Cypermethrin and Endosulfan, among others (CASAFE, 2013). Studies conducted in

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agricultural environments in Argentina reported glyphosate residues from 0.5 to 5 mg/Kg in sediments and soils after one application (Aparicio et al., 2013), and recently similar values were found in sediments of the Saladillo and Lujan rivers, two tributaries of the Parana river, in the central-east of Argentina (Ronco et al., 2016). Additionally, Primost et al. (2017) found maximum concentrations of glyphosate and its main metabolite AMPA in the Pampas of Argentina, among the higher reported in the world in soil: 8105 and 38,939 μg/kg, and other environmental compartments such as sediments: 3294 and 7219 μg/kg and suspended particulate matter: 584 and 475 μg/kg, respectively. Regarding END, a study conducted by Regaldo et al. (2017) in central-eastern Argentina (the Colastiné - Corralito stream system) measured "total Endosulfan" (α-, β- and ensodulfan sulfate) and registered a maximum value of 0.132 μg/L in water. Besides, Marino and Ronco (2005) indicated cypermethrin concentrations between 0.2 and 3.58 μg/L in water and from 1 to 1075 μg/kg in sediment in the *Pampa Ondulada* Region of Argentina.

The use of biomarkers of early warning is an alternative of increasing interest to measure the effects of pesticides on environmentally exposed species. Previous works performed by our group demonstrated the genotoxic and immunotoxic effects of pesticides and pesticide mixtures on *C. latirostris* (Latorre et al., 2016; López González et al., 2017). In a recent study, Poletta et al. (2016) characterized a new set of OS biomarkers in *C. latirostris* blood, in order to evaluate OD induced by exogenous agents. The aim of the present work was to assess oxidative damage to lipids through thiobarbituric acid reactive substances (TBARS) and to DNA through the modified Comet assay, as well as antioxidant defenses capacity by the determination of CAT and SOD activities, on *C. latirostris* neonates exposed to Glyphosate, Endosulfan and Cypermethrin formulations during embryonic stage.

## 2. Materials and methods

### 2.1. Chemicals

Pesticides formulations tested were obtained by courtesy of Establecimiento La Matuza S.A., Santa Fe, Argentina and included: (1) Roundup® Full II (RU, 66.2% GLY), a liquid water soluble (12,000 mg/l) herbicide, containing GLY potassium salt [N-(phosphonomethyl) glycine monopotassium salt, $C_3H_7KNO_5P$] as its active ingredient (a.i.) (CAS No. 70901-12-1); (2) PanzerGold® (PANZ; 60.2% GLY), isopropylamine salt of glyphosate-based [N-(phosphonomethyl) glycine; CAS 1071-83-6] commercial formulation; (3) CYP Atanor® (25% CYP), a liquid water-insoluble (0.01 mg/l) mixture of different CYP isomers ($C_{22}H_{19}C_{l2}NO_3$, CAS No. 52315-07-8); and (4) END Galgofan® (35% END) a liquid practically water- insoluble (0.32 mg/l) formulation, containing END as a.i. ($C_8H_6C_{l6}O_3S$, CAS No. 115-29-7) (EXTOXNET, access 2016). Ethanol was used as a vehicle control for END and CYP formulations. Hydrogen peroxide ($H_2O_2$), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), butylatedhydroxytoluene (BHT), and SOD Kit (19160-1KT) were from Sigma-Aldrich (St. Louis, MO, USA). Potassium dihydrogenphosphate ($KH_2PO_4$) and potassium hydrogen diphosphate ($K_2HPO_4$) were from Cicarelli (Argentina).

### 2.2. Caiman latirostris eggs collection

This study was evaluated and approved (N° 04–12) by the 'Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral (Santa Fe, Argentina). *C. latirostris* eggs were collected from different nests in the Natural Managed Reserve "El Fisco" (30° 11′ 26″ S; 61° 0′27″ W; Dpto. San Cristobal, Santa Fe Province, Argentina, as part of "Proyecto Yacare" (PY) ranching program activities (Larriera et al., 2008). This is a Protected Natural Area of approximately 1800 ha, free of any contaminating activity or source of contamination within it that belong to the natural distribution of the species. Even when the size of the reserve is considerable big to ensure that the activities in the surrounding areas do not affect natural populations living there, it is important to note that all these areas are dedicated to ranching, with no use of products that cause diffuse contamination. For this reason, caiman populations living there have been used as controls in many studies (Poletta et al., 2008, 2009; Latorre et al., 2016; López González et al., 2017) and were chosen here too, to ensure that eggs had not been environmentally exposed to any xenobiotic.

Six clutches collected during 2013–2014 nesting season (December 2013) with a minimum of 32 eggs each, were used to carry out the experiment. All nests were collected within 5 days after oviposition, under the same conditions from harvest to treatment assignment, and egg viability was determined by analyzing the opaque eggshell banding (Larriera et al., 2008). The average weight of eggs used in experiments was 67.8 ± 4.75 g.

### 2.3. Experimental design and treatments

One hundred and ninety-two eggs from six clutches (32 eggs per clutch), were equally distributed into 16 experimental groups of 12 eggs each (with two replicates of six eggs each). Experimental groups were: **1–3)** three groups exposed to 500, 750, 1000 μg/egg of Roundup®; **4–6)** three groups exposed to 500, 750, 1000 μg/egg of PanzerGold® (Poletta et al., 2009); **7–10)** 4 groups exposed to 1, 10, 100, 1000 μg/ egg Endosulfan formulation (Beldomenico et al., 2007); **11–14)** 4 groups exposed to 1, 10, 100, 1000 μg/ egg Cypermethrin formulation (Anwar, 2003); **15)** a water control (WC), as reference for GLY-based formulations, treated with distilled water; **16)** an ethanol control (EC), as reference for END and CYP formulations, tested with ethanol (50 μl).

These concentrations were chosen from previous studies made in *C. latirostris* and other species such as bird and mammals, adapting them to the average weight of *C. latirostris* eggs (70 g approximately) and to our experimental conditions. These concentrations are environmentally relevant considering data available in the literature on pesticide residues reported in different environments in Argentina, as above mentioned. Moreover, the presence of different kind of pesticides in eggs was demonstrated for this species by Stoker et al. (2013) concerning organochlorine pesticides, while pyrethroids occurred in chickens from a commercial farm and home egg production (Parente et al., 2017) and in wild birds eggs from a National Park in Spain (Corcellas et al., 2017). Up to our knowledge, there are no reports for glyphosate or AMPA residues in eggs for any species, but taking into account values reported in environmental matrices, the concentrations used are likely to be received by a caiman nest in the proximity of crops, especially considering repeated applications done in those environments.

Pesticide solutions were prepared in water for GLY formulations while for CYP and END we used ethanol solutions considering they are not soluble in water. For that reason, a second control group, the vehicle control, had to be included to ensure any effect for ethanol itself. All the solutions were applied on the eggshell (by topical application) at the embryo implantation zone within the first 5 days of incubation. Each experimental group was placed separately in a plastic container, using vermiculite as substrate and covering them with vegetal material similar to the nesting material, free of any exogenous substance. All eggs were incubated under a temperature of 31.5 ± 0.5 °C and 95% humidity in the "PY" incubator. They were checked periodically during the experiment in order to identify and discard those which became non-viable.

When hatchlings started to call within the eggs, the corresponding eggs were removed from the incubator and if hatching did not occur spontaneously during the following 24 h., they were assisted, considering the possibility they do not have enough strength to do it or have not the egg tooth well developed. The same process is done by the female in the nature with those eggs that do not hatch by their own after some hours of the pre-hatchling calling from inside. After 72 h.,

the remaining unhatched eggs of the same clutch were helped to avoid they die inside the eggs, because lack of oxygen (Larriera et al., 2008).

### 2.4. Developmental parameters

Hatching success was recorded for all groups. Then, all hatchlings were individually identified, weighed (OHAUS® Compact scale CS200, precision 0.1 g) and measured in total length (TL) and snout-vent length (SVL) (tape measure, precision 0.1 cm) in order to evaluate hatchlings size in each experimental group.

### 2.5. Blood samples

Immediately after hatching, blood samples (300 µl) were obtained from the spinal vein of all hatchlings with heparinized syringes (Myburgh et al., 2014) to analyze oxidative stress biomarkers. This sampling technique was previously used for hatchlings in many studies, without any damage to the animals observed immediately or even several months after sampling (López González et al., 2017). Peripheral whole blood was used immediately for the modified comet assay; while for the rest of the techniques, blood was centrifuged during 10 min at 450 × g, erythrocytes were washed with physiological saline solution (NaCl 0.9%) twice, then lysed with ice-cold demineralized ultrapure water (MilliQ plus reagent grade) at a 1:10 dilution and stored at − 20 °C until analysis (Poletta et al., 2016).

### 2.6. Oxidative damage

#### 2.6.1. Lipid peroxidation in erythrocytes (TBARS)

Lipid peroxidation in red blood cells was determined by measuring the formation of the color produced during the reaction of TBA with TBARS (TBARS Assay), according to a modification of the method of Buege and Aust (1978) adapted for this species by Poletta et al. (2016): lysed erythrocytes dilution was mixed with potassium chloride buffer (0.154 M) plus protease inhibitors and distilled water. Then it was thoroughly mixed with four volumes of the solution: 15% w/v TCA, 0.375% w/v TBA, 0.25 mol $l^{-1}$ HCl acid and 4% BHT. The mixture was heated in a dry bath at 95 °C for 45 min. After cooling, the flocculent precipitate was removed by centrifugation and the sample absorbance was determined at 535 nm. TBARS concentration in erythrocytes was calculated using the extinction coefficient $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$ and expressed as nmol/g Hb.

#### 2.6.2. Detection of oxidative DNA damage in erythrocytes through the modified comet assay

The alkaline comet assay (CA) was performed as developed by Poletta et al. (2008) for *C. latirostris* erythrocytes, with the modifications introduced later for oxidized bases determination, described also by our group (Poletta et al., 2016). Three slides were made for each animal. After lysis, slides were washed three times with enzyme buffer and excess liquid was dabbed off with tissue. Then, 50 µl of Formamidopyrimidine-DNA glycosylase (FPG) or Endonuclease III (ENDO III) enzyme solution, or enzyme buffer alone as control were placed on the gel, covered with a cover slip, and incubated into a moist box (to prevent desiccation) for 30 min at 37 °C. Unwinding was carried out in alkaline buffer during 10 min and then electrophoresis was performed in the same buffer during 15 min at 0.90 V/cm. One hundred randomly selected comet images were analyzed, classified into five arbitrary classes, and a single DNA damage index (DI = n1 + 2 n2 + 3 n3 + 4 n4) was calculated for each animal (Poletta et al., 2008). An increase in DI after incubation with the enzyme, compared with incubation with buffer alone, indicates the presence of oxidized bases. So, oxidative damage to DNA was calculated by subtracting breaks with buffer from breaks with FPG or ENDO III as follows:

FPG = Endo III sites = DI with FPG/Endo III - DI with enzyme buffer alone (Collins, 2009)

### 2.7. Antioxidant defense capacity

#### 2.7.1. Catalase (CAT) activity in erythrocytes

CAT activity in lysed erythrocytes was measured spectrophotometrically by monitoring the decrease in $H_2O_2$ concentration over time (Aebi, 1984) with some modifications for this species (Poletta et al., 2016). The specific activity of each sample was calculated on the basis that one unit of enzyme activity is defined as the activity required degrading 1 mol of hydrogen peroxide during 60 s/g Hb. $H_2O_2$ was added to a final concentration of 54 mM and absorbance was measured at 240 nm, 25 °C during 60 s in the spectrophotometer. Results are expressed in arbitrary units as the Activity of CAT (KU/g Hb).

#### 2.7.2. Superoxide dismutase (SOD) activity in erythrocytes

SOD was determined using the commercial kit 19160-1KT (SIGMA) (Poletta et al., 2016). SOD Assay Kit-WST utilizes a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl) − 3-(4-nitrophenyl) − 5-(2,4-disulfophenyl) − 2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with $O_2$ is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity, as an inhibition activity, can be quantified by measuring the decrease in the color development at 440 nm. SOD activity is expressed as percentage of inhibition (%).

### 2.8. Statistical analysis

Statistical analysis was performed using the Software SPSS 14.0 for Windows. Data were tested for normality with Kolmogorov-Smirnov test and homogeneity of variances between groups was verified by Levene's test. We first proved absence of differences between replicates for each variable (*Test t*), so treatment was considered as grouping variable. Then all variables were analyzed by a General Lineal Model were the grouping variables were the clutch of origin (to test the presence of *clutch effect*) and treatment, while the response variables were TBARS, CAT and SOD activities, FPG and ENDO III sites, weight, TL, SVL and hatching success. After verifying lack of clutch effect for all variables, the only grouping variable considered was the treatment. Results obtained for TL, SVL, weight, TBARS and CAT were evaluated through a one-way ANOVA followed by Dunnet test when difference was observed. Hatching success, SOD activity, FPG and ENDO III sites were analyzed by Mann-Whitney *U*-test (non-parametric data). For all variables, exposed groups were compared to their corresponding control: the water control for groups exposed to the glyphosate formulations (which are water soluble and were prepared in water) and the ethanol control for groups exposed to Endosulfan and Cypermethrin formulations (which are not water soluble and had to be prepared in an ethanol solution). On the other hand, concentration-effects relations within treatments and between oxidative stress parameters and animal size at birth were calculated by linear regressions.

## 3. Results

From the total number of eggs exposed, thirty-four resulted nonviable and were discarded during incubation period, being less than 20% for all groups. The period went from treatment to hatching was between 66 and 72 days. Number of caiman helped to born showed no influence of treatment and were as follows: WC = 1, END1 = 1; END100 = 2; CYP1000 = 1 and RU500 = 1. Hatching success was analyzed considering only animals born by themselves, and showed no statistically significant difference among groups ($p = 0.395$).

Results showed a statistically significant increase in TBARS for END10 ($p = 0.002$), 100 ($p = 0.007$) and 1000 ($p = 0.008$), CYP1 ($p = 0.013$), 10 and 1000 ($p^{<}0.0001$ compared to the EC, and for RU500 ($p = 0.001$), RU1000 ($p = 0.006$), PANZ500 ($p = 0.039$) and

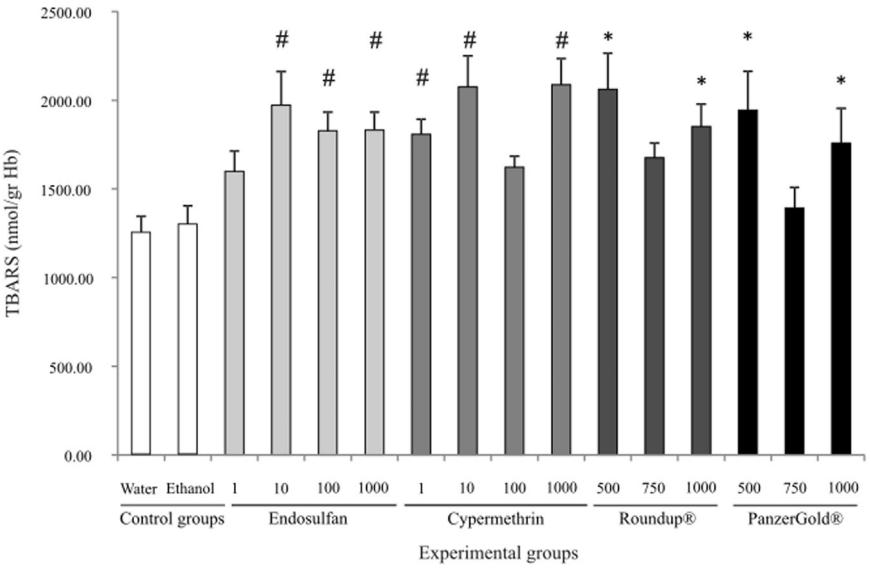


**Fig. 1.** Lipid peroxidation measured through the Thiobarbituric acid reactive substances assay (TBARS: mean ± SE, expressed as nmol/g Hb) in erythrocytes of *Caiman latirostris* neonates exposed *in ovo* to 1, 10, 100 and 1000 μg/egg of Endosulfan and Cypermethrin formulations, and 500, 750 and 1000 μg/egg of glyphosate-based formulations Roundup® and PanzerGold® (number of samples, n = 156). *Statistically different respect to water control and #to ethanol control (ANOVA-Dunnett test, $p < 0.05$).

**Table 1**
Endonuclease III (ENDO III) and Formamidopyrimidine-DNA glycosylase (FPG) sites (mean ± standard error) in erythrocytes of *Caiman latirostris* neonates exposed *in ovo* to 1, 10, 100 and 1000 μg/egg of Endosulfan and Cypermethrin formulations, and 500, 750 and 1000 μg/egg of glyphosate-based formulations Roundup® and PanzerGold® (n = 156).

| Experimental Groups | ENDO III-sites | FPG-sites |
|---|---|---|
| **Negative controls** | | |
| Water control | 21.33 ± 0.88 | 19.33 ± 2.19 |
| Ethanol control | 22.33 ± 5.78 | 32.35 ± 19.43 |
| **Endosulfan (μg/egg)** | | |
| 1 | 51.00 ± 8.38 | 47.25 ± 13.38 |
| 10 | 72.40 ± 4.84# | 53.40 ± 8.14 |
| 100 | 43.40 ± 4.58# | 44.20 ± 6.78 |
| 1000 | 59.20 ± 7.62# | 44.80 ± 8.12 |
| **Cypermethrin (μg/egg)** | | |
| 1 | 42.00 ± 6.05# | 41.20 ± 7.55 |
| 10 | 47.00 ± 5.13# | 41.66 ± 10.08 |
| 100 | 45.33 ± 8.95 | 44.67 ± 7.31 |
| 1000 | 46.40 ± 13.42 | 35.40 ± 8.44 |
| **Roundup® (μg/egg)** | | |
| 500 | 51.05 ± 22.56 | 56.25 ± 12.73 |
| 750 | 51.33 ± 19.74 | 41.76 ± 8.62 |
| 1000 | 52.75 ± 11.61 | 40.00 ± 14.44 |
| **PanzerGold® (μg/egg)** | | |
| 500 | 62.00 ± 17.43# | 42.50 ± 26.50 |
| 750 | 71.67 ± 13.97# | 80. 67 ± 17.65 |
| 1000 | 40.00 ± 20.66 | 39.67 ± 12.67 |

# Statistically different respect to the ethanol control (Mann-Whitney *U-test, $p < 0.05$*).

PANZ1000 ($p = 0.04$), respect to the WC (ANOVA $F_{4.062}$, $df_{155}$, $p<0.0001$ -Dunnet; Fig. 1). The results of the modified CA indicate oxidation of pyrimidines showed by ENDO sites at END10 ($p = 0.036$) and END100, 1000, CYP1 and 10 ($p = 0.016$), compared to the EC, and PANZ 500 and 750 ($p = 0.036$) compared to the WC (Mann-Whitney *U*-Test; Table 1). Considering antioxidant defenses, there was a statistically significant increase in SOD activity for END1 ($p = 0.05$), CYP10 ($p = 0.019$) and CYP1000 ($p = 0.032$), compared with the EC (Mann Whitney *U*-test; Fig. 2). CAT activity showed no statistically significant differences for any treatment (ANOVA, $F_{0.809}$, $df_{155}$, $p = 0.673$; Table 2). There were no statistically significant differences in weight ($F_{0.666}$, $df_{155}$, $p = 0.823$), TL ($F_{0.889}$, $df_{151}$, $p = 0.673$) or SVL ($F_{1.116}$, $df_{155}$, $p = 0.346$) for any treatment (Table 3; ANOVA).

No concentration-effect was found for any variable or compound tested ($R < 0.200$, $p > 0.05$ in all cases) and neither a relationship was found between OE parameters and size of animals at birth (Linear regressions, $R_{TBARS} = 0.042$; $R_{CAT} = 0.132$; $R_{SOD} = 0.213$, $p > 0.05$ in all cases). No differences were observed between the WC and the EC for any of the variables analyzed and no clutch effect was found in any of the analysis performed ($p > 0.05$ in all cases).

## 4. Discussion

Early development is a life stage where oxidative stress levels are high due to the presumed linkage between high metabolic activities required for growth and the consequent ROS generation (Monaghan et al., 2009). This is particularly relevant in reptile species, which have normally a very low metabolism rate. Any external factor that increases ROS production even more at this stage, could produce serious damage in these organisms. In the present study, we evaluated the effect of Glyphosate, Cypermethrin, and Endosulfan formulations through oxidative stress parameters (lipid peroxidation, oxidative DNA damage, catalase and superoxide dismutase activities after *in ovo* exposure of *C. latirostris*.

The results showed that all the formulations studied at most of the concentrations tested produce lipid peroxidation (Fig. 1). Another important finding was the induction of oxidative DNA damage at pyrimidines in caiman exposed to different concentrations of Endosulfan (END), Cypremethrin (CYP) and PanzerGold® (PANZ) (Table 1). Concerning antioxidant defense capacity, SOD activity responded with a significant increase in END1, CYP10 and CYP1000, but not in the groups exposed to Glyphosate (GLY) formulations, while CAT seemed to be less sensible with no significant variation at any treated group. Besides, no statistically significant differences were noted in hatching success or size of the animals in any treatment at the moment of hatching.

Agrochemicals may produce ROS through different mechanisms, by interference in electron transport in the mitochondrial membrane with the consequent accumulation of reactive intermediates, by inactivation of antioxidants enzymes, as well as by the deterioration of non-enzymatic antioxidants and lipid peroxidation. Because of ROS increase, antioxidant enzymes, particularly SOD and CAT, are activated to detoxify and counteract their deleterious effects. The first step in the detoxification process involves conversion of the superoxide anion ($O^{2-}$) to hydrogen peroxide ($H_2O_2$) by the SOD enzyme and the second step consists in the conversion of $H_2O_2$ to water ($H_2O$) and oxygen ($O_2$) by the CAT enzyme (Halliwell, 2012). Nevertheless, SOD and CAT can be inactivated by these 2 components ($O^{2-}$ and $H_2O_2$). Finally, hydrogen peroxide, when not converted into water and oxygen, is metabolized

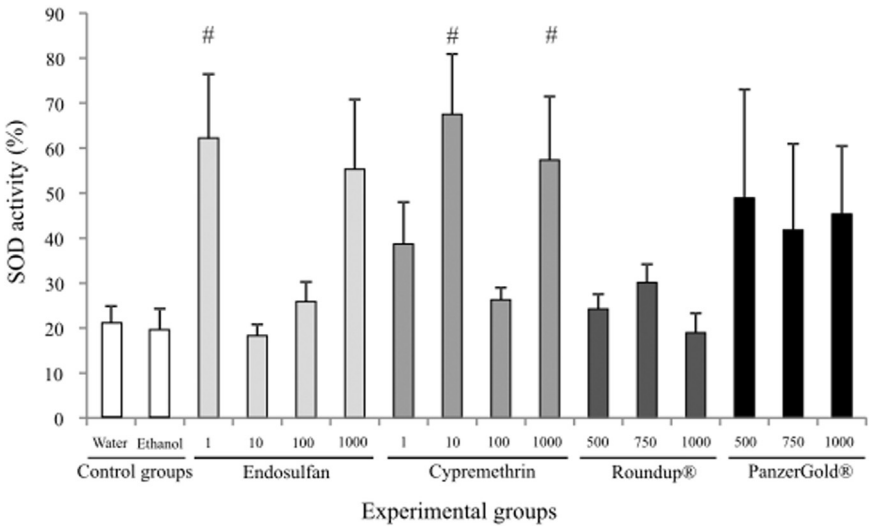


**Fig. 2.** Superoxide dismutase activity (SOD: mean ± SE, expressed as %) in erythrocytes of *Caiman latirostris* neonates exposed *in ovo* to 1, 10, 100 and 1000 μg/egg of Endosulfan and Cypermethrin formulations, and 500, 750 and 1000 μg/egg of glyphosate-based formulations Roundup® and PanzerGold® (n = 156). #Statistically different respect to ethanol control (Mann-Whitney *U*-test, $p < 0.05$).

**Table 2**
Catalase activity (mean ± standard error, expressed as kU/g Hb) in erythrocytes of *Caiman latirostris* neonates exposed *in ovo* to 1, 10, 100 and 1000 μg/egg of Endosulfan and Cypermethrin formulations, and 500, 750 and 1000 μg/egg of glyphosate-based formulations Roundup® and PanzerGold® (n = 156). No statistically significant differences were observed respect to the controls (ANOVA-Dunnett test, $p > 0.05$).

| Experimental Groups | Enzymatic activity of Catalase (kU/g Hb) |
|---|---|
| **Negative controls** | |
| Water control | 863.60 ± 96.50 |
| Ethanol control | 969.65 ± 92.70 |
| **Endosulfan (μg/egg)** | |
| 1 | 631.70 ± 119.70 |
| 10 | 840.30 ± 217.20 |
| 100 | 1004.70 ± 158.90 |
| 1000 | 927.90 ± 94.20 |
| **Cypermethrin (μg/egg)** | |
| 1 | 878.10 ± 322.10 |
| 10 | 902.90 ± 116.10 |
| 100 | 661.10 ± 70.60 |
| 1000 | 1161.90 ± 122.40 |
| **Roundup® (μg/egg)** | |
| 500 | 891.90 ± 130.80 |
| 750 | 956.50 ± 105.90 |
| 1000 | 705.80 ± 101 |
| **PanzerGold® (μg/egg)** | |
| 500 | 683.47 ± 289.28 |
| 750 | 419.40 ± 60.16 |
| 1000 | 540.56 ± 110.05 |

into hydroxyl radicals ($OH^-$) through the Fenton reaction. Hydroxyl radicals can react with lipids in the cell membranes, which can cause extensive damage (Hermes-Lima, 2004). In the current study, SOD showed significant changes at END1, CYP10 and CYP1000 formulations with an activity 215%, 240% and 190% higher than the ethanol control, respectively. Contrary to expectation, there were no significant differences for CAT activity at any formulation tested. The differences between the two enzyme activity profiles might be explained by SOD activation through $O^{2-}$ at END1, CYP10 and CYP1000, and its inactivation through $H_2O_2$ in caiman exposed to the other treatments. On the other hand, CAT activity could have been inhibited by $O^{2-}$ production. Such approaches are consistent with the results obtained for lipid peroxidation, which was significantly higher respect to the controls at most treatments.

All the results together suggest that: 1) at the lowest concentration of Endosulfan, caiman avoid lipid peroxidation and genetic damage by means of an increase in SOD activity, but at higher concentrations, antioxidant systems could possibly not avoid ROS generation resulting in lipid peroxidation and genetic damage; 2) at the lowest concentration of Cypermethrin, it seems that enzymatic activities were inhibited for the excess of radicals, therefore, the reactive substances induced DNA oxidation; 3) despite SOD activity increase at CYP10 and 1000, there was no change in CAT activity, maybe because of ROS inhibition, and consequently POL and genetic damage (at CYP10) were registered; 4) Roundup® 500 and 1000 (μg/egg) induced not significant differences at the enzymatic level, but damage to lipids exists, which indicates impaired ability of antioxidant defenses; 5) PanzerGold® 500 and 1000 (μg/egg) showed similar results for lipid peroxidation, and PANZ500 and 750 for DNA oxidation, which could demonstrated that both SOD and CAT were inhibited by ROS.

These findings are similar to those of Dzul-Caamal et al. (2016) who compared biomarkers of oxidative stress (TBARS, SOD and CAT) of inorganic and organic pollutants on *Crocodylus moreletii* in pristine and polluted localities. They detected statistical differences for TBARS and significant increase of SOD (1.33 fold) and CAT activities (1.44 fold) in animals from polluted areas compared to those from pristine areas. On the other hand, a study conducted in the Nile crocodile (*Crocodylus niloticus*) exposed to aquatic contaminants reported an efficient antioxidant system in which CAT, GSH and GPx suggest an elevated capability to reduce lipid hydroperoxides and hydrogen peroxide (Arukwe et al., 2015).

Differences of CAT and SOD enzymes activity profiles were also reported in other studies. Recently, Héritier et al. (2017a) compared the effects of high concentrations of glyphosate-based formulations (Roundup® and Clinic® 30 mg/L) in juveniles of the freshwater turtle (*Trachemys scripta elegans*). They measured gene expression of CAT and SOD, as well their activities and lipid peroxidation after 12 and 96 h of exposure. CAT activity was 1.5-fold higher and its expression 2.8-fold higher in the glyphosate-96-h group. There were no differences for SOD activity and lipid peroxidation. Opposite to the results obtained in the present work, the authors conclude that probably, SOD enzyme was inactivated whereas CAT enzyme was activated in turtles challenged with high concentrations of glyphosate-based herbicide, thus lipid peroxidation was not registered. Comparable to our results for glyphosate formulations, in another study, Héritier et al. (2017b) showed that enzymatic activity of SOD and CAT was inactivated on turtles (*Mauremys leprosa*) in degraded water with glyphosate and AMPA.

Other studies available in the literature have also contradictory results, as Theodorakis et al. (2017), who examined blood samples of gopher tortoises (*Gopherus polyphemus*) collected from different levels of habitat quality (high-quality and low-quality habitat) and military activity (high, low, and no military activity). They found a possible

**Table 3**
Total length, Snout-vent length and weight measures (mean ± standard error) of *Caiman latirostris* neonates exposed *in ovo* to 1, 10, 100 and 1000 μg/egg of Endosulfan and Cypermethrin formulations, and 500, 750 and 1000 μg/egg of glyphosate-based formulations Roundup® and PanzerGold® (n = 156). No statistically significant differences were observed respect to the controls (ANOVA-Dunnett test, $p > 0.05$).

| Experimental Groups | Total length (cm) | Snout-vent length (cm) | Weight (g) |
|---|---|---|---|
| **Negative controls** | | | |
| Water control | 23.42 ± 0.18 | 11.2 ± 0.70 | 44.19 ± 1.39 |
| Ethanol control | 23.02 ± 0.24 | 10.81 ± 0.12 | 44.45 ± 1.02 |
| **Endosulfan (μg/egg)** | | | |
| 1 | 23.46 ± 0.27 | 11.09 ± 0.17 | 43.96 ± 1.04 |
| 10 | 21.93 ± 1.58 | 12.5 ± 1.43 | 44.68 ± 1.33 |
| 100 | 23.60 ± 0.23 | 11.22 ± 0.10 | 44.58 ± 0.84 |
| 1000 | 23.34 ± 0.12 | 11.3 ± 0.06 | 44.54 ± 0.46 |
| **Cypermethrin (μg/egg)** | | | |
| 1 | 23.49 ± 0.19 | 11.18 ± 0.18 | 45.00 ± 1.38 |
| 10 | 23.35 ± 0.15 | 11.01 ± 0.08 | 43.83 ± 1.03 |
| 100 | 23.29 ± 0.22 | 11.11 ± 0.10 | 44.05 ± 0.62 |
| 1000 | 23.31 ± 0.20 | 11.04 ± 0.08 | 45.11 ± 0.74 |
| **Roundup® (μg/egg)** | | | |
| 500 | 23.48 ± 0.26 | 11.27 ± 0.10 | 45.04 ± 0.75 |
| 750 | 0.25 ± 1.40 | 11.09 ± 0.07 | 44.73 ± 0.89 |
| 1000 | 22.96 ± 0.19 | 11.01 ± 0.09 | 44.31 ± 0.88 |
| **PanzerGold® (μg/egg)** | | | |
| 500 | 22.55 ± 0.55 | 10.87 ± 0.22 | 41.32 ± 1.21 |
| 750 | 23.55 ± 0.54 | 11.15 ± 0.26 | 44.42 ± 0.98 |
| 1000 | 23.59 ± 0.30 | 11.44 ± 0.68 | 44.58 ± 0.96 |

relationship between military activity and level of oxidative stress in the low-quality habitat, with elevated parameters in the low activity groups, respect to the no military activity groups. They suggested that exposure to energetic compounds is responsible for the observed patterns of oxidative stress, but this could not explained what they found in high-quality habitat where the level of oxidative stress increases as the level of habitat disturbance decreases, contrary to the results obtained in the present work for TBARS. In the named article, authors reported lower values of lipid peroxidation in *G. polyphemus* (1300 and 1400 nmol/mg Hb) than those obtained for *C. latirostris* in the present research (1800 and 2000 nmol/mg Hb).

In the present work, we found a significant increase on lipid peroxidation at different concentrations of Endosulfan (10,100 and 1000 μg/egg), Cypermethrin (1, 10 and 1000 μg/egg), Roundup® (500 and 1000 μg/egg) and PanzerGold® (500 and 1000 μg/egg). Lipid peroxidation is considered one of the best biomarkers of oxidative damage because it indicates indirectly, the incidence of free radicals on the biological membranes. The exposure to Glyphosate, Endosulfan and Cypermethrin was made *in ovo,* thus we can expect a high susceptibility to oxidative damage on embryos due to their high content of unsaturated fatty acids and deficiencies in antioxidant enzymes, as a consequence of immaturity. This was explained by Hilscherova et al. (2003) when studied OS induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on embryos of *Gallus domesticus.*

The modified Comet assay demonstrated oxidative damage on pyrimidines in Endosulfan (10, 100 and 1000 μg/egg), Cypermethrin (1 and 10 μg/egg) and PanzerGold® (500 and 750 μg/egg). Similar results were found previously by other authors to different environmental mutagens as diesel exhaust particles and arsenate (Risom et al., 2007). In relation to pesticides, Zhao et al. (2015) observed an increase in DNA base oxidation in the goldfish (*Carassius auratus*) exposed to monocrotophos, and Pérez-Iglesias et al. (2017) reported that Imazethapyr induced oxidative DNA damage on *Hypsiboas pulchellus* tadpoles at purines but not at pyrimidines, contrary to what we found for Endosulfan, Cypermethrin and PanzerGold®. There were no previous studies reporting the oxidative DNA damage produced by pesticides or any other contaminant in any crocodilian species.

Cooke et al. (2003) indicated a direct relationship between oxidative damage to DNA and the production of the hydroxyl radical ($OH^{\bullet}$), derived from reactive oxygen species. Lipoperoxidation can produce a chain effects, and products derived from it can trigger a complex reaction causing DNA damage (Hulbert et al., 2007). In this study, a significant increase in TBARS was observed, thus DNA base oxidation could be an indirect effect of OS through lipoperoxidation in Endosulfan (10, 100 and 1000 μg/egg), Cypermethrin (1 and 10 μg/egg) and PanzerGold® (500 μg/egg).

Moreover, ROS can react with cellular components such as nucleic acids, thus a dysfunction in antioxidant system can be the result of an alteration in gene expression (Hilscherova et al., 2003), altering the enzyme synthesis in neonates exposed to different stress. Kryston et al. (2011) stated that the first border of cellular defense against DNA damage consists of endogenous non-enzymatic scavengers such as GSH, vitamins C and E, and antioxidant enzymes including SOD, CAT and glutathione peroxidase, as well as the highly specific and sophisticated DNA repair pathways. Results obtained on DNA oxidative damage go further from others previously reported by our group in terms of genotoxicity, and warns about the effects that may produce the constant exposure to pesticides, even at low concentrations, over the life span of the caiman (Poletta et al., 2009; López González et al., 2017).

The modifications in the oxidative state have been recognized as an important mechanism of the damage caused by pesticides (Halliwell, 2012). This work, using markers of oxidative stress applied in blood of *C. latirostris* neonates, allowed to link imbalances in the oxidative state with factors that could directly affect animal health, especially important in this species of great commercial and ecological interest for many regions of South America. For *C. latirostris,* the adaptation of these techniques for their application in peripheral blood, avoiding any damage to the animals (Poletta et al., 2016) represents a major advance over many previous studies reported in other crocodile species, where organs or tissues were used, with the consequent slaughtering of the animals. In this work, it was possible to use these techniques as markers of OS in caiman exposed *in ovo* to different pesticides, and this is the first report of oxidative damage and impaired antioxidant defenses produced particularly by pesticides in embryos of all crocodilian species. Considering the particularity of the reptiles in terms of its antioxidant capacity systems, it is expected that there is variability in responses among individuals of different population for sensitivity or resistance to various stressors. This variability, including gene expression of various enzymes, could derive from the genotypic differences between one population and another. Besides, the different responses found in this and the named works can also be related to the intensity, duration and route of exposure and the type of xenobiotic studied. Given these preliminary data, we intend to continue with the characterization of oxidative damage and antioxidant systems in this species, to expand existing knowledge on pesticides effect, in order to incorporate these biomarkers in the biomonitoring of environmentally exposed caiman populations in Argentina for really understanding the effects of long-term exposure to these compounds.

## 5. Conclusions

This work has determined imbalances in the oxidative state of *C. latirostris* neonates exposed during incubation period to different pesticide formulations widely used in Argentina and other many countries in the world. The endpoints of oxidative stress applied in blood demonstrated to be early sensible to these xenobiotics. Therefore, they are highly promising for the biomonitoring of natural populations of the broad-snouted caiman environmentally exposed to pesticides.

These results expand other previously reported by our group in terms of the effect that pesticide formulations have on animal health, warning about the consequence of a long term exposure to them, even at low concentrations, over the life span of the caimans living in exposed areas. This is of great importance for a species with great

commercial and ecological value for many countries in South America, as it is the broad snouted caiman.

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