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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.,