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# Alterations in the Expression of Antioxidant Enzyme Genes in Response to Pesticide Exposure During Embryonic Development in the Native Reptile Species *Caiman latirostris*

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**Abstract**
The aim of this study was to quantify the expression levels of Catalase (*cat*) and copper, zinc Superoxide dismutase (Cu, Zn-*sod*) genes involved in the antioxidant response in *Caiman latirostris* (broad-snouted caiman) blood, after embryonic exposure to the formulations cypermethrin (CYP), chlorpyrifos (CPF), glyphosate (GLY), and their binary and ternary mixtures. Experimental groups were: negative control (NC-distilled water), vehicle control (VC-ethanol), GLY-2%, CYP-0.12%, CPF- 0.8%, a ternary mixture of them (TM), and three binary mixtures. The applications were made on the nest material in contact with the eggs at the beginning of the incubation period. After hatching, RNA was isolated from blood and expression levels analyzed through qPCR. The results showed downregulation in the expression of *sod* and *cat* genes in the three binary mixtures studied, compared to the controls. In addition, we found a possible antagonistic effect between different pesticides in the TM on the expression of both genes.

**Keywords** Agrochemicals · Mixtures · Gene expression · Catalase · Superoxide dismutase · Reptiles

## Introduction

Reptiles are important components of ecosystems, both as prey and predators, and also have utility as bioindicators of environmental pollution. In recent decades, the role of environmental pollution as a possible reason for the decline of reptile populations has prompted researchers to evaluate the effects of xenobiotics on these vertebrate groups (Weir et al. 2010). In Argentina, there are two species of crocodilians, the most common being *Caiman latirostris* inhabits the north-central region of the country (Poletta et al. 2011). This species is of great commercial and ecological interest for a large part of South America (Bolivia, Paraguay, Uruguay and Brazil), however, most of its habitat has been fragmented or overlapped by soybean crops. As a result, natural populations of caimans are frequently exposed to pesticide including herbicides based on glyphosate (GLY) and insecticides such as cypermethrin (CYP) and chlorpyrifos (CPF), among others (CASAFE, 2022). On the other hand, the period of the year of maximum pesticide application coincides with the reproductive season of this species, implying a particularly important risk of contamination during the first months of life (Poletta et al. 2011). Different studies have shown that *C. latirostris* is a sentinel for the assessment of pesticide-induced effects (Poletta et al. 2011; Burella et al. 2018). Specifically, genotoxicity, oxidative damage and alterations in antioxidant enzymatic activity have been reported in blood of *C. latirostris* during embryonic development and in yearlings exposed to sublethal concentrations of CYP, CPF and GLY formulations (Poletta et al. 2011; Burella et al. 2018; LópezGonzález et al. 2021a, 2022). Current approaches survey for changes in mRNA patterns determine the role of gene expression in the context of toxicity. Numerous studies in fish and mammals

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have demonstrated that gene expression patterns are useful tools for biomonitoring and ecotoxicology tests (Zhang et al. 2017; Velki et al. 2019). Particularly, they have shown that pesticides alter the expression levels of genes encoding for antioxidant systems (Paravani et al.2019; Ghelichpoura et al. 2019). Although some studies on the expression of catalase (*cat*) and superoxide dismutase (*sod*) genes in response to xenobiotic exposure are available in reptiles (Héritier et al. 2017a, 2017b; Morao et al. 2022), at present, there were no reports in crocodilians. Recently a protocol for RNA extraction was adapted and genes belonging to the antioxidant system were identified in blood of *C. latirostris* to identify new molecular biomarkers of pesticide exposure (López González et al. 2021b, Odetti et al. 2021). The possibility of applying molecular markers of gene expression in this species will allow understanding the specific meaning of many alterations produced by pesticides and observed through markers of DNA damage, oxidative stress and immunological alterations. At the same time, the application of these biomarkers on blood represents a huge advantage for the study of wild populations since it allows assessing the effects of xenobiotics through non-lethal sampling to the animals (Poletta et al. 2016). In the present work, the gene expression levels of *cat* and Cu, Zn-*sod* genes were analyzed in blood of *C. latirostris* after embryonic exposure to GLY, CYP and CPF-based formulations, separately and in mixtures, in an experiment that attempt to simulate conditions of real exposure for *C. latirostris* eggs.

## Materials and Methods

### Experimental Setup and Treatments

Research Ethics and Safety Advisory Committee of the Facultad de Bioquímica y Ciencias Biológicas, UNL (Santa Fe, Argentina) evaluated and approved this study (N°: 01–15). This study was carried out at the Proyecto Yacaré (PY) facilities. All eggs were harvested from the Natural Managed Reserve "El Fisco" (San Cristóbal Department, Santa Fe, Argentina), a Protected Natural Area under PY ranching program activities and that has been used by our group as a control area several times (Poletta et al. 2011; Odetti et al. 2020; López González et al. 2022). Embryonic exposures to GLY (Roundup® Full II, 66.2% active ingredient [a.i.], CAS N°. 70901-12-1), CYP (Atanor®, 25% a.i., CAS N°. 52315-07-8) and CPF (Lorsban®, 48% a.i., CAS N°. 2921-88-2) formulations were performed separately and in mixtures by spraying on the nest material in contact with the eggs (Odetti et al. 2020). A total of 135 eggs from 5 nests (27 eggs per nest) were used. They were randomly distributed in 9 experimental groups (EGs) with three replicates of 5 eggs each (total eggs per EGs = 15), placed in a plastic tray using vermiculite as substrate and covering them with vegetal material similar to the nesting material, free of any exogenous substance (Odetti et al. 2020). EGs were as follows: a negative control (NC) treated with distilled water; a vehicle control (VC) treated with ethanol; three groups exposed to CYP (0.12%), CPF (0.8%) and GLY (2%) formulations, three groups exposed to the binary mixtures of them (BM1: GLY + CYP; BM2: CPF + CYP; BM3: CPF + GLY) and one group exposed to the ternary mixture of the three formulations (TM: CYP + CPF + GLY). The concentrations applied correspond to those recommended for field application in soybean crops, considering the surface of the incubation container (0.034 $m^2$) as the area to fumigate (Odetti et al. 2020). The spraying was carried out at an early stage of embryonic development (within 5 days after laying). A single application was made, in which the nest material in contact with the eggs was sprayed with the corresponding solution by means of a manual sprayer, until the whole volume appropriate for the tray surface was finished (approximately 400 µl). Eggs were incubated as routinely done in the PY incubator, under a temperature of 31.5 ± 0.5 °C and 95% humidity (with an incubation period of about 65–75 days).

### Determination of Pesticides

Samples of nest material were taken to determine pesticide concentrations. The samples were analyzed at the PRINARC laboratory (FIQ-UNL). GLY determination was conducted by high-performance liquid chromatography (HPLC) with pre-column derivatization using 9-fluorenylmethyl chloroformate (FMOC-Cl). Derivatization was conducted under alkaline conditions with borate buffer. HPLC conditions were: room temperature, RP-18 5 mm (0.46 cm 15 cm i.d.) column, injection volume 20 ml, flow volume 1.2 ml/min, fluorescence detector (excitation 270 nm, emission 315 nm). The limit of quantification was 0.6 µg/L (Demonte et al. 2018). CYP and CPF determinations were done by Gas chromatographic method (GC-TOF) with a detection limit of 0.10 µg/L. A volume of 1.0 ul is injected into capillary GC in split mode, with flame ionization detection (Regaldo et al. 2017). The recovery rate of this method was: 58% GLY, 106% CYP and 120% CPF.

### Measurement of Expression Levels

Within 5 hours after hatchling, 6 caimans per experimental group were selected (2 per replicate following the order of hatch) and 500 µl of peripheral blood was obtained from the spinal vein with heparinized syringe and 25G x 5/8" needle (Myburgh et al. 2014). Blood was immediately preserved

in TRIzol® LS Reagent (1:4, Ambion, Life Technologies), flash frozen in liquid nitrogen and then stored at -80 °C until analysis. RNA extraction, treatment with RNase free DNAse I (PBL Bio Logical Products), first strand cDNA synthesis (Thermo Scientific®) and qPCR were performed according to our previously described methods (Paravani et al. 2020; López González et al. 2021b, Odetti et al. 2021). The primers (forward-F- and reverse -R-) used for qPCR were: *β-actin*F:5′-TCACGAGACCACTTTCAACTC-3; *β-actin*R:5′-AGGGCTGTGATTTCCTTCTG- 3′; Cu,Zn-*sod*F:5′-GATGAGAGGCATGTTGGAG-3′; Cu,Zn-*sod*R:5′-CCACCATGGTA CGTCCA-3; *cat*F:5′-TGAGCCTAGCCCTGATAAAATG-3′; *catR*:5′-CTCTGATAGT TAGCGACACGAG-3′. Each sample was processed on a StepOne™ Real-Time PCR System (Applied Biosystems™) in duplicated with the *β-actin* as the housekeeping gene (Paravani et al. 2020; Odetti et al. 2021). The percentage efficiency of each curve was: 102% *β-actin*, 96% Cu, Zn-*sod* and 101% *cat*. The $2^{-\Delta\Delta CT}$ method was used to determine the relative expression levels (Livak and Schmittgen 2001).

### Data Analysis

Statistical analysis was performed using SPSS 14.0 software for Windows (SPSS, 2008). Data were tested for normality using the Kolmogorov-Smirnov test and the homogeneity of variances between groups was verified by Levene test. Replicates were compared using the One-way ANOVA. As there were no differences among them, the experimental group was considered as the grouping variable. Mann-Whitney U-test was carried out for the comparison of Cu, Zn-*sod* and *cat* gene expression level. The control groups (NC and VC) were compared to each other and then each exposed group was compared to its corresponding control: NC (distilled water) for the group exposed to GLY (water soluble) and VC (ethanol) for the other groups (water insoluble). Comparisons were also made among each mixture and the individual compounds constituting them. In these cases, a Bonferroni correction was done depending on the number of comparisons made $p \leq 0.025$ (binary mixtures) and $p \leq 0.016$ (ternary mixture). Values were considered statistically significant with $p \leq 0.05$.

## Results and Discussion

For several years, caiman populations have been facing one of the main problems affecting many wild species: habitat loss as a consequence of land being used for agricultural, and potential exposure to pesticides used in such settings (Poletta et al. 2011). The caiman can be exposed as embryos by female transferred through the egg yolk or direct exposure of the eggs from the environment through vegetal material for example, while as juveniles or adults they can be exposed by the presence of pesticides in water and sediments or through dietary intake (Weir et al. 2010). In this work, selected pesticide concentrations corresponded to those recommended for application in soybean crops, simulating conditions faced during the embryonic development of *C. latirostris* under environmental exposure. Results obtained from pesticides analysis on the nest material were: 148.8 mg/kg of GLY, 128 mg/kg of CPF and 3.1 mg/kg of CYP. Different works have reported that GLY, CYP and CPF levels in sediment ranged from 0.035 to 38.0 mg/kg in agricultural environments in Argentina (Primost et al. 2017; Etchegoyen et al. 2017). These concentrations produced no egg mortality in any experimental group. The mean weight of all hatchlings was 42.78 g ± 0.43 (mean ± EE; OHAUS® Compact scale CS200, precision 0.1 g).

Pesticides can be potent inducers of oxidative stress (Liu et al. 2015). They act as prooxidants in a variety of tissues, producing accumulation of reactive oxygen species (ROS), DNA damage and alteration of antioxidant (superoxide dismutase -SOD- and catalase -CAT-) defenses, causing major perturbations to intra- and intercellular homeostasis (Ighodaro and Akinloy, 2018). A large number of studies in different species and tissues have shown that exposure to GLY, CYP and CPF produces ROS and oxidative damage, thus generating deficiencies in exposed organisms, such as inhibition or overproduction of antioxidant defenses (Kaur and Jindal, 2017; Rainio et al. 2019; Paravani et al.2019; Wang et al. 2020). This alteration in the response of the antioxidant system can modify the expression of genes that encode for these enzymes (Liu et al. 2015). These changes are immediate and have been found to be more sensitive than the endpoints traditionally used in toxicology (biomarkers of biochemical alteration, oxidative stress and genotoxicity), so they are relevant and complement markers under stressful conditions (Ghelichpour et al. 2019).

In a previous study in this species, we reported no significant differences in the enzymatic activities of CAT and SOD under the same exposure conditions that this work, but still found significant oxidation of purines and pyrimidines contributing to DNA damage (Odetti et al. 2020). In order to elucidate the molecular mechanisms underlying the toxicity of pesticides and continuing with the integrated evaluation of the effects generated by these compounds, in the present study we analyzed molecular responses of *sod* and *cat* gene expression in blood of *C. latirotris.* Expression levels for Cu, Zn-*sod* and *cat* gene expression levels varies among the treatment groups (Fig. 1). Gene expression of *sod* and *cat* was significantly lower in BM3 in relation to the VC ($p = 0.0357$ in both cases) and the same was observed for

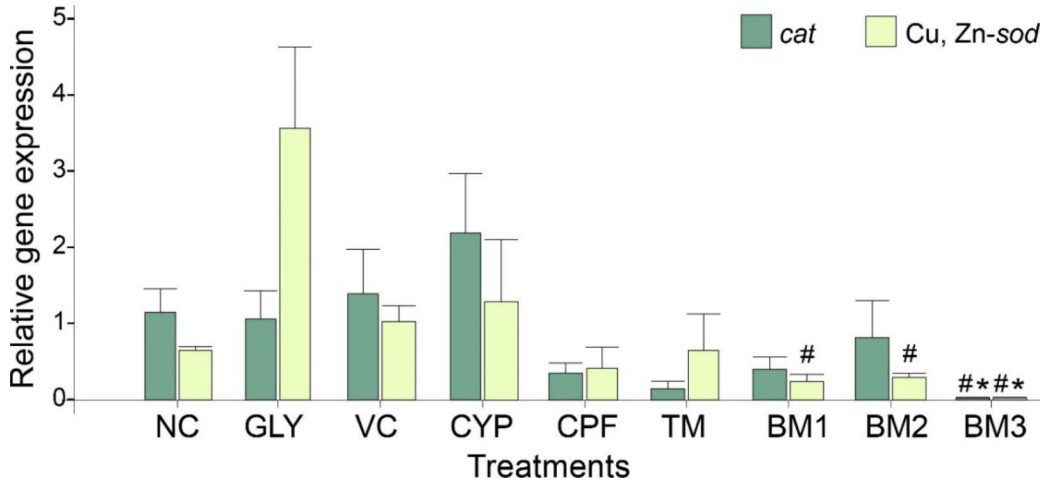


**Fig. 1** Cu, Zn-*sod* and *cat* mRNA abundance (mean ± SD) in blood of *C. latirostris* hatchlings exposed to different pesticide formulations and their mixtures during development. NC: Negative control; VC: vehicle control; GLY: glyphosate formulation at 2%; CPF: chlorpyrifos formulation at 0.8%; CYP: cypermethrin formulation at 0.12%; TM: ternary mixture at field concentrations: CYP + CPF + GLY; BM1: binary mixture of GLY + CYP; BM2: binary mixture of CPF + CYP; BM3: binary mixture of CPF + GLY. #Statistically significant compared to the VC; *Statistically significant compared to GLY.

*sod* in BM1 and BM2 ($p = 0.0357$ and $p = 0.0286$, respectively). No statistically significant effect was observed for any of the compounds separately. In the comparison of the mixtures with the individual compounds, we found a lower expression of both genes (*cat* and *sod*) in BM3 compared to GLY ($p = 0.0071$ and $p = 0.0014$, respectively).

The down-regulation of *sod* and *cat* genes could be due to an inhibition in the regulation of the expression of these genes caused directly by the toxic action of the xenobiotics (Ghelichpour et al. 2019). Possibly, when the antioxidant system cannot eliminate or neutralize the excess of ROS, there is an increased risk of oxidative damage, which in turn can decrease enzymatic activities or even degrade enzymes (Ma et al. 2017). The mechanisms by which an organism recognizes a signal to alter gene expression are diverse and may be due to several reasons. In general, the transcriptional alterations of target genes reflect the activation of defense mechanisms in organisms to counteract the toxicity of ROS against different xenobiotics (Wang et al. 2020). Generally, the gene expression encoding antioxidant enzymes is regulated when the cell is challenged by ROS and is under the control of numerous transcriptional factors activated by ROS (Liu et al. 2015). Some authors suggest that the mRNA levels are representative of cellular activity at a particular time, while protein activity could be regulated at the post-translational level, enzymatically and not by transcriptional control (Ma et al. 2017). Increasing evidence suggests that the alteration in *sod* and *cat* genes expression and the mispairing between activities of antioxidant enzymes and the transcriptional levels follow different patterns depending on the tissue, xenobiotic, and exposure time (Ghelichpour et al. 2019; Wang et al. 2020; Yang et al. 2020). The present results are in line with previous ones in blood of turtles exposed to different environmental pollutants (Héritier et al. 2017a, 2017b; Morao et al. 2022) and other species exposed to GLY, CYP and CPF (Paravani et al., 2019; Ghelichpour et al. 2019; Yang et al. 2020).

On the other hand, SOD requires a metal cofactor for its activity and various forms of the enzyme exist: Cu, Zn-SOD, iron SOD (Fe-SOD) and manganese SOD (Mn-SOD) (Ighodaro and Akinloy, 2018). This means that there are different subtypes of the gene, probably due to alternative splicing, that encode different isoenzymes of SOD. Cu, Zn-SOD is predominant in the cytosol, the intermembrane space and in the extracellular compartment in eukaryotes and in some prokaryotes (Sun et al. 2014). In this work, we use the Cu, Zn-*sod* gene and find significant differences in the three binary mixtures, confirming its sensitivity as a biomarker under different chemical stress conditions (Paravani et al. 2020).

Interactions between mixtures may cause complex changes that are different from the toxic effects exerted by the compounds separately (Varona-Uribe et al. 2016). Such combined toxicological effects may include potentiation, synergism, antagonism or absence of interaction (Brodeur et al. 2014). In this sense, we believe it is important to evaluate the effects that could arise from the combination of two or more chemical components, commonly applied as mixtures to the environment. Regarding the gene expression of the Cu, Zn-*sod* and *cat* genes, we found a possible antagonistic effect in MB3, where CPF seems to interfere with the effect of GLY. As a result, we observe a reduction in the predicted effect for individual compounds, particularly GLY.

Although there are reports in crocodilians on the toxic effects induced by various environmental pollutants through gene expression markers (such as androgen receptors, glucocorticoid receptor, heat shock protein; Kohno et al. 2008, 2019; Sun et al. 2018), this is the first study to use genes of antioxidants pathways in blood to assess pesticide toxicity. It is necessary to continue studying the mechanisms by which the cell detect ROS and induce specific responses at gene expression. Although many studies indicate that cells have the capability, the mode of action of xenobiotics and ROS regulators that activate genes is still unclear (Sun et al. 2014; Liu et al. 2015; Ghelichpour et al. 2019). If gene expression patterns reflect the mode of action of a toxicant, then they could be used as a tool for assessing the risk after short or long-term exposures (Sun et al. 2014). The present study provides new knowledge about the toxicity mechanisms of GLY, CPF and CYP formulations with special focus on interactions between their binary and ternary mixtures in a sentinel organism, a topic poorly studied in Argentina and worldwide. The use of biomarkers routinely applied by our group to evaluate genotoxicity, oxidative stress, biochemical parameters, and growth (Poletta et al. 2011; Burella et al. 2018; Odetti et al. 2020; López González et al. 2021a, 2022), together with the determination of gene expression levels in blood, allow us to perform an integrated and complementary analysis, giving a better perspective of the possible consequences of pesticide exposure for wild populations of *C. latirostris.* In turn, this new methodology is suitable for being used in all crocodilian species, and potentially other wild reptiles, to analyze the early response of individuals under stress, without any damage to the animals.

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