CROCODILE SPECIALIST GROUP
Student Research Assistance Scheme
FINAL REPORT

Project title: Gene expression patterns as biomarkers of pesticide exposure in *Caiman latirostris*

Student: Lucia M. Odetti

Institution: Cátedra de Toxicología, Farmacología y Bioq. Legal, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe Argentina.

Supervisor and member of CSG: Gisela L. Poletta

Introduction

Caiman latirostris is a crocodilian species that inhabits the north-central region of Argentina, south-east Bolivia, Paraguay, north-east Uruguay and south-east of Brazil, and it constitutes a valuable commercial and ecological item in South America (Larriera et al., 2008). Unfortunately, many natural populations of *C. latirostris* are affected by habitat fragmentation and chronic exposure to pesticides, such as glyphosate (GLY), cypermethrin (CYP) and chlorpyrifos (CPF) formulations, among others. Pesticide exposure of natural populations of *C. latirostris* is of great concern, considering the genotoxic and oxidative stress effects that have been found in hatchlings and juveniles under controlled conditions (López González et al., 2017; Burella et al., 2018). Pesticides may induce oxidative stress via a multi-step pathway and it is caused by an imbalance between reactive oxygen species (ROS) and antioxidant mechanisms, leading to a variety of physiological and biochemical changes that cause cell impairment or death (Díaz, 2001). This imbalance may cause damage to many components of the cell, including protein, lipids, DNA and RNA (Muniz et al., 2008). So, the measurement and quantification of oxidative damage is used to study the mechanisms that could explain the effects induced by pesticides. These toxicological endpoints are useful and convenient tools for monitoring environmental quality, assessing chemical risks and safety, investigating threshold values, and evaluating the vulnerability of organisms to specific chemicals or toxins, such as pesticides. Alterations in mRNA patterns offer new insights into the role of genes in the context of toxicity (Ghelichpoura et al., 2019). These changes are immediate and generally more sensitive than the endpoints traditionally used in toxicology, making them relevant markers in various stress conditions (Craig et al., 2007). Several studies in fish and mammals have demonstrated their usefulness as gene expression markers, establishing them as a useful tool for biomonitoring and ecotoxicology tests (Craig et al., 2007; Ghelichpoura et al., 2019). Among crocodilian species, Sujiwattanarat et al (2016) was the first report about molecular cloning and characterization of Siamese crocodile (*Crocodylus siamensis*) copper, zinc- superoxide dismutase gene (Cu, Zn-*sod*), and the analysis of its expression levels in different tissues (liver, pancreas, lung, kidney, heart and whole blood) by quantitative real-time PCR (qPCR). Then, we determined the presence, characterized molecular structure and quantified tissue specific expression of the same gene in *C. latirostris*, including liver, gonads, spleen, heart, and whole blood (Paravani et al., 2020). However, up to the moment, there is no information on modifications in the expression levels of catalase (*cat*) and superoxide dismutase (*sod*) genes in response to pesticide exposure in any crocodilian species. Taking into account that *C. latirostris* is an environmental and economic resource very representative of Argentina and its natural populations can be severely affected by the application of pesticides, it is important to incorporate biomarkers of molecular gene expression markers that, in combination with other types of biomarkers used in our research group, constitutes an important tool for environmental monitoring programs.

Objectives

- Identify and validate *cat* and *sod* genes as potential markers of xenobiotic exposure in *C. latirostris* blood.
- Analyze the expression levels of *cat* and *sod*, and how these relate with oxidative stress profile in blood of *C. latirostris* hatchlings exposed to pesticide formulations under *ex situ* and environmental conditions
- Characterize the oxidative status by determining the activity of the antioxidant enzymes (CAT and SOD) and lipid peroxidation in blood of *C. latirostris* exposed to pesticides under *ex situ* and environmental conditions.

Materials and Methods

1- Phase 1: Identification of genes of interest (*β-actin, cat and sod*)

A- RNA extraction, cDNA synthesis and primer design

We used eight *C. latirostris* hatchlings coming from two different clutches harvested by the *Proyecto Yacaré* (PY) sustainable used program (MAyCC, Gob. Santa Fe, Argentina). Five days after hatching, blood samples (0.5 mL) were taken from the spinal vein with heparinized syringes and 25G x 5/8 needles (Myburgh et al 2014). Blood was immediately preserved in TRIzol® LS Reagent (1:4, Ambion, Life Technologies), flash frozen in liquid nitrogen (LN_2) and then stored at -80 °C until analysis. RNA extraction, treatment with RNasa free DNAse I (PBL Bio Logical Products), first strand cDNA synthesis (Thermo Scientific®) and conventional PCR were performed according to our previously described methods (Paravani et al., 2020; López-Gónzalez et al., 2021a). The presence/absence was determined through conventional PCR of genes of interest such as *β-actin*, *cat* and *sod* in *C. latirostris* blood. Primers used were designed through Integrated DNA Technologies (IDT - https://www.idtdna.com/pages): *β-actinF*: 5´-TCACGAGACCACTTTCAACTC-3´; *β-actinR*: 5´- AGGGCTGTGATTTCCTTCTG- 3´; *sodF*: 5´-GATGAGAGGCATGTTGGAG - 3´; *sodR*: 5´-CCACCATGGTACGTCCA-3´; *catF*: 5´-TGAGCCTAGCCCTGATAAAATG- 3´; *catR*: CTCTGATAGTTAGCGACACGAG.

2- Phase 2: Exposure testing and biomonitoring

A- Exposure tests under *ex-situ* conditions.

A total of one-hundred caimans (20-day-old) from five different clutches (20 caimans from each one) from the raising stock of the PY were used. Each clutch was equally distributed between 5 experimental enclosures, each containing a total of 20 animals. The experimental groups (enclosures) were defined as follows: negative control (NC) treated with tap water; three groups exposed separately to CYP (Atanor®, 0.12%), CPF (Lorsban®, 0.8%) and GLY (Roundup® Full II, 2%) formulations, and one group exposed to the ternary mixture of the three formulations (Mx3: CYP + CPF + GLY). The concentrations were equivalent to those recommended for application in soybean crops. The enclosures were built outdoor within the PY facilities, one separated from each other by a sufficient distance to avoid cross-contamination (more than 100 mtrs). They were approximately 4.5 m^2 in size, closed at the top with small weave fabric to cover and prevent the entry of opportunistic predators (Figure 1). Each enclosure had an artificial water body, a feeder, a drinker and a cover to generate shade.



Figure 1. A) Outdoor enclosure. It can be seen, the effect of herbicide application outside the enclosure; B) hatchlings within the enclosure using the artificial water body.

The experiments were carried out during the period of warm weather (February to April), with a duration of 75 days, under semi-natural (*ex situ*) conditions. All caimans were acclimatized for 10 days in each enclosure under natural conditions before the beginning of the experiment. After acclimatization and immediately prior to pesticides application, all animals were removed from enclosures to avoid direct spraying on them and were returned 24 h later, including the NC, to keep the same conditions than the other groups. Two applications were made using a hand-held sprayer, covering the entire surface of the enclosure from a height of 60 cm. Every 2 days the animals were feed with a reptile formula (mixture of 50% ground chicken head and 50% dry reptile pellets) supplied ad libitum (Larriera et al., 2008).

Fifteen days after pesticide applications, soil and water samples were collected from the enclosures and analyzed by the PRINARC at the Facultad de Ingeniería Química, Universidad Nacional del Litoral. Liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) was used to determine GLY and AMPA and gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) for CYP and CPF (Demonte et al., 2018).

B - Biomonitoring

We obtained samples from animals living in two different natural areas in the north-central region of Santa Fe province, Argentina: one area exposed to pesticides, situated near to agricultural activities centres: “El Lucero” (29° 54’ S; 50° 21’W); and one natural protected area (Law N° 12930, Santa Fe, Argentina), free of any contamination activity “Fisco field” (30º 15’ S; 60º 50’ W), used as a control area. Eggs were collected after the end of the application schedule in the fields and females that were guarding the nest, as well of other adults and juveniles were captured.

3- Measurement of biological responses

A - Sampling

0.5 ml of peripheral blood was taken from the spinal vein of each specimen with a heparinized syringe and TERUMO® 25G x 5/8" (0.5 mm) needle (Myburgh et al., 2014). RNA extraction was performed as described in the previous section. For oxidative stress assessment, whole blood samples were centrifuged, and blood cells were washed twice with physiological solution, lysed using cold double distilled water (1:5) and then kept at -20 °C until analysis (Poletta et al., 2016).

B - Gene expression of *cat* and *sod*

A sample size of n=5 per experimental group was randomly selected to evaluate the stress-response candidate genes through the RT-qPCR technique. RNA extraction, DNAse RNasa free treatment (Invitrogen), and first strand cDNA synthesis (Thermo Scientific®) were performed as previously described. An optimized RT- qPCR protocol was used to analyze the mRNA level of

sod and *cat* genes in duplicate (Odetti et al., 2021). The StepOne™ Real-Time PCR System (Applied Biosystems™) was used, with *β-actin* gene as an internal reference to normalize the expression of the target genes (Odetti et al., 2021). The relative expression level was determined according to the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) and mRNA level of *sod* and *cat* genes was determined.

C- Enzyme activity (CAT and SOD) and Lipid peroxidation (LPO)

CAT (KU/mg protein) and LPO (nmol/mg protein) were determined following the protocol adapted for *C. latirostris* in blood by Poletta et al. (2016). On the other hand, SOD activity (% inhibitions) was determined using the commercial kit 19160-1KT (SIGMA) (Poletta et al. 2016).

D - Statistic analysis

Statistical analysis was performed using SPSS 22.0 software for Windows (SPSS, 2019). For all the parameters analyzed, the data were presented with the values of the mean ± SE. The data were evaluated in normality using the Kolmogorov-Smirnov test and the homogeneity of variance between the groups was verified using the Levene test. One-way ANOVA followed by Tukey's test was used for comparisons of TBARS, CAT and SOD while, the Mann-Whitney test was used for comparisons of the expression of the *sod* and *cat*. For all variables, the exposed groups were compared with the NC. The mixture was also compared with the three individual constituent components. Differences were considered statistically significant at a $p \leq 0.05$.

Result and discussion

A- Identification of genes of interest (*β-actin, cat and sod*)

The integrity of the RNA was evaluated by means of an agarose gel under denaturing conditions, which indicates two well-defined bands corresponding to rRNA (28s and 18s), in addition, the band corresponding to 28s was observed to have an intensity approximately twice than that of the 18s band (Figure 2A). In turn the *β-actin*, *sod* and *cat* genes were identified by conventional PCR (Figure 2B). The presence of the genes and the effectiveness of the designed primers were verified before being used in the qPCR technique.

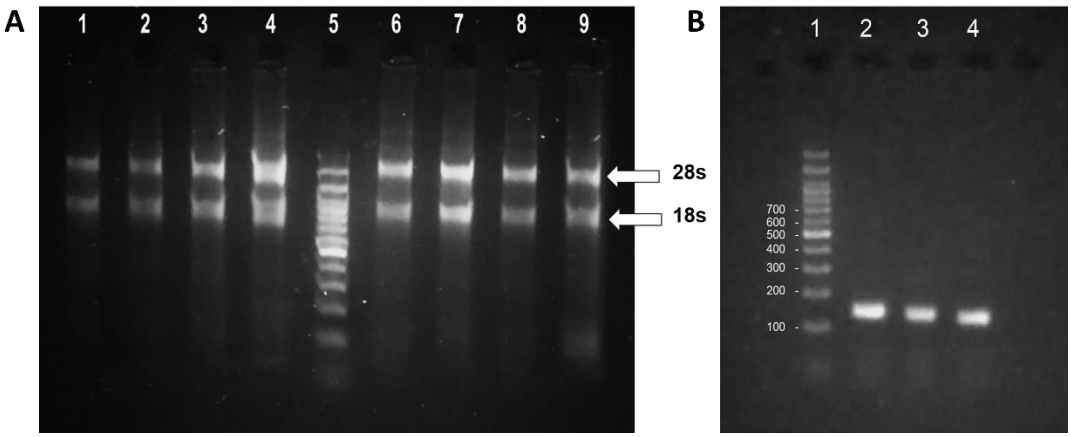


Figure 2. Agarose gel electrophoresis (2%). A) showing RNA extraction of all samples through the protocol adapted for C. latirostris. Lane 1–4 and 6–9: Blood RNA extractions without genomic DNA contamination; Lane 5: ladder 100 bp (Plus DNA - TransGen Biotech). B) Lane 1: ladder 100 bp (Plus DNA - TransGen Biotech), Lane 2: *β-actina* - 138 pb; Lane 3: *cat* - 135 pb and Lane 4: *sod*- 124 pb.

B- Exposure under semi-natural conditions

In order to have a more realistic ecological approach to the toxicological effects of pesticides in the environment, an *ex-situ* study was performed using the concentrations and application schedule recommended in soybean crops. In this type of study, other variables (biotic and abiotic) begin to intervene and is considered an intermediate instance of evaluation between controlled condition studies and biomonitoring. The concentrations of GLY, AMPA, CYP and CPF in soil were 19. 2, 0.02 and 0.35 mg/kg, respectively. In turn, the water levels detected were: 10 mg/kg in GLY; 0.5 mg/L in AMPA, 0.12 mg/L in CYP and 1.8 mg/L in CPF. In the NC, the presence of pesticides was not detected in the soil or water samples.

The *cat* and *sod* mRNA were detected in all samples. Figure 3 A and B depicts the results of mRNA level of these genes. A significant overexpression of *cat* gene was observed in CYP with respect to the NC, but not in the other groups. When Mx3 was compared with the individual compounds, we found differences with respect to CYP in antioxidant genes, being the expression in the mixture significantly lower (Figure 3A and B).

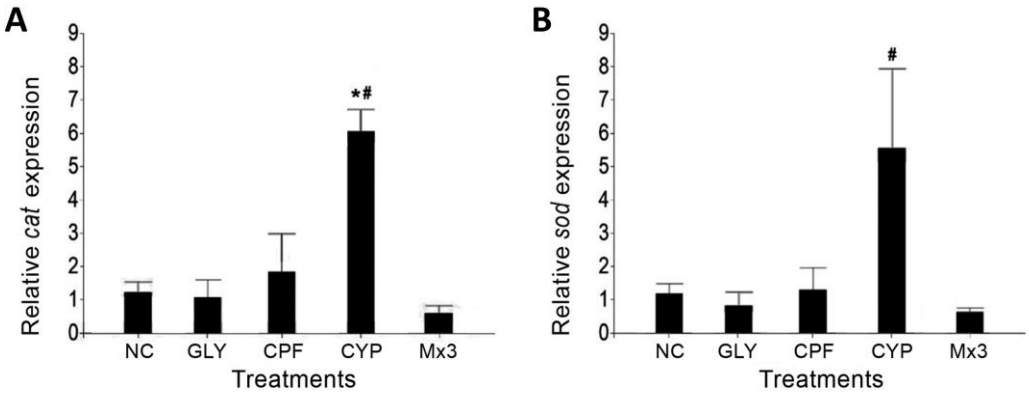


Figure 3: A) *cat* and B) *sod* gene expressions in *C. latirostris* neonates exposed to different pesticide formulations and their mixture under seminatural condition. NC: Negative control; GLY: 2% glyphosate formulation; CPF: 0.8% chlorpyrifos formulation; CYP: 0.12% cypermethrin formulation; Mx3: Mixture of the three formulations at field concentrations: CP+ CPF+ GLY. *$p < 0.05$ statistically significant compared to the NC. #Statistically significant compared to Mx3.

These results suggest that CYP induces the antioxidant system by *de novo* synthesis of CAT enzyme, acting in the first line of response to ROS. This could signify a higher toxicity effect of CYP compared to the other pesticides, or a different mechanism of action of this compound. Similar results have been found in other species (zebrafish, Swiss albino mice; Jin et al., 2010; Paravani et al., 2019). The response observed could be considered as a homeostasis adjustment to protect exposed organisms, as an increase in gene expression could lead later to a higher enzyme activity (Chen et al., 2016). However, in this work, increased expression of the *cat* gene did not correlate with a significant increase in enzyme activity and lipoperoxidation (Table 1). In this sense, Jin et al. (2010) suggest that gene expression may be controlled by post-transcriptional regulation. The non-correlation between antioxidant enzyme activities and transcription of their corresponding encoding genes could be attributed to a delay effect between transcription and translation or post-translational modifications (Singh et al., 2011). Concerning *sod* gene expression, although no significant differences were evidenced between the exposed groups, an increase was observed also in CYP. The lack of difference may be due to the great variation observed in data, that could evidence the need for more extensive sampling. Similarly, other studies made on turtle blood after exposure to different environmental pollutants, a variety of gene expression (*cat* and *sod*) and antioxidant enzyme responses have been observed (Héritier et al., 2017a, 2017b; Morao et al., 2022).

Table 1. Oxidative stress parameters (mean ± SE) analyzed in *C. latirostris* hatchlings for the different experimental groups exposed to formulations of GLY, CYP, CPF and the ternary mixture under *ex-situ* conditions.

Experimental groups	TBARS [nmol/g prot]	CAT [KU/mg prot]	SOD [% inhibitions]
NC	745.43 ± 141.01	66.88 ± 16.27	57.76 ± 8.82
GLY	618.70 ± 79.81	106.21 ± 10.93	61.86 ± 2.34
CPF	842.39 ± 93.23	97.97 ± 17.53	73.42 ± 4.80
CYP	860.03 ± 113.11	94.32 ± 16.96	63.18 ± 7.49
Mx3	782.70 ± 198.51	103.93 ± 9.32	64.86 ± 6.93

TBARS: Thiobarbituric Acid Reactive Substances; CAT: Catalase enzyme activity; SOD: Superoxide dismutase enzyme activity; NC Negative control; GLY: 2% glyphosate formulation; CPF: 0.8% chlorpyrifos formulation; CYP: 0.12% cypermethrin formulation; Mx3: Mixture of the three formulations at field concentrations (CYP + CPF + GLY).

C - Biomonitoring

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 into hydroxyl radicals (OH^-) through the Fenton reaction. Hydroxyl radicals can react with lipids in the cell membranes, which can cause extensive damage (Halliwell, 2012). In the biomonitoring, CAT activity was significantly higher in newborns from exposed areas compared to those for the control area. Contrary, there were no significant differences for CAT activity in adults and SOD activity in adults and newborns (Figure 4 A and B).

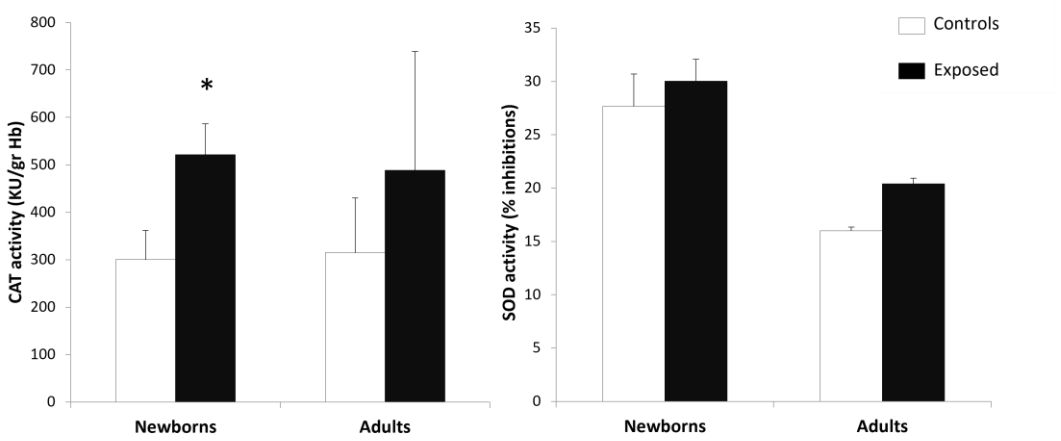


Figure 4: A) Catalase activity (mean ± standard error, expressed as kU/g Hb) and B) Superoxide dismutase activity (mean ± SE, expressed as % of inhibition) in erythrocytes of newborns and adults *C. latirostris*. *Statistically different respect to controls ($p<0.05$).

In turn, the results of lipid peroxidation showed a statistically significant increase in newborns and adults from exposed areas with respect to the controls (Figure 5).

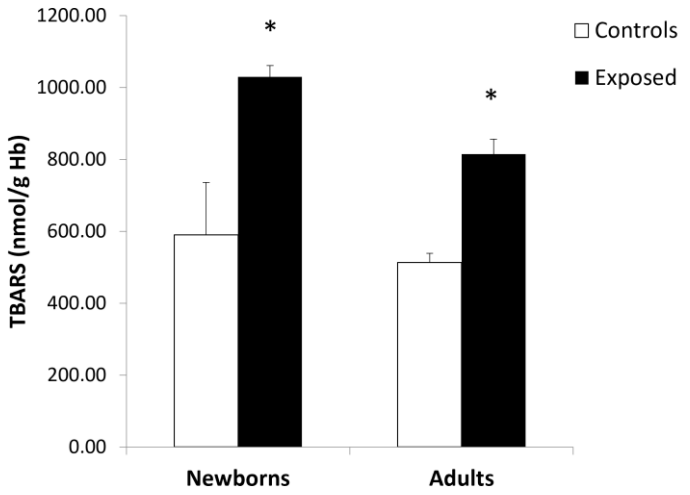


Figure 5: Lipid peroxidation measured through the Thiobarbituric acid reactive substances assay (TBARS: mean± SE, expressed as nmol/g Hb) in blood of newborns and adults *C. latirostris*. *Statistically different respect controls ($p<0.05$).

Studies made in *C. latirostris* under controlled conditions, reported lipid peroxidation and imbalances in the activities of the antioxidant enzymes CAT and SOD in hatchlings and yearlings after exposure to sublethal concentrations of the formulations GLY, ES and CYP (Burella et al., 2018; López González et al., 2021b). In turn, Dzul-Caamal et al. (2016) 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 and CAT activities 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). This work, using markers of oxidative stress in populations environmentally exposed to pesticides, allowed us to link imbalances in oxidative status with factors that could affect health. It demonstrated the need to continue monitoring natural populations of caimans and to increase the number of samples in order to avoid adverse health

effects derived from chronic toxicity of pesticide mixtures. As for the future perspectives of this work, it is expected to contrast the results obtained so far with other markers of different endpoints, specifically with the results obtained from the expression levels that are still being processed.

Reference

Arukwe, A et al (2015) Biotransformation and oxidative stress responses in captive nile crocodile (*Crocodylus niloticus*) exposed to organic contaminants from the natural environment in South Africa. PLoS One 10, e0130002.

Burella, P.M et al (2018) Oxidative damage and antioxidant defense in Caiman latirostris (Broad snouted caiman) exposed in ovo to pesticide formulations. Ecotoxicol. Environ. Saf. 161, 437–443. https://doi.org/10.1016/j.ecoenv.2018.06.006.

Craig, P.M et al (2007) Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*), Am. J. Physiol. Regul. 293,1882–1892.

Demonte, L et al (2018) Determination of glyphosate, AMPA and glufosinate in dairy farm water from Argentina using a simplified UHPLC-MS/MS method. Sci. Total Environ. 645, 34–43. https://doi.org/10.1016/j.scitotenv.2018.06.340

Díaz, L (2001) Radicales Libres en la biomedicina y estrés oxidativo. Rev Cubana Milit 30, 36–44 scielo.sld.cu/scielo.php?script=sci_arttext&pid=S0138-65572001000100007.

Dzul-Caamal, R et al (2016) Usefulness of oxidative stress biomarkers evaluated in the snout scraping, serum and Peripheral Blood Cells of *Crocodylus moreletii* from Southeast Campeche for assessment of the toxic impact of PAHs, metals and total phenols. Comp. Biochem. Physiol. A 200, 35–46.

Ghelichpoura, M et al (2019) Expression of immune, antioxidant and stress related genes indifferent organs of common carp exposed to indoxacarb, Aquat. Toxicol. 208, 208–216.

Halliwell, B., 2012. Free radicals and antioxidants: updating a personal view. Nutr. Rev.70, 257–265.

Héritier, L et al (2017a) Oxidative stress induced by glyphosate-based herbicide on freshwater turtles. Environ. Toxicol. Chem. 3343–3350. https://doi.org/10.1002/etc.3916

Héritier, L et al (2017b) Oxidative stress biomarkers in the Mediterranean pond turtle (*Mauremys leprosa*) reveal contrasted aquatic environments in Southern France. Chemosphere, 332-338.

Jin, Y et al (2010) Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). Chemosphere 78, 846-852. https://doi.org/10.1016/j.chemosphere.2009.11.044

Larriera A, Imhof A, Siroski P (2008) Estado actual de los programas de conservación y manejo de género Caiman en Argentina. In: Castroviejo J, Ayarzaguena J, Velasco A. (Eds.), Contribución al conocimiento del Genero Caiman de Suramerica, Public. Asoc. Amigos de Doña Ana 18, Sevilla, España, pp. 139–179

López González, E.C et al (2017) Micronuclei and other nuclear abnormalities on Caiman latirostris (Broad-snouted caiman) hatchlings after embryonic exposure to different pesticide formulations. Ecotoxicol. Environ. Saf. 136, 84–91.

López González, EC et al (2021b) Biomarkers of genotoxicity, immunotoxicity and oxidative stress on *Caiman latirostris* (Broad-snouted caiman) hatchlings exposed to pesticide formulations and mixtures widely used in agriculture. Environmental Advances 5, 100114 https://doi.org/10.1016/j.envadv.2021.100114

López-González E.C et al (2021a) Optimizing protocols for high-quality RNA extraction from blood and liver tissues of the Broad-snouted caiman. Russ. J. Herpetol. 28: 197-204.

Morao, I et al (2022). Stress response markers in the blood of Sao Tomé green sea turtles (*Chelonia mydas*) and their relation with accumulated metal levels. Environ. Pollut. 293. https://doi.org/10.1016/j.envpol.2021.118490

Muniz, J.F et al (2008). Biomarkers of oxidative stress and DNA damage in agricultural workers: a pilot study. Toxicol. Appl. Pharmacol. 227, 97–107. https://doi.org/10.1016/j.taap.2007.10.027.

Myburgh J.G et al (2014) The post-occipital spinal venous sinus of the Nile crocodile (*Crocodylus niloticus*): its anatomy and use for blood sample collection and intravenous infusions. J. S. Afr. Vet. Assoc. 85: 1–10

Odetti L.M et al (2021) Identification and evaluation of antioxidant and reference genes for quantitative real-time PCR in blood of Caiman latirostris. Heliyon. 7, e06253

Paravani E.V et al (2020) Molecular analysis and bioinformatic characterization of cooper, zinc-superoxide dismutase (Cu/Zn-*sod*) gene of *Caiman latirostris*. Molecular Biology Reports. 47: 8849–8857

Paravani, E.V et al (2019) Cypermethrin induction of DNA damage and oxidative stress in zebrafish gill cells. Ecotoxicol. Environ. Saf. 173, 1-7. https://doi.org/10.1016/j.ecoenv.2019.02.004

Poletta G.L et al (2016) Biomarkers of oxidative damage and antioxidant defense capacity in Caiman latirostris blood. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 179:29-36

Poletta, G.L et al (2016) Biomarkers of oxidative damage and antioxidant defense capacity in Caiman latirostris blood. Comp. Biochem. Phys. C 179, 29-3.

Sujiwattanarat P et al (2016) Molecular cloning and characterization of Siamese crocodile (*Crocodylus siamensis*) copper, zinc superoxide dismutase (CSI-Cu, Zn-SOD) gene. Comparative Biochemistry and Physiology, Part A. 191: 187 – 195