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Research article

A comprehensive approach using multiple biomarkers to detect damage induced by pesticides in broad-snouted caiman (*Caiman latirostris*) under *ex-situ* conditions

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HIGHLIGHTS

- Wild caiman populations under risk of environmental exposures to pesticides.
- *Caiman latirostris* juveniles exposed *ex-situ* during 75 days to pesticides and mixture.
- Pesticides produced DNA damage, MN and NAs, individually and in mixture.
- TWBC counts and growth parameters showed effects mainly at the mixture.
- Biomarkers of genetic damage were more sensible to the effect of pesticides.

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ABSTRACT

Caiman latirostris is one of the two species of the order Crocodylia that inhabit Argentina and is considered a species of vital ecological and economic importance in the north-east of Argentina. In this region, pesticides are the most common contaminants in natural environments and wild caiman populations are subject to this contamination constantly. The aim of this study was to evaluate the effects the main pesticides used in the region: glyphosate (GLY), cypermethrin (CYP) and chlorpyrifos (CPF) -based formulations, as well as the mixture of them, on *C. latirostris* juveniles under semi-controlled condition of exposure (*ex-situ*) during 75 days. One hundred yearling caimans (10-month-old) were equally distributed into five experimental groups (20 animals per group): a negative control (NC -tap water), GLY 2% (Roundup® Full II formulation -RU), CYP 0.12% (Atanor® formulation), CPF 0.8% (Lorsban® formulation), and a mixture of the three pesticides (Mx3: GLY 2% + CYP 0.12% + CPF 0.8%). We applied early warning biomarkers to detect damage induced by these chemicals in peripheral blood: activity of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD), analysis of lipid peroxidation (LPO) by the thiobarbituric acid reactive substances (TBARS), DNA damage and specific base oxidation through the standard and modified comet assay (CA), chromosome damage by micronucleus (MN) test and other nuclear abnormalities (NAs), hematological and growth parameters. Results showed a statistically significant increase in MN and NAs frequency, DNA damage, with an important contribution of base oxidation for all exposed groups compared to the NC. Total white blood cells count (TWBCC), and growth parameters showed effects mainly at the Mx3. The principal component analysis (PCA) demonstrated more sensitivity for biomarkers associated to genetic damage, including base oxidation to DNA than LPO, antioxidant enzyme modulation, immunotoxicity or growth parameters, to detect pesticides effects, applied under conditions similar to that found in natural environments.

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1. Introduction

Pesticides are a major concern in Argentina, as in many other countries in South America. The intensification of productive systems is mainly associated with frequent and increasing use of synthetic pesticides, so that residues have been detected in different environmental compartments worldwide and the potential risk of environmental contamination is growing (Ronco et al., 2016; Etchegoyen et al., 2017). Particularly, Primost et al., (2017) determined the presence of GLY and aminomethylphosphonic acid (AMPA) in soil and water in agro-ecosystems of the Mesopotamian Pampa, Argentina. The maximum concentration levels reported in soil samples were 8105 and 38939 μg/kg for GLY and AMPA, respectively. In the water samples, a maximum value of 1.8 μg/L of GLY and 1.9 μg/L of AMPA was recorded. In turn, Hunt et al. (2016) measured the concentrations of 17 insecticides in sediments collected from streams belonging to the main soybean producing regions of South America (Argentina, Paraguay and Brazil) during periods of high application. The concentrations found ranged from: 1.2–7.4 μg/kg of CPF; 0.9–8.3 μg/kg of CYP; 0.42–16.6 μg/kg of lambda-cyhalothrin and 0.49–2.1 μg/kg of endosulfan (END). It is also important to note that pesticide contamination is a result of complex interactions between the physicochemical properties of the individual constituents of each pesticide commercial formulations, and they can probably act synergistically, antagonistically or additively towards the induction of different alterations in the organisms, and in combination with the environmental conditions (Gaona et al., 2019).

Several studies have reported that systematic exposure to pesticides can cause genotoxicity, biochemical and hematological alterations in different species of vertebrates (Schaumburg et al., 2016; Luaces et al., 2017; López González et al., 2017, 2019; Mestre et al., 2019; Peluso et al., 2020; Odetti et al., 2020; Rossi et al., 2020; Ferré et al., 2020). Among the most widely used pesticides in the world, and particularly in Argentina, are GLY (broad spectrum systemic herbicide) and the insecticides CYP (synthetic pyrethroid) and CPF (organophosphorus).

Caiman latirostris plays vital roles in the ecosystem due to their high trophic status, long life span, and high site fidelity (Larriera et al., 2008). In Argentina, caiman populations are now immersed in areas highly impacted by agricultural activity in the littoral region (planted mainly with soybean, maize, and wheat) that have caused extensive habitat loss and degradation. Besides, the water courses that surround these areas, have residues of different pesticides including GLY, CPF, CYP (Etchegoyen et al., 2017; Gaona et al., 2019) and even endosulfan (END) (Lupi et al., 2019) that is forbidden since 10 years in Argentina. The fact that caimans can be exposed to contaminants in all life stages, their susceptibility to them and possible accumulation, has made it a sentinel species to assess environmental contamination stress.

In this context, in previous studies under laboratory-controlled conditions, it has been shown that pesticides used in predominant agriculture have negative effects on the physiology of broad-snouted caimans, exerting modifications at the genetic level, oxidative balance, and in some components of the immune system, with possible alterations at multi-organ level (Poletta et al., 2009, 2011, 2017; Latorre et al., 2013, 2016; López González et al., 2013, 2017, 2019, 2021; Burella et al., 2017, 2018; Siroski et al., 2016; Odetti et al., 2020). Besides, all these variations could influence the susceptibility of animals to the causative agents of different types of infections and/or diseases, giving rise to a population deterioration of these species.

In comparison to laboratory-controlled condition test, *ex-situ* tests are more realistic and give more relevant information in ecological approaches to evaluated toxicity. In this type of evaluation, we can consider the interactions with some biotic variables, physical variables (such as light intensity, temperature, and water flow rate) and chemical variables (toxic substances: complex mixtures). On the other hand, we can choose developmental stage of the animals controlling natural predators and the exact duration of exposure. In this way, these studies are important for evaluating the sensitivity of different species to particular toxic compounds because they allow to achieve more ecological realism than in controlled laboratory conditions (Costa et al., 2011). Moreover, they represent an intermediate situation between controlled experiments and biomonitoring studies (*in situ*) (Pollo et al., 2019; Bonifacio and Hued, 2019). The CA and the frequency of MN (FMN) have been applied in erythrocytes of the broad-snouted caiman to evaluate primary DNA damage and clastogenic/aneugenic effects produced by different pesticides under controlled conditions in different stages of development (Poletta et al., 2009, 2011, 2017; López González et al., 2013, 2019, 2021; Odetti et al., 2020). Besides, the description of other NAs in erythrocytes of caimans (López González et al., 2017) has become, together with the MN frequencies and the CA, a common procedure for genotoxicity assessment. These abnormalities in the nucleus have been successfully applied in erythrocytes of many vertebrate species exposed to different genotoxic agents and considered in some cases analogous to MN formation (Fenech et al., 2016; Schaumburg et al., 2016; Bonifacio and Hued et al., 2019; Pollo et al., 2019; Benvindo-Souza et al., 2020). LPO is probably the most studied ROS-induced process, which affects structures rich in polyunsaturated fatty acids, while oxidation of bases (purines and pyrimidine) is one of the most common types of DNA damage caused by ROS (Ázqueta et al., 2014). To counteract these actions, antioxidant defenses such as CAT and SOD act continuously in cells (Orbea et al., 2002). Besides, a wide variety of toxicants can act altering the immune system (IS). White blood cells (WBCs) are important components involved in a significant amount of processes associated with the defense mechanism. A wide range of xenobiotics can alter WBC amounts, even at much lower concentrations than needed to have impact on target organs in the short term. Therefore, TWBCC and differential WBC counts (DWBCC) can serve as very sensitive indicators of immunotoxicity (Burns et al., 1996).

The measurement of all the markers mentioned above can provide integrated information for the understanding of the multiple responses caused by pesticides in an organism, and the ecotoxicological consequences of pesticide use (Iturburu et al., 2018). Therefore, the aim of the present study was to evaluated the toxic effect of GLY -RU, CPF Lorsban 48E®, CYP Atanor® formulation, and a complex mixture of them commonly applied in agricultural activities in our country, through biomarkers of genotoxicity, oxidative stress (OS) and immunotoxicity, on peripheral blood of *C. latirostris* juveniles after *in vivo* exposure under semi-natural (*ex-situ*) conditions. A multivariate approach was included in order to assess the contribution of each group of variables to the toxic evaluation of pesticides and decide which are more sensitive for future *in situ* assessment of natural populations.

2. Materials and methods

2.1. Chemicals and reagents

Pesticide formulations were obtained from "Establecimiento La Matuza S.A." (Santa Fe, Argentina) and included: RU (66.2% GLY), CPF Lorsban 48E® (48%) and CYP Atanor® (25%). RU is a liquid water-soluble herbicide (12.000 mg/l), containing GLY potassium salt [N-(phosphonomethyl) glycine monopotassium salt, $C_3H_7KNO_5P$] as its active ingredient (a.i.) (CAS No. 70901-12-1). CPF Lorsban 48E® is a liquid water-insoluble (2 mg/l) insecticide (O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS N°: 2921-88-2). CYP Atanor® is a liquid water-insoluble (0.01 mg/l) insecticide: mixture of different CYP isomers ($C_{22}H_{19}Cl_2NO_3$, CAS No. 52315-07-8) (EXTOXNET, access 2020). Ethanol was used as a vehicle substance for insecticides (CYP and CPF), due to the low solubility of these compounds in water.

2.2. Ethics Committee

The Ethics Committee and Security (ECAS) of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (#258/16, Santa Fe, Argentina) evaluated and approved all experimental protocols. All

animals were managed in accordance with the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals (NSTRC, 2005), and following the "Best Management, Practices for Crocodilian Farming" – Version 1 (Manolis and Webb, 2016).

2.3. Animals

A total of one hundred caimans (10-month-old) were used for this experiment. These animals came from five different clutches (20 caiman from each one) collected during 2017 nesting season, under the ranching program activities of the Proyecto Yacaré (PY) (MAyCC, Gob. Santa Fe–MUPCN, Argentina). The eggs from five clutches were harvested in the Natural Managed Reserve "El Fisco" (30° 11′ 26″ S; 61° 0′27″ W; Dpto. San Cristóbal, Santa Fe, Argentina). This area is a Protected Natural Area (Provincial Law 12,930; 2008) situated at least 20 km far from any pesticide application or other contaminant activity and is part of the natural distribution of caimans (Larriera et al., 2008). After collection, eggs were immediately transported to the PY facilities (Laboratorio de Zoología Aplicada: Anexo Vertebrados, FHUC-UNL/MAyCC, Santa Fe) for artificial incubation under controlled conditions of temperature (30 ± 2 °C) and humidity (100%). After hatching, the caiman were individually identified by a cut in tail scutes, which correspond to the number of the nest and the individual number of each animal (Larriera, 1998). Then, they were maintained under appropriated controlled conditions of raising (cleaning, feeding three times a week, water supply, constant temperature (29 ± 1 °C) and natural light) at holding facilities of the PY, until the beginning of the study.

We used animals from different clutches to control the "*clutch effect*" considering that this effect is one of the most important causes of variability observed in crocodilians (Webb et al., 1987; Verdade, 1997). Also, we used animals of both sexes and the same age (juveniles, 10 months old) to avoid any possible variability between them.

2.4. Experimental design and treatments

The experiment was carried out in a semi-natural condition (*ex-situ*) and were conducted during 75 days, in the summer season (December to February), coinciding with the time of maximum pesticides application in soybean crops in our region and also with the reproduction season of this species.

Animals from each of the five clutches were individualized (as mentioned before) and then were equally distributed into five enclosures: five experimental groups (EGs), with 20 individuals each (four animals from each clutch to each EG), giving a total of 100 animals in the experiment.

The enclosures were built under the PY facilities (experimental area). Each one has approximately 2.5 m in diameter and 1.8 m in height (base area = 4.5 m^2), completely closed at the top with shade cloth to prevent the entry of predators and the escape of the animals. Each enclosure had a shelter, a feeder and an inclined big recipient with water permanently to provide a big space to permit caimans access into the water by volunteer immersion.

All caimans were acclimated for 10 days into each enclosure under natural conditions of temperature, raining regime, humidity and photoperiod. During all the experiment, the animals were fed every 2 days with a mixture of minced chicken and dry pellets for reptiles (50/50) (Larriera et al., 2008), supplied *ad libitum*. Water was refill every day. After acclimation and immediately before the pesticide spraying, the animals were removed from the enclosures to avoid direct spraying over them, and placed again 24 h later. The control group was also removed to keep the same conditions for all the animals.

Three EGs were exposed to pesticides formulations separately: GLY, CPF and CYP, the fourth group consisted in the ternary mixture of all pesticides together (GLY + CPF + CYP), while the last group was used as control, sprayed with tap water. Each enclosure (EG) was separated from the other by a distance of at least 100 m. Concentrations and schedule of application of all pesticides formulations used in this study were determined in relation to agricultural practices recommendations for their application in soybean crop; Table 1. GLY formulation was applied at 2% (2 L/100 L H_2O/ha) twice (day 1 and 30, simulating the pre- and post-plant emergence application). CPF formulation was applied at 0.8% (0.8 L/100 L H_2O/ha) and CYP formulation at 0.12% (0.12 L/100 L H_2O/ha), also in two different moment of the experiment (day 30 and 45). In all cases, application was done considering the surface of each enclosure (4.5 m^2) as the area to be sprayed and the solutions were applied covering the whole surface from a height of 0.5 m. Table 1 described the experimental design applied.

2.5. Pesticides analysis

Soil and water samples were collected from each enclosure 15 days after the first application of pesticides at each EGs. The samples were analyzed at the PRINARC laboratory (FIQ-UNL). The determination of GLY and aminomethylphosphonic acid (AMPA) was carried out by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC MS/MS) following the methodology of Demonte et al. (2018). The CYP and CPF determinations were done by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) according to the procedure of Regaldo et al. (2018). The methodologies were validated according to the guidance document of the European Commission (2017) on analytical quality control and the method validation procedure for pesticide residues, SANTE/2017/11813.

2.6. Blood sampling and variables analyzed

After the exposure period (75 days), the animals were removed by hand, from the enclosures, one per time, to minimize stress by handling, and peripheral blood samples were taken from the spinal vein (Myburgh et al., 2014) within 5 min of capture, using heparinized syringes. Blood samples (0.5 ml per animals) were preserved immediately in eppendorf tubes and kept cool until biomarkers process.

Table 1. Experimental groups of *Caiman latirostris* yearlings exposed to glyphosate-, chlorpyrifos-, cypermethrin-pesticide commercial formulations and their ternary mixture.

Experimental group	Compound	Concentration applied (%)	Time of application (days)	Nº animals per clutch/ experimental group	Nº total animals/ experimental group
Negative control (NC)	Tap water	100 mL	1, 30 and 45	4	20
GLY	Glyphosate: Roundup Full II®	2%	1 and 30	4	20
CPF	Chlorpyrifos Lorsban 48E®	0.8%	30 and 45	4	20
CYP	Cypermethrin Atanor®	0.12%	30 and 45	4	20
Mx3	GLY + CPF + CYP	2% + 0.8%+ 0.12%	1: GLY 30: GLY, CPF and CYP 45: CYP and CPF	4	20

2.6.1. Genotoxicity biomarkers

The MN and other NAs tests were performed on *C. latirostris* erythrocytes according to the techniques described by Poletta et al. (2008) and López González et al. (2017).

Immediately after sampling, a small amount of blood from each animal was smeared over two slides, fixed with ethanol for 10 min and stained with Giemsa (10%) for 15 min.

A total of 1,000 erythrocytes per animal were analyzed under the optical microscope mentioned before at 1,000x magnification and the frequencies of MN/NAs were determined (FMN/FNAs: number of cells with MN or other NAs/1,000 erythrocytes counted).

The NAs was classified in five categories: notched nuclei (NN, appreciable depth into a nucleus that does not contain nuclear material), nuclear buds or "budding" (nuclear evaginations), binucleated cells (BiN, cells under uncompleted division, with two completely separate nuclei in the same erythrocyte cytoplasm), eccentric nuclei (EN, cells with the nucleus in an abnormal peripheral position), and the sum of total NAs observed (TNAs).

The protocol used of CA was based on the technique proposed by Poletta et al. (2008). A single DNA damage index (DI = n1+2 n2+3 n3+4 n4) was calculated for each animal, where n is the number of nucleoids of each category (1–4), classified among 100 analyzed nucleoids.

2.6.2. Oxidative stress biomarkers

2.6.2.1. Determination of lipid peroxidation. The TBARS method adapted for application on *C. latirostris* blood (Poletta et al., 2016) was used. The absorbance of the sample was determined at 535 nm by spectrophotometer and is expressed as nmol/mg protein.

2.6.2.2. Catalase activity (CAT). The method described was adapted in blood for this species by Poletta et al. (2016). One unit of CAT is considered to be equivalent to the amount of protein required to decompose 1μM of H2O2 per minute under specific conditions. The specific CAT activity was referenced to the amount of protein in the sample and expressed in KU/mg protein.

2.6.2.3. Superoxide dismutase (SOD) activity. SOD activity was determined using the commercial kit 19160-1KT (SIGMA). The activity is quantified by the decrease in color at 440 nm (Poletta et al., 2016).

2.6.2.4. Protein determination. Protein concentration was determined with the commercial kit 1690007 Proti U/LCR (Wiener Lab®, Rosario, Argentina).

2.6.2.5. Oxidative DNA damage. To discriminate DNA damage produced by base oxidation, Formamidopyrimidine-DNA-glycosylated (FPG) and Endonuclease III (ENDO III) were used following the technique of Poletta et al. (2016).

2.6.3. Immunological biomarkers

TWBCC and DWBCC were determined as biomarkers of immunotoxicity. The TWBCC was done with a Neubauer chamber in a dilution of 1:200 of whole blood in NaCl (0.6%). The samples were examined under the optical microscope Nikon Eclipse E200 at 400x magnification, determining the amount of WBC per mm3 blood (Lewis et al., 2008). For the DWBCC, two blood smears were made per animal, fixed with ethanol (10 min), stained with May Grünwald - Giemsa solution (50%; 3 min and 10%; 15 min, respectively) and the proportion of each WBC type/100 (heterophils, lymphocytes, monocytes, and eosinophils) was registered under the same optical microscope mentioned before, at 1000x magnification. Heterophil/lymphocyte ratio was then determined and used as a stress indicator (Lance and Elsey, 1999).

2.6.4. Growth

All animals were measured in snout-vent length (SVL) and total length (TL) (precision 0.1 cm) and weighed (Electronic Compact Scale, TH 5000, precision 0.1–1 g), before and after exposure to evaluate the effect of pesticides on caiman growth (final - initial values).

2.6.5. Statistical analyses

Statistical analysis was performed using the SPSS statistical package software for Windows (SPSS, 2013). For all the parameters analyzed, data were presented as mean values ±standard error (S.E.), calculated from all the animals of each experimental group. Variables were tested for normality with Kolmogorov-Smirnov test and homogeneity of variances between groups was verified by Levene test. To analyze the *clutch effect,* the nest of origin was considered as a grouping variable. The difference between nests for all variables were analyzed using the ANOVA following by Tukey's test or Kruskal-Wallis followed by Dunn-Bonferroni post hoc test, depending on the distribution of data. In order to compare the effects of pesticide treatment, the ANOVA followed by Tukey's post hoc multiple comparison test was applied for the DI of the CA, the most of NAs frequencies (buds, NN, EN, and TNAs) eosinophils, TBARS, CAT, SOD and TWBCC. In the case of non-parametric data, the Kruskal-Wallis followed by Dunn-Bonferroni post hoc test was carried out for the FMN, BiN, FPG- and ENDO III -sites, TL, SVL, and weight. Finally, a PCA was performed to evaluate the interaction of those variables that showed significant differences among experimental groups. Data included in the PCA analysis belong to 34 animals, those with have values for all the 8 variables analyzed: five of genotoxicity (DI, FMN, buds, NN, TNAs), one of immunotoxicity (TWBCC), and two of OS (ENDO III and FPG sites), as well as the five EGs (NC, GLY, CPF, CYP and Mx3). Total variability explained by the model was 94.5%, with a distribution of 66.1% for PC1 and 28.4% for PC2; while three components explained 99% of the total variability.

3. Results

3.1. Analytical pesticide determinations

The results obtained from the analytical determinations of pesticides are presented in Table 2.

3.2. Genotoxic biomarkers

Results indicated an induction of all genotoxicity parameters evaluated. The FMN showed a significant increase for all exposed groups respect to the NC (GLY: $p = 0.001$; CPF and CYP: $p = 0.002$; Mx3: $p < 0.001$, Figure 1). In the case of other NAs frequencies, it was demonstrated an induction in buds with significant differences between the three individual compounds (GLY, CPF, and CYP) compared to both the

Table 2. Analytical determination of pesticides in soil and water of the enclosures 15 days after application of the corresponding formulations.

Experimental group		Active ingredient - concentration [mg/L]			
		GLY	AMPA	CYP	CPF
Water	NC	ND	ND	ND	ND
	GLY	19.20	2.00	ND	ND
	CYP	NA	NA	0.02	ND
	CPF	NA	NA	ND	0.35
		Active ingredient - concentration [mg/Kg]			
Soil	NC	ND	ND	ND	ND
	GLY	10.50	0.5	NA	NA
	CYP	NA	NA	0.12	ND
	CPF	NA	NA	ND	1.8

NC: negative control; GLY: glyphosate-based formulation Roundup®, AMPA: aminomethylphosphonic acid, CPF: chlorpyrifos-based formulation Lorsban 48E® and CYP: cypermethrin-based formulation Atanor®; NA: not analyzed; ND: not detected.

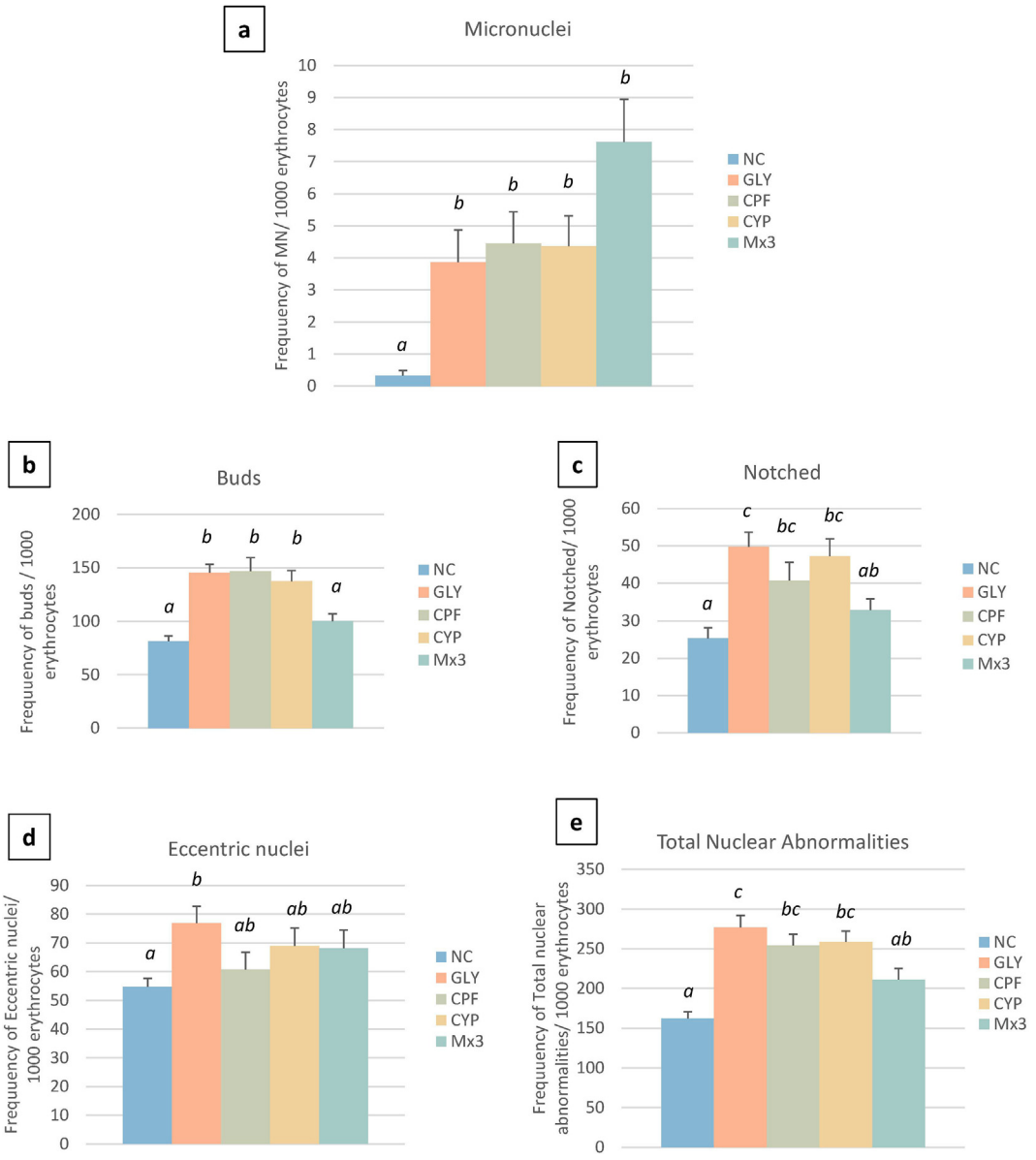

Figure 1. Frequency of nuclear abnormalities observed in *Caiman latirostris* erythrocytes at different experimental groups (a) Frequency of micronuclei (FMN) (b-e) Frequency of other nuclear abnormalities (FNAs). NC: negative control; GLY: glyphosate-based formulation Roundup®, CPF: chlorpyrifos-based formulation Lorsban 48E® and CYP: cypermethrin-based formulation Atanor®, and Mx3: mixture of the three commercial pesticides formulations (GLY + CPF + CYP). Different letters (*a, b* and *c*): indicate significant differences between experimental groups.

NC ($p < 0.001$) and Mx3 (GLY: $p = 0.001$; CPF and CYP: $p = 0.003$); while NN revealed a significantly higher frequency in GLY respect to the NC ($p < 0.001$) and compared to Mx3 ($p = 0.017$), as well as in CYP ($p < 0.001$) and CPF ($p = 0.046$), compared to the NC. Besides, in EN the difference was evident only in GLY compared with the control group ($p = 0.014$), and finally TNAs frequencies showed differences for GLY, CPF and CYP respect to the NC ($p < 0.001$), as well as in GLY respect to Mx3 ($p = 0.005$); while a tendency to increase was observed in the Mx3 respect to the NC but they did not reach a statically significant difference ($p = 0.056$) (Figure 1). No significant differences were observed in the BiN erythrocytes.

The results showed a significant increase in DNA damage index for all the groups with respect to the NC ($p < 0.001$, Figure 2), but no differences were observed between the mixture and the individual compounds.

3.3. Oxidative stress parameters

Figure 3 shows the significant differences observed in FPG and ENDO III in all treatments with respect to the NC ($p = 0.006$ and $p = 0.007$, respectively), but no differences were observed between the Mx3 and the individual compounds (Figure 3).

In relation to the other parameters evaluated for OS, no significant differences were observed in TBARS levels, CAT and SOD activities (Table 3).

3.4. Immunotoxic biomarkers

Immunological parameters are presented in Table 4. The analysis showed a significant higher TWBCC in GLY and CYP respect to the Mx3 ($p = 0.0037$ in both cases).

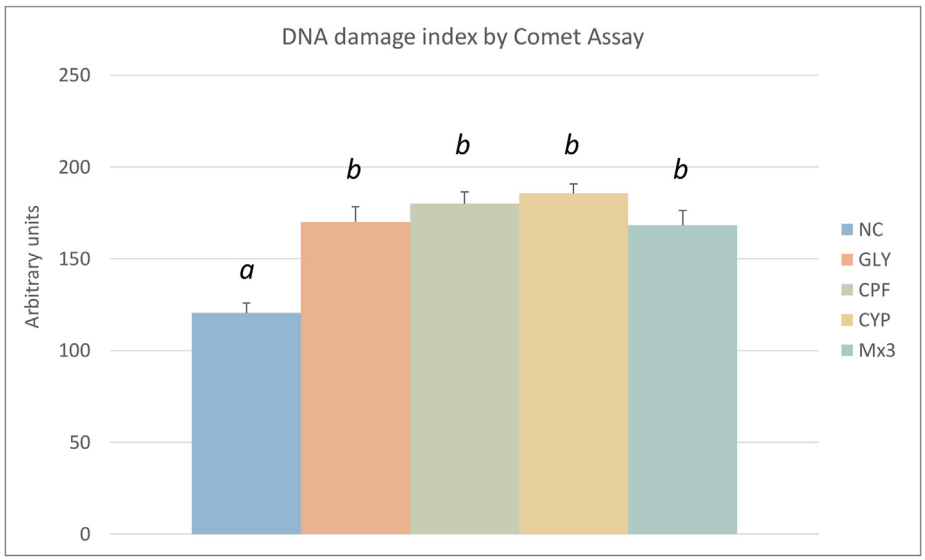

Figure 2. DNA damage detected through the comet assay observed in *Caiman latirostris* erythrocytes in the different experimental groups; NC: negative control; GLY: glyphosate-based formulation Roundup®, CPF: chlorpyrifos-based formulation Lorsban 48E® and CYP: cypermethrin-based formulation Atanor®, and Mx3: mixture of the three commercial pesticides formulations (GLY + CPF + CYP). Different letters (*a* and *b*): indicate significant differences at $p < 0.001$.

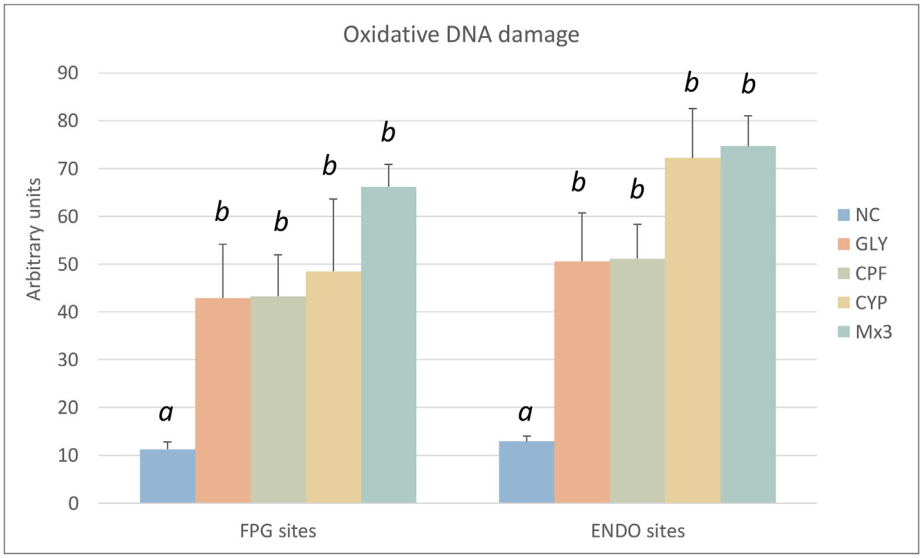

Figure 3. Oxidative DNA damage detected through the modified comet assay in *Caiman latirostris* erythrocytes of the different experimental groups; NC: negative control; GLY: glyphosate-based formulation Roundup®, CPF: chlorpyrifos-based formulation Lorsban 48E® and CYP: cypermethrin-based formulation Atanor®, and Mx3: mixture of the three commercial pesticides formulations (GLY + CPF + CYP). FPG: Formamidopirimidina-DNA-glycosylated sites; ENDO III: Endonuclease III sites. Different letters (*a* and *b*) indicate a significant difference Kruskal-Wallis/Dunn-Bonferroni post hoc test).

Table 3. Biomarkers of oxidative status (mean ± standard error) observed in erythrocytes of the control and treated caiman.

Experimental groups	TBARS (nmol/mg protein)	CAT (KU/mg protein)	SOD (% inhibition)
NC	2.27 ± 0.36	215.86 ± 37.42	65.81 ± 3.50
GLY	2.85 ± 0.32	162.50 ± 31.48	82.14 ± 4.72
CPF	2.95 ± 0.40	191.53 ± 34.61	71.52 ± 6.40
CYP	2.58 ± 0.50	224.25 ± 67.99	61.41 ± 1.49
Mx3	2.47 ± 0.26	166.87 ± 25.92	67.21 ± 6.32

TBARS: thiobarbiturate acid reactive substances; CAT: Catalase enzyme activity; SOD: Superoxide dismutase enzyme activity; NC: negative control; GLY: formulation of glyphosate (Roundup®), CPF: formulation of chlorpyrifos (Lorsban 48E®) and CYP: formulation of cypermethrin (Atanor®), and Mx3: mixture of the three commercial pesticides formulations (GLY + CPF + CYP).

3.5. Growth

Growth parameters of the animals were affected by the exposure to some of the pesticides (Table 5). We observed the same effect in TL and SVL parameters, where animals of the Mx3 showed a significant higher growth in comparison to the NC ($p_{TL} = 0.047$ and $p_{SVL} = 0.036$) and the group exposed to GLY ($p_{TL} = 0.001$ and $p_{SVL} = 0.011$), while a tendency to a higher growth (SVL) was observed in CYP respect to NC but without statically significant difference ($p = 0.057$).

In the case of weight of the animals, we observed a significant higher growth in animals of the Mx3 compared to those of GLY ($p = 0.040$) and CPF ($p = 0.011$).

No differences were found in any of the variables analyzed for genotoxicity, OS, immunotoxicity or growth considering the clutch of origin.

Table 4. Total and differential white blood cells count (mean ± standard error) evaluated in juveniles of *Caiman latirostris* at different experimental groups exposed to commercial pesticide formulations of glyphosate, cypermethrin, chlorpyrifos and the ternary mixture under semi-natural conditions.

Experimental groups	TWBCC/mm^3 blood	Heterophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
NC	22475 ± 1833.00 ^{ab}	18.67 ± 2.26	77.78 ± 2.45	1.17 ± 0.40	2.28 ± 0.47
GLY	29852.94 ± 1988.17 ^{b}	17.7 ± 3.03	79.2 ± 3.28	1.5 ± 0.54	1.6 ± 0.87
CPF	27192.31 ± 2273.56 ^{ab}	19.43 ± 3.62	75.71 ± 3.92	0.71 ± 0.64	4.14 ± 1.06
CYP	29571.43 ± 2190.85 ^{b}	19.45 ± 2.89	73.82 ± 3.13	2.36 ± 0.51	4.27 ± 0.93
Mx3	20076.92 ± 2273.56 ^{a}	17.55 ± 2.89	78.55 ± 3.13	1.27 ± 0.51	2.55 ± 0.41

NC: negative control; GLY: glyphosate-based formulation Roundup®, CPF: chlorpyrifos-based formulation Lorsban 48E®, CYP: cypermethrin-based formulation Atanor®, and Mx3: mixture of the three commercial pesticides formulations (GLY + CPF + CYP). TWBCC: Total white blood cells count. Different letters (*a* and *b*) indicate significant differences at p = 0.0037 between these groups (Mx3 vs CYP and GLY).

Table 5. Growth (mean ± standard error) parameters analyzed on yearlings of *Caiman latirostris* for the different experimental groups exposed to commercial pesticide formulations of glyphosate, cypermethrin, chlorpyrifos and in the ternary mixture under semi-natural conditions.

Experimental groups	TL (cm)	SVL (cm)	Weight (g)
NC	1.36 ± 0.58 ^{a}	0.50 ± 0.13 ^{a}	15.60 ± 5.85 ^{ab}
GLY	0.94 ± 0.58 ^{a}	0.41 ± 0.16 ^{a}	10.94 ± 6.48 ^{a}
CPF	2.04 ± 0.78 ^{ab}	1.19 ± 0.26 ^{ab}	14.08 ± 23.94 ^{a}
CYP	3.00 ± 0.70 ^{ab}	1.58 ± 0.35 ^{ab}	52.00 ± 27.51 ^{ab}
Mx3	3.27 ± 0.48 ^{b}	1.58 ± 0.30 ^{b}	98.38 ± 18.00 ^{b}

NC: negative control; GLY: glyphosate-based formulation Roundup®, CPF: chlorpyrifos-based formulation Lorsban 48E® and CYP: cypermethrin-based formulation Atanor®, and Mx3: mixture of the three commercial pesticides formulations (GLY + CPF + CYP). TL: total length; SVL: snout-ventral length; cm: centimeters; g: grams. Different letters (*a* and *b*) indicate significant differences at p ≤ 0.05 between these groups.

3.6. Multivariate integrated approach

The biplot obtained from the PCA performed with those variables that gave significant difference among treatment is shown in Figure 4. The PC1, was positively correlated with all variables while the PC2 was positively correlated with TWBCC (immunotoxicity) and most genotoxicity variables, while was negatively correlated with the FMN, DI, and oxidative damage to DNA (FPG and ENDO III sites). Besides, considering EGs, we found that the NC was separated from all the exposed groups and variables indicating damage, and that those variables concerning genotoxicity and oxidative damage to DNA seem to be the more efficient to detect the effect of pesticides.

Among nuclear abnormalities, the FMN seem to be more related to the effect of the Mx3, while pesticide formulations separately (GLY, CYP and CPF) induced preferably other type of NAs (buds, NN and TNAs).

4. Discussion and conclusions

The environmental conditions and contaminants produced by human activities may influence the health status of wildlife, and correlate to different alterations that can be investigated as biomarkers of their effect. The analysis of hematological alterations have been widely employed in ecotoxicological risk assessments of stress condition in native reptiles after exposure to pesticide compounds (Latorre et al., 2013; 2016; López González et al., 2017, 2019; Mestre et al., 2019). Overall, we consider that the integration of all hematological biomarkers included in the battery of tests applied here, are useful tools to detect the effects of pesticides and might indicate the health status of caiman populations in a representative manner.

The genotoxicity tests and oxidative damage to DNA evaluated in the present study, seem to be more sensitive tools in determining the potential toxicity of pesticides, considering that they responded better than other OS and the IS parameters.

MN is one of the most used methods to detect the early level of genetic damage induced by clastogenic or aneugenic agents in sentinel organisms exposed to environmental contaminants (Fenech et al., 2016). Besides, other NAs are indicative of adverse cellular reactions and/or control mechanisms used to eliminate cells with damaged DNA, due to toxico-kinetic of the contaminant, hematopoietic cycle speed or incorrect or inefficient DNA repair, and have been interpreted as nuclear lesions analogous to MN (Pollo et al., 2019; Benvindo-Souza et al., 2020). In the present work, there were a significant increase in the FMN and other FNAs (buds, NN, TNAs and EN) except for the BiN, in almost all exposed groups, respect to the NC. In previous studies, we validated the NAs together with MN formations as genotoxic endpoints in response to possible stressor compounds on *C. latirostris* (López González et al., 2017; Poletta et al., 2017). In turn, recently studies have reported a high increase in the FMN and FNAs in hatchlings and yearlings of broad-snouted caiman exposed under controlled conditions to environmentally relevant concentrations of the same pesticide formulations tested in this study and their mixtures (López González et al., 2019, 2021; Odetti et al., 2020). Previously, we also registered a significant difference in the FMN between the NC and all the groups of neonates exposed *in vivo* to the same commercial formulations of the herbicide GLY (RU 11 mg/L and 2.5 mg/L), showing a concentration-dependent effect (López González et al., 2013). Several studies have indicated an increase in MN and other NAs in different species exposed to pesticides (Bonifacio and Hued, 2019; Pollo et al., 2019; Benvindo-Souza et al., 2020).

Our findings also showed that buds increased at all EGs with a sensibility very similar to that observed for the FMN, but other NAs showed more variability in their responses. In the same way, the CA showed DNA strand breaks in *C. latirostris* yearlings exposed to GLY, CYP, CPF, and the mixture of them in semi-natural conditions, at concentrations equivalent to those recommended in agricultural practices for application in soybean crops. Collins (2004) define the CA as a sensitive test, since it can detect early damage to DNA, identifying single strand breaks and maximizing the expression of alkali-labile sites in the DNA molecule. Different studies have reported DNA damage on peripheral blood of *C. latirostris* hatchlings exposed to different concentration of GLY (PanzerGold® and Roundup® Full II), END (Galgofan®), CYP (Atanor®) and CPF (Lorsban®) formulations (Poletta et al., 2009, 2011; Burella et al., 2017, 2018; Odetti et al., 2020). These studies were applied during the embryonic period by topical application through the eggshell or by spraying on nesting material. Besides, in *in vivo* studies on broad-snouted caiman neonates, we could observe genotoxicity effects induces by the same complex mixtures applied in the present work (Poletta et al., 2017; López González et al., 2021). Both studies were performed under laboratory-controlled conditions during 60 days of exposure with concentrations that were progressively reduced in time during the experiment in order to simulate the degradation of the compounds in water. In the present study and in total agreement with previous reports on this species, the CA showed high sensitivity for assessing primary DNA damage. Besides, several studies also showed the same effect for CYP, CPF, GLY, and their mixtures in different combinations, on different

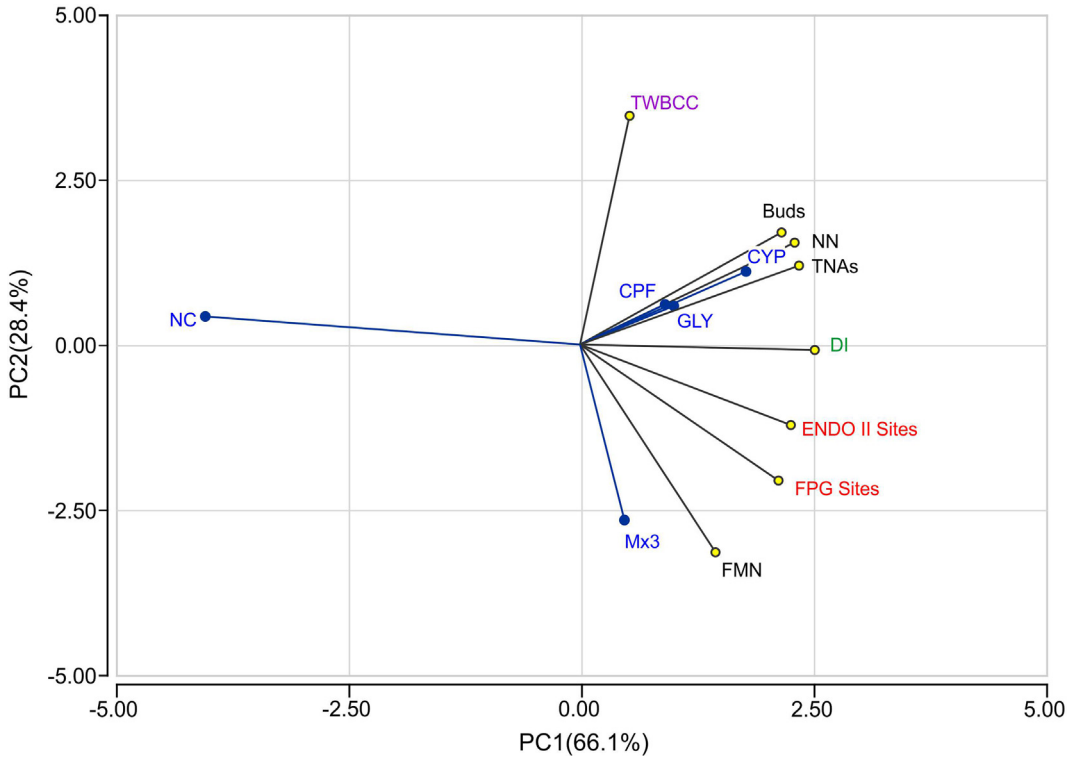

Figure 4. Biplot of the principal component analysis including genotoxicity, oxidative damage and immunotoxicity variables for the first two principal components (accumulated variability 94.5%) in *Caiman latirostris* peripheral blood samples. PC1: principal component one; PC2: principal component two; TWBCC: Total white blood cells count (violet); NN: Notched nuclei; TNAs: Total nuclear abnormalities; FMN: Frequency of micronuclei (black); ENDO sites: Endonuclease III sites; FPG sites: Formamidopyrimidine DNA glycosylase sites (red); DI: damage index (green); NC: Negative control; GLY: Glyphosate commercial formulation Roundup Full II®; CPF: Chlorpyrifos commercial formulation Lorsban 48E®; CYP: Cypermethrin commercial formulation Atanor®; Mx3: mixture of Glyphosate + Cypermethrin + Chlorpyrifos formulations (blue).

species such as fish, amphibians, reptiles and mammals (Mañas et al., 2009; Schaumburg et al., 2016; Paravani et al., 2019).

The MN and other NAs tests and the CA are the most used biomarkers to investigate genotoxic agents. Our results also probed that these biomarkers were very sensitive indicators of genetic damage induced by the pesticide formulations and mixtures tested here, even in conditions very similar to that expected for natural population exposure.

Environmental contaminants such as pesticides can cause OS by producing ROS. Under normal conditions, antioxidant enzymes provide adequate protection against free radicals and ROS (Mañas et al., 2009). However, deregulation of only one of these enzymes could seriously affect cell defense mechanisms. In this work, no significant differences were found in the activities of CAT and SOD, or in TBARS levels in any of the exposed groups compared to the controls. On the other hand, oxidation of purines and pyrimidines was observed though the modified CA with specific endonucleases ENDO III and FPG in all exposed groups. ROS are involved in various types of DNA lesions, being base oxidation one of the most frequent (Ázqueta et al., 2014). Several studies related this type of lesion with the exposure to pesticides at different concentrations (Soloneski et al., 2017; Ruiz de Arcaute et al., 2019). In this work, we observed that the results of LPO and antioxidant defenses are not correlated with the significant results in genotoxicity and oxidative damage to DNA. In this sense, our result allows us to suppose that excess of ROS has produced protein toxicity, affecting antioxidant enzymes and, as a consequence, the DNA was altered by base oxidation (Burella et al., 2018; Odetti et al., 2020). However, the effect of ROS was not enough to produce LPO. Regarding the comparison of the mixture with its constituent components, no interaction was observed between the components of the mixture in CA, FMN and the oxidation of the DNA bases.

The WBCs (heterophils, lymphocytes, monocytes, and eosinophils) participate in the defense mechanism of all vertebrates. Lymphocytes produce antibodies, while monocytes remove the debris of the injured cells. In the current study any of the exposed groups showed significant differences compared to the controls, but there were a significant lower value for the Mx3 when compared to GLY and CYP. This can be interpreted as some kind of antagonist effect among the individual compounds in the mixture, which showed the opposite tendency than that observed for the compounds separately, with respect to the control. In previous studies, we reported alterations in the TWBC on neonate caimans in an *in vivo* assay under controlled conditions and simulating the natural degradation of pesticides in water bodies, with the same formulations and mixture applied in the present work. We observed a significant increase in TWBC in GLY (RU) compared to the control group; and the contrary effect on the groups exposed to another GLY formulation (PanzerGold®), CPF, and Mx3. This difference in the responses may be explained by the fact that RU and PanzerGold® are herbicides with different formulations that include different types of additives and adjuvants, and although they use the same a.i. active principle, GLY, the mode of action of the complete formula, that is a mixture itself, could be somewhat different (López González et al., 2021). Besides, in another study, neonates exposed to the same commercial formulation of GLY applied in the present work (RU) showed a significant decrease in TWBCs related to NC, and in this case, authors pointed to a possible reason could be that newborns have there IS still immature (Latorre et al., 2013). Mestre et al. (2019) observed similar results in Argentinian tegu lizard (*Salvator merianae*) neonates exposed to the same complex mixture used in the present study (GLY + CPF + CYP) under similar semi-natural experiment applied here, postulating a possible immunosuppression event as a consequence of this effect. In non-reptile species, Modesto and Martinez (2010), evaluated the effects of Roundup Transorb® on the neotropical fish *Prochilodus lineatus*, observing a significant increase in TWBC after 96 h of exposure. All these different patterns of response

probably represent adaptive responses that would help the organisms to counteract the herbicide effects (Barreto-Medeiros et al., 2005). Concerning insecticide effect, Bacchetta et al. (2011), postulated that the leukocytosis they observed on the neotropical fish *P. lineatus* can be attributed to a greater leukocyte mobilization to protect the body against possible infections in the tissues damaged by the insecticide END. Other authors argue that an increase in TWBCC exposed to insecticides reflects a general state of toxicosis, which results in a deterioration of defense mechanisms (Ramesh and Saravanan, 2008). Pesticides formulations applied at sublethal concentrations can compromise the immunity of the organisms, disturb their normal physiology, even at the behavior, growth, development and/or reproduction, and can influences survival, or makes them more susceptible to infections with external agents (Ullah et al., 2019).

The growth is an integrated response of numerous physiological processes, which influence the performance of the individual (Amaral et al., 2012). Our results revealed different effects in the growth parameters. We found no significant differences in the size of the animals among different treatments at the initial of this study or in the growth of those animals exposed to the compounds separately. However, the Mx3 showed significant increases on length (SVL and TL) with respect to the NC and GLY as well as a higher weight compared to GLY and CPF. These results can be attribute to a possible synergism interaction of the compounds in the mixture. The growth parameters can be modified by pesticides, depending on the concentrations and the exposure time, the effects being in many cases not immediately evident (Michelmore et al., 2005; Amaral et al., 2012). Our experiment had a duration of 75 days of exposure, and no significant alterations in the growth of the animals were seen in most of the exposed groups, mainly in the groups exposed to individual compounds. Possibly in these cases, the time of exposure was not enough to create an imbalance on underlying bioenergetics processes such as energy assimilation and metabolic expenditures in these long-lived animals. We must consider that this is a relatively short exposure time for a generally negative effect on growth (delay or induction) to be observed in exposed animals. It should be noted, however, that in natural environments, these animals receive repeated exposures during the year with different compounds, depending on the existence of crop rotations, together with the lack of food availability in these widely fragmented environments. Many pesticides applications occur in early autumn, a period in which the animals must acquire energy to survive the winter, with extremely low temperatures, which induce the cessation of feeding and, therefore, the lack of nutritional reserves (Larriera et al., 2008; Amaral et al., 2012). The results obtained for the mixture applied coincides with results of previous studies reporting obesogenic activity of pesticides and its metabolites, which predicts that there is an inappropriate activation of receptors that would lead directly to adipocyte differentiation and metabolic disorders, that would decrease caloric expenditure, predisposing exposed individuals to obesity (Grün and Blumberg, 2006; Bingnan et al., 2020; Blanco et al., 2020). The exact mechanisms that may induce these chemicals to cause weight gain are not known but could be involve in the altered thyroid function metabolism and/or energy homeostasis (Newbold, 2010). Anyway, we consider that more studies are necessary to identify the type of disorders produced on the growth parameters by different pesticides, according to their chemical nature and in form of mixtures applied in the natural environments where this species live, keeping in mind this situation could be worse in long-term real situations.

The analysis of organisms in the natural environments provides information on the environmental health, generating data that are toxicologically relevant (Marques et al., 2013). In contrast, it is difficult to establish a direct cause-and-effect relationship with a specific element because of environmental complexity under certain natural conditions and the interactions between substances (Benvindo-Souza et al., 2020).

In relation to the result of analytical determination of pesticides in soil and water from each experimental enclosure, we found higher values than those recently reported in different agricultural areas in the Pampas region of Argentina (Hunt et al., 2016; Primost et al., 2017; Álvarez et al., 2019; Mac Loughlin et al., 2020, 2022; Pérez et al., 2021; Vera-Candioti et al., 2021), but we have to consider that in our study, determinations were made just two weeks after direct application to the enclosures. GLY and AMPA concentrations were reported in a range of 27.9–8105 and 270–38900 μg/kg respectively in soil, 8.28–1146 and 6.85–17.50 μg/kg respectively in sediments, and 0.2–110 μg/L and 0.2–4.5 μg/L respectively in surface water (Primost et al., 2017; Mac Loughlin et al., 2020, 2022; Pérez et al., 2021; Vera-Candioti et al., 2021). In the same region CPF concentrations were in a range of 1.2–7.4 μg/kg in soil and 0.0005–10.8 μg/L in water samples, while CYP was found in soil at 0.9–8.3 μg/kg (Hunt et al., 2016; Alvarez et al., 2019; Mac Loughlin et al., 2022).

Taking into account all this, a single biomarker is not sufficient to reflect the health status of an organism, so it is advisable to apply a battery of biomarkers when trying to explain the toxic effects of xenobiotics (Beliaeff and Burgeot, 2002). In order to integrate the results obtained from exposure to pesticides and biological responses, a multivariate analysis (PCA) was carried out. This analysis demonstrated more sensitivity of genotoxicity and OS biomarkers than immunotoxicity biomarker to detect the effect of the different pesticides separately and as a mixture. The mixture of the three pesticides induced a higher FMN than the compounds separately and is associated with the oxidation of bases in the DNA (ENDO and FPG sites), while GLY, CPF and CYP mainly induced higher frequencies of NN and buds in erythrocytes.

We can conclude that the PCA analysis allowed us to identify those variables more sensible to the effect of pesticides and contributes significantly to an integrated interpretation of the results, making the study more relevant.

The impact of pesticides on environmentally relevant concentrations can result in different alterations in non-target organisms, and the biomarkers applied in the present work showed different degrees of sensitivity according to the pesticide treatment (exposure to individual compounds or to a complex mixture).

Field studies may provide more representative, realistic and ecologically relevant data, but have some disadvantages as are more susceptible to noise variables that induce variability (Costa et al., 2011; Etchegoyen et al., 2017). In addition, the exact mode of action with which the compounds act within the mixture is difficult to estimate and might vary with various environmental conditions. The chemical properties of each formulation, the interaction with other compounds and the mechanism of action of each toxic, can also produce very variable effects. Besides, the individual susceptibility of each organism facing the exposure, add greater complexity to multiple situations (Amaral et al., 2012; Beyer et al., 2014).

Then, to determine the potential adverse effects of xenobiotics with an integrated approach, multiple biomarkers analysis is required to obtain further information on the health status of wild caiman populations. To the best of our knowledge, this is the first report of a multiple biomarker assessment carried out on crocodilians exposed under semi-natural (*ex-situ*) conditions to pesticides. We believe it is necessary to continue investigating the possible toxicity mechanisms underlying pesticide exposures, especially concerning mixtures, which can affect physiological responses of caiman populations, and may allow estimate the environmental impact of real situations. Finally, these findings provide useful information for future biomonitoring studies that could contribute to the assessment of environmental risks for the conservation of wild species and the development of sustainable management programs.

Declarations

Author contribution statement

López González E.C.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed analysis tools or data; Wrote the paper.

Odetti L.M.: Performed the experiments; Analyzed and interpreted the data; Contributed analysis tools or data; Wrote the paper.

Latorre M.A.: Performed the experiments; Contributed analysis tools or data.

Ávila O.B. & Contini L.E.: Analyzed and interpreted the data; Contributed analysis tools or data.

Siroski P.A.: Conceived and designed the experiments; Contributed reagents, materials.

Poletta G.L.: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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