Genotoxicity and oxidative stress in *Caiman latirostris* hatchlings exposed to pesticide formulations and their mixtures during incubation period

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ABSTRACT

Agricultural expansion and the consequent use of pesticides lead to the loss and fragmentation of natural habitats of several wild species. Then, many species are inevitably exposed to a wide amount of pesticide formulations. Glyphosate (GLY)-based formulations are the most used herbicide, whereas two of the most employed insecticides are chlorpyrifos (CPF) and cypermethrin (CYP). The aim of this study was to evaluate genotoxicity, oxidative damage, and the modulation of antioxidants defenses in peripheral blood of *Caiman latirostris* after embryonic exposure to pesticide formulations and their mixtures. Pesticides concentrations employed were equivalent to those recommended in agricultural practices for application in soybean crops and a half of them: GLY: 2% and 1%; CYP: 0.12% and 0.06%; CPF: 0.8% and 0.4%. Two similar experiments (E1 and E2) were carried out in consecutive years, where *C. latirostris* eggs were exposed to pesticide formulations separately and in different mixtures through application on the incubation material. After hatching, blood samples were taken and genotoxicity and oxidative stress was evaluated through the micronucleus (MN) test, the modified comet assay, the lipid peroxidation (LPO) levels and the activities of catalase (CAT) and superoxide dismutase (SOD) antioxidant enzymes. The results indicated the presence of DNA damage, oxidation of purines and pyrimidines, and increased frequency of micronucleus (FMN) in the case of GLY, CYP, and CPF formulations exposure, as well as in all the mixtures tested, with respect to the control groups. Specifically, the results observed for the mixtures would indicate independent action or antagonism of the components for DNA damage and base oxidation (purines and pyrimidines) and a possible potentiation interaction for the FMN in two binary mixtures. However, there were not differences regarding lipid peroxidation, the activity of antioxidant enzymes and growth parameters. This study proved that the use of pesticide formulations at concentrations used in the field generate deleterious genetic effects on this species, then, exposure to them could threaten its survival and health status.

1. Introduction

Agricultural activities and the associated use of pesticides have been increasing exponentially through the last three decades, and inevitably generate habitat degradation and fragmentation (Gaona et al., 2019). Glyphosate (GLY) is a non-selective herbicide, which has been widely proved to be efficient as growth inhibitor of perennial weeds (Van Bruggen et al., 2018). Its most known commercial formulation is Roundup®. On other hand, chlorpyrifos (CPF) is an organophosphate insecticide very used to control insect pests in agricultural fields. Its main toxic effect consists of non-reversible phosphorylation of esterases in the central nervous system (Mangas et al., 2016). Finally, cypermethrin (CYP) is a synthetic pyrethroid insecticide, used to control a wide range of insects (Carriquiriborde et al., 2007). These three pesticides are the most commonly used in non-tillage system in Argentinian genetically modified crops.

As a result of growing and uncontrolled application of many kinds of pesticides, several wild species may be affected in natural environments. Then non-target species may suffer from changes in development and growth, as well as physiological parameter alterations and genetic instability, so that the health of wild populations seemed threaten (Sparling et al., 2006; Glusczak et al., 2007; Capriglione et al., 2011; Jin et al., 2015; Paravani et al., 2019).

C. latirostris is a crocodilian species that inhabits the north-central

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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 GLY, CYP and CPF formulations, among others. In Argentina, its reproduction takes place between November and Jaanuary, and births occur in February–March (Larriera et al., 2008). Therefore, the period of maximum application of pesticides coincides with the reproductive season of this species, implying a greater risk of exposure for developing embryos and neonates (Poletta et al., 2017). López González et al. (2017) and Burella et al. (2018) reported genotoxicity, oxidative damage, and alteration in the activity of superoxide dismutase (SOD) enzyme in erythrocytes of *C. latirostris* exposed to sublethal concentrations of two GLY formulations: PanzerGold® and Roundup® Full II (500–1000 µg/egg); endosulfan (END) formulation Galgofan® and CYP formulation Atanor® (1–1000 µg/egg) by topical application through the eggshell at the beginning of the incubation period.

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. Among the parameters that can be measured, superoxide dismutase (SOD) which converts the superoxide anion into hydrogen peroxide; and catalase (CAT), which neutralizes hydrogen peroxide and converts it into water, are two of the most important antioxidant enzymes (Hedayati et al., 2014). On the other hand, lipid peroxidation (LPO) is probably the most studied ROS-induced process, which affects structures rich in polyunsaturated fatty acids, such as cell membranes, altering their permeability, causing edemas, and finally leading to cell death (Díaz, 2001). In addition, ROS are involved in various types of DNA lesions, including single and double strand breaks, alkali-labile sites, as well as the oxidation of bases that are easily detected by the comet assay (CA) (Azqueta et al., 2014). Standard CA is the most used method for measuring general DNA damage, and specifically it detects strand breaks and alkali-labile sites (Azqueta et al., 2014). The incorporation of the enzymes formamidopyrimidine DNA glycosylase (FPG) and Endonuclease III (Endo III) that recognizes the presence of oxidized purines and pyrimidine to the CA technique, increases its specificity and sensitivity (Collins, 2004). This study included the modified CA with the addition of the enzymes FPG which mainly detects the common oxidized purine 8-oxoGua and other purine oxidation products, and Endo III that recognizes oxidized pyrimidines (Azqueta et al., 2013). Finally, we applied the micronucleus (MN) analysis which is commonly used to detect the early level of genetic damage induced by clastogenic or aneugenic agents (Fenech et al., 2016), and it can allow the monitoring of the genetic effect of environmental pollutants (De Castilhos Guisi et al., 2016).

Taking into account the extensive use of pesticide formulations in current agricultural environments in Argentina, early detection of their effects through sensitive biomarkers provides high-valuable tools for assessment of medium- and long-term effects on wildlife. The aim of this study was to evaluate the effects of GLY-, CPF-and CYP-based pesticide formulations and their binary and ternary mixtures, on peripheral blood of *C. latirostris* hatchlings after *in ovo* exposure through spraying on nesting material.

2. Materials and methods

2.1. Pesticides

Pesticide formulations were obtained from "Establecimiento La Matuza S.A." (Santa Fe, Argentina) and included: 1) GLY Roundup® Full II (66.2%), water-soluble herbicide (12000 mg/L), containing potassium salt GLY [N-(phosphonomethyl) glycine monopotassium salt $C_3H_7Kno_5P$] as an active ingredient (a.i.) (CAS N° 70901-12-1); 2) CYP Atanor® (25% a.i.), a water-insoluble mixture (0.01 mg/L) of different cypermethrin isomers ($C_{22}H_{19}Cl_2No_3$), (CAS N° 52315-07-8); and 3) CPF Lorsban® (48% a.i.) water-insoluble insecticide (2 mg/L) (O, O-diethyl O-3, 5, 6-trichloro-2-Piridyl phosphorus) (CAS N° 2921-88-2) (EXTOXNET, access 2019).

2.2. Pesticide analysis

GLY determination was conducted by high-performance liquid chromatography (HPLC) with pre-column derivatization using 9-fluorenylmethyl chloroformate (FMOC-Cl), as described in Poletta et al. (2011). CYP and CPF determinations were done by Gas chromatographic method (GC-TOF) with a detection limit of 0.10 µg/L (CYP: protocols N° 340341 and 42; CPF: protocols N° 340343–44, Fox Laboratories, Venado Tuerto, Santa Fe, Argentina).

2.3. Experimental design and treatments

The present study was evaluated and approved by the Research Ethics and Safety Advisory Committee of the Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral (Santa Fe, Argentina) (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" (30° 11′ 26″ S; 61° 0′ 27″ W; San Cristóbal Department, Santa Fe, Argentina) a Protected Natural Area under PY ranching program activities (Provincial Law 12,930; Larriera et al., 2008).

Two experiments were conducted (E1 and E2) in consecutive years. The quantity of eggs used in each experiments was as follows: one hundred and twenty eggs from three different clutches (40 eggs per clutch) were randomly distributed into ten experimental groups with two replicas for each group in E1 and one hundred and eighty eggs from five clutches (36 eggs per clutch) distributed into twelve experimental groups with three replicas for each group in E2 (Table 1). We used animals from different clutches distributed in all experimental groups to control and homogenize any effect associated to the nest of origin, considering that the *clutch effect* is one of the most important causes of variability observed in crocodilians (Webb et al., 1992; Verdade, 1997).

Each replicate was placed in a plastic container separately, using vermiculite (mineral formed by silicates of iron or magnesium of the groups of micas) as substrate and covering them with vegetal material similar to the nesting material, free of any exogenous substance. The exposure of the eggs to pesticide solutions were carried out at an early stage of embryonic development (within 5 d after laying) by spraying them on nesting material (Fig. 1). The concentrations applied correspond to those recommended for field application in soybean crops. Therefore, as it is shown in Table 1, in the case of the GLY-based formulation Roundup® Full II, the higher concentration applied (that recommended for its application in soybean crop) is 2%, which means 2 L/100 L/ha (2 L of RU in 100 L H_2O per ha of crop), so we consider the surface of the incubation container (0.034 m^2) as the area to fumigate, and applied the corresponding amount of pesticide diluted in the corresponding amount of water. All eggs were incubated, under a temperature of 31.5 ± 0.5 °C and 95% humidity in the PY incubator. When vocalization of hatchlings started within the eggs, the corresponding eggs were removed from the incubator and if hatching did not occur spontaneously during the next 24 h, they were assisted (Larriera et al., 2008). Immediately after hatching, 0.5 ml of peripheral blood was taken from the spinal vein (Myburgh et al., 2014) of each hatchling with heparinized syringe and 25G x 5/8" needle, a method proved to cause no damage to the animals (López González et al., 2017; Burella et al., 2018). Peripheral whole blood was used immediately for the

Table 1

Experimental groups of *Caiman latirostris* exposed to glyphosate-, cypermethrin- and chlorpyrifos-based pesticide formulations and their binary and ternary mixtures during embryonic development evaluated in Experiment 1 and 2 (E1, E2, respectively).

Treatments	Compounds	Concentration	Number of eggs per replica		Number of eggs per experimental groups	
			E1	E2	E1	E2
NC	Distilled water	–	6	5	12	15
VC	Ethanol	10%	6	5	12	15
GLY1	Roundup® Full II (GLY 66. 2%)	2%	6	5	12	15
GLY2	Roundup® Full II (GLY 66. 2%)	1%	6	5	12	15
CPF1	Lorsban® (CPF 48%)	0.8%	6	5	12	15
CPF2	Lorsban® (CPF 48%)	0.4%	6	5	12	15
CYP1	Atanor® (CYP 25%)	0.12%	6	5	12	15
CYP2	Atanor® (CYP 25%)	0.06%	6	5	12	15
M1	GLY1 + CYP1	2% + 0.12%	–	5	–	15
M2	CPF1 + CYP1	0.8% + 0.12%	–	5	–	15
M3	GLY1 + CPF1	2% + 0.12%	–	5	–	15
Mx3.1	GLY1 + CYP1+ CPF1	2% + 0.12% + 0.8%	6	5	12	15
Mx3.2	GLY2 + CYP2 + CPF2	1% + 0.06% + 0.4%	6	–	12	–

NC: Negative control; VC: vehicle control; GLY1: glyphosate formulation at 2%; GLY2: glyphosate formulation at 1%; CPF 1: chlorpyrifos formulation at 0.8%; CPF2: chlorpyrifos formulation at 0.4%; CYP1: cypermethrin formulation at 0.12%; CYP2: cypermethrin formulation at 0.06%; M1: mixture GLY1 + CYP1; M2: mixture CPF1 + CYP1; M3: mixture CPF1 + GLY1; Mx3.1: mixture of the three formulations at field concentrations: CYP1 + CPF1 + GLY1; Mx3.2: mixture of the three formulations at half field concentrations: CYP2 + CPF2 + GLY2.

standard and modified CA and MN test; while for the rest of the techniques for oxidative stress evaluation we used lysed erythrocytes (Poletta et al., 2008; 2016). Hatching success per group was recorded in both experiments, and total length (TL), snout-vent length (SVL) (tape measure, precision 0.5 cm) and weight (OHAUS® Compact scale CS200, precision 0.1 g) of each neonate was measured.

2.4. Standard and modified comet assay with endonucleases

The CA was performed in erythrocytes of *C. latirostris* according the protocol of Poletta et al. (2008), and a single DNA damage index (DI = n1 + 2 n2 + 3 n3 + 4 n4) was calculated for each an imal, were n is the number of nucleoids of each category (1–4), classified among 100 analyzed nucleoids. The modified CA technique was applied following Poletta et al. (2016), using the endonucleases FPG and ENDO III in order to discriminate the damage to DNA produced by base oxidation. The oxidative damage to DNA was calculated by subtracting breaks with buffer from breaks with FPG or ENDO III as follows: FPG/Endo III sites = DI with FPG/Endo III - DI with enzyme buffer alone (Collins, 2009).

2.5. Micronucleus test

The MN test was conducted following the protocol of Poletta et al. (2008) adapted for *C. latirostris* erythrocytes. The criteria adopted for MN identification were based on Schmid (1975) as follows: (1) MN should be smaller than one-third of the main nucleus, (2) MN should be separated from the main nucleus, and (3) MN should be the same color and intensity of the main nucleus. Two slides were made for each animal, fixed with ethanol for 10 min and stained with Giemsa (10%) for 15 min. For each slide, 500 erythrocytes were analyzed under the optical microscope Nikon Eclipse E200 with a magnification of $1000 \times$, and the frequency of MN were determined for each animal (FMN: number of cells with MN/1000 erythrocytes counted).

2.6. Oxidative stress determinations: oxidative damage to lipids, catalase and superoxide dismutase activities

The techniques employed for measurements of LPO, SOD and CAT enzyme activities were conducted as described by Poletta et al. (2016). The LPO levels were measured by absorbance of substances reactive to thiobarbiturate acid (TBARS) using spectrophotometer at 535 nm. CAT

Fig. 1. Different experimental groups (replicates in each container) with *C. latirostris* eggs (A); Spraying application of pesticide formulations and their mixture on nests material (B).

activity was measured at 240 nm and 25 °C for 60 s in a spectrophotometer, by the decomposition of hydrogen peroxide. The specific activity of CAT and the concentration of TBARS were referenced to the amount of protein in the sample, determined with the kit 1690007 Proti U/LCR (Wiener Lab). SOD activity was determined using the commercial kit 19160-1 KT (Sigma) only in E1.

2.7. Statistical analysis

Statistical analysis was performed using SPSS 15.0 software for Windows (SPSS, 2008). Replicates were compared for all variables using the *t*-test in E1 and the One-way ANOVA in E2. As there were no differences among replicas in any of the two experiments all the analysis were performed considering the experimental groups as the grouping variable. For all the parameters analyzed, mean values ± standard error (SE) were calculated from data of all hatchlings of each experimental group in the two experiments (E1 and E2). Variables were tested for normality using the Kolmogorov-Smirnov test and the homogeneity of variances between the groups was verified by Levene test.

One-way ANOVA, followed by the Tukey test (p < 0.05), was used for the comparison of SOD, DI, FPG- and ENDO III-sites in E1 as well as weight, TL, SVL, CAT and LPO in E2. In the case of non-parametric data, the Mann-Whitney *U* test (p < 0.05) was carried out for comparison of the TL, SVL, Weight, LPO, CAT and FMN in E1; and DI, FMN, FPG- and ENDO III- sites in E2. For all variables, exposed groups were compared to their corresponding control: distilled water (negative control –NC) was used for groups exposed to the GLY formulations (water soluble), while ethanol was used as the vehicle control (VC) for CPF, CYP, and all the mixtures tested (water insoluble). In the same way, the two concentrations applied for each compound were compared between each other and all mixtures were compared to the individual compounds constituent them.

3. Results

Results obtained from analytical pesticide determinations done in the solutions prepared immediately before spraying on the nest material, gave the following recovery percentages in relation to the percentage of the active principle indicated in the formula: 92% for GLY formulation, 89% for CYP formulation, and 95% for CPF formulation.

Fig. 2 shows the results of the standard CA (mean ± SE). The results of E1 showed a significant increase in DNA damage index for all the groups with respect to their corresponding controls (p < 0.05, Tukey test, Fig. 2A). The same was observed in E2 (p < 0.05, Mann-Whitney *U* test, Fig. 2B) except for CPF2 and CYP2, compared to the VC.

In the comparison of the mixtures with the individual compounds, we found significant higher DNA damage in the Mx3.1 than for CPF1 or CYP1 alone in E1 (p < 0.05, Tukey test, Fig. 2A). In E2 differences were observed between M2 and the two individual compounds integrating the mixture: CYP1 and CPF1, but in this case individual pesticides cause a higher damage than the mixture (p < 0.05, Mann-Whitney *U* test, Fig. 2B). When different concentrations of the same compounds were analyzed (1 vs. 2), we found differences for GLY in both experiments and for CYP only in E2, being the damage higher at the higher concentration (Fig. 2A and B).

Fig. 3 shows the results observed in the modified comet assay (mean ± SE): in E1 FPG and ENDO III sites were significantly different in all exposed groups with respect to the NC or the VC (p < 0.05, Tukey test, Fig. 3A); in E2 oxidation of purines and pyrimidines was significantly higher in GLY1 compared to the NC and in CPF1, CYP1, M1, M2, M3 and Mx3.1 with respect to the VC; while GLY2 showed differences only in pyrimidines (p < 0.05, Mann-Whitney *U* test, Fig. 3B).

In relation to the mixtures, in E1 we observed significant differences only in ENDO III between Mx3.1 with GLY1 and CPF1, showing the

individual components, higher damage than the mixture (p < 0.05, Tukey test, Fig. 3A). In turn, in E2, significant differences were found in FPG between Mx3.1 with CPF1 and CYP1 but not with GLY1. In the binary mixtures, GLY showed significantly higher damage than M1 and M3, and the same was seen for CYP and M1; while CPF caused lower damage than M3 (p < 0.05, Mann-Whitney *U* test, Fig. 3B). In the case of ENDO III sites, M2 caused a significant lower damage than their constituent components: CYP and CPF (p < 0.05, Mann-Whitney *U* test, Fig. 3B). Considering the two concentrations applied of each individual compound, differences were found for the three compounds in ENDO III sites and for GLY and CPF in FPG sites in E1 (p < 0.05, Tukey test, Fig. 3A); while in E2 the three compounds (GLY, CYP and CPF) showed differences, both in FPG and ENDO III sites (p < 0.05, Mann-Whitney *U* test, Fig. 3B), being the damage always higher at the higher concentration.

Results of the FMN are presented in Fig. 4 (mean ± SE). In E1 the difference was evident only in CPF1 and Mx3.1 in comparison to the VC (p < 0.05, Mann-Whitney *U* test, Fig. 4A), while in E2 both groups exposed to GLY, both exposed to CYP and the three binary mixtures showed a higher FMN compared with their respective controls (p < 0.05, Mann-Whitney *U* test, Fig. 4B). When we compared the mixtures with its constituent components, we found a significant higher FMN in M2 and M3 than in the group exposed to CPF alone (p < 0.05, Mann-Whitney *U* test, Fig. 4B), but no differences were found with the other individual component of the binary mixtures (CYP or GLY, respectively) in E2. Moreover, there were no differences in the FMN induced by the two concentrations applied for each pesticide in any of the experiment (p > 0.05, Mann-Whitney *U* test, Fig. 4A and B).

Different letters (a, b, c and d) indicate a significant difference at p < 0.05 among the mixtures and their constituent individual components, or between the two concentrations applied for each pesticide separately.

No significant differences were observed in TBARS levels or CAT and SOD activities (Table 2), or in growth parameters (Table 3) between all exposed groups and their corresponding controls in the two experiments (E1 and E2).

4. Discussion

C. latirostris is considered a sentinel organism for the evaluation of the effect induced by pesticides (Poletta et al., 2009). In previous studies, we demonstrated immunotoxicity, genotoxicity, oxidative damage and enzymatic alterations in *C. latirostris* exposed to different formulations of GLY (PanzerGold® and Roundup® Full II), CYP (Atanor®) and END (Galgofan®) by topical application through the eggshell (Poletta et al., 2009; Siroski et al., 2016; López González et al., 2017; Burella et al., 2018). In this study, in agreement and complementing previous reports, *C. latirostris* exposed to GLY, CYP and CPF formulations, as well as to their mixtures, showed an increase in the FMN and DNA damage compared to the control group. Among the DNA damage observed, base oxidation was relevant in all groups. Even when field studies may be more representative of the real sceneries of exposures in natural environments, they are more susceptible to variables that introduce noise to the results (Costa et al., 2011; Etchegoyen et al., 2017). On the other side, laboratory exposure studies are considered useful tools for controlling these environmental variables (Etchegoyen et al., 2017). In the present study, exposure conditions tented to simulate the real situation of exposure taking place in natural caiman populations during the reproductive season, for both, the concentrations applied (those recommended for soybean crops) and the application method (spraying on nesting material).

CA showed to be an appropriate and highly sensitive method for detecting early DNA damage on sentinel organisms, identifying single strand breaks and maximizing the expression of alkali-labile sites in the DNA molecule (Mudry and Carballo, 2006; Azqueta and Collins, 2013; Demir et al., 2015). In the present study, we found a significant increase
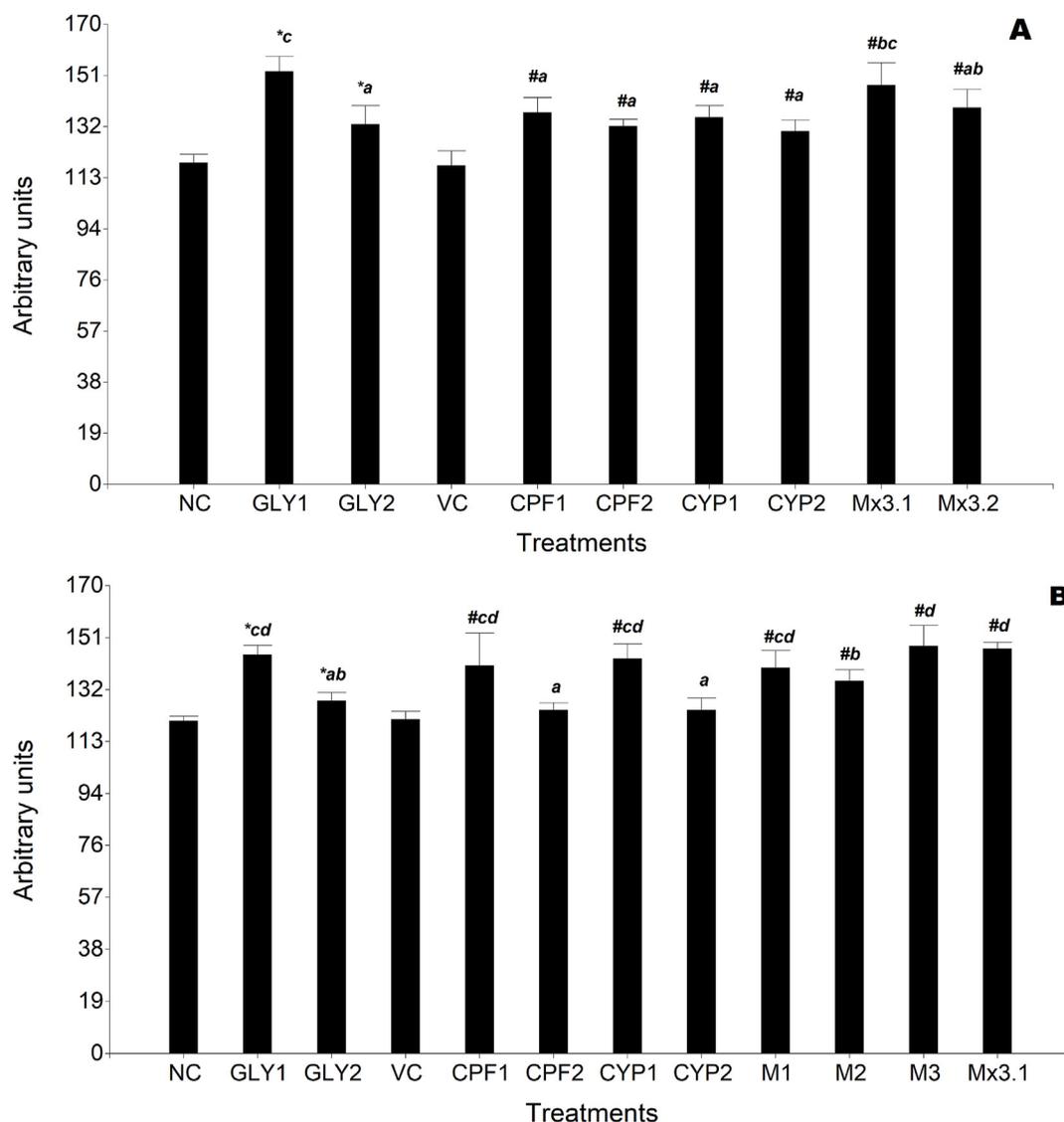
Fig. 2. DNA damage index of the Comet assay in *C. latirostris* hatchlings at different treatments in Experimental 1 (A) and 2 (B). NC: negative control; VC: vehicle control; GLY1: glyphosate formulation at 2%; GLY2: glyphosate formulation at 1%; CPF1: chlorpyrifos formulation at 0.8%; CPF2: chlorpyrifos formulation at 0.4%; CYP1: cypermethrin formulation at 0.12%; CYP2: cypermethrin formulation at 0.06%; M1: mixture GLY1 + CYP1; M2: mixture CPF1 + CYP1; M3: mixture CPF1 + GLY1; Mx3.1: mixture of the three formulations at field concentrations: CYP1 + CPF1 + GLY1; Mx3.2: mixture of the three formulations at half field concentrations: CYP2 + CPF2 + GLY2. *Statistically significant at p < 0.05 compared to NC. #Statistically significant at p < 0.05 compared to VC. Different letters (a, b, c and d) indicate a significant difference at p < 0.05 among the mixtures and their constituent individual components, or between the two concentrations applied for each pesticide separately.

in DNA damage in the groups exposed to pesticides separately and to their binary and ternary mixtures in both experiments (E1 and E2). There was a dose-response relationship in GLY and CYP, being DNA damage higher at the higher concentrations. Previous studies have reported DNA damage in *C. latirostris* blood exposed to Roundup® Full II (GLY-RU) at different concentrations. Poletta et al. (2009) found significant differences after *in ovo* exposure to the formulation RU (50–1750 μg/egg) by topication. In the same way, Burella et al. (2017) evaluated the potential stage-dependent effect of RU (750–1750 μg/egg) on *C. latirostris* embryos and found that the compound induced DNA damage during the whole development period, independently of the moment of exposure. Similarly, studies in other species demonstrated an increase in DNA damage with the CA in erythrocytes of different species exposed to CPF, GLY and CYP formulations (Yin et al., 2009; Simoniello et al., 2009; Schaumburg et al., 2016). In the same way, a significant increase in the FMN was observed after *in ovo* exposure to separate compounds and to the binary and ternary mixtures,

mainly at field concentrations. These results demonstrated that the FMN is also a sensitive indicator of damage to genetic material. These data coincide with previous studies made by our group reporting genotoxic effect and lower growth in *C. latirostris* after embryonic (*in ovo*) exposure by topical application through the eggshell, and postnatal (*in vivo*) exposure to the same pesticide formulations (Poletta et al., 2009; 2017; López González et al., 2013, 2017; 2019). Similarly, in the erythrocytes of *Odontophrynus americanus* tadpoles (Amphibia: Leptodactylidae) genotoxic effects were also evidenced after an *in vivo* exposure to CYP formulation (5–40 μg/L) (Cabagna- Zenklusen et al., 2006). The mechanisms by which pesticides induce genetic damage differ greatly with their chemical nature. Several authors have demonstrated that pesticides are involved in the imbalance of redox status and genotoxicity (Halliwell, 2002; Oruc and Usta, 2007; Zhao et al., 2015; Arrighetti et al., 2018; Burella et al., 2018). Pesticides could induce the generation of ROS such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (•OH) stimulating various oxidative
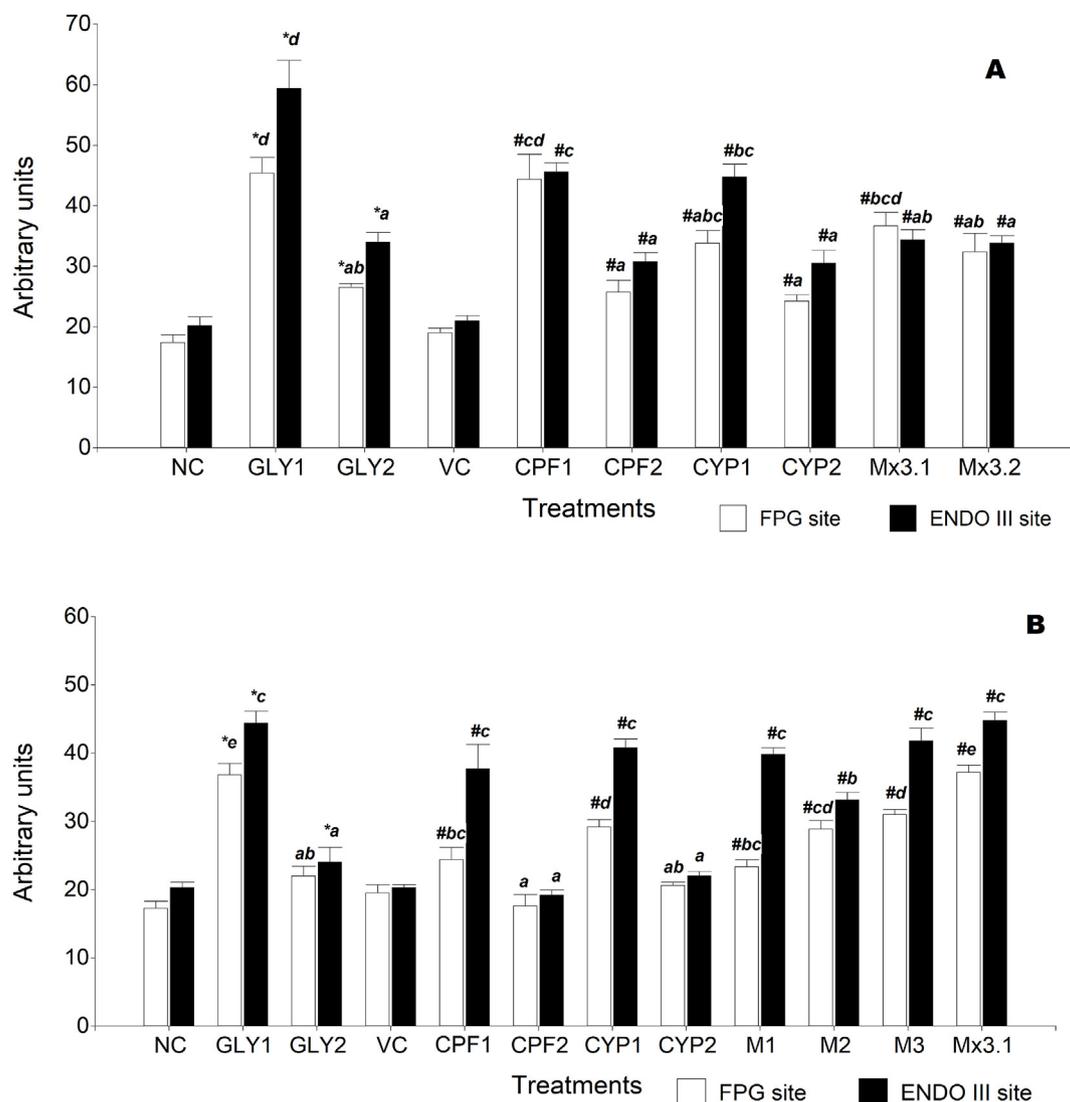
Fig. 3. DNA oxidative damage detected through the modified comet assay in *C. latirostris* hatchlings at different treatments in Experiment 1 (A) and 2 (B). NC: negative control; VC: vehicle control; GLY1: glyphosate formulation at 2%; GLY2: glyphosate formulation at 1%; CPF1: chlorpyrifos formulation at 0.8%; CPF2: chlorpyrifos formulation at 0.4%; CYP1: cypermethrin formulation at 0.12%; CYP2: cypermethrin formulation at 0.06%; M1: mixture GLY1 + CYP1; M2: mixture CPF1 + CYP1: M3: mixture CPF1 + GLY1; Mx3.1: mixture of the three formulations at field concentrations: CYP1 + CPF1 + GLY1; Mx3.2: mixture of the three formulations at half field concentrations: CYP2 + CPF2 + GLY2. *Statistically significant at p < 0.05 compared to NC. #Statistically significant at p < 0.05 compared to VC. Different letters (a, b, c and d) indicate a significant difference at p < 0.05 among the mixtures and their constituent individual components, or between the two concentrations applied for each pesticide separately.

stress markers (Li et al., 2015). This high production of ROS could inhibit enzymes involved in DNA repair, which can lead to both single and double-strand breaks, and later to MN formation (Saleha Banu et al., 2001; Zegura et al., 2004; Bonifacio and Hued, 2019).

Regarding crocodilians species, there are few studies related to oxidative damage and antioxidant defenses. Those works reported in crocodiles were made in tissues or organs (e.g. kidneys, muscles, gonads, or liver) where the animals had to be slaughtered, or samples were obtained from recently killed animals (Gunderson et al., 2004; Lance et al., 2006; Furtado Filho et al., 2007; Hermes-Lima et al., 2012). Poletta et al. (2016) was the first report to characterize the use of biomarkers of oxidative damage and antioxidant defense in *C. latirostris* blood, and later, Burella et al. (2018) applied them as biomarkers for pesticides evaluation. The authors reported imbalances in the oxidative state through lipid peroxidation and DNA damage, as well as in the activities of CAT and SOD antioxidant enzymes in *C. latirostris* embryos after a topical exposure to sublethal concentrations (1–1000 μg/egg) of pesticide formulations (GLY, END and CYP). On the

contrary, in the present work, there were no alterations in CAT and SOD activities or increase in lipid peroxidation in any exposed groups, so we suppose that individuals evaluated in this study were more capable of counteracting ROS effects by some other antioxidant mechanism, not evaluated here. In order to elucidate if base oxidation contributes significantly to the DNA damage caused by GLY, CYP and CPF formulations and their mixtures, the modified CA with lesion-specific endonucleases (ENDO III and FPG) was employed. The obtained results indicated oxidation in the purines and pyrimidines in all groups of caiman exposed to pesticide formulations separately and in the mixtures, at concentrations equivalent to those used in agriculture and the half of them, and in both experiments (E1 and E2). However, these results were not accompanied by modifications in the activity of the antioxidant enzymes CAT and SOD. One possibility to the lack of response of these enzymes is that the excess of ROS has produced protein toxicity. As a consequence, pesticide reactive substances affect DNA by base oxidation, as previously suggested by other authors in amphibians (Lajmanovich et al., 2015; Pérez-Iglesias et al., 2017) and in the broad-
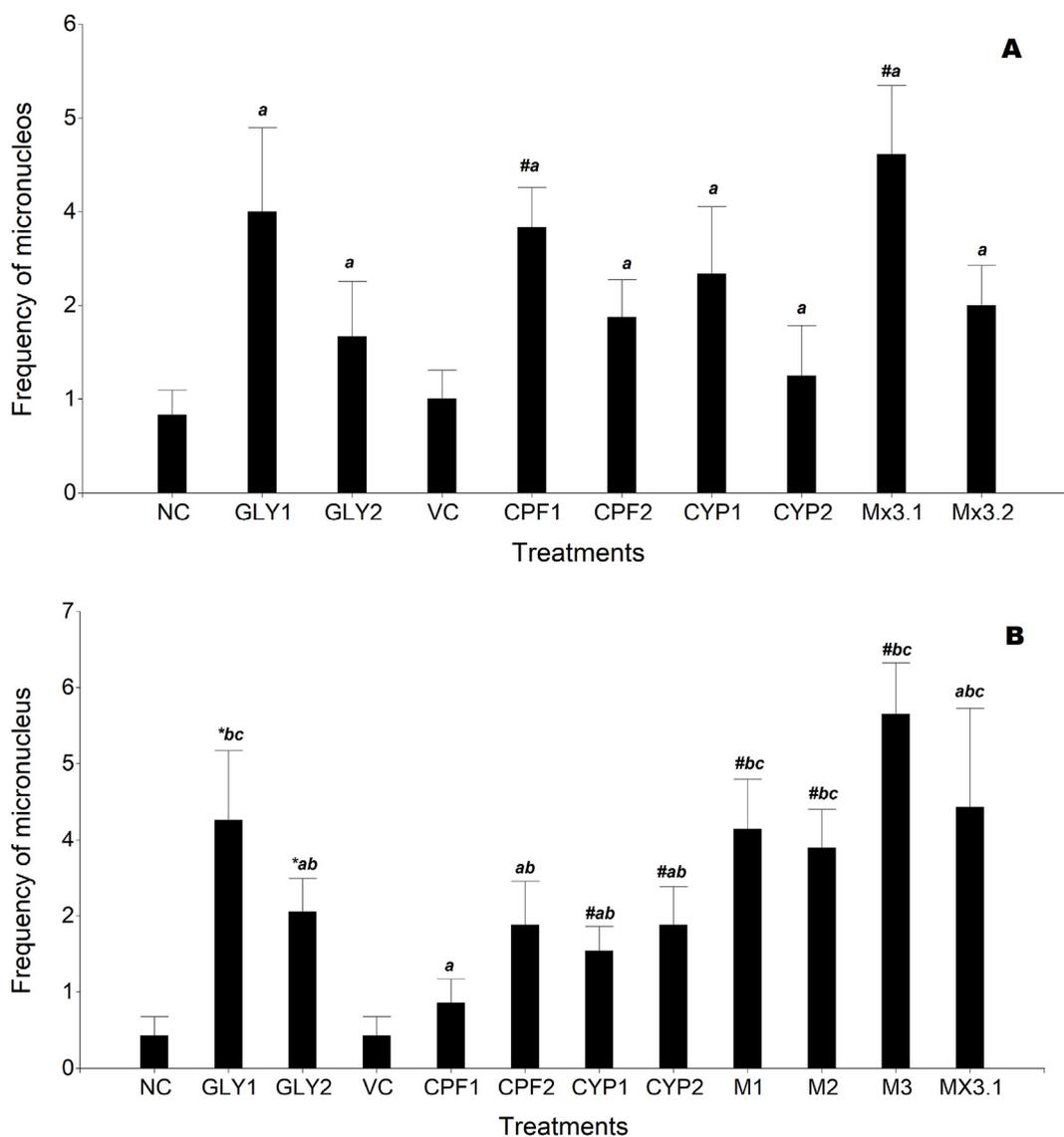
Fig. 4. Micronucleus frequencies observed in *C. latirostris* hatchlings at different treatments in Experiment 1 (A) and 2 (B). NC: negative control; VC: vehicle control; GLY1: glyphosate formulation at 2%; GLY2: glyphosate formulation at 1%; CPF1: chlorpyrifos formulation at 0.8%; CPF2: chlorpyrifos formulation at 0.4%; CYP1: cypermethrin formulation at 0.12%; CYP2: cypermethrin formulation at 0.06%; M1: mixture GLY1 + CYP1; M2: mixture CPF1 + CYP1: M3: mixture CPF1 + GLY1; Mx3.1: mixture of the three formulations at field concentrations: CYP1 + CPF1 + GLY1; Mx3.2: mixture of the three formulations at half field concentrations: CYP2 + CPF2 + GLY2. *Statistically significant at $p < 0.05$ compared to NC. [#]Statistically significant at $p < 0.05$ compared to VC.

snouted caiman (Burella et al., 2018). Oxidation of bases is one of the most common types of DNA damage caused by ROS (Hilscherova et al., 2003). The primary oxidizer responsible for DNA damage is hydroxyl radical (•OH), as it reacts directly to the DNA molecule (Cooke et al., 2003). Other authors also indicated similar explanations to the results obtained in our work, suggesting that oxidative damage cannot be predicted only based on antioxidant variations, because this association may be compromised when the consumption of low molecular weight antioxidants is counteracted by *de novo* synthesis, and/or when inhibitory actions impair the activity of enzymatic antioxidants (Ahmad et al., 2006; Guilherme et al., 2012). Besides, a dose-effect relationship has been found in both experiments (E1 and E2) for the compounds separately, where DNA oxidative damage was higher for GLY1, CYP1 and CPF1 at field concentrations than at the half of them.

The way in which the chemicals in a mixture influence general toxicity depends on many factors, including their concentration, target site and mechanism of action (Lydy et al., 2004). Hernandez et al. (2013) reported that the pesticide mixture can take one of three ways of combining the toxicological effects of one or more components of a

mixture: 1) independent; 2) dose addition or 3) interaction (potentiation, synergism and antagonism). In the present work, we found a possible independent action for the CA in the mixture of the three formulations (Mx3.1), where the effect of the mixture showed to be similar than that caused by GLY1 alone in E1 and almost the same than the three pesticides separately in E2 (Fig. 2A and B). Exactly the same was observed for FPG sites in E2 (Fig. 3B), being GLY the pesticide that contribute most to the toxicity of Mx3.1. However, for ENDO III sites, there seem to be an antagonistic effect, as Mx3.1 induced lower oxidized pyrimidines than GLY1 and CPF1 separately in E1 (Fig. 3A). Something similar was observed in the binary mixture M2 (CPF1 + CYP1) for both DNA damage and ENDO III sites in E2 (Figs. 2B and 3B) and in M1 (GLY1 + CYP1) for FPG sites. It is assumed that the two pesticides interfered with the effect of each other. As a result, we observe a reduction in the observed effect for individual compounds that do not need to be structurally similar (Zeliger, 2011). In the case of M3 we observed that FPG sites were lower than GLY1 but higher than CPF1 separately, the two pesticides constituting this mixture. This can be explained by a certain kind of antagonistic action of CPF on GLY,

Table 2
Biomarkers of oxidative status observed in erythrocytes of the control and treated caiman. All values are expressed as mean ± SE. E1: Experiment 1; E2: Experiment 2.

		TBARS (nmol/mg prot)	CAT (KU/mg prot)	SOD (% inhibition)
E1	NC	1.74 ± 0.11	207.02 ± 36.31	84.08 ± 3.31
	VC	2.64 ± 0.49	190.25 ± 40.52	85.19 ± 3.48
	GLY 1	1.78 ± 0.15	303.42 ± 75.78	79.39 ± 12.73
	GLY 2	2.03 ± 0.16	155.57 ± 33.08	74.84 ± 4.59
	CPF 1	1.73 ± 0.21	212.46 ± 34.11	87.23 ± 3.51
	CPF 2	1.61 ± 0.16	205.39 ± 27.44	78.68 ± 3.68
	CYP 1	2.01 ± 0.15	196.73 ± 33.96	85.66 ± 5.42
	CYP 2	1.87 ± 0.26	245.27 ± 32.69	77.98 ± 9.10
	Mx3 1	1.86 ± 0.21	147.68 ± 25.60	80.88 ± 10.46
	Mx3 2	1.77 ± 0.22	137.16 ± 27.13	69.04 ± 4.77
E2	NC	4.11 ± 0.76	130.60 ± 17.01	–
	VC	5.49 ± 0.60	113.72 ± 11.98	–
	GLY 1	4.28 ± 0.78	139.62 ± 12.73	–
	GLY 2	3.02 ± 0.71	208.42 ± 30.59	–
	CPF 1	4.05 ± 0.53	68.77 ± 22.39	–
	CPF 2	3.27 ± 0.32	131.67 ± 47.01	–
	CYP 1	6.67 ± 1.94	97.13 ± 26.45	–
	CYP 2	4.01 ± 0.80	153.1 ± 44.24	–
	M 1	3.39 ± 0.93	182.99 ± 26.05	–
	M 2	4.82 ± 0.80	135.06 ± 38.20	–
	M 3	4.58 ± 0.60	121.97 ± 13.20	–
	Mx3 1	4.02 ± 0.43	130.7 ± 41.27	–

NC: negative control; VC: vehicle control; GLY1: glyphosate formulation at 2%; GLY2: glyphosate formulation at 1%; CPF1: chlorpyrifos formulation at 0.8%; CPF2: chlorpyrifos formulation at 0.4%; CYP1: cypermethrin formulation at 0.12%; CYP2: cypermethrin formulation at 0.06%; M1: mixture GLY1 + CYP1; M2: mixture CPF1 + CYP1: M3: mixture CPF1 + GLY1; Mx3.1: mixture of the three formulations at field concentrations: CYP1 + CPF1 + GLY1; Mx3.2: mixture of the three formulations at half field concentrations: CYP2 + CPF2 + GLY2.

Table 3
C. latirostris growth paraments evaluated at birth in all experimental groups. All values are expressed as mean ± SE. E1: Experiment 1; E2: Experiment 2.

		TL (cm)	SVL (cm)	Weight (g)
E1	NC	23.47 ± 0.22	10.78 ± 0.22	25.77 ± 4.81
	VC	23.82 ± 0.14	11.05 ± 0.18	28.71 ± 4.11
	GLY1	23.44 ± 0.28	10.5 ± 0.25	42.69 ± 4.38
	GLY2	23.67 ± 0.24	11.11 ± 0.25	25.47 ± 4.24
	CPF1	23.22 ± 0.18	10.9 ± 0.15	22.74 ± 3.18
	CPF2	23.4 ± 0.21	11 ± 0.15	20.26 ± 1.67
	CYP1	23.75 ± 0.13	10.75 ± 0.17	31.57 ± 3.71
	CYP2	23.1 ± 0.40	10.6 ± 0.16	42.15 ± 3.97
	Mx3.1	22.99 ± 0.71	10.68 ± 0.29	38.06 ± 4.31
	Mx3.2	23.67 ± 0.13	11.08 ± 0.14	22.25 ± 1.46
E2	NC	23.65 ± 0.48	9.92 ± 0.08	43.17 ± 1.17
	VC	23.62 ± 0.24	9.83 ± 0.21	42 ± 1.97
	GLY1	22.93 ± 0.72	9.83 ± 0.17	40 ± 2.03
	GLY2	24.06 ± 0.33	10.43 ± 0.17	43 ± 1.31
	CPF1	23.90 ± 0.21	10.09 ± 0.11	42.75 ± 1.19
	CPF2	23.44 ± 0.29	10.06 ± 0.24	43 ± 2.38
	CYP1	24.03 ± 0.25	10.21 ± 0.18	43.57 ± 1.13
	CYP2	23.75 ± 0.14	10.15 ± 0.08	43.20 ± 1.32
	M1	23.66 ± 0.18	10 ± 0.09	44.38 ± 1.68
	M2	23.56 ± 0.21	10 ± 0.15	43.09 ± 1.28
	M3	23.40 ± 0.35	9.88 ± 0.08	42.13 ± 1.49
	Mx3.1	23.44 ± 0.21	10.18 ± 0.09	43.89 ± 1.65

NC: negative control; VC: vehicle control; GLY1: glyphosate formulation at 2%; GLY2: glyphosate formulation at 1%; CPF1: chlorpyrifos formulation at 0.8%; CPF2: chlorpyrifos formulation at 0.4%; CYP1: cypermethrin formulation at 0.12%; CYP2: cypermethrin formulation at 0.06%; M1: mixture GLY1 + CYP1; M2: mixture of CPF1 + CYP1; M3: mixture CPF1 + GLY1; Mx3.1: mixture of the three formulations at field concentrations: CYP1 + CPF1 + GLY1; Mx3.2: mixture of the three formulations at half field concentrations: CYP2 + CPF2 + GLY2; TL: total length; SVL: snout-vent length; cm: centimeters; g: grams.

that made the action of the individual pesticide is diminished in the mixture.

In relation to the FMN, the analysis of the mixtures showed that M2 and M3 induced a significantly higher FMN than CPF1, but similar to the other component of the mixtures (CYP and GLY, respectively). Even when the effects of the mixtures is not significantly different that those induced by CYP or GLY individually, it seems to be a certain kind of potentiation action of CPF on CYP and GLY in the binary mixtures, as the FMN is little higher in both cases (Fig. 4B).

Poletta et al. (2011) reported that the mixture of GLY, END and CYP formulations produced greater effects on genotoxicity in *C. latirostris* neonates, in addition to induce biochemical-enzymatic alterations after a semi-natural exposure of eggs inside nests. More recently, López González et al. (2019) simulated real conditions of exposure of *C. latirostris* yearlings during their first months of life, showing that the same pesticide formulations tested in this study and at environmentally relevant concentrations, increased the FMN and other nuclear abnormalities (NAs) in erythrocytes, with a possible antagonistic action between GLY and CPF in the binary mixture, a similar interaction between the two pesticides that was observed in the present study for FPG sites. Nevertheless, Bonifacio and Hued (2019) demonstrated different responses in mixtures treatments that reflected a complex interaction (antagonism and potentiation). This authors found no effect in the FMN of *Cnesterodon decemmaculatus* adult females exposed to Clorfox and Roundup Max (CPF and GLY commercial formulation, respectively): 0.84 nL/l and 8.4 nL/l of Clorfox®, and 0.2 and 2 mg/L of Roundup Max® and in the combinations of them (low environmentally relevant concentrations of both pesticides), during 42 d, but the effect was evidenced in all treatments by significant increase in other NAs and histopathological alterations in liver, showing a great variety of alterations in respect to the control group. In this way, we could suggest that the interactions observed in different pesticide complex mixtures often induce responses with different degrees of sensitivity.

In relation to growth parameters, *C. latirostris* was not affected by the formulations and mixtures tested in the present study. Similarly, López González et al. (2017) reported the lack of effect on growth of broad snouted caiman hatchlings exposed during development by topical application through the eggshell to GLY, CYP and END formulations at concentration of 1–1000 μg/egg. In agreement with the explanation given by other authors, we think that the concentration of pesticides applied, or the time of exposure were probably not enough to create an imbalance in the underlying bioenergetics processes affecting growth (Amaral et al., 2012; López González et al., 2019). However, previous studies on *C. latirostris* evidenced a smaller size at birth and a delay in growth at 3 months of life exposed *in ovo* to RU (Poletta et al., 2011), so we could suspect that the physiology associated to the growth of animals is not always affected at the same level, and maybe is mainly related to susceptibility of the animals use in different studies.

The use of sensitive biomarkers is essential to thoroughly evaluate genetic damage and oxidative stress, which could have significant at short and long-term consequences for wild species. In this sense, it is necessary to perform biomonitoring on natural caiman populations environmentally exposed, to expand the existing knowledge on the effect of pesticides.

5. Conclusion

The present study provided new information about the toxicity of GLY, CPF and CYP-based formulations and their possible interactions in mixtures, on *C. latirostris* hatchlings. Exposure conditions simulate the real situation faced by caiman natural populations during the reproductive season, considering both, the concentrations applied (those recommended for soybean crops) and the application method (spraying on nesting material). We demonstrated that the pesticides evaluated generated genotoxicity with a relevant incidence of base oxidation at concentrations and combinations recommended for application in

agricultural activities, associated mainly to soybean crops. The effects of mixtures differ depending on the parameter analyzed, showing independent action or antagonism of the components for DNA damage and base oxidation, while an apparent potentiation effect was seen in the case of the FMN. It is necessary to deepen the evaluation of the effects of pesticides mixtures, by means of different endpoints that allow us to assess the real conditions of exposure that this and many other species face daily in its natural environment.

CRediT authorship contribution statement

L.M. Odetti: Investigation, Formal analysis, Writing - original draft. **E.C. López González:** Investigation, Writing - original draft. **M.L. Romito:** Investigation. **M.F. Simoniello:** Writing - review & editing. **G.L. Poletta:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110312.

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