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Biomarkers of genotoxicity, immunotoxicity and oxidative stress on *Caiman latirostris* (Broad-snouted caiman) hatchlings exposed to pesticide formulations and mixtures widely used in agriculture

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ABSTRACT

Pesticide formulations are currently considered as one of the main factors responsible for environmental contamination worldwide. Consequently, several wild species can be affected by the over use of pesticides related to agriculture activity. The aim of this study was to evaluate genotoxicity, immunotoxicity and oxidative stress on *Caiman latirostris* hatchlings exposed *in vivo* to sub-lethal concentrations of three insecticide formulations: Endosulfan (END)-, Cypermethrin (CYP)-, and Chlorpyrifos (CPF)- based formulations, two Glyphosate (GLY)-based herbicide formulations and two different ternary complex mixtures of them.

C. latirostris hatchlings, 20 days old were exposed in plastics containers with pesticide solutions (by voluntary immersion) during two months, with a progressive decrease in pesticide concentrations through time, in order to simulate their degradation in water under natural conditions.

After exposure, blood samples were taken to all animals for the analysis of genotoxicity (Comet assay, Micronucleus and other nuclear abnormalities) immunotoxicity (Total and differential white blood cell counts) and oxidative stress (Lipoperoxidation and the antioxidant enzymes Catalase and Superoxide dismutase).

Results indicated that the five formulations tested, as well as the complex mixtures of them, induced genotoxicity, alteration in white blood cell counts and oxidative stress at environmentally relevant concentrations. Mixtures showed different interactions depending of the biomarker analyzed.

This study constitutes an integral evaluation of the effect of five pesticide formulations and two possible complex ternary mixtures widely used in soybean crops in Argentina, on different endpoints on a native reptile species, *C. latirostris*.

1. Introduction

Pesticides formulations are chemical mixtures extensively used for pest control in crops, and are currently considered as one of the main factors responsible for environmental contamination worldwide (Gaona et al., 2019).

In Argentina, the use of pesticides formulations in recent years has increased significantly, with negative consequences for several wild species living in environments affected by the overlapping of agricultural activity. Santa Fe is in the center-east of Argentina and is the third

Abbreviations: BiN, Binuclei erythrocyte; CA, Comet assay; CAT, Catalase; CPF1 and CPF2, two concentrations of commercial of Chlorpyrifos (Lorsban* 48E®); CYP1 and CYP2, two concentrations of commercial formulations of Cypermethrin (Atanor®); DI, Damage Index; DWBC, Differential white blood cells; E_1, Experiment 1; E_2, Experiment 2; EN, Eccentric nuclei; END1 and END2, two concentrations of commercial formulations of Endosulfan (Galgofan®); FNAs, Frequency of other Nuclear abnormalities; FMN, Micronucleus frequencies; GLY, Glyphosate; IS, Immune system; LPO, Lipoperoxidation; M_1, complex pesticide mixture RU CYP END; M_2, complex pesticide mixture RU CYP CPF; MDA, Malondialdehyde; MN, Micronucleus; NAs, other Nuclear Abnormalities; NC, negative control; NN, Notched nuclei; OS, oxidative stress; PANZ1 and PANZ2, two concentrations of commercial formulations of Glyphosate (PanzerGold®); RU1 and RU2, two concentrations of commercial formulations of Glyphosate (Roundup® Full II); SOD, Superoxide dismutase; SVL, snout-vent length; TBARS, Tiobarbituric acid reactive substances; TL, total length; TNA, the sum of total nuclear abnormalities; TWBC, Total white blood cells; VC, vehicle control; WBC, white blood cells..

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most important agricultural province in the country, considering soybean crops (Lacelli and Ybran, 2016). The expanding areas of extensive agriculture overlap with the natural geographic distribution of *C. latirostris,* so that caimans are exposed constantly and simultaneously to complex mixtures of several pesticides, including organophosphates, pyrethroids and organochlorines, among others. In the last years, many of these compounds have also been registered at high concentrations in environmental sites surrounding agricultural fields (rainwater or surface water bodies and soil) from the Pampean region (Ronco et al., 2016; Alonso et al., 2018; Peluso et al., 2019). Even GLY and its main metabolite AMPA, was considered as "pseudo-persistent" pollutants with a hight-toxic adverse effect in the ecosystem (Primost et al., 2017). The average half-life of GLY in natural freshwaters is about 60-70 days, with the most important route of degradation being mediated by aerobic and anaerobic microorganisms in soils and biofilms (Mercurio et al., 2014).

Additionally, the International Agency for Research on Cancer (IARC) has considered GLY as a compound "probably carcinogenic in humans" (category 2A) on the bases of *in vivo* and *in vitro* studies and primarily on research with the main formulated product Roundup® (Guyton et al., 2015; IARC, 2017), whereas the United States Environmental Protection Agency (US EPA) claimed that the herbicide is unlikely to be carcinogenic to humans (Benbrook, 2019).

Among the insecticides widely used in Argentina, CYP is employed in industries, domestic and agricultural setups. Due to its enormous global use, it is one of the most studied pyrethroid worldwide because the concern for keeping it within safe limits. CYP has low water solubility (10 μg/L) and aquatic persistence of 50 days. It has a moderate persistence in soils and degrades more quickly in those with a low content of organic matter. Under aerobic conditions, its half-life in soil is from 4 days to 8 weeks and it is subject to microbial degradation; being more persistent in anaerobic conditions with a strong tendency to adhere to soil particles (Ullah et al., 2018).

In the case of Chlorpyrifos (-CPF) is the most used insecticide for genetically modified soybean crops in the region (Bernal-Rey et al., 2020). CPF is an organophosphate is consider as a moderately persistent in the environmental, primarily due to its high absorption in soil, low water solubility (2000 μg/L) and aquatic persistence (154 days). This pesticide can breakdown in the environment through many routes by biotic and abiotic degradation, but it is still comparatively resistant to biodegradation and persists in the environment for 5–17 years (Mitkovska and Chassovnikarova, 2020). This pesticide is classified also as a moderately toxic agent according to the US EPA (Li et al., 2015).

Finally, the insecticide Endosulfan (-END) was banned in Argentina since 2013 (United Nations Environment Programme, 2011) with an extending legal use of the remaining circulating product until 2016, but still nowadays, high END levels are found in stream water exceeding the maximum permitted limit for aquatic biota protection (Lupi et al., 2019).

In addition, the period of maximum pesticide application takes place during the reproductive season of caimans (November to March), so that developing embryos and hatchlings can be particularly susceptible (Poletta et al., 2009). In relation to this, Lupi et al. (2019) reported that the air mean concentration of these chemicals might increase by 25 times during summer, compared to the winter period in agricultural regions.

Many of the effects of pesticides are chronic and may have consequences for the entire ecosystem, including lesions on animals and death, reproductive inhibition or failure, developmental disorders, suppression of immune system, disruption of endocrine system, teratogenic and carcinogenic effects, and cellular and molecular alterations including DNA damage (Islam and Tanaka, 2004; Beyer et al., 2014; Mitkovska and Chassovnikarova, 2020).

The biomarkers of genotoxicity are early indicators of damage to genetic material and/or associated structures. Among them, the analysis of micronucleus (MN) and other nuclear abnormalities (NAs), as well as DNA damage detected by the Comet assay (CA) are the most frequently used and recommended endpoints for detecting genotoxicity in environmental toxicology and have been adapted to be applied on broad snouted-caiman erythrocytes by our research group (Poletta et al., 2008; López González et al., 2017). NAs together with MN, have been considered good indicators of the clastogenic or aneugenic effects produced by toxic agents (Fenech, 2019).

Besides, several xenobiotics, and among them pesticides, can induced OS by increasing reactive oxygen species (ROS) production or affecting antioxidant molecules. Chronic and accumulative OS induces deleterious modifications to DNA, lipid peroxidation products (LPO), and modifications in endogenous oxygen free radical scavengers, including Superoxide dismutase (SOD) and Catalase (CAT), that are used as effective biomarkers to study pollutant mediated OS (Hermes-Lima, 2004; Ziech et al., 2010; Narra et al., 2017).

Likewise, the immune system (IS) is very sensitive to changes caused by environmental pollutants and the suppression of the IS can lead to increasing risk of diseases. Toxicants can cause immunotoxicity even at much lower concentrations than those needed to achieve an effect on target organs in the short term, so several immune parameters, like the value of selected blood components, could serve as very sensitive indicators of toxicity (Latorre et al., 2016; Ullah et al., 2019).

Our research group have demonstrated the deleterious effects of GLY, END, CPF and CYP based-formulations separately on *C. latirostris* and *Salvator merianae*, two native reptile species of Argentina, mainly in the early life stages, under controlled and semi-natural conditions (Poletta et al., 2009; 2011; López González et al., 2013; 2017; Latorre et al., 2013; 2016; Siroski et al., 2016; 2017; Schaumburg et al., 2016; Burella et al., 2017; 2018).

Pesticides are frequently applied in complex mixtures of different formulations and the effects of these mixtures in wild reptile species are still scarcely studied (Lopez González et al., 2019; Mestre et al., 2019; Odetti et al., 2020). In the present study we present for the first time, an integrated evaluation on *C. latirostris* during the first month of life, trough biomarkers of different endpoints, including herbicides and insecticides formulations separately as well as in ternary mixtures. We analyzed genotoxicity, immunotoxicity, oxidative stress and growth after *in vivo* exposure to sub-lethal concentrations of three insecticides: END-, CYP-, and CPF-based formulations, two GLY-based herbicide formulations and two different complex ternary mixtures commonly applied in soybean crops.

2. Material and methods

2.1. Chemicals

Pesticides formulations tested were obtained from *Establecimiento La Matuza S.A.*, Santa Fe, Argentina and included: (1) Roundup® Full II (RU; 66.2% GLY), a liquid water soluble (12000 mg/L) herbicide, containing GLY potassium salt [N-(phosphonomethyl) glycine monopotassium salt, $C_3H_7KNO_5P$] as its active ingredient (a.i.) (CAS No. 70901-12-1); (2) PanzerGold® (PANZ; 60.2% a.i., isopropylamine salt of GLY-based [N-(phosphonomethyl) glycine; CAS No. 1071-83-6]; (3) CYP Atanor® (CYP; 25% a.i.), a liquid water-insoluble (0.01 mg/L) mixture of different CYP isomers ($C_{22}H_{19}Cl_2NO_3$, CAS No. 52315-07-8); (4) END Galgofan® (END; 35% a.i.) a liquid practically water-insoluble (0.32 mg/L) formulation, containing END as a.i. ($C_8H_6Cl_6O_3S$, CAS No. 115-29-7); and (5) CPF Lorsban 48E® (CPF; 48% a.i.) O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, water solubility (2 mg/L) (EXTOXNET, 2020).

Ethanol (Cicarelli, Argentina) was used as a vehicle substance for END, CYP, CPF and the mixtures (M_1 and M_2).

Chemicals used as reagents for biomarker analysis included: potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen diphosphate (K_2HPO_4), sodium chloride (NaCl), 1,1,3,3′-tetraethoxypropane, ethylenediaminetetraacetic acid (EDTA), Tris–HCl, and sodium

hydroxide (NaOH) were also from Cicarelli (Argentina). Hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), acridine orange and SOD Kit (19160-1KT) were from Sigma-Aldrich (St. Louis, MO, USA. Normal and low melting point agarose were from Invitrogen® (Life Technologies Argentina).

2.2. Pesticide analysis

Analytical determination of GLY, CPF, END and CYP in aqueous solution and percentage of recovery of the a.i. after 30 and 60 days were described in Poletta et al. (2011) and López González et al. (2019). GLY was analyzed by high-performance liquid chromatography (HPLC) with pre-column derivatization using 9-fluorenylmethyl chloroformate (FMOC-Cl), and the insecticides by Gas chromatographic method (GC-TOF) with a detection limit of 0.10 μg/L (Fox Laboratories, Venado Tuerto, Santa Fe, Argentina).

2.3. Animals

All animals in this study were treated in accordance with the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals (CONICET, 2005), and the "Best Management, Practices for Crocodilian Farming Version 1 (Manolis and Webb, 2016), using non-invasive techniques of blood collection and minimizing stress and suffering by suitable management methods. This study was evaluated and approved (N° 258-16) by the Ethic and Safety Advisory Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Santa Fe, Argentina).

We used 20 days-old *C. latirostris*, hatched from eggs harvested from different nests immediately after oviposition (within 3-5 days), in the Natural Managed Reserve "El Fisco'' (30° 11' 26'' S; 61° 0' 27'' W; San Cristóbal Department, Santa Fe, Argentina), under the "Proyecto Yacaré" (PY) ranching program (Larriera et al., 2008). This Natural Managed Reserve was chosen as it is a Protected Natural Area (Provincial Law 12,930; 2008) and is part of the natural distribution of the species. This is located at least 20 km far from any pesticide application area so the risk of pesticides contamination by runoff, leach or drift is considered low enough to ensure no previous exposure of eggs. This area was previously used as a control area in several toxicological studies (Poletta et al., 2009, 2011, 2016; Latorre et al., 2013, 2016; López González et al., 2013, 2017, 2019; Siroski et al., 2016; Burella et al., 2017, 2018; Odetti et al., 2020).

2.4. Experimental design and treatments

The study was carried out at the PY - Laboratorio de Zoología Aplicada: Anexo Vertebrados (FHUC-UNL/MAC, Santa Fe) facilities. The experiments were performed in consecutive years (2014-2015) and were defined as Experiment 1 and Experiment 2 (E1 and E2). In both experiments, eggs coming from different clutches were used to homogenized clutch variation among experimental groups and controlled this factor at the moment of statistical analysis, bearing in mind that one of the principal causes of variability in studies on crocodilians is the "clutch effect" (effect associated to the clutch of origin, Webb et al., 1987; Verdade, 1997). Animals from each clutch were individualized by the cut of caudal scute (Larriera et al., 2008).

2.4.1. Experiment 1 (E1)

One hundred and thirty two animals, hatched from eggs coming from six different clutches were equally distributed into eleven experimental groups (12 animals per experimental group divided in two replicates): (1) a negative control (NC) treated with tap water; (2) a vehicle control (VC) treated with ethanol; **(3-4)** two groups exposed to different concentrations of RU (RU1 and RU2); **(5-6)** two groups exposed to different concentrations of PANZ (PANZ1 and PANZ2); **(7-8)** two groups exposed to different concentrations of END (END1 and END2); **(9-10)** two groups exposed to different concentrations of CYP (CYP1 and CYP2); and **(11)** a group treated with the complex mixture (M_1): CYP1 + END1 + RU1 (Table 1).

2.4.2. Experiment 2 (E2)

Sixty-four animals hatched from eggs coming from four different clutches, were equally distributed into four experimental groups (16 animals per experimental group divided in two replicates): 1) a vehicle control (VC) treated with ethanol; 2-3) two groups exposed to different concentrations of CPF; and 4) one group treated with the complex mixture (M_2): CYP1 + CPF1 + RU1 (Table 1). As in E1 we had tested both the NC and VC, we avoided using a NC in E_2 again, in order to reduce the number of animals under experimentation and because all treatments included water insoluble compounds.

Concentrations of GLY-based formulations were determine in relation to those recommended for their application in soybean crops (2%/ha), applied considering the base area of experimental plastic containers (75 cm long, 35 cm wide and 37 cm high, base surface = 0.2622 m^2) and diluted in a fixed volume of tap water (4 L) as described in previous studies (Latorre et al., 2013; López González et al., 2013, 2019; Siroski et al., 2016).

In the case of insecticide formulations (CYP, END and CPF), the amount recommended for their application in crops could not be used because when prepared to be applied in the bioassay containers, they gave concentrations higher than toxic reference doses, so we decided to applied an equal and lower range for the three of them (0.5-1 μg/L) based on the information available in other studies (Sharma et al., 2007; Simoniello et al., 2009; Vera Candiotti et al., 2013a). These concentrations were previously tested in the species in other studies made by our group (Latorre et al., 2016; López González et al., 2019).

All pesticides concentrations were progressively reduced in time during the experiment in order to simulate the degradation of the compounds in water (Table 1), previously determined as described in Section 2.2, in the same conditions of this experiment.

A sub-chronic exposure (60 days) was performed by voluntary immersion in plastic experimental containers, tilted to provide 60% dry and 40% water surface areas, with a maximum water depth of approximately 15 cm (fixed volume of 4 L). Temperature in the containers were maintained at 30 ± 2 °C and were monitored with Hobo data logger (Onset Computer Corp., Pocasset, MA, USA). Food was supplied *ad libitum* three times a week, consisting of a mixture of 50% minced chicken head and 50% dry pellets for reptiles. Water in the containers was renewed every two days alternating with feeding days.

At the end of the experiment, blood samples (0.5 ml) were taken from the spinal vein of all animals (Myburgh et al., 2014) with heparinized syringes and 25G x 5/8" needles. Samples were not taken at the beginning of the experiment to avoid any risk of death for caimans due to their initial small size.

Animals were measured in snout-vent length (SVL), total length (TL) and weighed at the beginning and at the end of the experiment to determine growth in each experimental groups.

2.5. Genotoxicity tests

2.5.1. Micronuclei and other nuclear abnormalities

MN and other NAs were analyzed on *C. latirostris* peripheral blood erythrocytes according to the procedures and criteria adopted by Poletta et al. (2008) and López González et al. (2017) respectively.

Two smears were made for each animal, fixed with ethanol and stained with Giemsa (10%). For each sample, 1,000 erythrocytes were analyzed under an optical microscope Nikon Eclipse E200 at 1000x magnification. The results were expressed as MN/NAs frequencies (FMN/FNAs: number of cells with MN or any other NAs/1000 erythrocytes counted).

The classification for other NAs included four nuclear lesions:

Table 1
Experimental groups and treatments applied in Experiment 1 (E_1) and Experiment 2 (E_2). *Caiman latirostris* hatchlings exposed to glyphosate-, cypermethrin-, chlorpyrifos- and endosulfan- based pesticide formulations separately and in ternary mixtures.

	EXPERIMENTAL GROUPS	Compound	Initial concentration	Final concentration	N° of animals per nest/EG	*N*
Experiment 1 (E1)	Negative control (NC)	Tap water	-	-	2	12
	Vehicle Control (VC)	Ethanol	200 µl/L	200 µl/L	2	12
	RU1	Roundup®Full II	5000 µg/L	500 µg/L	2	12
	RU2	Roundup® Full II	2500 µg/L	250 µg/L	2	12
	PANZ1	PanzerGold®	5000 µg/L	500 µg/L	2	12
	PANZ2	PanzerGold®	2500 µg/L	250 µg/L	2	12
	CYP1	Atanor®	0.5 µg/L	0.05 µg/L	2	12
	CYP2	Atanor®	1 µg/L	0.1 µg/L	2	12
	END1	Galgofan®	0.5 µg/L	0.05 µg/L	2	12
	END2	Galgofan®	1 µg/L	0.1 µg/L	2	12
	M_1	CYP1+END1+RU1	0.5 + 0.5 + 5000 µg/L	0.05 + 0.05 + 500 µg/L	2	12
Experiment 2 (E2)	Vehicle Control (VC)	Ethanol	200 µl/L	200 µl/L	2	16
	CPF1	Lorsban* 48E®	0.5 µg/L	0.05 µg/L	2	16
	CPF2	Lorsban* 48E®	1 µg/L	0.1 µg/L	2	16
	M_2	CYP1+CPF1+RU1	0.5 + 0.5 + 5000 µg/L	0.05 + 0.05 + 500 µg/L	2	16

NC: negative control; VC: vehicle control; EG: experimental group; RU1 and RU2: commercial formulation of Glyphosate (Roundup® Full II); PANZ1 and PANZ2: commercial formulation of Glyphosate (PanzerGold®); END1 and END2: commercial formulation of Endosulfan (Galgofan®); CYP1 and CYP2: commercial formulation of Cypermethrin (Atanor®); CPF1 and CPF2 commercial formulation of Chlorpyrifos (Lorsban* 48E®). M_1 and M_2: complex pesticide ternary mixtures from E_1 and E_2, respectively.

notched (NN- appreciable depth into a nucleus that does not contain nuclear material), nuclear buds or "budding" (nuclear evaginations), binucleate cells (BiN- cells under uncompleted division, with two completely separated nuclei in the same cytoplasm), presence of eccentric nuclei (EN, cells with the nucleus in an abnormal peripheral position), and finally, we registered the sum of total NAs observed (TNAs).

2.5.2. Comet assay

CA was applied on *C. latirostris* peripheral blood erythrocytes as described by Poletta et al. (2008). Unwinding was carried out in alkaline buffer during 10 min and then electrophoresis was performed in the same buffer during 15 min at 0.90 V/cm. One hundred randomly selected comet images were analyzed, visually classified into five arbitrary classes according to tail size and fluorescent intensity, from class 0 (undamaged) to class 4 (maximum damage) and a single DNA damage index ($DI = n_1 + 2n_2 + 3n_3 + 4n_4$) was calculated for each animal (Poletta et al., 2008).

2.6. Oxidative damage and antioxidant enzymes

2.6.1. Lipid peroxidation (LPO) in erythrocytes (TBARS)

Malondialdehyde (MDA) as a marker of LPO in red blood cells was determined by measuring the formation of the color produced during the reaction of TBA with MDA (TBARS Assay), according to Poletta et al. (2016). The sample absorbance was determined at 535 nm and TBARS concentration was calculated using the extinction coefficient $1.56 \times 10^5 M^{-1}$ cm^{-1}. MDA concentration in erythrocytes is expressed as nmol/mg protein.

2.6.2. Catalase (CAT) activity in erythrocytes

CAT activity in lysed erythrocytes was measured spectrophotometrically by monitoring the decrease in H_2O_2 concentration over time as described by Poletta et al. (2016). The specific activity of each sample was calculated on the basis that one unit of enzyme activity is defined as the activity required to degrade 1 mole of hydrogen peroxide during 60 s/g Hb, considering that the signal continues being linear over that period of time. Absorbance was measured at 240 nm, 25 °C during 60 s in the spectrophotometer. Results are expressed in arbitrary units as the Activity of CAT (KU/mg protein).

2.6.3. Superoxide dismutase (SOD) activity in erythrocytes

SOD was determined using the commercial kit 19160-1KT (SIGMA) as described by Poletta et al. (2016). The rate of the reduction with O_2 is linearly related to the xanthine oxidase activity, and is inhibited by SOD. Therefore, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity, as an inhibition activity, can be quantified by measuring the decrease in the color development at 440 nm.

2.6.4. Total protein determination

Total amount of protein in the samples was determined with the kit Proti

U/LCR (#1690007, Wiener Lab) and used for the corresponding calculation of TBARS and CAT activity.

2.7. Immunotoxicity test: total and differential white blood cells (WBC) counts

Total WBC count (TWBC) was performed using a Neubauer chamber. An aliquot of blood was diluted 1:200 with 0.6% NaCl (Latorre et al., 2013). The samples were observed under an optical microscope at 400x and results expressed as total cells/mm^3 blood. To perform differential counts (DWBC), two blood smears were prepared per animal, were air-dried and fixed with ethanol (96%, 10 min), and then stained with May Grünwald (50%, 3 min) - Giemsa (10%, 15 min) solution. The amount of each immune cell sub-type (*e.g.* heterophils, basophils, eosinophils, lymphocytes, monocytes) per 100 WBC analyzed was determined using a light microscope (Nikon Eclipse E200) at 1000x magnification.

2.8. Statistical analysis

Statistical analysis was performed using the software SPSS for Windows (2013).

The absence the differences between replicates for each variable was proved by *t-test,* so each treatment was considered as a grouping variable.

Variables were tested for normality with Kolmogorov-Smirnov test and homogeneity of variances between groups was verified by Levene test. For all the parameters analyzed, mean values ± standard error (S.E.) were calculated from data of all animals of each experimental group (E_1 and E_2).

The one-way ANOVA, followed by Tukey test were used for the comparison of buds, NN, EN and TNA in E_1 and buds, EN, TWBC count,

heterophils, eosinophils and CAT in E_2. The Mann Whitney U-test was used for the comparison of: CAT, TWBC count and DWBC counts in E_1; lymphocytes and monocytes in E_2; and the FMN, BiN, DI, SOD activity and TBARS in both experiments, between each exposed group and its respective control as well as between M and each individual pesticide included in the mixtures (RU, END, CYP, and CPF). In E_1, herbicides (RU and PANZ) were compared with the NC while insecticides (CYP and END) and the M were compared to the VC.

The difference between clutches (*clutch effect*) for all variables were analyzed using ANOVA followed by Tukey's test or Kruskal-Wallis/ Mann Whitney U-test, depending on the distribution of the variable analyzed.

Growth (final - initial values of SVL, TL and weight) were analyzed using the General linear model: univariate, in both experiments.

Significance level was set at $p \leq 0.05$.

3. Results

Results of genotoxicity tests are presented as mean ± S.E. of FMN, FNAs, and DI of the CA per experimental group. In E_1, data analysis showed that there were no significant differences in the DI, FMN or FNAs between the NC and VC, indicating that ethanol caused no genotoxic effect ($p > 0.05$).

There was a significant increase in the FMN in all the herbicides groups: RU1 ($p = 0.001$), RU2 ($p = 0.002$), PANZ1 and PANZ2 ($p < 0.01$) compared with the NC (Mann Whitney U-test). Also the FMN was

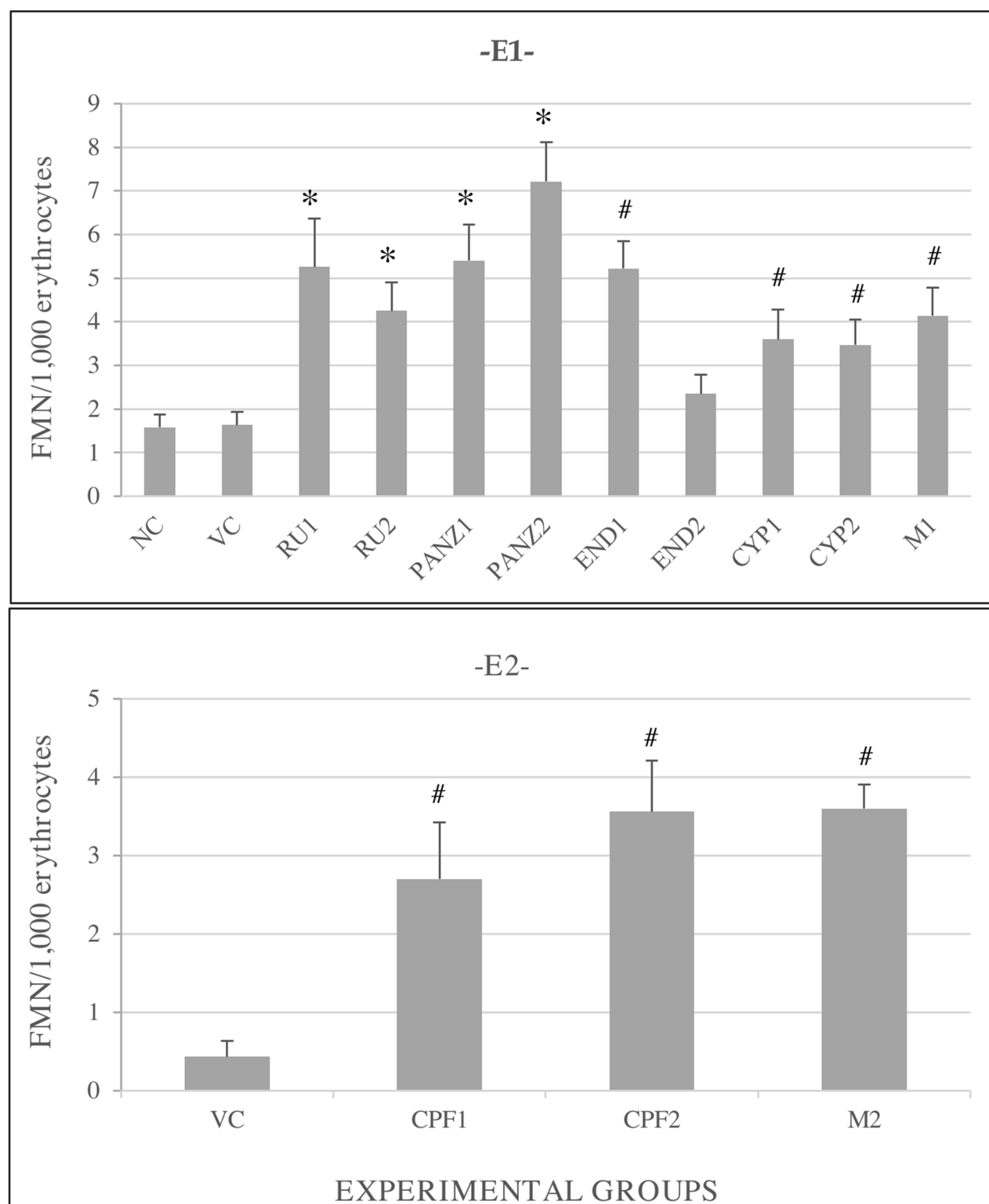

Fig. 1. Micronucleus frequencies (FMN) in 1,000 erythrocytes of *C. latirostris* observed in the different experimental groups. *Experiment 1 (E1):* NC: negative control; VC: vehicle control; RU1 and RU2: commercial formulation of Glyphosate (Roundup® Full II); PANZ1 and PANZ2: commercial formulation of Glyphosate (PanzerGold®); END1 and END2: commercial formulation of Endosulfan (Galgofan®); CYP1 and CYP2: commercial formulation of Cypermethrin (Atanor®); M_1: complex pesticide mixture RU+CYP+END. *Experiment 2 (E2)*: VC: vehicle control; CPF1 and CPF2 commercial formulation of Chlorpyrifos (Lorsban* 48E®); M_2: complex pesticide mixture RU+CYP+CPF. *: significantly different compared to NC. #: significantly different compared to VC.

significantly increased in the groups exposed to END1 ($p < 0.001$), CYP1 ($p = 0.03$), CYP2 ($p = 0.009$) and M_1 ($p = 0.002$) compared to the VC (Mann Whitney U-test -Fig. 1). When comparing the M_1 with each individual constituent components, no differences were observed ($p > 0.05$; Mann Whitney U-test -Fig. 1). Meanwhile in E_2, we observed similar results after exposure to both concentrations of CPF (CPF1: $p = 0.042$ and CPF2: $p = 0.002$) and to the M_2 ($p < 0.001$), respect to the VC (Fig. 1, Mann Whitney U-test).

When we analyzed the FNAs, the results in E_1 (Table 2) showed significant higher frequency of buds for PANZ1 ($p = 0.033$), while a tendency but not statistically significant difference was observed for RU1 ($p = 0.058$), compared to the NC (ANOVA/Tukey's test). For the BiN frequencies we found significant differences between the M_1 and the VC as well as all the individual component of the mixture (RU1, CYP1 and END1; $p \leq 0.01$, Mann Whitney U-test). In the case of EN the results showed differences only for the M_1 respect to the VC ($p = 0.002$; ANOVA/Tukey's test) while the TNAs presented significant differences in the group PANZ1 compared to the NC ($p = 0.028$; ANOVA/Tukey's test). In E_2, we observed a significant higher frequency of buds ($p = 0.028$; ANOVA/Tukey's test) and BiN ($p = 0.04$; Mann Whitney U-test) in the M_2 compared to the VC; and a higher frequency of EN in CPF2 respect to the VC ($p = 0.026$, ANOVA/Tukey's test; Table 2).

In relation to the CA, cell viability of all samples analyzed was in the range of 95-100%, indicating appropriate conditions for the application of the assay. In E_1 results demonstrated that pesticides induced a significant increase in DNA damage of caimans exposed to RU1 ($p = 0.003$) and RU2 ($p = 0.001$) compared to the NC, as well as to CYP1 ($p = 0.034$), CYP2 ($p = 0.001$), END1 ($p = 0.01$) and END2 ($p = 0.001$) compared to the VC (Mann Whitney U-test). However, no significant differences were found between M_1 and the VC ($p = 0.604$). When comparing M_1 to the individual component of the mixture, no significant differences were observed $p > 0.05$, Mann Whitney U-test; Fig. 2).

Besides, in E_2, CPF2 ($p = 0.012$) and M_2 ($p = 0.006$) also induced a significantly higher DI respect to the VC and the same was observed in CPF1 compared to M_2 ($p = 0.035$, Mann Whitney U-test; Fig. 2).

Considering OS parameters, in E_1 we found a significant increase in LPO (TBARS) only in RU1 compared with the NC ($p = 0.045$), while the M_1 caused a higher LPO compared END1 ($p = 0.017$; Mann Whitney U-test). In the case of CAT, there was a lower activity in the M_1 compared to the VC ($p = 0.037$) and CYP1 ($p = 0.019$; Mann Whitney U-test). SOD activity was significantly higher in PANZ1 compared with the NC ($p = 0.036$) and in the M_1 compared with the VC ($p = 0.012$) while the comparison of M_1 with individual compounds showed differences with RU1 ($p = 0.046$), and CYP1 ($p = 0.016$; Mann Whitney U-test; Table 3). In E_2 we observed only a significant LPO in M_2 compared with the VC ($p = 0.032$, Mann Whitney U-test; Table 3).

The results about IS parameters of both experiments are presented in Table 4. In E_1, total WBC count showed differences between the NC and RU1 ($p = 0.023$) and PANZ2 ($p = 0.046$), as well as between the VC and END2 ($p = 0.005$), CYP2 ($p = 0.05$) and M_1 ($p = 0.05$). The comparison of M_1 with the individual compounds showed a significant higher value for RU1 ($p = 0.003$) (Mann Whitney U-test, Table 4). Analyzing WBC types (DWBC), differences between the exposed groups and controls were observed in all subclasses as follows: heterophils NC vs. RU1 ($p = 0.029$), and the VC vs. CYP1 ($p = 0.003$), CYP2 ($p = 0.004$), END1 ($p = 0.001$) and END2 ($p = 0.025$). Lymphocytes: NC vs. RU1 and PANZ1 ($p = 0.011$ and $p = 0.023$, respectively), while the VC vs. CYP1 ($p = 0.005$), CYP2 ($p = 0.003$) and END1 ($p = 0.001$). In monocytes: NC vs. RU1 and RU2 ($p = 0.02$ and $p = 0.001$, respectively), and the VC vs. CYP1 ($p = 0.004$), END1 and END2 ($p = 0.001$ in both cases); and finally in eosinophils: NC vs. PANZ 1 and PANZ2 ($p = 0.011$ and $p = 0.008$, respectively), and the VC vs. CYP2 ($p = 0.004$) and the M_1 ($p = 0.005$). In the comparison between the M_1 and their constituent individuals compounds we found significant differences in heterophils with END1 ($p = 0.042$), in lymphocytes with RU1 ($p = 0.017$) and END1 ($p = 0.03$), in monocytes with RU1 ($p = 0.006$), END1 ($p = 0.001$) and CYP1 ($p = 0.003$), while in eosinophils M_1 showed differences with RU1 ($p = 0.008$), END1 ($p = 0.007$) and CYP1 ($p = 0.009$; Mann Whitney U-test -for all cases, Table 4).

In E_2 TWBC count showed differences between VC and all exposed groups: CPF1 and CPF2 ($p = 0.009$ in both cases) and M_2 ($p = 0.003$; ANOVA/Tukey's test, Table 4). In the case of DWBC no significant differences were observed among the experimental groups for any of the types of WBC ($p > 0.05$).

Finally, no effects were observed in length or weight of the caimans exposed to any pesticide formulation or complex mixtures tested in any of the experiments, compared with the control groups ($p > 0.05$ in all analysis performed).

Taking into account the clutch of origin as a grouping variable, no differences were found in any of the variables analyzed, so no "clutch effect" was observed.

4. Discussion

Evaluation of pesticide effects through integral approaches including complementary and non-destructive biomarkers of different endpoints

Table 2
Frequency of other Nuclear Abnormalities observed in *Caiman latirostris* hatchling in different experimental groups.

	EXPERIMENTAL GROUPS	Buds	NN	BiN	EN	TNAs
Experiment 1 (E1)	**NC**	134.05 ± 7.51	31.26 ± 3.32	0.63 ± 0.16	54.16 ± 5.14	220.10 ± 11.67
	VC	108.95 ± 7.52	24.26 ± 1.80	0.21 ± 0.10	52.80 ± 4.73	243.74 ± 10
	RU 1	145.69 ± 9.52	38.95 ± 3.40	0.58 ± 0.28[a]	50.47 ± 2.95	235.68 ± 11.56
	RU 2	150.25 ± 9.21	30.05 ± 2.75	1.05 ± 0.40	66.90 ± 4.55	248.25 ± 12.56
	PANZ 1	175.47 ± 11.49*	34.07 ± 3.40	0.60 ± 0.21	73.33 ± 9.23	283.50 ± 20.24*
	PANZ 2	147.71 ± 7.27	28.00 ± 2.43	0.50 ± 0.17	65.21 ± 5.18	241.43 ± 9.94
	END 1	129.95 ± 7.84	28.00 ± 3.62	0.22 ± 0.15[a]	94.83 ± 4.69	253.00 ± 11.68
	END 2	127.77 ± 7.81	23.24 ± 2.20	0.12 ± 0.12	94.00 ± 6.20	245.12 ± 13.40
	CYP 1	146.41 ± 9.36	27.53 ± 2.52	0.41 ± 0.21[a]	90.82 ± 7.06	265.18 ± 15.34
	CYP 2	133.90 ± 8.09	24.95 ± 2.25	0.47 ± 0.23	93.32 ± 6.44	252.63 ± 12.04
	M_1	106.21 ± 4.29	33.72 ± 2.83	2.79 ± 0.41[#]	75.14 ± 5.12[#]	217.86 ± 8.19
Experiment 2 (E2)	**VC**	108.00 ± 4.33	36.00 ± 3.39	0.14 ± 0.14	112.00 ± 10.60	256.00 ± 11.05
	CFP 1	134.20 ± 10.20	58.90 ± 9.04	1.30 ± 0.45	84.70 ± 6.68	279.10 ± 14.35
	CPF 2	128.10 ± 7.04	49.10 ± 5.70	0.55 ± 0.18	80.20 ± 4.95[#]	258.00 ± 12.12
	M_2	144.30 ± 8.36[#]	42.70 ± 2.85	1.20 ± 0.36[#]	88.60 ± 6.50	276.80 ± 12.26

All values are expressed as mean ± SE. NC: negative control; VC: vehicle control; RU1 and RU2: commercial formulation of Glyphosate (Roundup® Full II); PANZ1 and PANZ2: commercial formulation of Glyphosate (PanzerGold®); END1 and END2: commercial formulation of Endosulfan (Galgofan®); CYP1 and CYP2: commercial formulation of Cypermethrin (Atanor®); CPF1 and CPF2 commercial formulation of Chlorpyrifos (Lorsban* 48E®). M_1 and M_2: complex pesticide mixtures. Buds; NN: Notched; BiN: Binuclei; EN: Eccentric nuclei; TNA: sum of total nuclear abnormalities in 1,000 erythrocytes observed in all experimental groups. *: significantly different compared to NC. [#]: significantly different compared to VC. [a]: significantly different compared to M_1 ($p < 0.05$).

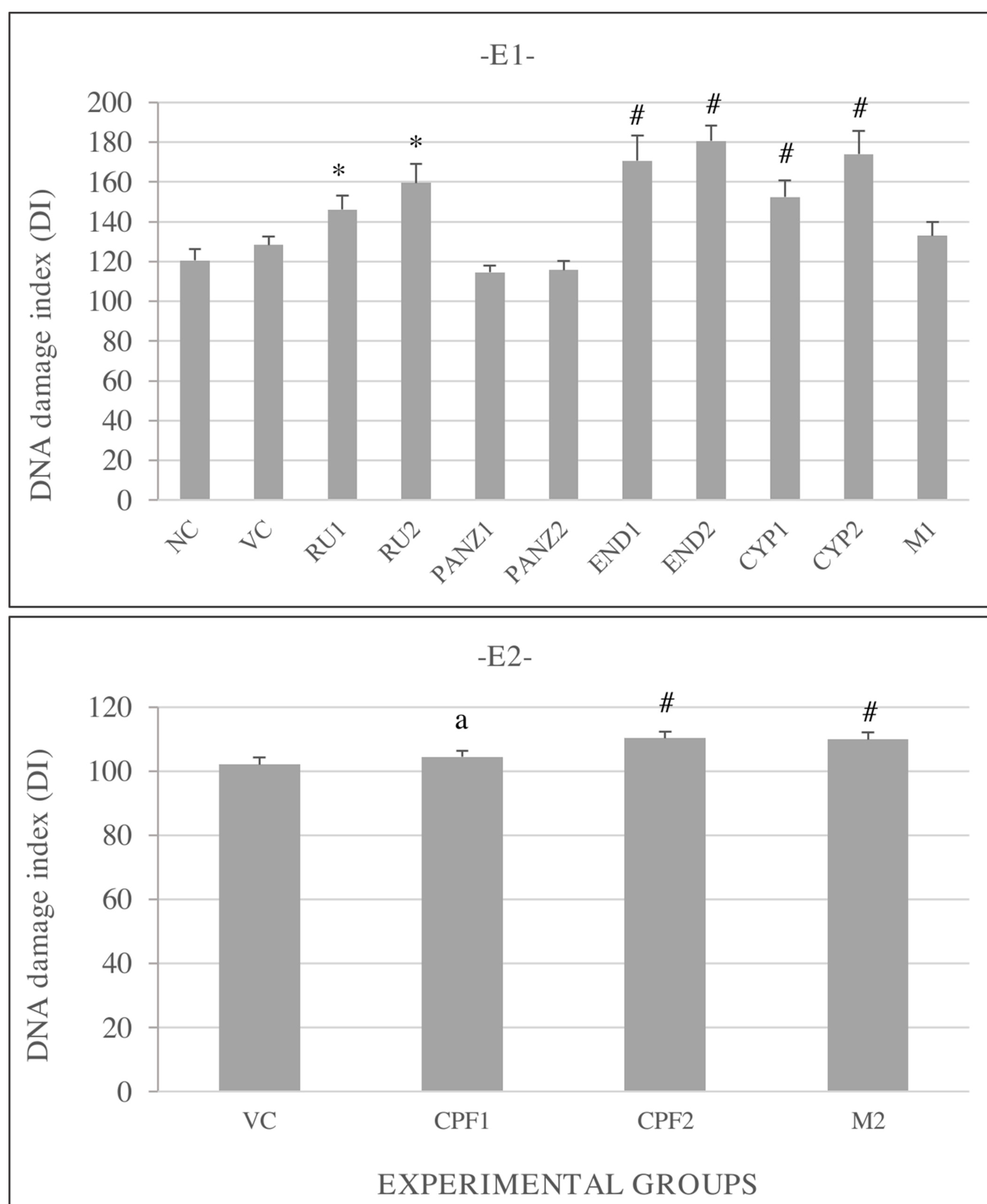

Fig. 2. Damage Index (DI) in *C. latirostris* exposed to different pesticides formulations and mixtures. NC: negative control; VC: vehicle control; RU1 and RU2: commercial formulation of Glyphosate (Roundup® Full II); PANZ1 and PANZ2: commercial formulation of Glyphosate (PanzerGold®); END1 and END2: commercial formulation of Endosulfan (Galgofan®); CYP1 and CYP2: commercial formulation of Cypermethrin (Atanor®); CPF1 and CPF2 commercial formulation of Chlorpyrifos (Lorsban* 48E®); M_1: complex pesticide mixture RU+CYP+END; M_2: complex pesticide mixture RU+CYP+CPF. *: significantly different compared to NC. $^{\#}$: significantly different compared to VC. ^{a}: significantly different ($p = 0.035$) compared to M_2.

constitutes the more reliable tools for understanding real exposure scenarios. Genotoxicity, oxidative stress, and immunotoxicity have widely demonstrated to be sensitive groups of biomarkers, of rapid response and potentially related among each other. In the current study, caiman blood was used to assess DNA damage based on its easy collection and processing and the benefit that no damage is done to the animals.

In the present study, our results highlighted the genotoxic effect on broad-snouted caiman exposed to sub-lethal concentrations of CYP, END, CPF and GLY formulations, as well as both complex ternary mixtures analyzed under laboratory-controlled conditions, evidenced by a significant increase in MN and some other NAs frequencies, as well as DNA damage by the CA. The DI and the FMN increased in the majority of the pesticide formulations and mixtures tested in both experiments (E_1 and E_2). Unlike this, all the other assessed abnormalities were more limited in their response to the effect of pesticide exposure, showing differences in few groups. However, they can provide complementary information on the mechanistic of actions of compounds together with the other indicators of genotoxic damage (FMN and CA).

Regarding the CA, we could observe a similar effect than the other genotoxicity biomarkers, mainly with the FMN. The results demonstrated that pesticides induced a significant increase in DNA damage of caimans exposed to all pesticides formulations separately, at both concentrations analyzed compared to the controls, except PANZ. Previous reports confirmed the genotoxic damage of RU in caiman neonates (after *in vivo* exposure) at similar experimental conditions but at a

Table 3
Lipoperoxidation (TBARS), and antioxidant enzyme activities (Catalase and Superoxide dismutase) in *Caiman latirostris* hatchling in the different experimental groups.

	EXPERIMENTAL GROUP	TBARS (nmol/mg prot)	CAT (KU/mg prot)	SOD (% activity)
Experiment 1 (E1)	NC	3.15 ± 0.20	216.77 ± 7.50	56.04 ± 2.99
	VC	4.33 ± 0.46	219.00 ± 17.85	59.16 ± 2.10
	RU1	5.78 ± 1.98 *	229.04 ± 60.79	67.58 ± 4.50[a]
	RU2	3.27 ± 0.21	221.78 ± 31.43	62.99 ± 4.60
	PANZ1	5.18 ± 1.47	197.31 ± 45.38	68.57 ± 3.72*
	PANZ2	4.74 ± 1.17	228.29 ± 39.33	63.89 ± 2.99
	CYP1	5.46 ± 1.29	255.31 ± 16.72[a]	70.35 ± 3.96[a]
	CYP2	5.15 ± 1.23	262.83 ± 13.78	73.73 ± 5.71
	END1	3.87 ± 0.16[a]	192.04 ± 46.58	70.59 ± 3.75
	END2	5.00 ± 0.73	211.33 ± 25.58	71.87 ± 1.58
	M_1	5.59 ± 0.46	248.82 ± 23.02[#]	90.64 ± 5.34[#]
Experiment 2 (E2)	VC	7.97 ± 0.26	292.78 ± 17.64	54.79 ± 3.28
	CPF1	8.85 ± 0.52	348.53 ± 27.70	63.26 ± 2.23
	CPF2	8.21 ± 0.41	385.01 ± 38.71	68.38 ± 1.89
	M_2	9.54 ± 0.45[#]	334.51 ± 28.19	84.66 ± 4.23

All values are expressed as mean ± SE. NC: negative control; VC: vehicle control; RU1 and RU2: commercial formulation of Glyphosate (Roundup® Full II); PANZ1 and PANZ2: commercial formulation of Glyphosate (PanzerGold®); END1 and END2: commercial formulation of Endosulfan (Galgofan®); CYP1 and CYP2: commercial formulation of Cypermethrin (Atanor®); CPF1 and CPF2 commercial formulation of Chlorpyrifos (Lorsban* 48E®). M_1 and M_2: complex pesticide mixtures. TBARS: Tiobarbituric acid reactive substances; CAT: Catalase; SOD: Superoxide dismutase.
[*] : significantly different compared to NC. [#]: significantly different compared to VC. [a]: significantly different compared to M_1 ($p < 0.05$).

concentration range of 21–5 mg/L and 11–2.6 mg/L, showing a concentration-dependent effect for the FMN (López González et al., 2013). The disparity observed in our study between the results obtained from both herbicides formulations could be explained by the fact that both commercial herbicides present a different GLY concentration and include one or more adjuvants that might contribute differently to the overall toxicity of the formulation. The polyethylene amine (POEA) is the main surfactant in most RU formulations and it has been demonstrated that it is more toxic to some species than glyphosate itself (Guilherme et al. 2012; Ghisi et al., 2016; Leveroni et al., 2016).

Other reports also indicate similar results in other species after *in vivo* exposure to comparable and lower concentrations of RU than those used in our study. Moreno et al. (2014) showed significantly higher DNA damage scores in erythrocytes and gill cells of the fish *Prochilodus lineatus,* exposed to two nominal concentrations of Roundup Transorb® (1 and 5 mg/L^{-1}) at 24 and 96 h. Likewise, individuals of the same species exposed to 10 mg/L of RU showed also DNA damage in erythrocytes, but did not show significant difference in the FMN and FNAs after 6 and 96 h of exposure (Cavalcante et al., 2008). Leveroni et al. (2016) evaluated the genotoxic response in peripheral blood, gill and liver cells of *Piaractus mesopotamicus* exposed to sub-lethal concentration of RU (2.75 mg/L) during 96 h, using CA, and the MN and other NAs test, with significant differences for all biomarkers. In the same way, Vera-Candioti et al. (2013b, 2013c) tested the toxicity of 48% GLY-based formulations, PANZ and Credit®, on *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). Credit® exposure showed an increase in the MN frequency after 96 h of treatment; whereas a similar increase was observe with PANZ between 48 and 96 h of exposure time at 3.9 and 7.8 mg/L. On the contrary, our data indicated lack of effect of the GLY-formulation PANZ in the DI, unlike the results obtained for the FMN, buds and TNAs (in the last two only at the lower concentration of PANZ).

Differences observed among the results of genotoxicity biomarkers for different GLY formulations tested in this and other studies may be due to the different mechanism of action of one or more components of the pesticide formula or to the mixture as a whole, in relation to the different endpoints of damage detect by each biomarker. While the CA detects low levels of damage at DNA molecule that may lead to gene mutations (Tice et al., 2000), the MN and other NAs test is a good indicator of both clastogenic and aneugenic effects (Fenech, 2019). The CA, in combination with the MN and other NAs test, with differences in sensitivity and type of information generated, constituted important

Table 4
Total and differential white blood cells count (mean ± SE) on *Caiman latirostris* hatchling in the different experimental groups applied in Experiment 1 (E_1) and Experiment 2 (E_2).

	EXPERIMENTAL GROUPS	TWBC	DWBC Heterophils	Lymphocytes	Monocytes	Eosinophils
Experiment 1 (E1)	NC	25578.95 ± 1534.81	22.20 ± 2.25	62.37 ± 5.50	0.42 ± 0.23	5.47 ± 0.76
	VC	26763.16 ± 1798.05	22.47 ± 1.60	62.66 ± 1.98	0.32 ± 0.11	4.96 ± 0.65
	RU 1	24305.56 ± 2296.32*[a]	24.33 ± 1.31*	69.33 ± 1.23*[a]	1.11 ± 0.24*[a]	4.33 ± 0.82
	RU 2	22500.00 ± 1092.24	27.37 ± 2.00	65.79 ± 2.15	1.32 ± 0.25*	4.79 ± 0.49
	PANZ 1	25055.56 ± 1512.46	24.27 ± 2.30	71.60 ± 2.44*	0.27 ± 0.12	2.93 ± 0.48*
	PANZ 2	20470.59 ± 1176.10*	28.15 ± 2.85	67.92 ± 2.90	0.23 ± 0.12	2.69 ± 0.67*
	END 1	32973.68 ± 2398.67	35.28 ± 2.68[#a]	55.78 ± 2.54[#a]	2.00 ± 0.41[#a]	6.00 ± 0.79[a]
	END 2	28552.63 ± 2146.30[#]	33.82 ± 2.23[#]	62.18 ± 2.38	0.82 ± 0.26[#]	5.18 ± 0.91
	CYP 1	25933.33 ± 1521.17	37.78 ± 3.73[#]	55.61 ± 3.65[#]	1.50 ± 0.33[#a]	4.22 ± 0.56[a]
	CYP 2	20464.29 ± 1158.42[#]	30.32 ± 1.46[#]	61.00 ± 1.86[#]	1.00 ± 0.30	6.68 ± 0.63[#]
	M_1	22214.29 ± 1105.32[#]	28.64 ± 1.78	63.71 ± 1.89	0.21 ± 0.11	6.57 ± 0.72[#]
Experiment 2 (E2)	VC	9187.50 ± 566.45	16.13 ± 3.31	80.00 ± 3.64	1.13 ± 0.35	1.88 ± 0.40
	CFP 1	6944.44 ± 367.46[#]	16.33 ± 2.49	79.78 ± 2.56	0.56 ± 0.18	2.67 ± 0.58
	CPF 2	6944.44 ± ± 467.29[#]	19.22 ± 1.74	77.33 ± 1.60	0.44 ± 0.18	2.11 ± 0.31
	M_2	6700.00 ± 528.10[#]	19.70 ± 2.30	76.20 ± 1.56	0.30 ± 0.153	2.80 ± 0.46

TWBC: Total white blood cells count. DWBC: Differential white blood cells count.NC: negative control; VC: vehicle control; RU1 and RU2: commercial formulation of Glyphosate (Roundup® Full II); PANZ1 and PANZ2: commercial formulation of Glyphosate (PanzerGold®); END1 and END2: commercial formulation of Endosulfan (Galgofan®); CYP1 and CYP2: commercial formulation of Cypermethrin (Atanor®); M_1: complex pesticide mixture RU+CYP+END; CPF1 and CPF2 commercial formulation of Chlorpyrifos (Lorsban* 48E®); M_2: complex pesticide mixture RU+CYP+CPF. *: significantly different compared to NC. [#]: significantly different compared to VC. [a]: significantly different compared to M_1 ($p < 0.05$).

complement tools to detect different aspects of genotoxicity (Frenzilli et al., 2009; Vasquez, 2010).

Besides herbicides, the results obtained in this study showed an increase in DNA damage and the FMN in erythrocytes of caimans exposed to different insecticide formulations (Figs. 1 and 2).

CPF induced a significantly higher FMN at both concentration tested and the same for the DI at the higher concentration, compared to the VC. The same commercial formulations was also tested in *C. decemmaculatus* (Vera-Candioti et al., 2013a) within the concentration range of 8-25 μg/L demonstrating an increased in the FMN at 48 and 96 h of treatment. Similar results were observed in the freshwater fish *Channa punctatus* exposed during 96 h to three different concentrations of CPF (203, 406 and 609 μg/L) under laboratory-controlled conditions (Ali et al., 2008). Ismail et al. (2014) observed an increase in DI induced by sublethal concentrations of CPF (221.4, 110.7 and 73.8 μg/L) in erythrocytes and gill cells of the fish *Labeo rohita* after 96 h of exposure. In the same way, Yin et al. (2009) demonstrated a genotoxic effect evidenced by the presence of MN, other NAs and increased of DI in anurans (*Bufo bufo gargarizands*) exposed to a series of five sublethal concentrations (0.32, 0.64, 0.72, 1.08 and 2.56 μg/L) of a formulation of CPF (40% i. a.) for 24, 48, 72, and 96 h. This last study is within the same concentration range applied in our study.

A recent experimental study in the common carp (*Cyprinus carpio L.*) revealed that CPF is toxic even at permissible levels (100%, 50% and 30% of Annual Average of Environmental quality standards), with considerable genotoxic and cytotoxic potential in peripheral erythrocytes (Mitkovska and Chassovnikarova, 2020).

Due to concerns over their persistence and toxicity of END, the United Nations Environment Program (UNEP) included this compound in the list of prohibited organic pollutants . In our country, a period of five years was established for phasing it out since the date of the effective Resolution 511/2011 (SENASA, 2011), including a total prohibition for using, importing, processing (synthesis), formulation, and marketing for the active ingredient and formulated products, thus extending legal use until 2016. In spite of this, at the moment this study took place, END was one of the main insecticides used for pest control in Argentina, and still now residues and metabolites are found in the environment (Ballesteros et al., 2014; Svartz et al., 2015; Astoviza et al., 2016; Lupi et al 2019).

In relation to this insecticide, both concentrations tested induced a significant DNA damage compared to the VC. Sharma et al. (2007) reported similar results in erythrocytes and other tissues of fish *Mystus vittatus* exposed *in vivo* to two sub-lethal concentrations of END (0.50 and 0.25 μg/L) and a non-lethal concentration (0.20 μg/L), one of them being equal to the lowest initial concentration tested in our study. Similar results were observed in zebrafish (*Danio rerio*) subchronically exposed (28 days) to four different concentrations of END, in a range including the same concentrations tested by us (0.01, 0.1, 1 and 10 μg/L) (Shao et al., 2012). Besides, Crupkin et al. (2013) reported an increase in the FNAs (0.02 μg/L) and FMN (5 μg/L) in *Australoheros facetus* after 24 h acute exposure. In our study, only the lower concentration of END (0.5–0.05 μg/L) induce an increase in the FMN, while at the highest concentration (1–0.1 μg/L) the FMN and some other NAs showed lower values, similar to the control. This effect was also observed at the higher concentration of CPF, in which EN showed values significantly lower than the VC, and at the higher concentrations of PANZ, in which the frequency of some NAs were lower than the lower concentration of the herbicide and, in some cases, even lower than the NC, but not statistically significant.

A possible explanation to this could be as suggested by several authors, that the frequency of nuclear lesions may be altered by several factors related to blood cell kinetic, such as cell cycle arrest for cytotoxic effects at the high concentration, with a reduction of damaged cell in the circulation; or the stimulation of erythropoiesis that leads to constant erythrocytes replacement with a “dilution effect” in the bloodstream (Barni et al., 2007; Burella et al., 2017; Jindal and Verma, 2015; López González et al., 2017; Polard et al., 2011; Udroiu, 2006; Vera-Candioti et al., 2013b).

In the case of CYP, our data clearly show that when caimans are exposed to sublethal concentrations of it, an increase in DNA damage and FMN are induced at both concentrations. These results are consistent with previous reports where the insecticide caused DNA damage in erythrocytes and gill cells of *P. lineatus* exposed *in vivo* to 0.300, 0.150 and 0.075 μg/L during 96 h (Simoniello et al., 2009; Poletta et al., 2013). Ansari et al. (2011) reported an increased in the FMN and a correlation with OS and disturbance of antioxidant enzymes in *Channa punctata* exposed to CYP at 0.4, 0.8 and 1.2 mg/L for 48 and 72 h.

The application of pesticides mixtures is a common agricultural practice. In this study we examined each pesticide alone and in different mixtures combinations M_1: GLY-CYP-END and M_2: GLY-CYP-CPF. Our data showed a higher frequency of MN, EN and BiN erythrocytes in individuals exposed to M_1, but no significant difference was observed in the DI. The same mixture (M_1) demonstrates a high level of damage in *C. latirostris* exposed *in ovo* under semi-natural conditions similar to that happening in natural environments near crops (Poletta et al., 2011). In the case of M_2, the genotoxic effect was evident through the DI, FMN, buds and BiN.

Additionally, a recent experimental study in caiman hatchlings (20 days old) sub-chronically exposed to binary mixtures of the same pesticide formulations and concentrations tested in the present study revealed an increase in the frequency of MN and other NAs in erythrocytes respect to the controls, with a possible antagonistic action between RU and CPF in that binary mixture. When we see the effect of the ternary mixture constituted by these compounds and CYP (M_2), it seems that the presence of CYP was determinant for the toxicity of this mixture as it would suppress the antagonistic effect observed between CPF and GLY as a binary mixture (López González et al., 2019). However, neither of the ternary mixtures showed to be more toxic than the individual compounds.

On the contrary, Odetti et al. (2020) found a significant increase in the FMN in caiman hatchlings exposed *in ovo* by spraying on the incubation material at concentration equivalent to those recommended in agricultural practices with a certain potentiation action of CPF on CYP and RU in the binary mixtures constituted by them (CPF+CYP and CPF+RU).

Moreover, the increase observed in the frequency of BiN erythrocytes in both ternary mixtures tested here is consistent with the increase in the FMN in the same groups, as both abnormalities can be originated by failure of tubuline polymerization and difficulty of the formation of mitotic fuse caused by aneugenic action of these toxic compounds (de Campos Ventura et al., 2008; Mitkovska and Chassovnikarova, 2020).

Results concerning DNA damage also showed interactions between the components but somehow different. In the ternary mixture where END is present (M_1: RU+CYP+END) some kind of antagonistic action is showed between the constituting compounds, as the genotoxic effect observed for all of them separately is not evident in the mixture any more. Odetti et al (2020) also reported an antagonistic effect in DNA damage and oxidized pyrimidines for the binary mixture of CPF+CYP and in oxidized pyrimidines for the ternary mixture (RU+CYP+CPF), as well as in purine oxidation in the mixture of RU+CYP. Authors assumed that the pesticides interfered with the effect of each other and, as a result, there was a reduction in the observed effect for individual compounds. On the other hand, they also reported an antagonistic action of CPF on RU in that mixture that made the oxidation of purines observed for RU alone is diminished in the mixture.

The activities of antioxidant enzymes are widely assessed as an early sign of intoxication or measure of cellular stress. In the present study, induction of SOD activity in peripheral blood of caimans could be due to increasing superoxide radicals (O_2^-) production, which in turn, is considered the first defense mechanism against OS caused by these pesticides. In our study, PANZ1 and M_1 showed a significantly higher activity of SOD compared to the controls, and there was LPO in animals

exposed to RU1 and M_2. These results suggest that in these two later groups of animals, antioxidant enzymes were not able to effectively scavenge ROS, as SOD activity showed an increase but not statistically significant, thus leading to LPO. On the contrary, in PANZ1 and M_1, the activity of SOD was enough to counteract the effect of ROS, protecting cells from LPO. Besides, CAT showed a lower activity in the M_1 compared to the VC, which can be interpreted as a toxicity effect of the mixture on the enzyme itself. This result was previously observed in the same species after *in ovo* exposure to the same formulations and mixtures tested here (Burella et al., 2018; Odetti et al., 2020).

In the present study, the insecticides seemed to cause no effects on OS parameters. The results of the comparison between the mixtures and the individual compounds would indicate a synergistic interaction in the case of SOD (E_1) and TBARS (E_2) while an antagonistic action in the case of CAT, that made in all cases, a significantly difference compared to the controls even when no effect was observed for the individual components separately.

In the analysis of mixtures, a recent *in situ* study evaluated the application of a current-use mixture of pesticides (the herbicide RU, the insecticide bifenthrin and the fungicides azoxystrobin and cyproconazole) on two native fish (*Markiana nigripinnis* and *Astyanax lacustris*) inhabiting a rice field. Authors reported that antioxidant system failed to prevent OS in liver and gills of *M. nigripinnis,* while caused an inhibition of the antioxidant defenses without LPO in *A. lacustris,* 21 days after a fumigation event (Rossi et al., 2020).

Hematologic investigations are important to wildlife because they can infer the health state of the populations, giving valuable information in relation to the IS (Gilbertson et al., 2003).

Furthermore, blood cells and plasma are recognized as a convenient vector for analysis since peripheral blood reflects the global health status of the individual and is considered to be in contact and under a close equilibrium with all tissues. Sublethal concentrations of pesticides may compromise the immunity of the organisms, and makes them susceptible to secondary infections (Ullah et al., 2019). Thus, an increase or decrease in the number of WBC is the normal reaction of the exposure to toxicants (Narra et al., 2017). In the present study, the exposure to RU1 induced an increase in the number of TWBC, and particularly in lymphocytes and monocytes, but a lower number of heterophils, compared with the NC. PANZ1 showed the same effect for lymphocytes but PANZ2 induced a decrease in both TWBC and eosinophils. In previous works under similar experimental design, that is *in vivo* exposure of neonates by voluntary immersion in water containers, considering environmentally relevant concentrations but higher than the present work and subsequently decay of the compound in water through time, we demonstrated a lower complement system activity in animals exposed to RU at higher decreasing concentrations (21 - 5 mg/L and 11- 2.6 mg/L), but no alterations in TWBC (Latorre et al., 2013; Siroski et al., 2016).

In relation to insecticides, END induced a decrease in the number TWBC (END1) and lymphocytes (END1), but an increase in heterophils (END 1 and 2), monocytes (END1 and 2) and eosinophils (END2). Bachetta et al. (2011) evaluated changes in hematological and oxidative stress markers on different tissues in fish exposed to sublethal concentrations of END. Differential leukocytes count was affected, showing higher proportion of monocytes and lower proportion of lymphocytes and neutrophils (known as heterophils for reptiles), similar results than those observed by us except for the contrary effect on heterophils.

CYP produced exactly the same effect than END in TWBC and DWBC, with little differences in lymphocytes (both concentrations of CYP produced effect) and monocytes (only CYP1 was significantly different to the VC). In contrast, Latorre et al. (2016) found no significant differences in TWBC of *C. latirostris* neonates exposed to END and CYP formulations, in the same concentrations used here, but they found differences in DWBC that are congruent to the findings of the present study. The authors attributed the lack of significant effects at the higher concentrations used to the appearance of a differential tolerance (Latorre et al., 2016). In the case of CPF, results showed only a decrease in the number of TWBC for both concentrations compared with the VC. Contrarily, Girón-Pérez et al. (2006) and Díaz-Resendiz and Girón-Pérez (2014) showed that CPF does not have any effect on hematological parameters evaluated in Nile tilapia.

The analysis of the effect of the mixtures showed that both of them (M_1 and M_2) induced a decrease in the number of TWBC and besides, M_1 (RU+CYP+CPF) showed an increase in the percentage of eosinophils. In the same way, Mestre et al. (2019) founded a reduction in TWBC in the Argentinian tegu lizard (*Salvator merianae*) juveniles in a semi-natural exposure with the same mixture (M_2), indicating a possible immunosuppression effect.

When compared the mixtures to the individual compounds, M_1 showed a significantly lower TWBC count, monocytes and lymphocytes compared to RU1 and for heterophils compared to END1. On the contrary, animals exposed to the mixture showed a higher number of lymphocytes than END1 and the same for eosinophils compared to all the individual compounds. These results allow us to propose a possible antagonistic action of CYP and END on the effect of RU for TWBC count and lymphocytes as well as a mutual antagonism between all the compounds for heterophils and monocytes. While in the case of eosinophils, it seems to be a synergistic effect. Liu et al. (2006) studied the interaction and dose-related effect of the mixture CYP-methyl parathion on endocrine and immune functions in the Wistar rats, reporting lower lymphocyte transformation rates and higher neutrophil phagocytosis rates in exposed rats. In the case of M_2 (RU+CYP+CPF), no differences were observed in any parameter with the groups exposed to the formulations separately.

A mixture of pesticides can produce different kind of interactions among their components (Brodeur et al., 2014) and the response depends on the chemical properties and modes of toxic action of the pesticides in addition to biological characteristic of the species (Lydy et al., 2004; Hernández et al., 2013). Bonifacio and Hued (2019) demonstrated different responses in mixtures treatments (in concentration environmentally relevant) that reflected a complex interaction (antagonism and potentiation). In this way, we could suggest that the interactions observed in different pesticide complex mixtures often induce responses with different degrees of sensitivity.

An integrated analysis using a broad variety of biomarkers and environmentally relevant concentrations of xenobiotics as we present in this study is required to obtain valid information on the stress undergoing by those organisms living in exposed environments. Moreover, the concentrations range applied in our study are similar to those currently found in agricultural basins in Argentina; indicating in many cases concentrations that exceed the reference levels for the protection of aquatic biota: GLY 0.2–700 μg/L, CPF 0.001–2.5 μg/L, CYP 0.01–6.62 μg/L, and END 0.001–0.9 μg/L (Etchegoyen et al., 2017; Peluso et al., 2019; Lupi et al., 2019; Mac Loughlin et al., 2020).

In the environment, organisms are simultaneously exposed to a great variety of chemicals with diverse properties. The way in which chemicals in a mixture influence the overall toxicity depends on many factors including their concentration, target site and mechanism of action. Contaminants with similar or different modes of action can influence each other's toxicity; resulting in an almost unlimited number of possible additive, synergistic or antagonistic combinations. In addition, non-chemical factors may also act as stressors and add to the complexity of multiple stressor situations (Beyer et al., 2014). In this context, pesticides have received much attention as possible combined toxicity stressors. Results of different kind of interactions found for the mixtures in the present work depending on the biomarker or parameter analyzed confirm these ideas.

5. Conclusion

Results of the present work indicated that the five formulations tested as well as the complex mixtures of them induced genotoxicity, alteration in total and differential white blood cell counts, as well as

oxidative stress. The effects observed for the mixtures revealed different kind of interactions among their components depending on the parameter analyzed, including synergistic, antagonistic and absence of interaction at all. The biomarkers applied showed different degrees of sensitivity to pesticides according to the concentrations used, so it is important to develop integrated approaches in biomonitoring, as the use of more parameters of different endpoints ensures more reliable and better interpretations of the results. Likewise, we highlight the importance of laboratory studies to elucidate mechanisms of toxicity of different pesticide formulations and their mixtures, in order to understand better the differences between possible answers in studies conducted under controlled conditions and those made *in situ* in natural populations environmentally exposed, to contribute to the ecosystem health assessment. Under field conditions, organisms can be exposed, in many cases, to different xenobiotics and other stress factors simultaneously, probably increasing the effects observed in controlled studies. Furthermore, these results could also contribute to the development of management and conservation actions for wildlife in these kinds of ecosystems with increasing environmental problems.

CRediT authorship contribution statement

E.C. López González: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **M.L. Romito:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **M.A. Latorre:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **P.A. Siroski:** Conceptualization, Resources, Supervision, Funding acquisition, Project administration. **G.L. Poletta:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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