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Micronuclei and other nuclear abnormalities on *Caiman latirostris* (Broad-snouted caiman) hatchlings after embryonic exposure to different pesticide formulations

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ABSTRACT

This study aimed to evaluate the embryotoxicity and genotoxicity of pesticide commercial formulations widely used in soybean crops through the Micronucleus (MN) test and other Nuclear Abnormalities (NAs) in erythrocytes of broad-snouted caiman (*Caiman latirostris*), exposed by topical application through the eggshell.

Embryos were exposed (during all incubation: 70 days approximately) to sub-lethal concentrations of two glyphosate formulations PanzerGold® (PANZ) and Roundup® Full II (RU) (500, 750, 1000 μg/egg); to the endosulfan (END) formulation Galgofan® and the cypermethrin (CYP) formulation Atanor® (1, 10, 100, and 1000 μg/ egg). Blood samples were taken at the moment of hatching from the spinal vein for the application of the MN test and analysis of other NAs in erythrocytes, as markers of genotoxicity.

Results indicated a significant increase in the frequency of MN for PANZ1000, END 10, CYP 1 and CYP 100 ($p < 0.05$), and in the frequency of other NAs including *Buds*: END 100, 1000 and CYP 10 ($p < 0.05$), eccentric nuclei: END 1, 10, 1000, CYP 10, 100, 1000 ($p < 0.01$) and END 100 ($p < 0.05$), notched nuclei: END 1, 10 ($p < 0.01$) and END 1000, CYP 10, 100,1000 ($p < 0.05$), and total nuclear abnormalities: END 1, 10, 100, 1000, CYP 10, 100 and 1000 ($p < 0.01$), and the positive control (PC) ($p < 0.05$), compared with the negative control. It was demonstrated a concentration dependent-effect in MN frequency only for PANZ (R^2=0.98; $p < 0.01$).

Our study demonstrated that commercial formulations of pesticides induced genotoxic effects on *C. latirostris*, and NAs are a good indicator of genotoxicity in this specie.

1. Introduction

The alterations of DNA integrity produced by toxic compounds such as pesticides have been thoroughly investigated in many organisms. These compounds can disrupt normal cellular processes and interact directly or indirectly with DNA, causing genetic instability (Carballo and Mudry, 2006).

The analysis of the blood cells morphology, particularly erythrocytes, became an important bioindicator of pollution, and thereby, providing important tools for the prediction of the potential long-term effects of xenobiotics in wild species. Over recent years, alterations in erythrocytes nuclei morphology have been increasingly used to evaluate genotoxic effects of different compounds (Strunjak-Perovic et al., 2010; Caliani et al., 2014; Furnus et al., 2014; Perez-Iglesias et al., 2014).

Among cytogenetic test systems, the assessment of MN is commonly used for evaluating structural and numerical chromosomal aberrations induced by clastogenic and/or aneugenic agents. MN originates from acentric chromosome fragments and/or whole chromosomes that are unable to engage with the mitotic spindle and/or fail to segregate properly to the daughter nuclei during anaphase. The lagging fragments or whole chromosomes are surrounded by membrane and become MN (Fig. 1). Acentric fragments are caused by failure of repair or mis-repair of DNA strand breaks which may be induced by chemicals that (i) damage the phosphodiester backbone of DNA, and/or (ii) inhibit the DNA damage response mechanisms or repair of DNA strand breaks and/or (iii) cause DNA replication stress due to DNA adduct or cross-link formation. MN originating from lagging whole chromosomes may be induced by chemicals that cause defects in centromeres or the mitotic machinery (Fenech et al., 2016).

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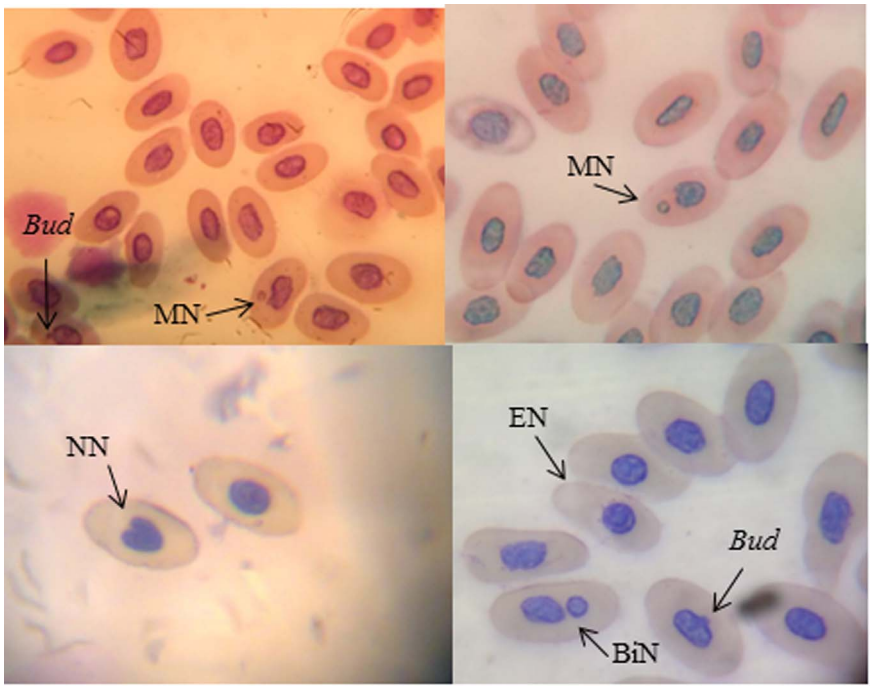

Fig. 1. Images of *C. latirostris* erythrocytes with Micronuleus (MN) and other Nuclear abnormalities (NAs) analyzed: Buds, Notched nuclei (NN), Binuclei (BiN) and Eccentric nuclei (EN) (arrows). 1000×.

In recent years, several studies have described the presence of other NAs, also considered to be induced by genotoxic agents. The formation mechanisms of many of these NAs are not yet fully understood but nowadays, many researches included them in the evaluations of genotoxicity as a complementary assay to the MN test (Crupkin et al., 2013; Lajmanovich et al., 2014; Pollo et al., 2015; Burella et al., 2016; Schaumburg et al., 2016). Carrasco et al. (1990) categorized these NAs into four groups (blebbed, lobed, notched nuclei and vacuolated nuclei). One of the most accepted explanations for those NAs concerning nucleus aspect suggested that when the cell detects an affected DNA region, a repair process and elimination of chromatin is initiated. The altered region is then moved to the periphery of the nucleus and eliminated by exocytosis. Before the process is completed, the nuclear membrane exhibits some imperfections, characterizing NAs. The presences of nuclear *buds* or *"budding"*, related to DNA amplification in the S phase of the cell cycle, is considered as a NA (Prieto et al., 2008; Fenech et al., 2011).

NAs included for this study were: *Buds*, Binuclei (BiN), Notched nuclei (NN), Eccentric nuclei (EN) and Total nuclear abnormalities (TNA) (Fig. 1).

In the last two decades, agricultural production in South America showed great expansions of genetically modified (GM) crops, specially soybean, driven by demand from the international market, and with the consequent increase in the application of different agrochemicals (López et al., 2012). GM soybean allows intensive use of GLY, along with several insecticides such as CYP and END, among others (EXTOXNET, updated, 2015), contributing to physical and chemical changes in water properties, which can be reflected in the biological integrity of organisms living there.

The direct effect of pesticides interferes with molecular mechanisms regulation in early life of development (Paganelli et al., 2010). The protective mechanisms available in adults, such as DNA repair mechanisms, a competent immune system, detoxifying enzymes, liver metabolism, and the blood/brain barrier, are not fully functional in early stages of developmental individuals (Uggini et al., 2010).

In Argentina, many natural populations of *C. latirostris* overlap with areas of intensive agriculture, mostly with soy crops. The maximal applications of all these pesticides coincide with the reproductive season of this species (November-March). During this period, females construct the nests using surrounding vegetation so that embryos may be exposed not only to residual pesticides in the nest material, but also through direct spraying during applications when nests are close to crops. The texture of eggshells allows substantial air and water exchange during development and may allow other compounds to cross through the eggshell (de Solla et al., 2014).

In these months (November–March), short but heavy rainfalls occur frequently and so they can cause intensive pesticide runoff to non target compartments such as aquatic ecosystems. In relation to this, pesticides such as GLY, CYP, END and clorpyrifos (CPF) were detected in sediments, suspended particles, and water (Peruzzo et al., 2003, 2008; Jergentz et al., 2005; Aparicio et al., 2013).

The aim of this study was to evaluate the embryotoxicity and genotoxicity of four pesticide formulations widely used in soybean crops through the Micronucleus (MN) test and Nuclear abnormalities (NAs) determination in erythrocytes of broad-snouted caiman (*Caiman latirostris*), exposed by topical application through the eggshell.

2. Materials and methods

2.1. Chemicals

Pesticides formulations tested were obtained by courtesy of *Establecimiento La Matuza S.A.*, Santa Fe, Argentina and included: (1) Roundup® Full II (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® (60.2% GLY, isopropylamine salt of glyphosate-based [N-(phosphonomethyl) glycine; CAS 1071-83-6] commercial formulations; (3) CYP Atanor® (25% CYP, 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); and (4) END Galgofan® (35% END) a liquid practically water- insoluble (0.32 mg/l) formulation, containing endosulfan as a.i. ($C_8H_6Cl_6O_3S$, CAS No. 115-29-7) (EXTOXNET, updated, 2015).

Ethanol was used as a vehicle control for END and CYP. Cyclophosphamide (CP; CAS 6055-19-2), an indirect alkylating agent

well known as a genotoxic substance, was used as an *in ovo* positive control (Poletta et al., 2009) and was purchased from Fluka.

2.2. C. latirostris eggs collection

C. latirostris eggs from different nests harvested in the Natural Managed Reserve "El Fisco" (30°11′26″ S; 61°0′27″W; Dpto. San Cristobal, Santa Fe Province, Argentina, as part of "Proyecto Yacare" ranching program activities (Larriera et al., 2008), were used. This is a Protected Natural Area, free of farming and urban activities which belong to the natural distribution of the species, and was chosen to ensure that eggs had not been environmentally exposed to any xenobiotic, as no contaminating activity is carried out there. A total of nine nests, collected during 2013–2014 nesting season (December 2013) with a minimum of 22 eggs each, were used to carry out the corresponding experiments. All nests used in the experiments were collected within 5 days after oviposition, under the same conditions from harvest to treatment assignment, and egg viability was determined by analyzing the opaque eggshell banding (Donayo et al., 2002).

The average weight of eggs used in experiments was 67.8 ± 4.75 g.

2.3. Ethics committee

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), using non-invasive techniques of blood collection and minimizing stress and suffering by suitable management methods. The study was evaluated and approved by the Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral (Santa Fe, Argentina) for animal experimentation (N° 04-12).

2.4. Experimental design and treatments

The study was carried out at the "Proyecto Yacaré" – Laboratorio de Zoología Aplicada: Anexo Vertebrados (FHUC-UNL/MMA, Santa Fe) facilities.

2.4.1. Experiment 1 (E_1)

Ninety-six eggs from three nests (32 eggs per nests), were randomly distributed into eight experimental groups (12 eggs per experimental groups with two replicates of 6 eggs each), as follows: **1)** a negative control (NC) group, treated with distilled water; **2–4)** three groups exposed to 500, 750, 1000 μg/egg of PANZ; **5–7)** three groups exposed to 500, 750, 1000 μg/egg of RU; and **8)** a positive control (PC) group treated with 700 μg/egg CP (Poletta et al., 2009).

2.4.2. Experiment 2 (E_2)

One hundred and thirty two eggs from six nests (22 eggs per nests), were randomly distributed into eleven experimental groups (N=132; 12 eggs per experimental groups with two replicates of 6 eggs each): **1)** a negative control (NC) group treated with distilled water; **2)** a vehicle control (VC) group treated with ethanol; **3–6)** four groups exposed to 1, 10, 100, and 1000 μg/ egg of END; **7–10)** four groups exposed to 1, 10, 100, and 1000 μg/ egg of CYP; and **11)** a positive control (PC) group treated with 700 μg/egg CP (Poletta et al., 2009).

Concentrations applied in our study were chosen taking as reference previous studies made in *C. latirostris* (Beldoménico et al., 2007; Poletta et al., 2009, 2011b) and other species such as bird and mammals (Sinha et al., 1997; Anwar et al., 2003; Patel et al., 2006), adapting them to the average weight of *C. latirostris* eggs (70 g approximately) and to our experimental conditions. CP concentration is based on previous studies made by our group in the same species (Poletta et al., 2009, 2011a).

In both experiments, the pesticide solutions were applied on the eggshell (by topical application) at the embryo implantation zone, within the first 5 days of incubation. Each experimental group was placed separately in a plastic container, using vermiculite as substrate and covering them with vegetal material similar to the nesting material, free of any exogenous substance.

All eggs were incubated, under a temperature of 31.5 ± 0.5 °C and 95% humidity in the "Proyecto Yacaré" incubator. They were controlled periodically during the experiment in order to identify and discard those which became non-viable.

When hatchlings started to call within the eggs, the corresponding eggs were removed from the incubator and if hatching did not occur spontaneously during the following 24 h., they were assisted (Larriera et al., 2008). In the case 72 h. after the hatching of the first egg of a certain clutch any egg of the same clutch remain unhatched, it was helped to do it.

2.5. Blood collection and genotoxicity tests

Peripheral blood samples (0.5 ml) were obtained immediately after hatching from the spinal vein of all neonates (Myburgh et al., 2014), with heparinized syringes and 25G ×5/8" needles.

MN test and other NAs were applied on peripheral blood erythrocytes. Two slides were made for each animal, fixed with ethanol for 10 min and stained with Giemsa (10%) for 15 min. For each slides, 500 erythrocytes were analyzed (Poletta et al., 2008) under the optical microscope Nikon Eclipse E200 with a magnification of 1000×.

The criteria adopted for MN identification were based on Schmid (1975) and Poletta et al. (2008) for *C. latirostris,* 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.

Other NAs were distinguished according to the classification proposed by Carrasco et al. (1990) and Fenech, (2000) in: NN (appreciable depth into a nucleus that does not contain nuclear material), nuclear *buds* or *"budding"* (nuclear evaginations), BiN (cells with two nuclei, in division), and presence of EN. The frequencies of the following nuclear abnormalities: MN (FMN); *Buds*, NN, BiN, EN and total nuclear abnormalities (TNAs- Oliveira et al., 2010; Lajmanovich et al., 2014, 2015; Schaumburg et al., 2016) were calculated from 1000 erythrocytes counted per animal (in two slides). Results were expressed as the frequency of each category: FMN and FNAs (separately or total NAs) (Fig. 1).

2.6. Developmental parameters

Hatching success was recorded for all groups. Then all hatchlings were individually identified, weighed (OHAUS® Compact scale CS200, precision 0.1) and measured in total length (TL) and snout-vent length (SVL) (tape measure, precision 0.5 cm).

After that, all the animals were maintained in plastic containers under common controlled conditions used in Proyecto Yacaré facilities. Food was supplied *ad libitum* three times a week, consisting of a mixture of 50% minced chicken head and 50% dry pellets for reptiles. At 3 and 6 months of age they were measured and weighed again in order to evaluate the effect of treatments on subsequent growth of the animals during the first months of life.

2.7. Statistical analysis

Statistical analysis was performed using the software SPSS 15.0 for Windows (SPSS, 2008).

Mean values ± standard error (S.E.) of MN and other NAs were calculated from data of animals of each experimental group. Variables were tested for normality with Kolmogorov-Smirnov test and homogeneity of variances between groups was verified by Levene test.

In both experiments the one-way ANOVA, followed by Dunnett's test were used for the comparison of *Buds* and BiN frequencies between

exposed groups and the NC, while the Mann Whitney U-test was used for the comparison of NN, EN, TNA and MN frequencies between each exposed groups with the NC.

Considering that the "*clutch effect*" is one of the most important causes of variability observed in crocodilians (Schulte et al., 1990; Verdade et al., 1997), we analyzed the difference between clutches for all variables, using the ANOVA or Kruskal-Wallis test depending on the distribution of the variable analyzed.

Linear regressions were done to determine the existence of a concentration-dependent effect on MN and NAs frequencies, to evaluate the relation between genotoxic biomarkers and size of the animals, and between the initial eggs weight with neonates weight.

Animal growth data (weight, TL and SVL) during the first months of life (at hatching, 3 and 6 months) were analyzed by General lineal model: repeated measures.

A difference of $p \leq 0.05$ was considered statistically significant except when the Bonferroni correction was applied, in which the p value depends on the number of pair analysis done (it is informed in each case).

3. Results

Results of genotoxicity tests are presented as mean ± S.E. of MN and NAs frequencies per experimental group (Table 1).

NC and VC showed no differences in the FMN or FNA, indicating that ethanol caused no genotoxic damage ($p > 0.05$; E_2).

On the other hand, results demonstrated an induction of MN caused by exposure to the PC, PANZ1000, END 10, CYP 1, and CYP 100 ($p < 0.05$), compared to the NC.

Results obtained from the other NAs showed significant differences respect to the NC in *Buds*: END 100, 1000 and CYP 10 ($p < 0.05$), EN: END 1, 10, 1000, CYP 10, 100,1000 ($p < 0.01$), and END 100 ($p < 0.05$) NN: END 1, 10 ($p < 0.01$), END 1000, CYP 10, 100, and 1000 ($p < 0.05$), and TNA: END 1, 10, 100 and 1000, CYP 10, 100 and 1000 ($p < 0.01$), and PC ($p < 0.05$).

There were differences among clutches (*clutch effect*) observed in the MN frequencies (Kruskal-Wallis / Mann-Whitney, applying the Bonferroni correction: $p=0.05/3 \leq 0.016$ for E_1 and $p=0.05/6 \leq 0.008$ for E_2), NN and TNA in E_1 (ANOVA; $p < 0.05$) and EN and TNA in E_2 (ANOVA; $p < 0.05$).

Table 2
Growth in total length (TL), snout-vent length (SVL) and weight of *C. latirostris* at birth, in all experimental groups in Experiment 1 (E_1) and Experiment 2 (E_2). All values are expressed as mean ± S.E. NC: negative control; VC: vehicle control; PC: positive control (Cyclophosphamide700 μg/eggs); PANZ (PanzerGold®) 500, 750, 1000 (μg/eggs); RU (Roundup®) 500, 750, 1000 (μg/eggs); END (Galgofan®) 1, 10, 100, 1000 (μg/eggs); CYP (Atanor®) 1, 10, 100, 1000 (μg/eggs).

	Experimental group	TL	SVL	Weight
Experiment 1 (E_1)	**NC**	23.4 ± 0.18	11.2 ± 0.07	44.2 ± 1.39
	PC	22.6 ± 0.05	10.1 ± 0.05	30.6 ± 0.47
	PANZ 500	22.6 ± 0.55	10.9 ± 0.22	41.3 ± 1.21
	PANZ 750	23.6 ± 0.54	11.2 ± 0.26	44.4 ± 0.98
	PANZ 1000	23.5 ± 0.29	11.4 ± 0.07	46.6 ± 0.96
	RU 500	23.4 ± 0.26	11.2 ± 0.11	45.5 ± 0.62
	RU 750	23.1 ± 0.13	11.1 ± 0.07	44.9 ± 0.81
	RU 1000	23 ± 0.17	11.0 ± 0.08	43.9 ± 0.91
Experiment 2 (E_2)	**NC**	22.2 ± 0.27	10.8 ± 0.12	38 ± 0.45
	VC	23.0 ± 0.24	10.8 ± 0.12	44.5 ± 1.02
	PC	23.0 ± 0.45	11.2 ± 0.11	43.0 ± 0.95
	END 1	23.5 ± 0.30	11.1 ± 0.19	43.9 ± 1.16
	END 10	23.5 ± 0.24	11.1 ± 0.11	44.8 ± 1.32
	END 100	22.4 ± 1.16	12.2 ± 1.03	44.8 ± 0.79
	END 1000	23.4 ± 0.14	11.3 ± 0.72	44.5 ± 0.50
	CYP 1	23.5 ± 0.18	11.2 ± 0.18	44.9 ± 1.40
	CYP 10	23.4 ± 0.16	11.1 ± 0.10	44 ± 1.01
	CYP 100	23.4 ± 0.19	11.0 ± 0.09	44.3 ± 0.47
	CYP 1000	23.2 ± 0.23	11.1 ± 0.10	44.5 ± 0.93

No significant differences were observed in growth between all experimental groups at birth (Table 2) or during the first 6 months of life, in any of the experiments (E_1 and E_2; GLM, Repeated measures,).

Regression analysis demonstrated a concentration-dependent effect only for PANZ formulation on the FMN (R^2=0.98; $p < 0.01$) (Fig. 2).

No relation was found between MN and NAs with caiman's weight or length neither in E_1 nor E_2 ($p > 0.05$ in all analysis performed).

Table 1
Micronucleus and other Nuclear abnormality frequencies observed in *C. latirostris* hatchlings at different experimental groups in E_1 and E_2.
All values are expressed as mean ± S.E. E_1: NC: negative control; PANZ (PanzerGold®) 500, 750, 1000 (μg/eggs); RU (Roundup®) 500, 750, 1000 (μg/eggs); PC: positive control (Cyclophosphamide 700 μg/eggs); and E_2: NC: negative control; VC: vehicle control; END (Galgofan®) 1, 10, 100, 1000 (μg/eggs); CYP (Atanor®) 1, 10, 100, 1000 (μg/eggs); PC: positive control (Cyclophosphamide 700 μg/eggs). MN/1000 cells: Micronucleus found in 1000 erythrocytes observed. TNA: sum of Total Nuclear Abnormalities in 1000 erythrocytes counted, excluding MN.

	Experimental group	MN	*Buds*	NN	BiN	EN	TNA
Experiment 1 (E_1)	**NC**	2.50 ± 0.62	89.3 ± 10.4	58.0 ± 7.45	1.21 ± 0.38	23.9 ± 8.11	173 ± 13.8
	PC	5.80 ± 1.10*	127 ± 11.6	56.2 ± 7.00	1.13 ± 0.31	32.0 ± 8.69	216 ± 16.1*
	PANZ 500	3.75 ± 1.03	139 ± 19.8	42.7 ± 9.31	0.75 ± 0.75	6.50 ± 2.63	189 ± 25.9
	PANZ 750	4.75 ± 1.11	162 ± 11.9	54.0 ± 9.86	0.50 ± 0.29	8.25 ± 2.29	225 ± 18.8
	PANZ 1000	5.80 ± 1.74*	158 ± 20.9	43.8 ± 10.5	0.60 ± 0.60	11.0 ± 2.28	213 ± 29.8
	RU 500	4.20 ± 0.98	122 ± 10.77	53.1 ± 6.16	0.40 ± 0.13	28.5 ± 7.92	204 ± 11.9
	RU 750	3.44 ± 0.65	117 ± 9.22	58.3 ± 5.78	0.78 ± 0.27	26.1 ± 6.87	203 ± 12.3
	RU 1000	3.13 ± 0.68	97.6 ± 10.2	51.7 ± 5.46	0.73 ± 0.25	24.1 ± 7.92	174 ± 13.3
Experiment 2 (E_2)	**NC**	2.90 ± 0.65	90.5 ± 11.5	61.2 ± 7.34	2.11 ± 0.45	25.0 ± 8.34	183 ± 14.5
	VC	2.88 ± 0.84	96.2 ± 9.38	58.9 ± 7.11	0.70 ± 0.25	24.9 ± 7.27	181 ± 11.7
	PC	6.33 ± 1.60*	130 ± 12.2	51.2 ± 6.07	1.63 ± 0.55	36.1 ± 10.3	224 ± 23.0*
	END 1	3.67 ± 0.80	134 ± 9.87	32.9 ± 3.56**	1.07 ± 0.37	81.4 ± 13.4**	249 ± 22.5**
	END 10	3.81 ± 0.45*	134 ± 11.1	33.7 ± 3.76**	1.00 ± 0.30	64.9 ± 6.64**	234 ± 14.5**
	END 100	3.93 ± 0.76	142 ± 14.7*	49.9 ± 6.54	0.93 ± 0.20	49.0 ± 10.2*	242 ± 20.9**
	END 1000	3.46 ± 0.52	141 ± 10.3*	38.5 ± 3.28*	0.87 ± 0.24	70.6 ± 8.10**	250 ± 16.2**
	CYP 1	4.29 ± 0.62*	108 ± 18.8	43.7 ± 6.75	0.93 ± 0.25	43.1 ± 11.9	196 ± 25.7
	CYP 10	3.89 ± 0.62	142 ± 12.9*	35.3 ± 4.69*	0.68 ± 0.20	66.4 ± 7.38**	245 ± 18.4**
	CYP 100	7.60 ± 0.62*	138 ± 8.26	32.7 ± 4.36*	0.87 ± 0.26	68.7 ± 6.87**	240 ± 12.9**
	CYP 1000	3.06 ± 0.62	137 ± 12.2	36.5 ± 3.95*	0.69 ± 0.18	67.8 ± 7.05**	242 ± 15.1**

* < 0.05 compared to NC.
** < 0.001 compared to NC.

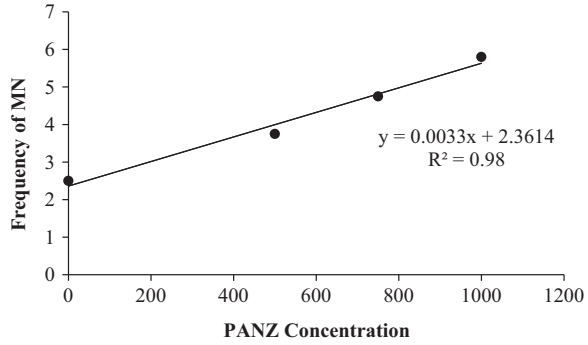

Fig. 2. Concentration-dependent effect of PANZ on the FMN in E_1 (R^2=0.98; $p < 0.01$).

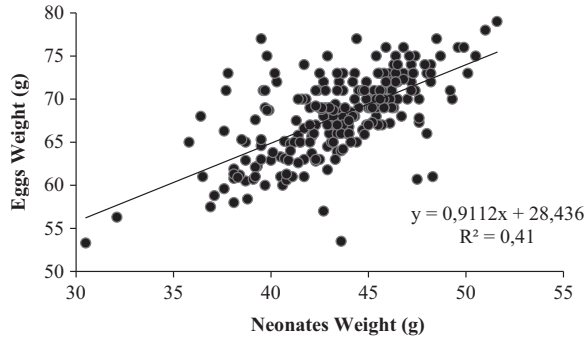

Fig. 3. Relation between eggs weight and hatchlings weight from E_1 and E_2 (R^2=0.41; $p < 0.01$).

Considering data from all animals (E_1 and E_2), there was a positive correlation between the egg weight and hatchlings weight (R^2=0.41; p= < 0.01; Fig. 3).

4. Discussion

The present study suggests possible mechanisms through which these commercial formulations of pesticides can exert embryotoxic and genotoxic damage in naturally exposed *C. latirostris*.

The results of our study revealed that the commercial formulation of pesticides widely applied in soy and other crops induces genotoxic effects in *C. latirostris* embryos at some of the concentrations tested in this study.

Enough information is known about the MN test applied in erythrocytes of different vertebrates exposed to genotoxic compounds. It is widely used in biomonitoring studies to detect chromosome damage in different wild species such as fish (Ramsdorf et al., 2012; Vera-Candiotti et al., 2013), amphibians (Babini et al., 2015; Pollo et al., 2015), mammals (Chibuisi and Adekunle, 2015) an particularly in reptiles (Poletta et al., 2009, 2011a, 2011b; López González et al., 2013a; Latorre and López González et al., 2015; Schaumburg et al., 2016). In all cases, this test demonstrated a high sensitivity to detect the effects of genotoxic agents.

Several studies have also determined the presence of other NAs induced by genotoxic agents (Cavalcante et al., 2008; Guilherme et al., 2010; Cabagna et al., 2011; Lajmanovich et al., 2014), as a complement biomarker together with the MN test. Among Crocodilians however, there were no previous data of NAs determination except for MN test, so this is the first report on the use of other NAs as biomarkers of genotoxicity on *C. latirostris* and all crocodilian species.

Many authors have previously reported genotoxicity by these pesticide formulations. For Gly-based formulations, Cavas and Könen (2007) observed high FMN, NAs and strand breaks in *Carassius auratus* after *in vivo* exposure to RU at concentrations of 5–15 mg/l. Guilherme et al. (2010) demonstrated that RU induces DNA damage to blood cells of *Anguilla anguilla* after short term exposure to environmentally relevant concentrations (58 and 116 µg/l).

Vera-Candioti et al. (2013) reported that 3.9 and 7.8 mg/l of PANZ formulation also increased the FMN in erythrocytes of *Cnesterodon decemmaculatus* after 48 and 96 h of *in vivo* exposure. Our results showed a positive relation in the concentration-dependent effect only for PANZ formulation. This can be attributed to the variability in response among individuals exposed to each pesticide formulations and is also in accordance with the proposal of Speit et al. (2000) who said that a genotoxic agent could has a threshold for three reasons: It is possible that low concentrations of the agent do not reach the target (DNA) because of the cell's effective clearance mechanisms. A second mechanism would be one based on the ability of some organisms to prevent damage caused by reactive oxygen species (ROS), as aerobic organisms constantly produce small amounts of ROS under physiological conditions, defense mechanisms have evolved to prevent ROS-induced damage. Chemicals that produce ROS may induce genotoxic effects when the formation of ROS is increased beyond the ability of the defenses to cope with them and/or if antioxidants are depleted thus leading to threshold effects. Finally, a third mechanism would be one based on an organism's repair ability, which could be enough to mask low concentrations of the agent. In addition, this assessment should also take into account variability in the experimental data, the intra- and inter species variation, the differences in exposure and a better understanding of the complex mechanisms and the profiles of genetic susceptibility is required before information on mutagen metabolism can be used to identify high risk groups and potential contributions of genetic variants to threshold effects (Elhajouji et al., 2011). It is also important to note that the individual constituents of each pesticide commercial formulation probably acted synergistically, antagonistically and additively towards the induction of the MN and NAs. These constituents exhibited differences in absorbability, complex formation and chemical reactivity in relation to the physiological uniqueness of the Broad-snouted caiman.

Recently, based on a review of the literature available on GLY effects in different organisms, The World Health Organization (March, 2015) in the last report of the International Agency for Research on Cancer (IARC,WHO) has classified Glyphosate into the Group 2A (*probably carcinogenic to humans*; Guyton et al., 2015).

Stanley et al. (2009) reported a neurotoxic response on embryo/larval Zebrafish (*Danio rerio*) induced by END at concentrations between 0.01 and 100 µg/l. Likewise, Mobarak and Al-Asmsari (2011) suggest an embryotoxic and teratogenic effects of END on developing chick embryos incubated during 24 h with a single dose of 7, 14 and 24 mg /eggs administered by topical application.

In the same way, Uggini et al. (2010 and Anwar (2003) observed severe teratological abnormalities in chick embryos exposed to 0.005–0.5 µg/egg and to 100–400 mg/Kg of CYP, respectively, as a single sublethal dose (final volume 50 µl) at day "0" of incubation. Cabagna et al. (2006) reported that 5, 10, 20 and 40 µg/L of CYP produced a significant increase in the frequency of micronucleated erythrocytes at 48 and 96 h of treatment in *Odontophrynus americanus* tadpoles.

Other studies conducted by our research group in broad snouted caiman, demonstrated the genotoxic effect of RU formulation and the active principle GLY after *in ovo* exposure by topical application (Poletta et al., 2009, 2011a). Similar results were found with the mixture of RU, END and CYP formulations after exposure of nests under semi-natural conditions similar to those which may happen in natural environments near crops, using the MN test and the comet assay (Poletta et al., 2011b). Other effects were also observed including enzymatic (Poletta et al., 2011b) and immunological alterations (Latorre et al., 2013; Siroski et al., 2016) after *in ovo* and *in vivo* exposure. Early life stages of oviparous organisms often exhibit a greater toxicological sensitivity to chemical contaminants than adults, often because repair and detoxification systems immaturity. This can increase the susceptibility of some hatchlings to factors that affects

their survival such as the response to different types of infections or contaminant-induced stress.

In relation to the insecticide END, for their toxic properties, bioaccumulation and persistence, the United Nations Environment Programme (UNEP) joined it to the list of prohibited organic pollutants (Stockholm Convention on POP's 2011). In Argentina, 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).

CP is an agent known to induce genotoxic effects in studies with this and other reptilian species (Poletta et al., 2009, 2011a, 2011b; López González et al., 2013b; Schaumburg et al., 2016), using the MN test and the Comet assay. Poletta et al. (2009) reported also teratogenic effects of CP, producing malformations in some caimans after *in ovo* exposure to 700 μg/egg. Moreover, it also demonstrated that the topical application is a suitable and efficient way of exposure, as the substance passed through the eggshell and induced its effects on embryos. In the present study, we demonstrated again that CP caused an increase in the FMN as well as in the FNA, being the latter, the first report for all reptile species.Other NAs analyzed in Broad-snouted caiman erythrocytes, apart from MN, showed a significantly higher frequency in many of the treatments applied compared to the NC, showing that they could be good indicators of genotoxic damage when applied with other biomarkers of genotoxicity. Previous reports have shown that these nuclear morphological abnormalities can be considered indicators of genetic damage and could be primary manifestations preceding the formation of micronuclei (Seriani et al., 2011, 2014), however, others propose different mechanisms of origin and few possible explanations are given (Cavas and Ergene-Gözükara, 2005; Pampalona et al., 2010; Fenech et al., 2011; Gökalp and Güner, 2011). The most accepted theories explain that micronuclei can originate during anaphase from lagging acentric chromosome or chromatid fragments caused by misrepair or unrepaired DNA breaks. Malsegregation of whole chromosomes at anaphase may also lead to MN formation as a result of hypomethylation of repeated sequences in centromeric and pericentromeric DNA, defects in kinetochore proteins or assembly, dysfunctional spindle and defective anaphase checkpoint genes. Nucleoplasmic bridges originate from dicentric chromosomes, which may occur due to misrepair of DNA breaks, telomere end fusions, and also, when defective separation of sister chromatids at anaphase occurs due to failure of decatenation. Meanwhile, Nuclear buds represent the process of elimination of amplified DNA, DNA repair complexes and possibly excess of chromosomes from aneuploid cells (Prieto et al., 2008; Fenech et al., 2011; Seriani et al., 2011), or due to a delayed mitosis and result of the malformation of spindle fibres (Melo et al., 2014). Lajmanovich et al. (2014) suggested that formation of erythrocytes with bilobed nuclei increase in situations of stress (*e.g.*, diet alterations, pathology, and metabolic damage). However, the exact mechanism of formation of other abnormalities such as notched nuclei is unknown yet. A decrease in the frequency of BiN in exposed groups can be interpreted as a reduction in mitotic index (less cells can entered division perhaps because of an arrest in a previous checkpoint). Particularly, some NAs can be induced by genotoxic compounds even when micronuclei are not induced, and other authors have also reported correlation between MN and other NAs in certain studies (Ayllon and Garcia-Vasquez, 2000; Kirschbaum et al., 2009). During incubation, vertebrate eggs experience loss or gain of weight. Under controlled incubation conditions, however, eggs generally undergo weight loss (Beldoménico et al., 2007). Several authors have inquired about a possible variation exists in some species of Crocodylia under controlled conditions of incubation (Manolis et al., 1987; Beldoménico et al., 2007). Manolis et al. (1987) suggested that in the Order Crocodylia, egg weight changes during incubation are also consequence of water-vapor balance established between the egg and the incubation chamber.

When humidity and temperature conditions are controlled, stable and homogeneous during incubation, any variation in the eggs weight should be the result of the influence of factors different from the physical condition in the incubation chamber.

We found that eggs weight, measured only at the beginning of the experiment, before pesticides application, showed a positive correlation with hatchlings weight, which is actually an expected result (Manolis et al., 1987; Webb et al., 1987). But there was no significant differences among experimental groups in eggs initial weight or hatchlings weight at birth, or during the first months of life (three and six month-measure). In contrast, Beldoménico et al. (2007) measured egg weights at the beginning and at the end of the incubation period and determined the decrease in weight during embryonic development and its relation to pesticide exposure, as well as hatchlings weight. They found that in exposed eggs, part of the egg weight loss was produced by a decrease in the mass of non-embryonic contents, the majority of what is caused by an inferior weight gain of the hatchlings. Greater egg weight loss during incubation was observed in eggs treated with an environmentally relevant dose of atrazine (0.2 mg/Kg) and relatively low doses of END (2 and 20 mg/Kg) ($p < 0.05$), respect to the NC, similar to concentrations applied in our study. They suggest that egg weight loss is related with some metabolic aspect of the embryo involved in the process of water evaporation through the eggshell, and the *in ovo* exposure to pesticides affects the functioning of several organs and/or disrupts signals that control development.

In our study, only the initial eggs weight were measured and then hatchlings at birth, but we do not measured eggs final weight so no effect of pesticide on eggs weight during incubation could be analysed.

Growth is an integrated response to numerous physiological processes, which influence the production budget of the individual. According to these studies, it is assumed that smaller hatchlings would have less chance of survival during their early years, thus affecting the population dynamic of the specie. Therefore, understanding the consequences of pesticides exposure and the causative levels of exposure during the critical periods of embryonic development is of prime significance. Our lack of effects on growth agree with those reported by Amaral et al. (2012) in the reptile species *Podarcis bocagei*. One possible explanation of the absence of effect on growth may be that the concentrations of pesticides applied was not enough to create an imbalance on underlying bioenergetics processes such as energy assimilation and metabolic expenditures. However, in previous studies we observed less size at birth and a growth delay at 3 months of life on broad-snouted caimans exposed *in ovo* to RU (Poletta et al., 2011a, 2011b). Likewise, Sparling et al. (2006) on *Trachemys scripta elegans* showed small body mass and reduced somatic index in hatchlings exposed *in ovo* to high concentrations of another GLY formulation (Glypro®) until 14 days of life. This has perhaps to do also with a more susceptibility of the animals used in previous studies.

5. Conclusions

In conclusion, these results add further evidence to the application of the MN test and other NAs in erythrocytes of Broad-snouted caiman, considered as useful parameters in the evaluation of the genotoxic effects of END, CYP and GLY-based formulations.

The use of a multimarker approach, combining the analysis of MNs and other NAs frequencies, reflecting potential alterations in cellular kinetics, metabolism, the structural profile of erythrocyte nuclei and genomic stability events, could provide valuable information on the stage of progression of some degenerative diseases significantly aiding in early predictive diagnosis.

C. latirostris is considered as useful sentinel for genotoxic monitor-

ing of environmental contamination, the use of MN test together with other NAs are sensitive tools in determining the potential genotoxicity of pesticides at subletal concentrations for *in ovo* exposure. We propose them to be included in the minimum battery of tests to characterize or indicate the potential environmental risk caused by pesticides.

Finally, our data indicated that different concentrations of commercial formulations of pesticides induce different MN and other NAs on *C. latirostris* erythrocytes. Further studies are needed to better understand the origin of these abnormalities in this species.

These findings provide useful information for future studies involving the biomonitoring of regions where this species is naturally and simultaneously exposed to multiple pesticide formulations, possibly altering the genetic integrity of the native populations.

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