Student Research Assistance Scheme

Project title: Evaluation of Chromosomal aberrations and Micronuclei in embryos of *Caiman latirostris* exposed to insecticides.

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Report

The aim of this project was to evaluate the embrotoxicity and genotoxicity of insecticides commercial formulations: Endosulfan (Galgofan® -END, 35%) and Cypermethrin (Atanor®: -CYP, 25%) on *Caiman latirostris* hatchlings, using the Chromosomal Aberration (AC), Micronucleus (MN) and Nuclear abnormalities (NA) tests, as biomarkers of genotoxicity.

According to the methodology described in the proposal, we used *C. latirostris* eggs harvested in different nests in the Natural Managed Reserve "El Fisco" (30° 11' 26'' S; 61° 0'27''W; Dpto. San Cristobal, Santa Fe Province, Argentina) an area free of farming and urban activities which belong to the natural distribution of this species. This area was chosen considering it is a Protected Natural Area (Law 12,930; 2008), to ensure that eggs had not been environmentally exposed to any xenobiotics, as no activity with an associated contamination risk is carried out there. Eggs were harvested as part of "Proyecto Yacare" (PY) ranching program activities (Larriera et al., 2008). Six nests collected during 2013-2014 nesting season (December 2013) with a minimum of 30 eggs each, were used to carry out the corresponding experiments. All nests used in the experiment were collected within 5 days after oviposition, maintained in the same conditions from harvest to treatment assignment and eggs viability determined by analyzing the opaque eggshell banding.

Experimental design and treatments

Eggs were randomly distributed in ten experimental groups (N=160; 16 eggs per experimental group with two replicates of 8 eggs each): **1)** a negative control (NC) treated with distilled water; **2)** a vehicle control (VC) treated with ethanol; **3-6)** 4 groups exposed to 1, 10, 100, and 1000 µg/ egg of END and 7-10) 4 groups exposed to 1, 10, 100, and 1000 µg/ egg of CYP formulations. Different pesticide solutions were applied on the eggshell (by topication) at the embryo implantation zone at the beginning 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, and free of any exogenous substance.
The eggs were incubated in the "Proyecto Yacare" incubator, under a temperature of 31.5 ± 0.5 °C and 95% humidity. Eggs were controlled periodically during the experiments in order to identify and discard those which became non-viable.

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

When hatchlings started to call within the eggs, the corresponding eggs were removed from the incubator and, if hatching did not occur spontaneously, then they were assisted (Larriera et al., 2008). In case 72 h. after the hatching of the first egg of a certain clutch any egg of the same clutch remain without hatch, it was helped to do it.

Developmental parameters

Immediately after hatching, hatching success was recorded on all groups and the hatchlings were 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), then they were individually identified and blood samples were obtained.

Then, all the animals were maintained in plastic containers under common controlled conditions used in PY 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.

Blood collection and tests applied

Peripheral blood samples (0.5 ml) were obtained from each hatchling from the spinal vein (Olson, 1977), with heparinized syringes.

Micronucleus test and Nuclear abnormalities test were applied on peripheral blood erythrocytes. Two smears were made for each animal, fixed with ethanol and stained with Giemsa (1:10). For each sample, 1000 erythrocytes were analyzed under an optical microscope with a magnification of 1000x (**Fig. 2** *a, b* and *c*) and the MN/NA frequencies were determined (FMN/FNA: number of cells with MN/1000 erythrocytes counted or number of cells with NA/1000 erythrocytes counted) (Poletta et al., 2008).

The criteria adopted for MN identification were the following (Schmid, 1975): (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.

NA were distinguished according to the classification proposed by Carrasco et al. (1990) and Fenech 2000 in: Notched (NN- appreciable depth into a nucleus that does not contain nuclear material), nuclear *buds* or *"budding"* (*Bud*- nuclear evaginations), Binuclei (BN- cells with two nuclei, in division), and Eccentric nuclei (EN), were also recorded.

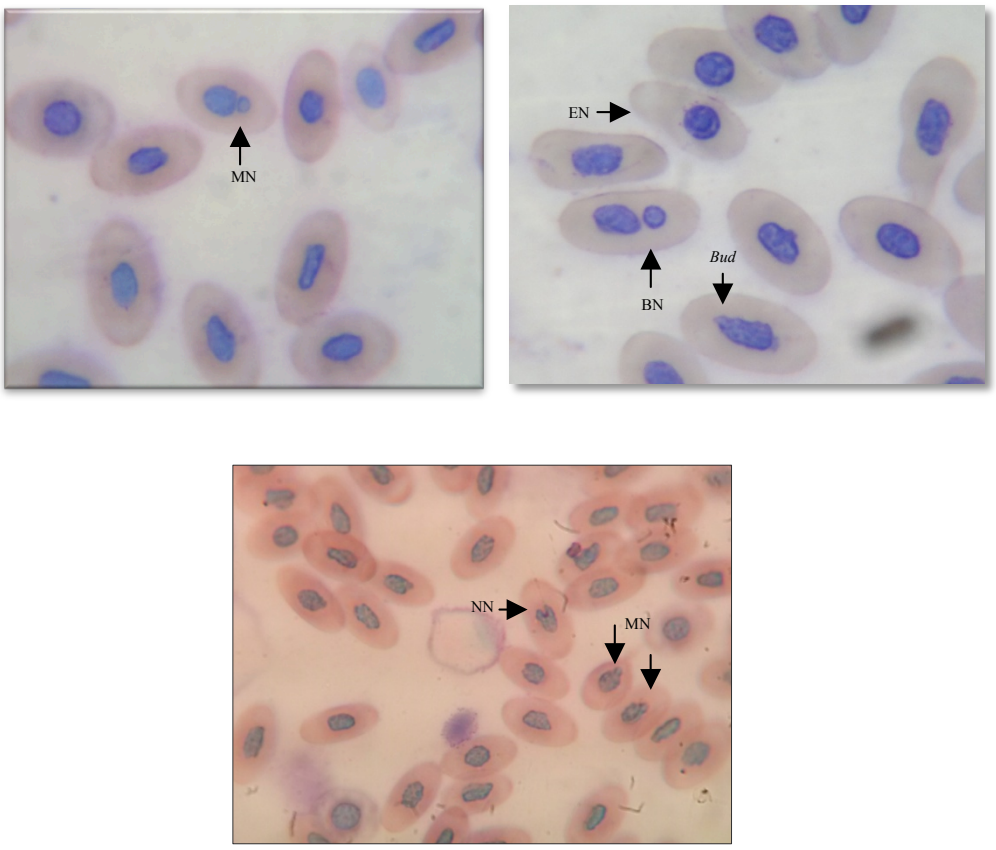


Fig 2. *a, b* and *c*. Images of micronucleus (MN) and nuclear abnormalities analyzed: eccentric nuclei (EN), binuclei (BN), *Buds*, and notched nuclei (NN). 1000x.

Chromosomal aberration test (CAT) was addapted to be applied in erythrocytes but cannot be used as a biomarker for this studies, as we continue doing with methodological adjustment until very short time. The following advances were obtained: the length of lymphocytes cell cycle was determined, the optimal culture time, temperature and required amount of mitogen for obtaining dividing cells in the first and second cycle were stabished. With this we obtained metaphases of good quality and numbre for microscopic analysis (**Fig. 3**).

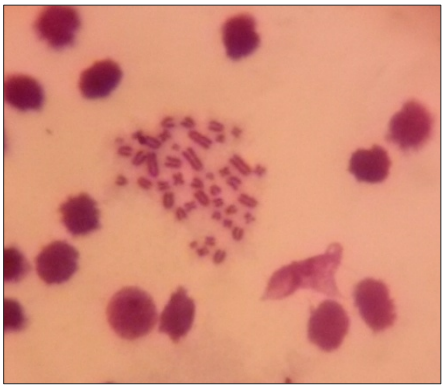


Fig 3. Metaphase of *C. latirostris.*1000x.

Results and Discussion

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

Experimental group	MN/1000 cells	Buds/1000 cells	Notched/1000 cells	Binuclei/1000 cells	Eccentric/1000 cells	ANT/1000 cells
NC (distiller water 5 µg/eggs)	2.50 ± 0.62	89.35 ± 10.38	58.00 ± 7.45	1.21 ± 0.38	23.93 ± 8.11	172.50 ± 13.80
VC (ethanol 5 µg/eggs)	2.88 ± 0.84	96.23 ± 9.38	58.94 ± 7.11	0.70 ± 0.25	24.88 ± 7.27	180.76 ± 11.70
END 1 (1 µg/eggs)	3.67 ± 0.80	133.67 ± 9.87	32.87 ± 3.56**	1.07 ± 0.37	81.40 ± 13.41**	249.00 ± 22.50**
END 10 (10 µg/eggs)	3.81 ± 0.45*	134.25 ± 11.08	33.69 ± 3.76**	1.00 ± 0.30	64.87 ± 6.64**	233.81 ± 14.52**
END 100 (100 µg/eggs)	3.93 ± 0.76	142.27 ± 14.71*	49.93 ± 6.54	0.93 ± 0.20	49.00 ± 10.25*	242.13 ± 20.93**
END 1000 (1000 µg/eggs)	3.46 ± 0.52	140.53 ± 10.30*	38.47 ± 3.28*	0.87 ± 0.24	70.60 ± 8.10**	250.47 ± 16.17**
CYP 1 (1 µg/eggs)	4.29 ± 0.62*	107.71 ± 18.81	43.71 ± 6.75	0.93 ± 0.25	43.14 ± 11.90	195.50 ± 25.71
CYP 10 (10 µg/eggs)	3.89 ± 0.62	142.16 ± 12.91*	35.32 ± 4.69*	0.68 ± 0.20	66.42 ± 7.38**	244.58 ± 18.40**
CYP 100 (100 µg/eggs)	7.60 ± 0.62*	138.13 ± 8.26	32.67 ± 4.36*	0.87 ± 0.26	68.73 ± 6.87**	240.40 ± 12.89**
CYP 1000 (1000 µg/eggs)	3.06 ± 0.62	136.62 ± 12.18	36.50 ± 3.95*	0.69 ± 0.18	67.75 ± 7.05**	241.56 ± 15.08**

Table 1. Micronucleus and Nuclear Abnormality frequencies observed in *C. latirostris* hatchlings in different experimental groups. All values are expressed as mean ± S.E. NC: negative control; VC: vehicle control; END 1, 10, 100, 1000 (µg/eggs): commercial formulation of Endosulfan (Galgofan®); CYP 1, 10, 100, 1000 (µg/eggs): commercial formulation of Cypermethrin (Atanor®); Micronucleus found in 1000 erythrocytes observed. ANT: sum of Total Nuclear Abnormalities in 1000 counted erythrocytes, excluding MN.
*<0.05 compared to control. **<0.001 compared to control.

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

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

Results obtained from the NA showed a significant difference in Buds: END 100, 1000 and CYP 10 ($p<0.05$), Eccentric nuclei: END 1, 10, 1000, CYP 10, 100 and 1000 ($p<0.01$), END 100 ($p<0.05$) and Notched: END 1 and 10 ($p<0.01$), END 1000, CYP 10, 100 and 1000 ($p<0.05$), Total Nuclear Abnormalities (TNA): END 1, 10, 100, 1000, CYP 10, 100 and 1000 ($p<0.01$), respect to the NC.

There were differences among clutches (*clutch effect*) in all examined variables: in the MN frequencies we found differences among all nest (1-6; $p<0.01$), in Notched we found statistical significant differences among nests 1, 2 and 5($p<0.01$), in Eccentric nucleus between nests 5 and 6 ($p<0.01$) while in TNA we observed differences between nests 1and 2 ($p<0.01$), and between nests 3 and 6 ($p<0.05$).

No relation was found between MN and NA with caiman's weight or length ($p>0.05$ in all analysis performed). The correlation between eggs weight with the neonates weight at birth was statistically significant ($R^2= 0.41$; $p<0.01$), being an expected result.

Among cytogenetic test systems, the assessment of micronuclei is commonly used for evaluating structural and numerical Chromosomal Aberrations induced by clastogenic and aneugenic agents. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Fenech et al., 1999; Carballo & Mudry, 2006).

In recent years, several studies have described the presence of nuclear abnormalities (NA), also considered to be induced by genotoxic agents. Carrasco et al. (1990) categorized these nuclear abnormalities into four groups (blebbed, lobed, notched and vacuolated nuclei). The presence of nuclear *buds* or *"budding",* relate to DNA amplification in the S phase of the cell cycle, is also considered as a NA (Bolognesi et al., 2006; Prieto et al., 2008; Fenech et al., 2011). The formation mechanisms of NA are not yet fully understood but nowadays, many researches included these abnormalities in the evaluations of genotoxicity as a complementary test to the micronucleus assay.

The CAT test is being currently used to determine others possible effects caused by these compounds at chromosome structure and number.

The results of this study demonstrate that *C. latirostris* is a sensitive testing experimental model for environmental pollution, currently associated with agricultural regions subject to application of pesticides. By possibly altering the genetic integrity of exposed organisms, these events represent a threat for natural populations.

The insecticide commercial formulations evaluated induced genotoxicity, so could lead to an increase in the susceptibility for different disease and disorders in this sentinel species for the Argentinian littoral.

Detail of expenditures in which funds were used:

Material & Reagents	***USD***
Syringes x 200	46
Needles x 200	29
Heparin x 2	17
Slides x 400	12
Giemsa	25
Medium RPMI	89
Fetal bovine serum	196
Penicillin-streptomycin	48
Phytohemagglutinin	107
Colcemid solution (Colchicine)	77
KCl 0.075 M	18
Acetic acid	19
Methanol	35
Ethanol	6
Hoescht solution 33258	277

References

Anwar K. Toxic effects of Cypermethrin on the biochemistry and methodology of 11th day chik embryo (*Gallus domesticus*). Pakistan Journal of Applied Sciences 3 (6): 432-445, 2003.

Beldoménico P. M., Rey F., Prado W.S., Villarreal J.C., Muñoz-de-Toro M., Luque E.H. *In ovum* exposure to pesticides increases the egg weight loss and decreases hatchlings weight of *Caiman latirostris* (Crocodylia: Alligatoridae). Ecotoxicology and Environmental Safety 68 (2007) 246–251

Bolognesi C., Perrone E., Roggieri P., Pampanin D. M., Sciutto A. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquatic Toxicology 78S (2006) S93–S98.

Carballo M.A., Mudry M.D. Indicadores y marcadores biológicos, in: M.D. Mudry, M.A. Carballo (Eds.), Genética Toxicológica, De los Cuatro Vientos Editorial, Buenos Aires, Argentina, 2006, pp. 83–108.

Carrasco, K.R., Tilbury, K.L., Myers, M.S., 1990. Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Can. J. Fish. Aquat. Sci. 47, 2123–2136.

Fenech M. The *in vitro* micronucleus technique. Mutation Research 455 (2000) 81–95

Fenech M., M. Kirsch-Volders, A. T. Natarajan, J. Surralles, J. W. Crott, J. Parry, H. Norppa, D. A. Eastmond, J. D. Tucker and P. Thomas, 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis vol. 26 no. 1 pp. 125–132.

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

Olson G., Hessler J., Faith R. Techniques for the blood collection and intravascular infusion of reptiles, Lab. Anim. Sci. 25 (1977) 783–786.

Patel S., Pandey A. K., Bajpayee M., Parmar D., Dhawan A. Cypermethrin-induced DNA damage in organs and tissues of the mouse: Evidence from the comet assay. Mutation Research 607 (2006) 176–183

Poletta G.L., Larriera A., Kleinsorge E., Mudry M.D. *Caiman latirostris* (broad snouted caiman) as a sentinel organism for genotoxic monitoring: basal values determination of micronucleus and comet assay, Mutat. Res. 650 (2008) 202– 209.

Poletta G.L., Kleinsorge E., Mudry M.D., Larriera A. Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and the Micronucleus test, Mutat. Res. 672 (2009) 95–102.

Poletta G.L., Kleinsorge E., Paonessa A., Mudry M.D., Larriera A., Siroski P.A. Genetic enzymatic and developmental alterations observed in *Caiman latirostris* exposed *in ovo* to pesticide formulations and mixtures in an experiment simulating environmental exposure, Ecotoxicol. Environ. Saf. 74 (2011) 852–859

Prieto Z., Leon-Incio L., Quijano-Jara C., Fernandez R., Polo-Benites E., Vallejo-Rodriguez R., Villegas-Sanchez L., 2008. Efecto genotóxico del dicromato de potasio en eritrocitos de sangre periférica de *Oreochromis niloticus* (Tilapia). Rev Peru Med Exp Salud Publica 25 (1): 51-58.

Schmid W. The micronucleus test, Mutat. Res. 31 (1975) 9–15.

Sinha N., Narayan R., Saxena D. K. Effect of Endosulfan on the Testis of Growing Rats. Bull. Environ. Contam. Toxicol. (1997) 58:79–86