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Genetic, enzymatic and developmental alterations observed in *Caiman latirostris* exposed *in ovo* to pesticide formulations and mixtures in an experiment simulating environmental exposure

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ABSTRACT

In South America, economic interests in last years have produced a constant increase in transgenic soybean cropping, with the corresponding rise in pesticide formulated products. The aim of this study was to determine the effects of pesticides formulations and mixtures on a South American caiman, *Caiman latirostris*, after *in ovo* exposure. We conducted a field-like experiment which simulates the environmental exposure that a caiman nest can receive in neighbouring croplands habitats. Experimental groups were Control group, Treatment 1: sprayed with a glyphosate herbicide formulation, and Treatment 2: sprayed with a pesticide mixture of glyphosate, endosulfan and cypermethrin formulations. Results demonstrated genotoxicity, enzymatic and metabolic alterations, as well as growth delay in caimans exposed *in ovo* to Treatments 1 and 2, showing a higher toxicity for the mixture. Integral evaluation through biomarkers of different biological meaning is highly informative as early indicators of contamination with pesticides and mixtures in this wildlife species.

1. Introduction

Current agricultural related practices affect natural ecosystems through their conversion (habitat destruction) into agricultural lands and through the utilisation of agrochemicals, which subsequent spread and runoff contaminate surrounding natural habitats (Johanson, 2004; Peruzzo et al., 2008). Agriculture expansion has lead to increased fragmentation of habitat due to deforestation and a great degradation of remnant ecosystems, with deep consequences for biodiversity. The use of pesticides formulated products, which are complex and variable chemical mixtures, has been increasing worldwide. It is estimated that pesticides reduce losses in soybean, maize and wheat crop to a 10–15%, allowing higher yields in food production. However, only a little amount of pesticides applied in agriculture reach target organisms directly, while the rest disperse through the environment, affecting wild flora and fauna populations of surrounding natural areas (Donald, 2004). Long-term, low level chronic exposure to chemicals may interfere with development and growth, haematological and physiological parameters and genetic stability of organisms living there (Glusczak et al., 2006).

In Argentina, economic interests in the last years has produced a constant increase in transgenic soybean single-cropping (glyphosate resistant), with the corresponding rise in pesticide use, including mainly the herbicide glyphosate (GLY) and the insecticides endosulfan (ES) and cypermethrin (CPT) (Table 1; EXTOXNET, updated 1996). In the last season, soybean cropping rose to more than 18 million ha cultivated and 200 million liters of pesticides released to the environment, with 170 millions corresponding only to glyphosate-based formulations (Bolsa de cereales de Buenos Aires, updated 2010; CASAFE, updated 2010). More than 50% of transgenic soybean cultivated in the last decade has extended over areas which corresponded previously to native forest (Paruelo et al., 2006).

In north-central region of Argentina, many natural environments where the broad-snouted caiman (*Caiman latirostris*) live are in the proximity of regions with high agricultural activity, where

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Table 1
Information and characteristics of glyphosate, endosulfan and cypermethrin.

Pesticide	Chemical name	Abbreviation	Chemical class	Use	Persistence in environment	Toxicological classification*
Glyphosate	*N*-phosphonomethyl glycine	GLY	Phosphonoglycine	Systemic herbicide for non-selective weed control in agriculture and non-agricultural environments.	Field half-life: from 1 to 174 days. Estimated average: 47 days in soil; from 12 days to 10 weeks in pond water.	General Use Pesticide (GUP). Class II, moderately toxic.
Cypermethrin	[(R,S)-alpha-cyano-3-phenoxybenzyl(1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate	CPT	Pyrethroid	Wide synthetic insecticide used to control many pests, mainly moths, in agriculture and multiple urban environments.	Moderately persistent in soil, half-life from 4 days to 8 weeks. Stable in water with a half-life from 50 to 100 days.	Some formulations classified as Restricted Use Pesticide (RUP). Class II—moderately toxic. Some formulations toxicity Class III—slightly toxic.
Endosulfan	[6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzadioxathiepin 3-oxide	ES	Organochlorine	Insecticide and acaricide used to control a wide variety of insects and mites on food crops, also as wood preservative.	Moderately persistent, average field half-life of 50 days in soil and from 30 to 150 days in water.	Restricted Use Pesticide (RUP). Class I, highly toxic.

Data obtained from EXTOXNET (updated 1996).

* Toxicological classification from US EPA.

GLY, ES and CPT are extensively used as a method for pest control in soybean crops. In addition, they are extensively applied in the same period of the year in which *C. latirostris* breeding season takes place, implying a contamination risk particularly important for developing embryos and neonates (Poletta et al., 2009).

Pesticides tend to be very reactive compounds that can trigger a whole cascade of biological responses in an organism, each of which may, in theory, serve as a biomarker (Mitchelmore et al., 2005). They can form covalent bonds with different nucleophilic centres of cellular biomolecules, and may induce reactive oxygen species (ROS) formation leading to protein, lipids, and DNA damage (Limón-Pacheco and Gonsebatt, 2009). Genotoxic agents usually disrupt normal cellular processes and can result in direct interactions of the toxic agent with DNA, inducing structural modifications in it or the associated machinery (Novillo et al., 2005). Various pesticides have also been shown to inhibit or enhance many endogenous enzyme activities and metabolic parameters (Becker et al., 2009; Begun, 2007; Glusczak et al., 2006; Jiraungkoorskul et al., 2003). Enzymes are of great value for diagnosis considering the precocity of their variation, rather than the specificity of their tissular origin. All these alterations may result in malfunction of normal cellular physiology at individual level, with possible long-term consequences depending on the severity of the damaged produced, and altered genotypic diversity as well as decreased reproductive success at population level (Acevedo-Whitehouse and Duffus, 2009; Mitchelmore et al., 2005).

To predict environmental effects of chemical substances, analyses use mostly data obtained from laboratory tests. However, natural ecosystems are more complex and variable than laboratory standardised systems. Therefore, toxicity bioassays done in the laboratory should be complemented with higher tier assessment conducted in field-like scenarios. A major strength of field-like studies is the incorporation of more realistic exposure regimes that allow a better understanding of the biological effects of chemical under natural conditions (Graney et al., 2003). In previous studies under laboratory controlled conditions, we have demonstrated genotoxic effect through the Micronucleus (MN) test and Comet assay (CA) in *C. latirostris* neonates after *in ovo* exposure to increasing concentrations of the GLY-based formulation Roundup®, applied directly on the eggshell (topication) (Poletta et al., 2009). In the present study, we aimed to model environmental conditions more closely through a field-like experiment simulating the exposure that a caiman nest can receive in neighbouring croplands habitats, using pesticide practises commonly applied in agriculture. In order to determine the effects of GLY, ES and CPT pesticide formulations on *C. latirostris* we analysed development, enzymatic and metabolic parameters, and genotoxic effects.

2. Materials and methods

2.1. Chemicals

Roundup® Full II (66.2% glyphosate, GLY), Cypermethrin Atanor® (25% cypermethrin, CPT) and Endosulfan Galgofan® (35% endosulfan, ES) formulations were obtained by courtesy of Establecimiento La Matuza SA, Santa Fe, Argentina. Roundup® Full II is a liquid water soluble (12 000 mg/l) herbicide, containing glyphosate potassium salt [*N*-(phosphonomethyl) glycine monopotassium salt, $C_3H_7KNO_5P$] as its active ingredient (a.i.) (CAS No. 70901-12-1). CPT Atanor® is a liquid water-insoluble (0.01 mg/l) mixture of different cypermethrin isomers ($C_{22}H_{19}Cl_2NO_3$, CAS No. 52315-07-8). ES Galgofan® is 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 1996).

Dimethyl sulphoxide (DMSO) was purchased from Fluka. Low melting point (LMP) agarose, normal melting point (NMP) agarose, ethidium bromide, the rest of the reagents for CA and MN test and general laboratory chemicals were provided by Sigma. RPMI-1640 medium was purchased from HyClone.

Aspartate aminotransferase (AST, *EC 2.6.1.21*), Alanine aminotransferase (ALT, *EC 2.6.1.21*), Alkaline phosphatase (ALP, *EC 3.1.3.1*), Lactate dehydrogenase (LDH, *EC 1.1.1.27*), Creatin kinase (CK, *EC 2.7.3.2*), Cholinesterase (ChE, *EC 3.1.1.8*), Total Protein (TP) and Serum Albumin (ALB) commercial kits were from Wiener Lab® (Rosario, Argentina).

2.2. *C. latirostris* eggs

We used *C. latirostris* eggs coming from nests collected during ProyectoYacaré (PY) ranching activities (Larriera et al., 2008), in an area free of cropping and urban activities in Santa Fe Province, Argentina (Natural reserve "El Fisco", 30°11′26″S; 61°0′27″W). All eggs were harvested within 5 days after oviposition, on the same day and maintained under the same conditions during transportation to PY facilities in Santa Fe city. Once in the laboratory, eggs viability was determine by checking the presence of the opaque eggshell banding (Iungman et al., 2008). Only eggs considered viable were included in the experiments.

2.3. Experimental design and treatments

2.3.1. Experiment 1 (E_1)

Three experimental groups of three artificial caiman nests each ($N=9$) were constructed separately in a field free of any contaminating activity, maintaining a distance of approximately 100 m between each group. Vegetal mounds resembling the nests constructed by caiman females, approximately 1 m wide and 0.60 cm high (Larriera and Imhof, 2006), were made with vegetal material obtained from the same non-contaminated area; based on our experience from the PY activities (www.mupcn.