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RESEARCH ARTICLE

Total and differential white blood cell counts in *Caiman latirostris* after *in ovo* and *in vivo* exposure to insecticides

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ABSTRACT

Agricultural activities associated mainly with soybean crops affect the natural environment and wildlife by habitat destruction and the extensive use of agrochemicals. The aim of this study was to evaluate immunotoxic effects of the insecticides cypermethrin (CYP) and endosulfan (END) in *Caiman latirostris* analyzing total blood cell count (TWBC) and differential white blood cell count (DWBC) after *in ovo* and *in vivo* exposure. Eggs (*in ovo*) and hatchlings (*in vivo*) from nests harvested in natural habitats were artificially incubated and reared under controlled conditions in the Proyecto Yacaré (Gob.Santa Fe/MUPCN) facilities. Exposure of embryos was performed by topication on the eggshell during the first stage of development. The treatments were distilled water (negative control; NC), ethanol (vehicle control; VC), four groups treated with different concentrations of CYP and four groups with END. *In vivo* exposure was performed by immersion; treatments were NC, VC, two groups exposed to CYP and two to END. After embryonic exposure to the insecticides, no differences were found in TWBC or DWBC among the neonates exposed to pesticides versus controls. In the *in vivo* scenario, similar results were obtained for TWBC, but DWBC data showed differences between NC hatchlings and CYP-1 hosts for heterophil, lymphocyte and monocyte levels, and between NC and END-1 hosts for lymphocyte and monocyte levels. Research on the effects of pesticide exposure on this species is of special interest not only to assess the impact on caiman populations, but also to further characterize the species as a potential sentinel of ecosystem health.

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Caiman; cypermethrin; endosulfan; immune system; *Caiman latirostris*

Introduction

In Argentina, economic interests in the last years have produced a constant increase in transgenic soybean mono-cropping (glyphosate-resistant). As of 2012, soybean cropping rose to >20 million ha cultivated, resulting in ≈200 million liters of pesticides being introduced into the environment (CASAFE [updated 2012]). In conjunction, there has also been a corresponding rise in pesticide use, including mainly that of the herbicide glyphosate (GLY) and the insecticides endosulfan (END) and cypermethrin (CYP) (CASAFE).

Caiman latirostris (broad-snouted caiman), one of the two species of caimans that inhabit Argentina, shows a great tolerance to low temperatures and occupies a greater diversity of environments (Siroski 2004). Considering agricultural frontier expansion over the years, many areas of the geographic distribution of *C. latirostris* have been affected by agricultural production. As a consequence, overlap has arisen between fumigated environs and those that the animal inhabits, resulting in scenarios wherein these hosts are near-constantly exposed to pesticides (Siroski 2004).

Under those conditions, reptiles are exposed to pesticides/other contaminants through different routes: primarily dermally, but also by inhalation and/or orally (ingestion) (Hopkins 2005). The relative importance of each route of exposure depends on numerous factors including the ecology and physiology of the organism, physico-chemical characteristics of the toxicant, and the environment in which the organism is exposed. As crocodilians are important components of South American wetlands (Messel et al. 1995), they are considered that they are likely to show any effects associated with environmental contamination (Brisbin et al. 1998). Based on their biological and ecological characteristics, these animals can be exposed to contaminants in all life stages, with the early stages being the most sensitive (Poletta et al. 2009). Contaminants accumulated in females can potentially be transferred to their embryos through the egg yolk, affecting *in ovo* development (Hall & Henry 1992). In the same way, crocodilian embryos might be directly affected by toxicants in the surrounding environment through their eggshell during incubation. Post-hatching, juveniles or adults may be exposed to the same agent via the food web, water and in sediments in the environment.

It is important to note that the period of maximum pesticide application in Argentina coincides with the reproductive season of this species (November to March), implying a risk of contamination particularly important to developing embryos and neonates (Poletta et al. 2009). Among the many potential adverse outcomes from exposures to pesticides, could be alterations in the crocodilian immune system (IS). Within the IS itself, white blood cells (WBC) are important components involved in a significant amount of processes.

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Low levels of toxicants can cause immunotoxicity, even at much lower concentrations than needed to achieve effects on target organs in the short term; thus, several immune parameters can serve as very sensitive indicators of toxicity (Burns et al. 1996). The IS system is sensitive to changes caused by environmental pollutants and immunosuppression can lead to an increased risk of diseases in reptiles (Tangredi & Evans 1997). In a previous study, we demonstrated that Roundup® (glyphosate 66.2%) caused alterations in some parameters of the IS (and growth) of young caimans (Latorre et al. 2013). Building upon those earlier findings, the aim of the study reported here was to evaluate the effect of the insecticides cypermethrin and END on total white blood cell count (TWBC) and differential white blood cell count (DWBC) after *in ovo* and *in vivo* exposure of *C. latirostris*, under controlled conditions.

Materials and methods

Animals

This study was evaluated and approved by the Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral (Santa Fe, Argentina). All animals were treated in accordance with the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm and Wild Animals (National Scientific and Technical Research Council 2005), using non-invasive techniques for blood collection and minimizing stress and suffering by suitable management methods.

Eggs (*in ovo*) and hatchlings (*in vivo*) from nest harvested under PY ranching activities were used. *Caiman latirostris* eggs were collected in the Managed Natural Reserve "El Fisco" (30°11′26″S 61°0′27″O), located in Santa Fe Province, Argentina. This is a Protected Natural Area (Law 12.930, 2008) free of farming and urban activities that may cause pollution, therefore minimizing possible environmental exposure of the eggs to any xenobiotics, prior to collection (Poletta et al. 2009).

Experimental design and treatments

Experiment 1 (E1)

A total of 120 eggs from six clutches were equally and randomly distributed into 10 experimental groups of 12 eggs each (six eggs per each of two replicates). The groups were a negative control (NC) treated with 50 µl distilled water/egg, a vehicle control (VC) treated with 50 µl ethanol/egg, four groups treated with different concentrations of CYP (Atanor®: 25% CYP; Atanor, Buenos Aires, Argentina) at 1, 10, 100 or 1000 µg/egg, and four with END (Galgofan®: 35% END; Chemotecnica, Buenos Aires, Argentina) at 1, 10, 100 or 1000 µg/egg (Table 1). Concentrations of insecticides were selected based upon previous *in ovo* studies (Patel et al. 2006; Beldoménico et al. 2007), and adapting to the average weight of caiman eggs (≈70 g) (Poletta et al. 2009). All treatments were applied by topication to the eggshell (Crews et al. 1991). A single application was done at the early embryonic stage (within first 5 days after oviposition) at the beginning of the experiment, based on the development of the opaque eggshell band (Donayo et al. 2002). VC was used to evaluate if the diluent (ethanol) itself imparted any effect on the host IS.

Eggs were incubated in an artificial room incubator under controlled conditions (31 ± 1 °C and 95% humidity) for a period between 65 and 75 days. Each experimental group was placed separately in a plastic container, using vermiculite as substrate. Eggs were covered with vegetal material similar to the nesting material, and free of any exogenous toxicants. Eggs were examined periodically during the experiments in order to identify and discard those that became non-viable. When caimans started to call within the eggs, the corresponding eggs were removed from the incubator and, if hatching did not hatch spontaneously, they were assisted (Larriera et al. 2008). After hatching, all neonates were weighed (precision to 0.1 g) and measured in total length (TL) and snout-vent length (SVL; precision 0.5 cm).

Experiment 2 (E2)

Caiman (20-day-old) from five different clutches ($n = 90$) were weighed and then randomly allocated into six experimental groups of 15 animals each (per replicates): a NC treated with tap water, a VC treated with 200 µl ethanol/L, two groups to face different concentrations of CYP, CYP-1 = 0.05–0.5 µg CYP/L and CYP-2 = 0.1–1.0 µg CYP/L; and, two to face different levels of END, END-1 = 0.05–0.5 µg END/L and END-2 = 0.1–1.0 µg END/L (Table 1). The test doses used were based on toxic doses reported earlier for these agents (Sharma et al. 2007; Simoniello et al. 2009; Bachetta et al. 2011). Based on these values, a preliminary study was conducted with 0.5 and 1 µg agent/L for each compound. Pesticide levels were progressively reduced over time in the study to simulate degradation of the compounds in water (without additional entry of new material into ecosystem). Levels at each point were based on data previously determined by gas chromatography (GC) of actual degradation kinetics in water samples (data not shown).

Table 1. Experimental groups and treatments applied in the *in ovo* and *in vivo* exposure assay.

	In ovo experiment (120 embryos)		*In vivo* experiment (90 hatchlings)		
Animals	Treatments	Time of application	Animal	Treatments	Time of application
NC	Water, 50 µl/egg	Within 5 days after oviposition	NC	Water, final volume 4 l	Whole experiment
VC	50 µl/egg		VC	200 µl/l	Whole experiment
Cypermethrin (CYP; µg/egg)	1		END1-CYP1 (µg/l)	END2-CYP2 (µg/l)	Days
	10		0.50	1.0	0–21
	100		0.25	0.5	21–28
	1000		0.17	0.34	28–35
Endosulfan (END; µg/egg)	1		0.12	0.24	35–42
	10		0.08	0.16	42–49
	100		0.06	0.12	49–56
	1000		0.05	0.10	56–63

NC: negative control; VC: vehicle control (ethanol).

For the treatments, these caimans were exposed by immersion during 2 months in plastic pens that were inclined to provide 60% dry and 40% water surface areas. Insecticides were applied in wet area, with a maximum water depth of ≈15 cm (fixed volume of 4 l). Food was offered *ad libitum* three times a week (i.e. a mixture of 50% minced chicken head and 50% dry pellets; Larriera et al. 2008). Pen cleaning was done every 2 days to ensure sanitary conditions. At the end of the experiment (≈60 days), the animals were measured for SVL and weight. Experiment 1 and Experiment 2 were performed separately.

Analytical pesticide determination

CYP and END were analyzed by GC with the following conditions described in Poletta et al. (2011). CYP determination was conducted following the AOAC Official Method 985.03 (AOAC 1995). Each sample was dissolved in methylene chloride (CH_2Cl_2) containing dicyclohexyl phthalate, and 1.0 ml was injected into a capillary GC in split mode, with flame ionization detection. Peak areas were measured for each CYP isomer and dicyclohexyl phthalate, and compared with those from standards that were also injected during the overall analyses.

END was measured by GC following AOAC Official Method 983.08 (AOAC 1995). Each sample was extracted with toluene, and α- and β-END isomers were determined separately by flame ionization GC, using di(2-ethylhexyl)phthalate as internal standard. Peak areas were measured for each isomer and compared with those from standards that were also injected during the overall analyses.

Blood collection

Immediately after hatching for neonates (E1) and on Day 63 for hatchlings (E2), peripheral blood samples were obtained from the spinal vein of all animals according to the method of Olson et al. (1977) using heparinized sterile syringes fitted with a 25-G needle. In all cases, a total volume of 0.5 ml was collected.

WBC count and TWBC

WBC counts on the blood samples were performed using a Neubauer chamber. In brief, an aliquot of blood was diluted 1:200 with 0.6% NaCl (Lewis et al. 2008) and then observed under an optical microscope (400× magnification); all results were expressed as total cells/mm^3 blood. To perform differential counts, two blood smears were prepared per animal. Each smear was then fixed with ethanol and stained with May Grünwald-Giemsa solution. The amount of each immune cell subtype (e.g. heterophils, basophils, eosinophils, lymphocytes, monocytes)/100 WBC analyzed was determined using a light microscope (1000× magnification). From the data, the heterophil/lymphocyte (H/L) index was then calculated and used as a marker of stress in the hosts (Morici et al. 1997; Lance & Elsey 1999).

Statistical analysis

All results are expressed as means ± SE. Experimental group was taken as a grouping factor because no differences were found among replicas in both experiments. All data were analyzed for normality using a Kolmogorov–Smirnov test and for homogeneity of variances using a Levene test. For the *in ovo* and *in vivo* exposures, the data for total WBC counts and H/L ratios were analyzed by a one-way analysis of variance (ANOVA) with a post hoc Dunnet's test. Differential counts of the heterophils, eosinophils and lymphocytes among the different experimental groups were analyzed using ANOVA/Dunnet's as well. All results were compared with the NC (note: analyses showed outcomes for NC versus VC to be non-significant, so only comparisons to NC are reported hereafter). To analyze monocytes and H/L ratios, a Kruskal–Wallis followed by a Mann–Whitney test was employed because the data could not pass the assumptions of the parametric tests. Results are expressed as means ± SE. A p-value < 0.05 was considered significant. All statistical analyses were performed using SPSS 14.0 for Windows software (SPSS, Chicago, IL).

Results

Hatchings success due to the different treatments in exposition *in ovo* is shown in Table 2; no differences were found as a function of treatment. In addition, there was no effect on the SVL or weights of the hatchlings exposed to either insecticide (at any concentration) (Table 3). After embryonic exposure to either insecticide, no differences were found between the exposed and controls neonates regarding the TWBC, DWBC and H/L indices (Table 4).

There was a similar non-effect on the weights or SVL or TL of the hatchlings exposed to either insecticide (at any concentration) (Table 5). This was also the same non-effect noted with the TWBC in yearling caimans after a 2-month subchronic exposure (Table 6). However, DWBC values were significantly different between the CYP-1 hosts and the NC – specifically in regard to levels of heterophils ($p = .013$), lymphocytes ($p = .029$) and monocytes ($p = .009$). In the END groups, there were significant differences in DWBC between END-1 and NC hosts with respect to levels of lymphocytes ($p = .018$) and monocytes ($p = .001$; Table 6). In the case of eosinophils, there were no significant

Table 2. Hatching success of different treatments *in ovo*.

Group	% Hatching success
NC	83.30
VC	91.65
END-1	83.30
END-10	66.60
END-100	91.65
END-1000	83.30
CYP-1	83.30
CYP-10	91.65
CYP-100	91.65
CYP-1000	83.30

Table 3. Body weight, total length (TL), and snout-vent length (SVL) in the *in ovo*-exposed animals.

Group	TL (cm)	SVL (cm)	Weight (g)
NC	23.42 ± 0.18	11.2 ± 0.07	44.19 ± 1.39
VC	23.02 ± 0.24	10.81 ± 0.12	44.44 ± 1.02
END1	23.45 ± 0.30	11.09 ± 0.20	43.92 ± 1.16
END10	23.49 ± 0.24	11.06 ± 0.10	44.76 ± 1.32
END100	22.42 ± 1.16	12.21 ± 1.03	44.77 ± 0.79
END1000	23.39 ± 0.14	11.32 ± 0.07	44.47 ± 0.50
CYP1	23.45 ± 0.18	11.16 ± 0.18	44.86 ± 1.41
CYP10	23.39 ± 0.16	11.09 ± 0.10	43.94 ± 1.01
CYP100	23.42 ± 0.19	11.04 ± 0.10	44.30 ± 0.48
CYP1000	23.17 ± 0.23	11.1 ± 0.10	44.52 ± 0.93

All values shown are means ± SE ($n = 12$/group). Values obtained immediately after hatching.

Table 4. Total white blood cell (TWBC) and differential white blood cell (DWBC) counts in *in ovo* study.

	NC	VC	END-1	END-10	END-100	END-1000	CYP-1	CYP -10	CYP-100	CYP-1000
TWBC	3.18 ± 0.62	3.64 ± 0.45	4.32 ± 1.08	4.08 ± 0.96	3.71 ± 0.29	4.70 ± 0.75	3.28 ± 0.39	4.48 ± 0.80	3.29 ± 0.33	3.93 ± 0.30
Heterophil	13.6 ± 3.3	19.7 ± 3.3	22.3 ± 3.2	21.0 ± 3.6	17.6 ± 2.1	17.2 ± 2.3	17.8 ± 2.1	23.0 ± 2.8	22.7 ± 3.4	20.0 ± 4.4
Lymphocyte	81.9 ± 3.9	75.7 ± 3.3	74.8 ± 3.7	75.9 ± 3.9	79.5 ± 2.4	78.6 ± 2.6	78.4 ± 2.3	75.0 ± 3.6	72.2 ± 3.4	76.2 ± 4.5
Monocyte	0.6 ± 0.4	0.6 ± 0.2	0.9 ± 0.4	0.6 ± 0.3	0.9 ± 0.4	0.4 ± 0.2	0.8 ± 0.3	0.5 ± 0.2	0.5 ± 0.3	0.5 ± 0.2
Eosinophil	2.3 ± 0.5	3.0 ± 0.7	2.3 ± 0.6	1.8 ± 0.4	1.0 ± 0.3	3.1 ± 0.6	2.0 ± 0.5	2.4 ± 0.5	3.4 ± 0.7	2.2 ± 0.5
H/L Index	0.18 ± 0.05	0.29 ± 0.06	0.32 ± 0.05	0.3 ± 0.07	0.23 ± 0.03	0.23 ± 0.04	0.23 ± 0.03	0.33 ± 0.06	0.35 ± 0.08	0.31 ± 0.10

All values are means ± SE ($n = 12$/group). TWBC reported as WBC [$\times 10^4$]/mm^3 blood. Except for Index and TWBC, all other parameters are in terms of %.

Table 5. Body weight, total length (TL), and snout-vent length (SVL) for *in vivo* exposure animals.

Group	TL (cm)	SVL (cm)	Weight (g)
NC	36.01 ± 0.95	17.41 ± 0.47	156.00 ± 12.75
VC	35.58 ± 0.75	17.15 ± 0.34	139.53 ± 9.44
CYP1	34.29 ± 1.08	16.73 ± 0.54	126.64 ± 10.37
CYP2	35.12 ± 1.17	17.2 ± 0.55	143.33 ± 15.55
END1	37.43 ± 0.87	18.47 ± 0.45	168.50 ± 9.72
END2	37.14 ± 0.8	18.18 ± 0.41	161.31 ± 12.5

All values shown are means ± SE ($n = 15$/group). Values obtained after young caiman (starting at Day 20 of age) had been exposed for 2 months.

differences noted among the experimental groups. Stress indices (H/L) were higher in hosts that were in the CYP-1 (1.11 [± 0.27]), END-1 (0.81 [± 0.07]), and END-2 (0.58 [± 0.07]) treatment groups as compared to in the NC animals (0.56 [± 0.10]; Table 6). Of these differences, only those between the NC and CYP-1 ($p = .012$), and NC and END-1 ($p = .008$) were significant. Vehicle alone gave rise to no significant changes in TWBC or DWBC or H/L ratios (vs. NC values), indicating that ethanol did not cause any immunotoxicity at the level used in the study. In no instance was the SVL or weight of the caimans exposed to either insecticide (at any concentration) affected.

Discussion

Although reptiles suffer many challenges when they are exposed to chemicals in the environment, these effects still remain poorly studied in ecotoxicology. Unlike most model species used in toxicology, reptiles do not typically exhibit short generation times and do not produce large numbers of offspring at short intervals. Ironically, the same characteristics that make reptiles difficult to study present scientists a singular opportunity to create new and ecologically meaningful paradigms in environmental toxicology (Hopkins 2000).

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). In recent years, several studies evaluated the impact of CYP and END formulations on the ISs of non-target organisms; these studies demonstrated that concentrations of these agents commonly applied in agriculture generated adverse effects in different wild species (Pushpanjali et al. 2005; Velisek et al. 2006; Beldoménico et al. 2007; Bachetta et al. 2011). Many studies have been made in order to assess exposure to pesticides in different crocodilian species at different life stages, including viable and non-viable eggs (Crain et al. 1999; Milnes et al. 2004), juveniles (Guillette et al. 1999; Lind et al. 2004) and adults (Crain et al. 1998). However, there have been few studies that evaluated the effects of pesticides on their ISs.

Leukocytes are important mediators of the IS, carrying out the tasks of sensing and ridding the body of pathogens. Similar to mammalian neutrophils, heterophils are the first cells to arrive at a site of infection, followed thereafter by mononuclear phagocytes (Jacobson 2007). Heterophils are mainly phagocytic cells and therefore significant increases in their counts are often associated with inflammatory diseases, infections or tissue damage (Martinez et al. 2011).

In a previous study, the effects to Roundup® (RU) on some parameters of the IS of *C. latirostris* were evaluated (Latorre et al. 2013). That study found that among young caimans exposed to RU there was an increase in the percentages of heterophils. This illustrated that exposure could caused alterations in the IS and growth of young caimans. In the present study, *in vivo* exposure to CYP induced a decrease in lymphocytes and an increase in heterophil and monocyte numbers (compared with values for the control counter-parts). On the other hand, exposure to END induced a decrease in lymphocytes and an increase in monocyte (compared with control levels).

The current results are in line with those of Bachetta et al. (2011) who evaluated changes in hematological and oxidative stress markers in different tissues in fish exposed to sublethal concentrations of END (1.2 and 2.4 µg/l). In the exposed hosts, differential leukocyte counts were affected, that is there were greater proportions of monocytes and lower proportions of lymphocytes and neutrophils. Christin et al. (2003) showed that exposure to a mixture of six pesticides (including END) in ambient conditions significantly reduced the proliferation of lymphocytes in leopard frog (*Rana pipiens*). Dorucu and Girgin (2001) studied CYP effects on some hematological parameters of carp (*Cyprinus carpio*); that study noted that erythrocytes, leukocytes, hemoglobin and hematocrit values decreased with increasing lengths of exposure.

Traditional hematological parameters may provide information about the general state of an individual host. Especially in birds and reptiles, an increase in the H/L index is a common response to stress caused by different factors (Morici et al. 1997; Lance & Elsey 1999). In the current study, the H/L index was significantly higher in the caimans from the groups exposed to CYP-1 and END-1 (compared to those in the NC), suggesting that pesticide exposure induced a state of stress. To our knowledge, there is a lack of any previous studies linking this index with exposure to pesticides.

With regard to stress in general, Lance and Elsey (1999) showed that handling and sequential bleeding of juvenile alligators (*Alligator mississippiensis*) generated a lot of stress, causing an increase in the H/L index. Morici et al. (1997), in studies of effects of corticosterone implants (hormone related to stress levels) in juvenile alligators, observed a higher H/L index in treated animals compared to in control hosts. Merchant et al. (2006) reported that *A. mississippiensis* injected with bacterial lipopolysaccharide had an increase in H/L index. Green turtles (*Chelonia mydas*) with fibro-papillomas also showed a significant increase in H/L ratios that was positively correlated with increases in corticosterone levels; this provided key evidence of the impact of

Table 6. Total white blood cell (TWBC) and differential white blood cell (DWBC) counts in *in vivo* study.

	NC	VC	CYP-1	CYP-2	END-1	END-2
TWBC	2.28 ± 0.11	2.33 ± 0.14	2.46 ± 0.30	2.25 ± 0.12	2.53 ± 0.19	2.05 ± 0.12
Heterophils	31.5 ± 2.7	22.2 ± 1.9	43.6 ± 3.4[a]	31.4 ± 1.7	40.1 ± 1.8	33.9 ± 2.3
Lymphocytes	62.4 ± 3.1	74.7 ± 2.0	50.3 ± 3.5[a]	59.9 ± 2.2	51.4 ± 2.0[a]	59.8 ± 2.7
Monocytes	0.5 ± 0.3	0.3 ± 0.1	1.8 ± 0.4a	1.3 ± 0.3	2.4 ± 0.4[a]	1.1 ± 0.3
Eosinophils	4.5 ± 0.7	3.9 ± 0.3	3.4 ± 0.4	6.3 ± 0.8	5.0 ± 0.7	4.2 ± 0.9
H/L Index	0.56 ± 0.09	0.33 ± 0.04	1.11 ± 0.27	0.54 ± 0.52	0.81 ± 0.07	0.58 ± 0.07

All values are means ± SE ($n = 15$/group). TWBC reported as WBC [$\times 10^4$]/mm^3 blood. Except for index and TWBC, all other parameters are in terms of %. DWBC were made in hosts that had been exposed (starting at Day 20 of age) for 2 months. Data analyzed using ANOVA–Tukey–heterophils, (B) ANOVA–Dunnet's–Lymphocytes and Kruskal Wallis–Mann Whitney–monocytes).
aValue significantly different from negative control (NC) at $p < .05$.

chronic stress on reptilian immune parameters (Aguirre et al. 1995).

We believe a key explanation for a lack of significant effects at the higher concentrations used here might be the appearance of a differential tolerance or existence of a threshold to induce an untoward response. Insecticide tolerance is almost exclusively described as a constitutive trait that arises from a microevolution of decreased susceptibility over time (Feyereisen 1995; Lopes et al. 2008). However, phenotypic plasticity is defined as a capacity of a single genotype to exhibit variable phenotypes in different environs (Schlichting & Pigliucci 1998). In this scenario, exposure to sub-lethal insecticide levels can induce increased tolerance to a lethal concentration of the same pesticide later in life so that higher concentrations could induce a quicker onset of tolerance than would lower concentrations (Hua et al. 2013). With regard to the threshold explanation, as no dose–response effects were noted in the experiments, it is apparent a toxic threshold had not been achieved for development of any "expected" response[s]; clearly, above this level, if there was any true toxicity, the magnitude of those responses would in some manner likely be related to subsequent increases in concentrations of the pesticides. However, it is equally likely that use of such "higher" doses would end up in increased host mortality. Thus, the possibility exists that it is just that these agents did not really have a "toxic" effect on the studied endpoints. Further studies are underway to provide greater clarification on this issue.

The reptilian immune response is profoundly affected by ecological factors, including population dynamics, stress, nutritional state, environmental temperature, seasonal variations, age and infectious pathogens. Researchers have reported strong correlations between animal exposure to pesticides or other environmental toxicants and suppression of immune responses to specific pathogenic challenge through anticholinergic and noncholinergic pathways, as well as other responses such as hypersensitivity and autoimmunity (Barnett & Rodgers 1994; Vial et al. 1996; Voccia et al. 1999). Histopathological changes in IS tissues and organs, pathologies at the cellular level, and alterations in lymphocyte population growth/function can also be attributed to pesticides exposure (Barnett & Rodgers 1994; Vial et al. 1996; Voccia et al. 1999). These effects caused by pesticides/environmental contaminants on the components and functions of the IS have been linked to immune compromise and reductions in disease resistance in exposed animals and humans (Vial et al. 1996; Voccia et al. 1999).

Though there have been significant laudable increases in concern about the misuse of pesticides (including insecticides, herbicides, algaecides, rodenticides), there remains a threat to wildlife resulting from indirect factors associated with pesticide use. Not only may pesticides directly and indirectly affect wildlife, but also might personal care products and pharmaceuticals that end up in surface waters and feedstuffs that local fauna consume. As global human populations continue to increase and expand into wild environs, there will continue to be increased risks posed to wildlife from pesticides and other contaminants. Ultimately, either directly through toxicity and/or indirectly through immunosuppression and other more subtle compromises to the animal ability to avoid infection, these will impact on the ability of these exposed hosts to thrive and survive (Presley et al. 2010).

Conducting research on the effects of pesticide exposure on this species is of special interest not only in regard to assessing the impact on caiman populations, but also to further characterize the species as a potential sentinel of ecosystem health. With the latter, this might enable detection of regions with high contamination burdens of pesticides in the absence of physical measures of the pesticide levels themselves.

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Disclosure statement

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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References

Aguirre A, Balazs G, Spraker T, Gross T. 1995. Adrenal and hematological responses to stress in juvenile green turtles (*Chelonia mydas*) with and without fibro-papillomas. Physiol Zool. 68:831–854.

[AOAC] Association of Official Agricultural Chemists. 1995. Official methods of analysis of AOAC International. In: Cunniff P, editor. Pesticide formulations. 16th ed. Arlington, VA: AOAC International, EEUU; p. 42–73.

Bachetta C, Cazenave J, Parma J. 2011. Responses of biochemical markers in the fish *Prochilodus lineatus* exposed to a commercial formulation of endosulfan. Water Air Soil Pollut. 216:39–49.

Barnett J, Rodgers K, editors. 1994. Pesticides. New York (NY): Raven Press.

Beldoménico P, Rey F, Prado W, Villarreal J, Muñoz del Toro M, Luque E. 2007. *In ovum* exposure to pesticides increases egg weight loss and decreases hatchling weight of *Caiman latirostris* (*Crocodylia: Alligatoridae*). Ecotoxicol Environ Safe. 68:246–251.

Brisbin I, Jagoe C, Gaines K, Gariboldi J. 1998. Environmental contaminants as concerns for the conservation biology of crocodilians. In: Proceedings of the 14th working meeting of the Crocodile Specialist Group of the Species Survival Commission of IUCN. Singapore: The World Conservation Union; p. 155–173.

Burns L, Meade B, Munson A. 1996. Toxic responses of the immune system. In: Klaasen C, editor. Casarett and Doull's toxicology. The basic science of poisons. New York: McGraw-Hill; p. 355–402.

[CASAFE] Chamber of Agricultural Health and Fertilizers Argentina. Acerca del Glifosato. Defienden el uso del herbicida Glifosato para la producción [Internet]. [cited 2013 Mar 18]. Available from http://www.casafe.org/blog/?tag=glifosato.

Christin M, Gendron A, Brousseau P, Ménard L, Marcogliese D, Cry D, Ruby S, Fournier M. 2003. Effects of agricultural pesticides on the immune system of *Rana pipiens* and on its resistance to parasitic infection. Environ Toxicol Chem. 22:1127–1133.

Crain D, Noriega N, Vonier P, Arnold S, McLachlan J, Guillette L. 1998. Cellular bioavailability of natural hormones and environmental contaminants as a function of serum and cytosolic binding factors. Toxicol Ind Health. 14:261–273.

Crain D, Spiteri I, Guillette L. 1999. The functional and structural observations of the neonatal reproductive system of alligators exposed *in ovo* to atrazine, 2,4-D, or estradiol. Toxicol Ind Health. 15:180–185.

Crews D, Bull J, Wibbel T. 1991. Estrogen and sex reversal in turtles: A dose-dependent phenomenon. Gen Comp Endocrinol. 81:357–364.

Donayo P, Piña C, Larriera A. 2002. Periodo de incubación, banda de calcificación, peso de los huevos y desarrollo embrionario de *Caiman latirostris* a tres temperaturas diferentes. In: Verdade L, Larriera A, editors. The conservation and management of caimans and crocodiles of Latin America – 2. Sao Paulo: Piracicaba; p. 96–109.

Dorucu M, Girgin A. 2001. The effect of cypermethrin on some hematological parameters of *Cyprinus carpio*. Aquat Int. 9:183–187.

Feyereisen R. 1995. Molecular biology of insecticide resistance. Toxicol Lett. 82:83–90.

Gilbertson M, Haffner G, Drouillard G, Albert A, Dixon B. 2003. Immunosuppression in the Northern Leopard frog (*Rana pipiens*) induced by pesticide exposure. Environ Toxicol Chem. 22:101–110.

Guillette L, Brock J, Rooney A, Woodward A. 1999. Serum concentration of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. Arch Environ Contam Toxicol. 36:447–455.

Hall R, Henry P. 1992. Assessing effects of pesticides on amphibians and reptiles: Status and needs. Herpetol J. 2:65–71.

Hopkins W. 2000. Reptile toxicology: Challenges and opportunities on the last frontier in vertebrate ecotoxicology. Environ Toxicol Chem. 19:2391–2393.

Hopkins W. 2005. Use of tissue residues in reptile ecotoxicology: A call for integration and experimentalism. In: Gardner S, Oberdörster E, editors. Toxicology of reptiles. New perspectives: Toxicology and the environment. New York: Taylor and Francis; p. 35–62.

Hua J, Morehouse N, Relyea R. 2013. Pesticide tolerance in amphibians: Induced tolerance in susceptible populations, constitutive tolerance in tolerant populations. Evol Appl. 6:1028–1040.

Jacobson E, editor. 2007. Infectious diseases and pathology of reptiles. Boca Raton (FL): CRC Press.

Lance V, Elsey R. 1999. Plasma catecholamines and plasma corticosterone following restraint stress in juvenile alligators. J Exp Zool. 283:559–565.

Larriera A, Imhof A, Siroski P. 2008. Current state of conservation and management programs of the *Caiman* genus in Argentina. In: Castroviejo J, Ayarzaguena J, Velasco A, editors. Contribution to the knowledge of Caiman genus in South America. Seville: Publicaciones de la Asociación Amigos de Doña Ana; p. 139–179.

Latorre M, López González E, Larriera A, Poletta GL, Siroski P. 2013. Effects of *in vivo* exposure to Roundup® on immune system of *Caiman latirostris*. J Immunotoxicol. 10:349–354.

Lewis S, Bates I, Bain B, editors. 2008. Dacie and Lewis. Hematology practice. 10th ed. Madrid: Elsevier; p. 41.

Lind P, Milnes M, Lundberg R, Bermudez D, Örberg J, Guillette L. 2004. Abnormal bone composition in female juvenile American alligators from a pesticide-polluted lake (Lake Apopka, FL). Environ Health Perspect. 112:359–372.

Lopes P, Sucena E, Santos M, Magalhaes S. 2008. Rapid experimental evolution of pesticide resistance in *C. elegans* entails no costs and affects the mating system. PLoS One. 3:e3741

Messel H, King F, Ross J. 1995. Introduction: The conservation and management of caimans and crocodiles. In: Larriera A, Verdade L, editors. The conservation and management of caimans and crocodiles of Latin America. Santo Tomé: Fundación Banco Bica; p. 1–3.

Milnes M, Allen D, Bryan T, Sedacca C, Guillette L. 2004. Developmental effects of embryonic exposure to toxaphene in the American alligator (*Alligator mississippiensis*). Comp Biochem Physiol C Toxicol Pharmacol. 138:81–87.

Martinez SA, Lavín S, Cuenca R. 2011. Hematology and blood cytology in reptiles. Clín Vet Pequeños Anim. 31:131–141.

Merchant M, Mills K, Williams S, Kleckley F, Sims A, Elsey R, Bushnell J. 2006. Effects of bacterial lipopolysaccharide on peripheral leukocytes in the American alligator (*Alligator mississippiensis*). Vet Immunol Immunopathol. 111:315–320.

Morici L, Elsey R, Lance V. 1997. Effects of long-term corticosterone implants on growth and immune function in juvenile alligators, *Alligator mississippiensis*. J Exp Zool. 279:156–162.

National Scientific and Technical Research Council. 2005. Reference ethical framework for biomedics research: Ethical principles for research with laboratory, farm and wild animals, Res. #1047. Buenos Aires: Anexo II. CONICET.

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

Patel S, Pandey A, Bajpayee M, Parmar D, Dhawan A. 2006. Cypermethrin-induced DNA damage in organs and tissues of the mouse: Evidence from the Comet assay. Mutat Res. 607:176–183.

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

Poletta G, Kleinsorge E, Paonessa A, Mudry M, Larriera A, Siroski P. 2011. 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 Safety. 74:852–859.

Presley S, Austin G, Dabbert B. 2010. Influence of pesticides and environmental contaminants on emerging diseases of wildlife. In: Kendall R, Lacher T, Cobb G, Cox S, editors. Wildlife toxicology. Emerging contaminant and biodiversity issues. Boca Raton (FL): CRC Press; p. 73–110.

Pushpanjali P, Prasad R, Prasad A, Singh S, Kumar A, Jadhao S. 2005. *In ovo* embryo toxicity of endosulfan adversely influences liver and brain metabolism and the immune system in chickens. Pest Biochem Physiol. 82:103–114.

Schlichting C, Pigliucci M, editors. 1998. Phenotypic evolution: A reaction norm perspective. Sunderland (MA): Sinauer Associates Inc. Publishers.

Sharma S, Nagpure N, Kumar R, Pandey S, Srivastava S, Singh P, Mathur P. 2007. Studies on the genotoxicity of endosulfan in different tissues of fresh water fish *Mystus vittatus* using the Comet assay. Arch Environ Contam Toxicol. 53:617–623.

Simoniello M, Gigena F, Poletta G, Loteste A, Kleinsorge E, Campana M, Scagnetti J, Parma M. 2009. Alkaline comet assay for genotoxic effect detection in neotropical fish *Prochilodus lineatus* (Pisces, *Curimatidae*). Bull Environ Contam Toxicol. 83:155–158.

Siroski P. 2004. *Caiman latirostris* and *Caiman yacare* population surveys in Formosa Province, Argentina. In: Proceedings of the 17th working meeting of the IUCN-Crocodile Specialist Group of the Species Survival Commission of IUCN. Gland: The World Conservation Union; p. 443–446.

Tangredi B, Evans R. 1997. Organochlorine pesticides associated with ocular, nasal, or otic infection in the Eastern box turtle (*Terrapene Carolina carolina*). J Zoo Wildl Med. 28:97–100.

Velisek J, Wlasow T, Gomulka P, Svobodova Z, Dobsikova R, Novotny L, Dudzuk M. 2006. Effects of cypermethrin on rainbow trout (*Oncorhynchus mykiss*). Vet Med. 51:469–476.

Vial T, Nicolas B, Descotes J. 1996. Clinical immunotoxicity of pesticides. J Toxicol Environ Health. 48:215–229.

Voccia I, Blakley B, Brousseau P, Fournier M. 1999. Immunotoxicity of pesticides: A review. Toxicol Ind Health. 15:119–132.