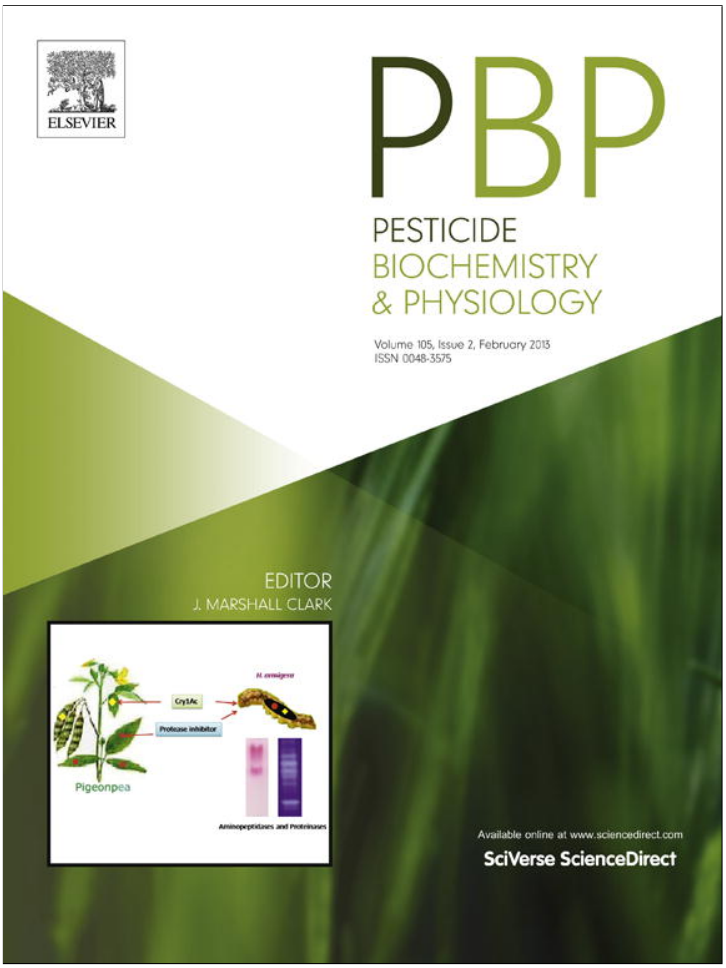

Pesticide Biochemistry and Physiology 105 (2013) 131–134

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

# Induction of micronuclei in broad snouted caiman (*Caiman latirostris*) hatchlings exposed *in vivo* to Roundup® (glyphosate) concentrations used in agriculture

E.C. López González [a,b,*], M.A. Latorre [a,b], A. Larriera [a], P.A. Siroski [a,c], G.L. Poletta [a,b,d]

[a] "Proyecto Yacaré" –Laboratorio de Zoología Aplicada: Anexo Vertebrados, Facultad de Humanidades y Ciencias, UNL/Ministerio de Aguas, Servicios Públicos y Medio Ambiente, Av. Aristóbulo del Valle 8700, CP 3000, Santa Fe, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, CP C1033AAJ, CABA, Argentina
[c] Laboratorio de Biología Celular y Molecular–Facultad de Ciencias Veterinarias, UNL-CONICET, R.P. Kreder 2805. CP S3080HOF – Esperanza, Santa Fe, Argentina
[d] Cátedra de Toxicología, Farmacología y Bioquímica Legal, Facultad de Bioquímica y Ciencias Biológicas, UNL/GIBE, IEGEBA (CONICET-UBA), Ciudad Universitaria, Paraje El Pozo S/N, CP 3000, Santa Fe, Argentina

ARTICLE INFO

*Article history:*
Received 19 August 2012
Accepted 28 December 2012
Available online 11 January 2013

*Keywords:*
Genotoxicity
Micronucleus test
Pesticides
Field concentrations
Glyphosate decrease

ABSTRACT

*Caiman latirostris* is one of the two crocodilian species living in Argentina. As a result of agricultural expansion produced in recent years, some populations are exposed to continuous pesticide discharge due to the proximity of its natural geographic distribution to extensive agricultural areas. The aim of this study was to evaluate genotoxicity and the effects on growth of Roundup® (RU; glyphosate based formulation) on *C. latirostris* hatchlings, considering concentrations commonly applied in crops and subsequently decay of the compound in water through time, as it might occur in the environment. *C. latirostris* 20 days old, from three different clutches, were exposed to two RU concentrations in plastic containers, during two months. RU concentration was progressively decreased through time, so experimental groups were: treatment 1: 11 mg/l (concentration at the beginning) to 2.5 mg/l (concentration at the end of experiment), treatment 2: 21 mg/l to 5 mg/l; and a control without RU. At the end of exposure period, blood samples were obtained and the micronucleus (MN) test applied in erythrocytes as a marker of genotoxicity. Results indicated a significant increase in the frequency of MN ($p < 0.05$) and a tendency to lower growth in the groups exposed to RU compared to the negative control. These results, together with those reported in previous studies; warn about the effect that *C. latirostris* wild populations continuously exposed to low concentrations of pesticides might be suffering.



## 1. Introduction

*Caiman latirostris* (Broad-snouted caiman, Crocodylia, Alligatoridae), is the crocodilian species with the southernmost distribution in South America, reaching to Santa Fe province, Argentina [1], and one of the two species of caimans living in this country.

Since 1996, the introduction of transgenic soy in Argentina led to a fast agricultural expansion over natural areas. As a consequence of this process, *C. latirostris* populations are exposed to continuous pesticide discharges in their natural geographic distribution because the proximity to intensive agricultural areas [2].

The area planted with transgenic soy (RR, resistant to the herbicide glyphosate) in Argentina, reached to more than 20 million ha in the 2010–2011 season, and continues increasing. This led to a steadily increment in the use of pesticides, particularly glyphosate, since it allows to control weeds during the entire cycle of the crop [3]. Because of the processes of drift, runoff and leaching, pesticides disperse in the environment, causing negative effects on the organisms living in adjacent natural areas [4].

Pesticides are often very reactive compounds that can disrupt normal cellular processes and interact directly or indirectly with DNA, causing genetic instability [5,6]. The micronucleus (MN) test is a biomarker to detect genotoxic effects of agents that modify the structure and/or segregation of chromosomes, allowing the detection of early biological responses, before the damage is irreversible and imbalances the organism health [7]. The MN test have been widely used as a biomarker of genotoxicity for environmental monitoring of wildlife populations exposed to different pollutants, considering them as sentinel organisms [8–12]. Studies made by our group revealed the induction of DNA damage in caimans after *in ovo* exposure (during the embryonic period) to the formulation Roundup® (RU; glyphosate), showing a concentration-dependent

* Corresponding author at: "Proyecto Yacaré" – Laboratorio de Zoología Aplicada: Anexo Vertebrados (Facultad de Humanidades y Ciencias, UNL/Ministerio de Aguas, Servicios Públicos y Medio Ambiente), Av. Aristóbulo del Valle 8700, CP 3000, Santa Fe, Argentina. Fax: +54 0342 4 579256.
*E-mail address:* evelynclg@hotmail.com (E.C. López González).

0048-3575/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pestbp.2012.12.009

effect [13]. Similar results were observed with the same formulation and a mixture including glyphosate, cypermethrin and endosulfan formulations in an experiment that simulated possible natural exposure of caiman nests in areas near herbicide applications [2]. It should be noted that the period of the year of highest pesticides use (November–March) coincides with the reproductive season of this species. During this period, females construct the nests using surrounding vegetation, eggs are incubated there and after hatching, caimans usually remain in surface waters near crops during the first months of life [2].

Up to our knowledge, no studies have been conducted yet evaluating the effect of glyphosate formulation in *C. latirostris* hatchlings, considering time of exposure, concentration and decrease of the compound in water as it might occur in the environment. The aim of this study was to evaluate the genotoxicity and effects on growth of *C. latirostris* hatchlings exposed *in vivo* to sub-lethal concentrations of Roundup®, studying exposure conditions, a route of exposure and a biological stage not previously evaluated.

## 2. Materials and methods

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* [14], 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.

We used 72 *C. latirostris* specimens, 20 days of age, hatched from eggs harvested in three different nests in the Natural Managed Reserve "El Fisco" (30°11′26″S, 61°0′27″O; Dpto. San Cristóbal, Santa Fe, Argentina), under the 'Proyecto Yacaré' ranching program. This area was chosen as it is a Protected Natural Area (Law 12,930; 2008), situated at least 20 km far from any pesticide application area or other contaminant activity.

Animals were randomly distributed into three experimental groups of 24 specimens each, with two replicates of 12 animals per group: a negative control (NC) without exposure, and two treatments exposed to different concentrations of RU: treatment 1 (RU1): 11 mg/l (initial concentration) to 2.5 mg/l (final concentration), and treatment 2 (RU2): 21 mg/l (initial) to 5 mg/l (final). A subchronic exposure (60 days) was performed by immersion [15] in plastic containers (75 cm long, 35 cm wide and 37 cm high, base surface = 0.2622 $m^2$), tilted to provide 60% dry and 40% water surface areas, with a maximum water depth of approximately 15 cm. Temperature in the containers were maintained at 30 ± 2 °C and were monitored with Hobbo data logger (Onset Computer Corp., Pocasset, MA, USA).

The RU concentrations chosen was that recommended for product application in crops (i.e., 2%/ha), considering the surface of the container base (0.2622 $m^2$) as the reference area for calculation of the amount of RU to be added to each container in a fixed volume of water (5 l), and then doubling this value (treatments 1 and 2, respectively). Water was renewed every two days and concentration of RU progressively decreased through time, taking into account glyphosate decay previously determined by HPLC, under the same conditions of the experiment. Through that previous study, we determined the duration of exposure as the time when the compound almost completely disappear (two months), as well as the progressive decreasing concentrations used for both treatments (Fig. 1). Therefore, the ranges of exposure concentrations were: RU1: 11 mg/l to 2.5 mg/l, and RU2: 21 mg/l to 5 mg/l.

All animals were individually marked with foot webbing tags (Monel Natl Band and Tag CO., Newport, Kentucky). They were

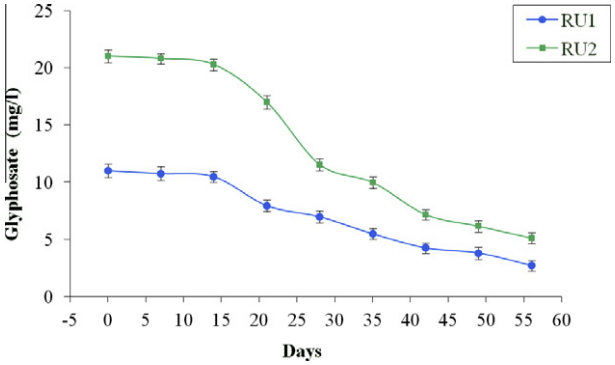


**Fig. 1.** Glyphosate (GFT) decrease in water analyzed by High Performance Liquid Chromatography (HPLC). Concentration was measured by triplicate and shows progressive GFT (mean ± standard deviation) decrease trough time.

measured in total length (TL) and weighed at the beginning and at the end of the experiment to determine growth in each experimental group. 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 the end of the experiment, blood samples (0.5 ml) were taken from the spinal vein [16] of all animals. Samples were not taken at the beginning of the experiment to avoid any risk of death for caimans due to their initial small size.

MN test was applied in erythrocytes [17] as a biomarker of genotoxicity; two smears were made for each animal, fixed and stained with Giemsa. For each sample, 1000 erythrocytes were analyzed under a microscope with a magnification of 1000× and the MN frequency determined (MNF: number of cells with MN/1000 cells counted). The criteria adopted for MN identification were the following [18]: (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 (Fig. 2).

Statistical analysis was performed using the software SPSS 14.0 for Windows [19]. The data were evaluated in normality by the Kolmogorov–Smirnov test and in homogeneity of variance using the Levene test. Considering that the "clutch effect" is one of the most important causes of variability observed in crocodilians [20,21], we analyzed the difference between clutches for all variables using a one-way ANOVA. Growth in TL and MNF were analyzed using the Kruskal–Wallis test followed by Mann–Whitney test to determine differences between experimental groups. We applied the Bonferroni correction according to the number of

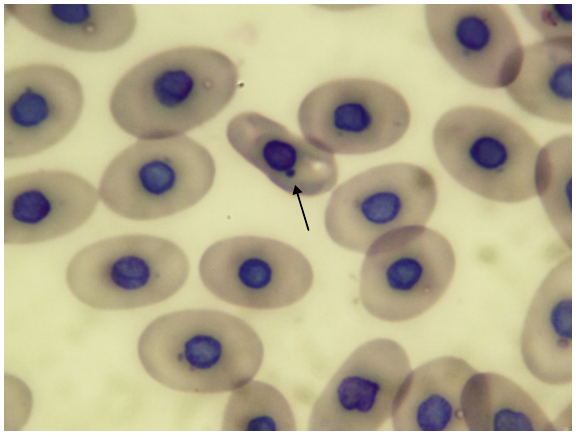

**Fig. 2.** Image of an erythrocyte of *C. latirostris* with MN (arrow). 1000×.

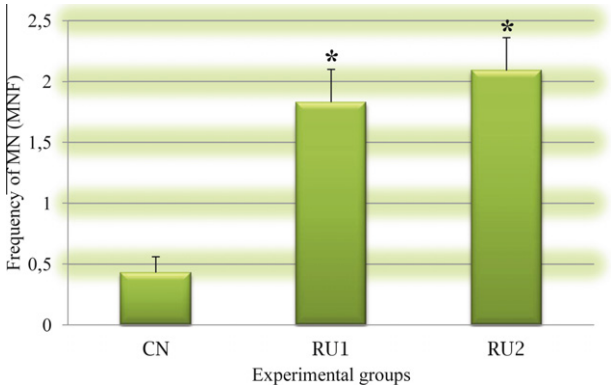


**Fig. 3.** Micronucleus frequency (mean ± standard error) observed in the different experimental groups. NC: negative control; RU1: 11 mg/l (initial concentration Roundup®) to 2.5 mg/l (final concentration Roundup®) and RU2: 21 mg/l (initial concentration Roundup®) to 5 mg/l (final concentration Roundup®). *Significantly different compared to the negative control (Mann–Whitney test).

analysis by pairs carried out, so a $p$ value < 0.016 was considered statistically significant. Growth in weight was analyzed by ANOVA followed by Tukey's test to determine the difference between treatments. To evaluate the existence of a relationship between the MNF and weight or length of the animals we conducted linear regressions. Results are expressed as mean ± Standard Error (SE).

## 3. Results

Results demonstrated an induction of genotoxicity caused by exposure to RU. There was a significantly higher MNF in RU1 (1.83 ± 0.27) and RU2 (2.09 ± 0.27) compared with the NC (0.43 ± 0.13, $p < 0.001$), but no difference was observed between RU1 and RU2 ($p = 0.494$) (Fig. 3).

Results of growth showed that hatchlings exposed to RU2 grew less in TL (5.64 ± 0.89 cm) and weight (54.13 ± 6.80 g) than those of the NC group (TL: 8.23 ± 0.61 cm, $p = 0.021$; weight: 69.44 ± 6.02 g, $p = 0.179$) and RU1 group (TL: 8.53 ± 0.40 cm, $p = 0.034$; weight: 82.16 ± 5.11 g, $p = 0.055$), but differences were not statistically significant. No differences in growth were observed between animals in RU1 and those of the NC (weight $p = 0.234$; length $p = 0.958$) (Fig. 4).

There were no differences between clutches in the MNF ($p = 0.639$), weight ($p = 0.136$) or length ($p = 0.344$) of the animals, and no relationship between animals weight or length and the MNF ($p = 0.113$, $R^2 = 0.036$ and $p = 0.092$, $R^2 = 0.041$, respectively).

## 4. Discussion and conclusions

In recent years, several studies evaluated the impact of glyphosate formulations on non-target organisms, demonstrating that concentrations commonly applied in agriculture generated adverse effects in different wild species [10,12,13,22–25].

The results of our study revealed that the formulation RU also induces genotoxic effects in hatchlings of *C. latirostris* exposed *in vivo*, with an increase in the MNF in both groups (RU1 and RU2) compared to the negative control (NC), and showing a concentration-dependent effect. These data are consistent with previous studies on different species of vertebrates exposed to RU. Grisolia [8] reported an increase in MNF in erythrocytes of *Tilapia rendalli* exposed *in vivo* while Cavas and Könen [10] observed high MNF, nuclear abnormalities and strand breaks in *Carassius auratus* at similar concentrations to those applied in our study (5–15 mg/l). Likewise, recent works in post metamorphic anuran *Rhinella arenarum* and *Odontophrynus cordobae* exposed to RU concentrations (100–800 mg a.i./l) 10–20 times lower than those typically applied to agricultural fields, demonstrated an increase in the MNF in both species, but with different sensitivity between them [12].

For reptiles, a scarce number of studies can be found on the genotoxicity of glyphosate. Sparling et al. [22] reported that glyphosate-based formulation GlyPro® induced dose-dependent genotoxic effects in neonates of *Trachemys scripta elegans* after *in ovo* exposure by topical application on the eggshells. In broad-snouted caiman, previous studies demonstrated the genotoxic effect of glyphosate and RU formulation after *in ovo* exposure by topical application [13,23]. In the same way, authors reported the effect of RU and its combination with endosulfan and cypermethrin formulations after semi-natural exposure of caiman nests with eggs inside, under conditions similar to those that might occur in natural environments near crops [2]. Hatchlings are particularly susceptible because they spend most of the time in small water bodies, many of which receive and concentrate pesticides discharges from neighboring crops. The results of this study demonstrated that RU formulation also induces genotoxic effects and a trend to lower growth in *C. latirostris* hatchlings exposed *in vivo*

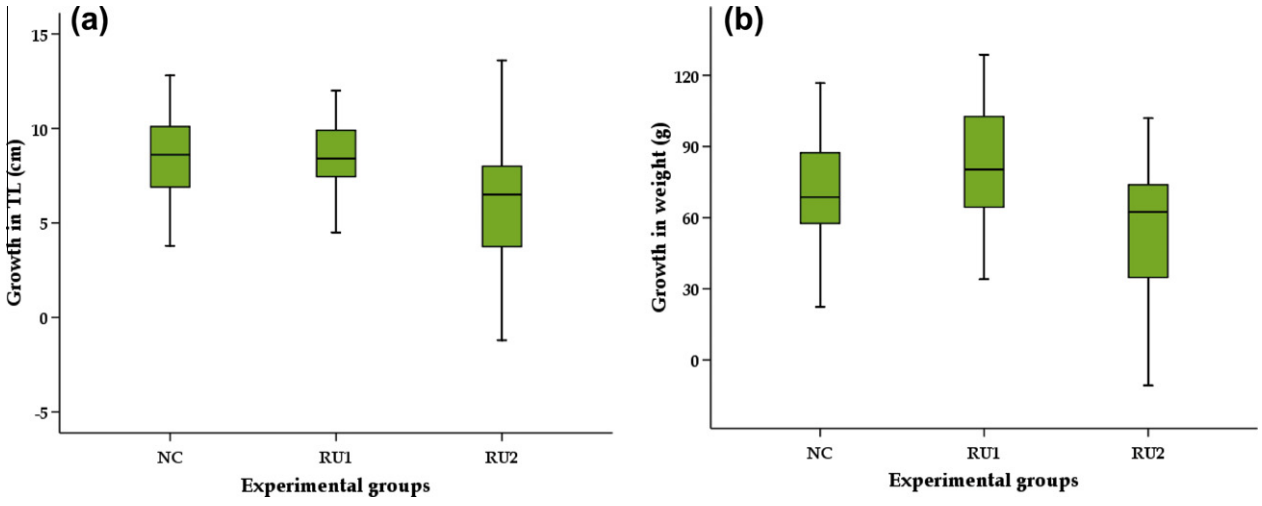


**Fig. 4.** (a) Growth in total length (TL) (Kruskal–Wallis - Mann–Whitney test) and (b) weight (ANOVA – Tukey's test) of the experimental groups. NC: negative control; RU1: 11 mg/l (initial concentration Roundup®) to 2.5 mg/l (final concentration Roundup®) and RU2: 21 mg/l (initial concentration Roundup®) to 5 mg/l (final concentration Roundup®).

during the first months of life. Glyphosate concentrations used include that typically applied in soy crops, as well as the progressive decrease of the compound as it can occur in water bodies.

Chronic sublethal exposure to contaminants has been shown to result in elevated Standard metabolic rate (SMR) in reptiles. Given no compensatory increase in feeding or assimilation, individuals having SMR elevated above normal would experience fitness costs associated with reduced growth, as a result of decreased energetic contributions to the production budget. In agreement to this study, Poletta et al. [2,25] reported a lower growth of caimans during the first months of life after *in ovo* exposure under controlled and semi-natural conditions. Likewise, Sparling et al. [22] observed a decrease in weight of newborns *T. scripta elegans* also after *in ovo* exposure to RU. In these species, early life stages have the higher growth rate. A decrease in growth during this period can have much influence on hatchlings survival under environmental conditions, as they are extremely vulnerable to cold temperature and predators, factors clearly dependent on body size [26].

No differences were observed between clutches in any of the variables analyzed. These data agree with those reported by Poletta et al. [13,2], concluding that the negative effect produced by RU is independent of the clutch of origin of animals. Moreover, we found no relationship between MNF and body size of the caimans. According with this, Poletta et al. [13,2] reported no relationship between size of the animals and genotoxic damage evidenced by the MN test and the comet assay. However, Schaumburg et al. [27] reported that smaller animals showed higher DNA damage, probably due to a poor nutritional status, which would involve a malfunction of protection and repair mechanisms.

The biological consequences of all these alterations are uncertain, but they could affect the normal function of physiological processes at the cellular and individual level, warning about the effect that wild populations of *C. latirostris* continuously exposed to low concentrations of these and other pesticides might be suffering.

## Acknowledgements

This work was supported by ANPCyT (GLP PICT 2011-1349) Argentina, Proyecto Yacaré and Yacarés Santafesinos (Gob. Sta. Fe/MUPCN). We would like to thank the members of Proyecto Yacaré (www.mupcn.com.ar/yacare).

This study is part of ECLG research as a doctoral fellow of CONICET.

## References

[1] P.A. Siroski, *Caiman latirostris, Caiman yacare* Population Surveys in Formosa Province, Argentina, in: Proceedings of the 17th Working Meeting of the CSG/IUCN–The World Conservation Union, Darwin, NT, Australia, 2004, pp. 443–446.
[2] G.L. Poletta, E. Kleinsorge, A. Paonessa, M.D. Mudry, A. Larriera, P.A. Siroski, 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.
[3] M. Aizen, L.A. Garibaldi, M. Dondo, Expansión de la soja y diversidad de la agricultura argentina, Ecol. Austral. 19 (2009) 45–54.
[4] P.F. Donald, Biodiversity impacts of some agricultural commodity production systems, Conserv. Biol. 18 (2004) 17–37.
[5] D.J. Ecobichon, Efectos Tóxicos de los pesticidas, in: C.D. Klaasseny, J.B. Watkins (Eds.), Casarett & Doull, Fundamentos de Toxicología, McGraw-Hill/Interamericana, Madrid, España, 2005, pp. 339–353.
[6] L. Glusczak, E. dos Santos Miron, M. Crestani, M. Braga de Fonseca, F. de Araujó-Pedron, M.Y. Frescura Durate, V.L. Pimentel Vieira, Effect of glyphosate herbicide on acetylcholinesterase activity metabolic and hematological parameters in piava (*Leporinus obtusidens*), Ecotoxicol. Environ. Saf. 65 (2006) 237–241.
[7] M.A. Carballo, M.D. Mudry, 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.
[8] C.K. Grisolia, A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides, Mutat. Res. 518 (2002) 145–150.
[9] R.C. Lajmanovich, M.C. Cabagna, P.M. Peltzer, G.A. Stringhini, A.M. Attademo, Micronucleus induction in erythrocytes of the *Hyla pulchella* tadpoles (Amphibia: Hylidae) exposed to insecticide endosulfán, Mutat. Res. 587 (2005) 67–72.
[10] T. Cavas, S. Könen, Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay, Mutagenesis 22 (2007) 263–268.
[11] B. de Campos Ventura, D. de Fransceschi de Angelis, M.A. Marin-Morales, Mutagenic and genotoxic effects of the Atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay, Pest. Biochem. Physiol. 90 (2008) 42–51.
[12] B. Bosch, F. Mañas, N. Gorla, D. Aiassa, Micronucleus test in post metamorphic *Odontophrynus cordobae* and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring, J. Toxicol. Environ. Health, Part A 3 (2011) 155–163.
[13] G.L. Poletta, E. Kleinsorge, M.D. Mudry, A. Larriera, 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.
[14] National Scientific and Technical Research Council–CONICET, Reference Ethical Framework for Biomedics Research: Ethical principles for research with laboratory, farm and wild animals, 2005.
[15] D.L. Eaton, C.D. Klassen, Principios de Toxicología, in: C.D. Klassen, J.B. Watkins (Eds.), Casarett and Doull, Fundamentos de Toxicología, McGraw-Hill/Interamericana, Madrid, España, 2005, pp. 7–22.
[16] G. Olson, J. Hessler, R. Faith, Techniques for the blood collection and intravascular infusion of reptiles, Lab. Anim. Sci. 25 (1977) 783–786.
[17] G.L. Poletta, A. Larriera, E. Kleinsorge, M.D. Mudry, *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.
[18] W. Schmid, The micronucleus test, Mutat. Res. 31 (1975) 9–15.
[19] SPSS for Windows, Version 14.0, SPSS Inc. Chicago, USA, 2005.
[20] D. Schulte, R. Chabreck, Effects of nest and egg characteristics on size and early development of American alligators, in: Proceedings 10th Working Meeting of the CSG-IUCN - TheWorld Conservation Union, Gland, Switzerland, 1990, pp. 177–187.
[21] L.M. Verdade, Morphometric analysis of the broad-snouted Caiman (*Caiman latirostris*): an assessment of individual's clutch, body size, sex, age, and area of origin, PhD Dissertation, University of Florida, Gainesville, Florida, USA, 1997, pp. 174.
[22] D.W. Sparling, C. Matson, J. Bickham, P. Doelling-Brown, Toxicity of Glyphosate as Glypro® and LI700 to Read-eared slider (*Trachemys scripta elegans*) embryos and early hatchlings, Environm. Toxicol. Chem. 25 (2006) 2768–2774.
[23] G.L. Poletta, A. Larriera, M.D. Mudry, E. Kleinsorge, Effects of chemical contaminants on wildlife: Identification of biomarkers in a sentinel species, in: J.D. Harris, P.L. Brown (Eds.), Wildlife: Destruction, Conservation and Biodiversity, Nova Science Publishers., New York, USA, 2009. Chapter 6.
[24] R.C. Lajmanovich, A.M. Attademo, P.M. Peltzer, C.M. Junges, M.C. Cabagna, Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors, Arch. Environ. Contam. Toxicol. 60 (4) (2011) 681–689.
[25] G.L. Poletta, A. Larriera, P.A. Siroski, E. Kleinsorge, M.D. Mudry, Integral aproach of Glyphosate-induced alterations in a South American caiman species, in: K.D. Piotrowski (Ed.), Herbicides: Properties, Crop Protection and Environmental Hazards, Nova Science Publishers, New York, USA, 2011. Chapter 5.
[26] C.L. Mitchelmore, C.L. Rower, A.R. Place, Tools for assenssing conteminant exposure and effects in reptiles, in: S.C. Gardner, E. Oberdörster (Eds.), Toxicology of reptiles, new perspective: toxicology and the environment, Taylor & Francis Group, Florida, 2005, pp. 63–122.
[27] L.G. Schaumburg, G.L. Poletta, P.A. Siroski, Ultraviolet radiation-induced genotóxico effects in the broad-snouted caiman *Caiman latirostris*, Mutat. Res. 700 (2010) 67–70.