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Evaluation of Stage-Dependent Genotoxic Effect of Roundup® (Glyphosate) on *Caiman latirostris* Embryos

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Abstract The agricultural expansion over the past decades, along with the associated increase in the use of pesticides, represents a high risk for many wild species. *Caiman latirostris* is a South American caiman with many features that make it highly vulnerable to pesticide exposure. Considering previous finding on the genotoxicity of the glyphosate-based formulation Roundup® in this species, the aim of this study was to evaluate the possible stage-dependent effect of this compound on *C. latirostris* embryos through the Comet assay (CA), micronuclei (MN), and nuclear abnormalities (NA) tests. Caiman eggs were exposed to three effective concentrations of Roundup® (750, 1250, 1750 μg/egg) in three different stages of the incubation period (total duration 70 ± 3 days at 31 ± 2 °C) of approximately 23 days each. A statistically significant difference in DNA damage determined by the CA was found between groups exposed to different concentrations of RU ($p < 0.05$) and the negative control, but no difference was observed among the three stages of exposure within any treatment ($p > 0.05$). There was no differences in the MN or NA frequencies between the different groups and the negative control ($p > 0.05$), nor among the different stages within each treatment. The results obtained in this study indicate that RU produce DNA damage on *C. latirostris* embryos independently of the developmental stage where the exposure occurs, implying an important risk for the species during all its period of development, when pesticide application is at maximum rate.

In Argentina, the agricultural frontier expansion has led to the loss of pristine habitat, with the consequent impairment of the biodiversity and the ability of the ecosystems to provide essential resources (Paruelo et al. 2004; Aizen et al. 2009). The genetically modified soybean, which achieved a global production of 257 millions tons between 2010 and 2012 (Ciani 2014), has become the most important crop in this country. This kind of transgenic crops are tightly associated with the application of different pesticide formulations, the most used worldwide, are glyphosate-base formulations (Pazos 2008).

Glyphosate [*N*-phosponomethyl glycine (GLY)] is a nonselective herbicide that inhibits plant growth and is the active ingredient of Roundup® (RU)—the most widely used formulated herbicide. Its broad-spectrum perennial weed control makes it a very effective product (Dill et al. 2010). In Argentina, during 2013–2014 more than 200 million liters of this herbicide were used (CASAFE 2015) and this tendency continue growing. GLY used to be considered slightly toxic to higher organisms as the enzyme inhibited by glyphosate (enolpyruvylshikimate phosphate synthase) is only present in plants, bacteria, and fungi (Williams et al. 2000; Dill et al. 2010; CASAFE 2015). However, studies on glyphosate and its commercial formulations reveal numerous morphological,

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physiological and biochemical disturbances in cells and organisms (Chłopecka et al. 2014). Different authors have studied the pro-oxidant effect of RU in animals (Costa et al. 2008; Cattaneo et al. 2011; Glusczak et al. 2007), and its genotoxic capacity and lethality in species of mammals, fish, amphibians, and reptiles (Bolognesi et al. 1997; Hook and Lee 2004; Relyea 2005; Sparling et al. 2006; Cavalcante et al. 2010). 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 International Agency for Research on Cancer, 2015) has classified Glyphosate into the Group 2A (*probably carcinogenic to humans*). Because of its high water solubility and its adherence to soil particles, this compound is associated to drift phenomena and has been reported in superficial water, sediments and soil samples in several environments in Argentina (Peruzzo et al. 2008; Aparicio et al. 2013; Ronco et al. 2016).

In this country, one of the species threatened by the loss of habitat for agricultural activities and the associated increase use of pesticides is *Caiman latirostris* (broad-snouted caiman, Crocodylia, Alligatoridae; Larriera et al. 2008). This is one of the two crocodilian species living in this country; its breeding season takes place during spring–summer, from late October–December (mating and copulation season) to March, when hatching occurs after an incubation period of 65–80 days approximately, depending on temperature (Larriera et al. 2008). Considering their biological and ecological characteristics, caimans can be exposed to contaminants in all life stages. These compounds can pass through the eggshell during incubation, being the embryos and neonates in particular risk (Poletta et al. 2009). Furthermore, contaminants accumulated by females can be transferred to the embryos through the egg yolk, affecting *in ovo* development. At the same time, juveniles and adults can be exposed to contaminants by trophic transfer (Russell et al. 1999) and via water and sediments (Ortiz-Santaliestra and Egea-Serrano 2013). The main problem that some of its wild populations are suffering is because of the temporal coincidence of its breeding season with the period of maximum application of pesticides, implying a contamination risk particularly important for developing embryos and neonates. Considerable scientific evidence demonstrates that early life stages of oviparous organisms often exhibit a greater toxicological sensitivity to chemical contaminants than adult life stages. Environmental biomonitoring make use of "sentinel" organisms living in their natural habitat and reflecting long-term, continuous exposure. Because of all these characteristics, this species has been used as a sentinel of pesticide contamination during the past years (Poletta et al. 2009, 2011; Latorre et al. 2012; López González et al. 2013; Siroski et al. under review).

Different studies have demonstrated the effect of a variety of pesticides during embryo development and its possible stage-dependent effect (Bentivegna and Piatkowski 1998; Howe et al. 2004; Aronzon et al. 2009; Mensah et al. 2011), but there is no information concerning this for any compound in *C. latirotris.*

In previous works, we reported genotoxic effect of RU through the micronucleus (MN) test and Comet assay (CA) on *C. latirostris* neonates after *in ovo* exposure by topical application on the eggshell, at the beginning of incubation period (Poletta et al. 2009). Likewise, genetic, enzymatic, and developmental alterations were observed in a semi-natural experiment after spraying of RU alone and in a mixture with endosulfan and cypermethrin formulations on nests (Poletta et al. 2011).

Considering our previous findings, the aim of this study was to determine the stage-dependent genotoxic effect of glyphosate-based formulation Roundup® through the CA, MN test, and NA test, in *C. latirostris* embryos exposed at three different stages of development (beginning, middle, and end).

Materials and Methods

Chemicals

Roundup® Full II formulation (66.2 % glyphosate) was obtained by courtesy of Agroservicios Humboldt, Santa Fe, Argentina. Roundup® Full II is a liquid water-soluble herbicide, containing glyphosate potassium salt [*N*-(phosphonomethyl) glycine monopotassium salt, C3H7KNO5P] as its active ingredient (a.i.) (CAS No. 70901-12-1). Roundup® is a registered trademark of Monsanto Company. Dimethyl sulfoxide (DMSO; CAS 67-68-5) and Cyclophosphamide (CP; CAS 6055-19-2) were purchased from Fluka. CP is an indirect alkylating agent wellknown as a genotoxic substance and was used as an in vivo positive control. Low melting point agarose (LMP; CAS 9012-36-6, A 9414), normal melting point agarose (NMP; CAS 9012-36-6, A 9539), and general laboratory reagents were provided by Sigma.

Experimental Design and Treatments

One hundred thirty eggs (average weight 70 g) from five nests of the "Proyecto Yacare" Ranching Program, Santa Fe, Argentina, were used. They were equally and randomly distributed in 13 experimental groups of 10 eggs each: 3 for each effective concentration of Roundup® determined in a

previous study (750, 1250, 1750 µg/egg) (Poletta et al. 2009), three exposed to CP as a positive control (700 µg/egg) (Poletta et al. 2009), and a negative control (NC) without exposure, treated with distilled water (Table 1).

The incubation period of this species is approximately 70 ± 3 days at 31 ± 1 °C. To evaluate the sensitivity of embryos at different developmental stages, the total incubation period was divided into three stages of approximately 23 days each (first, second, and third stages) and each concentration of RU and CP was applied by triplicate, at the beginning of these stages. Each group received the corresponding RU concentration or CP by topical application on the eggshell, at a final volume of 50 µl, diluted in distilled water (Table 1) (Poletta et al. 2009). Eggs were incubated in an artificial incubator, under controlled conditions: 31 ± 1 °C and 90 % humidity. Each single replicate of each treatment was placed separately in a plastic container (10 eggs), using vermiculite as substrate and covering them with vegetal material similar to the nesting material and free of any exogenous substance. Eggs were controlled periodically during the experiments to identify and discard those that became nonviable.

When hatchlings started to call within the eggs, they were removed from the incubator and, if hatching did not occur spontaneously, they were assisted within the following 72 h (Larriera et al. 2008). Immediately after hatching, blood samples were obtained (0.5 ml) from the spinal vein (Olson et al. 1977) with heparinized disposable syringes. After that individuals 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). The MN and NA tests and the CA were applied on peripheral blood erythrocytes as determined previously by our research group (Poletta et al. 2008; López González et al. 2013).

For MN and NA tests two smears were prepared from each animal and were stained with Acridine Orange (AO). The frequency of MN (FMN) and NA (FNA) were manually scored using a fluorescent microscope (Mikoba S350) equipped with a U-RFLT 50 excitation filter at 400×, analyzing 1000 erythrocytes for each individual, in two replicated slides (Poletta et al. 2008). The criteria adopted for MN identification was based on Poletta et al. (2008) for this species 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 (Fig. 1). All the other abnormalities observed in the nucleus characteristics including nuclear evaginations or *buds*, binucleated and excentric cells, among others, were classified together as NAs according Carrasco et al. (1990).

Regarding CA, cell viability was determined before its application by fluorescent DNA-binding dyes. A cell suspension was mixed with a dye-mix working solution of 100

Table 1 Experimental groups and treatments applied at three different stages (S1; S2; S3) of *C. latirostris* development

Experimental group	Treatment	Concentration (µg/egg)	Stage of exposure	No. of eggs exposed/clutch	No. of eggs exposed/stage	Total no. of eggs exposed/concentration
Negative control	Vehicle (distilled water)	0		2	–	10
Positive control	Cyclophosphamide	700				
CPS1			Beginning	2	10	30
CPS2			Middle	2	10	
CPS3			End	2	10	
RU750	Roundup®	750				
RU750S1			Beginning	2	10	30
RU750S2			Middle	2	10	
RU750S3			End	2	10	
RU1250	Roundup®	1250				
RU1250S1			Beginning	2	10	30
RU1250S2			Middle	2	10	
RU1250S3			End	2	10	
RU1750	Roundup®	1750				
RU1750S1			Beginning	2	10	30
RU1750S2			Middle	2	10	
RU1750S3			End	2	10	

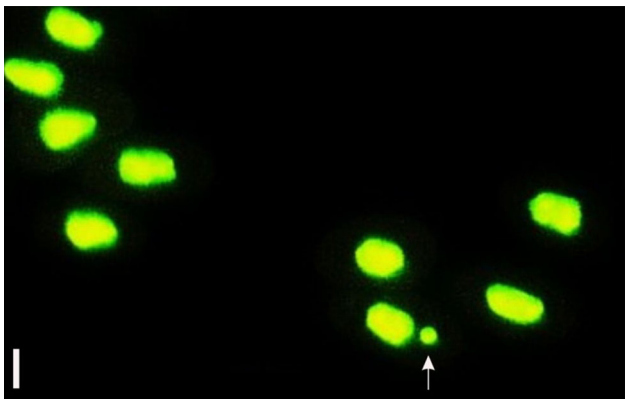

Fig. 1 *Caiman latirostris* erythrocyte showing a MN (*arrow*). *Bar* = 10 μm

μg/ml AO and 100 μg/ml ethidium bromide EB, prepared in Ca2+- and Mg2+-free phosphate buffered saline (PBS) and then examined under a fluorescent microscope (100×). A total of 100 cells were counted per sample and the percentage of viable cells was determined (Mercille and Massie 1994).

The alkaline Comet assay was performed as described by Poletta et al. (2008) for *C. latirostris* erythrocytes. Briefly, blood samples were diluted 1:19 (v/v) with RPMI-1640 medium and 1.5 μl of the dilution (4.0×10^3 erythrocytes, approximately) were used to prepared each of two slides per blood sample, following standard protocol. Then, slides were immersed in lysis buffer for 24 h, incubated in alkaline buffer for 10 min, and electrophoresed at 300 mA and 25 V (0.90 V/cm) during 15 min (Poletta et al. 2008).

All samples were coded for blind analysis, the slides were stained with AO and comet images were analyzed under the fluorescent microscope previously described. Images of 100 randomly selected nucleoids (50 from each of two replicated slides) were scored from each animal, and they were visually classified into five arbitrary classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4; Fig. 2). A single DNA damage score (Damage index, DI = $n1 + 2\ n2 + 3\ n3 + 4\ n4$) was calculated for each animal (Poletta et al. 2008).

Statistical Analysis

Mean values ± standard error of MN, NA, and DI were calculated from data of all animals of each experimental group. Statistical analysis was performed using the software SPSS 14.0 for Windows (2005). Variables were tested for normality with Kolmogorov–Smirnov test and homogeneity of variances between groups was verified by Levene test. A factorial ANOVA was applied to analyze simultaneously the effect of concentration and stage treatments, as well as the interaction between them, on DI, FMN, and FNA.

Depending on the assumption of homoscedasticity, either a Tukey or Games-Howell post hoc test was used. A difference of $p < 0.05$ was considered statistically significant.

Results

There were differences in DI between groups exposed to CP and to the different RU concentrations with the NC ($p < 0.05$), but no differences were found among the other groups ($p > 0.05$). A similar damage was observed at the three stages of each treatment ($p > 0.05$), indicating that both RU and CP produce DNA damage on *C. latirostris* embryos independently of the stage of development where the exposure occurs (Fig. 3). There was no differences in the frequency of MN (Table 2) or NA (Table 3) among the different treatment groups ($p > 0.05$), nor among the different stages within each treatment. No interaction was observed between treatment and stage for any of the biomarkers analyzed ($p > 0.05$). We found no differences in the size of animals among the different experimental groups at the moment of hatching ($p > 0.05$).

Discussion

In this study, we found significantly higher DNA damage in embryos exposed to the three concentrations of RU tested, compared with the NC, but no differences among the three stages of exposure. These results indicate that RU produces genotoxicity at the beginning, middle, and end of embryo development, which could be considered as evidence that the genotoxic effect is produced regardless of the moment in which exposure occurs. In their natural environment, embryos may receive pesticides during the whole developmental period as it take place during the moment of the year of maximum pesticides application rate, implying a higher probability of exposure. In studies conducted in agricultural environments in Argentina, values of glyphosate found in sediments and soils were from 1500 to 5000 μg/Kg after one application (Peruzzo et al. 2008; Aparicio et al. 2013) and recently similar values were found in sediments of the Saladillo and Lujan rivers, two tributaries of the Parana river, in central east of Argentina (Ronco et al. 2016). If this amount is applied on an egg of 70 g (in average, it would be approximately equivalent to 100–350 μg applied to an egg). Even when these concentrations are lower than those tested in the present study, it must be considered that caiman nests may receive repeated exposure to this and other compounds, as

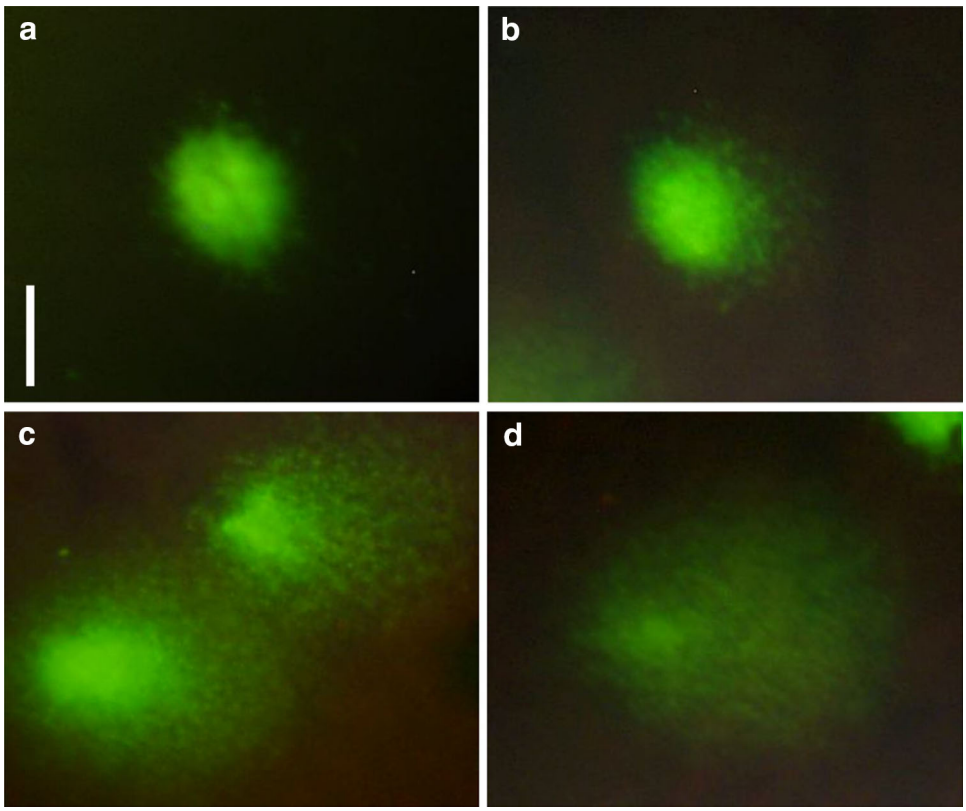

Fig. 2 Comet images of different damage classes obtained from peripheral blood erythrocytes. **a** Class 1, **b** class 2, **c** class 3, **d** class 4. *Bar* = 10 μm

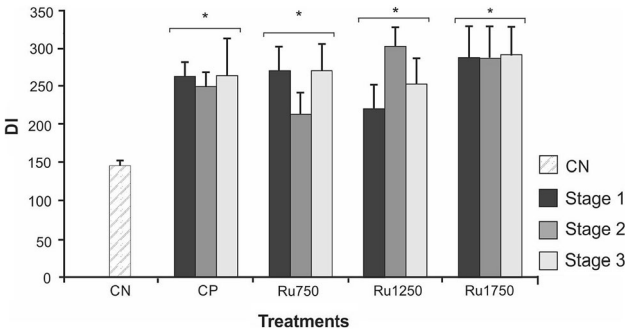

Fig. 3 DI of each experimental group (mean ± standard error) at three different stages of exposure. *Statistically significant compared with NC (factorial ANOVA, $p < 0.05$)

physiological processes in this species, with serious consequences at cellular, individual, and population level, especially under conditions of continuous environmental exposure. In a study by our group, we observed that embryos environmentally exposed to pesticides showed a delay in the time needed to repair the damage (Poletta et al. 2013).

Others studies that evaluated the effects of toxic compounds on developing organism found differences in the effects depending on the stage of development, suggesting that this was a consequence of differences in the capacity of DNA repair processes. Hook and Lee (2004) reported

Table 2 MN frequencies observed in *C. latirostris*

Treatments	Stage 1 (MN/1000 cells)	Stage 2 (MN/1000 cells)	Stage 3 (MN/1000 cells)
Negative control	0.86 ± 0.4		
Positive control	1 ± 0.58	0.83 ± 0.83	1.33 ± 0.8
Treatment 1 (RU750)	0.75 ± 0.25	1.33 ± 0.44	1.25 ± 0.62
Treatment 2 (RU1250)	2 ± 1.24	1.43 ± 0.57	1.22 ± 0.75
Treatment 3 (RU1750)	0.67 ± 0.49	2.25 ± 1.65	1.4 ± 0.98

Values expressed as mean ± standard error

pesticide spraying is conducted repeatedly on crops even as preventive action. The final fate of the alterations observed is uncertain, but they could affect the normal function of

that grass shrimp (*Palaemonetes pugio*) embryos exposed to various concentrations of benzo[α]pyrene (BαP), Cr (VI), and hydrogen peroxide showed a lower capacity to

Table 3 NA frequencies observed in *C. latirostris*

Treatments	Stage 1 (NA/1000 cells)	Stage 2 (NA/1000 cells)	Stage 3 (NA/1000 cells)
Negative control	90 ± 20.79		
Positive control	95.5 ± 6.01	115.17 ± 20.84	115.17 ± 19.08
Treatment 1 (RU750)	102.5 ± 8.92	103.11 ± 16.26	115.63 ± 24.28
Treatment 2 (RU1250)	106.17 ± 24.2	91.29 ± 13.75	122.8 ± 24.81
Treatment 3 (RU1750)	78 ± 5.27	110.5 ± 22.87	112.2 ± 28.56

Values expressed as mean ± standard error

repair DNA strand breaks, determined by the CA, at early-stage compare with later-stage embryos, which may contribute to the high sensitivity of early embryos to genotoxic compounds. Osman et al. (2007) studied the genotoxic effect quantified by the CA of three concentrations of lead nitrate in African catfish (*Clarias gariepinus*) embryos and larvae exposed at different times post-fertilization. Significant dose-related DNA damage response was observed in embryos exposed after 96 h post-fertilization, but after 144 h no remarkable genotoxic increase was observed at higher concentrations. The authors suggest that DNA repair processes took place after 144 h post-fertilization, preventing further DNA damage. Considering those results, it could be supposed that the enzymatic repair mechanisms in *C. latirostris* embryos are still immature and therefore deficient during all its development. Further efforts should be devoted in order to assess the DNA repair capacity of *C. latirostris*.

Exposure during critical stages of development can have severe and permanent consequences that can be evident much later in life (Hamlin and Guillette 2011). Caimans exposed to pesticides during the embryological stage showed developmental abnormalities, affected gonadal differentiation and, unlike the results obtained in our study, decreased hatchlings weight (Milnes et al. 2004; Milnes et al. 2005; Beldomenico et al. 2007).

Pesticides evaluated in those studies were organochlorines known to be highly toxic with potential to decrease hatchling weight, which is not altered by GLY based-pesticides.

In the present work, while the comet assay showed a positive response following RU exposure, the MN and NA test did not indicate any genotoxic effect of RU in *C. latirostris* embryos. Differences in the results found by the three techniques applied (CA, MN, and NA) can be explained by the endpoints they evaluated. While MN registers alterations at chromosomal level and NA are considered complementary to it, CA identifies single strand breaks and maximizes the expression of alkali-labile sites in the DNA molecule. Therefore, CA is more sensitive than MN test to detect damage at DNA level (Mudry and Carballo 2006).

Previously, we demonstrated a significant increase in both the MN frequency and DNA damage at 500–1750 μg/egg, compared with controls, as well as a concentration-dependent effect, after *in ovo* exposure to RU at the beginning of caiman development (Poletta et al. 2009). These results coincide with those obtained for CA in the present study at any of the three stages of development tested, but without a dose-dependent effect. The observation of similar results in two independent experiments is an evidence of DNA damage caused by RU on *C. latirostris* embryos and their supposed inefficient DNA repair mechanisms, as suggested by Hook and Lee (2004) and Osman et al. (2007) in other species. However, the lack of a dose dependent effect in DNA damage in the present study is a difference that must be taking into account. On the other hand, differences observed in the MN results of RU exposed groups between the two works could be explained by a higher susceptibility of the clutches used in the previous work or by the less sensitivity of the MN and NA tests to detect damage at these concentrations. Similar to the present study, the positive control alkylating agent Cyclophosphamide induced a significant difference compared to the NC in the CA in all assays conducted in two different reptile species, the broad snouted caiman and the tegu lizard (Poletta et al. 2009; Schaumburg et al. 2016). In the case of the MN and NA test, results were less consistent, showing positive results in some works (Poletta et al. 2009; López González et al. under review) but not in others (Schaumburg et al. 2016), as it was observed in the present study. A possible explanation to this could be the mechanism of action of this compound in relation to the kind of damage these biomarkers detect. The mechanisms of action of CP, as an alkylating agent, generally induced DNA fragmentation, a kind of damage detected by the CA, but rather frequently this may lead to clastogenic or aneugenic effects that are recognized by the MN or NA tests.

It is surprising the lack of information about the metabolic consequences in animal development after or during the exposure to different genotoxic compounds. This approach encourages further investigations to understand the final consequence of these alterations in *C. latirotris* as a sentinel of environmental pesticide contamination.

Conclusions

Exposure of *C. latirostris* embryos to different concentrations of RU produces genotoxicity demonstrated by the CA, independently of the moment of development in which exposure occurs. This could be a consequence of immaturity and deficiency of the repair mechanisms in *C. latirostris* embryos during development. The CA demonstrated to be more sensitive than MN or NA test to assess the genotoxic effects of RU formulations under the experimental conditions tested in the present study.

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Compliance with Ethical Standards

Conflict of interest Authors declare that they have no conflict of interest.

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