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Application of the micronucleus test and comet assay in *Trachemys callirostris* erythrocytes as a model for *in situ* genotoxic monitoring

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ABSTRACT

Trachemys callirostris is a turtle species endemic to northern South America. In northern Colombia it occurs in the middle and lower Magdalena River drainage and its principal tributaries (lower Cauca and San Jorge rivers) and in other minor drainages such as the lower Sinú River. In recent years, industrial, agricultural, and mining activities have altered natural habitats in Colombia where this species occurs, and many of the pollutants released there are known to induce genetic alterations in wildlife species. The micronucleus test and comet assay are two of the most widely used methods to characterize DNA damage induced by physical and chemical agents in wildlife species, but have not been employed previously for genotoxic evaluations in *T. callirostris*. The goal of this study was to optimize these genotoxic biomarkers for *T. callirostris* erythrocytes in order to establish levels of DNA damage in this species and thereby evaluate its potential as a sentinel species for monitoring genotoxic effects in freshwater environments in northern Colombia. Both genotoxic techniques were applied on peripheral blood erythrocytes from 20 captive-reared *T. callirostris* individuals as a negative control, as well as from samples obtained from 49 individuals collected in Magangué (Magdalena River drainage) and 24 individuals collected in Lorica (Sinú River drainage) in northern Colombia. Negative control individuals exhibited a baseline frequency of micronuclei of 0.78 ± 0.58 and baseline values for comet tail length and tail moment of 3.34 ± 0.24 μm and 10.70 ± 5.5, respectively. In contrast, samples from both field sites exhibited significantly greater evidence of genotoxic effects for both tests. The mean MN frequencies in the samples from Magangué and Lorica were 8.04 ± 7.08 and 12.19 ± 12.94, respectively. The mean tail length for samples from Magangué and Lorica were 5.78 ± 3.18 and 15.46 ± 7.39, respectively. Finally, the mean tail moment for samples from Magangué and Lorica were 23.59 ± 18.22 and 297.94 ± 242.18, respectively. The frequency of micronuclei in the samples was positively related to comet tail length and tail moment. Thus, this study showed that both genotoxicity biomarkers may be applied to *T. callirostris* erythrocytes as a sentinel organism for assessing the effects of environmental pollutants in freshwater ecosystems in northern South America.

1. Introduction

Chemical contamination of the environment has been implicated in the decline or disappearance of many natural populations of reptiles (Bickham et al., 2000; Campbell and Campbell, 2002; Gibbons et al., 2000). Reptiles often are closely linked to a particular habitat, making them more vulnerable to habitat degradation compared to birds and mammals (Hopkins, 2000). Although reptiles have proven to be excellent bioindicators of environmental contamination (Mitchelmore et al., 2006), they remain the group of vertebrates less studied in terms of genetic toxicology.

Even though turtles are important components of their ecosystems, they are often not included in environmental contamination studies or ecological risk assessments, with few studies employing freshwater turtles as biomonitors of chemical contaminants (Meyers-Schöne et al., 1993; Meyers-Schöne and Walton, 1994; Lamb et al., 1991, 1995). With their omnivorous diet, mid to high position in the food chain, long life spans, relatively sedentary life styles and high site fidelity, freshwater turtles are exposed to many sources of contamination and are ideal for monitoring of riparian zones.

The loss of a freshwater turtle population due to the effects of environmental contamination may have repercussions throughout the ecosystem. For example, the presence or absence of *Trachemys scripta elegans* has been shown to affect ecosystem functioning by

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altering processes and modifying different environmental variables (Lindsay et al., 2013). The loss of freshwater turtle species in pond ecosystems also may affect productivity due to decreased amounts of nutrients provided by the turtles directly, and also through the influence of their bottom activities resuspending sediments. Lindsay et al. (2013) suggested that freshwater turtles influence pond ecosystem function and trophic ecology by increasing resource availability for invertebrate communities.

The Colombian slider turtle (*Trachemys callirostris*, or *Trachemys ornata callirostris*, *sensu* Fritz et al. (2012)) is a moderate-sized freshwater specie (straight-line carapace length up to 33 cm) endemic to northern South America. The species is classified as Vulnerable in Colombia (Bock et al., 2015) due to the levels of hunting pressure and habitat loss it faces. In northern Colombia, it occurs in the middle and lower Magdalena River drainage and its principal tributaries (lower Cauca and San Jorge rivers), and in other minor drainages such as the lower Sinú River (Bock et al., 2010, 2015). In these rivers, high concentrations of mercury and other heavy metals have been reported, often at levels that exceed internationally permissible values (Mancera-Rodríquez and Álvarez-León, 2006). These pollutants can form complex mixtures that enter these aquatic ecosystems through discharges of industrial and municipal wastes.

Some contaminants (polycyclic aromatic and halogenated hydrocarbons, heavy metals, herbicides and solvents) can act as genotoxicants, inducing genetic alterations in the integrity and functioning of the DNA of wildlife species, but little is known about the exact mechanisms (Crespo-López et al., 2009; Hartwig, 1995; Léonard et al., 1983; Valko et al., 2005).

Evidence of genetic damage is a useful biomarker employed as an effective early warning tool in ecological risk assessments and environment monitoring (Park and Choi, 2007). The loss of DNA integrity can cause genotoxic effects, such as DNA base modifications, strand breaks, depurination and cross-linkages (Frenzilli et al., 2009; Jha, 2008; Lee and Steinert, 2003; Shugart, 2000, 1990; Shugart and Theodorakis, 1998). These types of damage have been associated with carcinogenesis and negative effects on fecundity, longevity, and growth rates in affected organisms that may have important demographic repercussions (Lee and Steinert, 2003; Theodorakis et al., 2000).

The Micronucleus (MN) test and the Comet assay (CA; or Single Cell Gel Electrophoresis, SCGE) are two sensitive, rapid and extensively used methods applied as genotoxic biomarkers for wildlife species. They allow characterization of DNA damage induced by physical and chemical agents due to their ability to detect chromosomal and DNA damage at an early stage (Jha, 2008). They have the advantages of being applicable for any nucleated cell type and requiring only small sample sizes (Ali et al., 2008, 2009; Carballo and Mudry, 2006; Dhawan et al., 2009; Jha, 2004).

The MN test is one of the biomarkers most widely used for in situ monitoring of genotoxic pollution in natural environments (Al-Sabti, 1994, 1995; Al-Sabti and Metcalfe, 1995; Bolognesi et al., 2006; Schaumburg et al., 2012; Strunjak-Perovic et al., 2010; Udroiu, 2006). This technique is based on quantification of whole chromosomes or fragmented chromosomes that are not incorporated into the main nucleus during mitosis due to aneugenic or clastogenic effects (Al-Sabti and Metcalfe, 1995). The CA is also an indicator of genotoxicity and an effective biomarker for detecting DNA strand breaks, cross-links and alkali-labile sites in aquatic organisms (Frenzilli et al., 2009; Tice et al., 2000). The advantages of the CA include the relative ease of application to most eukaryotic cell tissue types, its sensitivity for detecting low levels of DNA damage (1 break per 1010 Da of DNA), the detection of multiple classes of DNA damage with a small number of cells, and the generation of single-cell data (Da Rocha et al., 2009; Dhawan et al., 2009; Frenzilli et al., 2009; Lee and Steinert, 2003; Mitchelmore and Chipman, 1998; Tice et al., 2000).

Although the two methods measure different endpoints of damage, both tests generate complementary information and may be used for the simultaneous assessment of DNA damage in many tissues from the same individual and for the comparison of their responses under identical treatment conditions. Thus, these techniques may be used in combination for screening genotoxic effects of environmental pollutants and for investigating the implications of DNA damage in sentinel species.

Despite widespread use of both techniques for detection of genotoxic and mutagenic effects caused by chemicals and pollutants in fish (Al-Sabti, 1994, 1995; Al-Sabti and Metcalfe, 1995; Bolognesi et al., 2006; Çavaş, 2008; Crespo-López et al., 2011; Lee and Steinert, 2003; Palacio-Betancur et al., 2009; Peñalosa et al., 2003), few studies have applied the MN and CA methods to establish spontaneous and baseline values of DNA damage in reptile species such as lizards (*Tupinambis merianae*, *Iguana iguana, Ctenosaura pectinata*), crocodilians (*Caiman latirostris*), snakes (*Hierophis gemonensis, Mastigophis flagellum, Pituophis deppei, Boa constrictor, Lampropeltis* sp., *Python reticulatus, Crotalus molossus, Bothrops* sp.) and turtles (*Caretta caretta, Macrochelys temminckii*, *Kinosternon subrubrum*) (Caliani et al., 2014; Poletta et al., 2008, 2009; Schaumburg et al., 2012; Zúñiga-González et al., 2001, 2000). Also, some of these studies have determined concentration- and time-dependent genotoxic effects induced by physical or chemical agents in reptiles by employing both tests (Capriglione et al., 2011; Poletta et al., 2009; Schaumburg et al., 2010), confirming the usefulness of these species as good indicators of genotoxic activity.

Turtle species are often used as indicators of pollution levels by quantifying the accumulation of substances in their tissues, but only a few reports have assessed genotoxic effects through the application of the MN test or CA on erythrocytes of species inhabiting contaminated environments. *Trachemys scripta* and *Chelvdra serpentina* showed a genotoxic response with the CA of samples obtained from contaminated sites compared to a reference site, indicating these species are useful sentinels for genotoxic monitoring of environmental contamination (Meyers-Schöne et al., 1993). Also, *Emys orbicularis* and *Mauremys caspica* showed an increase in MN frequency in a contaminated site compared to a reference site (Matson et al., 2005).

Baseline levels of DNA damage vary depending on the study species and cell type, making it necessary to determine the optimal conditions for both tests. In turtles, the MN test and CA are usually performed in blood samples due to ease of sampling and a composition of 97% nucleated erythrocytes (Fossi et al., 1994; Clark et al., 2000; Mitchelmore and Chipman, 1998). The goal of this study was to optimize these two genotoxic biomarkers by developing protocols for *T. callirostris* erythrocytes in order to determine baseline values of DNA damage in this species and evaluate its potential as a sentinel species for monitoring genotoxic effects in freshwater environments in northern Colombia. The second goal of the study was to compare values obtained from two areas with different contamination sources.

2. Material and methods

2.1. Sample collection and preparation

Magangué is a city in the Bolivar Department in northern Colombia, located on the Magdalena River (9°13′N, 74°46′O) that drains a major portion of the country. The Magdalena receives input from the San Jorge, Cesar, and Cauca rivers in the northern lowlands that produces a complex floodplain of wetlands. Santa Cruz de Lorica, also known as Lorica, is a municipality of the

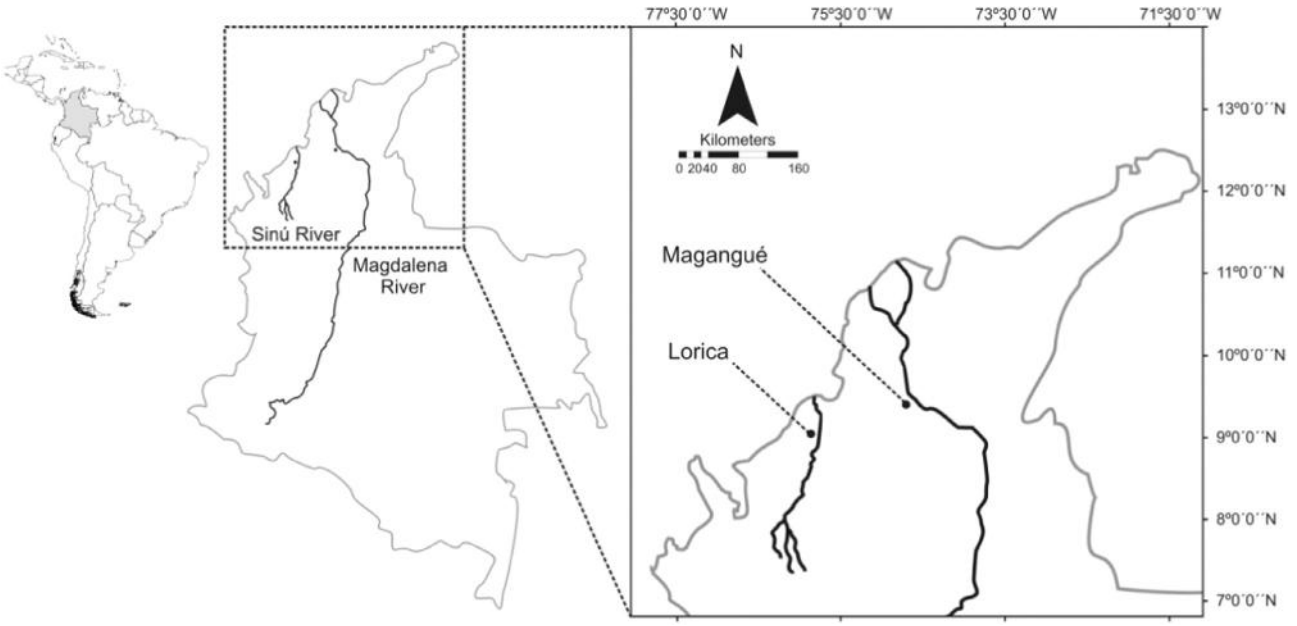

Fig. 1. Sampling site locations (black points) for *T. callirostris* individuals in Magangué (located along the Magdalena River) and Lorica (located along the Sinú River).

Córdoba Department (09°14′N, 75°48′W) in northern Colombia and is located on the lower portion of the Sinú River (Fig. 1).

These two sites were visited during the peak of the *T. callirostris* nesting season from February to May 2012. Samples collected in this study were part of a project that sought to assess mercury contamination in *T. callirostris* individuals. For this, samples from turtles at both sites were obtained directly from subsistence fishermen or at local marketplaces at the time the individuals were sacrificed for human consumption. All turtles from these two field sites were sacrificed within two days of their capture. Blood samples were collected from 49 individuals from Magangué and 24 individuals from Lorica. In addition, 20 *T. callirostris* adults from the Santa Fé Zoological Park in Medellín (Antioquia, Colombia) were used as a negative control group. These turtles had been confiscated from the illegal pet trade and had spent a minimum of three months in the zoo environment prior to sampling. Only individuals deemed to be in good physical condition by the zoo veterinarian were included in the sample.

Each individual was measured for straight-line carapace length (SCL), with median values of 28.33 ± 4.93 cm for the control negative group and 22.02 ± 4.41 cm and 22.77 ± 4.09 cm for individuals from Magangué and Lorica, respectively. Male and female *T. callirostris* mature sexually at different body sizes (Cortés-Duque, 2009), and many of the smaller turtles did not yet show secondary sexual characteristics useful for sexing adults, so gender was not included in the statistical analyses.

Peripheral blood samples (1 ml) were obtained from the cervical sinus of all turtles, as described by Olson et al. (1977), using heparinized syringes. At an initial phase of this study, blood samples from three turtles from the zoo were transported and stored (at 4 °C) mimicking the possible field conditions and analyzed within 24 and 48 h after the time of collection in order to study the effects of transportation and storage. There were no significant differences in MN frequency and comet tail length between both times (ANOVA, $p > 0.05$). For this, all field samples were immediately stored and transport at 4 °C until their analysis. All data samples from negative control group were analyzed within 24 h. Samples from Magangué were analyzed within 48 h. Due to greater difficulty in transporting samples from Lorica, samples from this site were analyzed 72 h after being obtained from those individuals.

2.2. Micronucleus (MN) test

Blood was smeared onto two clean glass slides, air dried for 48 h, and then fixed in absolute methanol for 10 min. After fixing, the same slides were stained in 10% Giemsa for 10 min. The Giemsa solution was centrifuged and filtered before staining to reduce precipitation that could interfere with the analyses. MN were identified and scored with an Olympus microscope (at $1000\times$ magnification). Two thousand erythrocytes were scored for each specimen (1000/slide) to determine the frequency of micronucleated erythrocytes. For scoring of MN, we adopted the criteria in Al-Sabti and Metcalfe (1995): MN had to be smaller than one-third of the main nuclei, clearly separated from the main nuclei, and had to be non-refractive small nuclei ($> 1/3$ of the main nucleus) with intact cytoplasm (Hooftmanand de Raat, 1982). The frequency of MN was calculated as: MN frequency=(number of cells containing micronuclei $\times$ 1000)/total number of cells counted.

2.3. Comet assay (CA)

Since there were no previous reports of the application of the CA in *T. callirostris*, we had to test critical parameters for this assay, such as cell suspension, unwinding and electrophoresis conditions. We conducted a preliminary standardization using the general procedures based on Singh et al. (1988), with some modifications established for erythrocytes in other reptile species (Poletta et al., 2008), varying critical parameters such as unwinding and electrophoresis conditions. For this, diluted blood samples were incubated at room temperature with 25, 50 and 100 μM of H_2O_2 during 30 min to induce strand breaks *in vitro*, and Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) was used as a negative control. Based on the extent of DNA migration in control and treated cells, the optimal time duration for unwinding and electrophoresis processes were 15 and 20 min, respectively. With these protocols, we obtained the characteristic comet shape in the case of the positive control cells, and a minimum migration in the case of the negative controls. To demonstrate the electrophoresis conditions and sensitivity, and to prevent false-negative results, cells treated during 30 min with 25 μM H_2O_2 were used as positive controls for each electrophoresis run conducted, and the results

only were included in the analyses if the positive controls demonstrated positive results.

Before conducting the CA, erythrocyte viability was determined by the Trypan Blue dye exclusion technique. Blood samples were diluted in a 1:100 (v/v) ratio with Ca^{2+} and Mg^{2+} free PBS at pH 7.4, and then approximately 4000 cells were mixed with 90 µl of 0.6% low-melting-point agarose (LMP; Sigma-Aldrich, St Louis, Mo.) at 37 °C. From this mixture, two replicates of 5 µl drops were layered on Gelbond® Films (Lonza, Basel, Switzerland) and allowed to solidify at 4 °C for 10 min (without coverslips). Each Gelbond film was incubated in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, with freshly added 1% Triton X-100 and 10% DMSO) for at least 1 h at 4 °C in the dark. After lysing, the Gelbond film was gently washed and then carefully placed in a horizontal electrophoresis chamber filled with freshly prepared cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 15 min to allow DNA unwinding. Immediately after unwinding, electrophoresis was conducted in the same buffer at 25 V (0.8 V/cm) and 300 mA at 4 °C for 30 min. After electrophoresis, the Gelbond film was washed three times for 5 min with a neutralization buffer (0.4 M Tris–HCl, pH 7.5). Subsequently, the Gelbond films were neutralized with pH 7.5 buffer (0.4 M Tris–HCl) with three changes every 5 min, air-dried, fixed in absolute ethanol for 10 min and stored for later scoring. Samples were soaked for 5 min in dH_2O and then dyed with 100 µL of ethidium bromide (20 µg/mL) and visualized under a fluorescence microscope (BOECO, BM-180; at 400 × magnification) equipped with a 420–490 nm excitation filter and a 520 nm barrier filter. DNA damage in 100 randomly selected cells per individual (two replicates of 50 cells) was scored using CASP software (public domain) (Końca et al., 2003). Apoptotic cells that showed a totally fragmented nucleus, or "clouds", were not scored. The parameters considered for quantification of the extent of DNA damage were tail length and tail moment (Frieauff et al.,2001; McKelvey-Martin et al., 1993). In addition, the cells were classified according to tail length into five classes ranging from undamaged (0) to maximally damaged (4), yielding a DNA damage score (Damage Index, DI) calculated as follows: $DI = n1 + 2n2 + 3n3 + 4n4$, where: $n1$, $n2$, $n3$ and $n4$ are the number of cells in class 1, 2, 3 and 4 of damage, respectively (Gedik et al., 1992). A measure of tail length, tail moment and DI was obtained for each turtle (data not shown) and averaged for each group.

2.4. Statistical analysis

All values were expressed as mean ± SD (standard deviation). Variables were tested for normality with Shapiro–Wilk's tests. Kruskal–Wallis tests were used to compare the MN test and CA data between sampling sites and with the negative control data. Spearman rank correlations were used to inspect for associations between the MN test and CA results, and also to examine the relation between these parameters and turtle body size. In all cases, values of $p < 0.05$ were considered statistically significant. Statistical analyses were performed using the statistical software Statgraphics Centurion.

3. Results

An image of a micronuclei observed in a blood sample is shown in Fig. 2. The mean frequency of MN cells in the negative control group was 0.78 ± 0.58 and ranged from 0 to 2 MN/1000. The mean frequency of MN in the samples from Magangué exceeded the control levels by a factor of 10, with a maximum value of 34.5 MN/1000. The mean frequency of MN in the samples from Lorica exceeded the control levels by a factor of 16, with a maximum of 45 MN/1000 (Table 1). The significant differences ($p < 0.001$) in mean frequency of MN among the groups is evident in Fig. 3. There were significant differences in mean MN frequency among the control group and both sites ($p < 0.001$), but not between the two field localities. There was no relationship between MN values and body sizes of the turtles inhabiting either field locality, or in the negative control group (all p values > 0.10).

Values of 85–100% cell viability prior to the CA indicated appropriate conditions for the use of this technique in *T. callirostris* erythrocytes, and allowed us to conclude that the DNA damage evaluated with the CA was the result of genotoxic processes rather than to degradation of the samples during transportation. The CA yielded images typical for this procedure that were classifiable into the five categories of damage according to Gedik et al. (1992).

The mean comet tail length in the negative control sample of *T. callirostris* individuals was 3.34 ± 0.24 µm, differing significantly from Magangué samples (5.78 ± 3.18 µm, $p < 0.001$) and Lorica samples (15.46 ± 7.39 µm, $p < 0.05$) (Table 1, Fig. 4). Lorica cells exhibited significantly longer tail sizes than Magangué cells ($p < 0.001$). The mean tail moment measure of DNA damage in the negative control erythorcytes of *T. callirostris* individuals was 10.70 ± 5.51, differing significantly from Lorica cells (297.94 ± 242.18, $p < 0.001$), but not from Magangué cells (23.59 ± 18.22) (Table 1, Fig. 4). Also, Lorica cells exhibited significantly higher values than Magangué cells ($p < 0.001$). The frequency of MN was positively related to tail length (Spearman's $r = 0.47$; $p < 0.05$), as was tail moment (Spearman's $r = 0.44$; $p < 0.05$). Again, there were no relationship between CA values

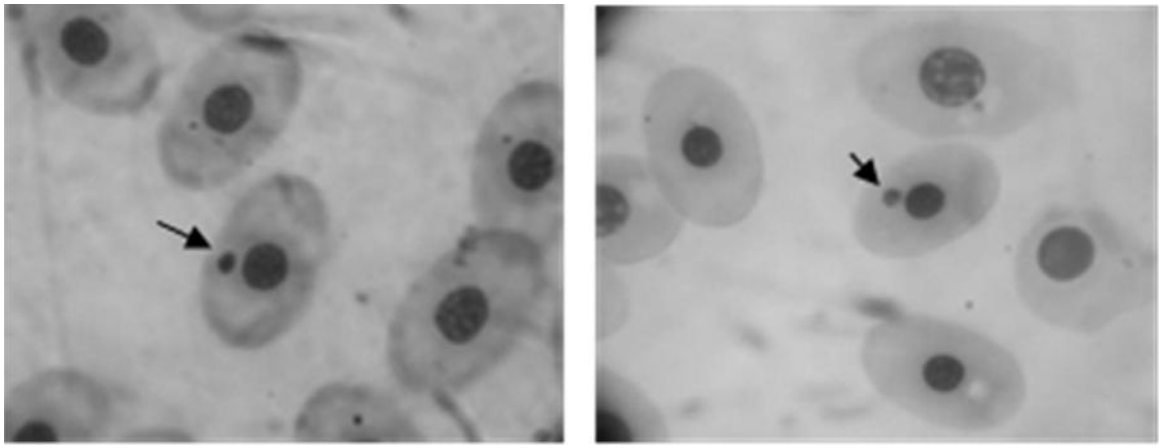

Fig. 2. *T. callirostris* blood sample with micronucleated erythrocytes (arrow) stained with Giemsa (1000 ×).

Table 1
Mean and standard deviation of body size (SCL), frequency of MN, tail length, tail moment and Damage Index of *T. callirostris* peripheral blood samples from negative control, Magangué and Lorica.

Site	*n*	SCL (cm)	Frequency of MN	Tail length (μm)	Tail moment	Damage index
Negative control	20	28.3 ± 4.93	0.78 ± 0.58	3.34 ± 0.24	10.70 ± 5.51	17.35 ± 6.71
Magangué	49	22.02 ± 4.41	8.04 ± 7.08	5.78 ± 3.18	23.59 ± 18.22	34.03 ± 12.41
Lorica	24	22.77 ± 4.09	12.19 ± 12.94	15.47 ± 7.39	297.94 ± 242.18	137.07 ± 87.04

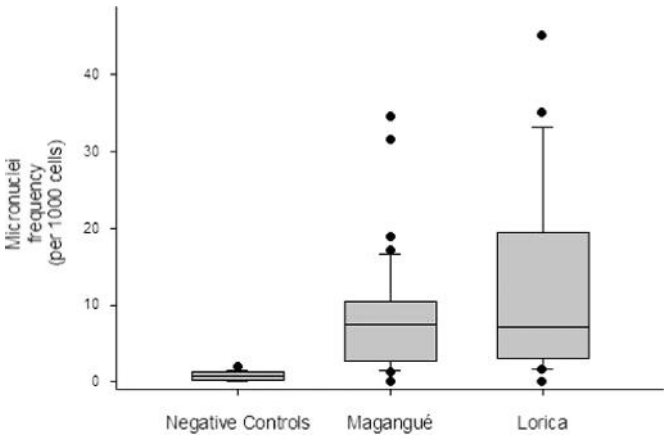

Fig. 3. Micronucleated erythrocytes (MN/1000 cells) of specimens of *T. callirostris* collected from negative controls, Magangué and Lorica.

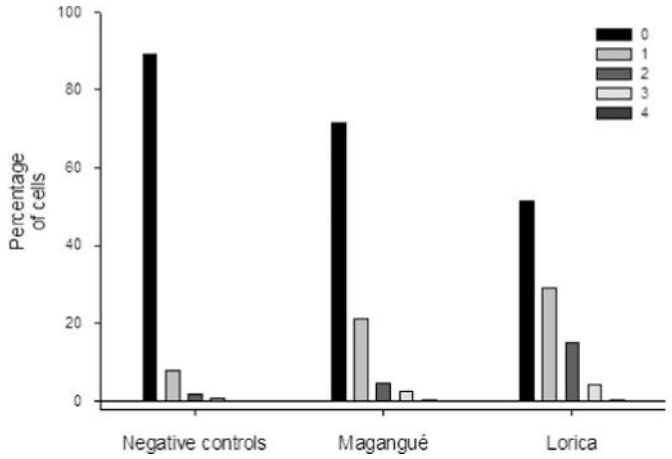

Fig. 5. Percentage of cells with different classes of genotoxic damage to their DNA (0–4) based on the erythrocytes of *T. callirostris* individuals from the negative control group and two study sites.

and the body sizes of the turtles inhabiting either field locality, or in the negative control group (all p values > 0.10).

Finally, the DI values for samples from Lorica and Magangué exceeded the DI of negative controls samples (17.35 ± 6.71) by 8 and 2 times, respectively. In turn, the DI value from the Lorica samples exceeded the Magangué samples by 8 times (Table 1).

As shown in Fig. 5, the percentage of cells with some class of damage (1–4) was significantly higher in samples from Magangué and Lorica compared to the negative controls, showing an increase in genotoxic effects for both localities. The frequency distribution of the cells within the categories of damage, from 1 to 4, was as follows: 8.01%, 1.85%, 0.78%, and 0.15% for negative control samples, 21.52%, 4.52%, 2.59%, and 0.25% for Magangué samples, and 29%, 15.01%, 4.40%, and 0.33% for Lorica samples.

4. Discussion

Genotoxicity and cytotoxicity are biomarkers useful for assessing the effects of chemicals in aquatic ecosystems, especially chemicals that are capable of affecting the health and demography of different aquatic organisms. Environmental pollutants can act selectively on sensitive phenotypes and consequently lead to a reduction in genetic diversity in an exposed population (Bickham et al., 2000; Carballo and Mudry, 2006; Geiszinger et al., 2009; Gibbons et al., 2000; Hall, 1980; Theodorakis et al., 2000). In consequence, genotoxic evaluation in wildlife species is a tool for documenting how exposure to xenobiotics may affect the health and status of natural populations (Carballo and Mudry, 2006).

Ectothermic organisms exhibit periods of decreased metabolic activity, with reduced efficiency in metabolizing or detoxifying pollutants; in consequence, reptiles such as turtles may be more sensitive to the effects of pollutants, making them more susceptible than endotherms to the presence of xenobiotics in the environment (Hall, 1980). However, reptiles have been under-represented in genotoxicological studies.

Some studies in reptiles have established baseline values of DNA damage using the MN test, focusing primarily on Squamates

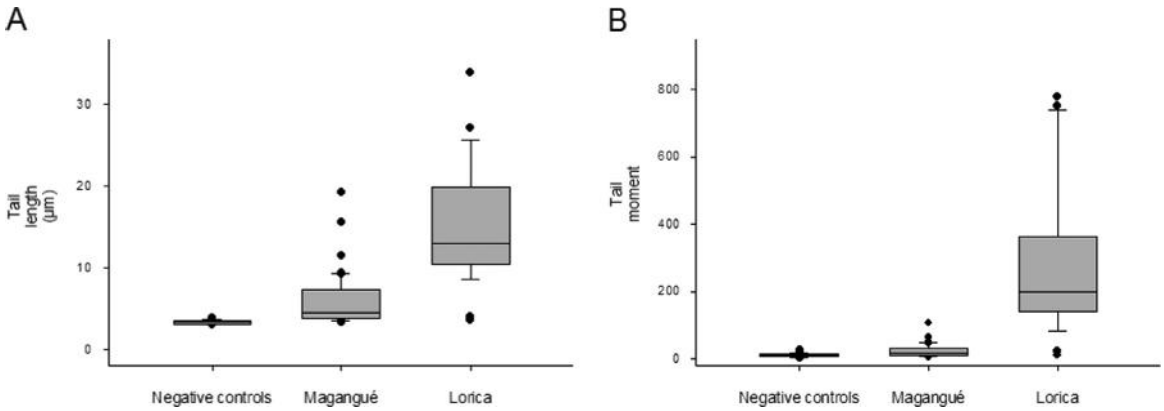

Fig. 4. Comet tail length (A) and tail moment (B) in *T. callirostris* erythrocytes from negative controls, Magangué and Lorica. There were statistically significant differences ($p < 0.05$) between each site compared to the negative controls and also between the two field sites. Bars represent standard deviations.

and Crocodylia, with values ranging from 0 to 5 MN/1000 cells and 0 to 3 MN/1000 cells, respectively (Poletta et al., 2008; Schaumburg et al., 2012; Strunjak-Perovic et al., 2010; Zúñiga-González et al., 2001). The results of MN test studies examining one specimen of *Macrochelys temminckii* and two specimens of *Kinosternon subrubrum* suggested that turtles might not be good indicators of genotoxic exposure using the MN test (Zúñiga-González et al., 2000; 2001). However, the results of this study showed a significant increase in MN frequencies in contaminated environments, with values as high as 45 MN/1000 cells compared to the control group, where MN frequencies were near 0. Matson et al. (2005) also found an increase in MN frequencies in *Emys orbicularis* individuals inhabiting contaminated environments as compared to a reference site, with mean values of 9.7 and 6 MN per 1000 red blood cells, respectively.

Species differ in their hematopoietic cycles and the time required for cell maturation, the lifespan of their erythrocytes and their capacities to eliminate damaged cells, which could influence the MN frequency found in tissues of different species (Udroiu, 2006). While micronucleated erythrocytes from the hemopoietical organs reflect genotoxic damage which occurred during a time equivalent to the cell cycle duration, those from the peripheral circulation reflect events that occurred in a time equal to the lifespan of the circulating erythrocytes (Schlegel and MacGregor, 1982). Therefore, the application of the micronucleus test on peripheral blood samples is particularly indicated for conditions of chronic exposure and ecotoxiciological studies conducted *in situ*. Nevertheless, species that have efficient systems for removing senescent or damaged cells from the blood also may not be appropriate sentinels because they tend to exhibit baseline MN frequencies near zero despite experiencing contact with xenobiotics (Corazza et al., 1990; Ramírez-Muñoz et al., 1999).

As in the MN test, the CA results showed elevated rates of DNA damage in individuals from the two study sites as compared with the control individuals. Two other turtle species, *Trachemys scripta* and *Chelydra serpentina,* have proven to be good indicators of genotoxic effects because they exhibited significant increases in DNA damage at contaminated sites compared to individuals from reference sites (Meyers-Schöne et al., 1993). Taken together, these results argue that turtles often may serve as good indicators of environmental contamination by genotoxic substances.

The effect of age on DNA repair efficiency in turtles is less known. Schwanz et al. (2011) found that erythrocytes from hatchling *Chrysemys picta* had a much higher ability to repair DNA damage caused by exposure to UV light and H_2O_2, compared to juvenile and adult individuals. These results suggest a risk to juveniles and adults who exhibit higher baseline values for DNA breaks coupled with a reduced ability to repair such damage caused by environmental pollutants. Future studies should evaluate the importance of age in *T. callirostris* individuals in terms of both their appropriateness as biosentinels and their abilities to confront stress from xenobiotics.

The evidence of genotoxic damage in *T. callirostris* is consistent with the human activities in each area. Lorica is located in the lower Sinú River drainage. Raciny et al. (1998) characterized this river from Monteria to Lorica as an area with serious environmental pollution problems due to discharges along its banks of garbage from human settlements, agricultural wastes, sand mining activities and cattle pastures bordering the shores of the river. In addition, discharges from mining of ferronickel, chromium, and gold aggravate the pollution problems in this area.

Open-cast coal extraction is another activity in this region that causes environmental contamination (Bian et al., 2010), producing a heterogeneous mixture of pollutants (Leffa et al., 2010) including hydrocarbons and heavy metals such as nickel, chromium, mercury and cadmium, that all have genotoxic potential (Bian et al., 2010; Celik et al., 2007; Crespo-López et al., 2009; Mugica et al., 2003; Tiwary, 2001). The presence of DNA damage in cells from biota inhabiting coal mining areas has been reported elsewhere (Leffa et al., 2010). Furthermore, DNA lesions attributable to metal exposure are known to be driven by mechanisms involving the generation of highly reactive oxygen species and interference with DNA repair processes (Hartwig, 1995; Kasprzak, 2002, 1995; Valko et al., 2005).

The other study site, Magangué, is located on the Magdalena River that drains a major portion of northern Colombia. Thus, this river receives effluent from large cities, industries, agrochemical runoff, and mining operations that occur along its banks. We expected to find higher genotoxic effects at this study site compared to Lorica, located on the smaller Sinú River. However, our results indicated lower levels of genotoxic damage in the Magangué turtles. Perhaps this was because Magangué is located further from focal contamination points in the larger Magdalena River drainage.

Alternatively, differences between levels of DNA damage in the two field sites may have been due to methodological procedures. In the initial phase of this study, we determined no significant increase of MN frequency or comet tail length due to transportation and storage time for analysis carried out within 24 and 48 h after the time of collection. Due to logistic difficulties related with transporting the samples from Lorica, these samples exceed this period and spent more time at 4 °C before being analyzed, which may have caused an increase in their genotoxic response produced during storage of the samples (Al-Salmani et al., 2011).

In a related study, tissue samples obtained from these same *T. callirostris* individuals were analyzed and shown to contain Hg levels that exceeded permissible limits for human consumption (Zapata et al., 2014). Most mercury contamination in this area is derived from commonly applied agricultural fungicides and pesticides, as well as artisanal gold mining activities (UNEP, 2013). The genotoxic mechanism of action of this metal is mainly clastogenic, and although the genotoxic effects observed in this study cannot be directly attributed to this heavy metal, it is certainly an important part of the complex environmental mixture of contaminants to which these populations are exposed. There are reports of the presence of other heavy metals and organophosphorus and organochlorine compounds in both study sites examined here, as well as in other organisms inhabiting in the Sinú and Magdalena river drainages (Alonso et al., 2014; Feria et al., 2010; Lans et al., 2011, 2008; Madero and Marrugo, 2011; Mancera-Rodríquez and Álavarez-León, 2006; Olivero and Solano,1998; Olivero-Verbel et al. 2011; Pinedo-Hernández et al., 2015). Exposure to these chemical substances has been associated to genotoxic effects in turtles inhabiting contaminated environments (Matson et al., 2005, 2009; Meyers-Schöne et al., 1993; Swartz et al., 2003).

In addition, in this study we documented a correlation between the frequency of MN and both of the CA parameters quantified (tail length and tail moment). Both types of tests measure DNA damage, but the MN test indicates clastogenic or aneugenic processes (Al-Sabti and Metcalfe, 1995; Bolognesi et al., 2006) while the CA identifies breaks in simple single and double stranded DNA, failures in base excision repair and high levels of oxidative stress, which makes this technique a more sensitive method than the MN test to detect primary DNA damage (Lee and Steinert, 2003). Thus, we recommend the use of both techniques for conducting environmental monitoring because they offer complementary information.

T. callirostris individuals from Magangué and Lorica exhibited considerable inter-individual variability, compared to negative controls (Figs. 3 and 4). Grisolia et al. (2009) argued that differences in baseline values in individuals inhabiting the same environment may be due to differences in their sensitivities. Some

authors affirm that there is a loss of sensibility in the CA conducted on field samples because of a development of tolerance by some individuals, suggesting a prolonged exposure to a genotoxic stress does not necessarily lead to an increase in DNA break frequencies. For this reason, it is important to examine an adequate number of individuals with both tests before reaching conclusions on the contamination levels and effects at a site.

Turtles usually mature at a specific size and not at a specific age (Páez et al., 2012), with the minimum size of maturity in *T. callirostris* being 10 cm SCL in males and 17 cm SCL in females (Cortés-Duque, 2009), with individuals in this size range or smaller being difficult to sex based on secondary sexual characteristics. Thus, in this study, we did not examine gender as a factor in determining the extent of DNA damage, because of the uncertainty of the sex of the smaller individuals in our samples. As sex is known to be an important factor in explaining variation in levels of DNA damage in other species, future research on *T. callirostris* should compare individual of known sex and similar SCL to inspect for gender effects in this species.

This study confirmed the potential of *T. callirostris* as a model organism for *in situ* monitoring using the MN test and CA as genotoxic biomarkers in natural environments and over short time periods. The data presented in this study also may serve as a reference for future biomonitoring in other regions inhabited by this species.

5. Conclusions

Environmental degradation of the Sinú and Magdalena river drainages may cause DNA damage in at least one native species. Our study revealed the presence of genotoxic effects in the habitats of *T. callirostris* near the towns of Magangué and Lorica, which suggests an environmental risk to this and other species inhabiting these areas. Our findings confirm the potential value of MN test and CA for being applied as biomarkers for evaluation of genotoxic effects caused by environmental contaminants on *T. callirostris* erythrocytes. In this study, we showed *T. callirostris* is a good model organism for *in situ* monitoring of local levels of genotoxicity and environmental contamination. Data provided here will be useful for future work involving the biomonitoring of other regions where *T. callirostris* occurs, with the possibility to apply both short-term tests on peripheral blood erythrocytes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.01.016.

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