Mercury levels and genotoxic effect in caimans from tropical ecosystems impacted by gold mining

José Marrugo-Negrete^{a,*}, José Durango-Hernández^a, Clelia Calao-Ramos^a,
Iván Urango-Cárdenas^a and Sergi Díez^{b,*}

^aUniversidad de Córdoba, Carrera 6 No. 76-103, Montería, Córdoba, Colombia

^bEnvironmental Chemistry Department, Institute of Environmental Assessment and Water Research, IDAEA-CSIC, E-08034 Barcelona, Spain.

* Corresponding autor:

E-mail address: jmarrugo@correo.unicordoba.edu.co (J.Marrugo-Negrete),
sergi.diez@idaea.csic.es (S.Díez)

Highlights:

- Hg in caimans was studied by means of 3 non-lethal tissues that correlate between them
- Higher Hg were found in tissues of caimans impacted by upstream mining gold sites
- Hg in scutes and claws are 15- and 8-fold significantly higher than in control
- Caiman shows higher genotoxic damage in blood cells in exposed reptiles vs. controls
- A highly significant correlation was found between Hg and damage in all the tissues

GRAPHICAL ABSTRACT

ABSTRACT

One of the most representative predator species in tropical ecosystems is caiman that can provide relevant information about the impact of mercury (Hg) associated with artisanal and small-scale gold mining. To evaluate the degree to which adverse effects are likely to occur in *Caiman crocodilus*, total Hg (THg) concentrations in different tissues and DNA damage in erythrocytes were determined. Samples of claws, scales, and blood were taken from 65 specimens in sites impacted by upstream gold mining, and in a crocodile breeding center as control site, located in a floodplain in northern Colombia. In all the sites, the highest THg among tissues was in the following order: claws>>scales>blood. High concentrations of THg were found in the different tissues of the specimens captured in areas impacted by mining activities, with mean values in claws (1100 ng/g ww), caudal scutes (490 ng/g ww), and blood (65 ng/g ww), and statistically significant differences compared to those of the control site ($p<0.05$). THg in scutes from impacted sites are 15-fold higher than in control, whereas for claws and blood are 8 times higher, and a high significant correlation with THg was found in all the tissues. The comet assay reveals significant differences in the DNA damage in the exposed reptiles compared to the controls ($p>0.05$). In sum, *C. crocodilus* from La Mojana floodplain presents a high ecological risk given its genotypic susceptibility to Hg levels present in its habitat, which could possibly influence vital functions such as reproduction of the species and the ecological niche that it represents within the ecosystem.

Keywords: Genotoxicity; *Caiman crocodilus fuscus*; Hg; comet assay; floodplain

1. INTRODUCTION

Due to its release during the artisanal and small-scale gold mining operations, mercury (Hg) contamination has been found in important ecosystems such as natural tropical wetlands, such as La Mojana floodplain in Colombia, where Hg has been reported in water, plankton, fish, aquatic plants, and sediments (Marrugo et al., 2008a; Marrugo et al., 2010; Olivero et al., 2014; Pinedo-Hernández et al., 2015; Marrugo-Negrete et al., 2015a). Tropical wetlands are the preferred habitat of large reptiles such as alligators and crocodiles, a species that suffers from a population deficit since they are prey of otter and hunting birds when young, and by jaguars when they are adults. Additionally, the pressure exerted by human is a determining factor in the maintenance of the population over time. Moreover, these reptiles are exposed daily to many physical, chemical, and biological agents present in the environment, which in one way or another affect their physiological functions (Ansoar-Rodríguez et al., 2015). Although reptiles have very important functions in ecosystems, they are generally undervalued and poorly documented. These include processes associated with their role as prey, predators, commensals, seed dispersers, pollinators, and other ecosystem services that contribute to the health and integrity of the ecosystem and the energy and matter flow between terrestrial and aquatic environments (Valencia-Aguilar et al., 2013, Urbina-Cardona et al., 2015). As a result, some reptiles are considered good sentinel specimens of the ecosystem health (Böhm et al., 2013), and they are often closely linked to a particular habitat that makes them more vulnerable to habitat degradation compared to birds and mammals (Hopkins, 2000). Although reptiles

have proven to be excellent sentinel specimens of pollution (Mitchelmore et al., 2006), they remain the least studied group of vertebrates in terms of genetic toxicology. In this sense, evaluation of genotoxicity in wild species employs the comet assay test, as this method is preferred for its sensitivity in detecting DNA damage (Mitchelmore and Chipman, 1998; Carballo and Mudry, 2006). Few studies have applied this method to reptile erythrocytes to determine the degree of DNA damage (Zuñiga et al., 2001; Boned et al., 2011). Among these, Poletta et al. (2011) evaluated the genotoxicity of glyphosate in erythrocytes of *Caiman latirostris*. Likewise, Schaumburg et al. (2012) studied DNA in reptile species by adjusting the protocols of genotoxicity techniques, including the comet and micronucleus assays, and demonstrated the sensitivity of these biomarkers.

In La Mojana wetland, most of the studies related to contamination from gold-mining waste that have been carried out thus far have focused on the ichthyofauna (Marrugo et al., 2008ab; Olivero et al., 2004; Olivero et al., 2014). However, studies conducted on the accumulation of Hg in *Caiman yacare* in Brazil showed that this species can be considered an Hg bioaccumulator (Lázaro et al., 2015). We currently lack information regarding Hg contamination in the species *Caiman crocodilus*, which, given its role as a predator in controlling the populations of prey species, is one of the main species involved in the maintenance and regulation of aquatic communities in Colombia (Morales-Betancour et al., 2013). For all the above reasons, the main objective of this study was to obtain detailed information on the concentrations and genotoxic effects of Hg on caimans in order to assess the impact of Hg in a mining area of Colombia.

2. METHODOLOGY

2.1. Study area

The samples for the present study were collected in the Momposina depression located in the north of Colombia (Figure 1), which is considered the most important wetland complex in the country and is characterized as being very productive for its ichthyofauna richness. This wetland complex serves the environmental function of regulating the channels of the Magdalena, Cauca, and San Jorge rivers, dampening floods, and facilitating the decantation and accumulation of their sediments. The source of Hg in La Mojana floodplain comes mainly from the waste generated by mining activities (INGEOMINAS, 1995; Veiga, 1997; Olivero et al., 2002; Marrugo et al., 2008b) that is transported by these three rivers that cross this region. Specimens of the species *Caiman crocodilus fuscus* (Cope 1868) were obtained from three sites: La Redonda marsh (S1) and La Encaramada marsh (S2), marked as Hg contaminated areas, and a crocodile breeding center, located far away from mining activities, that was used as a control site (S3) (Figure 1).

La Redonda marsh (S1), receives mining waste with Hg directly from an important mining area (mine of Santa Cruz) located in the municipality of Barranco de Loba, south of the department of Bolívar, Colombia. This mining area is one of the larger and most productive auriferous zones in the country, where Hg is used in great quantities to recover gold using the amalgamation process. THg levels exceeding 30 mg/kg have been reported in soils close to this mine (Vidal et al., 2010).

S2 (La Encaramada marsh), despite not having direct discharges from mining activities, is influenced by frequent floods caused by the overflow of the Cauca River that receives mining waste from the northeast of the department of Antioquia and the south of Bolívar that are considered the most important gold mining area in the country (Marrugo et al., 2008b; UPME-MME-UC, 2015; Pinedo et al., 2015). Some studies have shown mercury contamination in this region, with THg values in marshes sediments ranging from 196 to 1188 ng/g dry weight, dw. Average percentages of 2.6% of the bioavailable phase (Marrugo-Negrete et al., 2015b; Pinedo et al., 2015), and mean fish Hg concentrations were 0.50 ± 0.10 mg/kg (Marrugo et al., 2007).

The reference site (S3), is a crocodile breeding center located in the municipality of San Marcos, department of Sucre. The operation of the crocodile breeding center for the species *Caiman crocodilus fuscus* was established by the Corporation for the Sustainable Development of La Mojana and San Jorge in Resolution number 08262, dated June 4, 2004. This site was chosen as control site because the impact of mining activities is limited to sporadic flooding from the San Jorge River.

Taking into account the hydrological information of the study area, the drought period runs from the end of December to March and early April, whereas the flood period corresponds to the remaining months of the year, with low rainfall in June and November (Pinedo et al., 2015). The climate of the area is hot and humid, and the temperature varies between 28 and 35°C.

2.2. Sample collection

A total of 65 specimens were captured from the locations described above, S1 (n=23), S2 (n=22), and S3 (n=20), during February and April of 2016 (dry season). Crocodile specimens S1 and S2 were captured at night, with the help of a herpetological lace (Vanzolini and Nelson 1990). Once captured, they were firmly held by the nape and their muzzle was tied with a rubber band. At the control site (S3), the animals were supplied by the breeding center, selecting those reptiles with physiological age similar to those obtained in sampling sites S1 and S2. The reptiles were tied by the neck and immobilized, after which we took the following measurements: the length, measured by taking a tape measure to the back of the animal from the tip of the mouth to the end of the tail; the weight, determined using a clock weight; and the sex, identified by the cloacal examination of the genitals (Cabrera and García, 2004).

The biological tissue samples were collected by cutting the individual caudal scutes, and the claws of the right hind limb with the help of scalpels and pliers, respectively. The samples were packed in polyethylene bags, labeled and stored in a portable fridge at 4 °C for transport to the laboratory. Peripheral blood samples (5 mL) were obtained by puncture of the caudal vein under aseptic conditions, according to the technique described by Jacobson (1984), in vacutainer tubes into which we added K₂EDTA for the analysis of Hg or heparin (5 mL) for the development of the comet assay technique (Singh et al., 1988). The tubes with the samples were stored in a portable fridge at 4°C, for their preservation and transport to the laboratory.

Once the samples were taken, the caimans were released again at the sites where they were captured.

2.3. Analytical methods

The determination of total Hg concentrations in biological samples was performed by atomic absorption spectroscopy using a direct mercury analyzer (DMA80 TRICELL, Milestone Inc., Italy), using EPA method 7473 (USEPA, 2007) by weighting 0.1 g of blood, 0.02 g of claws and 0.5 g of tail scutes samples, respectively. Previous to lyophilization, claws and scutes were washed with abundant distilled water to remove any material adhering to the surface that could affect the analysis. The quantification in the equipment was done using a calibration curve, obtaining a determination coefficient of 0.9997. The detection limit determined as three times the standard deviation of 10 blank measurements was 0.05 ng Hg (0.5 ng/g THg for blood and 2.5 ng/g for claws and scutes) and the method was evaluated by analyzing, in triplicate, certified reference material DORM-2 dogfish muscle from the National Research Council Canada (certified value = 4.6 ± 0.26 µg/g dw), and IAEA-086 human hair from the Community Bureau of Reference (certified value = 0.573 ± 0.039 µg/g dw). The recovery percentage for DORM-2 (4.52 ± 0.18 µg/g dw) and IAEA-086 (0.565 ± 0.023 µg/g dw) was 98.2 and 98.6%, respectively, with variation coefficients of less than the 5%, within the 95% confidence limits.

2.4. Alkaline Comet Assay

In order to evaluate the appropriate status of the erythrocytes for the application of the assay, cell viability was determined using the Trypan blue dye exclusion method (Tice et al., 2000). A total of 100 cells were counted per sample and the percentage of viable cells was determined.

The alkaline comet assay was carried out as described by Singh et al. (1988), with some modifications following the protocol of Poletta et al. (2008), Schaumburg et al. (2012), and Caliani et al. (2014). Specimen blood was diluted 1:19 (v/v) in RPMI 1640 medium, and, for the positive controls, cells were treated for 30 min with 25 μ M H₂O₂. Treated cells were used for each electrophoresis instance. A volume of the aforementioned dilution was taken and mixed with 200 μ L of low melting point agarose. This mixture was distributed on two slides with a previously dehydrated layer of normal melting point agarose that was allowed to solidify at 4 C for 10 min. Afterwards, the slides were submerged in lysis solution for 18 h in order to break the cell membranes. Subsequently, these slides were incubated in the alkaline electrophoresis solution to allow the unwinding of the double strand of DNA, and electrophoresis conditions were always 300 mA and 25 V (0.90 V/cm). The whole process was carried out in the dark to avoid any further damage to the DNA.

The slides were washed with neutralizing solution Tris 0.4 M. Finally, the samples were stained with ethidium bromide solution (2 μ g/mL) and observed in an Olympus BX43F fluorescence microscope equipped with a 505-560 nm excitation filter and a 40x objective in the dark. 100 cells were analyzed per sample (50 from each duplicate). The images were analyzed using the visual scoring method (Collins, 2004) (Figure SI) (reported as Supplementary Information) and classified

into five categories of damage according to the fragments migration (length of the tail): Class 0, no damage, no fragments migration (no tail); Class 1, slight damage (tail length less than nucleus diameter); Class 2, moderate damage (length of the tail greater than a nucleus diameter but less than diameter of two); Class 3, high damage (tail of length greater than the diameter of two nucleus and less than of three); and Class 4, extremely high damage (tail length greater than the diameter of three nucleus).

Comets were individually scored according to class, and the results were expressed as DNA damage index comet assay (DICA) according to: $DICA = n_1 + 2n_2 + 3n_3 + 4n_4$, where n_1 , n_2 , n_3 and n_4 is the number of cells in damage category 1, 2, 3 and 4, respectively (Rodríguez-Ferreiro et al., 2002). Hence, DICA ranged from 0 (completely undamaged: 100 cells $\times$ 0) to 400 (maximum damage: 100 cells $\times$ 4) (Collins et al., 2008).

2.5. Statistical analysis

The results of THg concentrations in the different tissues are presented as the mean $\pm$ SD, in wet weight (ww). The results did not meet the normality and homoscedasticity tests of Shapiro-Wilk and Bartlett, respectively; therefore, nonparametric statistics were used as a data analysis tool. The Kruskal-Wallis test was used to evaluate significant differences in THg and DICA in different tissues and sampling sites, using multiple comparisons through the Mann-Whitney test. Likewise, Kruskal-Wallis test was used to evaluate differences in the weights and lengths of the captured specimens, while the Mann-Whitney test was used to analyze variation in THg concentrations in the studied tissues relative to sex. A

Spearman's correlation analysis (r) was performed in order to determine correlations between the THg concentrations in tissues, DICA, and the size of the animal. The analyses were performed using the statistical program IBM SPSS Statistics Version 23.0.0.0, at a significance level of $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Concentrations of THg in different tissues

The number of specimens, length, weight, sex, and THg concentrations at each of the sampling sites are shown in Table 1. The number of female specimens was higher than the number of males at all the sampling sites except for the control site. Nevertheless, no statistically significant differences were found in the THg concentrations of the different tissues between males and females ($p > 0.05$), and therefore, sex was not considered for any statistical analysis. Several studies have found no significant mean differences by gender, in tissues of reptiles of the order Crocodilia (Jagoe et al., 1998; Schneider et al., 2012; Lázaro et al., 2015; Nilsen et al., 2016). The length of the specimens captured was between 50 and 68 cm, with a single individual exceeding 80 cm. Given the small variation in size between specimens, which may indicate the same chronological age, these lengths correspond to those of juvenile reptiles (Rodríguez 1989; Buenfil-Rojas et al., 2015). Moreover, individuals in this life stage are characterized by their high metabolism and assimilation of environmental pollutants (Kant and Tansel, 2000).

As expected due to standardization of animal sizes, no statistically significant differences were found for weight and length of the captured specimens among the three sampling sites ($p > 0.05$). Similarly, no significant correlation was found between the THg concentrations in the tissues and the size or weight of crocodilians at the different sampling sites ($\rho < 0.1$; $p > 0.05$). Several studies have found a positive relationship between the THg concentration in tissues such as muscle, scales, blood, and claws and the size of *Alligator mississippiensis*, *Melanosuchus niger*, and *Caiman crocodilus* (Yanochko et al., 1997; Burger et al., 2000; Schneider et al., 2012, 2015; Lázaro et al., 2015; Nilsen et al., 2016). However, other studies on *Crocodylus moreletii* and *Crocodylus acutus* have found a different pattern (Rainwater et al., 2002). This discrepancy can be explained by ontogenetic variation and habitat-related variation in the diet, which determine individual and inter-species differences, especially in blood that indicates recent toxic exposure (Lázaro et al., 2015; Buenfil-Rojas et al., 2015).

Highest THg concentrations were found in claws, followed by scutes and blood (Table 1). Similar findings were previously reported in other species of alligators and caimans (Lázaro et al., 2015; Schneider et al., 2015; Buenfil-Rojas et al., 2018). Moreover, the highest THg in all the biological samples correspond to samples located at S1. THg concentrations were about 18 times higher in claws than in blood in all three sampling sites. Statistical analysis (i.e. Kruskal-Wallis test) shows significant differences in THg for the three tissues ($H(2, N=60, 69 \text{ and } 66) = 52.4619, 45.4090 \text{ and } 51.4297$) for S3, S2, and S1, respectively, with $p < 0.001$) in all sites. Multiple comparisons using the Mann-Whitney test indicate that the mean

THg in biological samples was significantly different among all tissues, except between claws and scales at S2. Weight and length in crocodilians were tested and showed colinearity ($r^2 = 0.935$; $p < 0.01$); however, no relationships were found between THg in any tissue and the length, probably due to the low variation in size in the collected juvenile crocodilians ($N=65$; mean length= 58 ± 4 cm).

The highest concentrations of THg found in the claws and scales versus blood are related to its high content of β -keratin. This protein is rich in amino acids with sulfhydryl groups that form complexes with Hg of the metallothioneins type, facilitating the toxic bioconcentration of Hg. In fact, these tissues have shown a high potential for serving as a better indicator of contaminant levels in internal tissues than muscle and bone (Buenfil-Rojas et al., 2018; Schneider et al., 2015). On the other hand, in the caudal scutes of crocodiles, the content of THg in the keratinized epidermis can be diluted by the bone dermis of the scales (Schneider et al., 2015); in addition to the losses due to the natural erosion of the skin, and the effect that dietary changes have on the THg concentration in both blood and scales, due to continuous metabolic exchange with the rest of the body (Rainwater et al., 2007; Richardson et al., 2002; Nilsen et al., 2016). These physiological differences could explain the differences in THg concentrations between blood, scutes, and claws. Similarly, several studies have reported that claws are better contamination biomonitors due to their ability to store information over time, and to their lack of susceptibility to dietary variations in Hg, because they act as sinks for nutrients and other dietary materials (Hopkins, 2007; Lázaro et al., 2015). Blood,

meanwhile, has been reported as an indicative of recent exposure, as it acts as a means of transporting the contaminant to all tissues (Khan and Tansel, 2000).

A significantly correlation was found between the different tissues evaluated at the three sampling sites (Figure 2), suggesting a high positive relationship among blood vs. scutes ($r = 0.889$; $p < 0.01$) and blood vs. claws ($r = 0.925$ $p < 0.01$). Also, THg in scutes and claws were significantly related ($r = 0.924$ $p < 0.01$). As has been described previously (Schneider et al., 2015), keratin is a better predictor of exposure to Hg than muscle and bone tissues. Even though muscle tissues have been traditionally used to monitor Hg concentrations in the environment, the high affinity of Hg to keratin (because the prevalence in keratin of the sulfur-containing amino acid cysteine), better reflects Hg exposure over time. Some researchers identify the bloodstream as a possible route of distribution of toxic substances such as Hg, being Hg in blood directly related to the body burden of Hg in organisms, and when the exposure is recurrent, it becomes a persistent body burden (Burger et al., 2005; Buenfil-Rojas et al., 2015).

The presence of high concentrations of THg in cocodriles is a cause of great concern, because of species preservation, and for public health risk, because many villagers often consume them as a source of meat. Several studies have indicated that high concentrations of Hg in animals can alter the endocrine system by inhibiting the action or normal functioning of hormones, given the ability of methylmercury to bind sulfhydryl groups and the formation of Hg complexes with selenium-enzymes in the pituitary and thyroid gland, which interferes with the synthesis, secretion, transport, action, or elimination of natural hormones in the

body that are responsible for processes such as homeostasis, reproduction, development, and behavior (Schneider et al., 2012; Meyer et al., 2014). Besides this, *C.crocodilus fuscus* is in category II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), and hunting has been the primary reason for population decline in Colombia, as it is a target of illegal trafficking to commercialize its skin. The database analysis of the regional autonomous corporations shows that 99% of seizures belong to this subspecies (Morales-Betancourt et al., 2013). *C.crocodilus* is part of a family of long-lived reptiles, and this longevity could pose a risk in terms of Hg toxicity, because the animals live in aquatic habitats, such as wetlands and marshes, where the production of toxic methylmercury is high (around 10%), and they also feed on invertebrates and higher order fish, accumulating Hg in their tissues for years (Meyer et al., 2014). Finally, this accumulated Hg can, in turn, be consumed by the inhabitants of the region, given the high hunting rate of this species and its incorporation into the natives' diet (De la Ossa & De la Ossa-Lacayo, 2011; De la Ossa-Lacayo & De la Ossa, 2012).

3.2. Spatial variability

The results show higher concentrations of Hg in S1, followed by S2, and finally S3 (control), for all the evaluated tissues (Table 1). Average THg in the control S3 for blood and claws is about 2 and 8 times lower than in S2 and S1, respectively. In the case of the scales, 15- and 8-fold higher concentrations are observed in the specimens captured in S1 and S2 compared to those at the control site, respectively. Statistically significant differences were found between the means of

THg concentrations for the three sampling sites in all the evaluated matrices ($p < 0.01$), except between the captured specimens at S3 and S2 for blood ($p = 0.097$). The highest concentrations of THg found in *C. crocodilian* are from the specimens located at S1 (La Redonda marsh), that should be attributed to the fact that this site receives wastes generated by the upstream gold mining activities of the Santa Cruz mine district, directly related with Hg. In fact, the mining district of Santa Cruz is a site considered a 'hotspot' site of Hg contamination in Colombia (Vidal et al., 2010; Perez-Vargas et al., 2014; UPME-MME-UC, 2015; UPME-MME-UC, 2016). Several studies report high Hg concentrations in domestic animals, plants, and soils in this area (Argumedo et al., 2013; Perez-Vargas et al. (2014). Moreover, some authors have reported values similar to those found in this study at sites in the Brazilian Amazon that have been highly contaminated with Hg by mining activities, with values of 2176 ± 725 ng/g ww and 387 ± 136 ng/g ww in claws and scales, respectively, for *C.yacaré* (Lazaro et al., 2015), and values of 348 ± 298 ng/g ww (range 70-770) and 205 ± 158 ng/g ww (range 70-570) in scales of *C.crocodilus* and *M.niger*, respectively (Schneider et al., 2015). Nonetheless these values are lower than those found in the Florida Everglades for juvenile alligators, with values of 5830 ± 104 ng/g and 160-680 ng/g for blood and scales, respectively (Khan and Tansel, 2000; Nilsen et al., 2016). On the other hand, in sites with low Hg contamination in Central America, like Belize and Costa Rica, values up to 100 ng/g in the scales of *Crocodylus acutus* and *Crocodylus moreletii* have been reported (Rainwater et al., 2007; Tellez et al., 2017).

Despite the controlled living conditions present in the crocodile breeding center, the low THg concentrations found in the three tissues evaluated in the control species (S3) can be linked to the composition of the food products and/or Hg depositions (dry and humid) transported by air from the mining area.

According to our results, claws, scales, and blood of *C. crocodilus fuscus* can be used as good target tissues to assess both levels and spatial patterns of Hg. Among them, the preferred tissue is claw, since it is a less invasive tissue to analyze Hg concentrations in caimans in comparison with scutes and blood. Although all could be used as precision tools in the spatial analysis of Hg exposure, some of these tissues are susceptible to the ontogenetic and dietary variations of the animals and represent the time-integrated Hg exposure in their habitat (Lazaro et al., 2015). In fact, it has been reported that Hg concentrations in crocodiles can vary considerably between sites (Jagoe et al., 1998).

The results of this study remarkably show the effect of mercury-contaminated ecosystems on fauna. In this case, it is shown how the natural wetland system, which receives Hg wastes from gold mining activities, possibly promotes methylation conditions of toxic materials (given the anoxic conditions prevailing during periods of flooding and the high load of organic matter in sediments), and favors the uptake of Hg in the body burden of *C. crocodilus fuscus* through bioaccumulation and biomagnification processes. Once again, this study demonstrated the problems related with artisanal and small-scale gold mining, by showing significant differences between sites affected by different degree of Hg-contamination.

Similarities with other studies are difficult to draw because of different studied species as well as the particular characteristics of every environment. Table 2 shows recent studies on different alligators and caimans. In general, Hg concentrations found in biological tissues claws of our specimens was similar or even lower than those that have previously been reported.

3.3. Comet Assay

Assessing the genotoxicity of wildlife species is a tool to document how exposure to xenobiotics can affect the health and condition of natural populations (Carballo and Mudry, 2006). The comet assay has been applied to multiple wild species to detect DNA damage, and this technique has also proven to be a sensitive method for the detection of the genotoxicity of chemicals and complex mixtures, as well as a method for environmental biomonitoring (Ansoar-Rodríguez et al., 2015). Among the wild species that have been used to assess DNA damage from environmental contaminants are the reptiles. Several studies showing a high sensitivity in these species for detecting the effects of genotoxic agents (Poletta et al., 2009; Strunjak-Perovic et al., 2010; Poletta et al., 2011; Zapata et al. 2016). Among them, Zapata et al. (2016) reported evidence of genotoxic effects on the species *Trachemys callirostris* in the Magdalena and Sinú river basins, that is close to our study sites..

In our comet assay, the cell viability was in the range of 95 to 100% for all the analyzed samples, indicating that all blood samples were in good condition for the assay. Therefore, the comet assay was carried out in order to evaluate the toxicological susceptibility of *C. crocodilus* in La Mojana floodplain.

Results show that a significant increase in DNA damage was found in crocodilians at S1 and S2 compared to the control site, S3 (Figure 3). It can be observed that the greatest damage to DNA occurred in the specimens collected in S1, compared to S2 and S3. We found significant differences between the mean values of DICA in these sampling sites ($p < 0.01$). On the other hand, no correlation was found between DICA values and the length or weight of the animals. Again, this finding allows us to argue that the homogeneity in sizes of the captured specimens does not allow us to draw any conclusion regarding the time of exposure, as evaluated through toxic exposure at different life stages of the animal.

Finally, relationships between DICA and THg concentrations in the biological tissues were evaluated for the specimens at each of the sampling sites, and for the total of the specimens. A highly significant correlation was found between the concentrations of THg in all the tissues and DICA when we evaluated all analyzed specimens together ($p = 0.878$, $p = 0.00$; $p = 0.813$, $p = 0.00$; $p = 0.633$, $p = 0.00$; for blood, claw, and scale, respectively) that shows that the degree of exposure to Hg by *C. crocodilus fuscus* has a high influence on genomic alterations presented by these organisms. On the other hand, S1 showed a high correlation in all the tissues in relation to DICA, whereas S2 only showed a correlation with blood ($p = 0.829$, $p = 0.001$). This finding suggests that a continuous and recent exposure to Hg occurs in crocodilians living near these areas, especially those surrounding the mining district that is considered a hotspot site (UPME-MME-UC, 2015). According to Nilsen et al. (2016), high Hg exposure of the *C. crocodilus fuscus* in this area could potentially indicate a decrease in overall DNA methylation. According to this

supposition, this scenario could have very serious consequences for the species, because DNA methylation, which consists in the covalent addition of a methyl group to the carbon 5 of the cytosine, is an epigenetic modification that allows functions that include gene expression regulation, the promotion of chromosomal stability, and the silencing of transposon transcription (Di Giacomo et al., 2013, Parrott et al., 2014, Rodríguez et al., 2006). In addition, Hg causes reproductive and neurological deterioration as a function of direct exposure (Crump and Trudeau, 2009; Frederick and Jayasena, 2011; Khan and Tansel, 2000) that can contribute to population decline and species loss.

CONCLUSIONS

C. crocodilus living in the aquatic ecosystems of La Mojana floodplain, allows us to demonstrate its usefulness as a potential sentinel species for assessing bioaccumulation of Hg. In fact, THg concentrations of different biological tissues (blood, claws, and scales) showed high Hg concentrations that are higher in those animals captured on sites receiving wastes generated by the upstream gold mining activities that use Hg.

One important finding was that claws, scutes, and blood provided useful information about the Hg exposure of crocodilians, since they are significantly correlated. In fact, all tissues were found to be suitable as non-lethal predictor for measuring Hg bioaccumulation in reptiles. Results proved that among the three

non-lethal methods employed, claws seem to be the best predictor, because these provide the same information, and is even less invasive than scutes and blood.

According to the comet assay to detect DNA damage, there is a statistical difference between DICA of crocodilians impacted by upstream artisanal and small-scale gold mining operations in comparison with control site. Moreover, a highly significant correlation was obtained between DICA and THg in all biological tissues. This fact confirms an environmental impact downstream from mining districts, with toxic effects that probably cannot be limited to the evaluated animals, but likely extend to the rest of the species that inhabit these ecosystems, and further indicating the potential risk to the human inhabitants who consume their meat.

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ACKNOWLEDGEMENTS

The authors wish to thank the Colombian Institute for the Development of Science and Technology "Francisco José de Caldas" (COLCIENCIAS) for the financing of the project: Spatial distribution of heavy metals and nutrients in flooded soils of the Mojana region: environmental implications and Recovery strategies, identified with the code: 1112-569-35214.

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Figure 1. Map of the sampling sites location in La Mojana floodplain. La Redonda marsh (S1), and La Encaramada marsh (S2) are impacted by gold mining; S3 is the reference site

Figure 2. Correlation among THg concentrations in blood, claws and scales

Figure 3. Damage Index Comet Assay (DICA) at the different samples sites. La Redonda marsh (S1) (n=23), and La Encaramada marsh (S2) (n=22) are impacted by gold mining; S3 (n=20) is the reference site.

Table 1. Biometric values, sex ratio, damage index comet assay (DICA), and total mercury (THg, ng/g ww) concentrations in blood, claws and caudal scutes of *C. crocodilus fuscus* captured in La Mojana floodplain, Colombia. Mean values $\pm$ standard deviation.

Site	N	Weight (g)	Length (cm)	Sex Ratio (F:M)	Hg-blood	Hg-claw	Hg-scute	DICA
S1	22	468 $\pm$ 49 ^A	57.2 $\pm$ 3.5 ^B	2:1	65.0 $\pm$ 21.6 ^a	1100 $\pm$ 492 ^d	490 $\pm$ 216 ^g	134 $\pm$ 63
S2	23	5448 $\pm$ 172 ^A	57.5 $\pm$ 6.8 ^B	3:2	14.1 $\pm$ 6.9 ^b	267 $\pm$ 117 ^e	247 $\pm$ 98 ^e	25 $\pm$ 38
S3	20	496 $\pm$ 13 ^A	59.0 $\pm$ 1.6 ^B	2:3	8.4 $\pm$ 3.1 ^c	131 $\pm$ 38 ^f	31.5 $\pm$ 5.8 ^h	5 $\pm$ 3

Different uppercase letters represent differences in size (weight and length) for the different sampling sites. Different lowercase letters represent significant differences in THg content between sites and tissues.

Table 2. THg (ng/g, ww) from different species of alligators and caimans worldwide in different tissues

Species	THg	N	Tissue	Location	Reference
<i>A. mississippiensis</i>	56±13	31	skin	Florida, USA	Burger et al. (2000)
	1012±741	13	keratin	Amazonia, Brazil	Schneider et al. (2015)
<i>C. crocodilus</i>	882±564	7	keratin	Amazonia, Brazil	
<i>C. yacare</i>	96±92	17	scutes	Paraguay river Pantanal, Brazil	Lazaro et al, 2015
<i>C. yacare</i>	846±623	17	claws	Paraguay river Pantanal, Brazil	
<i>C. yacare</i>	264±159	22	scutes	Bento Gomes river Pantanal, Brazil	
<i>C. yacare</i>	1945±704	22	claws	Bento Gomes river Pantanal, Brazil	
<i>C. crocodilus</i>	60±63	11	blood	Puris river, Amazonia, Brazil	Eggs et al., 2015
<i>C. crocodilus</i>	3527±3095	8	keratin		
<i>M. niger</i>	48±32	12	blood		
<i>M. niger</i>	2092±1052	13	keratin		
<i>P. unifilis</i>	4±10	18	blood		
<i>P. unifilis</i>	148±70	9	keratin		
<i>P. expansa</i>	1±1	5	blood		
<i>P. expansa</i>	169	1	keratin		
<i>C. crocodilus</i>	39±30	45	blood	Impacted areas, La Mojana, Colombia	Present study
<i>C. crocodilus</i>	674±547	45	claws		
<i>C. crocodilus</i>	366±205	45	scutes		
<i>C. crocodilus</i>	8±3	20	blood	Control site, La Mojana, Colombia	Present study
<i>C. crocodilus</i>	130±38	20	claws		
<i>C. crocodilus</i>	32±6	20	scutes		

SUPPLEMENTARY INFORMATION

Figure SI. Comet assay images of different damage classes obtained from the peripheral blood of the crocodilians. (a) No obvious damage Class 0; (b) Damage at the cellular level class 0 and 1; (c) Damage at the cellular level Class 3 and 4; (d) Damage at the cellular level Class 4