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Bats are an excellent sentinel model for the detection of genotoxic agents. Study in a Colombian Caribbean region

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A B S T R A C T

Wildlife animals have been affected by human activities and the diminution of the areas needed to develop wildlife. In Colombia, artisanal and industrial mining focuses on gold extraction, which uses mercury and causes contamination in water sources. Bats may be susceptible to chemical contamination and primarily to bioaccumulated heavy metal contaminants in the food chain. The primary source of exposure is contaminated food and water ingest, followed by dermic exposition and inhalation. The objective was to evaluategenotoxic damage and mercury concentration in bats. Forty-five samples of blood and organs of bats captured in Ayapel and Majagual were collected. Erythrocytes were searched for micronuclei by peripheral blood smear. Mercury concentration in 45 liver and spleen samples was determined by atomic absorption spectroscopy (DMA80 TRICELL, Milestone Inc, Italy). Bats from four families were studied: *Phyllostomidae* (6 species), *Molossidae* (3 species), *Vespertilionidae* (1 species), and *Emballonuridae* (1 species). Mercury was found in all bat species from the different dietary guilds. Insectivores had the highest concentration of mercury in the liver (0,23 μg/g) and spleen (0,25 μg/g) and the highest number of micronuclei (260 micronuclei/10,000). The specimens captured in Majagual had the highest frequency of micronuclei (677 micronuclei/10,000), and those captured in Ayapel had the highest mercury concentration (0,833 μg/g). This is the first study in Colombia to report that bats could act as sentinels to the environment's genotoxic chemical agents. Mercury and a high frequency of micronuclei were found in the tissues of captured bats. In addition to mercury contamination, there could also be other contaminants affecting *Chiroptera*.

1. Introduction

The significant increase in human activities generates changes and alterations in the balance of the ecosystem; activities such as habitat fragmentation and pollution threaten biodiversity in regions with high species richness (Quintero et al., 2012). All of the environmental contaminants cause great affectations in organisms and ecosystems. One of the contaminants that cause damages to exposed organisms is heavy metals. This may be due to their acute toxicity or bioacumulation r capability in plant and animal tissues through repeated exposure (Racero et al., 2017; Romero et al., 2009).

Mercury (Hg) is a pollutant that can spread through the atmosphere over long distances and be deposited in terrestrial and aquatic ecosystems far from the primary emission sources (Chételat et al., 2018). Atmospheric deposition and human activities such as gold mining, coal-fired power stations, agriculture, and wildland fires pollute soil and water with Hg (Becker et al., 2017; Hsu-Kim et al., 2018). Several studies have reported heavy metals in sediments, waters, plants, fish, wild birds, and humans in several regions of the country, especially in the zone of this study (Feria et al., 2010; Gracia et al., 2010; Marrugo-Negrete et al., 2010; Marrugo-Negrete et al., 2015; Marrugo-Negrete et al., 2016). Likewise, heavy metal genotoxicity studies have been reported in mammals such as rodents, jaguars, and reptiles such as turtles (León et al., 2007). Chronic exposition to Hg may affect immunological functions, reducing the capacity of animals to resist or recover from infections (Becker et al., 2017).

Bats are the only flying mammals, so they have extensive geographic distribution. The highest abundance and richness of species occur in tropical and subtropical regions (Ballesteros et al., 2007). In Colombia, 209 species of bats have been reported (Ramírez-Chaves et al., 2021)

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with different ecological roles: seed dispersal, plant pollination, and control of insects and small vertebrates. Despite their resilience capacity, bats are affected by anthropic processes associated with deforestation (McCartor and Becker, 2010), homogenization of environments (Hsu-Kim et al., 2018), pesticide contamination (Cruz-Esquivel et al., 2017), pollution, and contamination of water sources (Marrugo-Negrete et al., 2010). These environmental conditions do not favor the maintenance of bats' diversity and increase the susceptibility to population-level extinctions. That is why bats are animals that bioaccumulate heavy metals such as mercury (Jones et al., 2009; Zukal et al., 2015).

Ecological characteristics of bats (high mobility, presence in human-modified habitats) and their life-cycle (long life, fast metabolism, and high-level dietary guild) could increase the exposition and bioaccumulation of heavy metals (Zukal et al., 2015). Bats are excellent models for the examination of trophic transference of Hg. Several bat species consume food equivalent to a high proportion of their body mass per night while seeking foodstuff and obtaining it from aquatic and terrestrial ecosystems (Salvarina, 2016).

Living in agricultural habitats could have a high cost for these species if bats are exposed to Hg during feeding (Becker et al., 2017). In the last decades, a decrease in bat populations from *Pipistrellus sp*., *Rhinolophus hipposideros, Rhinolophus ferrumequinum*, and *Myotis myotis* species have been documented in Europe and North America (Dietz et al., 2009; Jones et al., 2009; Stebbings, 1988). These decreases in populations could be attributed to the exposition of several organic chemical products. Namely, the decrease in the most significant population of Greater Horseshoe Bats (*Rhinolophus ferrumequinum*) in Germany was linked to lindane and DDT in agriculture and silviculture (Dietz et al., 2009).

Evaluation of genotoxicity biomarkers is an efficient methodology for assessing the potentially toxic effect of a high number of chemicals and complex mixes (Calao and Marrugo, 2015). Micronucleus assay (MN) is a sensitive, fast, and broadly used method in studying genotoxic biomarkers for wild species. Micronuclei develop during the mitosis of acentric fragments or complete chromosomes that are not included in the central nucleus of the daughter cell. These fragments or chromosomes may have a clastogenic origin (induced by substances that provoke rupture of the chromosome) or may surge through an aneugenic process when chemical agents affect the mitotic fusion mechanism (Sarpal et al., 2016; Benvindo-Souza et al., 2019a). Moreover, the micronucleus method allows the characterization of DNA damage induced by chemical and physical agents because of its capacity to detect chromosomic and DNA damage in the early stages (Zapata-Restrepo et al., 2017).

The Mojana region is an area of the Colombian Caribbean where large bodies of fresh water converge. However, this region is also affected by multiple factors that cause environmental deterioration, such as increased agricultural and livestock frontier. Also alteration in natural watercourses through dikes, the mercury contamination produced by artisanal mining in southern Bolívar and northern Antioquia, and the deficiency in essential sanitation services for pollutants that contaminate the water resource, the biota, and the soil contribute to the contamination of this area. As a result of this imbalance in the ecosystem, the hydrobiological and fauna resources have suffered a drastic decrease (Paternina and Chejne, 2012). As a consequence of the contaminant releasing processes of mining activity previously described, the carrying these to the Mojana region by Cauca and San Jorge rivers, and auriferous exploitation done in the Ayapel swamp, has generated an environmental pollution problem. Thus, increased health risks related to heavy metal exposition (mercury being among these), previous research (Paternina and Chejne, 2012; Pinedo-Hernandez et al., 2015) reported high Hg and methylmercury concentrations in water source sediments from the Mojana Therefore, the risk that bioaccumulation of these concentrations in aquatic organisms is evident. Essential levels of total mercury concentration (THg) and methylmercury have been reported in fishes (Marrugo-Negrete et al., 2008a; Marrugo-Negrete et al., 2008b), presence of Hg in water, seston, phytoplankton, zooplankton, fish, as well as high levels in human hair related to feeding on contaminated fish. The objective of this study was to evaluate the mercury concentration and genotoxic damage in bats from two areas of La Mojana in the Colombian Caribbean.

2. Materials and methods

2.1. Type of study, area, and sample size

A descriptive cross-sectional study was carried out between September 2016 and June 2017. The work area was in the Mojana region, located in the Momposina depression, in the southern Colombian Caribbean. In the Mojana, the Magdalena, Cesar, Cauca, and San Jorge rivers converge. The set of rivers forms a complex system of wetlands vital for fauna and flora. However, in this great wetland, many pollutants and sediments from the Andean region accumulate due to the discharge of untreated wastewater (Calao and Marrugo, 2013). The river also collects and sediments waste containing toxic metals from illegal mining areas in southern Bolívar, the San Jorge Basin, and northeast Antioquia (Marrugo-Negrete et al., 2008a; Calao and Marrugo, 2015; Pinedo et al., 2015; Marrugo-Negrete et al., 2015). In the region of La Mojana are the municipalities of Ayapel, the Department of Córdoba, and Majagual, the Department of Sucre, places where the specimens were obtained (Fig. 1).

2.2. Ethical aspects and collection of bats

The ethics committee approved the Faculty of Veterinary Medicine study of the University of Córdoba, Colombia (Record # 029, June 17, 2014). Bats were captured utilizing five mist nets installed from 17:30 until 23:59, placed in strategic places associated with water sources and native vegetation in rural zones of the municipalities. The less frequent species, juvenile specimens, pregnant or lactating females were released. Captured bat specimens were euthanized with acetylpromazine/ketamine (0,02mg/g-0,05mg). They were later identified using dichotomous taxonomic keys (Díaz et al., 2016). 6-10 μL of blood were collected, and three replicates of peripheral blood smear were done for the micronucleus technique. Liver and spleen samples were also taken for the analysis of metals (mercury). The samples were kept refrigerated and transferred to the Environmental Management and Toxicology Laboratory of the University of Córdoba, frozen at -4°C.

2.2.1. Peripheral blood smear analysis for micronucleus

A peripheral blood smear was done to observe morphological alterations of uniform blood elements by using Giemsa stain (Cromakit™). Blood was collected from the radial arteria of each bat, and aliquots were extended in a clean glass microscope slide in triplicates for each animal. Slides were air-dried, fixed with methanol, and stained with Giemsa stain (Cromakit™) (Cruz-Esquivel et al., 2017). All of the smears were read by the same person.

Micronuclei observation and counting were done in 10,000 red blood cells under an optical microscope (Olympus BX43) and a 100x objective with 505-560nm/objective filter under immersion oil. Micronuclei were defined as (i) structures similar to the principal nucleus but no more significant than 1/16 – 1/3 of its size, (ii) the absence of any connection to the principal nucleus, (iii) with the same texture, (iv) staining intensity, and (v) round or oval shape (Benvindo-Souza et al., 2019a).

2.2.2. Mercury determination

Determination of total mercury concentrations (THg) was done in liver and spleen samples; mercury is capable of bioaccumulating in the liver because of the lipophilic capability of this metal, methylmercury is bioaccumulated and biomagnified in the food chain, and as a result, species such as bats and humans may get exposed to contaminants (Zukal et al., 2015; Eagles-Smith et al., 2018). The spleen is susceptible

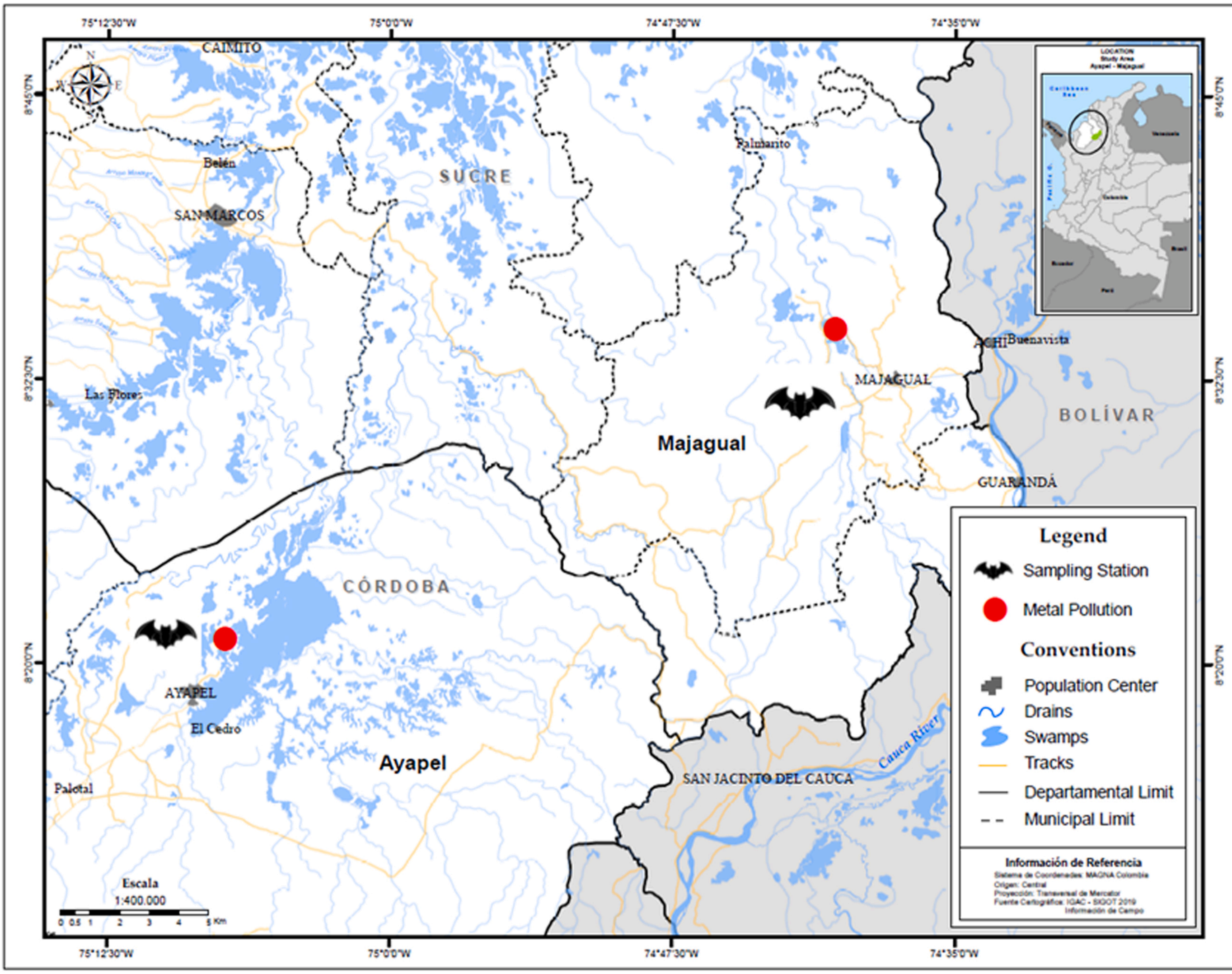

Fig. 1. Localization of the research area, La Mojana Region, Colombian Caribbean.

to toxicity caused by heavy metals, and it has been confirmed that the spleen's oxidative stress and inflammatory responses could be caused by metals like mercury (Zukal et al., 2015).

Mercury quantification was done by atomic absorption spectroscopy (DMA80 TRICELL, Milestone Inc, Italy), following EPA 7473 method (EPA-US, 2007) by weighing 0,1g of sample. Quantification in the machine was carried out using a calibration curve and obtaining a determination coefficient of 0,9997. Limit of detection (LOD) was determined three times the standard deviation of 10 blank sample measurements was 0,05 ng Hg. This method was evaluated by analyzing triplicates' reference material DORM-2 (dogfish muscle, from the National Research Council, Canada, certified values =4,64±0,26 μg/g dry weight). The recovery percentage for DORM-2 was 98,2% with a variation coefficient lower than 5%, between the 95% confidence intervals (Marrugo-Negrete et al., 2019).

2.3. Statistical analysis

The difference between the species' feeding habits was evaluated for the variables, micronuclei and mercury concentration in the liver and spleen. The Shapiro-Wilk statistical test was used to determine the normality of the data obtained. As the data did not have a normal distribution, the Kruskal-Wallis test evaluated statistical differences. A Sperman correlation analysis (r) was performed to determine correlations between the THg concentrations in tissues and frecuency of micronuclei. In addition, for these variables, the confidence interval was estimated for each of the sampled localities. Statistical significance was defined as $p<0,05$.

3. Results

Forty-five bat samples were analyzed in of Ayapel (n = 20) (longitude 8° 17′53.8 ''; latitude -75° 9′20.6′') and Majagual (n = 25)

Table 1
Bat species and their main diet in Majagual and Ayapel.

Species	Dietary guild	Majagual	Ayapel	Total
Artibeus lituratus	Frugivorous	1	2	3
Artibeus planirostris	Frugivorous	7	6	13
Carollia perspicillata	Frugivorous	2	5	7
Eptesicus brasiliensis	Insectivorous	1	0	1
Eumops glaucinus	Insectivorous	0	1	1
Molossops temminckii	Insectivorous	1	0	1
Molossus molossus	Insectivorous	5	2	7
Phylostomus discolor	Omnivorous	5	3	8
Saccopteryx leptura	Insectivorous	1	0	1
Sturnira lilium	Frugivorous	2	0	2
Uroderma bilobatum	Frugivorous	0	1	1
Total		25	20	45

(longitude 8° 32′35.7′'; latitude -74° 34′31′'). Table 1 shows the bat species, their primary diet, and the number of specimens captured in Ayapel and Majagual. The most representative species of the two municipalities studied were *Artibeus planirostris*, followed by *Phylostomus discolor* and *Molossus molossus*, the latter with greater frequency in Majagual.

The micronucleus count in peripheral blood showed genotoxic damage, demonstrated with Giemsa and ethidium bromide stains (Fig. 2)

The insect-eating bats had higher micronuclei count and a higher mercury concentration in the liver and spleen. Table 2 shows the results by types of feeding. There is a statistical difference in the group of insectivores compared to the other dietary guild studied, as the principal component analysis shows (Fig. 3).

In the liver and spleen of the bats, mercury concentrations were higher in Ayapel than in Majagual. Values of 0,833 μg/g and 0,712 μg/g were found in the liver and spleen, respectively. Regarding the number of micronuclei / 10,000, the highest genotoxic damage values occurred in Majagual bats' organs (Table 3).

The Spearman correlation between the concentration of mercury and the frequency of micronuclei between both zones showed no statistical difference. It was determined that there is no correlation between the frequency of micronuclei and mercury concentrations in Ayapel (Fig. 4).

4. Discussion

This is the first study in Colombia that shows that bats could act as sentinels to genotoxic agents of chemical origin present in the environment. The micronucleus test application allows evaluating genotoxic damage in wildlife, although, in bats, it has been little used (Meehan et al., 2004). Genotoxic damage as a biomarker has been widely used in fish and aquatic ecosystems (Jaramillo-García et al., 2020; Hussain et al., 2018). In bats and other mammals, it detects environmental pollution in terrestrial ecosystems (Meehan et al., 2004).

In animals, metals are bioaccumulated and excreted by the liver, and minimal amounts could imply severe organic dysfunctions (Racero et al., 2017). Insectivorous bats occupy high trophic levels, being sensitive to pesticides and other toxins (Jones et al., 2009). The bioaccumulation of pollutants is evidenced in organs such as the liver, where concentrations are slightly higher than the spleen because the liver is the main organ for storage, regulation, and detoxification (Racero et al., 2017). Metals have been reported to bioaccumulate in bats' livers (De Souza et al., 2020; Walker et al., 2007).

The mercury concentrations in the liver in the current study were higher than those reported in a previous study (Racero et al., 2017). The maximum value of mercury was 0,0887 μg/g in an omnivorous *Phyllostomus discolor* bat. In our study, mercury's maximum value was found in the liver of a frugivorous bat, *Artibeus planirostris* (0,833 μg/g). These results indicate the degree of contamination of two nearby areas in the Colombian Caribbean. This study was carried out in an agricultural area of the department of Córdoba, where there is no high exposure to polluting sources such as the gold and nickel exploitation contrary to the La Mojana region. Pollutants reach water bodies and settle in ecosystems and flow through food chains such as plants and insects that manage to develop their life cycles with contaminated water (Racero et al., 2017).

For determining if dietary guild had an effect concerning studied variables, body mass index was estimated (weight/forearm length, FMI) for each collected individual, and Spearman correlations were done in general and for each dietary guild of body mass index versus the variables evaluated in this study, resulting in no significant differences (P > 0,05). This suggests that analyzed variables are presented in any dietary guild and body size. Furthermore, the effect of sex upon studied variables was assessed by applying the Mann-Whitney U test, showing no significant differences (P > 0,05)

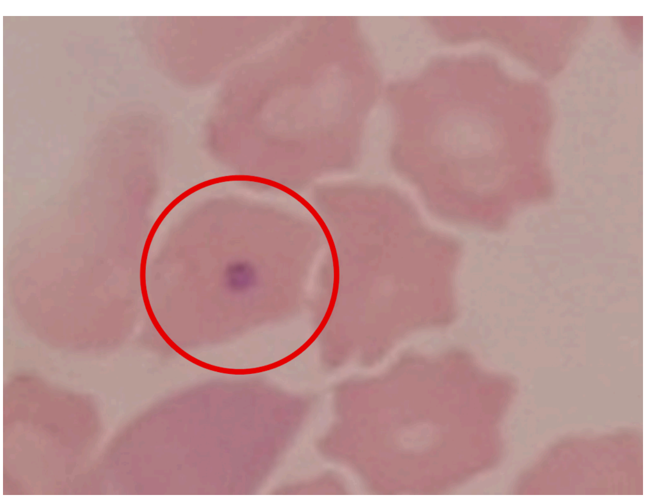

Fig. 2. Peripheral blood smears with Giemsa stain, the cell with micronucleus at the center as observed in an optical microscope (Olympus BX43), and a 100x filter of 505-560 nm/objective.

Table 2
Relationship of the dietary guild and bioindicators in liver and spleen.

Dietary guild	Micronuclei median/10,000 cells	Median [Hg] (μg/g)	
		Liver	Spleen
Frugivorous	13,5^{b}	0,02^{a}	0.03^{a}
Insectivorous	260^{a}	0,23^{a}	0,25^{a}
Omnivorous	20^{a}	0,07^{ab}	0,04^{a}

Medians in the same column with the same letters are not signicantly different (p > 0,05).

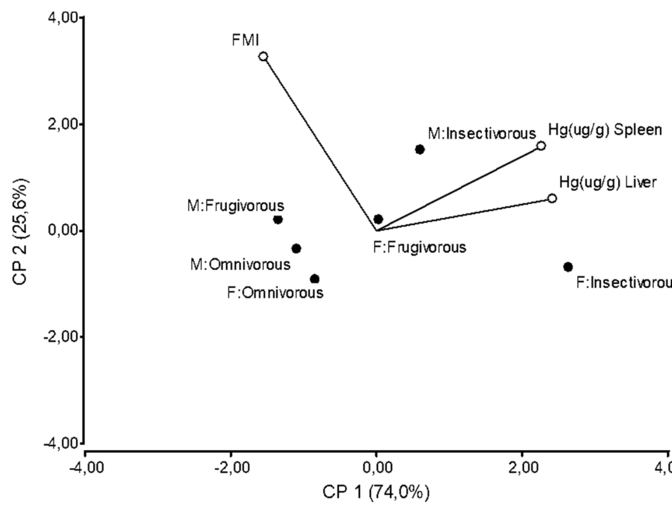

Fig. 3. Biplot graph extracted from principal component analysis (PCA). Body mass index (FMI), Female (F), Male (M), and concentration of mercury in the spleen and liver for different types of feeding bats.

Table 3
Mercury concentration in liver and spleen and frequency of micronuclei in bats from municipalities of Majagual and Ayapel.

		Hg (μg/g) median (min-max.)		Micronuclei/10,000 median (min-max.)
Municipality	N	Liver	Spleen	
Majagual	25	0,049 (0,001 – 0,522)	0,05 (0,001 – 0,412)	29.5 (0 - 677)
Ayapel	21	0,05 (0,009 – 0,833)	0,04 (0,002 – 0,712)	14 (0 - 16)

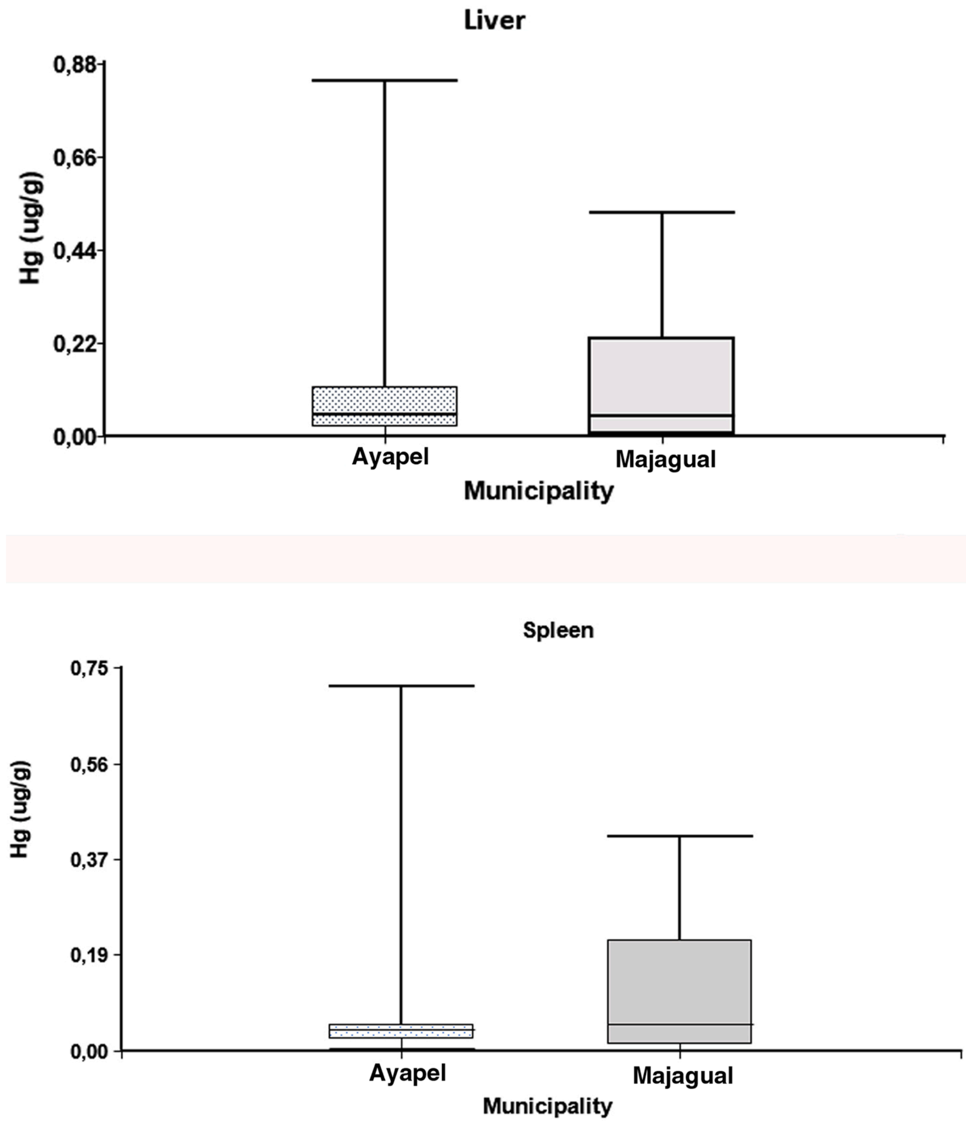

Fig. 4. Total concentration of mercury in the liver and spleen in bats from Ayapel and Majagual.

In the present study, the highest concentration of mercury was found in the liver of insectivorous bats. This result is similar to those obtained in Peru, where the highest bioaccumulation was observed in insectivores and carnivores compared to omnivores and frugivores. Higher concentrations (1,39 ± 0,87μg/g) have been reported in livers of Canadian bats, which may be due to the high concentrations present in the atmosphere (Chételat et al., 2018). Mercury absorption in bats occurs mainly through the digestive tract, so its presence is directly related to the type of diet used (Racero et al., 2017, Walker et al., 2007). Likewise, rains can influence heavy metal concentrations due to increased food availability (Racero et al., 2017). Bat species that feed in agricultural areas are exposed to pesticides by ingesting fruits (De Souza et al., 2020; Oliveira et al., 2017) or contaminated insects (Bayat et al., 2014; Stahlschmidt et al. 2017).

On the other hand, by presenting an average frequency of at least six micronucleated erythrocytes (MNEs), bats can be considered environmental genotoxic bioindicators non-sinusoidal spleen that does not filter old, abnormal erythrocytes or with inclusions such as micronuclei (Kuzukiran et al., 2021; Benvindo-Souz et al., 2019b). In the present study, insectivorous bats presented the highest frequency of 260 MND compared to other studies (Olopade et al. 2020), where an increase in micronuclei and other genotoxicity indicator cell levels was reported. The presence of micronuclei has been reported in frugivorous bats sampled in agricultural and urban environments, as well as insectivorous bats from urban areas (Benvindo-Souz et al., 2019b). The results obtained in the current study demonstrate the presence of genotoxic damage in bats.

Regarding the two sampled areas of the Mojana region, it was possible to show that the area with the most severe genotoxic damage was Majagual, with the highest frequency of micronuclei in bats' blood cells. The genotoxic damage in Majagual is probably present due to the small concentrations of mercury and other pollutants, such as the pesticides used indiscriminately in the agricultural production of that region (Morante and Negrete, 2018). A research carried out in 3 cave colonies in Colima and Jalisco, center-west Mexico, reported a high frequency of micronuclei in bats with different exposition levels to pesticides, demonstrating the genotoxicity of these contaminants (Sandoval-Herrera et al., 2021). The risks to human populations associated with contamination in this region have been previously documented, finding high mercury concentration in fish, rice, and the inhabitants' hair (Marrugo-Negrete et al., 2018).

Pesticides could put the bat population at risk if conservation and environmental awareness strategies are not implemented (Vargas et al., 2012). Exposure to these pollutants poses a threat that decreases bat populations (Bayat et al., 2014). Exposure to heavy metals affects energy metabolism and tissue damage (Ferrante et al., 2018). The risks associated with pesticides' persistent exposure are similar to heavy metals due to their transfer and bioaccumulation along the food chain (Bayat et al. 2014; de Souza et al., 2020). It is urgent to implement acceptable agricultural production practices to reduce the indiscriminate use of pesticides (Varona et al., 2005; Sánchez et al., 2019).

In the Ayapel area, mercury concentrations were higher than in Majagual. It is likely because the area is affected by atmospheric exposure to mercury generated during the non-technical and illegal extraction process of gold in southern Bolívar, North Antioquia, and in the San Jorge River basin Córdoba. Besides, there is nickel mining located in the municipality of Montelibano, where activities are carried out that can release mercury into the environment, such as the removal of the earth's crust and the burning of fossil fuels such as carbon (Marrugo-Negrete et al. 2015).

In conclusion, the bats of La Mojana act as bioindicators by presenting genotoxic damage due to environmental pollutants. The high mercury concentrations indicate the bioaccumulation present in these animals, which threatens ecosystem sustainability considering the multiple functions of this group of flying mammals.

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