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Abstract:

Mercury (Hg) is a contaminant that is impacting ecosystems worldwide. Its toxicity is threatening wildlife and human populations, leading to the necessity of identifying the most affected ecosystems. Therefore, it is essential to identify pertinent bioindicator organisms to monitor Hg contamination. In this study, we determined the stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios in the red blood cells (RBCs), and the total Hg concentration in total blood of 72 *Melanosuchus niger* in French Guiana. The goals of our study were to assess the level of Hg contamination in total blood of Black caimans and to further investigate the influence of individual traits (i.e., sex, size/age, diet) on Hg concentrations. Mercury concentration in total blood of Black caimans ranged from 0.572 to 3.408 $\mu g.g^{-1}$ dw (mean ± SD is 1.284 ± 0.672 $\mu g.g^{-1}$ dw) and was positively correlated to individual body size and trophic position ($\delta^{15}N$). We did not find any sexual or seasonal effects on Hg concentrations in the blood. The use of blood of *M. niger* is relevant to determine Hg concentrations within the population and suggests that this species can be used as a bioindicator for environmental contamination. In addition, our results emphasize trophic position as a major source of Hg variation and further suggest that it is essential to take trophic position ($\delta^{15}N$) into account for future studies.

Keywords: Crocodilians, Trace metals, Tropical ecosystems, Diet, Bioaccumulation

1. Introduction

Mercury (Hg) is one of the major contaminants that affect human and wildlife around the world with increasing levels due to anthropogenic activities (Ericksen *et al.*, 2003; Scheuhammer and Sandheinrich, 2007; Hsu-Kim *et al.*, 2018). In anoxic conditions, microorganisms can transform inorganic Hg into methylmercury (MeHg), the most toxic and bioavailable form of Hg (Compeau and Barta, 1985; Benoit *et al.*, 2003). Because of its strong bioavailability, MeHg is highly absorbed and retained in biota. Hence, it accumulates within organisms during their lifetime and biomagnifies through food webs, resulting in an increasing Hg contamination level throughout most living organisms (Mason *et al.*, 1995; Atwell *et al.*, 1998; Power *et al.*, 2002).

Due to their life history traits, crocodilians (caimans, true crocodiles, alligators, gharials) are potentially good bioindicators of environmental contamination. Indeed, they are long-lived predators, resulting in the accumulation of Hg over a lifespan of several decades. As ectothermic vertebrates, crocodilians display relatively low metabolic rates, but relatively high tissue conversion rates; two features that are expected to favor the bioaccumulation of significant levels of Hg (Cook *et al.*, 1989; Camus *et al.*, 1998; Jagoe *et al.*, 1998; Twining *et al.*, 1999; Schneider *et al.*, 2015; Lázaro *et al.*, 2015; Nilsen *et al.*, 2017). Maternal transfer, an elimination pathway of Hg, which is already known from a variety of reptiles, can also be found in crocodilians (Sakai *et al.*, 2000; Day *et al.*, 2005; Nilsen *et al.*, 2020). However, some studies reported similar Hg concentrations in males and females, whereas females that have already reproduced should theoretically have lower concentrations than males (Burger *et al.*, 2000; Eggins *et al.*, 2015; Nilsen *et al.*, 2020).

The geographic range of caimans is altered by intense gold mining, an anthropogenic activity that is a major source of Hg deposition to the aquatic ecosystems of equatorial South America, and represents approximately 70% of local Hg emissions that are increasing the availability throughout food webs (De Lacerda, 2003; Rocha *et al.*, 2018; Ottenbros *et al.*, 2019). Contrarily to highly mobile individuals such as migratory birds or fishes (Fréry *et al.*, 2001; Fort *et al.*, 2014), crocodilians are rather sedentary (Hutton, 1989; Magnusson *et al.*, 1991; Fujisaki et al., 2014; Caut *et al.*, 2019). Hg concentrations in their tissues reflect the contamination of their environment at a relatively small and precise spatial scale when measured in the blood. In equatorial South America, this taxon could provide the opportunity to assess environmental Hg contamination due to its wide distribution. In addition, sample collection from crocodilians is rather uncomplicated as there is sufficient tissue (i.e., blood, scutes, claws) that can be sampled with comparatively little impact on individuals.

Stable carbon and nitrogen isotope analyses provide information on the diet composition and the trophic position of organisms via the variation of $\delta^{13}C$ and $\delta^{15}N$ levels. Carbon stable isotopes ($\delta^{13}C$) are used as a proxy to discriminate different types of habitat and to provide information on primary production (Pinnegar and Polunin, 2000; Post, 2002). Nitrogen stable isotopes ($\delta^{15}N$) are discriminating the trophic position, as consumers are predictably enriched in ^{15}N in relation to their diet (Minagawa and Wada, 1984, Post, 2002; Vanderklift and Ponsard, 2003). Among crocodilians, it has been shown that increased values of $\delta^{15}N$ reflect a change in the trophic position, linked to a change in their diet (Radloff *et al.*, 2012; Bontemps *et al.*, 2016; Caut *et al.*, 2019). Therefore, adding analyses of stable carbon and nitrogen isotopes to the quantification of Hg levels is extending information on the feeding habitat and trophic positions.

In this study, we assessed Hg concentrations in the only known population of Black caimans (*Melanosuchus niger*) in French Guiana (De Thoisy *et al.*, 2006). This French territory suffers from illegal, artisanal, small-scale gold mining. The goals of our study were to determine the levels of Hg contamination in the blood of Black caimans and the factors influencing the Hg concentrations. We investigated a potential variation between seasons, and the influence of size, sex and foraging ecology on the individual contamination level.

2. Material and Methods

2.1. Study area

The study was conducted in the Nature Reserve "Réserve Naturelle Nationale de Kaw-Roura", French Guiana (4°36'N, 52°07W) (*Fig.1*), a 94.700 ha protected area situated approximately 90 Km southwest of the city of Cayenne. Animals were captured in "Agami Pond", an area situated in the middle of the Nature Reserve, (04°38'N, 52°09'W), a patchwork of herbaceous savannah, swamp forest and open water. Black caimans were sampled during three sampling periods: once during dry season (October 2013), and two samplings during rainy season (May 2014 and May 2015). Caimans were located at night between 19:00pm to 04:00am, using a head lamp, and further captured with a noose.

2.2. Sample collection

A total of 72 individuals were captured, among which 49 adults and subadults were sexed with a ratio of 30 males and 19 females. The body (from the tip of the snout to the cloaca) and total length (including the tail) of each individual were measured with a flexible ruler. We collected a blood sample (~2 ml) through occipital venous sinus puncture, using a syringe with a 30

gauge heparinized needle (heparin sodium). Black caimans were released at the location of their capture directly after biometric measurements and sample collection were performed. Blood samples were separated as following: 1 mL of each blood sample was centrifugated in order to separate the red blood cells (RBCs) and plasma for isotopic analyses (see below), an additional 1 mL of the total blood was kept in 70 % alcohol until further processed at the laboratory for Hg assays. Samples were initially collected to investigate the dietary ecology of Black caimans (Caut *et al.*, 2019). We took the opportunity to further use this sampling set to investigate the Hg concentration in the blood of *Melanosuchus niger*. As a result, the protocol that had been originally applied did not allow us to assess water blood content, and Hg values are therefore presented as dry weight (dw) of the total blood (see below).

2.3. Mercury analysis

One mL of each total blood sample was freeze-dried and grounded to a fine powder. Total Hg concentration (hereafter Hg) in the blood was determined by direct measurement using an atomic absorption spectrometer AMA-254 (Advanced Mercury Analyser-254; Altec®). Analyses were made on at least two replicates of ~3.0 mg dry weight (dw) for each individual. The reproducibility for duplicate samples was approved when Relative Standard Deviation (RSD) was below 10%. The method was validated by the analyses of certified reference material (CRM) produced by the National Research Council of Canada: TORT-2 (Lobster hepatopancreas; certified Hg concentration: 0.27 ± 0.06 $\mu g.g^{-1}$ dw) and TORT-3 (Lobster hepatopancreas; certified Hg concentration: 0.29 ± 0.02 $\mu g.g^{-1}$ dw). CRMs were analyzed at the beginning and at the end of the analytical cycle, and between every 10 samples (Chouvelon *et al.*, 2009). Recovery rates of certified reference material were 97.3 ± 1.0 % for TORT-2 (n=4) and 102.0 ± 1.5 % for TORT-3 (n=5). Blanks were included at the beginning of

each analytical run and the limit of quantification was 0.05 ng. Hg concentrations in caiman blood are presented in $\mu g.g^{-1}$ dw.

2.4. Stable isotope analysis

An analysis of nitrogen and carbon stable isotopes was conducted on red blood cells (RBCs) separated from plasma by centrifugation. RBC samples were freeze-dried and then grounded to a fine powder. Aliquots of 0.3-0.4 mg were placed in tin capsules. Stable isotopes were analyzed using a mass spectrometer (IsoPrime 100, Isoprime, UK) associated to a C-N-S elementary analyser (vario MICRO cube, Elementar, Germany). Stable carbon and nitrogen isotope ratios are expressed as $\delta^{15}N$ or $\delta^{13}C = [(R_{sample}/R_{standard})-1]x1000$, where R is $^{15}N/^{14}N$ or $^{13}C/^{12}C$ for $\delta^{15}N$ or $\delta^{13}C$. IAEA-CG-6 (-10.4‰) was used as a standard reference for carbon, and IAEA-N1 (+0.4‰) for nitrogen. Ten replicate assays of internal laboratory standards indicated maximum measurement errors (SD) of ± 0.2‰ and ± 0.15‰ for the nitrogen and carbon isotope measurements, respectively. Further details on isotope analysis are available in Caut *et al.*, 2019.

2.3. Statistical analyses

All analyses were performed using the software R, v.3.2.4 (*R development Core Team 2013*). The data was first checked for normality and homogeneity of variances. The relationship between Hg concentration and animal total length was assessed by parametric linear regression. Paired t-tests were used to compare Hg concentrations between the sex, and the season. A linear regression model was used to determine the relationship between Hg concentrations, $\delta^{15}N$ and $\delta^{13}C$ values and to further determine the relationship between

caiman length and δ^{15}N and δ^{13}C values. The significance for statistical analyses was always set at $p < 0.05$.

3. Results

The Hg concentrations in the 72 sampled individuals ranged from 0.299 to 3.408 $\mu g.g^{-1}$ dw (Table 1). There was a significant, positive relationship between Hg concentration and total body length (Linear regression, $F_{1,70} = 92.37$, $p < 0.0001$, $r^2 = 0.56$, Fig. 2). Adult females and males had a similar size range, respectively 162.9 ± 60.4 cm and 171.0 ± 53.1 cm (Paired t-test, $t = 0.48$, $p = 0.64$) and displayed similar Hg concentrations, respectively 1.660 ± 0.694 $\mu g.g^{-1}$ dw and 1.459 ± 0.502 $\mu g.g^{-1}$ dw (Paired t-test, $t = 1.09$, $p = 0.28$, Fig. 3).

Seasons (dry and rainy) did not influence Hg concentration (Paired t-test, $t = 0.38$, $p = 0.70$), with values of 1.499 ± 0.440 $\mu g.g^{-1}$ dw for dry season and 1.559 ± 0.661 $\mu g.g^{-1}$ for rainy season. The δ^{15}N was significantly and positively related to caiman total length (linear regression, $F_{1,70} = 68.33$, $p < 0.0001$, $r^2 = 0.49$) and Hg concentration (linear regression, $F_{1,70} = 58.74$, $p < 0.0001$, $r^2 = 0.45$, *Fig.4a*). However, the δ^{13}C was not related to caiman total length (linear regression, $F_{1,70} = 0.86$, $p = 0.36$, $r^2 = -0.002$) nor Hg concentration (linear regression, $F_{1,70} = 1.13$, $p = 0.27$, $r^2 = -0.003$, *Fig.4b*).

4. Discussion

Our results show that Black caimans in French Guiana bioaccumulate Hg. As a consequence, Hg concentration increases with body size and is further linked to their trophic position (δ^{15}N). We did not find any sexual or seasonal correlation with the Hg concentration.

Blood is a universally used matrix to measure Hg exposure in a wide array of organisms (Lommel *et al.*, 1992, Henny *et al.*, 2002, Eggins *et al.*, 2015). Our results show a significant positive relationship between the total length of Black caiman and the Hg concentration in the blood (Fig. 2). Mercury is being transported through the blood to different tissues, such as those involved in detoxification (mainly the liver), storage (muscles), excretion (kidneys) and elimination (keratinized tissues). In reptiles, Hg concentrations of blood are related to Hg concentrations of internal tissues because of the dynamic transfer between these matrices (Burger *et al.*, 2007; Eggins *et al.*, 2015; Nilsen *et al.*, 2017). Therefore, Hg values in the blood reflect an overall Hg concentration in the internal tissues. Our results also confirm the usefulness of blood to determine the Hg contamination in caimans (Eggins *et al.*, 2015; Marrugo-Negrete *et al.*, 2019).

Several studies in crocodilians already reported a linear increase of Hg with age in various tissues (Yanochko *et al.*, 1997; Burger *et al.*, 2000; Rumbold *et al.*, 2002; Schneider *et al.*, 2012). Age and size are generally correlated (i.e. Eaton and Link, 2011), yet, in wild populations, detailed information on age is rarely available. The relationship between crocodilian size and Hg concentration in tissues such as blood, scutes, claws, muscles and liver shows that Hg is bioaccumulated across the life of an individual (Burger *et al.*, 2000; Schneider *et al.*, 2015; Lázaro *et al.*, 2015; Marrugo-Negrete *et al.*, 2019). Although the Hg concentration in various tissues and the body size are positively correlated in some crocodilian species (i.e., *Alligator mississippiensis, Caiman crocodilus, Melanosuchus niger, Caiman yacare*), we emphasize that this pattern has not been detected in other species (i.e., *Crocodylus acutus, Crocodylus moreletii*, Yanochko *et al.*, 1997; Burger *et al.*, 2000; Rainwater *et al.*, 2007; Schneider *et al.*, 2015; Lázaro *et al.*, 2015; Marrugo-Negrete *et al.*, 2019). Such divergent

findings may highlight the importance of relatively large sample sizes associated with significant body size ranges in order to robustly assess the relationship between Hg and individual traits. Mercury concentrations are significantly linked to δ^{15}N values (a proxy used to discriminate the trophic level) (Fig. 4a). Results indicate that the Hg concentration is depending on the ontogenetic change in the trophic position in *M. niger* (Caut *et al.,* 2019). Our results do not show a relationship between Hg concentration and δ^{13}C (a proxy used to discriminate different types of habitat) for *M. niger* (Fig. 4b). This result shows that the ontogenetic change in the foraging ecology of *M. niger* does not induce a change in foraging habitats in the studied population. We did not find any seasonal influence on Hg concentrations, suggesting that the trophic ecology of *M. niger* at our study site does not significantly vary across seasons.

We did not find any variation in Hg concentrations between males and females (Table 1, Fig. 3). A possible mechanism of Hg elimination in females is the maternal transfer of Hg to the eggs: Females use their energy storage (e.g. body fat and proteins) during vitellogenesis, which may induce a transfer of the Hg stored in their tissues towards their eggs (Nilsen *et al.*, 2020) as reported for birds for instance (e.g., Lewis *et al.*, 1993). This process would lead to lower concentrations in the blood of females that recently laid eggs, compared to males. Therefore, our results suggest that either 1), Hg remobilization did not occur, or more likely, 2) this process is not significant enough to be detected in the blood of female Black caimans. Future studies should investigate the level of Hg transfer to the eggs during vitellogenesis in female *M. niger*, as well as in other crocodilian species.

It is important to emphasize that the relatively low turn-over rates of erythrocytes in crocodilians may have obscured putative influences of sex and season on Hg levels. Indeed,

blood is known to reflect short-time Hg exposure in birds or mammals (Bearhop *et al*., 2000). For instance, the lifetime of erythrocytes is up to two months in birds and up to four months in mammals (reviewed in Rodnan *et al*., 1957; Monteiro and Furness, 2001). In crocodilians, the lifetime of erythrocytes can last up to 3 years (Cline and Waldmann, 1962). Future studies are required to assess whether low turn-over rates of erythrocytes in crocodilians influence short-scale, temporal variations in Hg concentrations.

Mercury concentrations in the blood of *M. niger* in French Guiana (1.284 ± 0.672 $\mu g.g^{-1}$ dw) are higher than in other South American species with a maximum value of 0.325 ± 0.105 $\mu g.g^{-1}$ dw (Table 2; Marugo-Negrete *et al*., 2019). However, limited data is available from this geographic area (Table 2). The values we report in the blood of *M. niger* are similar to what has been determinated in the muscle of the same species in Brazil (Table 2). This is especially interesting as Hg concentrations in the muscle usually tends to be higher than in the blood (Schneider *et al*., 2012). Insufficient data on Hg in crocodilians from the Americas make comparison difficult, except for the United States where the American alligator is well documented (Table 2). Clearly, future studies are required in order to provide a complete background to perform substantial comparisons. In addition, the use of other tissues where sampling is less invasive (i.e.., claws and scutes, Lázaro *al*., 2015; Marrugo-Negrete, *et al.,* 2019), should be considered, for it is already a conventional method in other reptile species (e.g., Slimani *et al*., 2018; Lemaire *et al.*, 2018; Beau *et al.*, 2019).

5. Conclusion

Overall, the use of blood of *M. niger* is informative regarding the Hg concentration and it extends the use of Crocodilians to monitor the environmental Hg contamination. To do so,

precise individual information (e.g., size and diet or trophic position) is required. For instance, the change of the trophic position has a significant impact on the level of Hg contamination between juveniles (feeding on low trophic level prey) and adults (feeding mostly on high trophic level prey). Therefore, it is essential to take their diet into account to compare levels of Hg contamination between different sites or populations. Additionally, future studies are required to assess whether the concentrations of Hg we have found in *M. niger* pose a threat to the species.

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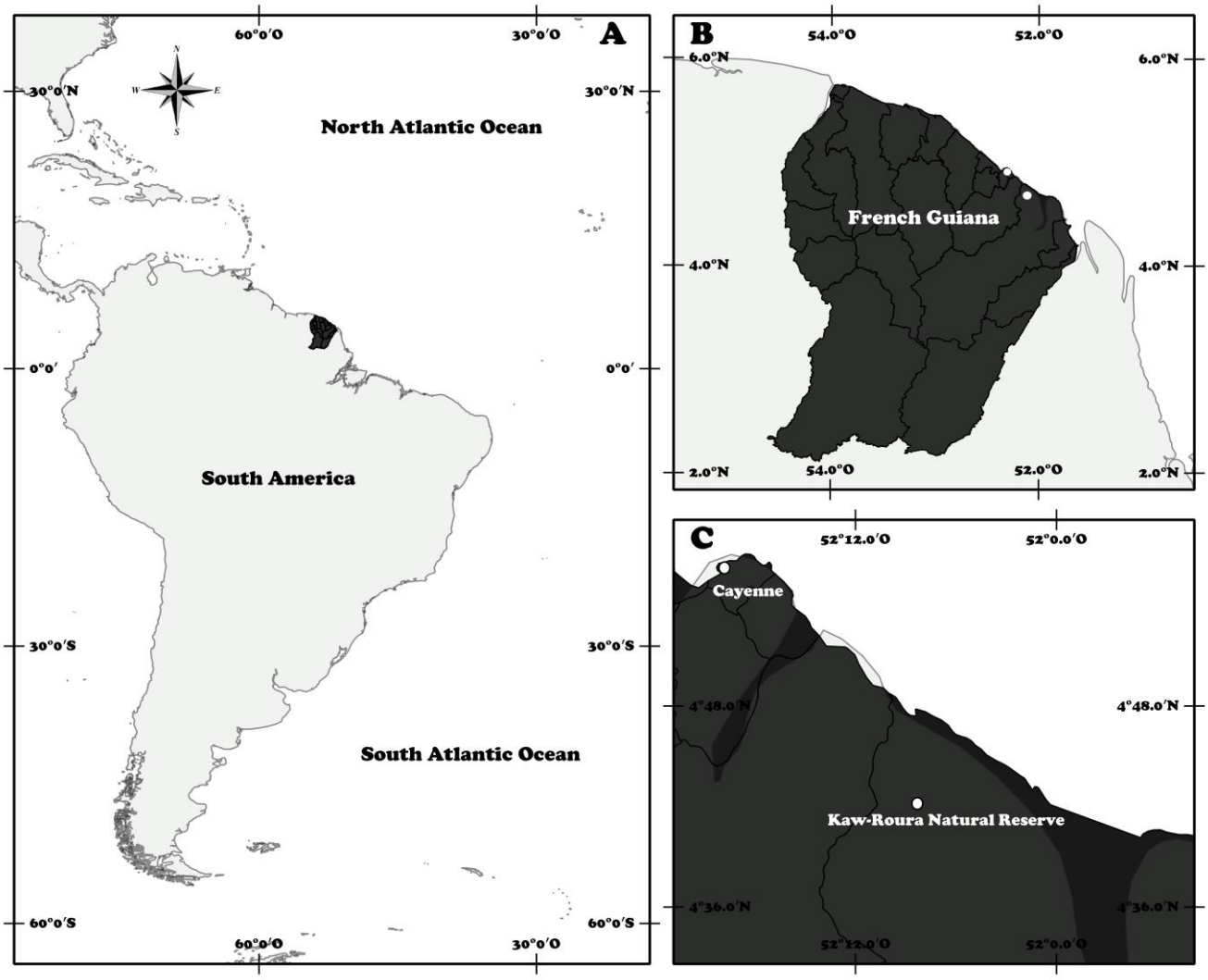

Figure 1. Geographic location of the study site in South America (A), French Guiana (B), Kaw-Roura Nature Reserve (C).

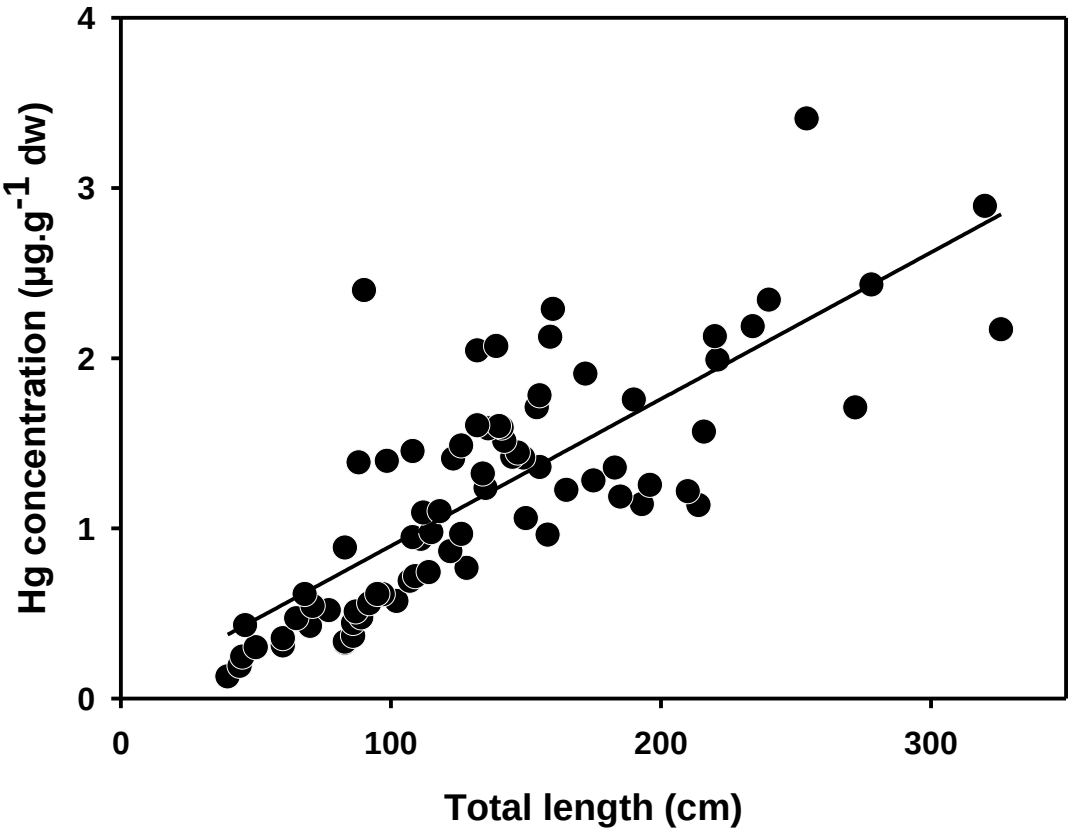

Figure 2. Relationship between the total length (in cm) and Hg concentration (µg.g^{-1} dw) measured in the blood of Black caiman *Melanosuchus niger* from French Guiana (Linear regression, $F_{1,70} = 92.37$, $p < 0.0001$, $r^2 = 0.56$).

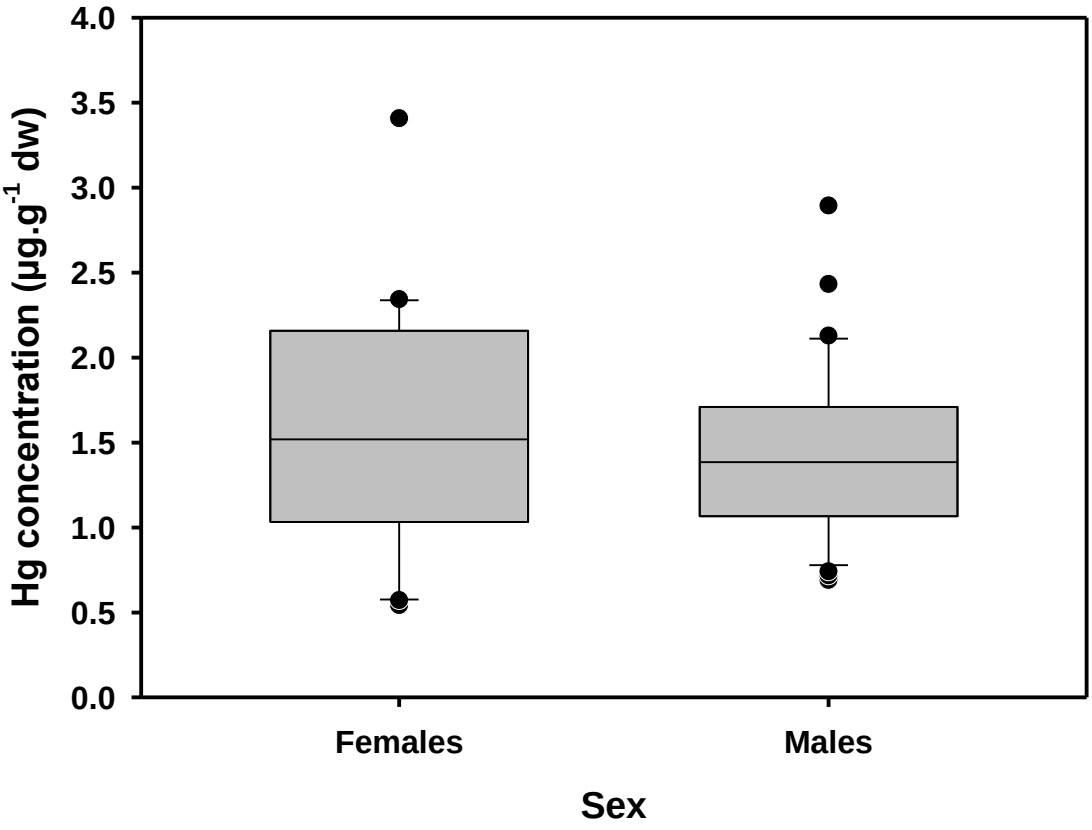

Figure 3. Hg concentrations in the blood of males and females Black caiman *Melanosuchus niger* from French Guiana. The top and bottom of the boxes represent the first and last quartiles, the line across the box represents the median, the whiskers represent the fifth and ninety-fifth percentiles, and the circles represent outliers.

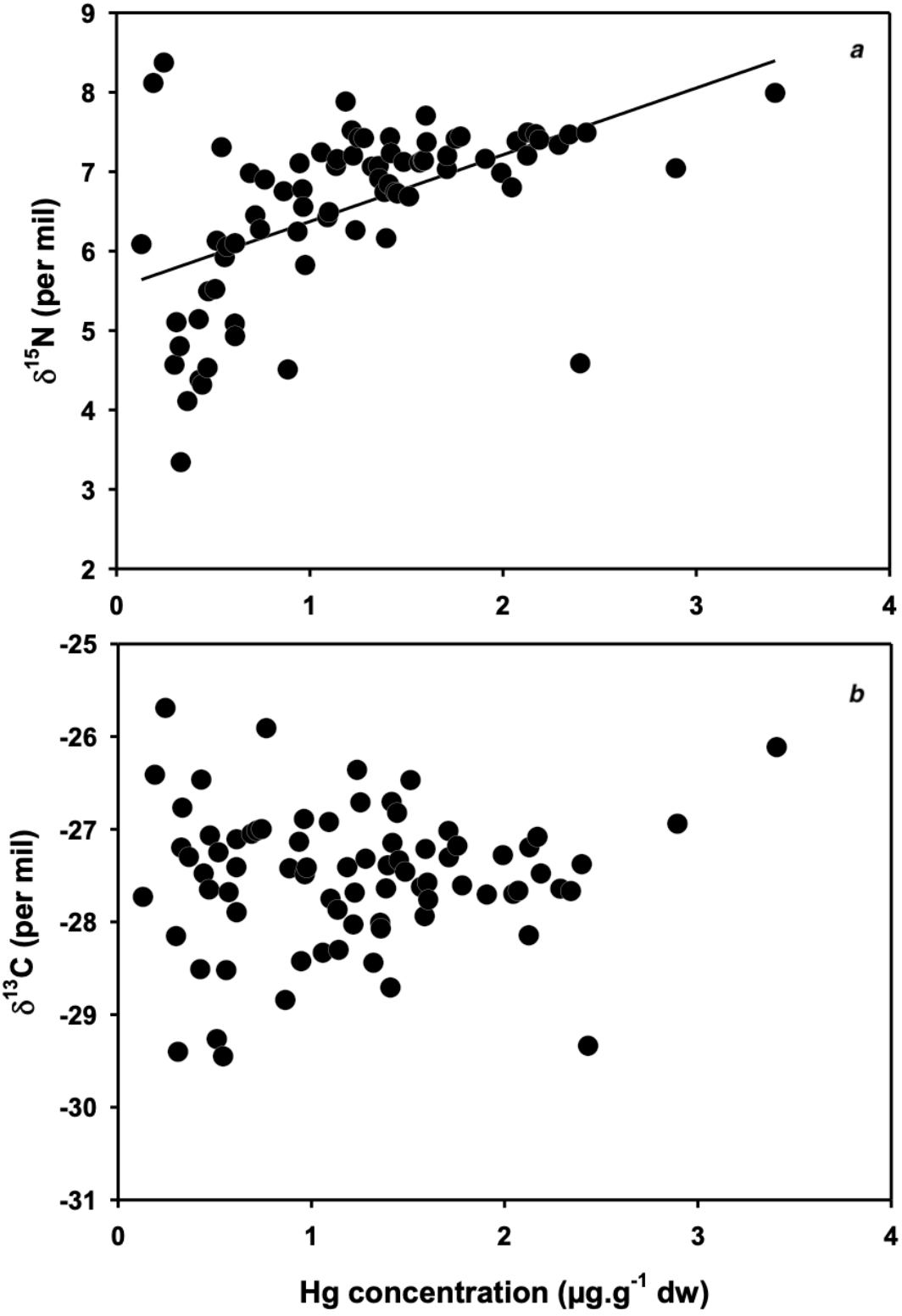

Figure 4. Relationship between Hg concentration in the blood (µg.g^{-1} dw) and stable isotope ratios of (a.) δ^{15}N (‰) (linear regression, $F_{1,70} = 58.74$, $p < 0.0001$, $r^2 = 0.45$) and (b.) δ^{13}C (‰) (linear regression, $F_{1,70} = 1.13$, $p = 0.27$, $r^2 = -0.003$) of Black caiman *Melanosuchus niger* from French Guiana.

Table 1. Sex, total length (cm) and blood Hg concentration (µg g^{-1} dw) of the Black caiman, *Melanosuchus niger*, from French Guiana.

	N (Males/Females)	Total Length		Hg concentrations	
		Mean ± SD	Min-Max	Mean ± SD	Min-Max
Season					
Dry	18 (11/7)	155.8 ± 35.8	114 - 278	1.499 ± 0.440	0.741 - 2.432
Rainy	31 (19/12)	174.9 ± 63.8	95 - 320	1.559 ± 0.661	0.572 - 3.408
Sex					
Male	30	171.0 ± 53.1	109 - 320	1.459 ± 0.502	0.717 - 2.894
Female	19	162.9 ± 60.4	95 - 254	1.660 ± 0.694	0.572 - 3.408

Table 2. Review of Hg concentration (µg.g^{-1} dw) reported in crocodilians. TL stands for Total Length and SVL for Snout-Vent-Length (cm). ([a] Original data reported in wet weight, transformed in dry weight with a factor 3.8 calculated by Jeffree *et al*., 2001 for muscles; [b] original data reported in wet weight, transformed in dry weight using a factor 5).

Species	**Location**	**Tissue**	**n**	**Length (cm)**		**Hg (µg.g^{-1} dw)**		**Reference**
				Mean ±SD	Min-Max	Mean ±SD	Min-Max	
Black caiman	Rio Purus, Brazil	Muscles	11	107.5 ± 31.4 (SVL)	75.3 – 190.9 (SVL)	1.93[a]	0.69 – 4.06[a]	Schneider *et al.*, 2012
Melanosuchus niger								
Black caiman	Rio Purus, Brazil	Muscles	16	102 ± 27 (SVL)	75 – 191 (SVL)	0.669 ± 0.369 [a]	-	Eggins *et al.*, 2015
Melanosuchus niger								
Black caiman	Mamirauá	Muscles	60	107 – 309 (TL)	107 – 309 (TL)	1.457 ± 0.433 [a]	-	Correia *et al.*, 2014
Melanosuchus niger	Reservoir, Brazil							
American Alligator	South Carolina	Total Blood	-	-	-	2.19 ± 0.38	-	Jagoe *et al.*, 1998
Alligator mississippiensis								
American Alligator	Florida	Total Blood	37	92.1 ± 31.6 (SVL)	43.9 – 153.5 (SVL)	0.965[b]	0.280 – 6.900[b]	Nilsen *et al.*, 2017
Alligator mississippiensis								
Spectacled Caiman	La Mojana,	Total Blood	22	57.2 ± 3.5 (TL)	-	0.325 ± 0.105[b]	-	Marrugo-Negrete *et al.*, 2019
Caiman crocodilus	Colombia							
Spectacled Caiman	La Mojana,	Total Blood	23	57.5 ± 6.8 (TL)	-	0.07 ± 0.04[b]	-	Marrugo-Negrete *et al.*, 2019
Caiman crocodilus	Colombia							
Black caiman	Kaw, French	Total Blood	72	143.2 ± 61.3 (TL)	46 – 326 (TL)	1.284 ± 0.672	0.30 – 3.41	Our study
Melanosuchus niger	Guiana							