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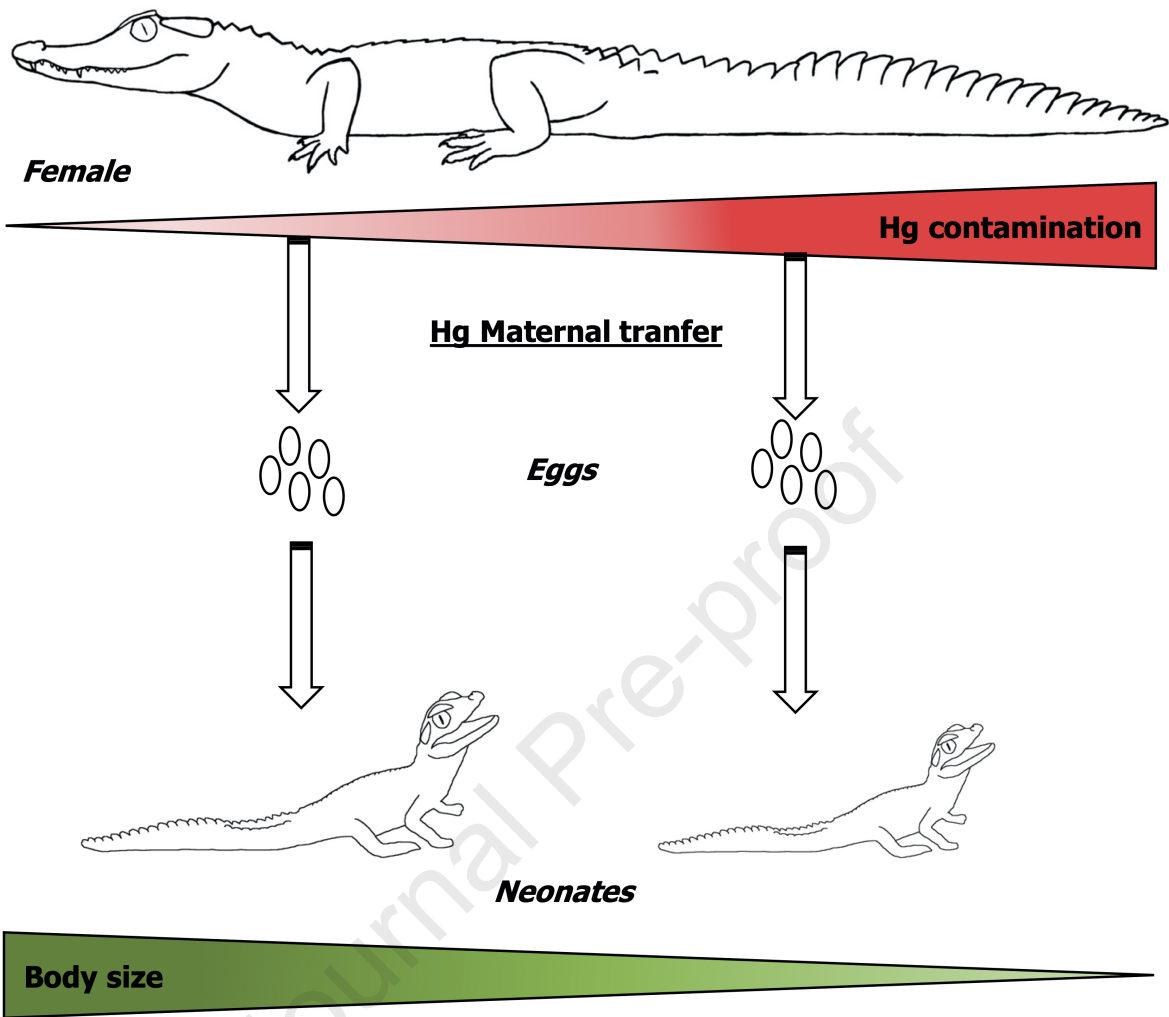
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I got it from my mother: Inter-nest variation of mercury concentration in neonate Smooth-fronted Caiman (*Paleosuchus trigonatus*) suggests maternal transfer and possible phenotypical effects

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Abstract

The deleterious effects of mercury (Hg) contamination are well documented in humans and wildlife. Chronic exposure via diet and maternal transfer are two pathways which increase the toxicological risk for wild populations. However, few studies examined the physiological impact of Hg in crocodilians. We investigated the Hg contamination in neonate Smooth-fronted Caimans, *Paleosuchus trigonatus*, and the use of keratinized tissues and blood to evaluate maternal transfer. Between November 2017 and February 2020, we sampled 38 neonates from 4 distinct nests. Mercury concentration was measured in claws, scutes and total blood. Highest Hg concentrations were found in claws. Strong inter-nest variations (Hg ranging from 0.17 ± 0.02 to $0.66 \pm 0.07 \mu\text{g}\cdot\text{g}^{-1}$ dw) presumably reflect maternal transfer. Reduced body size in neonates characterized by elevated Hg concentrations suggests an influence of Hg during embryonic development. We emphasize the use of claws as an alternative to egg collection to investigate maternal transfer in crocodilians. Our results demonstrated the need of further investigation of the impact of Hg contamination in the first life stages of crocodilians.

Key Words: Trace elements; Claws; Crocodilians; Keratinized tissues; Conservation.

1. Introduction

Mercury (Hg) is a worldwide environmentally dangerous contaminant (Chen *et al.*, 2018). Its chronic effects on humans and wildlife impact reproduction, offspring quality, embryonic development, hormonal synthesis and secretion, cellular respiration, metabolic processes and immune functions, and further cause neurobehavioral and neuronal dysfunctions (Fingerman *et al.*, 1996; Zahir *et al.*, 2005; Bergerons *et al.*, 2011; Hopkins *et al.*, 2013; Schneider *et al.*, 2013; Tartu *et al.*, 2013; Bridges *et al.*, 2016; Landler *et al.*, 2017; Withney and Criscol, 2017).

Since more than 30 years, several studies report Hg contamination in a variety of tissues of alligators, caimans and true crocodiles (e.g. Delany *et al.*, 1988; Jagoe *et al.*, 1998; Burger *et al.*, 2000; Almlí *et al.*, 2005; Vieira *et al.*, 2011; Eggins *et al.*, 2015; Nilsen *et al.*, 2017; Buenfil-Rojas *et al.*, 2020). In certain geographical areas (e.g., Amazon region), the abundant natural Hg in soil and biota (e.g. an average of $0.3 \mu\text{g}\cdot\text{g}^{-1}$ in forest soil in French Guiana, Richard *et al.*, 2000), human activities such as deforestation, gold mining activities and agriculture additionally contribute to increase Hg bioavailability (Roulet *et al.*, 1998; Maurice-Bourgoin *et al.*, 2000, Vieira *et al.*, 2011; Schneider *et al.*, 2012; Correia *et al.*, 2014; Eggins *et al.*, 2015; Lázaro *et al.*, 2015; Schneider *et al.*, 2015; Rivera *et al.*, 2016, Marrugo-Negrete *et al.*, 2019, Lemaire *et al.*, 2021).

Mercury concentrations obtained in crocodilian tissues have been shown to reflect the contamination of the individual's environment across different temporal scales (Lázaro *et al.*, 2015; Schneider *et al.*, 2015). Blood Hg concentration is thought to reflect relatively recent

exposure, while keratinized tissues (e.g., scales and claws) seem to reflect Hg concentration accumulated during longer time periods (Schneider *et al.*, 2015). Despite such tissue-specific variations in Hg burden, ingestion of contaminated food is thought to be the primary source of Hg exposure in crocodylians (Smith *et al.*, 2007; Lemaire *et al.*, 2021). Although relatively elevated Hg concentrations are found in crocodylians (e.g. up to $42.15 \pm 6.64 \mu\text{g}\cdot\text{g}^{-1}$ in liver and 6.33 ± 1.04 in scutes of the American alligator, *Alligator mississippiensis*, Yanochko *et al.*, 1997), few studies have investigated actual impact of Hg contamination on these taxa (Jagoe *et al.*, 1998; Almlı *et al.*, 2005; Nilsen *et al.*, 2017; Marrugo-Negrete *et al.*, 2019). A negative impact of Hg contamination on body condition has been shown in the American alligator *Alligator mississippiensis* (Nilsen *et al.*, 2017) and DNA damages related to Hg exposure have been indicated in the Spectacled caiman *Caiman crocodylus* (Marrugo-Negrete *et al.*, 2019).

In addition to the direct influence of trophic level on an individual's contamination, Hg can also be maternally transferred to the progeny in vertebrate species (Evers *et al.*, 2003; Bergeron *et al.*, 2010; Heinz *et al.*, 2010; Ackerman *et al.*, 2017). In mammals, Hg is transferred directly across the placenta and via lactation during nursing while in reptiles *sensu lato* (i.e., including birds), Hg can be transferred to the eggs during vitellogenesis. The maternal transfer of Hg is a particular source of contamination that is directly related to the female Hg burden (Akearok *et al.*, 2010, Nilsen *et al.*, 2020). Importantly, Hg contamination during early development has been shown to negatively affect embryonic development, embryonic mortality and can have relatively long-lasting effects on the physiology and behavior of neonates (Wolfe *et al.*, 1998; Scheuhammer *et al.*, 2007; Cusaac *et al.*, 2016, Nilsen *et al.*, 2020). Although a recent study reported a positive relationship between the Hg concentration of reproductive American Alligators, *Alligator mississippiensis*, and its eggs

indicating vertical (maternal) transfer of Hg during vitellogenesis (Nilsen *et al.*, 2020), to our knowledge, no study has examined the Hg concentration in hatchling crocodylians yet.

The Smooth-fronted Caiman (*Paleosuchus trigonatus*) is a small neotropical caiman living in rainforest and wetland habitats with a largely unknown ecology and biology (Magnusson and Lima 1991; Lemaire *et al.*, 2018). In French Guiana, few data is available on this species and the regional population is classified as decreasing (IUCN France, 2016). Because of their lifestyle and habitat, *Paleosuchus trigonatus* can be directly impacted by anthropogenic activities such as deforestation and gold mining that both lead to elevated values of available Hg. In this study, we assessed the total Hg concentration in neonate Smooth-fronted Caimans, *Paleosuchus trigonatus*, from different nests in French Guiana in order to investigate inter-nest variations that may be linked to maternal transfer of Hg to the progeny, to document Hg concentration in different tissues (blood, scutes and claws) of hatching caimans and to explore potential relationships between neonate phenotype (body size) at birth and Hg burden.

2. Material and Methods

2.1. Sample collection

From November 2017 to February 2020, we captured 38 neonates from 4 distinct nests in 3 different areas in French Guiana (Fig. 1). Neonates were found and caught in close proximity to the nests (< 5m), indicating that they hatched recently (Magnusson and Lima, 1991). We also collected eggshells and shell membrane remnants from each nest. For each individual, claw and scute samples were clipped using pliers and were then placed in dry plastic

containers; blood samples were collected on a subsample of 7 individuals from 3 of the 4 nests (due to the difficulty of blood sampling in neonates). Blood samples were taken in the tail vein with a heparinized (heparin sodium) needle of 27 gauge - 25 mm for neonates, and 21 gauge - 50 mm for adults. Blood samples were immediately put on ice and further stored at -28 °C. Each individual was measured for Total Length (TL) and Snout-Vent-Length (SVL). At two nest sites, the alleged mothers were caught in a less than 3-meter proximity to the nest. Biometric measurements and blood samples were taken from both adult individuals. All individuals (neonates and mothers) were released at the place of capture after sampling. *Paleosuchus trigonatus* is protected by the French law (Ministerial decree NOR: TREL1933710A of 08/10/2018) and a permission to capture individuals, draw blood, sample claws and scutes and further collect egg remnants was granted by the French authorities (Direction Régionale des Territoires et de la Mer) after evaluation by the CSRPN, the regional scientific committee (Permit: R03-2016-06-21-010; R03-2019-01-09-001; R03-2019-10-24-007, www.guyane.developpement-durable.gouv.fr).

2.2. Sample preparation

Before analysis, claws and scutes were cleaned in an ultrasonic bath for 5 minutes containing ultrapure water and rinsed 3 times to remove all external elements that could contaminate the sample (i.e. mud, sand...). Eggshells and shell membranes were cleaned in ultrapure water with a soft brush and rinsed. Clean samples were placed in a clean container and dried for 48 hours in an oven at a constant temperature of 45 °C. Total blood was freeze-dried to eliminate water and then grounded to a fine powder to avoid the water-mass variation which can occur between different tissues (Yanochko *et al.*, 1997).

2.3. Instrumental method and quality control

The quantification of total Hg (THg) of all samples was determined using an atomic absorption spectrometer AMA-254 (Advanced Mercury Analyser-254; Altec). At least two replicates of 0.2 – 1.0 mg dry weight (dw) were used for scute, blood and shell membrane samples, and 20 mg dw for eggshell samples, until the Relative Standard Deviation (RSD) was below 10% between measurements. Regarding claw samples, two different claws of each individual were used to calculate the RSD. At the beginning and the end of the analytical cycle and after every 10 samples, an analysis of certified reference material (CRM) TORT-3 (Lobster hepatopancreas from the National Research Council of Canada; certified Hg concentration: $0.292 \pm 0.022 \mu\text{g.g}^{-1}$ dw) was performed to validate the method. Measured values for TORT-3 were $0.292 \pm 0.008 \mu\text{g.g}^{-1}$ dw ($n=11$), with a recovery of $99.9 \pm 2.9 \%$. Blanks were included at the beginning of each analytical run and the limit of quantification was 0.05 ng. The Hg concentrations are further expressed in $\mu\text{g.g}^{-1}$ dw.

3. Statistical analysis

All analyses were performed using the software R, v.3.6.1 (*R development Core Team*).

The normality and the homogeneity of variance were first checked, and data were log-transformed when necessary. The comparison of Hg concentration and body size (SVL and TL) in neonates between the 4 nests was assessed by one-way ANOVAs, as well as for shell membranes. We did not perform statistical analysis for eggshells due to absence of variation. In order to assess the relationship between total blood and keratinized tissues, and further between different keratinized tissues, we performed linear regressions. The

relationship between egg membranes and keratinized tissues was performed using the mean of the tissues for each nest. The significance for statistical analyses was always set at $p < 0.05$.

4. Results

For all neonates, Hg concentrations in keratinized tissues were the highest in claws with average concentrations ranging from 0.171 to 0.663 $\mu\text{g}\cdot\text{g}^{-1}$ dw and the lowest in scutes with average concentrations ranging from 0.092 to 0.251 $\mu\text{g}\cdot\text{g}^{-1}$ dw (Table 1). The Hg concentration in eggshells did not vary between nests with a value of 0.001 $\mu\text{g}\cdot\text{g}^{-1}$ dw, while the Hg concentration in shell membranes varied between nests with average concentrations ranging from 0.020 to 0.040 $\mu\text{g}\cdot\text{g}^{-1}$ dw (Table 1). Our results show a significant difference of Hg concentration in claws (ANOVA: $F_{3-34} = 225.44$, $p < 0.001$), scutes (ANOVA: $F_{3-34} = 154.4$, $p < 0.001$) and egg membranes (ANOVA: $F_{3-29} = 42.3$, $p < 0.001$) between the 4 nests (Fig. 2).

Although our sample sizes were low to perform statistical analyses, it is important to highlight that blood Hg concentrations of the alleged mothers seems to relate to Hg burden of their offspring (Table 1).

The Hg concentration in total blood of neonates showed a significant positive relationship with scutes ($F_{1-5} = 30.44$, $R^2 = 0.83$, $p = 0.003$) and claws ($F_{1-5} = 23.68$, $R^2 = 0.79$, $p = 0.005$).

Mercury concentration in claws showed a significant positive relationship with scutes ($F_{1-36} = 334.2$, $R^2 = 0.90$, $p < 0.001$). Egg membranes showed a significant positive relationship with claws ($F_{1-2} = 21.15$, $R^2 = 0.870$, $p = 0.044$) and scutes ($F_{1-2} = 105.7$, $R^2 = 0.972$, $p = 0.009$).

A significant difference of SVL and TL of neonates between the 4 nests was found (respectively, ANOVAs: $F_{3-34} = 8.5$, $p < 0.001$ and $F_{3-34} = 17.0$, $p < 0.001$); with nests with

individuals having higher Hg concentrations having produced smaller hatchling caimans both in terms of SVL and TL (Fig. 3, data not shown for SVL).

5. Discussion

Overall, we found relatively high Hg concentrations in hatchling caimans. Our results show that inter-nest differences in Hg concentration may indicate that this metal is transferred from the mother during vitellogenesis. This process seems further supported by the relationship between alleged mother and offspring Hg concentrations on a limited sample. Finally, we showed that nests having higher Hg concentrations produced smaller hatchling caimans.

5.1. Hg concentrations in the tissues

Our results show relatively high concentrations of Hg in the keratinized tissues of *Paleosuchus trigonatus* neonates (Table 1, Fig. 1). The results are similar to what is found in the keratinized tissues of *Caiman yacare* (between 95.7 ± 92.2 and $0.263 \pm 0.158 \mu\text{g}\cdot\text{g}^{-1}$ in scutes, Lázaro et al., 2015) and *Caiman crocodilus* (between 0.131 ± 0.038 and $0.647 \pm 0.547 \mu\text{g}\cdot\text{g}^{-1}$ in claws, Marrugo-Negrete et al., 2019) sub-adults in South America. Keratinized tissues are known to accumulate Hg due to its affinity to cysteine residues in beta-keratin, where Hg fixes on SH groups (Alibardi, 2003; Alibardi and Toni, 2007). In our samples, claws had 2.1 times higher Hg concentrations than scutes which reflect the fact that our scute samples were composed of keratin layer as well as the underlying connective tissues with presumably lower Hg concentrations. Keratinized tissues, by the immobilization of Hg, reflect the long-term Hg exposure in crocodylians (Lazaro et al., 2015; Schneider et al., 2015).

The significant relationship between Hg concentrations in the total blood and the keratinized tissues claims for the use of keratinized tissues as a less invasive sampling method to provide information on Hg concentration in caimans. In the specific case of neonates, in which blood sampling can be complicated due to their small body size, keratinized tissues such as claws or scutes provide an efficient sampling method to assess Hg exposure. In addition, the use of keratinized tissues in hatchling crocodiles could be an alternative to egg collection to investigate maternal transfer in crocodiles, which is particularly important for species with declining populations. Finally, Hg concentration in egg membranes seems to be related to Hg concentration in keratinized tissues and could therefore provide an additional less-invasive sampling method.

5.2. Hg concentration between nests

In neonates, keratinized tissues reflect Hg contamination due to maternal transfer during vitellogenesis and the exposure during embryonic development. Our results show a significant variation of Hg in claws, scutes and shell membranes of *Paleosuchus trigonatus* neonates between different nests. The Hg variations observed in neonates between different nests are likely the result of maternal transfer and presumably indicate that the most contaminated female produced the most contaminated eggs. Maternal transfer of Hg into eggs has already been documented in several species where the concentration found in the eggs is directly influenced by the female Hg burden, which depends on its habitat and diet (Heinz et al., 2010; Ackerman et al., 2017).

Mercury concentrations differ between egg compartments, with higher concentration found in the protein-rich albumen compared to the lipid-rich yolk. In crocodylians, several

studies reported Hg contamination in different egg compartments (Rainwater *et al.*, 2002; Xu *et al.*, 2006; Du Preez *et al.*, 2018), but the relationship with the female Hg burden was never investigated. The first confirmation of vertical Hg transfer from the female to the eggs was recently shown in crocodylians with a concentration of Hg in egg yolk corresponding at approximately 12.5% of the Hg concentration in the blood of the alleged mother (Nilsen *et al.*, 2020). So far, no variation in the Hg concentration of males and females was found, suggesting that the elimination of Hg by the female through egg laying could be marginal or non-detectable (e.g. Jagoe *et al.*, 1998; Burger *et al.*, 2000; Vieira *et al.*, 2011; Riveira *et al.*, 2016; Lemaire *et al.*, 2021).

5.3. Relation between Hg concentrations and body size of neonates

Our results show a significant difference in Hg concentration in all tissues, and a significant difference in body size (SVL and TL) of neonates between the 4 nests. The most contaminated nests yielded the smallest neonates (Fig. 3). In several animal species, Hg has effects on reproduction such as hormonal disruption, low quality of semen and altered embryonic development (Hammerschmidt *et al.*, 2002; Frederick *et al.*, 1997; Homma-Takeda *et al.*, 2001; Goutte *et al.*, 2014ab). The reduction of body size we measured might be associated with impaired embryonic development. Mercury alters the endocrine system in vertebrates and acts as an endocrine disrupter (Colborn *et al.*, 1993; Wada *et al.*, 2009; Meyer *et al.*, 2014). Hg accumulates in the thyroid, testes and pituitary gland, where its concentrations are generally higher than in keratinized tissues (Tan *et al.*, 2009). Hormones have an important function during the embryonic development, and a disruption of their regular activity has major consequences. The survival rate of hatchling crocodylians is very

low: in the first stage of their life, they are easy prey for birds, mammals and adult crocodiles due to their small size (Somaweera *et al.*, 2013). We emphasize that the relationship we found between Hg contamination and hatchling size may affect their survival. In addition, the relatively high Hg concentrations we found could have significant long-lasting effects into adulthood in interaction with later chronic exposure.

6. Conclusion

Our results highlight the use of keratinized tissues, particularly claws, to quantify the fetal Hg exposure and to evaluate maternal transfer in crocodylians. The variation of Hg concentrations between nests reinforces the fact that the contamination of the reproductive female has a direct effect on the concentration in its eggs. Finally, Hg concentration in the egg may influence hatchling morphology, thus potentially reducing survival and increasing susceptibility to later chronic exposure. For a long-lived species such as *Paleosuchus trigonatus*, the consequences of Hg mediated survival in neonates undoubtedly require a long-term survey of exposure and population dynamics. In order to assess the conservation challenges of Hg contamination, it is necessary to establish long-term studies on the populations that associate Hg levels and offspring success.

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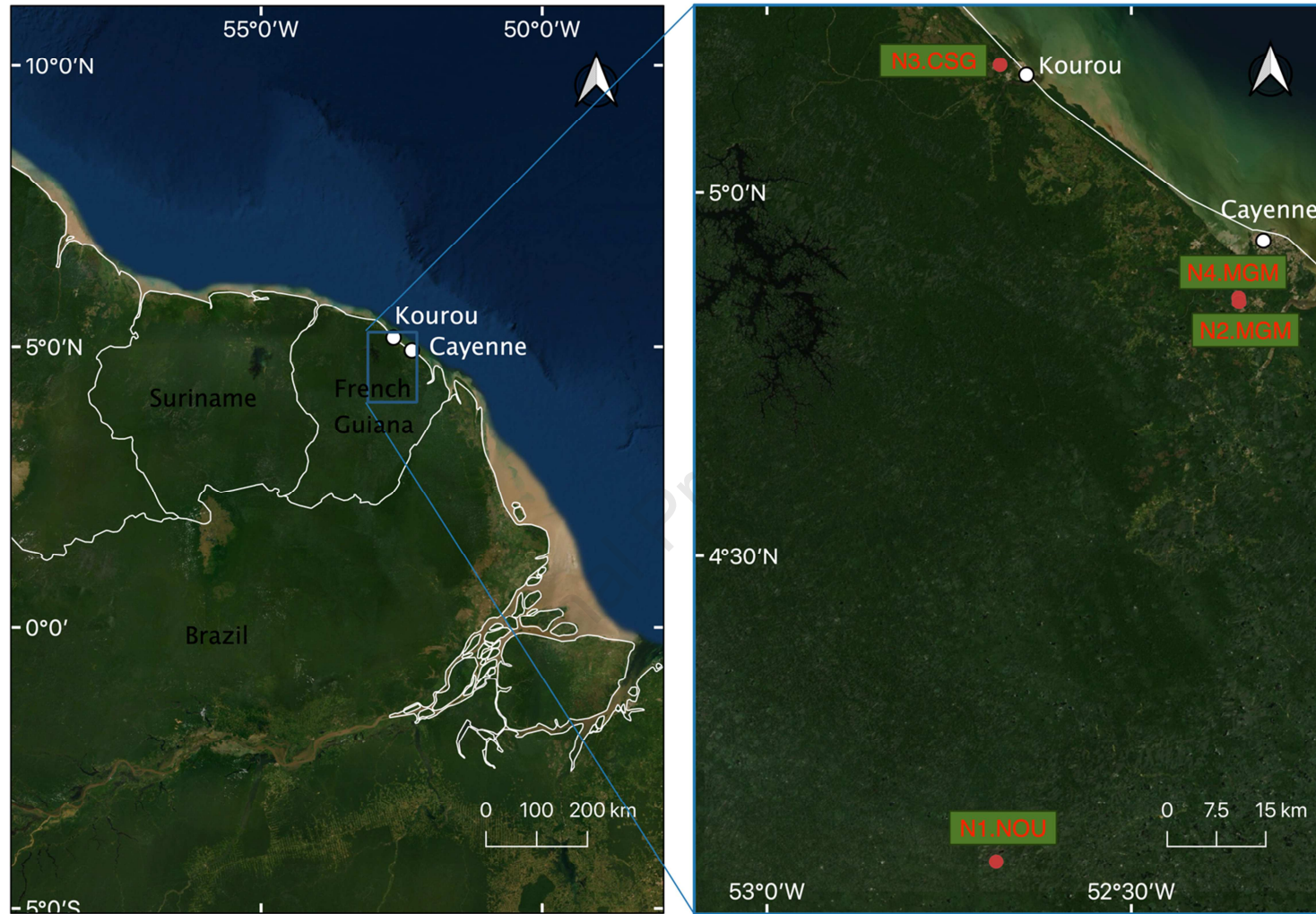


Figure 1. Locations of the 4 nests sampled in French Guiana. CSG = “Centre Spatial Guyanais”; MGM = “Réserve naturelle nationale du Mont Grand Matoury”; NOU = “Réserve naturelle nationale des Nouragues”.

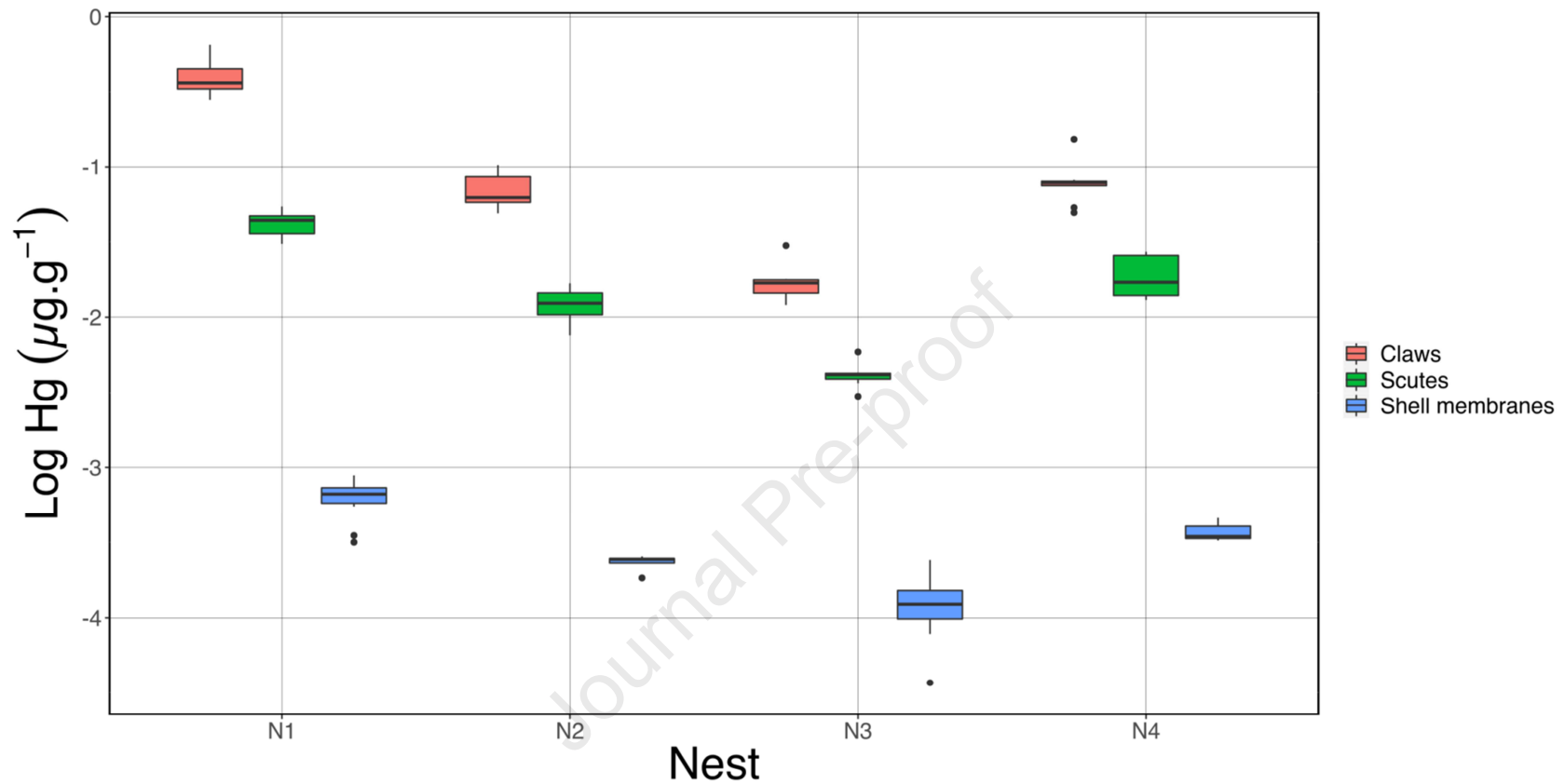


Figure 2. Log₁₀ Hg concentrations measured in claws (n = 38) and scutes (n = 38) of neonate Smooth-fronted Caimans, *Paleosuchus trigonatus*, and shell membranes (n = 33) from 4 different nests (N1, N2, N3 and N4) in French Guiana. The top and bottom of the boxes represent the first and last quartiles, the line across the box represents the median, the whiskers represent the fifth and ninety-fifth percentiles, and the circles represent outliers.

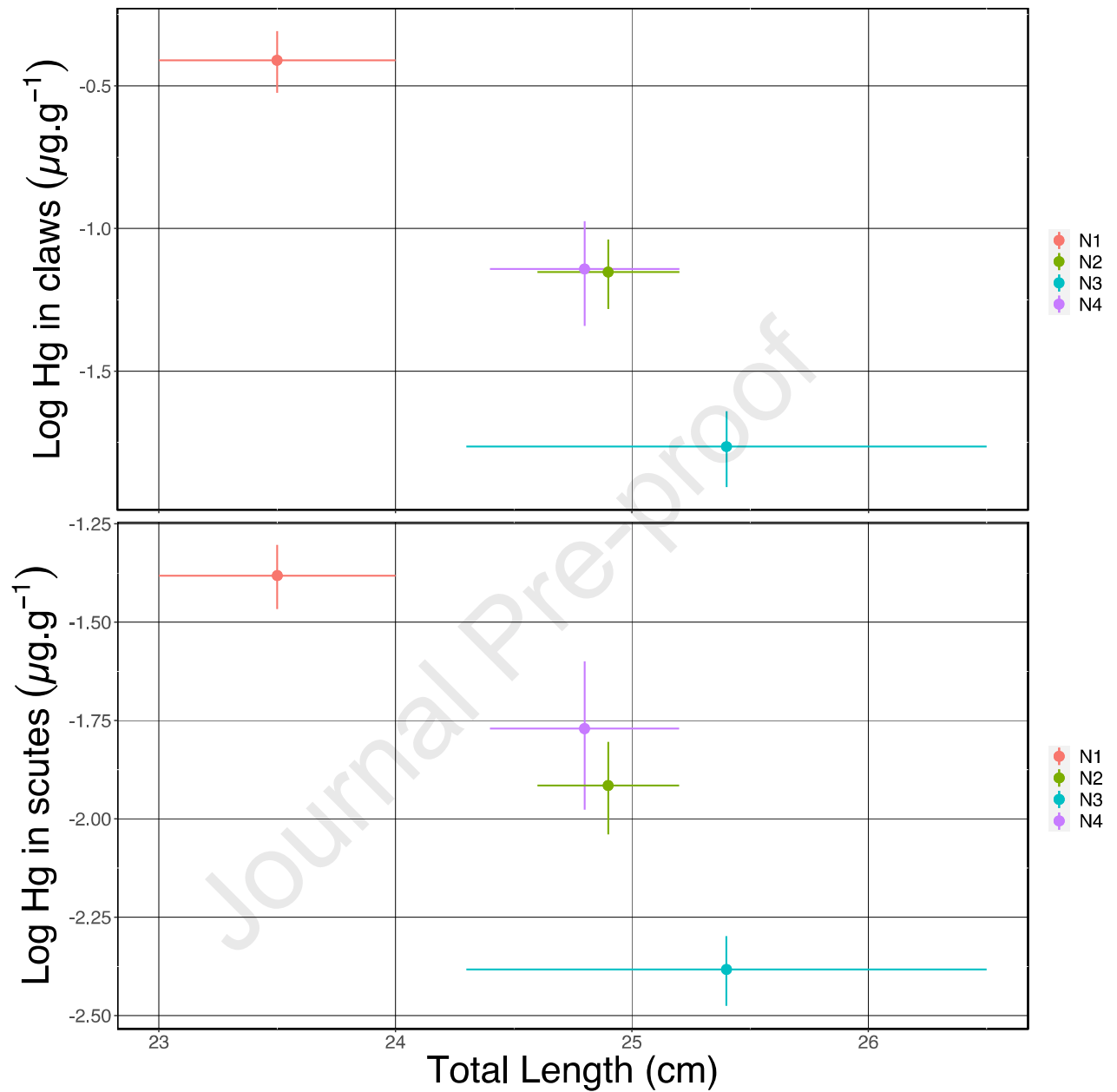


Figure 3. Log_{10} Hg concentrations (in $\mu\text{g.g}^{-1}$ dw) measured in claws and scutes and Total Length (cm) of neonate Smooth-fronted Caiman, *Paleosuchus trigonatus*, from French Guiana from 4 different nests. Values are Mean \pm SD.

Table 1. Biometric data (in cm, Mean \pm SD) and Hg concentrations (in $\mu\text{g}\cdot\text{g}^{-1}$ dw; Mean \pm SD) in the tissues of Smooth-fronted Caiman *Paleosuchus trigonatus* neonates, and the eggshells and shell membranes from 4 different nests in French Guiana, and the Hg concentration (in $\mu\text{g}\cdot\text{g}^{-1}$ dw; Mean \pm SD) in total blood of the alleged mothers. TL: Total Length; SVL: Snout-Vent Length; n: Number of samples.

<i>Nest</i>	<i>TL</i> (<i>n</i>)	<i>SVL</i> (<i>n</i>)	<i>Hg Claws</i> (<i>n</i>)	<i>Hg Scutes</i> (<i>n</i>)	<i>Hg Total blood</i> (<i>n</i>)	<i>Hg shell membranes</i> (<i>n</i>)	<i>Hg eggshells</i> (<i>n</i>)	<i>Hg Total blood</i> <i>alleged mother</i>
<i>N°1</i>	23.5 \pm 0.5 (15)	12.5 \pm 0.03 (15)	0.663 \pm 0.071 (15)	0.251 \pm 0.020 (15)	0.056 \pm 0.007 (4)	0.040 \pm 0.005 (11)	0.001 \pm 0.000 (3)	-
<i>N°2</i>	24.9 \pm 0.3 (7)	13.0 \pm 0.3 (7)	0.316 \pm 0.038 (7)	0.147 \pm 0.017 (7)	0.054 (1)	0.026 \pm 0.002 (5)	0.001 \pm 0.000 (2)	-
<i>N°3</i>	25.4 \pm 1.1 (7)	13.1 \pm 0.4 (7)	0.171 \pm 0.023 (7)	0.092 \pm 0.008 (7)	0.032 \pm 0.001 (2)	0.020 \pm 0.004 (11)	0.001 \pm 0.000 (2)	0.296
<i>N°4</i>	24.8 \pm 0.4 (9)	13.0 \pm 0.3 (9)	0.331 \pm 0.048 (9)	0.177 \pm 0.024 (9)	-	0.032 \pm 0.002 (6)	0.001 \pm 0.000 (5)	0.640

- Total mercury concentration in neonates *Paleosuchus trigonatus*
- Claw sampling to quantify mercury through maternal transfer
- Effects of mercury concentration on body size of neonates

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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