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Metallic elements in aquatic herpetofauna (Crocodylia; Testudines) from a lentic Atlantic rainforest environment in northeastern Brazil

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Abstract In the present study, Fe, Cu, Cr, Cd, Pb, Ni, and Al concentrations in *Caiman latirostris* and Testudines blood from the Tapacurá reservoir, Pernambuco, Brazil, were investigated. Blood was acid digested with HNO_3, and metals were determined by ICP-OES and FAAS. Lead showed concentrations below the established limit of detection. Eighty animals were evaluated, forty from each group. The levels of all elements were statistically significant when compared between the two studied taxa ($p < 0.05$). In caimans, significant differences between young and adults were observed for chromium ($p = 0.0539$) and aluminum ($p = 0.0515$). Testudines showed no statistically significant differences for the variable age structure. Gender did not influence metal concentrations detected in the present study for either group. Differences between species of testudines were significant for Fe between *Mesoclemmys tuberculata* vs *Phrynops geoffroanus* ($p = 0.0932$) and *Kinosternon scorpioides* vs *Phrynops geoffroanus* ($p = 0.063$). The inter-elementary correlations showed statistically significant differences between the elements Cr vs Al ($R^2 = 0.52$), Cr vs Cd (0.43), Cd vs Cu ($R^2 = 0.41$), Ni vs Cu ($R^2 = 0.31$), Ni vs Cr ($R^2 = 0.30$), Al vs Cd ($R^2 = 0.27$), and Cd vs Fe ($R^2 = 0.26$). It is concluded that blood is an excellent predictor of metals in crocodilians and testudines in the Tapacurá reservoir, with statistically significant differences when correlated to concentrations such as size and species studied. In addition, it evidenced data that prove the exposure of these animals to metals, with strong inter-elementary correlations and opening doors for future studies that seek to understand possible biological effects caused in the studied taxa.

Keywords Metals · ICP-OES · FAAS · Chelonia · Crocodylia

Introduction

The use of organisms sensitive to environmental variations in biomonitoring studies is extremely relevant

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in the effective diagnosis of ecosystem health, allowing for the development of laws that protect water bodies, aquatic life, and, consequently, humans (Aragão & Araújo, 2006). Although reptiles are listed as non-sensitive a variety of chemical agents, they are known as contaminant bioaccumulators (Domingues & Bertoletti, 2006). Several species of reptiles display long life cycles, territorial behavior, and resistance to environmental changes and are important key species for maintaining ecosystem homeostasis. Since the 1980s, they have been applied in the detection and monitoring of contaminated sites, mainly lentic environments such as lakes, ponds, and reservoirs (Tellez & Merchant, 2015; Schneider et al., 2015; Nilsen et al., 2017).

Despite adequate responses, the use of organs in the detection of chemical contaminants requires animal sacrifice. Thus, the use of non-lethal matrices (muscle, blood, scale, carapace, skin) has been continuously stimulated, especially for studies using animals that are on the List of Species Threatened with Extinction (Schneider et al., 2011). Non-lethal samples, such as blood, provide responses regarding recent contamination, reflecting circulating metal levels (Schifer et al., 2005). In addition, research evidences that this matrix is positively correlated with concentrations in other reptile body regions, such as shells, muscle, and liver (Schneider et al., 2011), the latter commonly used in the detection of environmental markers due to its ability to sequester contaminants in order to detoxify the organism.1802

Three freshwater turtle species occur in Pernambuco, Brazil, three of which inhabit freshwater environments in the Caatinga and Atlantic rainforest biomes, namely, *Kinosternon scorpioides* (Linné, 1766), *Mesoclemmys tuberculata* (Luederwaldt, 1906), and *Phrynops geoffroanus* (Schweigger, 1812) (De Melo Moura et al., 2014), while one land species (*Chelonoidis carbonaria* Spix, 1824) (Red-footed tortoise) also inhabits this region. Crocodilians are represented in Pernambuco by *Caiman latirostris* (Daudin, 1802) and *Paleosuchus palpebrosus* (Cuvier, 1807). No record of contamination studies in Northeastern Brazil for both groups is available. In this context, the main objective of this study was to measure levels of Fe, Cu, Cr, Cd, Pb, Ni, and Al present in blood samples of three freshwater turtles species (*Kinosternon scorpioides*, *Mesoclemmys tuberculata*, and *Phrynops geoffroanus*) and in one caiman species (*Caiman latirostris*) from the Tapacurá reservoir, located in the municipality of São Lourenço da Mata, Pernambuco, Brazil, and correlate metal with sex and age group.

Material and methods

Study areas

The Tapacurá River comprises a drainage area of 470.5 km^2 and includes six municipalities: Vitória de Santo Antão, Pombos, São Lourenço da Mata, Gravatá, Moreno, and Chã Grande (Aprile & Bouvy, 2010). Located at latitude 8° 10′, longitude 35° 11′, and at 102 m above sea level, the dammed lake covers 9.5 km^2 and presents a water capability storage of about 94,200 m^3, contributing with more than 36% of the water consumed in the metropolitan region of Recife (Andrade et al., 2009) (Fig. 1).

An important Atlantic Rainforest remnant that forms part of the Tapacurá Ecological Station, a conservation unit belonging to the Federal Rural University of Pernambuco, is located adjacent to the reservoir. This remnant occupies 776 ha, separated in two forest portions around the Tapacurá dam lake (Lyra-Neves et al., 2007).

Animal capture and processing

Blood samples were collected from 40 Testudines and 40 caimans captured between 2014 and 2017 within the limits of the Tapacurá reservoir. The caimans were manually captured using a steel cable attached to a telescopic rod with a 4-m reach, ropes, seals, and adhesive tapes. Animal jaws and limbs (anterior and posterior) were immobilized for transport (Magnusson, 1982; Bayliss, 1987). Funnel traps or "covos" were used for Testudines capture and were occasionally captured by active search with the aid of a net (Bossle, 2010).

After capture, the animals were taken to the Tapacurá Ecological Station laboratory for biometric assessments, sexing (Balestra et al., 2016; Webb et al., 1984), weighing, and caudal scale removal or sawing their marginal shields (Cagle, 1939) for animal identification.

Biometric measurements were made using a measuring tape, caliper, and a pesola scale adequate from 1 to 100 kg. The caimans were classified into the following size classes: young (class I, ≤ 49 cm),

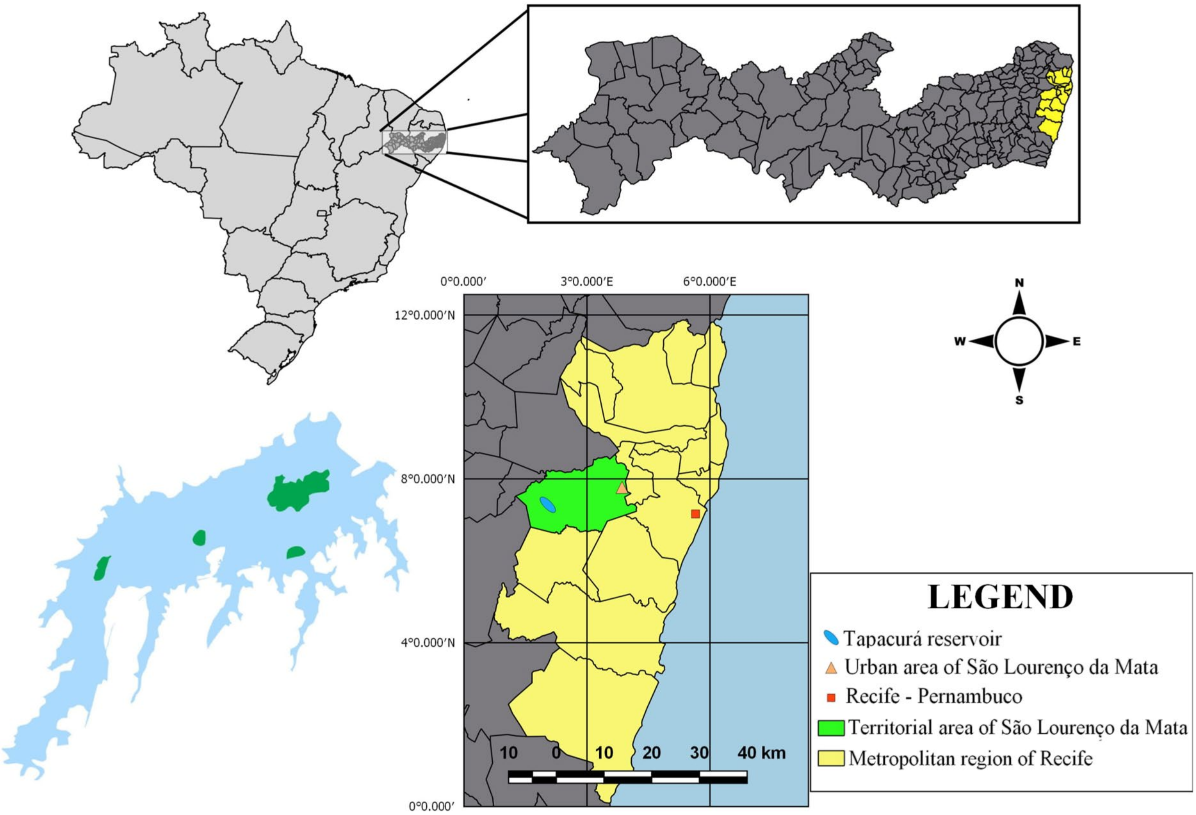


Fig. 1 Location of the Tapacurá dam, in the municipality of São Lourenço da Mata, Pernambuco—Brazil (8° 2′59 "S, 35° 11′5" O)

sub-adult (class II, 50–119 cm), adult (class III, 120—179 cm), and larger adult (class IV > 180 cm), adapted from Velasco and Ayarzagüena (1995). The Testudines specimens were classified to the carapace length of each species studied into the size classes "adults" and "young" (*K. scorpioidis*: ≥ 10 cm; *M. tuberculata*: ≥ 207 cm*; P. geoffroanus*: ≥ 24 cm) (Molina, 1998; Santana et al., 2016; Vogt, 2008).

For blood collection, the animals were placed in sternal recumbency, and blood was obtained through an access to the post-occipital and caudal vein region, performed with conventional hypodermic syringes (various calibers) (Myburgh et al., 2014). The puncture site was cleaned with iodized alcohol to clean and remove particles adhered to the animal's skin. Then, iodine was placed on the spot to prevent the spread of microorganisms. The blood was immediately stored at −20° C for subsequent heavy metal analyses. After processing, all animals were released near the capture sites. Anticoagulant was not used to avoid possible interference.

This proposal has the authorizations and licenses for data collection with wild animals; they are Approval of the Research Project by the UFRPE Technical Administrative Council (CTA) N° 18/2014, License of the Ethics Committee on Animal Use—CEUA N 068/2014 and Authorization of SISBIO (Authorization and Information System for Biodiversity) Nos. 39929-4 and 61664-2.

Blood sample preparation

The blood samples remained chilled until their acid digestion, where they were withdrawn from storage until reaching an ambient temperature of 28ºC. Blood sample preparation was performed by adapting the methodology developed by Neto and Barreto (2011). Digestion was carried out by mixing 0.25 mL of blood to 3 mL nitric acid 65% (HNO_3) (Modern

Chemistry ®) in glass tubes, which were then placed in an electric resistance oven for 72 h at 67 °C. The digested samples were diluted in 25-mL volumetric flasks, made up with distilled water, filtered (Micropore®) with 25 micron quantitative filtering paper (Micropore®), homogenized and transferred to plastic containers, where they remained chilled until analysis by atomic flame absorption spectrometry (FAAS) and inductively coupled plasma optical emission spectrometry (ICP-OES).

Elemental determinations by (ICP-OES and FAAS

Pb, Cd, Cu, Cr, Ni, Fe, and Al were determined by ICP-OES using an OES Optima 7000 DV ICP-OES (Perkin Elmer, USA) with axial configuration, and Zn was determined by FAAS, using an Agilent Technologies (200 Series AA) FAAS. All analyses were carried out in triplicate. The instrumental parameters and analytical lines employed in multielemental determination using ICP-OES were performed as standardized: RF power (kW)—1.3; nebulizer gas flow rate (L min^{-1})—0.8; auxiliary gas flow rate (L min^{-1})—0.2; plasma gas flow rate (L min^{-1})—15; sample aspiration rate (mL min^{-1})—1.0; number of replicates—1; nebulizer—concentric; nebulizer chamber—cyclonic; detection wavelength (nm): Pb 220353, Cd 228,802, Cu 327,393, Cr 267,716, Ni 231,604, Fe 238,204, Al 396,153.

The limit of detection (LOD) and quantification (LOQ) were calculated according to standard deviation of ten blank samples for each element, according to the angular calibration. The LODs were calculated according to INMETRO (2020):

$$\text{LOD} = 3.3s/b$$

where "s" is the standard deviation of ten blank samples and "b" the slope of analytical curve. Likewise, the limit of quantification (LOQ) was estimated to the equation:

$$\text{LOQ} = 10s/b$$

where "s" is the standard deviation of the white response and "b" the slope of analytical curve.

The multi-element standard solutions (Merck Certipur®) containing 1000 mg L^{-1} (Fe, Cu, Cr, Cd, Pb, Ni, and Al) for the ICP-OES analysis were obtained from the dilution of the analyzed elements in HNO_3. The limits of detection and quantification for each element were, respectively, Pb (1.4, 4.7), Cd (0.05, 0.17), Cu (0.9, 3.0), Cr (0.25, 0.83), Ni (0.4, 1.3), Fe (0.005, 0.017), and Al (0.2, 0.07) (μg L^{-1}). The data collected from the ICP instrument, expressed in mg L^{-1} solution, was multiplied by the volume of the sample. As carrier gas and plasma former, argon grade 5.0 (purity of 99.999%) (White Martins).

Statistical analyses

Data normality and homogeneity of variances were tested using the Shapiro–Wilk and Bartlett test. A variance analysis (ANOVA) was used to correlate reptile age, sex, and species with elemental concentrations, followed by the post hoc Tukey test when homogeneity assumptions were observed. Otherwise, the Kruskal–Wallis test followed by the post hoc Mann–Whitney test was used. Student's *t* test was also used when normality assumptions were observed. If they were not, the Mann–Whitney test was used to evaluate variable medians. In addition, Pearson's correlation test was also applied to assess inter-elemental correlations (X^2). Statistical significance was considered at a 5% significance level. The R software (R DEVELOPMENT CORE TEAM, 2016) was used for all statistical analyses.

Results

From snout-vent length (SVL), Caimans were classified into the following age classes: class II ($n = 22$), class III ($n = 13$), and class IV ($n = 5$), with mean of total length 118.36 (62–211 cm) and snout-vent length of 55.34 ± 23.02 (20–104.5). Testudines were classified as young ($n = 13$) and adults ($n = 27$) and presented total shell length and mean weight of 16.9 cm ± 4.82 (9–29) and 744.31 g ± 723.39 (95–3200), respectively.

The seven elements analyzed here were detected in all blood samples. Statistically significant differences between median levels the two taxa were identified only for all elements ($p < 0.05$) (Mann–Whitney). Pb showed concentrations below the established limit of detection (LOD). The average concentrations for the elements analyzed were as follows: Fe (126.29 ± 94.03), Al (6.32 ± 5.99), Cu (2.00 ± 1.47), Ni (0.91 ± 0.35), Cr (0.83 ± 0.86), Cd (0.60 ± 0.42) (Fe > Al > Cu > Ni > Cr > Cd). All results were obtained in mg L^{-1}.

Crocodilians showed higher values when compared to Testudines averages for the elements, Cu (Crocodylia: 2.60 ± 1.67; Testudine: 1.45 ± 0.99), Fe (Crocodylia: 134.36 ± 59.05; Testudines: 118.2 ± 119.54), and Cd (Crocodylia: 0.72 ± 0.39; Testudines: 0.15 ± 0.06) ($p<0.05$). In contrast, Testudines showed higher values for the elements Cr (Crocodylia: 0.48 ± 0.82; Testudine: 1.06 ± 0.82), Ni (Crocodylia: 0.79 ± 0.30; Testudines: 1.37 ± 0.32), and Al (Crocodylia: 3.18 ± 4.44; Testudines: 8.93 ± 6.55). The age group of alligators, based on the rostrum-cloacal length (SVL), showed statistically significant differences for Cr (class II: 0.01 ± 0.35; class III: 0.50 ± 0.93) ($p=0.0539$) and Al (class II: 3.57 ± 5.66; class III: 1.89 ± 1.78) ($p=0.0515$) (Kruskal–Wallis post hoc Fisher) (Fig. 2). The Testudines showed no statistically significant differences for the variable age structure.

When the concentrations between the three species of Testudines were compared, a significant difference was observed in the average Fe concentrations in *Mesoclemmys tuberculata* vs *Phrynops geoffroanus* (*M. tuberculata*: 111.82 ± 87.69; *P. geoffroanus*: 76.71 ± 31.84) (p = 0.0932) and *Kinosternon scorpioides* vs. *Phrynops geoffroanus* (*K. scorpioides*: 95.15 ± 140.94; *P. geoffroanus*: 76.71 ± 31.84) (p = 0.063) (Fig. 3). The inter-elementary correlations showed statistically significant differences between the elements Cr vs Al ($R^2=0.52$), Cr vs Cd (0.43), Cd vs Cu ($R^2=0.41$), Ni vs Cu ($R^2=0.31$), Ni vs Cr ($R^2=0.30$), Al vs Cd ($R^2=0.27$) and Cd vs Fe ($R^2=0.26$) (Kruskal–Wallis post hoc Fisher) (Table 1; Fig. 4). There were no statistically significant differences for the gender variable.

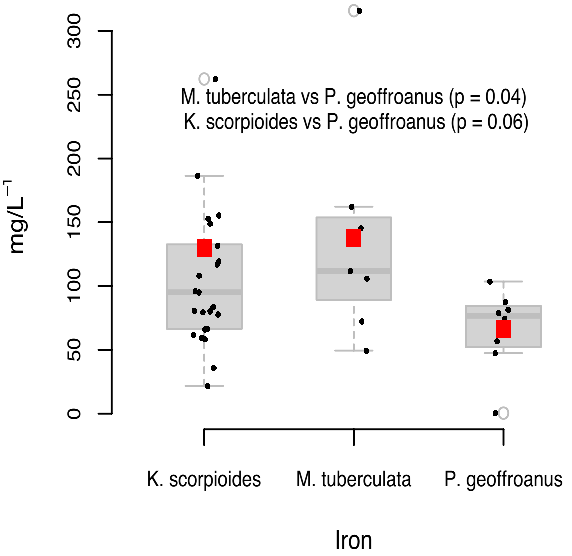


Fig. 3 Comparison of Fe concentrations in blood samples ($p\leq0.05$) between Testudines species: *K. scorpioides*, *M. tuberculata*, and *P. geoffroanus* captured at the Tapacurá Ecological Station. The dots outside the graph represent the outliers, in red, highlighting medians

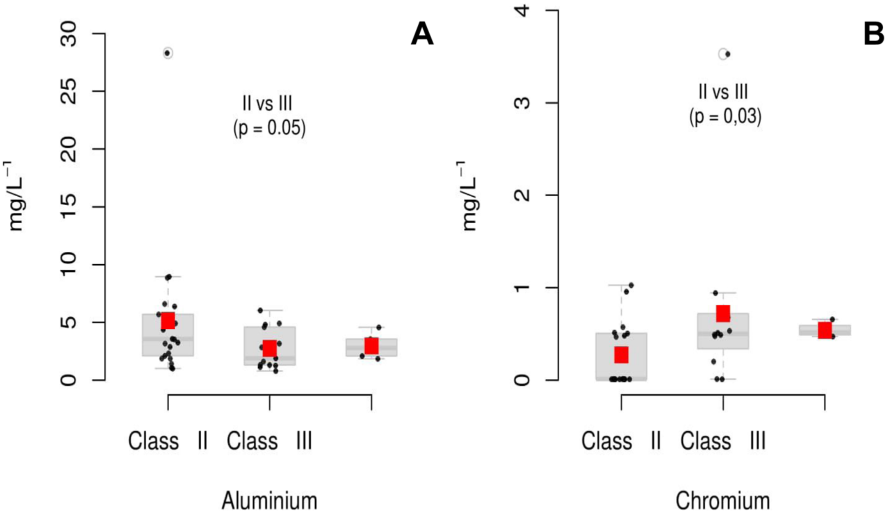


Fig. 2 Statistically significant differences in heavy metal concentrations detected by age group (class II, III, IV) in Crocodilians for Al (**a**) and Cr (**b**) captured in the Tapacurá reservoir, Pernambuco, Brazil ($P<0.05$). The dots outside the graph represent the outliers, in red, highlighting medians

Table 1 *P* values among inter-elemental concentrations of trace elements in blood of *Caiman latirostris* and Testudines (*Phrynops geoffroanus*, *Kinosternon scorpioides*, *Mesoclemmys tuberculata*) captured in the Tapacurá reservoir

	Cu	Fe	Cd	Cr	Ni	Al
Cu	1.0000	0.2825	**0.0038**	0.7080	**0.0568**	0.1830
Fe	0.124	1.0000	**0.0643**	0.8935	0.6961	0.1277
Cd	0.414	0.264	1.0000	**0.0043**	0.6503	**0.0562**
Cr	−0.056	−0.019	−0.432	1.0000	0.0793	**0.0001**
Ni	0.312	0.063	−0.073	0.296	1.0000	**0.0019**
Al	0.154	0.173	−0.272	0.515	0.471	1.0000

Highlight in bold are statistically significant *p* values

Discussion

No reference values were found for iron in blood for reptiles, making it more difficult to compare data with the present study (where Fe levels were 94.67 mg L^{-1}). Possibly, this is due to the direct relationship that this element has with the blood, since it is found in Fe-heme proteins, precursors of hemoglobin, myoglobin, Fe-sulfur enzymes, among others (Fraga, 2005; Fraga & Oteiza, 2002). The available articles use this element in other biological matrices such as muscle (22.76 μL g^{-1}—Delany et al., 1988), eggs (13 μL g^{-1}—Heinz et al., 1991), osteoderms (3.9 μL g^{-1}—Jeffree et al., 2001), and liver (352.4 μL g^{-1}—Xu et al., 2006); therefore, there is no way to make an effective comparison of the results obtained.

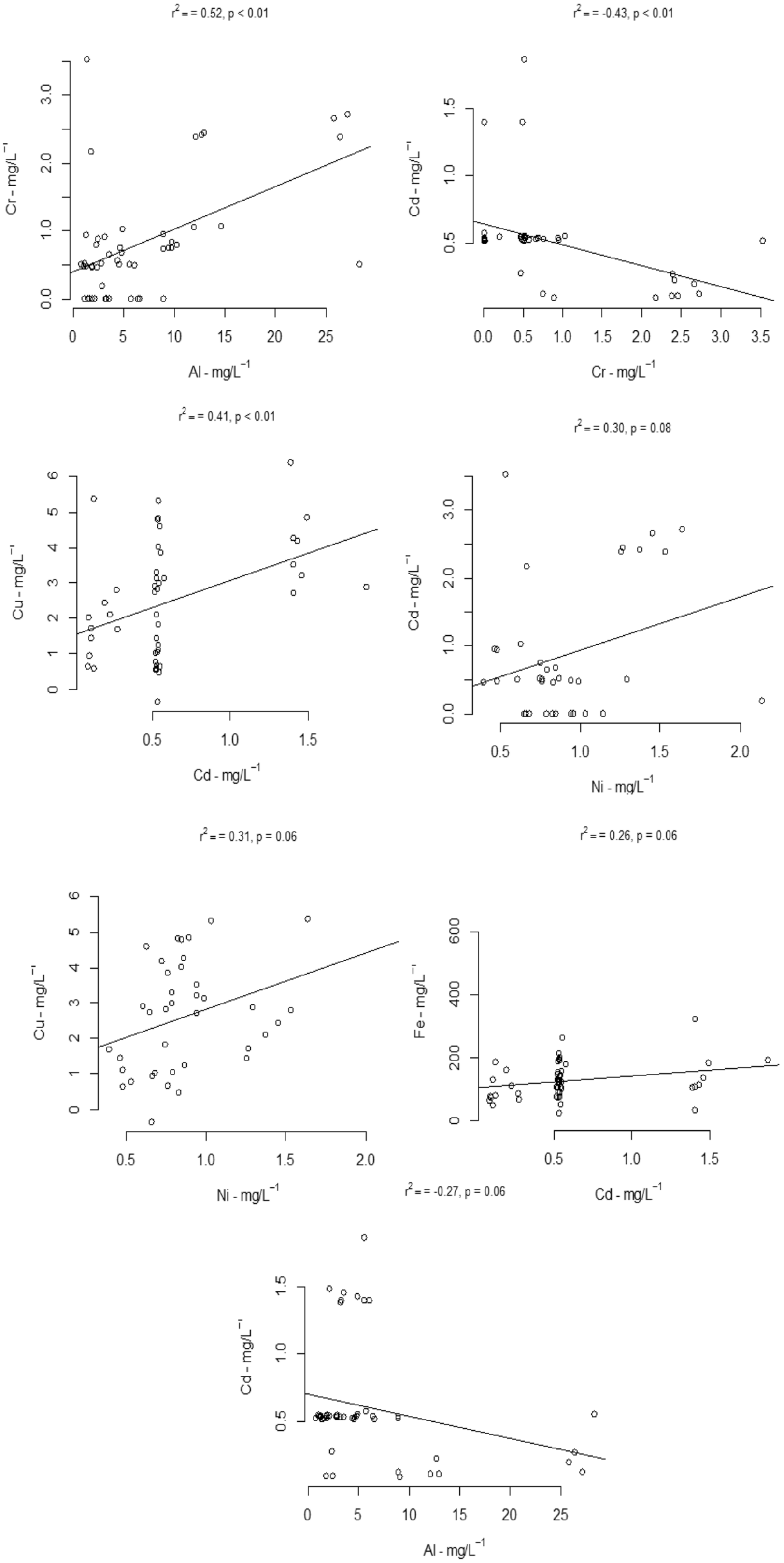
This may lead to overlaps in what are considered normal concentrations and high concentrations due to the absence of parameters that define these internal doses for wildlife crocodilians. Fe normally appears in water in its oxidation forms (Fe^{+2} and Fe^{+3}), the ferrous ion being more soluble than the ferric ion (Marcelino et al., 2017). Interesting finding observed by Aprile and Bouvy (2003) is that the Tapacurá River sediments act as a heavy metal accumulator, with very heterogeneous results and point sources of contamination. The authors point out that during the drought period, iron concentrations increased from 1.458 to 9.563 μg g^{-1} during drought, representing an increase of 556%. Years later, the same authors pointed out that there is disposal of organic effluents that are discharged along the Tapacurá basin, a fact that directly influences these levels (Aprile & Bouvy, 2010).

Considered a transition element, aluminum was the second element that presented the highest concentrations in the studied samples. The element presented significant concentrations for both taxa of 4.10 mg L^{-1} for Crocodylia and 8.59 mg L^{-1} for Testudines. In Florida, Nilsen et al. (2017, 2019) found values much lower than those of the present study in *Alligator mississipiensis* blood samples (40.7 ng L^{-1} and 66 ng L^{-1}, respectively), with differences statistically influenced by gender. The author emphasizes the lack of literature that explains the toxicity patterns of this element in reptiles.

Statistically significant differences were observed between the concentrations of estimated elements and age classes of the captured caimans only for Al and Cr, which presented significance between two of the three age classes (class II and class IV) (Kruskal–Wallis post hoc Fisher). Class II animals presented median Al concentrations of 3.57 mg L^{-1} (1.01—28.3 mg L^{-1}), higher than the levels detected for class III individuals 1.89 mg L^{-1} (0.8 – 6.04 mg L^{-1}) ($p=0.0515$). Regarding chromium, larger animals (class III) showed higher concentrations than did younger animals (class II) ($p=0.0538$). The Testudines showed no statistically significant differences for the variable age structure.

The literature points out that larger animals tend to have higher concentrations of chemical contaminants. This is because these animals have been exposed to the studied environment for a longer time; they are top predators and carnivores. The reverse of this fact is explained by the fact that smaller animals have a faster metabolism, feeding more often for an efficient development, and for that reason, they can present higher levels. Because the growth rates of large alligators are slower, the frequency with these animals feed is lower, consequently generating less exposure

Fig. 4 Pearson's correlation coefficients among inter-elemental concentrations of trace elements in blood of *Caiman latirostris* and Testudines (*Phrynops geoffroanus*, *Kinosternon scorpioides*, *Mesoclemmys tuberculata*) captured in the Tapacurá reservoir. Cd vs Cr presented a negative correlation



to contaminants (Khan & Tansel, 2000; Vieira et al., 2011; Schneider et al., 2012).

As pointed out in the results, we observed significant differences only in the iron concentration average in *Mesoclemmys tuberculata* vs *Phrynops geoffroanus* vs *Kinosternon scorpioides*. *M. tuberculata* displays carnivorous feeding behavior, consuming mainly fish, while the others species are considered omnivores and feed on fish, crustaceans, and aquatic insects (Souza, 2004; Vanzolini et al., 1980), which may explain the higher levels detected in this species. Similarly, Schneider et al. (2011) observed that Hg differences evaluated for six Amazonian Testudines were due to variations in diet and foraging behavior. Burger et al. (2009) assessed six metals in the blood of four Amazonian Testudines and pointed out no significant differences among species from the Podocnemididae family.

Chemical elements interact differently, presenting synergic, potentiation, additive, inhibitor, or antagonistic effects. Studies involving these relationships are widespread among plants and aquatic invertebrates (Naddy et al., 2015; Puga et al., 2015; Rainbow, 2017). However, studies in vertebrates in this regard are less abundant, mostly in toxicity assays carried out in mice and fish (Green & Planchart, 2018).

No studies were found evaluating inter-elementary correlations for reptiles, especially in Testudines and crocodilians for comparative purposes; however, there are studies carried out mainly with cetaceans that evaluate and point out these types of relationships (Agusa et al., 2008; Seixas et al., 2008). The results of the correlations obtained for this study are based only on statistics, i.e., the existence of these correlations is speculated based on previous research with organisms from other zoological groups. However, for specific, safe inference about metal interactions, specific studies with rapid indicators of acute contamination are required, applying specific doses and exposure periods. Sparling et al. (2010) states that metals usually present synergistic, additive, or antagonistic effects that can have serious consequences for exposed amphibians and reptiles. These mixtures lead to potentiated toxic effects more harmful than the elements isolated and studied.

Khangarot and Ray (1990) pointed out in their toxicity tests concerning Ni and Cr in *Poecilia reticulata* that both elements display an additive effect when mixed. A previous study (1981) by the same authors evaluated the toxicity of Zn–Ni–Cu mixtures for the same species and observed synergistic effects.

Cd, Pb, and Fe have similar absorption mechanisms. A higher concentration of Cd and Pb occurs in animals with low Fe stores (antagonism), while Pb inhibits the functioning of ferrochelatase, the enzyme responsible for inserting Fe into the organic molecule protoporphyrin IX (heme group), causing anemia (Souza & Tavares, 2009). Likewise, Cd may also decrease Fe uptake through the gastrointestinal tract (ATDSR, 2004). Thus, Pb and Cd may produce additive effects, or more than additive in hematological parameters (ATSDR, 2004).

More recently, in the study by Land et al. (2018), quite significant and positive correlations were observed in the elements studied of *Geophagus brasiliensis* livers in which Fe × Al ($r=0.91$), Co × Al ($r=0.86$), Se × Al ($r=0.93$). The authors emphasize how metallic and trace elements have different distributions in fish tissue, and this correlation is extremely important for understanding the effects on biological functions and homeostasis of organisms, in order to improve the management of aquatic environments and their biota.

Conclusion

In addition to complement existing data in literature about analysis of metallic elements in biological matrices of continental Crocodilians and Testudines, the present work is unprecedented in confirming the exposure of these populations and elucidating information that can help in diagnosing these animals' health in northeastern Brazil, opening doors for future research that seeks to evaluate possible effects caused by long-term exposure to studied chemical contaminants. It also encourages research that seeks to understand other factors that contribute to contaminant bioaccumulation in the biota, such as inter-elementary relationships and in combination with other known chemicals, concentrations in environmental matrices (soil, surface and sedimentary water), presence of industry close to water bodies, and the use of other organisms in the food chain as biomonitors of aquatic ecosystems.

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Data availability Data available on request from the authors.

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