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Negative metal bioaccumulation impacts on systemic shark health and homeostatic balance

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ABSTRACT

Contamination by metals is among the most pervasive anthropogenic threats to the environment. Despite the ecological importance of marine apex predators, the potential negative impacts of metal bioaccumulation and biomagnification on the health of higher trophic level species remains unclear. To date, most toxicology studies in sharks have focused on measuring metal concentrations in muscle tissues associating human consumption and food safety, without further investigating potential impacts on shark health. To help address this knowledge gap, the present study evaluated metal concentrations in the gills, muscle, liver and rectal gland of coastal sharks opportunistically sampled from Brazilian waters and tested for potential relationships between metal bioaccumulation and general shark health and homeostatic balance metrics. Results revealed high metal concentrations in all four tissue types, with levels varying in relation to size, sex, and life-stage. Metal concentrations were also associated with serum biomarkers (urea, lactate, ALT, triglycerides, alkaline phosphatase, and phosphorus) and body condition, suggesting potential negative impacts on organismal health.

1. Introduction

As a taxonomic group, sharks are particularly vulnerable to anthropogenic stressors, such as exploitation, habitat degradation and climate change, due to life-history traits that feature late maturity and relatively low reproductive output (Gallagher et al., 2012; Worm et al., 2013; Pacoureau et al., 2021). While research into threats to sharks has primarily focused on mortality due to fishing, a growing concern on the possible sublethal impacts of bioaccumulated toxic pollutants (e.g., metals) on shark health and fitness is noted (Turoczy et al., 2000; Rumbold et al., 2014). While previous research has tested shark tissues for the presence and concentration of metals (e.g., Shipley et al., 2021), this has usually been addressed from a food safety perspective to ascertain whether metal levels would be safe for human consumption (e.g., Souza-Araujo et al., 2021; Hammerschlag et al., 2016; Anandkumar et al., 2018).

Some studies have evaluated the mechanistic consequences of metal contamination on shark physiological processes, including alterations in osmoregulatory function (Kinne-Saffran and Kinne, 2001; De Boeck et al., 2001, 2010; Grosell et al., 2003) as well as cellular and fluid composition (Ballatori and Boyer, 1996; De Boeck et al., 2001, 2010; Grosell et al., 2003). For example, Pb accumulation in the gills, rectal gland, muscle and liver has been shown to affect osmoregulation, respiratory capacity and energy metabolism in the dogfish shark (*Squalus acanthias*) (Eyckmans et al., 2013), while Ag and Cu accumulation in the gills, liver, kidney, rectal gland, intestine, muscle and skin tissues have been reported to cause respiratory disturbance, hyperventilation, blood alkalosis, altered anaerobic metabolism, lactate accumulation,

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erythrocyte swelling, and hemolysis in dogfish sharks (*Scyliorhinus canicula* and *S. acanthias*; De Boeck et al., 2001, 2010). Recently, Norris et al. (2021) evaluated mercury concentrations in blood, muscle, liver, and kidney samples from neonatal and juvenile blacktip sharks (*Carcharhinus limbatus*) in Southwest Florida. The study found that melanomacrophage presence and lipid deposition exhibit a significant association with Hg concentrations, suggesting that Hg exposure may lead to potentially negative effects to blacktip liver. In contrast, an evaluation of plasma levels for 14 heavy metals and 12 trace elements in white sharks, *Carcharodon carcharias*, did not find any negative effects of metal concentrations upon the determined shark health parameters, including total leukocytes, granulocyte to lymphocyte ratios, and shark body condition (Merly et al., 2019). Taken together, these studies suggest that the sublethal effects of metal concentrations on shark health are likely to depend on the species, life-stage, type and concentration of the accumulated metals, tissue type and associated physiological processes.

The present study evaluated the concentration levels of cobalt (Co), manganese (Mn), nickel (Ni), copper (Cu), iron (Fe), and mercury (Hg) in four tissues (gill, muscle, liver and rectal gland) opportunistically sampled from coastal sharks in Brazil, and tested for potential relationships between metal bioaccumulation and general shark health and homeostatic balance metrics. The reason for choosing these elements is multifold. First, Hg is an important contaminant routinely detected in sharks worldwide, resulting in negative health effects to both sharks and humans through shark meat consumption. Ni, is traditionally considered non-essential and has been reported as toxic, although mounting evidence has indicated it may be essential to fish (Pyle and Couture, 2012). Although all other elements are essential (Co, Mn, Cu, Fe), high concentrations are being detected in many regions worldwide, probably due to climate change effects, which are more devastating in coastal areas (EC, 2021), leading to increasing leaching from substrate to the water column and subsequent alterations in metal bioavailability (Machado et al., 2016). Furthermore, all the determined essential metals contribute to homeostasis in most vertebrates, as an integral part of proteins and enzymes. However, even essential elements might become potentially toxic when in concentrations above biological thresholds (Hauser-Davis and Lavradas, 2018).

To this end, four study hypotheses were empirically tested. First, that metals would show bioaccumulation in shark tissues, with concentrations increasing with shark size (Alves et al., 2016). Second, that among the four tested tissues, metal concentrations would be higher in the liver as organs containing lipid deposits tend to accumulate higher metal contents (Terrazas-López et al., 2016). Third, that overall shark health and homeostatic balance metrics would be impacted by high tissue concentrations of metals (Alves et al., 2016). Specifically, individuals with higher metal concentrations would exhibit evidence of decreased body condition and impaired osmoregulatory capacity, acid-base balance, metabolic energy, and liver functioning. Fourth and finally, we hypothesized that sex (Lopez et al., 2013) and life-stage (Frías-Espericueta et al., 2015) would influence differences in bioaccumulation patterns and their consequent effects upon systemic health (i.e., physical and nutritional condition).

2. Materials and methods

2.1. Specimen collection

Twenty sharks from eight species (Supp. Table 1) were incidentally caught by artisanal fisheries in São Luís, Maranhão (Brazilian Amazon Coast) and opportunistically obtained by researchers. Prior to the necropsies, biometrics, sex and life stage (considering size at maturity previously described in the literature) were determined. To avoid major *post-mortem* alterations in the assessed physiological parameters, only recently deceased animals (i.e., the presence of reddish gills and absence of *rigor mortis*, ocular retraction and blood coagulation) were sampled. The freshness index was set considering parameters commercially used to assess food quality and safety.

After the biometrics, sharks were necropsied for gill, hepatic, muscular and rectal gland tissue sampling. Blood was also collected by puncture of the caudal vein (~7 mL). After collection, blood samples were immediately centrifuged for 7 min at room temperature (20 °C) at 2000 ×*g*. Serum was separated and maintained frozen at −20 °C for further analysis. The sampling was conducted in accordance with approval by the Brazilian Ministry of Environment (IBAMA/ICMBio-SISBIO # 60306-1).

2.2. Metal analyses in tissues

The gill, muscle, liver and rectal gland samples were first weighed on a precision scale (wet weight). These tissues were then minced and dried at 50 °C for 96 h. The samples were then weighed again (dry weight) and 0.1 g of each sample was dissolved in 2 mL of HNO_3 (65% nitric acid, P. A, ISOFAR, ref. 0107, Brazil) at 25 °C for 48 h. After heating the capped (closed) tubes in a water bath at 50 °C for 30 min, thus avoiding losses (USP, 2013), the samples were made up with ultrapure water to 15 mL for subsequent analysis by inductively coupled plasma optical emission spectrometry (ICP OES) employing a 720-ES spectrometer (VARIAN, United States).

Metal quantifications were performed by external calibration using nine concentration levels (0, 0.02, 0.05, 0.1, 0.25, 0.5, 1.0 2.5, and 5.0 mg L^{-1}) prepared from a multielement standard solution (100 mg L^{-1}) (Specsol, Quimlab, Brazil). All determinations were performed in triplicate. Analytical curve correlation coefficients were always above 0.995. Method accuracy was verified by analyzing procedural blanks and two certified reference materials (CRM) (TORT 3 - Lobster hepatopancreas, National Research Council Canada, Halifax, Nova Scotia, Canada; ERM-CE278k - Mussel tissue, European Commission, Joint Research Center, Geel, Belgium). Recoveries ranged from a minimum of 95.62% for Ni and maximum of 104.1% for Cr in the ERM-CE278k CRM and 96.4% for Cr and 119.3% for Mn in the TORT 3 CRM. The certified reference material recovery values were considered adequate for this type of study, as per Eurachem standards (Eurachem, 1998; Ishak et al., 2015), including for Hg (ERM-CE278k, 98.59% and TORT-3, 95.89%). The complete recovery values are presented in Supplementary Table 2.

2.3. Fulton's condition factor

To access individual body condition, the Fulton (K) condition factor was estimated for each specimen following the equation as per Blackwell et al. (2000):

$$K = \left(\frac{W}{TL^3}\right) \times 10^3$$

Where W is weight (g), TL is the total length (mm), and 10^3 is a constant value used for scaling purposes. The resulting K value must be close to one digit and one decimal place.

The Fulton's condition factor is traditionally used for fish in general, including sharks, and has been validated for several species (Parsons and Hoffmayer, 2005; Rosa et al., 2014; Logan et al., 2018), as it is a cheap, quick and, when required, non-lethal method to assess fish health status.

2.4. Serum markers and analysis

To generate osmoregulatory capacity and homeostasis maintenance metrics, urea and phosphorus concentrations were determined in serum. Sharks are ureotelic and urea plays a key role in osmoregulation (Hammerschlag, 2006). When allostatic overload occurs and homeostatic balance is lost, alterations in urea concentrations are observed (Wosnick et al., 2017). Consequently, this osmolyte becomes a valuable tool to assess the physiological effects of extrinsic stressors. Phosphorus plays an important role in metabolic processes and acts as a buffer in osmoregulatory dynamics (Ferreira and Baldisserotto, 2007), becoming a promising marker when increases in circulating levels are observed, indicating allostatic overload (Wosnick et al., 2017).

Serum lactate levels were measured to evaluate acid-base balance. Lactic acidosis is a physiological condition in which excess circulating lactate causes a significant reduction in plasma pH (Skomal and Bernal, 2010). This condition is related to reductions in oxygen supply and/or uptake and may indicate severe metabolic stress levels (Robergs et al., 2004). To determine metabolic energy profiles, triglyceride and total cholesterol concentrations in serum were measured. Triglycerides are an important source of metabolic energy and indicative of nutritional condition and systemic health (Gallagher et al., 2017). Cholesterol also plays a crucial role in vertebrate metabolism and the synthesis of steroid hormones (Gallagher et al., 2017). Therefore, increasing circulating concentrations are important signals indicative of nutritional and metabolic alterations.

To evaluate liver functioning, alkaline phosphatase (ALP) and alanine transaminase (ALT) activities and circulating bilirubin levels were determined. Alkaline phosphatase is an enzyme synthesized in the lining membranes of bile ducts and plays the physiological role of dephosphorylating compounds, including proteins, nucleotides and alkaloids (Sharma et al., 2014). Activity is expected to increase with major bile duct obstructions, intrahepatic cholestasis, or infiltrative liver diseases (Kasarala and Tillmann, 2016). Alanine transaminase is an enzyme synthesized in hepatocytes, increasing dramatically in acute liver injuries, such as viral hepatitis (Kasarala and Tillmann, 2016). Bilirubin is an effective hepatobiliary marker since increases in activity are expected when structural and functional damage is observed, such as bile duct obstructions, impaired hepatocyte, and reduction of bilirubin conjugation and/or secretion (Méndez-Sánchez et al., 2017). Moreover, to evaluate potential hepatorenal syndrome, creatinine activity was also assessed. Creatinine is produced as a metabolic waste from creatine which supports muscle cells during anaerobic energy delivery. Creatinine synthesis occurs at constant rates and is excreted by the kidneys. As kidneys begin to lose function, blood creatinine levels rise (Gowda et al., 2010), becoming a valuable marker to access renal activity and homeostatic balance.

Urea (Labtest, Brazil, catalog n. 27; wavelength 600 nm), phosphorus (catalog n. 42; wavelength 650 nm), lactate (catalog n. 138–1/50; wavelength 550 nm), triglycerides (catalog n. 87; wavelength 505 nm), total cholesterol (catalog n. 76; wavelength 500 nm), alkaline phosphatase (catalog n. 40 wavelength 590 nm), alanine aminotransferase (ALT) (catalog n. 108; wavelength 340 nm), bilirubin (catalog n. 31; wavelength 525 nm) and creatinine (catalog n. 35; wavelength 510 nm) were assayed from serum samples strictly following the protocols established by the manufacturer (Labtest Diagnóstica S.A., Brazil). All markers were quantified colorimetrically (Visible UV Spectrophotometer Q898U2M5 Quimis, Brazil).

2.5. Statistical analyses

Due to the low number of specimens sampled from each species as a consequence of opportunistic sampling from fishery captures, our sample size for each species was low, preventing us from evaluating metal concentrations or physiological markers at a species-specific level. Thus, descriptive statistics were provided for each species, and the data analysis was conducted following the approach reported by Deming (2018), by grouping species and evaluating potential general relationships between tissue metal concentrations and various shark health and homeostasis metrics.

To test for the influence of shark size (considering total length) on metal concentrations in each tissue, data were analyzed using a Generalized Linear Mixed Model (GLMM) including species as a random effect. The overall relationships between the different tissues sampled were assessed using a Principal Component Analysis (PCA), which included the tissue-specific metal concentrations. Tissue-specific concentrations were then analyzed for each metal using independent analysis of variance (ANOVA) applying *posthoc* Tukey tests to potentially identify the groups of tissues with similar metal concentrations.

The effects of the bioaccumulation of each element on health indicators and homeostatic balance were investigated using GLMMs again including species as random effects. Due to the relationships between the chosen physiological markers and adequate organ/tissue functioning, specific serum components were respectively considered for liver (creatinine, triglycerides, alkaline phosphatase, bilirubin, and ALT/GTP), gills (urea, phosphorus, and lactate), rectal gland (phosphorus) and muscle (lactate). As body condition typically reflects energy storage (Gallagher et al., 2014), the possible influence of metal concentrations upon shark condition factor (K) and serum triglycerides and total cholesterol were also analyzed. For this purpose, a GLMM including both species and tissue as random effects was applied and included the candidate interactive effects between the respective metal concentrations and the biological variables sex (i.e., male x female) and life stage (i.e., juvenile x adult). All analyses were conducted using the R software (version 3.5.1), and the vegan package (Oksanen et al., 2018) was used for the PCA analysis.

3. Results

3.1. Influence of shark size on metal concentrations

The GLMM (Fig. 1) showed tissue-specific patterns for each metal when considering shark total length. A positive correlation was observed for Co, with higher concentrations detected in larger sharks in gills and muscle. The same pattern was observed for Cu and Fe, with similar higher concentration in the gills and muscle of larger individuals. For Hg, a positive correlation was observed only for the gills. Interestingly, a negative correlation was observed for Ni and Fe in the liver, with the lowest concentrations detected in the largest sharks.

3.2. Tissue-specific accumulation patterns and species-specific descriptive information

The PCA analysis (total variance explained = 67.1%) indicated a considerable similarity between the trace metal concentrations in shark muscle and gills, with both tissues showing overall lower concentrations in comparison with the rectal gland and liver (Fig. 2).

As for specific patterns for each tissue analyzed, the mean metal element concentrations in the muscle, gills, rectal gland and liver samples for each of the investigated shark species are displayed in Table 1.

Liver elemental concentrations were higher for all elements among the four investigated organs, across all species, with the exception of Co in *G. cirratum, R. porosus, I. oxyrhynchus* and *C. leucas*, where this metal was higher in the rectal gland, and in *C. porosus*, which was higher in the gills, followed by muscle. When evaluating muscle metal contents, several species displayed higher concentrations compared to the gills and rectal gland, indicating potential bioaccumulation. In addition, Cu was higher in the rectal gland in *C. porosus* compared to the other investigated organs. Hg was higher in the rectal gland in *Sphyrna lewini*. Mn was higher in the gills, followed by the liver in *G. cuvier*, and very similar in both gills and liver in *C. limbatus* and *G. cirratum*. *S. lewini* exhibited the highest content for this metal in muscle.*R. porosus* exhibited higher Mn contents in the rectal gland, followed by the liver, while the same was noted for *Carcharhinus leucas*, followed by muscle. Regarding Ni, the lowest levels were observed in *S. lewini* liver compared to the other organs evaluated in this species. Lower levels were also noted in *C. porosus* and *I. oxyrhynchus*, following the same trend of higher concentrations compared to the rectal gland but lower than in gills and muscle.

No statistical differences were found between tissue types for Co (F-value = 1.93; p-value = 0.132), Mn (F-value = 0.82; p-value = 0.485) or

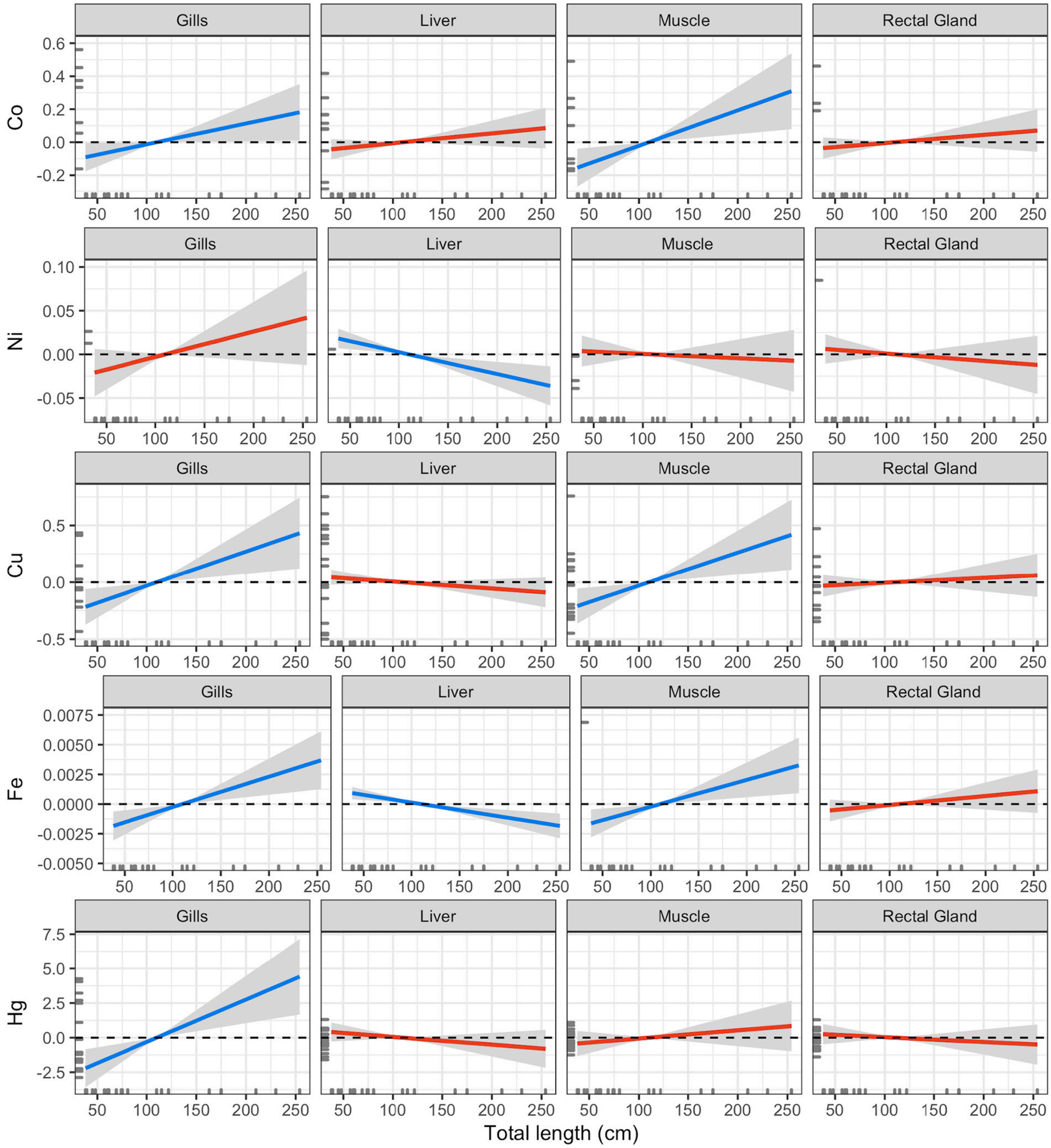

Fig. 1. Influence of shark size on concentrations of cobalt (Co), nickel (Ni), copper (Cu), iron (Fe) and mercury (Hg) in each tissue. Manganese (Mn) was excluded from the figure as no correlation was observed. The y-axes represent the modelled standardized partial residuals for each metal. Red and blue lines represent non-significant and significant regressions, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ni (F-value = 3.84; p-value = 0.062), whereas significant differences were identified for Cu (F-value = 10.95; p-value < 0.001), Fe (F-value = 17.35; p-value < 0.001) and Hg (F-value = 6.75; p-value < 0.001) (Fig. 3). Specifically, higher Cu and Fe concentrations were observed in shark liver as compared to the other tissues. Moreover, Hg concentrations in shark gill were significantly lower than in the other investigated tissues (Fig. 3).

3.3. Metal accumulation and shark overall health

As for the correlation between metal accumulation and overall health, the GLMM analysis indicated positive correlations between the tissue-specific concentrations of some metals and different physiological markers (Fig. 4). Specifically, shark liver Co was positively correlated with alanine transaminase, Fe was positively correlated with triglycerides and alkaline phosphatase, and Hg was correlated with alkaline phosphatase (Supp. Table 3). In shark gills, Fe and Hg were positively correlated with urea and lactate levels, respectively (Supp. Table 4), while phosphorus was positively correlated with Co, Mn, and Hg in the rectal gland (Supp. Table 5). No significant correlations were detected between any of the assessed metals and total cholesterol or bilirubin serum concentrations. For future comparative purposes, the highest and lowest concentrations (urea, phosphorus, lactate, triglycerides, total cholesterol, bilirubin and creatinine) and activities

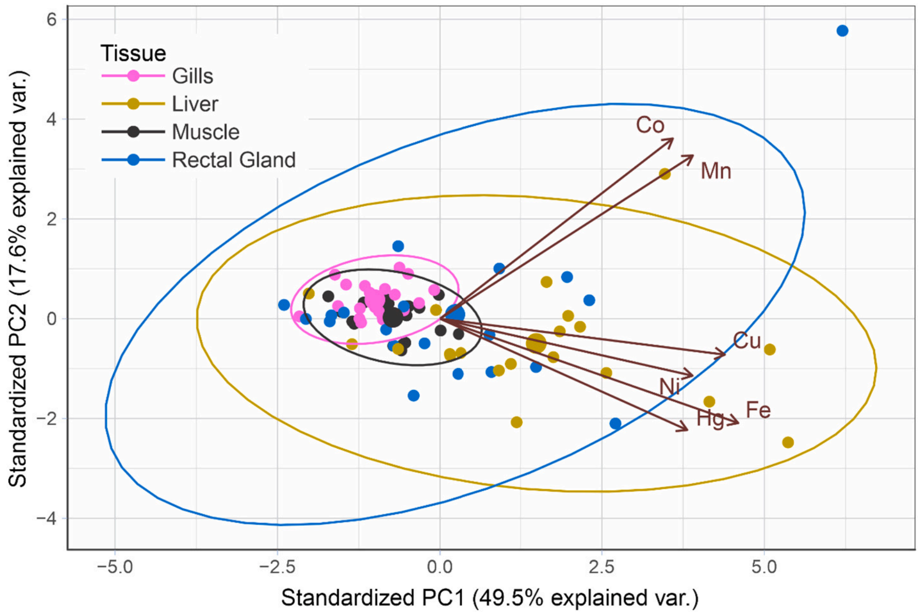

Fig. 2. Principal component analysis (PCA) for total concentration of metals per shark tissue types.

Table 1
Mean metal element concentrations (μg.g^{-1}, wet weight) in muscle, gills, rectal gland and liver samples of the eight investigated shark species.

Species (N)	Matrix	Co (μg.g^{-1})	Cu (μg.g^{-1})	Fe (μg.g^{-1})	Hg (μg.g^{-1})	Mn (μg.g^{-1})	Ni (μg.g^{-1})
Galeocerdo Cuvier (*N* = 1)	Muscle	1.43	0.90	87.74	0.167	3.43	10.54
	Gills	1.11	2.74	130.35	0.169	13.62	9.02
	Rectal gland	0.28	1.73	74.27	0.675	7.64	6.98
	Liver	8.92	3.66	367.73	0.134	12.15	30.41
Carcharhinus limbatus (*N* = 2)	Muscle	3.69	1.57	155.57	0.19	7.34	8.94
	Gills	3.94	0.96	104.19	0.15	8.42	12.24
	Rectal gland	1.79	1.77	153.98	0.53	3.46	11.44
	Liver	7.75	2.92	284.38	0.160	8.89	16.73
Sphyrna lewini (N = 2)	Muscle	3.89	1.89	156.66	0.220	10.18	8.32
	Gills	2.13	1.07	113.61	0.140	7.97	6.88
	Rectal gland	1.74	2.15	175.04	0.180	3.47	13.75
	Liver	3.95	6.80	255.29	0.160	3.03	3.42
Ginglymostoma cirratum (*N* = 3)	Muscle	2.98	1.16	100.15	0.140	3.66	9.13
	Gills	2.87	1.08	103.60	0.100	6.63	2.71
	Rectal gland	4.13	2.34	122.44	0.180	5.32	12.98
	Liver	2.58	2.95	238.50	0.230	7.66	23.74
Carcharhinus porosus (N = 3)	Muscle	3.93	1.53	146.02	0.240	5.72	13.12
	Gills	4.70	1.65	129.68	0.140	5.54	9.59
	Rectal gland	0.73	3.14	159.30	0.220	3.13	5.13
	Liver	1.91	2.93	246.33	0.390	8.23	9.35
Rhizoprionodon porosus (N = 3)	Muscle	2.37	1.40	114.09	0.170	7.04	1.89
	Gills	2.92	1.27	123.70	0.130	7.98	10.11
	Rectal gland	20.99	3.89	215.37	0.300	19.38	15.52
	Liver	5.33	4.06	291.07	0.420	15.43	28.40
Isogomphodon oxyrhynchus (N = 3)	Muscle	0.84	1.22	105.07	0.240	5.19	11.95
	Gills	3.96	1.16	98.02	0.100	5.22	11.78
	Rectal gland	5.90	0.83	137.45	0.110	3.97	3.19
	Liver	3.50	3.43	295.58	0.280	8.87	8.69
Carcharhinus leucas (N = 3)	Muscle	1.03	1.28	133.01	0.200	7.64	18.74
	Gills	3.98	0.99	117.85	0.120	2.15	8.88
	Rectal gland	8.62	2.31	220.79	0.290	13.03	24.23
	Liver	7.70	3.37	428.08	0.370	7.30	26.26

(alkaline phosphatase and ALT) detected in serum are displayed in Supplementary Table 6. The means and standard deviations for each marker are also presented.

Shark condition factor (K) was significantly influenced by interactions between sex and Co, Ni, Cu, and Fe concentrations (Supp. Table 7) (Fig. 5A). Among female sharks, higher metal concentrations were correlated with lower condition factor values, except for Fe (Supp. Table 7). In male sharks, higher Cu and Fe concentrations were positively correlated with body condition (Supp. Table 7). Regarding life stage, serum triglycerides were influenced by specific Co, Ni, and Fe concentrations (Supp. Table 8) (Fig. 5B). Adult sharks exhibited higher serum triglyceride concentrations positively correlated with Co and Fe, whereas a negative correlation between serum triglycerides and Ni concentrations was observed for juveniles (Supp. Table 8).

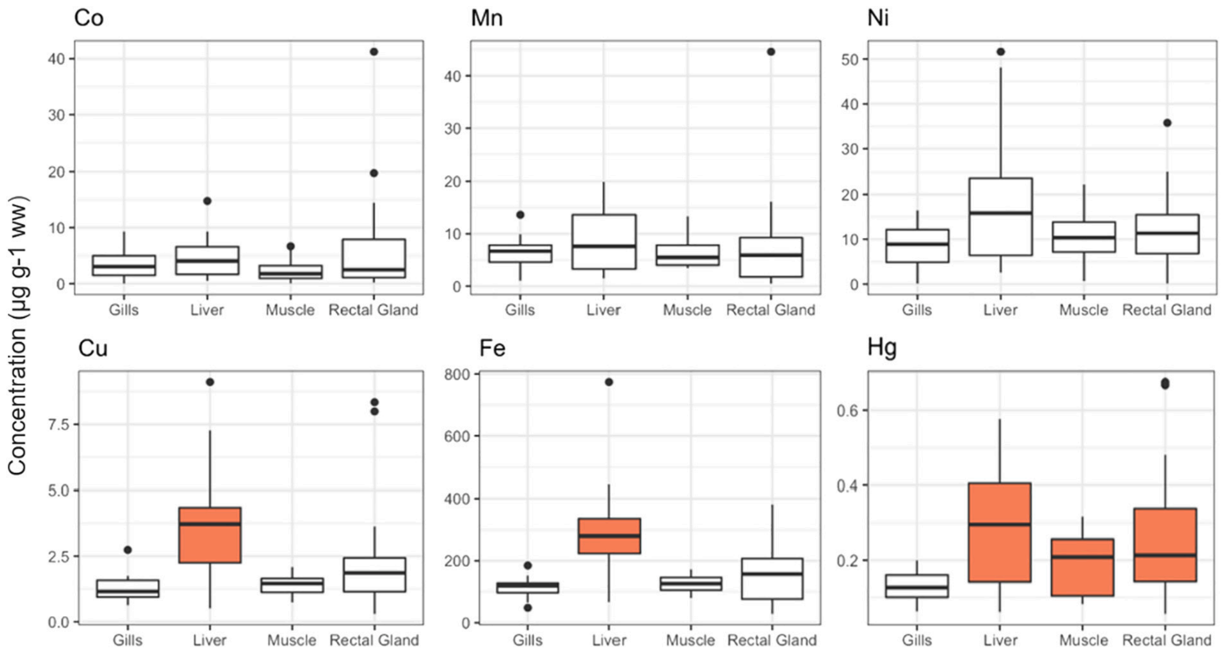

Fig. 3. Shark tissue-specific variations in cobalt (Co), manganese (Mn), nickel (Ni), copper (Cu), iron (Fe) and mercury (Hg) concentrations. Significant groups identified by the post-hoc Tukey tests (lower panel) are represented by the colored boxplots.

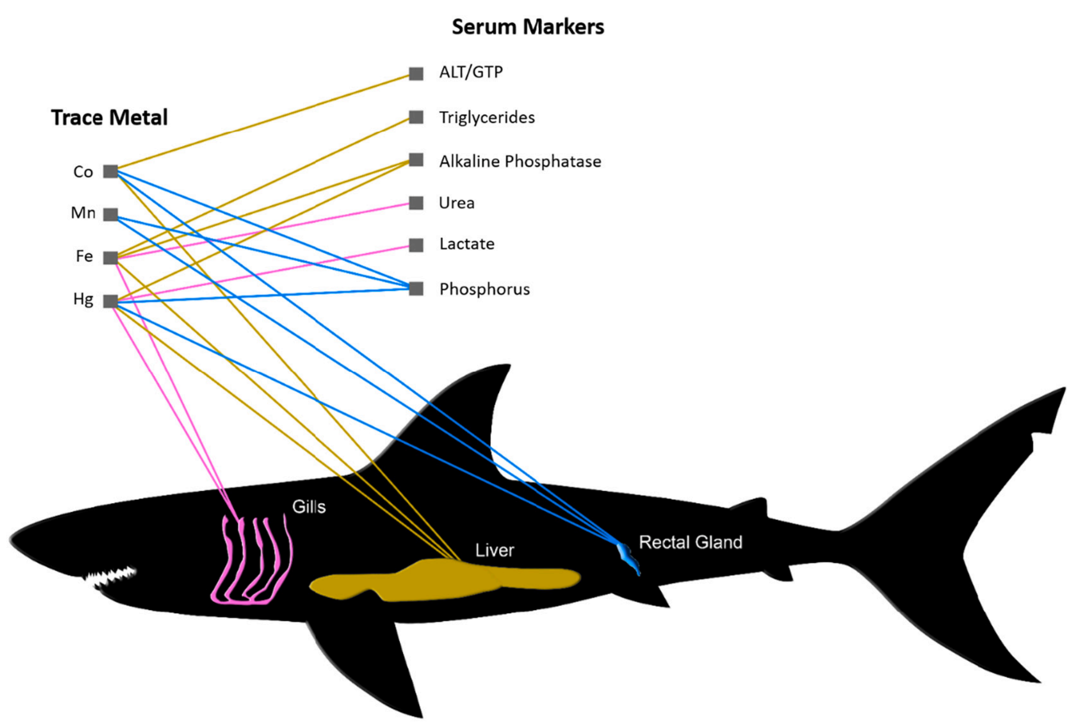

Fig. 4. Positive tissue-specific relationships between shark metal concentrations and physiological markers in the gills, liver and rectal gland. The pink lines represent the associations between urea and lactate concentration alterations with Fe and Hg bioaccumulations in gills. The yellow lines represent the associations between alterations in the physiological markers ALT, triglycerides, and alkaline phosphatase with Co, Fe, and Hg bioaccumulations in liver. The blue lines represent the associations between phosphorus concentration alterations and Co, Mn, and Hg bioaccumulations in the rectal gland. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Shark size

We found mixed evidence for our first hypothesis of an expected positive relationship between shark size and metal concentrations. The positive correlations found between Co, Cu and Fe concentrations and shark size for gills and muscle suggests a lower detoxification potential for these elements. As fish age is typically positively correlated to fish length (Trudel and Rasmussen, 1997; Van Walleghem et al., 2007), slow elimination rates, or, alternatively, slow growth rates, (Shipley et al., 2021) may lead to higher metal concentrations with increasing fish age,

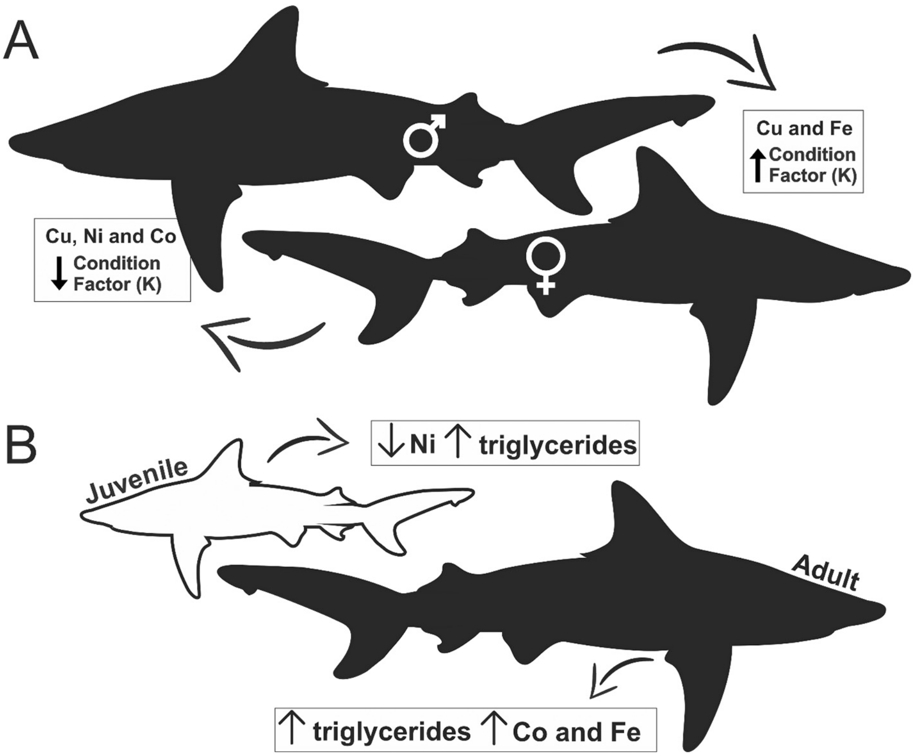

Fig. 5. Correlations between metal concentrations and physical and nutritional condition indicators between shark sexes and life stages. (A) In females, Co, Ni, and Cu bioaccumulation were related to lower physical condition scores. In males, Cu and Fe bioaccumulation was associated to higher physical condition scores. (B) In juveniles, a relationship between Ni bioaccumulation and lower triglyceride concentrations was observed, while in adults, the Co and Fe bioaccumulation led to higher triglyceride concentrations.

as fish consume more contaminated prey through time and/or are chronically exposed to environmental metal contamination (Castro-González and Méndez-Armenta, 2008; Chumchal and Hambright, 2009; Piraino and Taylor, 2009). This has been observed in several shark species for different metals, such as Al, Cr and Cu in *Prionace glauca* from the southeast-south coast of Brazil (Vignatti et al., 2018), and Hg, Fe and Se in several shark species from Jeju Island, Korea (Kim et al., 2019).

For Hg, a positive correlation with size was observed only in the gills. As Hg gill assessments in sharks are still scarce, no basis for this correlation was found in the literature, expect for the possibility of high environmental Hg concentrations in the water column and a primary Hg accumulation pathway through the gills, as postulated for sharks from the Bahamas (Shipley et al., 2021). Other alternative explanations have also been postulated, for example, when assessments evaluate mostly juveniles, a limited range of assessed body length is obtained, such as reported for several elasmobranch studies, i.e., Silvertip sharks (*C. albimarginatus*) from Japan (Endo et al., 2009), or due to relatively low sample size numbers, as reported for the Smooth hammerhead shark, *S. zygaena*, from the Mexican Pacific Ocean (Escobar-Sánchez et al., 2010). This lack of correlations has also been recently reported for juvenile *R. lalandii* and *R. porosus* from southeastern Brazil concerning both liver and muscle Hg concentrations (Amorim-Lopes et al., 2020). Therefore, any lack of correlation between Hg concentrations in shark tissues and organism lengths should not be applied as evidence of effective detoxification (Escobar-Sánchez et al., 2010), and further studies are required in this regard.

Ni and Fe concentrations in relation to shark size exhibited the opposite pattern in the liver (negative correlation). Accordingly, we suggest that sharks may develop more efficient hepatic detoxification mechanisms (e.g., metallothionein synthesis) for these elements. As Ni is essential to energetic metabolism and endocrine dynamics (He et al., 2014), it is also possible that the negative correlations are a result of organismal assimilation. Regarding Fe, this may also be due to alterations in ferritin and transferrin levels, which store and transport Fe, respectively (Andersen, 1997), although such a pattern has yet to be reported in sharks. With respect to the elements for which no correlations were detected with shark size, this is possibly the result of the balance between their absorption and secretion as detoxification mechanisms are expected to take place for essential trace elements (Nishito and Kambe, 2018).

4.2. Tissue concentrations

We found support for our second hypotheses that metal concentrations would be higher in the liver. However, high concentrations were also observed in the rectal gland. As expected, the gills and muscle exhibited the lowest concentrations for all the elements analyzed, indicating two clusters (liver and rectal gland; gills and muscle) grouped by their similarity regarding accumulation potential (Fig. 2).

The hepatic accumulation observed herein (Table 1) corroborates results from previous studies, as the liver is the main homeostatic organ for higher vertebrates, thus leading to elevated tissue metal concentrations (Grosell et al., 2003; De Boeck et al., 2010; Terrazas-López et al., 2016; Endo et al., 2017; Adel et al., 2018; Boldrocchi et al., 2019; Amorim-Lopes et al., 2020). Such a pattern is probably related to its lipid-rich composition and metabolizing function (Ballantyne, 1997; Speers-Roesch and Treberg, 2010).

The rectal gland plays an important role in shark osmoregulation and associated homeostatic balance (Hammerschlag, 2006; Ballantyne and Fraser, 2012). The accumulation of Hg in the rectal gland found herein (Table 1) may be related to its high vascularization, suggesting target organ concerning metal toxicity. Similar results were observed for the Spotted dogfish, *Scyliorhinus canicula,* with high concentrations of Ag, Cu and Pb detected in the rectal gland, indicating bioaccumulation potential (De Boeck et al., 2010).

The overall lower concentrations of metals found in the gills (Table 1) indicate that bioaccumulation occurs mostly through dietary intake rather than direct environmental input, corroborating results previously published by Mathews and Fisher (2009) for *S. canicula*. Moreover, the higher metal concentrations detected in the rectal gland compared to gills may be also due to the former's primary role in ion-oregulation as compared to the latter (De Boeck et al., 2010).

Muscle tissue exhibited relatively low concentrations of all metals compared to the other analyzed tissues (Table 1), corroborating data previously reported for several shark species, including those analyzed in the present study (Endo et al., 2008, 2017; Alves et al., 2016; Terrazas-López et al., 2016). Low muscle lipidic content and perfusion rates could be the main contributing factors for the reduced concentrations observed in muscle tissue (Wood, 2011).

4.3. Physiological impairment

We found empirical evidence in support of our third hypothesis that systemic health and osmoregulatory capacity would be compromised from metal toxicity. Hepatic alterations caused by metal bioaccumulation have previously been reported for sharks using oxidative stress markers (Barrera-García et al., 2013; Vélez-Alavez et al., 2013). In contrast, the effective use of physiological markers as indicative of structural and functional damage in the liver has yet to be validated for most shark species (Wosnick et al., 2020). The results of this study suggest that hepatic functioning alterations may be linked to metal bioaccumulation based on the examination of key hepatic markers and metabolites measured in serum. To date, data on the negative impacts of metal accumulation on liver functioning are available only for Blacktip sharks (Norris et al., 2021). However, different from our results, metal accumulation (i.e., Hg) did not affected the activity of ALT in the species. In the present study, hepatic Co accumulation (0.58–14.76 μ g g^{-1} ww) was directly associated with increased ALT activity (75.7–2389 U L^{-1}), suggesting damage to hepatocyte membranes. Despite being an essential element, Co was shown to be potentially toxic to rodents, with high concentrations related to increased ALT activity (Mohammed et al., 2014; Rasool et al., 2020). Moreover, hepatic Fe (66.84–773.79 μ g g^{-1} ww) and Hg (0.061–0.576 μ g g^{-1} ww) concentrations were correlated with an increased activity of alkaline phosphatase (1.7–55.1 U L^{-1}), also suggesting alterations in liver/kidney functioning. Increased alkaline phosphatase activity can also be indicative of gallbladder damage or cholestasis (Wosnick et al., 2020). However, this was ruled out, since serum bilirubin levels did not exhibit significant changes concerning hepatic metal accumulation. Previous studies have found that liver disorders can lead to higher triglyceride concentrations, indicative of metabolic dysfunctions (Sabesin et al., 1980). Our findings of increasing Fe concentrations (66.84–773.79 μ g g^{-1} ww) in shark liver associated with higher serum triglycerides (101.3–413 mg dL^{-1}) could also be related to metabolic impairment from toxicity, with potential negative effects on general health and long-term individual survival.

One of the main roles of gills in sharks is to maintain the acid-basic balance and prevent systemic acidosis or alkalosis (Shuttleworth, 1988). Additionally, gills play a major role in urea regulation, the main osmolyte for elasmobranch homeostatic balance (Hammerschlag, 2006). Traditionally, Na^+/K^+ATPase activity and expression are used to evaluate the sublethal effects of exposure to metals in the gills and rectal gland (De Boeck et al., 2001, 2007; Grosell et al., 2003; Eyckmans et al., 2013). However, lactate and urea have also been validated and applied as physiological markers of metal bioaccumulation in gills (De Boeck et al., 2001, 2007, 2010; Eyckmans et al., 2013). Our results suggest a direct relationship between the dysregulation of both urea (265.7–698.3 mmol L^{-1}) and lactate (2.1–16.2 mmol L^{-1}) markers with Fe (48.25–184.76 μ g g^{-1} ww) and Hg (0.069–0.169 μ g g^{-1} ww) concentrations in gills, respectively. This finding is an indication of potentially compromised gill functioning (i.e., loss of acid-basic balance and reduction in urea regulation capacity) due to metal toxicity, leading to reduced homeostatic maintenance and systemic health disruption.

Although not directly regulated by the rectal gland, phosphorus plays a key role in saline secretion (NaCl). More specifically, phosphorus acts as a substrate for the phosphorylation of Na^+/K^+-ATPase and Na-K-Cl cotransporter, both essential for proper ionic regulation (Forrest Jr, 2016). Consequently, the associations between higher Co (0.27–41.23 μg g^{-1} ww), Mn (1.88–44.57 μg g^{-1} ww), and Hg (0.08–0.324 μg g^{-1} ww) concentrations in the rectal gland and elevated levels of circulating phosphorus (8.2–16.9 mmol L^{-1}) observed in the present study, may lead to direct effects on adequate enzyme functioning and subsequent osmoregulation. Considering the high concentration of phospholipids in this organ (Gerzeli et al., 1976), positive correlations between metal bioaccumulation and circulating phosphorus may also be indicative of cell membrane denaturation due to metal toxicity.

4.4. Sex and life stage

Consistent with our fourth hypothesis, we detected influences of sex and life stage on the relationships between metal bioaccumulation and shark physical/nutritional condition. Herein we detected the highest Co, Ni and Cu concentrations and associated lower condition factors in female sharks. While the effects of such metal concentrations on shark physiology require additional study, it is plausible that potential greater toxicity in females could lead to negative demographic and population consequences from contamination, since lower health could impair fecundity and reproductive success (Naulleau and Bonnet, 1996). Several metals have been reported as directly disrupting reproductive functions in fish, for example, leading to decreased estrogenic and androgenic secretions (Ebrahimi and Taherianfard, 2011). Toxic thresholds are, however, only available for bony fish. The lower condition found in females could also be related to an imbalance in metabolic pathways associated with food acquisition. Regardless, the liver appeared to be the most affected tissue in females, with potentially negative consequences to reproduction given the liver's important role in vitellogenesis, maternal-fetal transfer, and yolk quality (Hussey et al., 2010).

Co and Cu are considered essential elements and are required for adequate homeostasis and protein/enzymatic functions (Carvalho et al., 2005). However, even essential elements when present in excess of normal concentrations may lead to deleterious effects (Hauser-Davis and Lavradas, 2018). In teleost fishes, exposure to all three elements has been reported to have deleterious effects (Moiseenko and Kudryavtseva, 2001), and associations between these elements and morphometric indices in fish (e.g., condition factor), have been previously reported. For example, both Ni and Co have been associated with decreased condition factors in the bony fishes *Catla catla*, *Labeo rohita* and *Cirrhina mrigala* (Javed, 2013). Moreover, a significant association between Cu and the condition factor has been recently demonstrated for *Dules auriga*, indicating that increasing Cu may decrease morphometric indexes (Hauser-Davis et al., 2021). This corroborates the data reported herein for sharks, although further assessments are still required in this regard, as studies are still scarce and most have been conducted for teleosts.

Interestingly, higher Cu concentrations did not appear to negatively affect the physical condition of males. Concentrations of both Cu and Fe were, in fact, positively correlated with condition factor. Higher Cu concentrations are related to higher body weight in male rodents (Kowal et al., 2010), and this essential element plays a key role in male fertility, as it modulates spermatogenesis and androgenic pathways (i.e., hypothalamic-pituitary-testis) (Ogórek et al., 2017). Fe is also imperative for males, not only during spermatogenesis but also for steroid synthesis throughout life (Gabrielsen et al., 2018). Although not directly related to physical condition, Cu and Fe roles mentioned above may, at least partially, explain the positive correlation observed for males in the present study. However, further studies are needed to elucidate the possible interactions between Cu and Fe and the physical condition of males, considering not only sex, but also the stages of sexual maturation and associated endocrine responses.

Previous studies have noted significant relationships between life stage and metal bioaccumulation (Maz-Courrau et al., 2012; Terrazas-López et al., 2016; Matulik et al., 2017). However, these results may be more related to animal size rather than age/life stage itself. According to our results, serum triglycerides in adults were higher when Co and Fe concentrations were more elevated. Consequently, adults may be more affected by the cumulative effects of Co and Fe than juvenile sharks, with potentially negative consequences upon energy metabolism and storage mobilization for daily activities. In juveniles, higher Ni concentrations led to lower concentrations of circulating triglycerides. This is an essential element of great importance in urease activity and metabolism in plants and ruminants (Spears and Hatfield, 1978; Polacco et al., 2013), and its role in osmotic balance in aquatic organisms has only been briefly discussed (Muyssen et al., 2004). Given the ureotelic profile of sharks and the high costs of urea production (Speers-Roesch et al., 2006), it is plausible to assume that the negative relationship found between triglycerides and Ni in juveniles might be linked to the energetic demands of urease during this critical life development period, where the costs of osmoregulation must be balanced against the costs of growth. Further studies are, however, necessary to better elucidate these still unknown relationships in sharks.

Some important limitations must be considered. As this study was opportunistically performed with sharks incidentally captured by artisanal fishers, our sample size was low. Therefore, in order to be able to carry out the statistical analyses necessary for testing the proposed hypotheses, it was necessary to group all individuals, thus precluding the evaluation of any species-specific effects. Future studies should consider including larger sample sizes to address such a knowledge gap. Considering the need for non-lethal methodologies in studies with sharks (Hammerschlag and Sulikowski, 2011), toxicology studies could benefit from using animals captured by commercial fisheries. However, considering that many sharks are landed beheaded and eviscerated (Wosnick et al., 2019), sampling of internal organs and blood may be compromised. Besides, given than *post-mortem* alterations are expected for some of the evaluated serum markers (i.e., urea and phosphorus) (Donaldson and Lamont, 2013), results should be compared with reference intervals when available and interpreted with caution.

It is becoming increasingly necessary to understand the effects of metal bioaccumulation on the systemic health and well-being of top predators as marine pollution is reaching alarming rates. This is particularly relevant considering that health impairments may compromise fitness, thus impacting population recruitment, even if the sublethal effects are not directly linked to reproduction. Many shark species are under imminent threat of extinction, with pollution being an additional stressor to overexploitation. Therefore, further research into their physiological vulnerability may aid in elucidating key aspects for the implementation of contingency plans aimed at conservation. Health monitoring of individuals and populations through physiological markers sampled using non-lethal approaches could help unravel the long-term effects of pollution and their real impacts for shark conservation.

CRediT authorship contribution statement

NW and APC were responsible for the material collection and analysis performed. RCCR was responsible for data structuring and metal concentration calculations. YN was responsible for the statistical design. JLSN and MJB provided logistical and financial support for the study. NH and RHD were responsible for the structuring and extensive revision of the manuscript. All authors contributed intellectually to the manuscript.

Declaration of competing interest

Authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112398.

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