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Biliary and hepatic metallothionein, metals and trace elements in environmentally exposed neotropical cichlids *Geophagus brasiliensis*

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ABSTRACT

One of the metal detoxifying mechanisms that occurs in fish is metallothionein (MT) induction and metal binding. Hepatic MT induction has been well described, but biliary MT metal detoxification has only recently been described in fish. In this scenario, metal-metal interactions have been increasingly evaluated to further understand the behavior of these contaminants regarding homeostasis and biological functions, as well as their toxic effects. Studies, however, have been mainly conducted concerning the elemental pair Se-Hg, and scarce reports are available concerning other metal pairs. Therefore, this study aimed to evaluate biliary and hepatic MT metal detoxification mechanisms in a territorial neotropical cichlid, *Geophagus brasiliensis*. Fish were sampled from the anthropogenically impacted estuarine Rodrigo de Freitas Lagoon, located in Southern Rio de Janeiro, and trace elements and MT were determined by inductively coupled plasma mass spectrometry (ICP-MS) and UV-Vis spectrophotometry, respectively, in fish liver and bile. MT in bile were significantly lower than in liver. Significant differences between bile and liver were observed for many trace elements, and, although most were higher in liver, Cd and Ni were significantly higher in bile, indicating efficient excretion from the body via the biliary route. A significant correlation was observed between MT and Fe in bile, and between MT in liver and Cu and Zn in bile. Molar ratio calculations demonstrated protective elements effects against Al, As, Cd, Hg, Pb and V in both bile and liver, as well as some novel interrelationships, indicating the importance of these investigations regarding the elucidation of element detoxifying mechanisms. Furthermore, investigation of other elemental associations may aid in decision-making processes regarding environmental contamination scenarios linked to public health.

1. Introduction

Aquatic environmental pollution by metals and trace-elements has become a worldwide problem, since these compounds show potential toxic effects and can bioaccumulate in aquatic ecosystems [1]. To counteract the negative effects caused by the presence of toxic elements or essential elements in excess, organisms present certain biochemical defenses, such as increased metallothionein (MT) synthesis, that binds to free elements [2]. MT have been implicated in the homeostasis of essential elements, such as Cu and Zn, as well as in the detoxification of toxic metals, such as Ag, Cd, Pb and Hg [3], although this metalloprotein has also displayed protective free radical scavenging activity, playing an active role in the capture of harmful oxidant species [4], both capabilities due to abundant cysteine residues. Because of these properties, MT are considered adequate biomarkers for metal and trace-element exposure, and have been increasingly applied to detect oxidative stress.

Recently, biliary fish MT have been shown to follow the same trend as hepatic MT, demonstrating an alternative detoxifying mechanism for metals and trace-elements [5]. In addition, fish bile has also been

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validated as a biomarker for metal and trace-element excretion, and certain elements have been reported as excreted preferentially by this route, before reaching a certain threshold and accumulating in the liver [6]. However, studies in this regard are scarce, and many variables in this process are still unknown. Thus, further analyses are required to better investigate this detoxification route.

Elemental interactions in living organisms may lead to different effects, by inducing synergistic (increased), antagonistic (decreased) or additive (independent) behavior [7], depending on element bioavailability, uptake from the environment and different distribution patterns in fish tissues [8]. Because of this, interactive effects of elemental pairs have been increasingly evaluated in both in vivo and in vitro studies, in the laboratory or in the field, and contribute to further understanding of the behavior of these contaminants both in homeostasis and biological functions and concerning toxic effects. Studies in this regard, however, have been mainly conducted concerning the Se-Hg pair, and not many reports are available concerning other elemental pairs. In addition, to the best of our knowledge, no studies in this regard have been conducted in bile.

In this context, the aim of the present study was to characterize biliary and hepatic metallothionein-mediated element detoxification mechanisms and interactions of both essential and non-essential metals and trace-elements by metallothioneins in environmentally exposed territorial neotropical cichlids *Geophagus brasiliensis*.

2. Methodology

2.1. Study area

The estuarine ecosystem of the Rodrigo de Freitas Lagoon is located in the highly urbanized south region of Rio de Janeiro (22°5700200S; 043° 1100900 W), southeastern Brazil (Fig. 1). It is connected to the sea by a narrow channel, although this channel is frequently blocked by sand deposits and, thus, does not allow for free water exchanges, restricting circulation and water renewal [9]. Because of this, water stratification occurs, with the deeper water layer frequently becoming anaerobic and rich in hydrogen sulfide (H_2S) gas, due to oxidation of the organic matter present in the bottom of the lagoon. This, in turn, results in low water quality level, leading to many episodes of low dissolved oxygen levels, anoxic conditions and frequent fish mortality [10]. These characteristics present favorable conditions for pollutant accumulation in the lagoon's substratum. In addition, the lagoon illegally receives untreated domestic sewage enriched in organic matter, detergents, synthetic organic material and metals and trace-elements on a daily basis, and is also exposed to huge hydrocarbon and metal and trace-elements inputs originated from incomplete combustion of fossil fuels, due to the approximately 190 thousand vehicles that pass through the area each day [11], as well as the presence of several surrounding gas stations [9].

Fig. 1. Map of the study area, with the Rodrigo de Freitas Lagoon displayed in the inlay, located in Southeastern Brazil.

2.2. Sampling

Adult *G. brasiliensis* specimens (n = 17) were sampled from the Rodrigo de Freitas Lagoon by local fishermen and maintained alive in underwater cages until taken to the laboratory (time between capture and transport was no longer than 1h30 min). This species was chosen because it is considered an adequate sentinel species regarding environmental monitoring, since it is non-migratory, territorial and limnobenthofagous, and, therefore, particularly exposed to sediment-associated contamination, such as metals and trace-elements [12].

At the laboratory, fish were quickly sacrificed by spinal cord severing. After weighing and measuring each individual, bile was collected by direct puncture of the gallbladder with a disposable syringe, and livers were dissected, removed and weighed. Bile samples were immediately aliquoted and stored at −80∘C in sterile eppendorfs, while liver samples were freeze-dried for 48 h (Liotop 101, Liobrás, São Paulo, Brazil) and powdered with mortar and pestle.

2.3. MT purification and determination

MT purification was carried out by taking advantage of the heat-stable properties of these metalloproteins. Briefly, liver and bile samples (approximately 100 mg aliquots) were homogenized in a buffer solution (Tris−HCl 20 mmol L^{-1}, pH 8.6 containing phenylmethylsulphonylfluoride at 0.5 mmol L^{-1} as an antiproteolytic agent and β-mercaptoethanol 0.01% (w/v)) as a reducing agent, and centrifuged at 20,000 x *g* for 1 h at 4 °C in a Mikro 220R refrigerated centrifuge (Hettich, Germany). The supernatants were then heated at 70 °C for 10 min, to denature most proteins, centrifuged again in the same conditions for 30 min to precipitate the denatured proteins, and then collected and frozen at −80 °C [13]. MT quantification in the purified supernatants followed the protocol reported by Viarengo et al. [14], with modifications, where samples are homogenized in a buffer solution (1 mol L^{-1} HCl, 4 mol L^{-1} EDTA, 2 mol L^{-1} NaCl, 0.43 mol L^{-1} DTNB in a 0.2 mol L^{-1} Na_2PO_4, pH 8.0), incubated for 30 min to form a colored solution [15] and then analyzed by UV–vis spectrophotometry at 412 nm, carried out using a Perkin Elmer Lambda 35 spectrophotometer. MT concentrations were estimated using GSH as an external standard by applying an equivalence ratio of 20 mol GSH:1 mol MT [16].

2.4. Metal and trace-element determinations

Approximately 250 mg of each sample (liver in powdered form and bile in liquid form) were weighed in 15 ml screw-capped polypropylene tubes. Concentrated subboiled bidistilled nitric acid (Vetec, Rio de Janeiro) was added to each sample (1.5 mL) and the mixtures were left to stand, closed, overnight at room temperature. The following day, the acid digestions were completed by heating the samples at 100 °C, for approximately 4 h in the closed vessels, to avoid volatilization of certain elements, such as Hg and Se. Samples were then diluted with ultra-pure water (resistivity > 18.0 MΩ cm) to 10 mL. Metals and trace-elements (Al, V, Cr, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Hg and Pb) were determined, in triplicate, using multielemental external calibration, by appropriate dilutions of a mixed standard solution (Merck IV). The determinations were conducted on an ELAN DRC II ICP-MS (Perkin-Elmer Sciex, Norwalk, CT, USA), using ^{103}Rh as an internal standard, at 20 mg L^{-1}. Method accuracy was verified with procedural blanks and by the parallel analysis of DORM-4 (dogfish muscle tissue) certified reference material (National Research Council of Canada), in triplicate. Table 1 displays the observed and certified values for the DORM-4 certified reference material (in mg kg^{-1} dry weight), their recoveries (%) and the LODs (in mg kg^{-1}) for each determined element.

Table 1
Observed and certified values for the DORM-4 certified reference material (in mg kg^{-1} dry weight), recoveries (%) and LODs (in mg kg^{-1}) for each element determined in the present study.

	Observed value	Certified Value	Recovery (%)	LOD
Al	1308 ± 91.7	1280 ± 340	102	0.05
V	1.52 ± 0.14	1.57 ± 0.14	97	0.08
Cr	1.75 ± 0.27	1.87 ± 0.18	94	0.05
Fe	351 ± 35.3	343 ± 20	102	0.39
Co	0.25 ± 0.018	0.25	100	0.0003
Ni	1.29 ± 0.11	1.34 ± 0.14	96	0.004
Cu	16.3 ± 0.87	15.7 ± 0.46	104	0.011
Zn	54.7 ± 5.65	51.6 ± 2.8	106	0.08
As	7.03 ± 0.20	6.87 ± 0.44	102	0.005
Se	3.63 ± 0.25	3.45 ± 0.40	105	0.04
Cd	0.36 ± 0.025	0.299 ± 0.018	87	0.001
Hg	0.43 ± 0.035	0.412 ± 0.0036	104	0.002
Pb	0.40 ± 0.13	0.404 ± 0.062	99	0.001

2.5. Statistical analyses

All data were normalized by fish length, in order to remove interferences due to differences in size and weight. Thus, parametric tests were subsequently applied. Pearson's correlation test was used to evaluate the degree of associations between the elements themselves and between MT determined in liver and bile, while Student's *t*-test was used to assess the differences between the elemental concentrations in the different organs. The significance level for all statistical tests was set at $p < 0.05$. To elucidate the overall relationship between elements and MT in liver and bile, a Principal Component Analysis (PCA) was carried out, in addition to a Cluster analysis (CA), to further classify the elements into groups representing the different variables (MT and elements) in liver and bile. The variables used in these statistical techniques were standardized by means of Z-scores. Euclidean distances were calculated and Ward's method was performed. A dendrogram was then constructed to assess the cohesiveness of the formed groupings, in which correlations between the elements and MT in the different tissues could be observed. The statistical analyses were performed on the Statistica 7 (StatSoft) and R version 3.0.2 software packages for Windows.

3. Results and discussion

3.1. MT, metal and trace-element levels in G. brasiliensis bile and liver

As anticipated, MT levels in bile were significantly lower than liver concentrations (Fig. 2), corroborating previous studies conducted in fish [5]. This is expected, since the liver is the main detoxifying organ of the body and can accumulate high levels of contaminants, whereas only

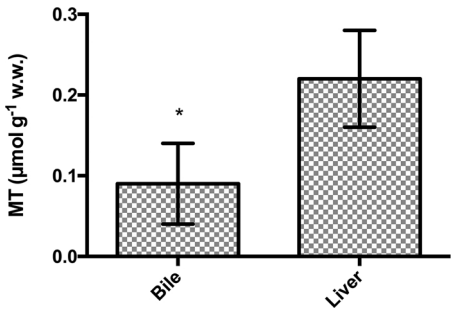

Fig. 2. Biliary and hepatic metallothionein levels in *G. brasiliensis* sampled from the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil. Mean values and standard deviations are presented; n = 17.

excess bile is stored in the gallbladder, in contact with xenobiotics being constantly detoxified by the liver [5,17].

Regarding metal and trace-element levels, significant differences between bile and liver were observed for Cd, Fe, Ni, Cu, Zn, As, Se and Hg. Table 2 displays the metal and trace-element concentrations determined in the present study for both liver and bile. Liver data, although obtained from freeze-dried samples, was transformed into wet weight to carry out comparisons with bile, by calculating 70% water content.

Table 2
Metal and trace-element concentrations in *G. brasiliensis* bile and liver from the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil (data are displayed as means ± standard deviation, in mg L^{-1} wet weight for both liver and bile, n = 17).

	Bile	Liver
Al	< LOD	2.256 ± 1.650
V	< LOD	0.631 ± 0.221
Cr	0.240 ± 0.135	0.476 ± 0.272
Fe	11.163 ± 4.145	539.7 ± ± 195.7[a]
Co	0.380 ± 0.235	0.312 ± 0.122
Ni	0.568 ± 0.337[a]	0.104 ± 0.052
Cu	6.166 ± 1.809	11.15 ± 6.695[a]
Zn	1.299 ± 1.444	36.11 ± 7.116[a]
As	0.198 ± 0.058	0.473 ± 0.098[a]
Se	0.425 ± 0.161	5.308 ± 1.421[a]
Cd	0.790 ± 0.66[a]	0.030 ± 0.023
Hg	0.012 ± 0.010	0.022 ± 0.005[a]
Pb	0.978 ± 0.530	< LOD

[a] Indicates statistically significant differences between bile and liver.

Al and V were below the LOD in bile, while Pb was below the LOD in liver. Fe, Cu, Se, As, Hg and Zn were not efficiently removed by the biliary pathway, since concentrations were significantly higher in liver. Cd and Ni, however, were significantly higher in bile, indicating efficient excretion from the body through this pathway.

Regarding toxic effects to exposed organisms, some studies do not state in which tissue certain toxicity thresholds for aquatic biota are reported, while others state this is specifically in muscle. Thus, we will compare levels reported in the literature for each element with both liver and bile and briefly discuss comparisons with limits reported in the literature. In addition, not all elements have literature-reported values regarding toxicity thresholds in aquatic organisms.

Among those that have been described, Se has been reported as showing adverse effects on aquatic biota in the range of 0.117 to 0.75 mg kg^{-1} (wet weight) in tissues in general, while As is considered damaging for fish and other aquatic organisms at over 0.09 mg kg^{-1} wet weight in tissues in general [18–20]. Se in the present study was significantly higher than the level of concern in liver, and in the cited range in bile, while As levels where in the concern range for both organs. As was substantially higher than the average found in biota, of 0.09 mg kg^{-1} wet weight, indicating exposure to this element from pesticides, herbicides, or mine wastes [21]. Regarding Pb, there is no toxic threshold established for this metal, and, thus, any lead concentration in the body is considered harmful [22]. Thus, levels detected in bile indicated at least some exposure to this metal in the environment, which may lead to deleterious effects.

Hg concentrations as low as 0.008 mg kg^{-1} wet weight in muscle are enough to affect biochemistry (for example, nervous system enzymatic activity), as well as gene expression, and thresholds for effects on reproduction, histology, and growth have been reported as approximately 0.135 mg kg^{-1} wet weight in muscle [23]. Thus, in the present study, Hg concentrations may pose a threat to the analyzed fish species, since both liver and bile metal concentrations were slightly higher than these thresholds.

With regard to Cd, acute lethal effects for marine organisms have been noted at concentrations of approximately 4.8 mg kg^{-1} [24]. Thus, the values observed in the present study do not indicate concern related to exposure to this metal, as both liver and bile concentrations were orders of magnitude lower than this toxicity threshold.

Tissue levels above 1.2 mg kg^{-1} total Cr dry weight should be viewed as presumptive evidence of Cr contamination [25], which, in the present study, does not seem to be the case, as concentrations observed in both liver and bile were much lower than this limit.

Regarding Al, Ni, Cu, Fe, Zn and Fe, the literature is extremely conflicting, and most reports generally state in many different ways that "these elements are essential but may become toxic at higher concentrations" (Hauser-Davis, *Pers. Comm*). Thus, no concrete comparisons regarding fish toxicity could be conducted with the values observed in the present study.

3.2. Statistical correlations between MT and metals and trace-elements in *G. brasiliensis* bile and liver

The significant ($p < 0.05$) Pearson correlations observed between MT and metals and trace-elements in liver and bile are displayed in Table 3.

Table 3
Significant Pearson correlations between MT and metals and trace-elements in *G. brasiliensis* bile and liver from the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil (n = 17).

Matrix	Relationship	Strength of the association
Bile	MT x Fe	r = 0.97
Liver x bile	MT (l) x Cu (b)	r = 0.93
	MT (l) x Zn (b)	r = 0.98

All significant correlations were very strong, observed in bile or between liver and bile. No associations were found for liver. Removal by the biliary route seem to be very efficient for Fe MT-detoxification, while in liver, Cu and Zn seem to be similarly bound to MT, noted by similar associations strengths, as expected, since both elements bind easily to MT for organism homeostasis [26].

The correlations between liver and/or bile MT and/or metals and trace-elements indicate an interesting link between these variables. On hypothesis to explain these correlation would be that free liver MT, excreted alongside excess bile, may be able to also bind to metals and trace-elements present in stored gallbladder bile. In fish, as in higher vertebrates, bile is produced continuously by the liver to aid in digestion, and the excess is stored and concentrated in the gallbladder by continuous water extraction, until the next feeding episode, where bile is then diluted and released into the small intestine [17]. As in the present study the bile ducts were carefully removed, it is improbable that any MT present in biliary fluid would be present in the liver samples. Thus, the significant, positive, strong and direct correlations observed between hepatic MT and Cu and Zn in bile indicate that these elements present in bile already removed by the liver for excretion may also continuously stimulate MT synthesis in liver, perhaps due to direct contact of this fluid with the liver through the biliary ducts.

3.3. Statistical correlations between metal pairs in *G. brasiliensis* bile and liver

In the present study, several positive strongstrong and very correlations were observed between elemental pairs in both bile and liver, as well as between elements in each of these organs (Table 4).

Organisms are exposed to environmental contaminants mostly encountered as mixtures [27] but, unfortunately, water quality criteria, toxicity assays and reference guidelines are usually established by

Table 4
Significant correlations between elements involved in homeostasis and biological functions and between essential and toxic elements in *G. brasiliensis* from the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil. (l) - liver; (b) bile.

Significant relationships between elements involved in homeostasis and biological functions		
Matrix	Relationship	Strength of the association
Liver	Se x Cu	r = 0.92
	Co x Cr	r = 0.99
	Zn x Cr	r = 0.89
	Cu x Se	r = 0.92
Liver x bile	Ni (l) x Fe (b)	r = 0.93
	Ni (l) x Zn (b)	r = 0.95
Significant relationships between essential and toxic elements		
Matrix	Relationship	Strength of the association
Liver	Fe x Al	r = 0.91
	Co x Al	r = 0.86
	Se x Al	r = 0.93
	Cu x As	r = 0.99
Liver x bile	Cu (l) x Cd (b)	r = 0.88
	Cr (b) x Cd (l)	r = 0.95
	Cu (b) x Cd (l)	r = 0.93
	Fe (b) x V (l)	r = 0.89
	Zn (l) x Hg (b)	r = 0.95
	Se (l) x Cd (b)	r = 0.94
	Fe (b) x Hg (l)	r = 0.90
	Co (b) x Hg (l)	r = 0.94
	Co (l) x Pb (b)	r = 0.90

considering acute and chronic bioassays of individual chemicals only [28,29]. Co-solutes, however, may induce either synergistic (increased) or antagonistic (decreased) effects, as well as additive (independent) behavior [7]. Interactions between metals and trace-elements are related to their competitive uptake from the environment and different distribution patterns in fish tissues, and recent studies have suggested that interactive effects between metals and trace-elements must be considered in order to improve environmental monitoring and risk assessment efforts [8,30,31] and that investigations of correlations between metals and trace-elements, both between those involved in homeostasis and biological functions and between essential and toxic elements, are paramount.

Metals and trace-elements enter the organisms via common routes and interact with each other affecting uptake, bioaccumulation and toxicity. The type of interaction depends on which elements are involved, their external concentration, bioavailability and exposure scenario, length of exposure, the studied species and the examined organs [8]. Statistical correlations between pairs of elements have been suggested as indicative of common sources of exposure, storage pathways or detoxification processes [32,33]. In the present study, all associations were positive and either strong or very strong, both between elements involved in homeostasis and biological functions and between essential and toxic elements.

Both positive and negative correlations between a specific element and different tissue types have been observed previously in fish [34], and seemingly indicate movement of elements between tissue. Thus, the positive correlations observed between elements in liver x bile (both between essential and essential and toxic elements) indicate excretion from the liver into the increasingly concentrated gallbladder bile, for subsequent excretion.

3.4. Molar ratios for essential and toxic elemental pairs presenting statistically significant correlations in G. brasiliensis bile and liver

Regarding the associations observed between essential and toxic elements, protection by essential elements against deleterious effects of toxic elements is still not well investigated [35], and only some associations have been described in the literature in this context. To further discuss these effects, the molar ratios for the significant associations between essential and toxic elements were calculated (Table 5).

The molar ratio calculations indicate protective effects against several toxic elements, with essential to toxic element molar ratios higher than 1 [36] in liver and in liver x bile, described in the next sections.

3.4.1. Protective effect against Cd

A protective effect was observed against Cd present in both bile and liver. For Cd in bile, this was demonstrated by Cu:Cd and Se:Cd molar ratios of 21:1 and 9:1, respectively, while for Cd in liver, a Cr:Cd ratio of 24:1 and a Cu:Cd of 3635:1 also indicated protective effects against Cd deleterious effects.

Cd bioavailability is very high; hence, it tends to bioaccumulate throughout the trophic food web [37]. Several different routes for Cd toxicity have been reported, and the basic underlying mechanisms are oxidative stress caused by Cd exposure [38,39] and interactions between Cd and essential metals, mainly zinc, iron, calcium, and copper, by interfering with Zn metabolism and competing for gastrointestinal Zn absorption, decreasing Fe absorption and metabolism, possibly binding to ferritin and transferrin, decreasing intestinal Ca transport and interfering with Cu metabolism, possibly by decreasing Cu absorption [40–42]. Cd initselfi does not directly generate reactive oxygen species, but may alter intracellular reduced glutathione (GSH) levels, inducing MT expression in liver [43].

Se has been previously reported as exhibiting a protective effect against Cd exposure in aquatic organisms. For example, female zebrafish exposed to 0.4 mg L^{-1} Cd in water and supplemented Se at 2 mg

Table 5
Molar ratios between essential and toxic elements in *G. brasiliensis* from the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil.

Matrix	Elements	Molar ratio	Probable effect
Liver	Fe x Al	115:1	Protective effect against Al toxicity
	Co x Al	0.06:1	No protective effect against Al toxicity
	Se x Al	0.80:1	No protective effect against Al toxicity
	Cu x As	27:1	Protective effect against As toxicity
Liver x bile	Cu (l) x Cd (b)	24:1	Protective effect against Cd toxicity
	Cr (b) x Cd (l)	24:1	Protective effect against Cd toxicity
	Cu (b) x Cd (l)	3635:1	Protective effect against Cd toxicity
	Fe (b) x V (l)	17:1	Protective effect against V toxicity
	Zn (l) x Hg (b)	7497:1	Protective effect against V toxicity
	Se (l) x Cd (b)	9:1	Protective effect against Cd toxicity
	Fe (b) x Hg (l)	1921:1	Protective effect against Hg toxicity
	Co (b) x Hg (l)	58:1	Protective effect against Hg toxicity
	Co (l) x Pb (b)	1.3:1	Slight protective effect against Pb toxicity

kg^{-1} for 21 days via diet, displayed reversed Cd-induced toxicity in liver and ovaries, suggesting that the protective mechanism against Cd-induced oxidative stress is dependent on the correction of protein biological activities [44]. This has been confirmed in rats, where Se supplementation decreased Cd-induced nephrotoxicity and hepatotoxicity alterations [45], while also decreasing Cd-induced oxidative stress in rat liver and kidneys [46].

The protective effect of Cu against Cd has been demonstrated in several organisms, and the mechanism seems to be via antioxidant effects of ceruloplasmin, a Cu-carrier in plasma that acts as an antioxidant [47] and has been shown to be upregulated in fish serum in the presence of copper [48], as well as the homeostasis and antioxidant effects of MT, also upregulated and increasing protection against Cd toxicity in the presence of this essential element [49].

Concerning Cr, no protective effects of this elements concerning Cd toxicity were found in the literature, indicating further research is necessary in this regard.

3.4.2. Protective effect against Hg

Regarding Hg, a Zn:Hg molar ratio of 7497:1 was observed for Hg in bile, indicating protective Zn effects in liver, while for Hg in liver protective effects were observed by a Cu:Hg ratio of 1599:1, a Fe:Hg ratio of 1921:1 and a Co:Hg ratio of 58:1, with Cu, Fe and Co in bile.

Hg is considered the most toxic element in the environment, particularly known to bioaccumulate throughout the trophic web [50]. Exposure to this metal can damage to the nervous system and kidneys [51], and, when in the form of organic mercury, it easily permeates across biomembranes and, as a lipophilic compound, accumulates in most species of fatty fish and in the liver of lean fish [52]. Regarding protective effects of essential elements against this toxic metal, certain relationships have been described in the literature, some more studied than others.

Fe has been shown to exert a protective effect against Hg toxicity, although only scarce studies are available in this regard and, to the best of our knowledge, only regarding rats [53–56]. Thus, further investigations in environmentally exposed organisms, especially fish, regarding this association are of interest.

Concerning Zn-Hg antagonism, Zn seems to have a significant impact on Hg distribution, [57], while the toxic effects of Hg have been shown to be exacerbated in Zn-deficient animals [58]. As discussed previously, increases in Zn levels induce MT synthesis. This occurs because the zinc-dependent metal-responsive transcription factor 1 (MTF-1) plays a role in activating MT transcription. In fact, it has been demonstrated that Hg is capable of displacing Zn to form the more stable Hg-MT complex, activating the synthesis of more MT and, in turn, increasing detoxification efficiency [59–61]. However, not many studies are available in this regard in the literature. Most have been, again, conducted with rats, and demonstrate different mechanisms of action regarding Zn protective effects. For example, in one of study, rats were exposed to Zn and Hg and kidneys were examined at 2, 6, and 12 h after treatment. Increases in GSH levels were observed, apparently related to the activation of some GSH-associated enzymes. The authors indicate that the response of the protective function involving GSH and GSH-associated enzymes depended on the magnitude of Hg toxicity but appeared to be independent of the Zn dosage. Thus, the data suggested that increased GSH levels in kidneys resulting from the activation of GSH-associated enzymes seems to play a role in the protective effect of Zn against Hg toxicity [62]. In another study, female rats exposed to Hg were subcutaneously injected with Zn 12 or 48 h after Hg exposure, and the results indicate that Zn pretreatment prevented renal weight increase, partly prevented decreases in body weight gain and increases in creatinine levels, in addition to totally preventing renal δ-aminolevulinic acid dehydratase inhibition, demonstrating different Hg effects and Zn protection mechanisms [63]. In the few studies conducted with fish, the authors do not propose a mechanistic pathway, but indicate that decreases in glucose and protein levels were observed in the intestine of catfish *H. fossilis*, as well as increases in alkaline phosphatase during Hg treatment for 30 days, which recovered to basal levels after Zn exposure, again suggesting a protective role of Zn against Hg toxicity [64].

Regarding the Co:Hg association, no reports are available in the literature citing that Co is effective in protecting against Hg toxic effects. However, Co has been shown to act synergistically in the presence of Se [65], perhaps showing the same protective effects against Hg due to the presence of Se. In addition, Co, alongside Se and Zn, has also been implicated in the induction of MT synthesis [66], which may also justify protective effects indicated by the high Co:Hg ratio observed in *G. brasiliensis*.

Until recently, few papers reporting molar ratios are available, mainly Se:Hg, and even less studies with regard to the associations between this toxic element and other metals are found [67]. The presence of the significant associations between several metals, as well as molar ratios indicative of protective effects against Hg, demonstrate the need for further research in this regard, since most of the research conducted to date focuses mainly on the relationship between Se and Hg. In fact, governments have even acted on the data with regard to the antagonist effects of Se on Hg, and in some Nordic countries, such as Sweden, entire lakes have been treated with Se to combat Hg toxicity [68]. Thus, further investigation of other metal and trace-element associations against the toxic effects of Hg may also aid in decision-making processes regarding environmental contamination scenarios.

3.4.3. Protective effect against As

A Cu:As molar ratio of 27:1 was observed in liver, indicative of protective effects against As toxicity. As is prominently toxic and carcinogenic, and cells accumulate As by using an active transport system normally used in phosphate transport [21]. This element affects primarily the sulphydryl group of cells causing malfunctioning of critical cellular pathways, such as cell respiration, cell enzymes and mitosis [37,69,70], in addition to producing excess reactive organic species, contributing to oxidative stress [71]. Even though As is mainly found in its less toxic organic forms in aqiatic organisms in general, since inorganic As species are metabolized by methylation into organic forms and excreted much faster than inorganic species [72,73], liver has been shown as a major target of As toxicity [70]. For example, exposure to $NaAsO_2$ was found to cause liver chromosomal DNA fragmentation in *Channa punctatus* in hepatocytes [70].

Regarding Cu-As interactions, antagonist effects have been reported between these elements, although reports are scarce in this regard, have been mainly conducted with rats and deal only with the effects of As exposure on Cu levels. For example, one study reported decreases in Cu levels in liver after exposure to As, in a dose-dependent manner, while exacerbating Cu deficiency in other tissues [74]. No reports citing protective effects of Cu regarding As toxicity, however, are available in the literature, and further studies are of importance in this regard, since a protective relationship might be in effect.

3.4.4. Protective effect against Al

In liver, a Fe:Al molar ratio of 115:1, was observed indicating protective effects against Al, while Co and Al ratios of 0.06:1 and 0.80:1 were noted, indicating no protective effects against Al toxicity.

Al as the free metal cation is highly biologically reactive, and biologically available Al is known as non-essential and essentially toxic [75]. In addition, no homeostasis mechanisms concerning Al are available, and no element-specific biological responses to its presence and its availability are known [75]. Some studies indicate decreased bone mineralization after Al exposure, due to alterations in Ca influx and efflux from bone cells [76]. In fish, Al is highly toxic in acid waters, with the gills as the main target, leading to a combination of ionoregulatory, osmoregulatory and respiratory dysfunctions [77]. Low-molecular weight inorganic Al forms of are believed to be the most important Al species concerning fish toxicity [78].

Fe and Al share important biological pathways, and it has been

reported that these elements display direct effects on both metabolisms [79,80]. Concerning the protective effect of Fe against Al, Fe has been reported as interfering with Al absorption in humans, due to high serum ferritin levels [81], while experiments carried out in iron-depleted rats demonstrated significant increases in gastrointestinal Al absorption and brain Al content, while iron-overloaded animals seemed to display protection against Al accumulation, which was also observed in studies carried out with human epithelial intestinal and bone cells [79]. However, no literature on the protective effect of Fe against Al in fish was found.

3.4.5. Protective effect against V

A molar ratio for Fe:V of 17:1 was observed for V in liver, indicating protective effects against V.

V is a redox-active metal, and, thus, may be involved in oxidative injury mechanisms. In certain conditions, it may enhance the generation of oxygen-derived reactive species and stimulate lipid peroxidation [82]. The most toxic form of V for mammals seems to be pentavalent vanadium (Roshchin, 1967). Although some accumulation of this element has been reported in rats (Shroeder & Balassa, o1967), this metal is also excreted readily, mostly via urine (Dimond et al.,1963). Aquatic toxicity of this element to fish, however, has not been well characterized [83]. In the few studies observed in the literature, exposure to V was shown to decrease larvae growth and survival in adult American Flagfish (*Jordanella floridae*) [84], while V toxicity to *Heteropneustes fossilis* led to increased glucose values and decreased total protein content in liver, muscle and kidney [85]. However, again, no literature was found concerning the protective effect of Fe against Al toxicity in fish, thus, indicating that further studies are also required.

3.4.6. Protective effect against Pb

A Co:Pb molar ratio of 1.3:1 was observed for Pb in bile and, seemingly indicating a certain amount of protective effects against Pb, although this ratio was low compared to the other elements observed herein. Thus, the protective effect in this regard, if any, should be very slight. However, no literature records on a protective role in this regard were found. As Co is a redox-active element, it may play a role in dealing with the effects of Pb bonding to protein sulphydryl groups and glutathione depletion, as reported elsewhere [86], which may lead to increased reactive species in the cell, although further studies are necessary.

3.5. PCA and cluster analysis

The PCA (Fig. 3) distinctly separated the elements found in liver from those found in bile, comprising Factor 1, responsible for 44.87%, and Factor 2, responsible for 22.59% of the total data variability. The former is more strongly correlated to variables V, Fe, Cu, Zn, As, Se and MT, while the latter is more strongly correlated to Cd, Co, Pb and Ni, confirming their importance with regard to separation between tissues.

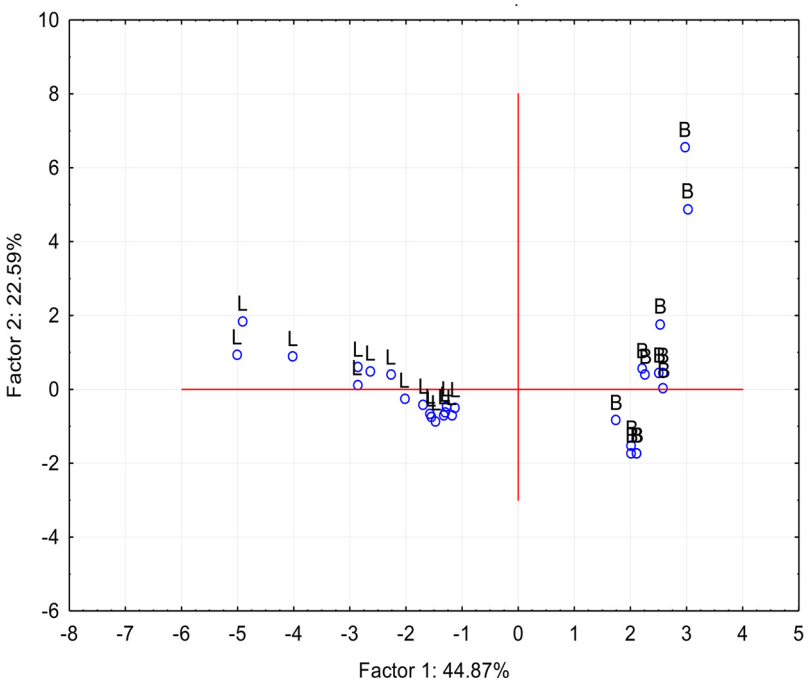

Fig. 3. PCA plot of *G. brasiliensis* liver and bile data. L-Liver; B – Bile.

The cluster grouping further corroborated the PCA results, where Pb, Cd, Co and Ni were present in lower concentrations in liver, evidencing that data separation was due to the elements present in lower levels in each matrix. Thus, this further confirms the detoxification processes that occur between these tissues and the investigated elements, and data separation seems to be due to, in fact, the elements more efficiently detoxified from the liver, confirming the previous discussions conducted in this regard (Fig. 4).

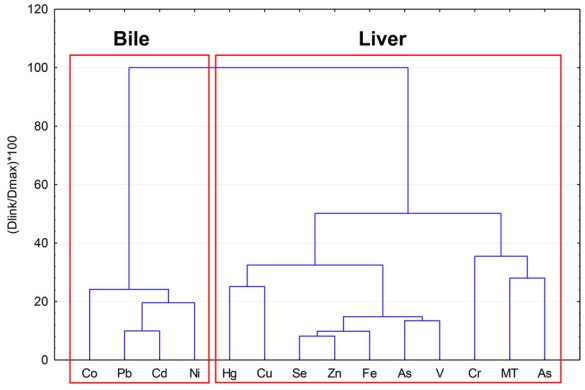

Fig. 4. Cluster analysis of *G. brasiliensis* liver and bile metal and MT data.

4. Conclusions

Metal and trace-element detoxifying routes in fish include the biliary and hepatic pathways. Taken together, the analysis of metals and trace-elements and MT levels in bile instead of liver is indeed viable to indicate the presence of these contaminants, in the environment, although some differences between liver and biliary metals and trace-elements are expected.

In addition, molar ratio calculations demonstrated protective metal and trace-elements effects against Al, As, Cd, Hg and Pb in both bile and liver, demonstrating inter-elemental relationships that seem to aid in protection against the effects of toxic elements. This includes some interrelationships that either have been scarcely reported, or even not at all, indicating the importance of these investigations regarding the elucidation of metal and trace-element detoxifying mechanisms. Moreover, the investigation of other metal associations against the deleterious effects of toxic elements may also aid in decision-making processes regarding environmental contamination scenarios.

Conflict of interest

The authors declare no conflict of interest.

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