CrossMark

Identification of Biomarkers of Mercury Contamination in *Brachyplatystoma filamentosum* of the Madeira River, Brazil, Using Metalloproteomic Strategies

João Vitor de Queiroz[1] · José Cavalcante Souza Vieira[2] · Grasieli de Oliveira[2] · Camila Pereira Braga[3] · Izabela da Cunha Bataglioli[1] · Janaína Macedo da Silva[2] · Wellington Luiz de Paula Araújo[1] · Pedro de Magalhães Padilha[1,2]

Abstract
Predator fish can accumulate high levels of mercury, which qualifies them as potential indicators of this toxic metal. The predatory species *Brachyplatystoma filamentosum*, popularly known as filhote, is among the most consumed species in the Brazilian Amazon. Continuing the metalloproteomic studies of mercury in Amazonian fishes that have been developed in the last 5 years, the present paper provides the data of protein characterization associated with mercury in muscle and liver samples of filhote (*Brachyplatystoma filamentosum*) collected in the Madeira River, Brazilian Amazon. The mercury concentration in the muscle and liver samples was determined by graphite furnace atomic absorption spectrometry (GFAAS). The protein fraction was extracted in an aqueous medium, and later, a fractional precipitation procedure was performed to obtain the protein pellets. Then, the proteome of the tissue samples of this fish species was separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and a mercury mapping of the protein spots was carried out by GFAAS after acid digestion. Protein spots that had mercury were characterized by mass spectrometry with electrospray ionization in sequence (ESI-MS/MS) after tryptic digestion. It was possible to characterize 11 mercury-associated protein spots that presented biomarker characteristics and could be used to monitor mercury in fish species of the Amazon region. Thus, the metalloproteomic strategies used in the present study allowed us to characterize 11 mercury-associated protein spots. It should be noted that the protein spots identified as GFRP, TMEM186, TMEM57B, and BHMT, which have coordination sites for elements with characteristics of soft acids, such as mercury, can be used as biomarkers of mercury contamination in monitoring studies of this toxic metal in fish species from the Amazon region.

Keywords Biomarkers · Proteomics · System biology · Mercury · Proteins

Introduction

Chemical pollution in the rivers of the Brazilian Amazon represents one of the main environmental problems of the region. Mercury contamination, which was reported in the Brazilian Amazon in the 1980s to mid-1990s due to environmental liabilities left by gold mining, and the potential for contamination that these liabilities cause, must be discussed in light of the construction of hydroelectric plants in the Amazon River. The construction and implementation of plants in the Amazon River basin could, because of environmental changes, modify the chemical species of mercury, making them available to the aquatic environment. Mercury species, such as methylmercury, which are available to aquatic life, can be absorbed by aquatic biota, accumulate in organisms, and magnify up through the food chain [1–3].

Several studies developed over the past two decades point to high concentrations of mercury in certain Amazonian fish. In general, these surveys point to bioaccumulation along the food chain as responsible for the high mercury content in Amazonian fish. Natural and anthropogenic mercury, upon

✉ José Cavalcante Souza Vieira
jcavalcante–s@bol.com.br; jcavalcante@ibb.unesp.br

1   School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu, Brazil

2   Institute of Biosciences, São Paulo State University (UNESP), Botucatu, Brazil

3   Biochemistry Department, University of Nebraska, Lincoln, NE, USA

entering aquatic ecosystems, participate in biogeochemical cycles mediated by microorganisms which will be chemically transformed and bioaccumulated in the food chain. Thus, predatory fish such as *Brachyplatystoma filamentosum* accumulate high levels of mercury and may serve as vehicles in this chemical form for its consumers, such as reptiles, birds, and humans. The fish species *Brachyplatystoma filamentosum* is also the most consumed by the riverside population of the Madeira River. Thus, consumption of *Brachyplatystoma filamentosum* may pose a risk to the health of these coastal communities from the Brazilian Amazon [3–10].

In this context, developing biomarkers related to mapping and expression of proteins associated with mercury can indicate the risks related to fish contamination of Amazonian rivers before reaching the riverine population that consumes these fish [1, 2, 4]. Metalloproteomics, a recent research area, has enabled the integration of analytical and biochemical studies which are highly complex, comprising the sequencing and characterization of proteins associated with metals [5–7]. This new area of scientific knowledge has contributed to elucidating the physiological and functional aspects of proteins responsible for transporting metal ions in living organisms [8]. The elucidation of mercurial species complexed by proteins and enzymes using the metallomics allow detailed study of an increase or decrease in the expression of these metalloproteins, compared to changing limnological characteristics and the availability of mercury species in the environment of the Amazon Rivers [2, 4, 9]. Thus, metalloproteins and metal-binding proteins characterized under these study conditions may be used as biomarkers of mercury exposure in fish from the Amazon region.

Considering the above, this paper presents the quantitative metalloproteomic results in the muscle tissue and liver of *Brachyplatystoma filamentosum* as corroborating with the results of recent metallomics studies of mercury developed by Braga et al. and Vieira et al. [2, 4], with the final aim of contributing to the elucidation of possible biomarkers of mercury exposure in Amazonian fish.

Materials and Methods

Collection and Sample Preparation

The metallomics study of *Brachyplatystoma filamentosum* was carried out considering the trophic level of this fish species of the Madeira River. The fish were captured using a fixed fishing net with a rectangular structure measuring 2 to 3 m long, 150 m in length, and mesh of 10 cm between the opposite sides. Six individual fish, with an average length of 72.40 ± 3.70 cm and average weight of 20.40 ± 2.30 kg, were collected in 2014 (in the flood period of the Madeira River—November to March) in the Jirau Hydroelectric Power Plant,

located in the Brazilian state of Rondônia (lat S—9° 15′ 17.96″ and long W—64° 38.40′ 13″). The captured fish were anesthetized with a benzocaine solution (100 mg L^{-1}) and killed by sectioning the cervical spine to collect samples [4]. The tissue samples (white muscle and liver) were pooled and homogenized using liquid nitrogen, placed in 25-mL polypropylene flasks, and stored in a freezer at − 20 °C until analysis (stored for approximately 30 days) [2].

Sample Preparation for 2D-PAGE

By using a mortar and pestle, about 2 g of the pooled sample of the muscle and liver tissue was macerated in an equivalent volume of ultrapure water (1:1). Then, the extract containing the proteins was separated from the solids by centrifugation for 30 min at 16.000g and 4 °C in a refrigerated centrifuge. The protein extracts obtained from these samples were transferred into 2-mL vials and centrifuged again at 16.000g and 4 °C for 30 min. The obtained supernatant (transparent extracts) was transferred into 2 mL vials that were later used to quantify the total protein content, total mercury, and the precipitation of the proteins that would be utilized in the electrophoretic runs [3, 4].

Fractional Precipitation and Solubilization of Protein Pellets

Obtaining protein pellets of the aqueous extracts was performed using fractional precipitation according to the procedure described by Braga et al. [2], with some modifications. Initially, aliquots of ethanol-chloroform solution and protein extract were transferred to 2-mL vials, in the proportion 1:1 (v/ v) and homogenized in a Vortex Stirrer. The homogeneous mixture was kept in a refrigerator for 30 min to precipitate protein formation (proteins with molecular weight > 90 kDa). Then, the heterogeneous mixture was centrifuged for 30 min at 16.000g at 4 °C. The supernatant was separated and again kept in the refrigerator for 30 min and centrifuged again for 30 min at 16.000g at 4 °C. The obtained supernatant (transparent extracts) was transferred to 2-mL vials to be subsequently submitted to a second precipitation step. The precipitates obtained in this step (proteins with molecular weight > 90 kDa) were transferred to 2-mL vials and stored in a freezer at − 20 °C for the subsequent determination of total mercury. The masses of the pellets were determined by the difference of the initial weights of empty vials and final masses of vials with pellets.

In the second stage of precipitation, in 2-ml vials, 1 mL of the supernatant removed from the first stage and 400 μL of hydrochloric acid-ethanol solution in the ratio 2.5:1 (v/v) were added. The homogeneous mixture was maintained overnight in a freezer at − 20 °C to ensure that precipitation occurred quantitatively. Then, the heterogeneous mixture was

centrifuged at 16.000*g* in a centrifuge refrigerated at 4 °C for 30 min, discarding the supernatant. The protein precipitate (pellet lower molecular weight) was washed four times with 1-mL ice-cold ethanol (− 20 °C) to remove traces of hydrochloric acid. This procedure was conducted with three repetitions to ensure obtaining pellets that would be used for the quantification of total protein and total mercury, as for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) runs. The mass of the pellets was determined as described in the first precipitation step. For quantification of total protein, the precipitate was resolubilized in 0.50 mol L^{-1} NaOH. For the determination of total mercury, acidic mineralization of the precipitate was carried out as described by Moraes et al. [1]. For electrophoretic separations, the pellet was resolubilized in specific buffer [5–7].

Determination of Total Protein

Determination of total protein in the extracts of the pellets obtained in the second step of the protein precipitation process was performed using the Biuret method, using bovine serum albumin as standard [7]. Determination of total protein in the pellets of the extracts allows for calculating the volume of protein extract, which must be diluted to contain 375 μg of protein, to be applied to each IEF strip used in the first stage of the two-dimensional electrophoresis [5–7].

Electrophoretic Runs

Pellets with a molar mass of less than 90 kDa were solubilized in a solution containing urea 7 mol L^{-1}, thiourea 2 mol L^{-1}, CHAPS 2% (*w/v*), ampholytes 0.5% (*v/v*) at pH ranging from 3 to 10, and bromophenol blue 0.002% (*w/v*). Solubilization of the pellets was done in such a way that the resulting concentration of total protein was the same for all samples: 1.50 μg/μL. A volume of 250 μL of this solution was added to the strips 13 cm containing polyacrylamide gels and with a pH gradient from 3 to 10 so these were rehydrated with a solution containing the sample itself. The strips were rehydrated for 12 h in a hydration box at room temperature and then taken to the isoelectric focusing system (IEF), EttanTMIPGphorTM 3. The IEF runs were performed under conditions described by Lima et al. [7].

After the IEF run, the strips were equilibrated in two steps. First, we used 10 mL of solution containing urea 6 mol L^{-1}, 2% SDS (*w/v*), 30% glycerol (*v/v*), Tris-HCl 50 mmol L^{-1} (pH 8.8), bromophenol blue 0.002% (*w/v*) DTT, and 2% (*m/v*) to keep the proteins in their reduced forms. Second, we used a solution similar in composition. However, DTT was replaced by iodoacetamide 2.5% (*m/v*) to obtain alkylation of the thiol groups of the proteins and thereby prevent a possible re-oxidation. After the reduction and alkylation steps, the tapes were applied to a polyacrylamide gel 15% (*m/v*) previously

prepared plate 180 × 160 × 1.5 mm. The molecular weight standards β-phosphorylase (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were applied beside the tape, and both were sealed with 0.5% agarose solution (*w/v*). The program used in the run, performed in the vat SE 600 Ruby (GE Healthcare Bioscience AB), was the same as described by Lima et al. and Santos et al. [6, 7].

After the 2D-PAGE runs, the proteins in the gels were shown with colloidal Coomassie stain. After stain removal, the gels were scanned with a scanner (GE Healthcare, Uppsala, Sweden) and analyzed using ImageMaster 2D Platinum 7.0 software (GE Healthcare 2007) as described by Lima et al. and Santos et al. [6, 7].

Determination of Total Mercury

Determination of total mercury in samples of the muscle tissue and liver of filhote in the protein pellets and protein spots was done by graphite furnace atomic absorption spectrometry (GFAAS). For this purpose, we transferred to digestion flasks of 5 mL an average mass of 50 mg of samples (the muscle or liver tissue and protein pellets) and 12 protein spots obtained from three replicates of 2D-PAGE runs (in each three 2D-PAGE run, four gels were obtained). Then, the acid mineralization of the samples was carried out according to the optimized procedure described by de Moraes et al. [1]. Briefly, the steps in the procedure were as follows: 1 mL of concentrated sulfuric acid and 0.25 ml of hydrogen peroxide 30% (*w/w*) were added to the sample tubes. The mixtures were allowed to stand for approximately 2 h and were then heated to 40 °C and 135 W in an ultrasonic bath until complete mineralization of the samples (clear extract). Then, the acid extracts were transferred to 5-mL volumetric flasks, and the final volume was adjusted with ultrapure water. A sample portion of the gel without any protein spot was mineralized under the same conditions and used for a negative background value (blank).

Total mercury determinations were made using atomic absorption spectrometer Shimadzu Model AA-6800, equipped with background absorption broker with deuterium lamp and self-reverse system (SR), pyrolytic graphite tube with integrated platform, and ASC-6100 automatic sampler, using the optimized procedures by Moraes et al. [1]. Validation of the analytical method used was carried out using certified standard Fish Protein DORM 4-NRC containing 410 ± 55 μg kg^{-1} of total mercury.

Characterization of Protein Spots by ESI-MS/MS

For characterization by electrospray ionization tandem mass spectrometry (ESI-MS/MS), protein spots were extracted from the gel using a scalpel and cut into segments of approximately 1 mm^3. The segments were transferred to 2-mL

microtubes containing 1% (*v/v*) acetic acid and kept in the refrigerator until the subsequent steps described in Technical Bulletin of the Walters, which are summarized in four steps [10]: (1) removal of dye; (2) reduction and alkylation; (3) tryptic digest of the protein; and (4) eluting the peptides. The extract aliquots containing the peptides obtained from the tryptic digestion step were analyzed to obtain the mass spectra using UPLC Platform mass spectrometer with ESI source ionization and Xevo G2 Q-TOF analyzer hybrid masses (Waters, Milford, MA, USA) coupled with nanoACQUITY Ultra Performance LC system (Waters, Milford, MA, USA) [10]. The proteins were identified by their homology to proteins in the UniProt database; the species used was the "Otophysi" [2, 4].

Results and Discussion

Determination of Total Protein

The total protein concentration in the extracts of the lower molecular mass of pellets was (g L^{-1}): muscle tissue—11.80 ± 0.34 and liver tissue—18.60 ± 0.43. Based on the results of total protein concentration present in the muscle tissue extracts, it was possible to calculate the volume of extract to be applied on IEF strips to obtain a mass of 375 μg protein. Based on the IEF system manual, this protein mass allows better separation of proteins in the pH gradient 3–10 [7].

Fractionation of Proteins by 2D-PAGE

Correlation analysis between repetitions of the gels and protein spots obtained in each gel was made using the parameters to obtain the images, as described in previous papers [2, 4–7, 11, 12]. Figures 1 and 2 show a sample obtained in each electrophoresis gel run of three replicates of the samples of muscle and liver tissue of *Brachyplatystoma filamentosum*. The circle with prominent protein spots shows those in which GFAAS determined the presence of mercury and the concentrations of this element, which are also displayed in three dimensions. The average results of correlation analysis between repeats of the gels (matching) are 80 ± 5% and 93 ± 3%, and the average number of protein spots in the gels were 338 ± 13 and 308 ± 7, respectively, for the muscle and hepatic tissue. The optimized conditions of 2D-PAGE were more efficient compared to other run conditions presented in the literature [5–7, 11, 12], which presented values matching less than 80%.

Quantitative Assessment of Mercury

Table 1 shows the mercury concentrations in the protein pellets obtained. The concentration of mercury in the muscle tissue samples of fish species and the concentration of mercury in fish muscle protein certified standard DORM 4-NRC can be used to validate the method for the determination of optimized mercury.
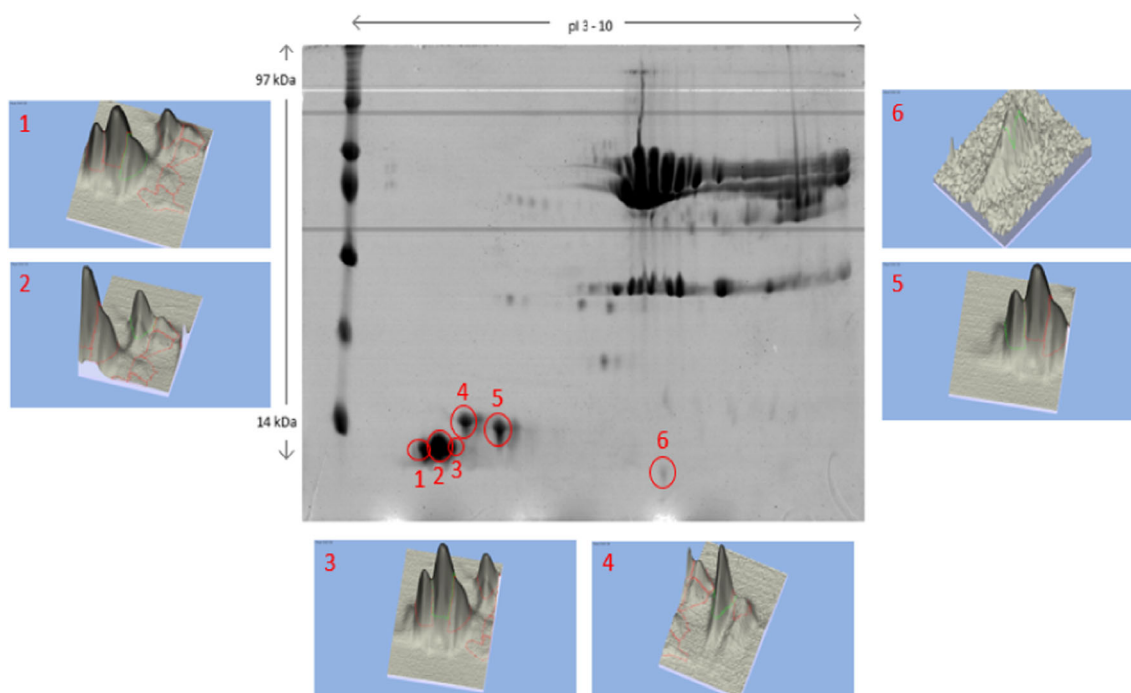

Fig. 1 Gel 15% (*w/v*) obtained by 2D-PAGE (pH range 3–10) to the muscle tissue of filhote (*Brachyplatystoma filamentosum*). The numbers and circles in red indicate spots which identified the presence of mercury and were characterized by ESI-MS/MS
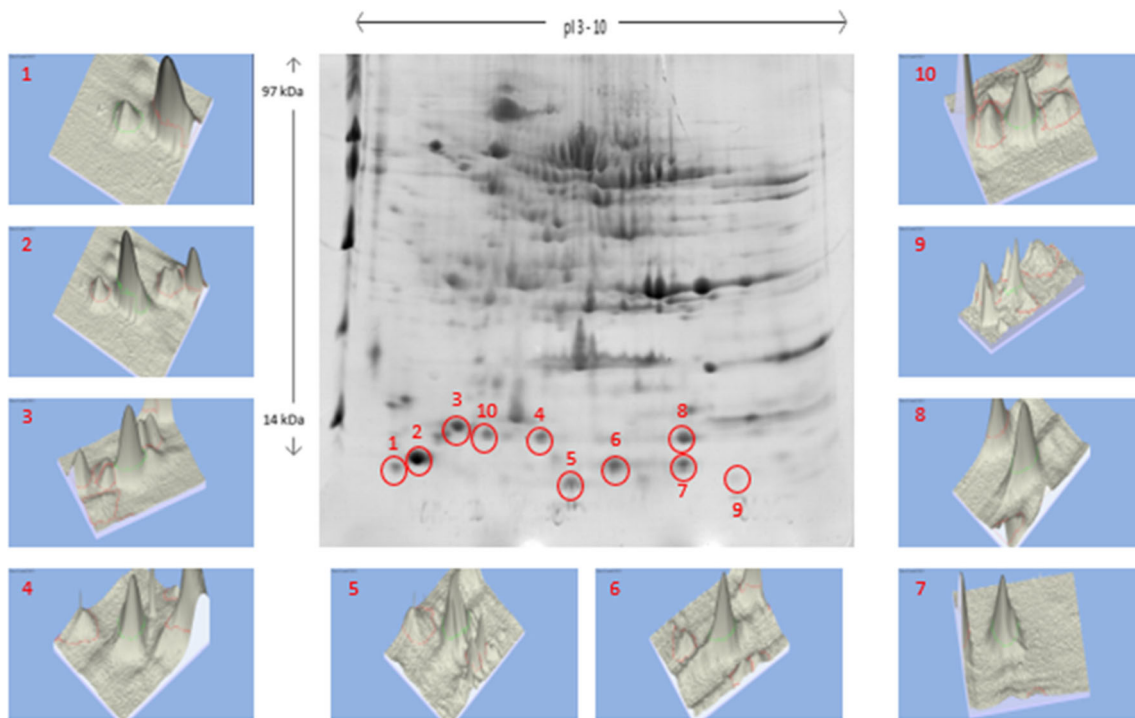
Fig. 2 Gel 15% (*m/v*) obtained by 2D-PAGE (pH range 3–10) to the liver tissue of filhote (*Brachyplatystoma filamentosum*). The numbers and circles in blue indicate spots which identified the presence of mercury and were characterized by ESI-MS/MS

The data in Table 1 show that the muscle and liver pellets present mercury concentrations of 402 ± 7 and 248 ± 3 µg kg^{-1}, respectively. This data indicates that approximately 65% of total Hg determined in the samples (378 ± 9 and 617 ± 11 µg kg^{-1} to the muscle and liver tissue, respectively) is bound in protein pellets with molecular mass less than 90 kDa. As mercury was not detected in protein pellets with a molecular mass higher than 90 kDa, it can also be inferred that 65% of the mercury present in hepatic and muscle tissue samples from the fish studied is connected to the protein fraction, and 35% can be linked to other macromolecules, such as lipids. The data presented in Table 1 were validated based on results obtained in the determination of mercury in the certified standard DORM 4-NRC fish muscle protein. As shown in Table 1, the experimental value of the relative standard deviation in the samples is less than 1%, and the absolute value of the certificate experimental pattern (408 µg kg^{-1}) is about 0.50% lower than the absolute value of standard certificated (410 µg kg^{-1}), thus proving the accuracy of the method.

The results presented in Table 2 show that mercury concentrations are in the range of 13.90 to 16.50 mg g^{-1} and 12.50 to 83.40 mg g^{-1} for the protein of muscle and liver spots, respectively. The results corroborate those obtained recently by Braga et al. and Vieira et al. [2, 4], who determined the mercury content in the muscle tissue and liver samples of Dourada and Tucunaré (other fish species from the Brazilian Amazon) and reported values in this concentration range. Based on the estimation of molecular weights of protein spots and in the mercury concentration in this spots, we calculated the number of mercury atoms per protein spot molecule as in the procedure described by Braga et al. [2]. The results obtained in these calculations are also shown in Table 2.

Table 1 Results obtained in the determination of mercury in the pellets of higher and lower molecular mass (Mm) in samples of the muscle tissue (TM) and liver (TH)

Fish species	Pellets of TM (> Mm)	Pellets of TH (> Mm)	Pellets of TM (< Mm)	Pellets of TH (< Mm)	Muscle tissue	Liver tissue
Filhote	n.d.	n.d.	248 ± 3	402 ± 7	378 ± 9	617 ± 11
DORM 4-NRC[a]	–	–	–	–	408 ± 3	–

Mm molecular mass > 90 kDA; concentration of Mercury in micrograms per kilogram

n.d. not detected

[a] Standard certified of protein of muscle fish containing 410 ± 55 µg kg^{-1}

The results obtained by stoichiometric calculations allowed the estimation of spots of the muscle tissue M1-M5 and L1-L10, with approximately one mercury atom per protein molecule spot. These results, although suggesting that there may be a stoichiometric relationship between the number of mercury atoms and the number of molecules of protein spots, corroborate those obtained by Braga et al. and Vieira et al. [2, 4] in similar studies with Dourada and Tucunaré fish species.

Proteins Identified by ESI-MS/MS

Protein spots with the presence of mercury were analyzed by ESI-MS/MS (Table 3). In total, nine different proteins were identified: four in muscle and six in the liver tissue of filhote. In some cases, a protein spot can have one or more proteins, and points such as score, coverage, molecular mass, and pI experimental/theoretical are considered to decide what proteins are in the spot. The UniProt database was used to obtain molecular mass and theoretical pI and information about each protein. FASTA sequences were used to analyze each protein in the Blast2GO program (B2G) considering the separation in three levels: molecular function, biological process, and cellular component [13].

Analyses using Blast2GO (Fig. 3) related the sequences of proteins at the molecular level divided by catalytic activity (betaine–homocysteine S-methyltransferase 1 and N-terminal Xaa-Pro-Lys N-methyltransferase 1), structural molecule activity (ubiquitin-40S ribosomal protein S27a), transport activity (glycolipid transfer protein), and binding (glycolipid transfer protein, parvalbumin beta, ubiquitin-40S ribosomal protein S27a, betaine–homocysteine S-methyltransferase 1).

At the cellular level, the sequences were divided as follows:

- organelles (ubiquitin-40S ribosomal protein S27a, GTP cyclohydrolase 1 feedback regulatory protein, macoilin-2, and N-terminal Xaa-Pro-Lys N-methyltransferase 1),
- cells (ubiquitin-40S ribosomal protein S27a, macoilin-2, GTP cyclohydrolase 1 feedback regulatory protein, glycolipid transfer protein, betaine–homocysteine S-methyltransferase 1, and N-terminal Xaa-Pro-Lys N-methyltransferase 1),
- membrane-enclosed lumen (ubiquitin-40S ribosomal protein S27a),
- membrane (ubiquitin-40S ribosomal protein S27a, macoilin-2, GTP cyclohydrolase 1 feedback regulatory protein, macoilin-2, and transmembrane protein 186), and
- macromolecular complex (ubiquitin-40S ribosomal protein S27a, GTP cyclohydrolase 1 feedback regulatory protein, macoilin-2, and N-terminal Xaa-Pro-Lys N-methyltransferase 1).

A multi-organism process divided the biological process sequences, localization, signaling, reproduction, immune system process, and the following:

- response to stimulus (ubiquitin-40S ribosomal protein S27a),

Table 2 Values obtained in the determination of mercury concentration by GFAAS in protein spots of muscle and liver tissue samples of filhote
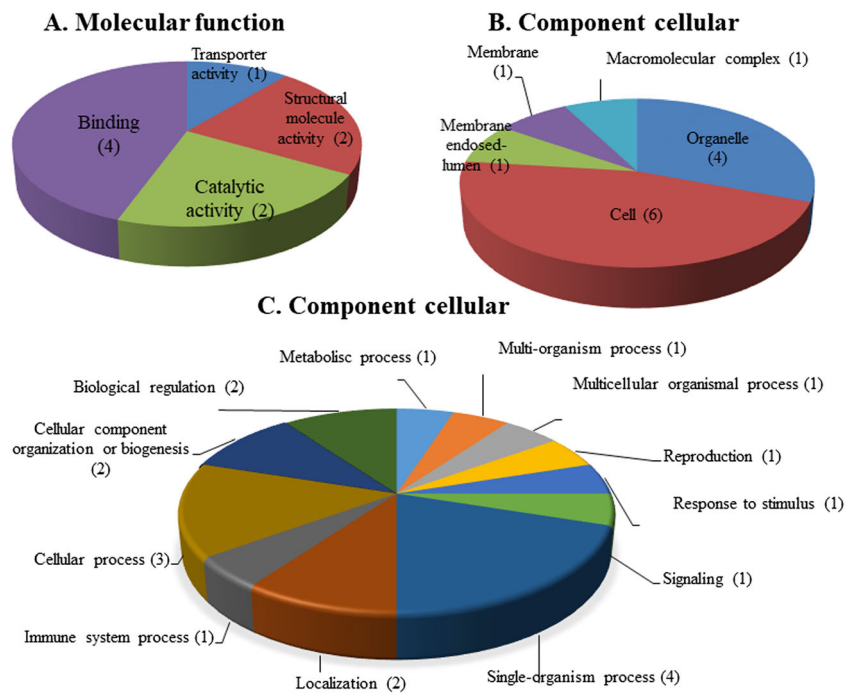
Protein spots	Molecular mass (kDa)	pI	Protein mass (µg)	Concentration of Hg (mg g^{-1})	Number of molecules in the protein spot $\times 10^{12}$	Number of Hg atoms $\times 10^{12}$
M1	13.00	3.50	4.40	15.20	0.204	0.201
M2	13.10	3.80	22.40	15.50	1.030	1.042
M3	13.00	4.00	9.30	14.90	0.430	0.416
M4	14.40	4.20	5.40	13.90	0.230	0.225
M5	14.00	4.70	5.00	13.60	0.215	0.204
M6	12.00	7.30	0.12	16.50	0.006	0.006
L1	12.20	3.50	2.30	14.50	0.110	0.100
L2	12.70	3.80	8.50	14.90	0.400	0.380
L3	14.10	4.90	5.90	13.00	0.250	0.230
L4	13.60	5.70	3.20	12.50	0.140	0.120
L5	11.50	6.20	4.30	33.90	0.230	0.440
L6	12.30	6.90	6.10	15.40	0.300	0.280
L7	12.20	8.00	4.70	14.20	0.230	0.200
L8	13.30	8.00	5.90	14.10	0.270	0.250
L9	10.50	8.90	0.60	83.40	0.040	0.150
L10	13.70	4.90	2.40	12.50	0.100	0.090

M1–M5 spots of filhote muscle; L1–L10 spots of filhote liver

Table 3 Proteins identified by ESI-MS/MS in muscle and liver tissue samples of filhote

Spot gel	Protein	Access	Score	pI/Mm experimental	pI/Mm theoretical	Coverage (%)	Sequenced peptides
Muscular tissue							
M1	Parvalbumin beta	PRVB_SQUCE	53	3.50/13,000	4.88/11,263	9.43	SGFIEEELK
M2	Parvalbumin beta	PRVB_SQUCE	221	3.80/3,100	4.88/11,263	9.43	SGFIEEELK
M3	GTP cyclohydrolase 1 feedback regulatory protein	GFRP_DANRE	145	4.00/13,000	5.53/10,018	28.09	LETGPTMVGDEYSDPSIMNYLGARK
M4	Not identified	–	–	4.20/14,400	–	–	–
M5	Transmembrane protein 186	TM186_DANRE	51	4.70 /14,000	10.69/25,720	8.77	DIYVPVSDVVTLGDSGDSR RDIYVPVSDVVTLGDSGDSR
M6	Ubiquitin-40S ribosomal protein S27a	RS27A_ICTPU	303	7.30/12,000	9.68/17,999	25.00	ESTLHLVLR TLTGKTITLEVEPSDTIENVK
M7	Glycolipid transfer protein	GLTP_DANRE	13	3.50/ 12,200	8.40/23,950	6.7	FIQVLLQSLVDGDK
Hepatic tissue							
L2	Not identified	–	–	3.80/12,700	–	–	–
L3	Not identified	–	–	4.90/14,100	–	–	–
L4	Not identified	–	–	5.70/13,600	–	–	–
L5	Ubiquitin-40S ribosomal protein S27a	RS27A_ICTPU	101	6.20/11,500	9.68/18,000	11.54	ESTLHLVLR EGIPPDQQR
L6	Betaine--homocysteine S-methyltransferase 1	BHMT1_DANRE	1442	6.90/12,300	6.65/44,100	9.5	AGPWTPEAAAEHPEAVR AGSNVMQTFTFYASDDK AGPWTPEAAAEHPEAVRQLHR
L7	Macoilin-2	MACO2_DANRE	15	8.00/12,200	9.10/78,300	2.58	QEREAEEAAAAAAAAASK
L8	Not identified	–	–	8.00/13,300	–	–	–
L9	N-terminal Xaa-Pro-Lys N-methyltransferase 1	NTM1A_DANRE	72	8.90/10,500	4.85/25,300	11.66	DVPPTVDGMLGGYGSISSIDINGSKK
L10	GTP cyclohydrolase 1 feedback regulatory protein	GFRP_DANRE	268	4.90/13,700	5.53/10,020	11.24	PYILISTQIR

Fig. 3 Analysis of proteins using the Blast2GO program in the muscular and hepatic tissue: molecular function (**a**), cellular component (**b**), and biological process (**c**)

A. Molecular function

Transporter activity (1)
Structural molecule activity (2)
Binding (4)
Catalytic activity (2)

B. Component cellular

Membrane (1)
Macromolecular complex (1)
Membrane endosed-lumen (1)
Organelle (4)
Cell (6)

C. Component cellular

Metabolisc process (1)
Multi-organism process (1)
Biological regulation (2)
Multicellular organismal process (1)
Cellular component organization or biogenesis (2)
Reproduction (1)
Response to stimulus (1)
Cellular process (3)
Signaling (1)
Immune system process (1)
Localization (2)
Single-organism process (4)

- single organism process (ubiquitin-40S ribosomal protein S27a, betaine–homocysteine S-methyltransferase, N-terminal Xaa-Pro-Lys N-methyltransferase 1, and glycolipid transfer protein),
- metabolic process (ubiquitin-40S ribosomal protein S27a, betaine-homocysteine S-methyltransferase, N-terminal Xaa-Pro-Lys N-methyltransferase 1, and GTP cyclohydrolase 1 feedback regulatory protein),
- localization (ubiquitin-40S ribosomal protein S27a and glycolipid transfer protein),
- cellular process (ubiquitin-40S ribosomal protein S27a, betaine–homocysteine S-methyltransferase, and N-terminal Xaa-Pro-Lys N-methyltransferase 1),
- multicellular organismal process (ubiquitin-40S ribosomal protein S27a and betaine–homocysteine S-methyltransferase),
- cellular component organization or biogenesis (ubiquitin-40S ribosomal protein S27a and N-terminal Xaa-Pro-Lys N-methyltransferase 1), and
- biological regulation (ubiquitin-40S ribosomal protein S27a and GTP cyclohydrolase 1 feedback regulatory protein).

Parvalbumin beta is an isoform of parvalbumin and binding proteins, as shown by other studies performed by our group [2, 4]. This protein was shown to be a mercury-binding protein, and it was related that this isoform of the protein had a cysteine residue that can form specific linkages with mercury (soft acid). Parvalbumins are typical proteins present in the muscle tissue of fish [14], with studies showing

higher expression in light than dark muscle tissue [15]. Parvalbumins are EF-hand proteins involved in binding divalent metal ions, with two EF-hand motifs for calcium and magnesium [16], related by regulation of the intracellular calcium concentration during muscle relation [17]. Two protein spots were identified as parvalbumin beta in the muscle tissue (M1 and M2) and had a significant stoichiometric ratio, with one mercury atom per molecule of protein. This evidence added to other studies and shows that parvalbumin beta is a mercury biomarker in different species of fish.

GTP cyclohydrolase I (GTPCHI) is involved in the biosynthesis of tetrahydrobiopterin (BH$_4$), a cofactor for aromatic amino acid hydroxylases and NO synthases [18]. GTP cyclohydrolase mediates the feedback inhibition I feedback regulatory protein (GFRP); defects affecting GFRP activity can cause hyperphenylalaninemia and neurological disorders [19]. The study showed that the rat GFRP monomer contains one zinc ion which binds conserved cysteines (132 and 203) at the active site [20]. These sites coordinated with zinc have characteristics similar to mercury (soft acid). Mercury can move zinc ions of thiol groups and establish binding at these sites. In muscle (M3) and hepatic tissue (L10) identified as GFRP, the stoichiometric ratio showed one mercury atom per molecule of protein. The literature does not contain any reports regarding the interaction of GFRP and mercury, so our results can infer that GFRP may be a biomarker of mercury.

Transmembrane protein 186 and macoilin-2 are macromolecules implicated in biological processes, ion transport, and diseases [21]. The peptide sequence is formed of glycine, proline, histidine, and hydrophobic amino acids such as

cysteine that has a thiol group in its structure [22]. Thiol groups are known to bind with soft acids as mercury. The presence of this group, along with the characteristic of ion transport and the presence of one mercury atom per molecule of transmembrane protein 186 in M5 and macoilin-2in L7 spots by GFAAS analyses, may indicate that these bind mercury and may be a biomarker of this metal.

Ubiquitin-40S ribosomal protein S27a is a metal-binding protein; recent studies by our group [2, 4] indicate the presence of mercury in this protein and associated the presence of mercury with its zinc-finger metal-binding domains and the characteristics that are similar between mercury and zinc. In this study, the protein spots M6 and L5 had mercury in their structure. Analyses by GFAAS showed one and two mercury atoms per protein molecule, respectively, corroborating the data in other studies that support the hypothesis that this protein acts as a biomarker for this element.

Glycolipid transfer protein (GLTP) is a small soluble protein reported in various species; it is 24 kDa, cytosolic, involved in the catalysis, and transport of sphingoid and glycerol [23]. Previous studies reported that this protein has three cysteine residues in its structure, with two reside inside the protein and the third on the surface. The authors suggest that the two internal cysteines form an intramolecular disulfide bond and the third cysteine bonds with another GLTP [24, 25]. This suggests that mercury can bind in its structure in thiol groups; in our study, the protein spot M7 showed mercury after analysis by GFAAS, indicating that GLTP can be a mercury-binding protein.

Betaine–homocysteine S-methyltransferase 1 is a major zinc metalloenzyme in the liver involved in the remethylation pathway, forming dimethylglycine and methionine by transfer of methyl groups from betaine to homocysteine [26]. In this case, as in parvalbumin beta and ubiquitin-40S ribosomal protein S27a, betaine–homocysteine S-methyltransferase 1 has specific domains linked to zinc and can bind to other divalent ions such as mercury. The protein spot L6 had a stoichiometric ratio of one mercury atom per betaine–homocysteine S-methyltransferase 1 molecule, so it can be inferred that it may act as a biomarker of this element. In the literature, there is no discussion of the possibility that betaine–homocysteine S-methyltransferase 1 can be bound to mercury, such as GLTP, transmembrane protein 186, and macoilin-2.

N-terminal Xaa-Pro-Lys N-methyltransferase 1 (NTM1A) is involved in the methylation of the N-terminus of target proteins that contain the sequence (Ala/Pro/Ser)-Pro-Lys [27]. Its structure is formed of alanine, lysine, proline, serine, and S-adenosyl-L-methionine linker [27]. The presence of methionine (soft base) is correlated with the binding of a soft acid such as mercury. In our study, the protein spot L9 had a stoichiometric ratio of four mercury atoms per NTM1A molecule, suggesting that it can be a mercury-binding protein and a biomarker of mercury in fish.

Conclusion

The analysis using 2D-PAGE and the quantification of mercury showed that mercury is bound in proteins with a molecular weight of less than 15 kDa. The analysis by ESI-MS/MS characterized 11 spots of proteins in which the presence of mercury was confirmed by GFAAS. This suggests possible new biomarkers of mercury such as GFRP, TMEM186, TMEM57B, and BHMT, which have specific domains linked to zinc atoms and characteristics similar to mercury (soft acid). Generally, the protein spots characterized presented biomarker characteristics, which means they may be used in monitoring the proteomic level of the toxic concentrations of mercury in fish species in the Amazon region after validation using controlled experiments of the mercury concentrations in relation to the expression of these protein spots.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interest.

References

1. Moraes PM, Santos FA, Cavecci B, Padilha CCF, Vieira JCS, Roldan PS, Padilha PM (2013) GFAAS determination of mercury in muscle samples of fish from Amazon, Brazil. Food Chem 141: 2614–2617. https://doi.org/10.1016/j.foodchem.2013.05.008

2. Braga CP, Bittarello a C, Padilha CCF et al (2015) Mercury fractionation in dourada (Brachyplatystoma rousseauxii) of the Madeira River in Brazil using metalloproteomic strategies. Talanta 132:239–244. https://doi.org/10.1016/j.talanta.2014.09.021

3. Vieira JCS, Braga CP, de Oliveira G, do Carmo Federici Padilha C, de Moraes PM, Zara LF, de Lima Leite A, Buzalaf MAR, de Magalhães Padilha P (2017) Correction to: mercury exposure: protein biomarkers of mercury exposure in Jaraqui fish from the Amazon region. Biol Trace Elem Res 183(1):172. https://doi.org/10.1007/s12011-017-1195-8

4. Vieira JCS, Cavecci B, Queiroz JV, Braga CP, Padilha CCF, Leite AL, Figueiredo WS, Buzalaf MAR, Zara LF, Padilha PM (2015) Determination of the mercury fraction linked to protein of muscle and liver tissue of Tucunaré (Cichla spp.) from the Amazon region of Brazil. Arch Environ Contam Toxicol 69:422–430. https://doi.org/10.1007/s00244-015-0160-9

5. Silva FA, Cavecci B, Baldassini WA, Lima PM, Moraes PM, Roldan PS, Padilha CCF, Padilha PM (2013) Selenium fractionation from plasma, muscle and liver of Nile tilapia (Oreochromis niloticus). J Food Meas Charact 7:158–165

6. dos Santos FA, Lima PM, Neves RCF et al (2011) Metallomic study on plasma samples from Nile tilapia using SR-XRF and GFAAS

after separation by 2D PAGE: initial results. Microchim Acta 173: 43–49. https://doi.org/10.1007/s00604-010-0522-y

7. Lima PM, Neves RDCF, dos Santos FA et al (2010) Analytical approach to the metallomic of Nile tilapia (Oreochromis niloticus) liver tissue by SRXRF and FAAS after 2D-PAGE separation: preliminary results. Talanta 82:1052–1056. https://doi.org/10.1016/j.talanta.2010.06.023

8. Garcia JS, De Magalhães CS, Arruda MAZ (2006) Trends in metal-binding and metalloprotein analysis. Talanta 69:1–15. https://doi.org/10.1016/j.talanta.2005.08.041

9. Cavalcante J, Vieira S, Braga CP et al (2017) Mercury exposure: protein biomarkers of mercury exposure in Jaraqui fish from the Amazon region. Biol Trace Elem Res 183:164–171. https://doi.org/10.1007/s12011-017-1129-5

10. Shevchenko A, Tomas H, Havlis J et al (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860. https://doi.org/10.1038/nprot.2006.468

11. Moraes PM, Santos FA, Padilha CCF, Vieira JCS, Zara LF, de M. Padilha P (2012) A preliminary and qualitative metallomics study of mercury in the muscle of fish from Amazonas, Brazil. Biol Trace Elem Res 150:195–199. https://doi.org/10.1007/s12011-012-9502-x

12. Neves RCF, Lima PM, De Medicina F et al (2012) Artigo 35:493–498

13. Conesa A, Götz S (2008) Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics 2008: 619832–619812. https://doi.org/10.1155/2008/619832

14. Sharp MF, Lopata AL (2014) Fish allergy: in review. Clin Rev Allergy Immunol 46:258–271. https://doi.org/10.1007/s12016-013-8363-1

15. Kobayashi A, Tanaka H, Hamada Y, Ishizaki S, Nagashima Y, Shiomi K (2006) Comparison of allergenicity and allergens between fish white and dark muscles. Allergy: European. J Allergy Clin Immunol 61:357–363. https://doi.org/10.1111/j.1398-9995.2006.00966.x

16. Arif SH (2009) A Ca2+-binding protein with numerous roles and uses: parvalbumin in molecular biology and physiology. BioEssays 31:410–421. https://doi.org/10.1002/bies.200800170

17. Schwaller B (2009) The continuing disappearance of "pure" Ca2+ buffers. Cell Mol Life Sci 66:275–300. https://doi.org/10.1007/s00018-008-8564-6

18. Li L, Rezvan A, Salerno JC, Husain A, Kwon K, Jo H, Harrison DG, Chen W (2010) GTP cyclohydrolase i phosphorylation and interaction with GTP cyclohydrolase feedback regulatory protein provide novel regulation of endothelial tetrahydrobiopterin and nitric oxide. Circ Res 106:328–336. https://doi.org/10.1161/CIRCRESAHA.109.210658

19. Ichinose H, Ohye T, Matsuda Y, Hori TA, Blau N, Burlina A, Rouse B, Matalon R, Fujita K, Nagatsu T (1995) Characterization of mouse and human GTP cyclohydrolase I genes: mutations in patients with GTP cyclohydrolase I deficiency. J Biol Chem 270: 10062–10071

20. Maita N, Okada K, Hatakeyama K, Hakoshima T (2002) Crystal structure of the stimulatory complex of GTP cyclohydrolase I and its feedback regulatory protein GFRP. Proc Natl Acad Sci U S A 99: 1212–1217. https://doi.org/10.1073/pnas.022646999

21. de Brevern AG (2010) 3D structural models of transmembrane proteins. Methods Mol Biol 654:387–401. https://doi.org/10.1007/978-1-60761-762-4_20

22. von Heijne G (1994) Membrane proteins: from sequence to structure. Annu Rev Biophys Biomol Struct 23:167–192. https://doi.org/10.1146/annurev.bb.23.060194.001123

23. Mattjus P (2009) Glycolipid transfer proteins and membrane interaction. Biochim Biophys Acta 1788:267–272. https://doi.org/10.1016/j.bbamem.2008.10.003

24. Abe A, Sasaki T (1989) Formation of an intramolecular disulfide bond of glycolipid transfer protein. BBA-Biomembranes 985:45–50. https://doi.org/10.1016/0005-2736(89)90101-6

25. Abe A, Sasaki T (1989) Sulfhydryl groups in glycolipid transfer protein: formation of an intramolecular disulfide bond and oligomers by Cu2+-catalyzed oxidation. BBA-Biomembranes 985:38–44. https://doi.org/10.1016/0005-2736(89)90100-4

26. Ratnam S, Wijekoon EP, Hall B, Garrow TA, Brosnan ME, Brosnan JT (2006) Effects of diabetes and insulin on betaine-homocysteine S-methyltransferase expression in rat liver. Am J Phys Endocrinol Metab 290:E933–E939. https://doi.org/10.1152/ajpendo.00498.2005

27. Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M (2010) Synthetic therapeutic peptides: science and market. Drug Discov Today 15:40–56. https://doi.org/10.1016/j.drudis.2009.10.009