UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CÂMPUS DE BOTUCATU

ELUCIDAÇÃO DE ASPECTOS FISIOLÓGICOS E FUNCIONAIS DE PROTEÍNAS ASSOCIADAS AO MERCÚRIO EM PIRARUCU (*Arapaima gigas)*

IZABELA DA CUNHA BATAGLIOLI

Dissertação apresentada ao Programa de Pós-graduação em Zootecnia como parte dos requisitos para obtenção do título de Mestre em Zootecnia.

Botucatu – SP
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Orientador: Prof. Dr. Pedro de Magalhães Padilha
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Elucidação de aspectos fisiológicos e funcionais de proteínas associadas ao mercúrio em pirarucu (*Arapaima gigas)*

RESUMO GERAL

Nas últimas décadas estudos científicos tem dado destaque as altas concentrações de mercúrio (Hg) encontradas em solo, sedimentos, peixes e seres humanos na Amazônia. No entanto, encontram-se ainda escassos estudos relacionados aos mecanismos de toxicidade do mercúrio em nível celular na ictiofauna e populações ribeirinhas. Neste sentido, a investigação dos níveis de expressão de proteínas poderá agir como peça chave na elucidação de biomarcadores de exposição ao mercúrio, facilitando o estudo da contaminação no ambiente e seres vivos por este metal tóxico. O mapeamento destas proteínas associadas ao mercúrio poderá indicar previamente possíveis riscos de contaminação do pescado, bem como evitar a exposição humana ao mercúrio. Estudos metaloproteômicos recentes com peixes da região amazônica, desenvolvidos por grupo de pesquisadores da UNESP de Botucatu, permitiram o fracionamento e caracterização de algumas proteínas associadas ao mercúrio com características de biomarcador. No entanto, os aspectos fisiológicos e funcionais dessas proteínas associadas ao mercúrio nos peixes amazônicos e os mecanismos de adaptação apresentados por estes animais frente à exposição ao metal ainda não foram elucidados. Assim, o presente estudo teve por objetivo utilizar uma nova estratégia de preciptação (fracionada) de proteínas para avançar no conhecimento em busca de biomarcadores proteicos/enzimáticos da contaminação de mercúrio em amostras de tecido hepático de pirarucu (*Arapaima gigas*) coletados em áreas próximas do reservatório da Usina Hidrelétrica de JIRAU - Rio Madeira utilizando-se as seguintes estratégias metaloproteômicas: fracionamento do proteoma das amostras por 2D PAGE (eletroforese bidimensional); mapeamento do mercúrio nos *spots* proteicos por GFAAS (espectrometria de absorção atômica em forno de grafite); caracterização das proteínas associadas ao mercúrio por ESI-MS-MS (espectrometria de massas) e utilização de procedimentos de bioinformática que poderão contribuir no conhecimento dessas proteínas em níveis fisiológicos e funcionais, bem como suas vias metabólicas. Os resultados obtidos mostram que dos 105 *spots* proteicos obtidos, 18 apresentaram concentrações de Hg. Nestes 18 spots, foram caracterizadas 10 proteínas que interagiram com mercúrio: Cyb5a protein, Cystathionine beta-synthase a, Fatty acid binding protein 1-A, liver, GTP-binding nuclear protein Ran, Triosephosphate isomerase, 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1

alpha (TCF1), Novel protein similar to zebrafish hemoglobin alpha-adult 1 (Hbaa1), Hemoglobin subunit alpha, Superoxide dismutase, Perforin 1.8. Todas essas proteínas encontradas apresentam caracteristicas moleculares que favorecem a ligação a metais e podem ser usadas futuramente como biomarcadores de exposição ao mercúrio.

Palavras chaves: Mercúrio em peixes amazônicos; Proteômica; Metalômica; Toxicidade; Pescado e Meio Ambiente.

Elucidation of physiological and functional aspects of mercury-associated metal-binding protein in pirarucu (*Arapaima gigas*)

ABSTRACT

Over the recent decades scientific studies have pointed to the high concentrations of mercury found in soil, sediment, fish and humans in the Amazon. However there are still few studies on the mercury toxicity mechanisms at cellular level in ichthyofauna and riverine populations. The investigation of protein expression levels may be a key element in the elucidation of mercury exposure biomarkers in living beings, which would aid the mapping of environmental contamination by this toxic metal. The early screening of these mercury-associated proteins may indicate the risk of fish contamination at a given area and possibly prevent human exposure to mercury. Recent metalloproteomic studies in fish from the Amazon region developed by a group of researchers from Botucatu UNESP have achieved the fractionation and characterization of some mercury-associated proteins with biomarker characteristics. However they have not elucidated the physiological and functional aspects of these mercury-associated proteins and these animals adaptation mechanisms towards this metal exposure.

This study aimed to increase the knowledge of possible protein biomarkers of mercury contamination in pirarucu (Arapaima gigas) collected in areas close to JIRAU - Rio Madeira Hydroelectric Power Plant reservoir. Liver tissue samples were analysed using a novel protein precipitation strategy (fractionation) and the following metalloproteomic strategies: 2D PAGE proteome fractionation of samples; mapping of mercury in protein spots by GFAAS (graphite furnace atomic absorption spectrometry); characterization of mercury-associated proteins by ESI-MS-MS (mass spectrometry) and bioinformatics procedures that may contribute to the knowledge of these proteins at physiological and functional levels, as well as their metabolic pathways. The results show that 18 of the 105 protein spots contained Hg (mercury), leading to the characterization of 10 mercury-interacting proteins: Cyb5a protein, Cystathionine beta-synthase A, Fatty acid binding protein 1-A, liver, GTP-binding nuclear protein Ran, Triosephosphate isomerase, 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1), Novel protein similar to zebrafish hemoglobin alpha-adult 1 (Hbaa1), Hemoglobin subunit alpha, Superoxide dismutase and Perforin 1.8. All

of these proteins have molecular characteristics that favor metal binding and have the potential to be used in the future as mercury exposure biomarkers.

Keywords: Mercury in Amazonian fish; Proteomics; Metallomics; Toxicity; Fish and Environment.

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CAPÍTULO II

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Hg	Mercúrio
GFAAS	Espectrometria de absorção atômica em forno de grafite
ESI-MS-MS	Espectrometria de massas
2D-PAGE	Eletroforese bidimensional

SUMÁRIO

CAPÍTULO I

Elucidação de aspectos fisiológicos e funcionais de proteínas associadas ao mercúrio em pirarucu (Arapaima gigas)

ELUCIDAÇÃO DE ASPECTOS FISIOLÓGICOS E FUNCIONAIS DE PROTEÍNAS ASSOCIADAS AO MERCÚRIO EM PIRARUCU (*Arapaima gigas*)

CONSIDERAÇÕES INICIAIS

Na Amazônia as altas concentrações de mercúrio encontradas em solo, sedimentos, peixes e seres humanos têm sido reportadas em diversos trabalhos nas últimas décadas. No entanto, estudos relacionados à elucidação dos mecanismos de toxicidade do mercúrio na ictiofauna e nas populações ribeirinhas em nível celular, de fundamental importância socioambiental, ainda são poucos. O desenvolvimento de biomarcadores relacionados ao mapeamento e expressões de proteínas associadas ao mercúrio poderá indicar previamente possíveis riscos de contaminação do pescado e da exposição humana. Neste contexto, a metalômica, área científica proposta recentemente, apresenta-se como proposta inédita e inovadora na identificação de biomarcadores de mercúrio associados às proteínas. Estudos metaloproteômicos recentes com peixes da região amazônica, desenvolvidos por grupo de pesquisadores da UNESP de Botucatu, permitiram o fracionamento e caracterização de algumas proteínas associadas ao mercúrio com características de biomarcador. No entanto, os aspectos fisiológicos e funcionais dessas proteínas associadas ao mercúrio nos peixes amazônicos e os mecanismos de adaptação apresentados por estes animais frente à exposição ao metal ainda não foram elucidados. Assim, a proposta de trabalho busca utilizar uma nova estratégia de preciptação (fracionada) de proteínas para avançar no conhecimento em busca de biomarcadores proteicos/enzimáticos da contaminação de mercúrio em amostras de tecido hepático de pirarucu (*Arapaima gigas*) coletados em áreas próximas do reservatório da Usina Hidrelétrica de JIRAU - Rio Madeira utilizando-se as seguintes estratégias metaloproteômicas: fracionamento do proteoma das amostras por 2D-PAGE; mapeamento do mercúrio nos spots proteicos por GFAAS; caracterização das proteínas associadas ao mercúrio por ESI-MS-MS e utilização de procedimentos de bioinformática que poderão contribuir no conhecimento dessas proteínas em níveis fisiológicos e funcionais.

1. REVISÃO DE LITERATURA

1.1. Espécie estudada

Os critérios de escolha da espécie, pirarucu (*Arapaima gigas*) para este estudo, levaram em consideração seu nivél trófico e sua importância para o estudo metaloproteômico com mercúrio, por ser uma espécie muito consumida pela população ribeirinha e bastante comercializada nos grandes centros comerciais do Brasil (MURRIETA, 2001).

1.1.1. *Arapaima gigas*

O *Arapaima gigas*, popularmente conhecido como pirarucu ou arapaima (Figura 1), pertence a família *Arapaimidae* da ordem *Osteoglossiformes* (REIS, 2003). São animais piscívoros, ou seja, alimentam-se de outros peixes (SÁNCHES, 1969), podendo atingir até 3 metros de comprimento com aproximadamente 200kg, sendo considerado a maior espécie de peixe de escama do mundo em comprimento (ARANTES et al., 2010; NELSON, 2006). Seu nome tem origem tupi (pira=peixe e urucu=vermelho) e é atribuído devido sua intensa coloração em algumas partes do corpo, que variam sua intensidade de acordo com o sexo e período reprodutivo (VENTURIERI; BERNARDINO, 1999).

O pirarucu é um peixe de água doce que habita as regiões tropicais da América do Sul, podendo ser encontrados no Peru, Bolívia, Guiana e Brasil (bacias hidrográficas do rio Amazonas e Tocantins-Araguaia) (SANTOS; FERREIRA; ZUANON, 2006). Vivem em várzeas, áreas que sofrem constante influência dos ciclos hidrológicos regionais (GOULDING; BARTHEM; FERREIRA, 2003) e possuem estratégias para se adaptarem a essas variações do nível d'água (JUNK, 1997). O animal em sua fase adulta consegue retirar oxigênio do ar atmosférico através de uma bexiga natatória muito vascularizada. A cada 20 a 30 minutos, o animal sobe à superficie para respirar. Mesmo sendo um animal que precisa buscar oxigênio fora d'água para manter sua respiração, o pirarucu não consegue viver por muito tempo fora do rio, a não ser que sejam constantemente irrigado com água (BARD; IMBIRIBA, 1986; CASTELLO, 2004). Da mesma forma, quando malhado na rede de pesca, se seu resgate for demorado, o animal não resiste a falta de oxigênio e morre.

O pirarucu é uma espécie de peixe muito apreciada e com grande aceitação de mercado regional e nacional. É explorado na Amazônia desde o século XVIII, tendo sido na região uma das espécies mais comercializadas durante a segunda metade do século XIX. A partir da década de 1970 estudos citam a espécie como importante nos desembarques pesqueiros e

comercialização em mercados dos principais centros urbanos da Amazônia brasileira. Alguns destes estudos avaliaram os tamanhos dos pirarucus, com base em informações indiretas como por exemplo: dados de desembarque e comercialização, medidas de línguas e mantas, e indicaram que a espécie encontrou-se sobrexplorada em diversas partes da bacia amazônica. Uma das formas em se tentar conter a exploração descontrolada do pirarucu foi por meio da implementação de medidas restritivas adotadas pelo Instituto Brasileiro de Recursos Naturais Renováveis (IBAMA), estipulando tamanhos mínimos de captura, período de defeso reprodutivo, além de permitir as capturas apenas em áreas provenientes de manejo ou produzidas em cativeiro. No entanto, mesmo havendo medidas de proteção, a pesca do pirarucu está colocando em risco a sobrevivência da espécie, pois é praticada de forma predatória. A intensidade da pesca, determinada pelo alto valor comercial, tem estimulado a captura de exemplares jovens que ainda não atingiram a maturidade sexual, prejudicando assim os estoques naturais (GOULDING, 1980; SANTOS; SANTOS, 2005).

Figura 1: Exemplar de *Arapaima gigas* (popularmente conhecido como pirarucu)
Fonte: http://seafoodbrasil.com.br/especie/pirarucu-arapaima-gigas/

1.2. Problemática do Mercúrio

O rio Madeira é o principal afluente do rio Amazonas, banhando os estados de Rondônia e Amazonas, sendo rico em sedimentos e matéria dissolvida, com altas cargas de sólidos em suspensão (SIOLI, 1968; VAUCHEL et al., 2017). A instalação do complexo Hidrelétrico de Jirau (HE JIRAU), na região de Porto Velho-RO, poderá modificar a dinâmica do mercúrio nesse ambiente (BASTOS; LACERDA, 2004; BASTOS et al., 2006), por meio de processos de remobilização de espécies mercurais que até o momento apresentavam-se indisponíveis, podendo modificar sua forma química, sendo disponibilizados para a biota aquática (BASTOS; ALMEIDA; ZARA; ROCHA; SANTOS, 2009; PFEIFFER et al., 1993), e os

processos de metilação, com transformação da forma inorgânica para forma orgânica, o metilmercúrio (MeHg), poderiam ser favorecidos.

Entre as décadas de 80 e 90 , toda a região amazônica passou por intensa corrida do ouro, causando preocupação com a contaminação de mercúrio na água e solo (OLAF, 1998; PFEIFFER; LACERDA, 1988), concomitante à esse problema, alguns estudos tem observado que altas concentrações de mercúrio são encontradas em latossolos de florestas, de origem natural e não antropogênica (ROULET; LUCOTTE, 1995), e que a disponibilização desse elemento para o corpo d'água estaria relacionado principalmente com processos de erosão do solo, decorrentes do desmatamento e práticas agrícolas, responsáveis pela perturbação do ciclo natural do mercúrio (ROULET et al., 1999).

1.3. Mercúrio nos rios amazônicos

Podemos encontrar no ambiente o mercúrio em sua forma inorgânica (metáliaca) e na forma orgânica (metilmercúrio e dimetilmercúrio), sendo esta última muito mais tóxica aos organismos aquáticos por sua facilidade de absorção, elevada taxa de captação e baixa taxa de eliminação pelo organismo (BIDONE et al., 1997; PFEIFFER et al., 1993). Concentrações mais altas de mercúrio são frequentemente encontradas nas espécies que compõem o topo da cadeia alimentar. Os fatores que podem influenciar as variações dos níveis de concentração de mercúrio são: idade do peixe, taxa de crescimento individual, extensão da cadeia alimentar e fase de desenvolvimento do peixe, já que os hábitos alimentares podem variar de acordo com crescimento do mesmo (AULA; BRAUNSCHWEILER; MALIN, 1995; BARBOSA et al., 1995). A medida que aumenta o nível trófico, além de aumentar o acúmulo de mercúrio no indivíduo, a taxa de conversão em metilmercúrio também aumenta. Quanto ao acúmulo desse metal em peixes com maior longevidade pode se relacionar ao fato do mercúrio apresentar baixa taxa de eliminação pelo organismo, ou seja, quanto mais velho o organismo, maior o acumulo em seu sistema (DOLBEC et al., 2001; WASSERMAN; HACON; WASSERMAN, 2001).

Com mais de 1300 espécies descritas, a Amazônia possui a ictiofauna mais rica do mundo; porém, a avaliação dessa diversidade de peixes ainda é de dificil mensuração, pois os estudos existentes encontram-se dispersos nos institutos de pesquisa (CÁUPER, 2006). Com tamanha riqueza pesqueira, pode se imaginar que o pescado é a principal fonte de proteína animal da população ribeirinha da Amazônia e quando contaminados com mercúrio, podem

apresentar riscos a saúde humana (BIDONE et al., 1997; PFEIFFER et al., 1993). Na corrente sanguínea esse metal fixa-se às proteínas e aos glóbulos vermelhos, tendo acesso a vários sistemas do organismo (SHENKER; GUO; SHAPIRO, 2000), podendo causar alterações na mitocôndria, indução de apoptose em céculas T (GUO et al., 1998), mutações em nível cromossômico e gênico podendo dar origem à cânceres e doenças hereditárias (QUEIROZ et al., 1999), além de transpor a barreira placentária, causando grandes danos à saúde do feto, inclusive ao sistema nervoso (SHENKER; GUO; SHAPIRO, 2000).

Ao entrar nos ecossistemas aquáticos, o mercúrio de origem natural e/ou de origem antropogênica, participam de ciclos biogeoquímicos mediados por microorganismos, transformando-se quimicamente em mercúrio iônico ou mercúrio orgânico que pode ser bioacumulado e biomagnificado na cadeia trófica (Figura 2). Portanto, peixes predadores que estão no topo da cadeia trófica, ao acumularem altos níveis de mercúrio, tornam-se transportadores dessas espécies mercurais para seus consumidores como répteis, aves e seres humanos (LEBEL et al., 1998).

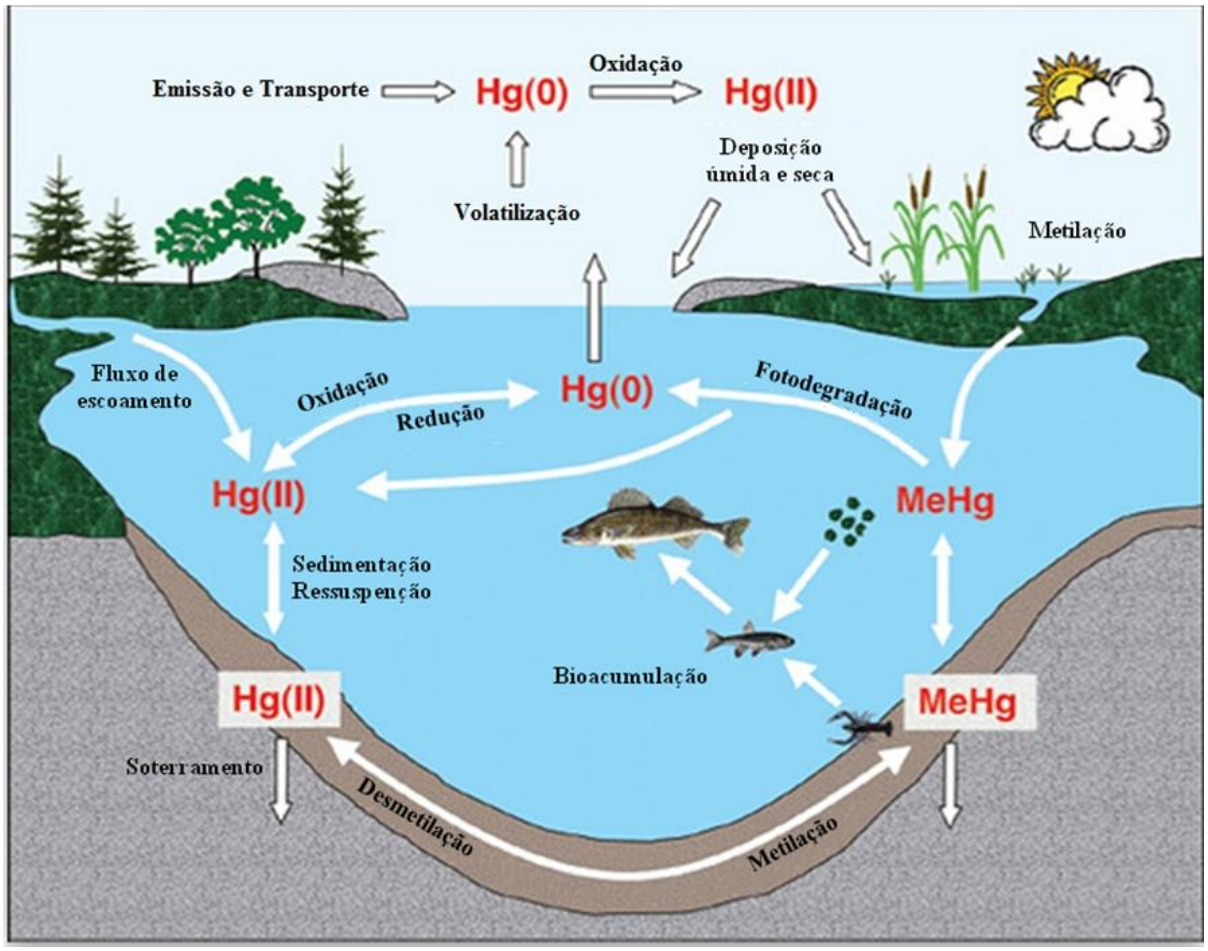

Figura 2: Ciclo do mercúrio em uma bacia hidrográfica. As emissões de mercúrio podem ser transportadas por longas distâncias, principalmente em sua forma inorgânica gasosa [Hg (0)],

o elemento [Hg (II)] oxidado na atmosfera pode ser depositado por precipitação e pelo contato superficial (deposição seca). Bactérias anaeróbicas podem converter uma pequena porção do Hg (II) em metilmercúrio (MeHg), sendo então bioacumulado na cadeia alimentar aquática. Várias reações bióticas e abióticas interconvertem as diferentes formas de mercúrio (Hg), afetando a absorção, a deposição e a evasão de volta à atmosfera (ENGSTROM, 2007).

1.4. Proteômica e Metalômica

A proteômica tem por objetivo analisar as proteínas sintetizadas nas células, tecidos e fluídos biológicos, bem como sua identificação e quantificação. Atua também na identificação da alteração de expressão dessas proteínas, na caracterização de modificações pós-traducionais, identificação de proteínas com interação mútua (interação proteína-proteína), entre outras diversas aplicações (BERANOVA-GIORGIANNI, 2003; VISPO, 2004).

As pesquisas proteômicas tiveram início em meados dos anos 90, devido ao desenvolvimento simultâneo de três áreas: eletroforese bidimensional (2D-PAGE); espectrometria de massas para a análise de proteínas separadas por 2D-PAGE com alta sensibilidade e especificidade; e pesquisa em alta-escala do genoma, que gerou um grande número de sequências peptídicas que foram catalogadas em vários bancos de dados, tornando necessária a utilização de programas cada vez mais avançados de bioinformática para suas análises. A utilização dessas técnicas para obtenção de perfis de expressão de proteínas são ferramentas-chave para o estudo proteômico (MANSO et al., 2005).

Dentro da proteômica temos a metalômica, vertente que busca informações sobre as interações e conexões funcionais entre os genes, metabólitos, proteínas e outras biomoléculas do organismo com espécies metálicas (GÓMEZ-ARIZA et al., 2004; MOUNICOU et al., 2009; SZPUNAR, 2005). Esta área de pesquisa busca esclarecer o papel biológico dos íons metálicos que ligam-se as biomoléculas e sua função no organismo, podendo ser utilizada como ferramenta valiosa no estudo de toxicologia ambiental, envolvendo metais tóxicos tais como o mercúrio (MOUNICOU; SZPUNAR; LOBINSKI, 2009).

1.5. Metaloproteômica

As metaloproteínas são proteínas cuja função é modulada por um metal. A presença do metal é essencial para que ela desempenhe atividade catalítica, participe de reações redox em oxidoredutases, podendo também estabilizar estruturas terciárias ou quaternárias, etc. A determinação da função de metaloproteínas e características de seu sítio de ligação, podem ser

elucidadas por análise conjunta de sua sequência por técnicas de bioinformática e a identificação de seu metal intrínseco (GÓMEZ-ARIZA et al., 2004; MOUNICOU; SZPUNAR; LOBINSKI, 2009; SHI; CHANCE, 2011; SZPUNAR, 2005).

As metaloproteínas fazem parte de um grupo onde existe a necessidade de um íon metálico incorporado as proteínas por meio de ligações específicas para exercerem suas funções biológicas. Se caracterizam pela alta afinidade da interação metal-proteína. Elas são diferentes das proteínas ligadas a metais, que fazem parte de um grupo onde as proteínas incorporam íons metálicos por meio de ligações não-específicas, se caracterizando pela fácil quebra dessa ligação devido a baixa afinidade de interação metal-proteína. No grupo que constituem uma fraca ligação proteína-metal, podemos encontrar os íons monovalentes como o potássio e o sódio, de moderada intensidade temos o cálcio e o magnésio, já dentre os metais que apresentam forte ligação encontramos com maior frequência os de transição como o cobalto, manganês, zinco, ferro, cobre e molibidênio, que estão ligados à maioria das metaloproteínas devido às suas propriedades como densidade, forças eletrostáticas, raio atômico pequeno e interação via eletromagnética (FÁBIO ARLINDO SILVA, 2009).

1.6. Biomarcadores

Biomarcadores são parâmetros mensurados em fluidos corporais, células ou tecidos que indicam modificações bioquímicas ou celulares que prejudicam a homeostase de um organismo, como a presença de substâncias tóxicas ou de condições patológicas (GOLDSTEIN et al., 1987). No contexto de organismos aquáticos, a definição foi modificada por Adams (ADAMS, 1990), incluindo as respostas desses organismos, populações ou comunidades às mudanças no ambiente. As respostas dos organismos foram classificadas em três grupos de biomarcadores: exposição, efeito e susceptibilidade. Com a crescente identificação de novos biomarcadores, se tornou aparente que alguns biomarcadores podem pertencer a mais de um desses grupos. Numa perspectiva mais recente relacionada aos biomarcadores, um efeito resultante da exposição a agentes de estresse pode ser definido como um evento não-patogênico adaptativo ou como um evento funcional alterado mais sério, o que depende da toxicocinética e mecanismo de ação desses agentes. Assim, os biomarcadores de exposição e de efeito podem muitas vezes ser combinados em uma única classificação, com ocorrência de susceptibilidade em qualquer estágio (DECAPRIO, 1997).

Normalmente, a ocorrência das perturbações manifesta-se inicialmente nos níveis mais baixos da organização biológica, antes de surgirem nos níveis mais altos (populações,

comunidades ou ecossistemas). A avaliação de efeitos adversos ou de estresse sinalizados por biomarcadores permite a observação dos mesmos ainda na fase inicial da perturbação (ADAMS, 1990).

Nessa fase inicial, o estresse causa ativação do sistemas de defesa celular, como um modo de adaptação do organismo ao contaminante após a exposição. No entanto, caso a resposta de defesa seja falha, podem ser desencadeados danos em níveis mais altos (histológico ou tecidual). Quando a falha nesses processos ocorre em períodos críticos do desenvolvimento do organismo, podem ser afetadas sua reprodução ou mesmo sua sobrevivência, acarretando em mudanças populacionais e, possivelmente, nos níveis de comunidade da organização biológica (SCHLENK, 1999). Desse modo, o reconhecimento de biomarcadores em níveis hierárquicos mais baixos permite a identificação precoce do efeito, antes que seja propagado aos níveis mais altos (Figura 3) (BAYNE et al., 1985).

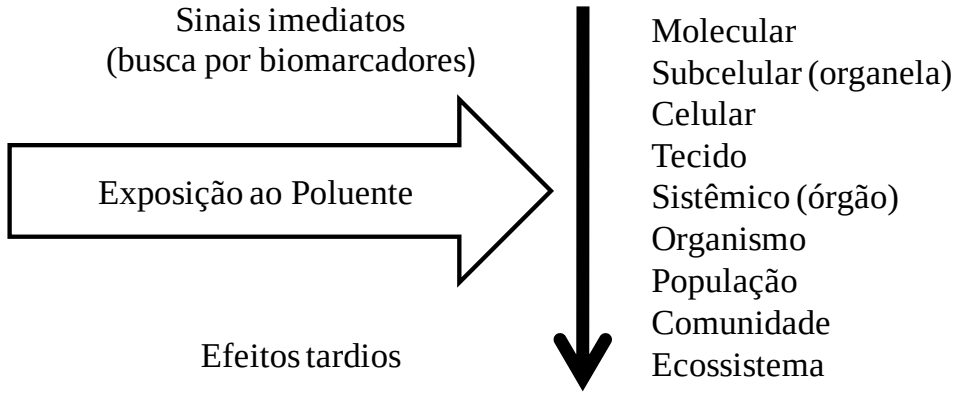

Figura 3. Níveis de respostas do sistema biológico mediante a exposição a poluentes (xenobióticos). Adaptado de Bayne et al., 1985.

2. OBJETIVO GERAL

Fracionar e analisar o proteoma do tecido hepático da espécie de peixe amazônico *Arapaima gigas* (pirarucu) para identificar metal binding protein de mercúrio e elucidar os seus aspectos fisiológicos e funcionais por meio de análises de bioinformática utilizando o software Blast2Go.

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CAPÍTULO II

Physiological and functional aspects of metal-binding protein associated with mercury in theliver tissue of pirarucu (Arapaima gigas) from the Brazilian Amazon

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Physiological and functional aspects of metal-binding protein associated with mercury in the liver tissue of pirarucu (*Arapaima gigas*) from the Brazilian Amazon☆,☆☆

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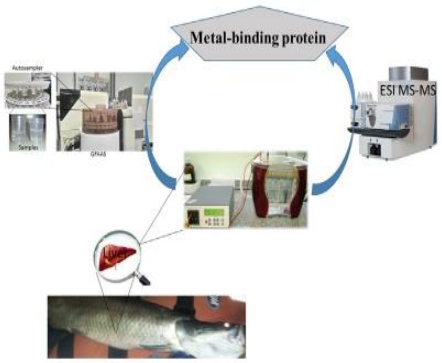
HIGHLIGHTS

- Metalloproteomic study in samples of hepatic tissue from *Arapaima gigas*.
- Mercury-associated metal binding protein in Amazonian fish.
- Elucidation of mercury toxicity mechanisms in ichthyofauna at the molecular and metallomic.
- Mercury detected in 18 protein stains at different concentrations.

GRAPHICAL ABSTRACT



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ABSTRACT

High concentrations of mercury found in soils, sediments, fish, and humans of the Amazon region have gained prominence in scientific studies during the last decade. However, studies related to the elucidation of mercury toxicity mechanisms in ichthyofauna at the molecular and metallomic levels that seek to elucidate physiological and functional aspects, as well as the search for biomarkers of mercury exposure, are still sparse. In the search for these answers, the present study analyzed the hepatic tissue

☆ *Concentration in mg kg^{-1}.

☆☆ #Results expressed as an mean value ± standard deviation (n = 5, P < 0.05).

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proteome of the *Arapaima gigas* (pirarucu) fish species collected in the Jirau hydroelectric power plant reservoir in the state of Rondônia state, Brazil, in order to identify mercury-related metal-binding proteins and to elucidate their physiological and functional aspects. The proteomic profile of the hepatic tissue of *Arapaima gigas* was obtained by two-dimensional electrophoresis (2D-PAGE) and the presence of mercury was mapped in the protein SPOTS by graphite furnace atomic absorption spectrometry(GFAAS). Mercury was detected in 18 protein SPOTS with concentrations ranging from 0.13 ± 0.003 to 131.00 ± 3 mg kg^{-1}. The characterization of the protein SPOTS associated with mercury was performed by electrospray ionisation tandem mass spectrometry (ESI–MS/MS), and 10 proteins were identified. Bioinformatics analyses showed that most of the proteins found linked to mercury were involved in cellular component processes and biological processes. For the most part, protein sequences have cellular functions comprising catalytic, binding, sense of localization, and metabolic processes.

1. Introduction

During the gold rush, due to the use of mercury to amalgam gold in artisanal mining, great amounts of this toxic metal entered the Amazon basin through its rivers and the atmosphere, contributing to high concentrations of this pollutant found in the ecosystem (Lacerda and Marins, 1997). In addition, the occupation of the Brazilian Amazon resulted in severe environmental degradation caused by widespread deforestation, environmental imbalances, hydroelectric construction, and the exploitation of other mineral resources, resulting in the release and remobilization of substances toxic to the environment and the subsequent contamination of biota and humans by mercury (de Lacerda and Malm, 2008). Scientific studies conducted in the Amazon during recent decades have highlighted high concentrations of mercury in fish tissues (Aula et al., 1995; Bourdon et al., 2000; Dolbec et al., 2001; Lebel et al., 1998a; Roulet et al., 2000). The results of these studies point to bioaccumulation along the trophic chain as responsible for the high mercury content in Amazonian fish (Aula et al., 1995; Dolbec et al., 2001). Higher concentrations of mercury in fish with greater longevity may be related to the fact that this element presents a low rate of elimination by the organism, that is, as the body ages, the concentrations of mercury continue to increase. Thus, predatory fish, by accumulating high levels of mercury, act as carriers of the mercurial species to those that consume them, including reptiles, birds, and humans (Lebel et al., 1998b).

However, the toxicity of mercury in the body is dependent on a number of variables, and the determination of only the total mercury concentration in the environment and in the organism is not sufficient to infer such a situation. Often, deleterious effects on populations are difficult to detect in wild organisms, and when they are presented clearly, the destructive process may have progressed beyond the reach of corrective actions. Therefore, there must be a connection between the external and internal levels of exposure, organ-specific bioaccumulation, and sub-lethal and molecular-level effects.

In Brazil, most of the studies related to mercury in freshwater environments, particularly in the Amazon region, have focused on the quantification of the metal in water, sediments, and/or fish tissues, especially those destined for human consumption. Studies of the effects of mercury-related biomarkers on mercury exposure in fish are still few in number and even more so in the case of wild fish (Amorim et al., 2000; Azevedo-Silva et al., 2016; Bastos et al., 2009, 2006; Maurice-Bourgoin et al., 2000; Van der Oost et al., 2003). Among the most studied species of Amazonian fish are those of commercial and ecological interest, such as *Arapaima gigas, Brachyplathystoma filamentosum, Plagioscion squamosissimus, Colossoma macropomum, Cichla* spp, among others. It is also important to note the great importance of these fish for food since fish is the main source of protein and represents the daily food of the riverine population (Parente, 1996).

Based on the proteome of living organisms, several lines of research have emerged seeking information about the functional interactions and connections of metal/metalloid species with genes, proteins, metabolites and other biomolecules of the organism and, therefore, the elucidation of the biological role of the ions metals bound to these biomolecules (Gao et al., 2003; Garcia et al., 2006; Escobar, 2001; Kazmi and Krull, 2001). In this sense, metal ions bound to proteins and metalloproteins represent a large portion of the total number of proteins (Garcia et al., 2006). Metalloproteins are considered different from metal-binding proteins. The first is characterized by the high affinity of the metal–protein interaction, and the metal confers biological activity to the protein (Garcia et al., 2006). Already metal-binding proteins, the metal–protein interaction may not exhibit high affinity, and thus, the coordinated metal does not confer biological activity to the protein (Garcia et al., 2006). The investigation of metalloproteins and/or metal-binding proteins present in the tissues of living organisms is of fundamental importance in both structural and functional aspects (Herton Escobar, 2001). In this way, metallomics, a recently proposed research area, has the objective of verifying the distribution of metal and/or metalloid species, as well as the elucidation of the physiological and functional aspects of the biomolecules containing metal ions in their structures (Gao et al., 2003; Kazmi and Krull, 2001).

In the last years, Brazilian researchers have published the previously unpublished results of a metalloproteomic study in fish species of the Brazilian Amazon (Braga et al., 2015; Cavalcante et al., 2017; de Queiroz et al., 2018, 2019; Moraes et al., 2012, 2013, Vieira et al., 2015, 2017). In these studies, researchers identified the following metal-binding proteins associated with mercury: parvalbumin (with isoforms: alpha and beta); ubiquitin-40S; ribosomal protein S27a; N-alpha-acetyltransferase 20; zinc finger and BTB domain-containing protein 24; dual-specificity protein phosphatase 22-B; fatty acid-binding protein 10-A, liver basic; mediator of RNA polymerase II transcription subunit 11; betaine–homocysteine S-methyltransferase; Mth938 domain/containing protein; protein NLRC5; and 39S ribosomal protein L36 mitochondrial protein. However, more-detailed studies related to the physiological and functional aspects of these metal-binding proteins are required (Braga et al., 2015; Cavalcante et al., 2017; de Queiroz et al., 2018, 2019; Moraes et al., 2012, 2013, Vieira et al., 2015, 2017), as well as research that extends to other species of fish.

Based on the above, the present work has as its objective the fractionation and analysis ofthe hepatic tissue proteome of the Amazonian fish species *Arapaima gigas* (pirarucu) in order to identify metal-binding proteins bound to the mercury and to

elucidate its physiological and functional aspects through bioinformatics analyses using software such as Blast2GO.

2. Material and methods

2.1. Experimental animals, sample collection and processing

Hepatic tissues from five individuals of the *Arapaima gigas* (pirarucu) species were collected and stored in liquid nitrogen in the area of influence of the reservoir of the Jirau hydroelectric power plant in the state of Rondônia, Brazil. The collection points located along the Madeira river were marked usingGPS as follows: (a) (S 09°47′18,3″, W 065°31′12,4″); (b) S 09°41′0,06″, WE 064°58′43.9″; and (c) S 09°16′12,8″, W 064°41′14″).

The captured *Arapaima gigas* (nine animals with a mean weight of approximately 6 kg) were euthanized by section of the cervical spine, and hepatic tissue samples were placed in vials of 50 mL and stored in liquid nitrogen for the proteomic and mercury analyses performed in the Bioanalytical and Metalloproteomic Laboratory (LBM) of the Institute of Biosciences of UNESP Botucatu, São Paulo. In the laboratory, the still-frozen samples were ground in a mixer to obtain pools of hepatic tissue of the nine individuals. The experiment was approved by the Committee on Ethics in the Use of Animals –CEUA, protocol number 0186/2017.

2.2. Protein extraction and precipitation

The extraction process of the protein fraction was conducted based on the procedure described by Braga et al. (2015); Vieira et al. (2015), with some modifications. Approximately 200 mg of pirarucu hepatic tissue pool were weighed and homogenized in an OMNI BEAD RUPTOR 4 cell disruptor with 3 mL of extraction solution (20 mmol L^{-1} of tris, 0.5 mol L^{-1} of sucrose, and 8.4 mg of dithiothreitol (DTT), pH 8.5). After homogenization, the suspensions obtained were allowed to stand for 5 min in the refrigerator and were then centrifuged at 13,000 rpm for 60 min in a Hettich Universal 320 R refrigerated (4 °C) centrifuge. The protein extract was collected and aliquoted for the fractionation precipitation process, which will be briefly described below: 1 mL of protein extract was transferred to a new 2 mL microtube, 1 mL of ice-cold solution containing ethanol-chloroform in ratio of 12.5:1 (v/v) to obtain protein pellets of molecular mass greater than 90 kDa obtained by centrifugation at 13,000 rpm at 4 °C of the mixture. The pellets obtained in this stage did not show mercury according to the analyses conducted by GFAAS and were discarded. The supernatant obtained was collected for re-precipitation of proteins of lower molecular mass (molecular mass less than 90 kDa) using hydrochloric acid/ethanol solution (1:3 v/v). Protein pellets were obtained using a refrigerated centrifuge as described previously and washed three times with a 1 mL extraction solution, and the total protein content was quantified using the Biuret method (Carl Burtis and Edward Ashwood, 2005).

2.3. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

After the total protein content was determined, the pellets were solubilized with a specific buffer (7 mol L^{-1} urea; 2 mol L^{-1} thiourea; 2% (m/v) of CHAPS (3-[(3-cholamidopropyl) dimethylammonio] -1-propanesulfonate); 0.5% (v/v) ampholytes pH 3 a 10; 0.002% (m/v) bromophenol blue). Then, 250 μL of protein extract (corresponding to 375 μg protein) was applied in strips of 13 cm, with a pH range of 3–10, which were used in isoelectric focusing, the first step of 2D-PAGE electrophoresis. Posteriorly, the strips were allowed to rest for 12 h. After this process, the strips were focused on an Ettan IPGphor isoelectric focusing apparatus (GE Healthcare, Uppsala, Sweden) according to the protocol previously established by Braga et al. (2015); Vieira et al. (2015). After the isoelectric focusing, the strips were equilibrated in a solution containing 6 mol L^{-1} urea, 2% (m/v) sodium dodecyl sulphate (SDS), 30% (v/v) glycerol, 50 mmol L^{-1} Tris-HCl, 0.0025% (m/v) bromophenol blue, and 2% (m/v) dithiothreitol (DTT) for 15 min. After this step, the strips were again equilibrated in a solution of similar composition but replacing the DTT with 2.5% (m/v) iodoacetamide (IAA) also for 15 min. The second step of the electrophoretic process (SDS-PAGE) was conducted using 15% (m/v) polyacrylamide gels. This step of the electrophoretic run had a duration of 5 h and 41 min, with 30 min in the first phase with a voltage of 100V and 5 h and 11 min in the second phase with a voltage of 180V. After the end of the run, the gels were contacted with fixative solution (acetic acid, ethanol and deionized water) for 40 min, and then left for 72 h in Coomassie Blue G-25 dye on a shaker table. Dye removal was performed by washing the gels with ultrapure water, and the images were digitized and treated using Image Master 2D Platinum 7.0 software (GE Healthcare, Uppsala, Sweden). At the end, two triplicates of gels were obtained (an example of a gel is shown in Fig. 1). Three gels were used for the quantification of total mercury in their protein SPOTS and three gels for characterization by ESI-MS/MS of protein SPOTS in which GFAAS determinations indicated the presence of mercury.

2.4. Determination of total mercury

The determination of total mercury in the hepatic tissue sample (pellets and protein SPOTS) was performed by GFAAS according to the procedure described by Moraes et al. (2013), Braga et al. (2015) and Vieira et al. (2015). For this, the samples were digested with a mixture of 1.00 mL concentrated sulfuric acid and 0.25 mL 30% (w/w) hydrogen peroxide in digestion tubes with light heating in a digester block until complete mineralization of the samples (Moraes et al., 2013). The obtained acid extracts were added to 5 mL deionized water (ultrapure 18.2 MΩ cm^{-1}). These determinations were performed using a SHIMADZU AA-6800 atomic absorption spectrometer equipped with an ASC-6100 autosampler and background absorption corrector with a deuterium lamp and also self-reverse system (SR). Pyrolytic graphite tubes with integrated platforms were used in the mercury determinations. These tubes had their internal walls covered with tungsten carbide, which acted as a permanent chemical modifier (Silva and PadilhaPezzato, 2006; 2007). A Shimadzu hollow cathode mercury lamp was used and operated at a current of 12 mA. The wavelength was 253.7 nm, and the spectral resolution was 0.5 nm. Argon was used as the inert gas and the flow rate was kept constant at 1 L min^{-1} throughout the heating program except in the atomization stage, when the gas flow was interrupted. The absorbance values were measured in peaks area. The validation of these determinations was performed by analysis of DOLT-4 Dogfish Liver Certified Reference Material for Trace Metals (National Research Council Canada/Measurement Science and Standards, Ottawa-Canada). The certified value for mercury in the DOLT-4 is 2.58 ± 0.22 mg kg^{-1}. An analytical curve was done in the concentration range 0.15 and 1.50 μg L^{-1}, using the adjustment conditions optimized by Moraes et al. (2013). The equation of the analytical curve for mercury corresponds there is:

$$C(Hg - \mu gL - 1) = Abs. + 0.000071/0.1129 \quad (1)$$

The linear correlation coefficient (r) obtained for the analytical curve was 0.9999. The limits of detection (LOD) and quantification (LOQ) of the determination method, calculated based on the standard deviation of 10 readings of the standard solution blank and on the slope of the analytical curve (LOD = 3/slope and LOQ = 10/

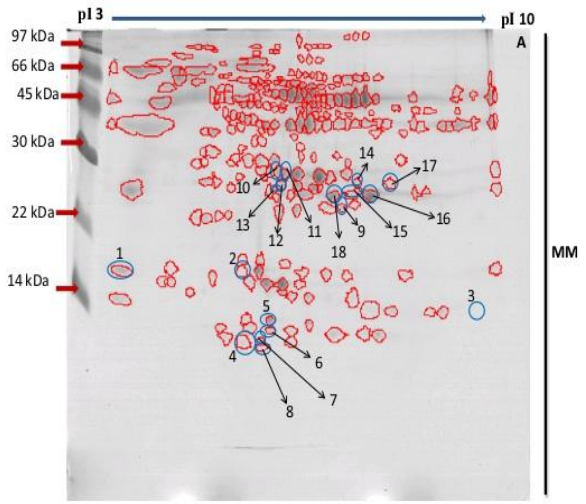

Fig. 1. Polyacrylamide gel obtained by 2D PAGE from the hepatic tissue *pool* of *Arapaima gigas* fish specie. The numbers and circles in blue indicate the spots that have been identified for the presence of mercury by GFAAS. Experimental conditions: 15% (m/v) polyacrylamide gels; strips with 3–10 pH gradient; molecular mass standard of 14–97 kDa; programming of the electrophoretic run - stage 1: 100V, 30 min, stage 2: 180 V, 5 h and 11 min.

slope), as described by Currie (1999), were 0.011 and 0.039 μg L^{-1} mercury, respectively. The LOD and LOQ determined in relation to the certified standard, using 0.01 g of DOLT-4, were 0.096 and 0.32 mg kg^{-1}, respectively (Moraes et al., 2013).

2.5. Characterization of protein SPOTS by ESI-MS/MS

The protein SPOTS that demonstrated the presence of mercury were extracted from the gels using a scalpel, then cut into segments of approximately 1 mm^3 and transferred to microtubes containing 1 mL of acetic acid 5% (v/v), after which the following steps were performed on the SPOTS: dye removal, protein reduction and alkylation, and tryptic digestion using 10 ng mL^{-1} trypsin solution as described by Vieira et al. (2015). Peptide sequences were characterized by ESI-MS/MS according to the procedure described by Shevchenko et al. (2006). The identification of the proteins was done by means of a search in UniProt database (UniProt, 2016).

After identification of the proteins, bioinformatics was performed using Blast2GO software (B2G), where the *FASTA sequence* of the proteins was used to classify them into three levels according to molecular function, cellular component, and biological function (Braga et al., 2017, 2016).

3. Results and discussion

Of the 105 protein SPOTS with a molecular mass less than 90 kDa obtained in the gel (Fig. 1), the determinations made by GFAAS indicated the presence of mercury in 18 SPOTS. The concentration of mercury in protein pellets of molecular mass less than 90 kDa was 170 ± 3.00 μg kg^{-1} (Table 1). The mercury concentrations determined for DOLT-4 (n = 5), as shown in Table 1, were approximately 4.26% lower than the certified values (determined values - 2.47 ± 0.042 mg kg^{-1}; certified values - 2.58 ± 0.22 mg kg^{-1}), which represents a recovery percentage of 95.74%, showing excellent accuracy. As shown also in Table 1, in pellets of molecular mass greater than 90 kDa, GFAAS determinations did not detect the presence of mercury. These results align with those obtained in studies described in the literature (Bittarello et al., 2019; Braga et al., 2015; Cavalcante et al., 2017; de Queiroz et al., 2018, 2019; Moraes et al., 2012, 2013, Vieira et al., 2015, 2017), which highlight that the molecular SPOTS of the lower molecular mass have mostly sulfhydryl groups, functional groups with soft base characteristics that have a great affinity for mercury, an element with a soft acid characteristic (Pearson, 1963).

As shown in Table 2, mercury was detected in 18 protein SPOTS of hepatic pirarucu tissue, with mean concentrations in the range of 0.13 ± 0.003 to 131.00 ± 3 mg kg^{-1}. This is because mercury bioaccumulates and biomagnifies in the organism and, as a result, connects to the protein fraction (Bittarello et al., 2019; Braga et al., 2015). Another factor that may exacerbate this bioaccumulation is that pirarucu isa predatory species at the top of the food chain of the Madeira river in Rondônia, Brazil, a region that, besides the availability of mercury due to the process of gold mining (anthropogenic activity) that dominated the region in the last decades and persists ona smaller scale, still counts on the natural mercury leached from Andean rocks to the bed of Amazonian rivers (natural activity) (Bittarello et al., 2019). In addition, the bioaccumulation of mercury in living organisms can be influenced by the construction of large hydroelectric reservoirs in the Amazonian rivers that can provide mercury species already inert in the environment to the aquatic environment(Dorea, 2004; Dórea et al., 2013). Previous studies conducted with other species of fish from the Madeira river in Rondônia, including predatory species, have shown the presence of mercury inseveral important fish metalloproteins, as well as human hair and milk (Braga et al., 2015; Vieira et al., 2015, 2017; Santos et al., 2015; Cerbino et al., 2017; de Queiroz et al., 2018, 2019).

Table 3 shows the results obtained in the characterization of the protein SPOTS associated with mercury. According to the results, it was possible to observe that most of the protein SPOTS characterized had more than one protein per SPOT; this can occur in the protein characterization process since some of them have isoelectric points and very close molecular masses that can overlap in the gel during the electrophoretic run (Braga et al., 2015; Vieira et al., 2015). For reliability of the analyses, theoretical molecular mass data (UniProt database) were correlated with molecular mass and pI data obtained by 2D-PAGE (Braga et al., 2016; UniProt, 2016).

As shown in Table 3 (the Molecular function column), several proteins have as their main function binding to essential metals, and depending on the essential metal species bound to the protein, mercurial species can substitute for the essential metal at the protein-binding site. The proteins *fatty acid binding protein 1-A, liver;GTP-binding nuclear protein Ran; triosephosphate isomerase;6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)*; *novel protein similar to zebrafish hemoglobin alpha-adult 1 (Hbaa1)*; *hemoglobin subunit alpha;superoxide dismutase (SOD)*; and *perforin 1.8* are examples of proteins found in the present study that have, among their functions, binding to essential metals. In this case, it is possible to emphasize the *superoxide dismutase* that presents copper, zinc, or manganese ions as cofactors and *perforin 1.8* that presents as cofactor calcium ions. In both cases, the essential metal ions may be replaced by mercurial species in an affinity dispute for the binding site (Vieira et al., 2017, 2015).

Using the FASTA sequences of the mercury-associated proteins identified by ESI-MS/MS (Table 3), the graphs representing the functions of each protein were generated in the Blast2GO software (Fig. 2). Analyzing the graphs generated by the Blast2GO software for Cell Component function, it can be verified that 08 sequences of these identified proteins have functions in some cellular parts and that one of these sequences are involved in cellular organelles. Still further on the Cell Component function graph, other activities in which these proteins are involved can be observed including the following: sequence involved in forming part of organelles; cell

Table 1
Concentration of total mercury in the protein *pellets* of hepatic tissue with molecular mass less than and higher of 90 kDa of *Arapaima gigas* fish specie and DOLT-4 fish liver protein (Standard certified).

Pellets	Average Protein mass of *pellet* (mg)	Average Mercury concentration (μg kg^{-1})
Pellet MM > 90 kDa	170	ND
Pellet MM < 90 kDa	20	170 ± 3.00#
DOLT-4	–	2.47 ± 0.042*#

MM = Molecular mass; ND = not detected; DOLT-4: Standard certified of Fish liver protein 2.58 ± 0.22 mg kg^{-1}

Table 2
Mercury concentration in protein spots with molecular mass less than 90 kDa of the hepatic tissue samples of *Arapaima gigas* fish specie.

Spot ID	pI/MM Experimental	Mercury concentration (mg kg^{-1})
001	3.23/16.00	0.44 ± 0.007
002	5.40/16.00	59.70 ± 0.35
003	9.70/14.05	0.95 ± 0.02
004	5.44/11.41	0.18 ± 0.004
005	5.94/12.62	0.13 ± 0,003
006	5.94/12.057	2.10 ± 0.07
007	5.76/11.49	1.40 ± 0.03
008	5.77/11.11	1.70 ± 0.05
009	7.25/21.97	29.70 ± 0.53
010	6.10/26.72	39.10 ± 0.46
011	6.21/26.72	5.50 ± 0.07
012	6,14/25.18	1.80 ± 0.04
013	6.01/24.44	4.20 ± 0.06
014	7.52/25.41	131.00 ± 3
015	7.49/24.02	10.50 ± 0.14
016	7.79/23.25	4.30 ± 0.07
017	8.16/25.26	20.50 ± 0.35
018	7.10/23.31	0.50 ± 0.008

pI - isoelectric point; MM-molecular mass in kDa; Results expressed as an mean value ± standard deviation (n = 5, P < 0.05).

membrane sequences; two forming membranes; sequences forming part of the macromolecular complex; one sequence involved in extracellular regions; sequence involved in extracellular region parts and sequence acting in extracellular regions (Fig. 2).

When analyzing the Molecular Function plot generated by the Blast2GO software, five important functions of the characterized proteins can be highlighted. Four of these participate in cell transport activities; six participate in catalytic activity; one participates in antioxidant activity, in binding, and in transcription factor activity; and seven sequences participate in binding activities (Fig. 2). It is observed that some proteins have more than one function inside the cell, which makes finding bound mercury in their structures more worrisome considering the possibility of this connection to mercury being unfeasible for metabolic functions important for the organism.

The Biological Process function graph generated by the Blast2GO software also provides important information such as the following: six of the proteins found have sequences that participate in important metabolic processes in the body; six are responsible for responses to stimuli; one is involved in the regulation of negative biological processes and in regulating positive biological processes, and the sequence also participates in detoxification processes; and two sequences participate in immune system processes. Another group of very important proteins are those involved in the localization sense; these proteins are responsible for the migratory orientation of the fish at the time of spawning, which allows the population of the species to increase and, thereby, avoid extinction. For this function, three of the 10 mercury-bound proteins were identified, being these: GTP-binding nuclear protein Ran (SPOTS 15 and 18), Novel protein similar to zebrafish hemoglobin alpha-adult 1 - Hbaa1 (SPOT 03) and Hemoglobin subunit alpha (SPOT 03).

Of the SPOTS associated with mercury (Table 2), metalloproteins that are dependent on metal species to exert biological activities and enzymes that perform important biological functions were identified. Cyb5a is an iron-bearing protein linked to the heme group, whose main function is the cellular response to xenobiotic stimuli. This protein reduces iron hemoglobin (methemoglobin) to ferrous hemoglobin, required for stearyl-CoA desaturase activity (Sacco and Trepanier, 2010). CYB5A protein was found in SPOT one, which showed 0.44 ± 0.007 mg kg^{-1} of mercury, a relatively high concentration that may limit the function of this metalloprotein. Cystathionine-beta-synthase was found for the first time in zebra fish encoded by the cbsa gene. His report in the animal species literature is still scarce, but in humans this enzyme participates in important biological activities including the following: cysteine and serine biosynthetic processes; cysteine biosynthetic process via cystathionine; DNA protection; protection; homocysteine catabolic and metabolic processes; hydrogen sulfide biosynthetic process, L-cysteine catabolic process, L-serine catabolic and metabolic processes; and transsulfuration. Moreover, it is believed that, because it is the same enzyme, in fish it performs these same functions or a number of them (Gerhard et al., 2004; Porto et al., 2005). In pirarucu this enzyme was found in SPOT two, which presented 59.70 ± 0.35 mg kg^{-1} of mercury. The fact that this enzyme is associated with the biosynthesis of cysteine, an amino acid in the sulfhydryl group, can explain its affinity to mercury atoms (Bittarello et al., 2019; Braga et al., 2015; Vieira Rocha et al., 2014).

Another protein that called attention in this study was a novel protein similar to zebra fish hemoglobin alpha-adult 1 (Hbaa1), identified in SPOT three, which presented 0.95 ± 0.02 mg kg^{-1} of mercury. This is a cytosolic protein that has two heme sites in its structure, which explains the identification of hemoglobin subunit alpha also in this site (Vieira et al., 2017, 2015). In fish, there are reports of this protein in zebrafish, where it has molecular functions of heme binding, iron ion binding, oxygen binding, and oxygen transport activity (Uniprot, 2018). The occurrence of mercury binding can be explained by the presence of methionine (an amino acid rich in the sulfhydryl groups, with a soft base that shows a high affinity for mercury species, with soft acid) in its FASTA sequence (Pearson, 1963). Studies have reported the interaction of these metalloproteins with mercury, having classified this interaction as the metal-binding type (Vieira et al., 2017, 2015). Another protein with a similar function, fatty acid binding protein 1-A, liver (FABP10a), found in the liver of fish and encoded by the fabp1a gene, has the function of binding to free fatty acids and derivatives of coenzyme A, bilirubin, and intracellular lipid transport. This protein was identified at SPOTS five and six with 0.13 ± 0,003 and 2.10 ± 0.07 mg kg^{-1} of mercury, respectively. These results align with those obtained by Vieira et al. (2017), who, in studies of hepatic tissue of *Brachyplatystoma rousseauxii*, a species of fish also found inthe Brazilian Amazon, identified a mercury-associated protein SPOT as FABP10a. Studies developed by Chmurzyńska (2006) report the presence of n-acetylmethionine in the structure

Table 3
Results of the characterization of the protein spots by ESI-MS/MS of hepatic tissue samples *Arapaima gigas* fish specie.

Spot ID	Protein	Access	Score	pI/MM experimental	pI/MM (DA) Theoretical	Coverage (%)	Molecular function	Peptide sequence
Main liver proteins of pirarucu								
001	Cyb5a protein	A8WGS0	662.2113	3.23/16.003	4.71/ 15.392	6.57	Heme binding (interacting selectively and non-covalently with heme, any compound of iron complexed in a porphyrin (tetrapyrrole) ring). Metal ion binding (interacting selectively and non-covalently with any metal ion).	STWIIIHNK
002	Cystathionine-beta-synthase a	E9QG18	191.2754	5.40/16.003	7.79/9.183	13.1		NLSGTNNVDKK
003	Novel protein similar to zebrafish hemoglobin alpha-adult 1 (Hbaa1)	Q6ZM17	936.5629	9.70/14.054	8.81/ 15.565	20.28	Heme binding (interacting selectively and non-covalently with heme, any compound of iron complexed in a porphyrin (tetrapyrrole) ring). Iron ion binding (interacting selectively and non-covalently with iron (Fe) ions). Oxygen binding (Interacting selectively and non-covalently with oxygen (O_2)). Oxygen carrier activity (enables the directed movement of oxygen into, out of or within a cell, or between cells).	ADEIGAEALAR MLTVYPQTK LRVDPANFK MLTVYPQTK
	Hemoglobin subunit alpha	Q90487	936.5629		7.97/ 15.522	20.28	Heme binding (interacting selectively and non-covalently with heme, any compound of iron complexed in a porphyrin (tetrapyrrole) ring). Iron ion binding (interacting selectively and non-covalently with iron (Fe) ions). Oxygen binding (Interacting selectively and non-covalently with oxygen (O2)). Oxygen carrier activity (enables the directed movement of oxygen into, out of or within a cell, or between cells).	ADEIGAEALAR MLTVYPQTK LRVDPANFK MLTVYPQTK
004	6-pyruvoyl-tetrahydropterin synthase/ dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)	Q6PBI6	167.1621	5.44/11.410	5.73/ 11.822	23.53	4-alpha-hydroxytetrahydrobiopterin dehydratase activity (catalysis of the reaction: (6R)-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-4a-hydroxypterin = (6R)-6-(L-erythro-1,2-dihydroxypropyl)-7,8-dihydro-6H-pterin + H(2)O).	MDHHPEWFNVYNK DFNQAFGFMSR
005	Fatty acid binding protein 1-A, liver	Q1AMT3	223.5709	5.94/12.620	5.06/ 14.080	11.02	Bile acid binding (interacting selectively and non-covalently with bile acids, any of a group of steroid carboxylic acids occurring in bile). Fatty acid binding (interacting selectively and non-covalently with fatty acids, aliphatic monocarboxylic acids liberated from naturally occurring fats and oils by hydrolysis).	YQLESHENFEAFMK YQLESHENFEAFMK
006	Fatty acid binding protein 1-A, liver	Q1AMT3	192.9632	5.94/12.057	5.06/ 14.080	11.02	Bile acid binding (interacting selectively and non-covalently with bile acids, any of a group of steroid carboxylic acids occurring in bile). Fatty acid binding (interacting selectively and non-covalently with fatty acids, aliphatic monocarboxylic acids liberated from naturally occurring fats and oils by hydrolysis).	YQLESHENFEAFMK YQLESHENFEAFMK
007	6-pyruvoyl-tetrahydropterin synthase/ dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)	Q6PBI6	2699.871	5.76/11.493	5.73/ 11.822	33.33	4-alpha-hydroxytetrahydrobiopterin dehydratase activity (catalysis of the reaction: (6R)-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-4a-hydroxypterin = (6R)-6-(L-erythro-1,2-dihydroxypropyl)-7,8-dihydro-6H-pterin + H(2)O).	DFNQAFGFMSR VALQAEK VQITLSTHECGGLSQR DFNQAFGFMSR LQAEK ALQAEK
008	6-pyruvoyl-tetrahydropterin synthase/ dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)	Q6PBI6	625.7964	5.77/11.113	5.73/ 11.822	26.47	4-alpha-hydroxytetrahydrobiopterin dehydratase activity (catalysis of the reaction: (6R)-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-4a-hydroxypterin = (6R)-6-(L-erythro-1,2-dihydroxypropyl)-7,8-dihydro-6H-pterin + H(2)O).	VQITLSTHECGGLSQR DFNQAFGFMSR DFNQAFGFMSR
009	Superoxide dismutase	Q6P980	388.922	7.25/21971	8.29/ 25.008	10.27	Metal ion binding (interacting selectively and non-covalently with any metal ion). Superoxide dismutase activity (catalysis of the reaction: 2 superoxide + 2 H+ = O2 + hydrogen peroxide).	NVRPDYVK HHATYVNNLNVTEEK
010	Triosephosphate isomerase	A0A0R4I9C4	1160.381		6.90/ 26.827	20.16	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	LAYEPVWAIGTGK HVFGESDELIGQK GAFTGEISPAMIK FFVGGNWK KFFVGGNWK

Table 3 (*continued*)

	Triosephosphate isomerase	E9QBF0	1160.381	5.63/ 21.811	24.75	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK HVFGESDELIGQK GAFTGEISPAMIK FFVGGNWK
	Triosephosphate isomerase	A0A0R4IGG3	1160.381	6.45/ 26.526	20	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK HVFGESDELIGQK GAFTGEISPAMIK FFVGGNWK
	Triosephosphate isomerase	I3IS66	338.1218	4.72/ 17.583	30.67	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	DCGIDWVILGHSER IGVAAQNCYK HVFGESDELIGQK GAFTGEISPAMIK
	Perforin 1.8	I3IS28	74.6957	8.25/ 63.675	1.57	Calcium ion binding (interacting selectively and non-covalently with calcium ions (Ca^{2}+)).	ANGLYGDVR YGDVR ANGLYGDVR
011	Triosephosphate isomerase	A0A0R4I9C4	347.2775 6.21/26.722	6.90/ 26.827	11.29	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK GAFTGEISPAMIK
	Triosephosphate isomerase	E9QBF0	347.2775	5.63/ 21.811	14.14	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK GAFTGEISPAMIK
	Triosephosphate isomerase	A0A0R4IGG3	347.2775	6.45/ 26.526	11.43	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK GAFTGEISPAMIK
012	Triosephosphate isomerase	A0A0R4I9C4	5396.335	6.90/ 26.827	27.82	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK HVFGESDELIGQK FFVGGNWK VVFAQTK LDEREAGITEK KFFVGGNWK RHVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	E9QBF0	5396.335	5.63/ 21.811	34.34	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK HVFGESDELIGQK FFVGGNWK VVFAQTK LDEREAGITEK RHVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	A0A0R4IGG3	5396.335	6.45/ 26.526	27.76	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK HVFGESDELIGQK FFVGGNWK VVFAQTK LDEREAGITEK RHVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	I3IS66	4513.838	4.72/ 17.583	31.29	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	IGVAAQNCYK GAFTGEISPAMIK DCGIDWVILGHSER HVFGESDELIGQK RHVFGESDELIGQK GAFTGEISPAMIK
013	Triosephosphate isomerase	A0A0R4I9C4	1033.191	6.90/ 26.827	16.53	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK HVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	E9QBF0	1033.191	5.63/ 21,811	20.71	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK HVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	A0A0R4IGG3	1033.191	6.45/ 26,526	16.73	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK HVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	I3IS66	683.5021	4.72/ 17,583	30.67	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	IGVAAQNCYK HVFGESDELIGQK GAFTGEISPAMIK DCGIDWVILGHSER
014	Triosephosphate isomerase	A0A0R4I9C4	353.1822 7.52/25.408	6.90/ 26.827	11.29	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK
	Triosephosphate isomerase	E9QBF0	353.1822	5.63/ 21.811	14.14	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK

(*continued on next page*)

Table 3 (*continued*)

	Triosephosphate isomerase	A0A0R4IGG3	353.1822		6.45/ 26.526	11.43	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK
	Triosephosphate isomerase	I3IS66	232.2603		4.72/ 17.583	14.11	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	IGVAAQNCYK GAFTGEISPAMIK
015	GTP-binding nuclear protein Ran	P79735	228.9305	7.49/24.016	6.60/ 24.460	11.16	GTPase activity (catalysis of the reaction: GTP + H2O = GDP + phosphate). GTP binding (interacting selectively and non-covalently with GTP, guanosine triphosphate).	LVLVGDGGTGK VCENIPIVLCGNK
016	Triosephosphate isomerase	A0A0R4I9C4	192.9388	7.79/23.245	6.90/ 26.827	9.27	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK FFVGGNWK
	Triosephosphate isomerase	E9QBF0	192.9388		5.63/ 21.811	11.62	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK FFVGGNWK
	Triosephosphate isomerase	A0A0R4IGG3	192.9388		6.45/ 26.526	9.39	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK FFVGGNWK
017	Triosephosphate isomerase	A0A0R4I9C4	1134.855	8.16/25.258	6.90/ 26.827	19.76	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK HVFGESDELIGQK FFVGGNWK GAFTGEISPAMIK
	Triosephosphate isomerase	E9QBF0	1134.855		5.63/ 21.811	24.75	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK HVFGESDELIGQK FFVGGNWK GAFTGEISPAMIK
	Triosephosphate isomerase	A0A0R4IGG3	1134.855		6.45/ 26.526	20	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK VVLAYEPVWAIGTGK HVFGESDELIGQK FFVGGNWK GAFTGEISPAMIK
	Triosephosphate isomerase	I3IS66	701.1451		4.72/ 17.583	22.09	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	GAFTGEISPAMIK IGVAAQNCYK HVFGESDELIGQK GAFTGEISPAMIK
018	Triosephosphate isomerase	A0A0R4I9C4	845.3638	7.10/23.314	6.90/ 26.827	16.53	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK GAFTGEISPAMIK HVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	E9QBF0	845.3638		5.63/ 21.811	20.71	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK GAFTGEISPAMIK HVFGESDELIGQK GAFTGEISPAMIK
	Triosephosphate isomerase	A0A0R4IGG3	845.3638		6.45/ 26.526	16.73	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	VVLAYEPVWAIGTGK GAFTGEISPAMIK HVFGESDELIGQK GAFTGEISPAMIK
	GTP-binding nuclear protein Ran	P79735	546.9617		6.60/ 24.460	31.16	GTPase activity (catalysis of the reaction: GTP + H2O = GDP + phosphate). GTP binding (interacting selectively and non-covalently with GTP, guanosine triphosphate).	LVLVGDGGTGK VCENIPIVLCGNK NLQYYDISAK SNYNFEKPFLWLAR KYVATLGVEVHPLVFHTNR
	Triosephosphate isomerase	I3IS66	377.2552		4.72/ 17.583	22.09	Triose-phosphate isomerase activity (catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate).	IGVAAQNCYK GAFTGEISPAMIK HVFGESDELIGQK GAFTGEISPAMIK

of this protein, which may justify the binding of mercury species to FABP10. The SPOTS four, seven and eight were identified as the enzyme 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1), presenting mercury 0.18 ± 0.004, 1.40 ± 0.03, and 1.70 ± 0.05 mg kg^{-1}, respectively. Protein encoded by the pcbd gene has the molecular function of 4-alpha-hydroxytetrahydrobiopterin dehydratase activity and the biological biosynthetic process of tetrahydrobiopterin. Because it presents cysteine and methionine in its FASTA sequence, this enzyme can associate with mercurial species through metal-binding interactions (Vieira et al., 2017, 2015).

In addition to those previously mentioned herein, other proteins that act in the fight against oxidative stress caused by aggressive agents, such as the protein superoxide dismutase (SOD), an enzyme of the antioxidant system that works to protect cells from harmful reactions of superoxide, have been characterized (Piper, 1999). Superoxide dismutase may contain zinc, copper (Cu–Zn-SOD), manganese, or iron in its structure. Damage to this enzyme can lead to serious problems to the body due to the accumulation of free radicals and the emergence of several chronic pathologies. Under conditions of oxidative stress caused by environmental factors, this enzyme presents altered expression in the organism, indicating a biological disturbance (Pascual et al., 2003; Piper, 1999). This enzyme was identified in SPOT nine, presenting 29.70 ± 0.53 mg kg^{-1} of mercury. Considering the high concentration of mercury determined in this SPOT, its presence can be

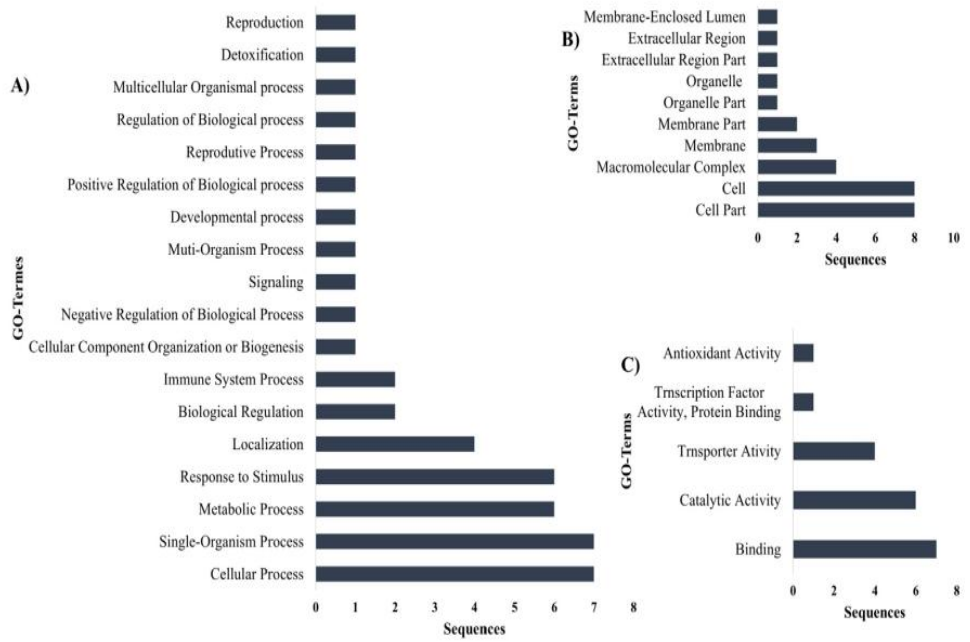

Fig. 2. - Graphics representing of the functions: Biological Process (A), Celular Components (B) and Molecular Functions (C), generated by Blast2Go software. The graphics represents how many proteins or part of these proteins found by ESI-MS/MS analysis are involved in the biological process, celular components and molecular functions.

inferred as an attempt to protect the organism from damage caused by mercury. The enzyme triosephosphate isomerase (TPI) was found in eight SPOTS (SPOTS 10, 11, 12, 13, 14, 16, 17, and 18) with mercury concentration in the range of $0.50 \pm 0.008 - 131.00 \pm 3$ mg kg^{-1}. It is a glycolytic enzyme encoded by the tpi1b gene, which is involved in gluconeogenesis, carbohydrate biosynthesis pathway, biochemical functions, protein binding, triosephosphate isomerase activity, and protein ubiquitin ligase binding (Schneider, 2000). With an active site in the center of the molecule, triosephosphate isomerase has a residue of glutamic acid and histidine that allows this enzyme to act in catalysis processes (Rozovsky et al., 2001; Schneider, 2000). The literature does not report the presence of coordinated metal ions to the active site of TPI; however, the presence of methionine in the FASTA sequence of this enzyme allows the binding of mercurial species with their active sites through metal-binding interactions. The high concentrations of mercury determined in the protein SPOTS of this enzyme (SPOTS 10, 14 and 17) suggest a high affinity for mercury species. Considering the various functions that TPI performs in biological systems, more-detailed studies of TPI with mercurial species are needed. Perforin 1.8 is a calcium ion binding protein, encoded by the prf1.8 gene, and a participant in the body's immune system (Liu et al., 1995; Varela et al., 2016). In fish, its role is still being studied; however, in mammals, perforins have been identified as playing roles in cell death, in the defense against viruses and neoplastic cells, and in the recognition of unknown molecules by the immune system (Varela et al., 2016). Because it is a protein involved in the immune system and binds to calcium ions, it can be inferred that mercury may be replacing the calcium-binding site to associate with the molecules of that protein. It is observed, that SPOTS identified as perforin 1.8 has a high concentration of mercury, which reinforces the need for more in depth studies of the association of this protein with mercury species. In the SPOT 18 besides triosephosphate isomerase the GTP-binding protein Ran was identified too. This protein encoded by the run gene in fish has as its cofactor magnesium ions (UniProt, 2016). Among its molecular functions are GTPase activity, GTP binding, and magnesium ion binding. In addition, this protein participates in important biological processes such as GTP metabolic processes, the segregation of mitotic sister chromatids, the development of neural retina, the transfer of proteins into the nucleus, the nuclear export of ribosomal subunits, and the import of snRNA into the nucleus (Liao et al., 1997; UniProt, 2016). The GTP-binding nuclear protein Ran is also involved in nucleocytoplasmic transport, participating in both the import and export of core proteins and RNAs (Liao et al., 1997). The high concentration of mercury (10.50 ± 0.14 mg kg^{-1}) determined in the SPOT 15, SPOT also identified as GTP-binding nuclear protein Ran, may to compromise the processes involved in cell division and contribute to congenital malformations or to mitotic disorders in adult organisms. The presence of cysteine (soft basis) in the FASTA sequence of the GTP-binding nuclear protein Ran allows the coordination of mercurial species (soft acids) in their active sites (Pearson, 1963).

As can be observed in Table 3, the data presented draw attention not only to the number of protein SPOTS with the presence of mercury bound to proteins but also to the diversity of proteins that were identified with the presence of this toxic element. The data presented in this study do not yet allow us to establish the metalloprotein profile of mercury, which makes it difficult to conclude if it occurred an increase or decrease of mercury bioaccumulation in the organism of this species. More-detailed and specific studies on mercury metal-binding proteins identified in the hepatic tissue of *Arapaima gigas* are needed. It should be emphasized that, because of the fact that this species of fish is highly commercialized in the Brazilian Amazon and other regions of Brazil, their mercury contamination may to cause serious public health problems. In addition, the mercury-bound proteins identified in present study, corroborate with works of the literature that also identified similar proteins bound to mercury (Bittarello et al., 2019; Braga et al., 2015; Cavalcante et al., 2017; de Queiroz et al., 2018, 2019; Moraes et al., 2012, 2013, Vieira et al., 2015, 2017). Thus, the data presented in this study enables the initiation of a discussion on proteins associated with mercury and the identification of its metabolic pathways, which allows for the inference of possible damages to the organism.

4. Conclusion

The metalloproteomic study of muscle tissue of *Arapaima gigas*, demonstrated that mercury-bound proteins identified in this tissue could become biomarkers of mercury exposure. Knowledge of their functions in the organism, metabolic pathways, and affinities for making connections with essential metals may help to consolidate them as biomarkers. *Arapaima gigas* presented a great diversity of proteins linked to mercury, perhaps because it is a species at the top of the food chain that can biomagnify this element in its tissues, and as it is a species widely consumed by the human population, it may cause serious public health problems. The bioinformatics analysis using the Blast2GO software showed that proteins, among their most outstanding functions, present catalytic activity, binding, cell division, localization, cellular regulation, and development processes among others. This indicates their diversity of functions in the body and their importance as future biomarkers of exposure to mercury.

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CAPÍTULO III

IMPLICAÇÕES

A metaloproteômica se apresenta como uma importante ferramenta de pesquisa em busca de biomarcadores moleculares de exposição ao mercúrio em peixes, sinalizando proteínas que interagem com este metal. A técnica utilizada de separação eletroforética bidimensional (2D-PAGE), se mostrou eficiente na busca por possíveis biomarcadores de exposição ao mercúrio, a padronização do protocolo se fez necessária devido a estrátegia de precipitação utilizada (fracionada) não ter sido desenvolvida até então para a espécie estudada (*Arapaima gigas*).

O presente estudo trás contrubuições para a área, no entanto, apesar dos avanços que foram obtidos por meio deste trabalho, ainda será necessário mais estudos com as proteínas identificadas, caracterizar sua ligação ao mercúrio e entender o que esse fato pode causar nos organismos a curto e longo prazo, bem como estudar outras espécies de peixes expostos ao mercúrio para comparar os efeitos desse metal em diferentes organismos que tenham dietas variadas (onívoros, detritívoros e carnívoros). Estudos futuros que confrontem os resultados obtidos em diferentes espécies de peixes expostos ao mercúrio, serão de suma importância, bem como novos experimentos que possam ser realizados estudando mais a fundo as proteínas identificadas encontradas nesse trabalho que apresentaram associações entre concentração de mercúrio e proteínas, podendo ajudar na consolidação das mesmas como biomarcadores de exposição a este metal.