UNIVERSIDADE VILA VELHA - ES

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA DE ECOSSISTEMAS

EVIDÊNCIAS DE ALTERAÇÕES BIOQUÍMICAS E REPRODUTIVAS EM *Astyanax lacustris* (LUTKEN, 1875) (TELEOSTEI: CHARACIFORMES) DO RIO DOCE APÓS DESASTRE AMBIENTAL EM MARIANA/MG

JULIA MERÇON FERNANDES MOREIRA

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Tese apresentada à Universidade Vila Velha, como pré-requisito do Programa de Pós-graduação em Ecologia de Ecossistemas, para a obtenção do grau de Doutora em Ecologia.

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Dedico à minha mãe, Maria José Fernandes, e às minhas tias Maria da Conceição, Alvanir, Neuza, Cleia e Etelvina que, mesmo diante de todas as dificuldades, sempre me apoiaram em tudo.

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LISTA DE FIGURAS

maiúsculas representam a diferença significativa encontrada entre os tecidos, na estação da Cheia.

SUMÁRIO

RESUMO

Moreira, Julia Merçon Fernandes, Dr., Universidade Vila Velha – ES, abril de 2021. **EVIDÊNCIAS DE ALTERAÇÕES BIOQUÍMICAS E REPRODUTIVAS EM *Astyanax lacustris* (LUTKEN, 1875) (TELEOSTEI: CHARACIFORMES) DO RIO DOCE APÓS DESASTRE AMBIENTAL EM MARIANA/MG.** Orientador: Dr. Levy de Carvalho Gomes.

No dia 5 de novembro de 2015, a barragem de Fundão, localizada em Mariana, Minas Gerais, se rompeu liberando mais de 50 milhões de metros cúbicos de rejeitos de minério de ferro, enriquecido em metais, no leito do Rio Doce. O objetivo desse estudo foi avaliar os efeitos deletérios na biologia reprodutiva e na resposta bioquímica dos organismos de *Astyanax lacustris* expostos aos metais contidos nos rejeitos de mineração despejados no Rio Doce. O estudo foi realizado no Rio Doce, na região de Baixo Guandu, Espírito Santo, Brasil. Amostras mensais foram coletadas no período de um ano. *Astyanax lacustris* apresentou reprodução múltipla, latência na formação das gônadas e correlação positiva entre o dano histológico da gônada e concentrações de Al e Fe. As gônadas masculinas apresentaram 47,36% de células imaturas invadindo o lúmen da gônada e, as gônadas femininas, apresentaram 39,64% de atresia. Além disso, foram observadas alterações bioquímicas no fígado de *A. lacustris,* com efeitos claros de sazonalidade, estando diretamente relacionadas à alta concentração de Al e Fe. Apesar disso, o processo de bioacumulação de metais, pelos organismos, apresentou efeitos de sazonalidade apenas nas brânquias, por serem o primeiro órgão de contato com a água contaminada. Os dados gerados no presente estudo fornecem uma visão geral da saúde do ecossistema da região, evidenciando os efeitos prejudiciais causados à população de *A. lacustris* afetada pelo rompimento da barragem de Fundão.

Palavras-chave: Enzimas, gônadas, lambari, barragem, desova.

ABSTRACT

Moreira, Julia Merçon Fernandes, Dr., University Vila Velha – ES, April, 2021. **EVIDENCES OF BIOCHEMICAL AND REPRODUCTIVE CHANGES IN *Astyanax lacustris* (LUTKEN, 1875) (TELEOSTEI: CHARACIFORMES) OF DOCE RIVER AFTER ENVIRONMENTAL DISASTER IN MARIANA/MG.** Advisor: Dr. Levy de Carvalho Gomes.

On November 5th, 2015, the Fundão Dam collapsed dumping more than 50 million/m^3 of iron ore tailings, enriched with metals, into the Doce River channel. The objective of this study was to evaluate the deleterious effects on reproductive biology and on the biochemical response of *Astyanax lacustris* organisms exposed to the metals present in the mining waste dumped into the Doce River. The study was carried out in Doce River, in the region of Baixo Guandu, Espirito Santo, Brazil. Monthly samples were collected over a period of a year. *Astyanax lacustris* showed multiple spawning, latency in the formation of gonads and a positive correlation between gonad's histological damage and concentrations of Al and Fe, in females. Male gonads presented a frequency of 47.36% of immature cells invading the cell lumen and female gonads presented 39.64% frequency of atresia. In addition, biochemical changes were observed in the liver of *A. lacustris*, with clear effects of seasonality, being directly related to the high concentration of Al and Fe. Despite that, the process of bioconcentration of metals by the organisms presented seasonality effects only by the gills, as they are the first organ of contact with contaminated water. The data generated in the present study provide an overview of the health of the region's ecosystem, showing the harmful effects caused to the population of *A. lacustris* affected by the rupture of the Fundão dam.

Palavras-chave: Enzymes, gonads, lambari, dam, spawning.

1. APRESENTAÇÃO DA TESE

No dia 5 de novembro de 2015, a barragem de Fundão, localizada em Mariana, Minas Gerais se rompeu. Cerca de 50 milhões de metros cúbicos foram despejados no leito do Rio Doce (Gomes et al., 2018). Os rejeitos de mineração foram carreados por cerca de 600km até chegar na foz do Rio Doce, localizada em Regência, no município de Linhares, Espírito Santo (Passos et al., 2020). Estudos anteriores como o de Gomes et al. (2017) relataram altas concentrações de metais, como Fe (57,2%), Al (1,3%), e metais traço como Cr e Pb, na região de coleta do Rio Doce depois do rompimento da barreira da Samarco. Dessa forma, esta tese visa relatar os a concentração atual de metal, no leito do Rio Doce, assim como verificar os possíveis efeitos causados pela bioacumulação dos metais, pelos organismos expostos. Essa tese foi dividida em dois capítulos para melhor entendimento e fluidez na escrita, além de proporcionar ao leitor os artigos já redigidos para publicação.

O capítulo 1 relaciona a deposição de metais, causados pelo desastre, com a biologia reprodutiva da espécie *Astyanax lacustris*. Os peixes foram coletados no Rio Doce, na região de Colatina, ES, próximo à hidrelétrica de Mascarenhas. Foi realizada a análise da concentração do metal presente na água e nas gônadas dos indivíduos expostos. Além disso, as gônadas foram submetidas a cortes histológicos a fim de determinar o período de maturação dos indivíduos, no momento de coleta, e de verificar a existência de histopatologias. Por fim, foi feita uma correlação entre os índices histopatológicos encontrados e a concentração do metal nas gônadas, para verificar a influência desses metais, na biologia reprodutiva do organismo estudado.

O capítulo 2 relaciona a deposição de metais, causados pelo desastre, com as respostas bioquímicas sazonais possivelmente geradas pelos organismos da espécie *Astyanax lacustris.* Os peixes foram coletados no Rio Doce, na região de Colatina, ES, próximo à hidrelétrica de Mascarenhas. Foi realizada a análise da concentração do metal presente na água, nas brânquias, no fígado e no músculo dos peixes coletados. Foi realizada a análise das enzimas antioxidantes Catalase e Glutationa-S-Transferase a fim de verificar seus níveis de atividade e correlaciona-los com a concentração dos metais encontrados nos respectivos tecidos determinando, assim, a influência desses metais na fisiologia do organismo estudado.

PROJETO “RECUPERAÇÃO DA BIOTA AQUÁTICA DO BAIXO RIO DOCE”

FINANCIAMENTO DE PESQUISA

2. INTRODUÇÃO GERAL

A água doce contribui com apenas 0,8% de toda a reserva do mundo, além de possuir apenas 0,02% dessa reserva, disponível e habitável às formas de vida (Tedesco et al., 2017). Levando isso em conta, sabe-se que a quantidade de água doce na superfície terrestre é limitada. Estudos prévios feitos por Wu et al. (2017) e Tedesco et al. (2017) indicam que a água doce é considerada um dos habitats mais ameaçados da Terra, levando em conta que a água é essencial para a sobrevivência de todas as formas de vida. A falta e o fornecimento inadequado da água são capazes de mudar o padrão de distribuição dos organismos e até humano. Sendo assim, a escassez de água, a destruição gradual dos ecossistemas aquáticos e a poluição dos mesmos, pode levar a degradação de todo o ecossistema circundante (Javed e Usmani, 2017) e, por isso, o estudo de águas contaminadas por metais não-essenciais se tornou um dos assuntos mais importantes para os pesquisadores ambientais, na atualidade.

Devido à característica dos metais não-essenciais - como estabilidade, toxicidade e potencial de bioacumulação - esses contaminantes são considerados perigosos poluentes para o ambiente aquático (Mirzabeygi et al., 2017). Quando os metais não-essenciais entram em contato com a superfície da água, ocorre a contaminação desse ambiente e, consequentemente, a redução da qualidade e deterioração do suprimento da água potável e para irrigação de alimentos (Bortey-Sam et al., 2015). A presença desses metais nos ecossistemas aquáticos pode causar impactos negativos nos organismos expostos, como danos genéticos, reprodutivos, redução da taxa de crescimento e patologias potencialmente fatais (Cantanhêde et al. 2016, Viana et al. 2018).

A indústria de mineração, hoje, é um dos maiores suportes para a economia nacional de diversos países, além de ser a base de inúmeras outras indústrias que fornecem matérias-primas à população (Dong et al., 2019). O aumento acelerado da mineração nas últimas décadas e o consequente aumento do volume dos rejeitos de mineração, ocasionaram o aumento gradual dos impactos nos ambientes circundantes (Dong et al., 2018). As operações de mineração, juntamente com o descarte de seus rejeitos, fornecem fontes de contaminação do ambiente por diferentes vias (Sun et al., 2018).

Além dos riscos ambientais causados pela existência indústria de mineração, ainda há o risco causado pelo armazenamento dos rejeitos sem reabilitação ou restauração adequada (Silveira et al., 2019). Somente a segurança na estabilidade geotécnica é insuficiente. Relatos recentes fornecem informações de barragens que passaram por diversos testes de estabilidade, o mais recente sendo alguns meses antes do rompimento da mesma (Cross et al., 2017). A falta de adequação no monitoramento das barragens pode acarretar seu rompimento e, consequentemente, a contaminação direta do ecossistema circundante. Estudos prévios relatam a existência de cerca de 50 barragens de rejeitos, somente no estado de Minas Gerais, sob risco de colapso (Meira et al. 2016).

No dia 5 de novembro de 2015, a barragem de Fundão, localizada em Mariana, Minas Gerais se rompeu. Cerca de 50 milhões de metros cúbicos foram despejados no leito do Rio Doce (Gomes et al., 2018). Os rejeitos de mineração foram carreados por cerca de 600km até chegar na foz do Rio Doce, localizada em Regência, no município de Linhares, Espírito Santo (Passos et al., 2020). Estudos anteriores como o de Macêdo et al. (2020) relataram altas concentrações de metais, como Al, Cr, Mn, Fe e Pb, na região de coleta do Rio Doce depois do rompimento da barragem de Fundão. Além do impacto financeiro, os rejeitos de mineração podem levar a sérios impactos ambientais, prejudicando a saúde do ecossistema e da população ribeirinha, com o soterramento de assembléias bênticas e a contaminação crônica dos ecossistemas circundantes (Gomes et al., 2017).

A correlação entre as concentrações dos contaminantes ambientais e as respostas biológicas dos organismos expostos a esses contaminantes fornecem um melhor entendimento sobre os riscos que essa contaminação oferece e o diagnóstico apropriado para o manejo ambiental necessário em cada situação (Vieira et al., 2019). As mudanças histopatológicas são biomarcadores utilizados na avaliação do status da saúde de organismos expostos em laboratório ou em seu habitat natural (Van der Oost et al., 2003). Stentiford et al. (2009) declararam que a análise de mudanças histopatológicas também é um ótimo indicador de contaminantes antropogênicos. Uma das vantagens desse biomarcador é que ele é utilizado para avaliar tecidos específicos, incluindo brânquias, fígado e gônada, órgãos responsáveis por funções vitais, como respiração, excreção e acumulação e biotransformação de xenobióticos, em peixes (Raj et al., 2018). Outro biomarcador utilizado na avaliação do status da saúde dos organismos, são as enzimas antioxidantes. A exposição a contaminantes também geram um aumento na produção de espécies reativas de oxigênio (EROs), causando estresse oxidativo. O estresse oxidativo ocorre quando contaminantes modificam o equilíbrio entre as defesas antioxidantes (Kim e Kang,2015), podendo resultar em danos oxidativos para biomoléculas (Jijie et al., 2020). Com a função interceptar e inativar os radicais das EROs produzidos pela exposição aos metais, algumas enzimas são ativadas como, por exemplo, a Catalase (CAT) e a Glutationa-S-Transferase (GST) (Davies, 1995). A Catalase, enzima de fase I, atua decompondo o H_2O_2 em água e oxigênio. A Glutationa-S-Transferase (GST), uma enzima de biotransformação de fase II, por sua vez, atua na desintoxicação dos poluentes ambientais (Masella et al., 2005), catalisando a conjugação da Glutationa reduzida (GSH) com compostos tóxicos exógenos e endógenos, fazendo com que estes se tornem mais solúveis na água, menos tóxicos e mais fáceis de serem excretados (Lee et al., 2006).

Originalmente, a espécie *Astyanax lacustris,* utilizada como bioindicador neste estudo, foi descrita por Lutken (1875). Porém, recentemente, Lucena e Soares (2016) sinonimizaram outras três espécies, *Astyanax jacuhienses*, *A. asuncionensis* e *A. altiparanae,* com a *A. lacustris*. Isso fez com que a distribuição geográfica dessa espécie se tornasse ainda maior. Além disso, a sinonimização resultou em um aumento na importância do estudo dessa espécie a fim de melhor entendimento na sua biologia reprodutiva (Súarez et al., 2017). As espécies do gênero *Astyanax* são relativamente pequenas (10-12cm, quando adultos), se movimentam em cardumes e possuem pequeno interesse comercial, porém, são consideradas importantes como espécies forrageiras (Pereira et al., 2016). A biologia reprodutiva de *A. lacustris* possui uma certa escassez de informações, dificultando a aplicação de projetos de manejo e conservação da espécie (Súarez et al., 2017). *Astyanax lacustris* é nativa do Rio Doce e tem maturidade sexual precoce (maturação em 0,7-1,0 anos e gerações com cerca de 18 meses). Como a maioria das espécies do gênero *Astyanax*, *A. lacustris* possui ampla distribuição geográfica e é uma espécie considerada dominante entre os peixes de água doce (Nascimento et al., 2020). *Astyanax lacustris* possui hábitos generalistas e são conhecidas por conseguirem sobreviver a águas correntes e de remanso (Macêdo et al., 2020). É considerada uma espécie onívora que habita a coluna d'água, porém, também se alimenta de macroinvertebrados localizados no sedimento (dos Santos et al., 2020). Além disso, a espécie também possui versatilidade ecológica, habilidade para se ajustar em situações adversas e competência adaptativa exploratória. Por possuírem prole numerosa, gerações curtas e de fácil reprodução e manejo (Jeffery 2001, Silva e Porto-Foresti 2020), *A. lacustris* é uma espécie geralmente utilizada como bioindicador e, consequentemente, considerada uma espécie sentinela em estudos ecotoxicológicos (Viana et al., 2013; Nascimento et al., 2020) (Figura 1).

Fig 1 Espécime de *Astyanax lacustris*. Foto retirada do FishBase. Autor: Thiago Nascimento da Silva Campos

3. CAPÍTULO I – Evidence of reproductive disturbance in *Astyanax lacustris* (Teleostei: Characiformes) from the Doce River after the collapse of the Fundão Dam in Mariana, Brazil


Evidence of reproductive disturbance in *Astyanax lacustris* (Teleostei: Characiformes) from the Doce River after the collapse of the Fundão Dam in Mariana, Brazil

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Abstract

On November 5th, 2015, the Fundão Dam collapsed dumping more than 50 million/m3 of iron ore tailings, enriched with metals, into the Doce River channel. The objective of this study was to evaluate the reproductive biology and histological damage in *Astyanax lacustris* exposed to the metals from the dam collapse. The study was carried out at Doce River, in Espírito Santo State, Brazil. Monthly samplings were carried out for a year. *Astyanax lacustris* had multiple spawning: females reproductive peak was in September, October, November, and December; and males between September, October, January, and February. There was a latency in the formation of gonads. For male gonads, it was necessary a 6 cm growth for it to increase from 30% to 50% and 4 cm for female gonads to increase from 40% to 50%. There is a positive correlation between gonad's concentration of Al and Fe and the rate of histological damage in females. Male gonads had a high rate of immature cells invading the cell lumen (47.36%) and female gonads showed higher frequency of atresia (39.64%). Fish exposed to the contaminated water showed high gonad histological damage. The observed changes can directly influence the organism development and reproduction in the long run, thus affecting *A. lacustris* population present in the region.

Keywords: Lambari; contamination; metals; bioconcentration; histological damage; spawning.

3.1. INTRODUCTION

On November 5th, 2015, the Fundão dam collapsed in Mariana, a municipality located in Minas Gerais State, Southeast of Brazil, causing a flood of mud and mining waste (55-62 million m^3) into the Gualaxo do Norte River. The mud flowed into the Doce River and spread for 600km until arriving at the Espírito Santo coast (Escobar 2015). Gomes et al. (2019) and Quadra et al. (2019) reported an increase in several metals in the Doce River shortly after the dam burst. Macêdo et al. (2020) observed high concentrations of metals, such as Iron (Fe), Aluminum (Al), Manganese (Mn), and Lead (Pb) in the Doce River, even three years after the dam broke. Xenobiotics, such as metals, can act in different pathways in organisms, involving different physiological processes (Tolussi et al. 2018), affecting sexual differentiation (Baroiller and Guiguen 2001) and gonadal development (Vested et al. 2014), and inducing vitellogenin synthesis in males (Vetillard and Bailhache 2006).

Unlike organic pollutants, metals do not suffer degradation or rapid elimination from the ecosystem, causing organisms exposed to these contaminants to accumulate them through the gills or by ingestion through the food chain (Merciai et al. 2015). When ingested, metals bind to the body's molecules such as water, proteins, and enzymes, forming stable biotoxic compounds and inactivating biomolecules (Duruibe et al. 2007). These biochemical changes, whether severe or prolonged, can cause structural changes (Van Dyk et al. 2009) and, thus, cause harmful reproductive effects (James 2011).

Yancheva et al. (2016) state that one of the methodologies that have a direct connection with physiological functions, such as reproduction, is histology. Greenfield et al. (2008) affirm that histological changes in target tissues are sensitive biomarkers for the effects caused by exposure to xenobiotics. These changes occur before phenotypic ones and provide a more in-depth assessment of the effects of water pollution on communities, evaluating the incidence and prevalence of abnormalities in target tissues of exposed organisms. Yancheva et al. (2016) also state that histological changes are a reflection of the health of the entire population within the ecosystem studied. Due to the Doce River disaster, it is important to study a representative population of the affected ecosystem so we can evaluate the changes occasioned by it; therefore, the species population chosen to represent the ecosystem in this study was *Astyanax lacustris*.

Astyanax lacustris is native to the Doce River and has an early sexual maturity (maturation in 0.7-1.0 years and generations about 18 months old). In addition to its ecological importance, *A. lacustris* is indicated as a sentinel species for environmental investigations concerning aquatic contamination and experimental tests in the laboratory (Prado et al. 2011), as they have numerous offspring, short generations and are easy to reproduce and manage (Silva and Porto-Foresti 2020). *Astyanax lacustris* does not migrate for reproduction; it can reproduce in both lotic and lentic environments and has a long reproductive period with multiple peaks of active reproduction (Godinho et al. 2010, Weber et al. 2012).

The populations of *A. lacustris* from the Doce River have been continuously exposed to metals throughout their life cycle, due to the environmental disaster that occurred after the Mariana dam collapsed in 2015 (Passos et al., 2020). With the hypothesis that the enrichment of metals in water can negatively influence the reproduction of fish (James 2011), this work aimed to evaluate the reproductive biology of *A. lacustris* in the lower Doce River and to verify the deleterious histological effects of a population chronically exposed to tailings from the dam rupture in November 2015.

3.2. METHODOLOGY

3.2.1. Sampling area

The Doce River Basin is divided into three physiographic regions. Both the upper and middle Doce River are located in Minas Gerais, while the lower Doce River is located in Espírito Santo, and it is responsible for approximately one-third of the state's water volume (Moretto 2001). Fish and water were sampled in the lower Doce River, downstream of the Mascarenhas Hydroelectric Power Plant (UHE), Baixo Guandu, Espírito Santo State (19°30'04.8 "S 40°53'23.2" W), close to the mouth of the Mutum Preto stream. The climate presents pluviometric seasonality with greater rainfall occurring between October and March, with variations from 50 to 300 mm and an annual total of 1019 mm (Silva et al. 2010, ANA 2020). In addition, Sales et al. (2018) and ANA (2020) determined that the largest drought occurs from April to September, with an average rainfall of less than 50 mm per month. The data obtained at the National Water Agency (ANA 2020) come from a pluviometric station (station code: 01941003) located along the course of the river in the municipality of Baixo Guandu.

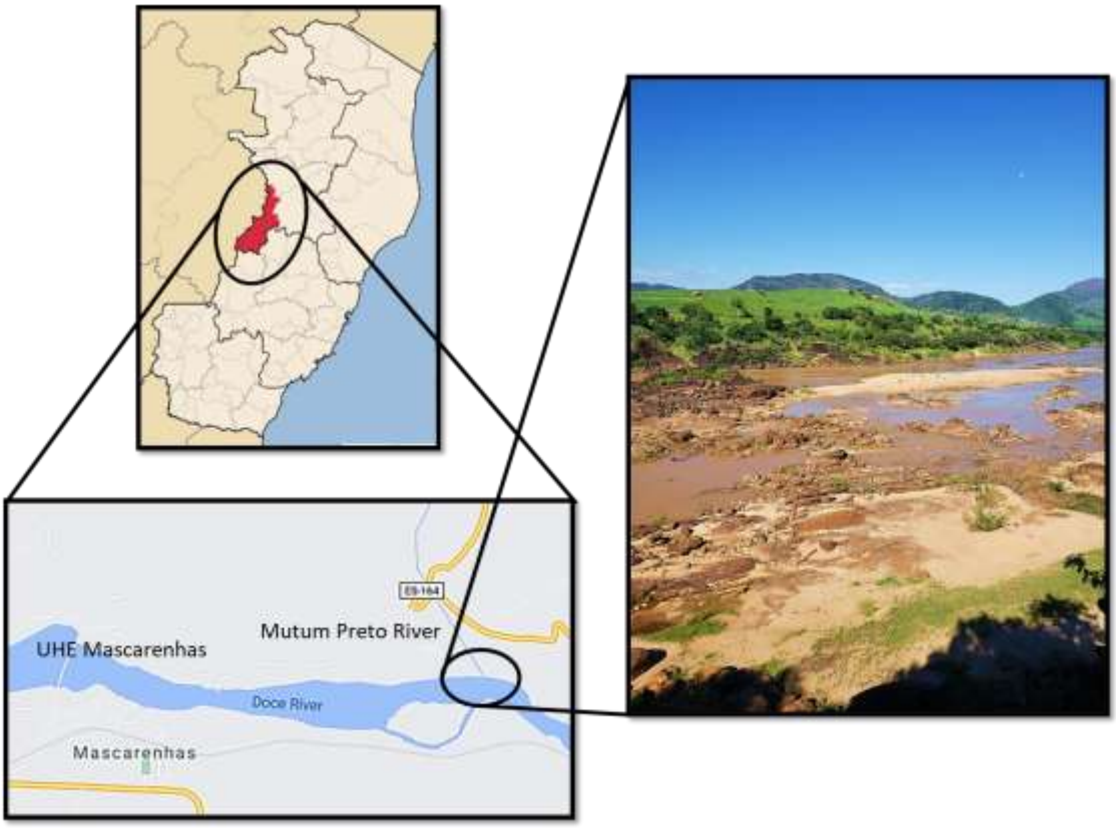


Fig 2 Sample area.

3.2.2. Fish sampling and biometric measurements

The project was carried out with the approval of the animal ethics committee (CEUA/UVV # 563-2018). Monthly samplings of *A. lacustris* were carried out for one year, from June 2018 to May 2019. The fish sampling was performed with a 12mm mesh ring net. Between 10 and 20 individuals were sampled monthly (224 individuals at the end of the study) (Table 1). The results of fish sampling are showed bimonthly.

Table 1. Number of mature, immature and unidentified individuals of *A. lacustris* exposed to the Doce River mining tailings.

Bimesters	Male			Female			UI
	Mature	Immature	Total	Mature	Immature	Total	
Jan-Feb	14	9	23	2	5	7	1
Mar-Apr	5	10	15	12	10	22	0
May-Jun	3	13	16	7	15	22	2
Jul-Aug	9	12	21	5	13	18	7

Sep-Oct	2	10	12	19	9	28	0
Nov-Dec	12	6	18	9	3	12	0
Total	45	60	105	54	55	109	10

The fish were anesthetized with Benzocaine solution (0.2 $g.L^{-1}$) and, afterward, euthanized by cervical section. For all specimens, sexing was performed based on the presence of spikes in the anal fin of the males and the shape of the body (Dos Santos et al. 2020), with the counterproof being made with the analysis of the gonads. The sex ratio of the population was assessed using the Chi-square test (X_2) with $p < 0.05$; furthermore, measurements of total length (TL), body weight (BW), and gonad weight (GW) were taken. Afterward, the following biological indices were calculated: gonadosomatic index ($GSI = GW \times 100/BW$) and Fulton's condition factor ($K = BW \times 100/(TL^3)$). Then, the gonads were removed; a part of them was immersed in glutaraldehyde 0.5%, where they were kept until histological analysis (Venturoti et al. 2019a, 2019b); the other part was frozen at -80ºC for later analysis of metals.**Water samplings and analysis**

Monthly water samplings of Doce River (collection site) were carried out in triplicate for physicochemical and metal analysis. The following physicochemical parameters of the water were measured: dissolved oxygen (DO), temperature, conductivity, and pH with the Horiba U53 multiparameter (Tokyo, Japan); and hardness and alkalinity were measured by titration, according to APHA (2005). For metal analysis, the collected water was preserved in nitric acid ($pH < 2$) for later analysis. Total Al, Cr, Pb, Mn, and Fe were analyzed encompassing the metals with toxic potential and evidenced in previous analyses of the Doce River's water and sediment according to Gomes et al. (2017) and Macêdo et al. (2020).

3.2.4. Study of gonads

Presence and absence of gonads

The probability of considering *A. lacustris* individuals capable of reproduction, at a certain body length, was obtained through gonads' presence (visible gonads) or absence (invisible gonads) (Blain and Sutton 2016). The collected fish were separated by size categories, and the number of mature and immature individuals in each category was described. Subsequently, the frequency of individuals of each sex with mature gonads was calculated.

The body length estimated at the first maturation (L_{50}) represents the body length at which 50% of the fish are in reproductive capacity. The following formula was used to obtain the percentage of growth that the population needs to move from X% of mature individuals to 50% of them: $Growth\,percentage\,(\%) = (L50 - LX) * (100/maximum\ length\ of\ the\ individuals)$. For males, we used X = 30% and for females, X = 40%, since the smallest individuals collected presented 30% and 40% of the population already sexually matured for males and females, respectively.

Histological analysis of gonadal maturation

For histological analysis, the gonads were prepared according to Venturoti et al. (2019a, b). Afterward, the stages of gonadal development were classified according to Prado et al. (2011) using a microscope (Leica Galen III model). The stages description of the gonadal development is detailed in the supplementary material (Table 2).

Table 2. Microscopic characteristics of the gonadal maturation stages of *A. lacustris* organisms exposed to Rio Doce mining tailings.

	Males	Females
Resting (1)	Seminiferous tubules with only sperm and occluded lumen.	Presence of early and advanced perinuclear follicles and oogonia nests.
Maturation (2)	Seminiferous tubules with sperm cell cysts and lumen full	Presence of perinuclear follicles, pre-vitellogenic

	of sperm and acidophilic secretion.	follicles, and vitellogenic follicles.
Parcial spawning (3)	Presence of few sperm cell cysts and partially empty tubular lumen.	Presence of perinuclear follicles, pre-vitellogenic follicles, vitellogenic follicles, and post-ovulatory follicles.
Total spawning (4)	Seminiferous tubules containing spermatogonia and empty tubular lumen with only a few remnants of sperm.	Presence of perinuclear follicles and post-ovulatory follicles.

Histological damage to the gonad

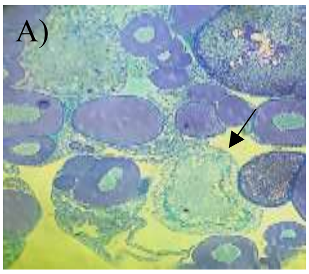
For both sexes, 8 gonad sections from each individual were read microscopically. A qualitative histological analysis was performed using a microscope. After this procedure, the results were transformed in a semi-quantitative way using a protocol as said by Agbohessi et al. (2015). Also, according to Agbohessi et al. (2015), the score and the importance factor for each alteration identified were multiplied and then added to obtain the gonad damage index of each individual. As stated by Bernet et al. (1999) and Agbohessi et al. (2015), the formula used to calculate these indices was: $\mathrm{I}_{org} = \sum_{rp} \sum_{alt} (\alpha_{org\ rp\ alt} \times \omega_{org\ rp\ alt})$, where org = organ (constant), rp = reaction pattern, alt = alteration, α = score, and ω = importance factor.

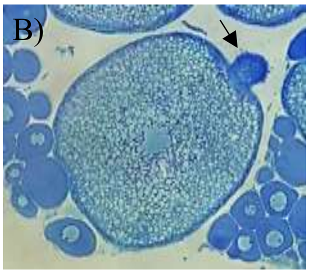
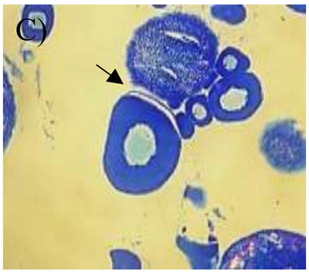
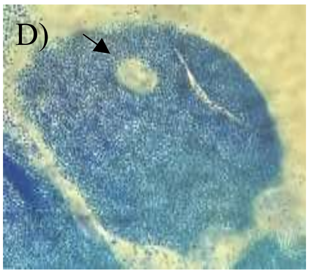
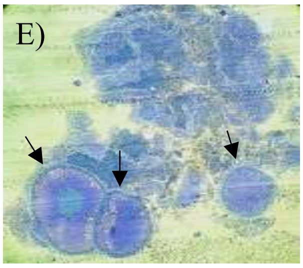
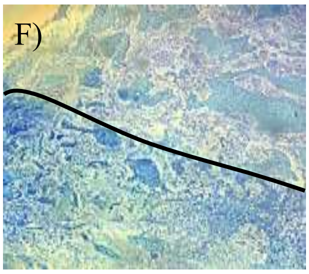
For females, the histological analysis and damage index determination were also carried out quantitatively. For this, a reading of all cells and all cuts was made. The numerical data obtained were later transformed into a percentage of damage (Prado et al. 2011).

These indices were used to compare the severity of each histological alteration found in the collected fish. To classify the severity of the damage index in the testicles (It) and the damage index in the ovaries (Io), the results were evaluated based on a classification system provided by Agbohessi et al. (2015): Class 1 (index <10): tissue with normal structure and few histological changes; Class 2 (index 10 - 20): tissue with normal structure and moderate histological changes; Class 3 (index 20 - 30): clear changes in organ tissue; Class 4 (index> 30): severe changes in organ tissue. In order to determine the severity index of each individual, the changes described in Table 3 were accounted for.

In addition to the indices calculated by score and pathological importance of the lesions, another point of interest was used for histopathological characteristics (Agbohessi et al. 2015). The prevalence of each change was calculated according to the percentage of occurrence among the collected fish. The formula used was: $Prevalence\ of\ histological\ changes\ =\ (Number\ of\ fish\ with\ the\ change\ /$ $Total\ number\ of\ fish)\ \times\ 100$ (Agbohessi et al. 2015).

Table 3. List of histological changes detected microscopically in the gonads of *Astyanax lacustris* exposed to mining waste from Rio Doce. Magnifications: (A, B, C, and D) 20x, (E and F) 10x.

Alteration		Description
	Females	
Atresia		Agglomeration or perforation of the radiated area, fragmentation of the nucleus, disorganization of the ooplasm, and reabsorption of the yolk by the perifollicular cells

Hyperplasia		Increase in the number of cells in the epithelium involved in follicular development
Basal membrane detachment		When the cytoplasm recedes and detaches from the epithelium involved in follicular development
	Male	
Immature cells invading lumen		It occurs when cells of primary development, such as spermatogonia, invade the lumen, staying between the spermatozoa
Intersex		Presence of one or more oogenic cells (individual or clustered), between the testicular cells; however, they are perinuclear, cortical alveoli, vitellogenic or attractive. There is no evidence of ovarian architecture
Desynchronized growth		Presence of more than one stage of sperm cell development in a single gonad cut

3.2.5. Metal analysis

The concentrations of metals in water and gonads were measured. For digestion of the gonads, ultrapure water, ultrapure nitric acid, and hydrogen peroxide (5:2:1), and a microwave energy digester (Ethos UP from Millestone) were used. We followed the protocol of the device for digestion: 30min at 200°C and maximum power of 1000W. After being digested, the samples were quantified for Al, Cr, Pb, Mn, and Fe in a graphite oven, in the Atomic Absorption spectrophotometer (Thermo ICE3500). The quality assurance and quality control (QA/QC) tests of the analyses were carried out to monitor and control the reliability of the analysis methodology. For each evaluated metal, a calibration curve was prepared and for Fe and Mn, a certified reference material (ERM® - BB422) was used (Table 4). The bioconcentration factor of each metal was also calculated, relating the concentration of metals in the water and the gonad of each fish, using the following formula: $BCF = Cg/Cw$, where Cg is the concentration of the metal in the gonad of each organism and Cw is the metal concentration in the collected water (Nenciu et al. 2016). According to the Registration, Evaluation, Authorization, and Restriction of Chemicals, regulation of the European Parliament (REACH # 1907/2006), a metal fulfills the bioaccumulation criterion when BCF > 2000. As for the United States Environmental Protection Agency (USEPA), a metal is considered a trigger for potential bioaccumulative effects when BCF > 1000. When BCF > 5000, the metal is considered highly bioaccumulative for both REACH and USEPA.

Table 4. Percentage of recovery of analytes present in the certified reference material (ERM-BB422), limits of quantification and detection practicable (mg.kg^{-1} wet weight), limits of detection (LD), and limit of quantification (LQ) of analytes.

Metal	Certified values (mg/kg)	Measured values (mg/kg)	Recovery (%)	LD (µg/L)	LQ (µg/L)	R^2
Al	-	-	-	40.473	122.65	0.99
Cr	-	-	-	10.945	33.168	0.99
Mn	0.37	0.44	118	6.730	20.396	0.99
Fe	9.4	9.9	105	44.035	133.44	0.99

Pb	-	-	-	0.420	1.274	0.99

3.2.6. Statistical Analysis

The results of metal concentration and the water quality parameters were grouped every two months and presented as mean and standard error. The results of metal concentration in the gonad were also grouped every two months and presented as mean and standard deviation. The concentration of the different metals in the gonads was compared – between sexes and bimonthly– by a two-way ANOVA ($p < 0.05$), followed by a Tukey test ($p < 0.05$). The values were logarithmic (Log 10) in order to normalize the results. The GSI and FC were compared between bimesters by ANOVA ($p <0.05$), followed by a Tukey test ($p < 0.05$).

To determine the correlation between the histological damage index and quantification of metals in the gonad, a Pearson correlation was performed ($p < 0.05$).

3.3. RESULTS

3.3.1. Physicochemical and metal analysis in water

The results of the water physicochemical parameters and the concentrations of Fe, Mn, Al, Cr, and Pb in water are described in Table 5. The water quality parameters did not present differences along the bimesters ($p < 0.05$ for all parameters). The dissolved oxygen average was 7.71 ± 0.34 $mg.L^{-1}$, with a higher concentration in May and June. Temperature had an average of 27.09 ± 1.54 ºC, with higher values in January and February, and, lower, in July and August. We obtained the same results for conductivity and alkalinity, with higher results in May and June (101.00 ± 0.008 and 80.9 ± 3.75 $mg.L^{-1}$, respectively). The average pH was 6.59 ± 0.29 and the average hardness was 44.21 ± 1.26 $mg.L^{-1}$, with higher values in Jan-Feb and Jul-Aug, respectively. Concerning metals, the ones with the highest concentration were Al and Fe, with averages of 543.78 ± 125.58 $\mu g.L^{-1}$ and 491.59 ± 97.36 $\mu g.L^{-1}$, respectively. Al was found in greater concentration in January and February, with 986.83 ± 237.08 $\mu g.L^{-1}$; and Fe was found in greater concentration in November and December, with 900.88 ± 131.78 $\mu g.L^{-1}$, both in the period of greatest rainfall.

Table 5. Annual physicochemical parameters and concentration of metals quantified in the Doce River. The data are presented as mean and standard error. Lower-case letters indicate a significant difference between bimesters.

Parameters	Bimesters					
	Jan-Feb	Mar-Apr	May-Jun	Jul-Aug	Sep-Oct	Nov-Dec
Dissolved oxygen ($mg.L^{-1}$)	7.71 ± 0.68 [a]	7.40 ± 0.23 [a]	8.90 ± 0.15 [a]	7.74 ± 0.15 [a]	7.73 ± 0.83 [a]	6.77 ± 0.03 [a]
Temperature (°C)	30.81 ± 1.27 [a]	27.06 ± 2.43 [a]	27.04 ± 1.97 [a]	24.46 ± 0.14 [a]	25.64 ± 0.95 [a]	27.51 ± 2.48 [a]
pH	7.41 ± 0.15 [a]	6.55 ± 0.41 [a]	7.37 ± 0.02 [a]	6.93 ± 0.09 [a]	6.06 ± 0.97 [a]	5.21 ± 0.11 [a]
Conductivity ($\mu s.cm^{-1}$)	60.00 ± 0.02 [a]	65.00 ± 0.010 [a]	101.00 ± 0.008 [a]	54.00 ± 0.002 [a]	57.00 ± 0.001 [a]	61.00 ± 0.001 [a]
Hardness ($mg.L^{-1}$)	35.48 ± 2.95 [a]	53.68 ± 0.53 [a]	36.67 ± 0.38 [a]	65.56 ± 0.50 [a]	34.49 ± 1.45 [a]	39.38 ± 1.78 [a]
Alkalinity ($mg.L^{-1}$)	39.59 ± 1.51 [a]	53.32 ± 5.35 [a]	80.9 ± 3.75 [a]	43.4 ± 5.62 [a]	46.00 ± 6.85 [a]	36.35 ± 3.22 [a]
Al ($\mu g.L^{-1}$)	986.83 ± 237.08 [a]	840.26 ± 202.77 [a]	284.15 ± 43.32 [b]	204.13 ± 57.02 [b]	289.70 ± 65.59 [b]	657.63 ± 147.69 [ab]
Cr ($\mu g.L^{-1}$)	0.42 ± 0.08 [c]	0.83 ± 0.12 [b]	5.86 ± 0.11 [a]	3.58 ± 0.85 [a]	0.24 ± 0.48 [c]	0.76 ± 0.05 [b]
Fe ($\mu g.L^{-1}$)	724.80 ± 164.01 [ab]	685.32 ± 101.35 [ab]	308.18 ± 92.52 [bc]	155.07 ± 46.09 [c]	175.32 ± 48.40 [c]	900.88 ± 131.78 [a]
Mn ($\mu g.L^{-1}$)	75.82 ± 18.38 [a]	64.70 ± 8.43 [ab]	29.33 ± 16.05 [ab]	22.42 ± 7.97 [b]	23.83 ± 8.47 [ab]	76.18 ± 17.37 [a]
Pb ($\mu g.L^{-1}$)	0.002 ± 0.001 [a]	0.0002 ± 0.0001 [a]	0.0012 ± 0.001 [a]	0.00001 ± 0.0001 [a]	0.0001 ± 0.00001 [a]	0.01 ± 0.001 [a]

3.3.2. Biological indices and sexual maturation

A total of 105 males and 109 females were collected, and no significant differences were found in the sex ratio of the population (X_2 = 0.075; gl = 1; p = 0.78). The average male weight was 9.14 ± 3.81 g, and the average length was 8.46 ± 1.44 cm. The average weight of the females was 11.89 ± 6.44 g, and the average length was 9.27 ± 1.75 cm.

Males obtained 30% gonadal maturation with 5 cm in length and 50% gonadal maturation with 11 cm in length. Between obtaining 30% of matured organisms and 50% of matured organisms, male fish had to grow 35.29% in relation to their maximum size. Females obtained 40% of gonadal maturation at 7 cm in length and 50% of gonadal maturation at 11 cm in length. Thus, between 30% and 50% of matured organisms, females needed to grow 22.22% (Table 6).

Table 6. Sexual maturation of *Astyanax lacustris* exposed to the Doce River mining tailings in different length classes (cm). M = frequency of mature individuals in each length.

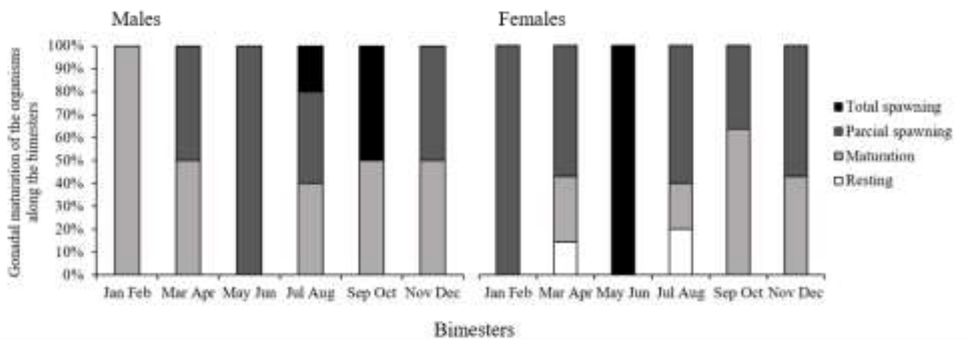
Size (cm)	Male				Female			
	n	Immature	Mature	M (%)	n	Immature	Mature	M (%)
5-6	3	2	1	33.3	0	-	-	-
6.1-7	8	5	3	37.5	0	-	-	-
7.1-8	34	22	12	35.3	33	20	13	39.9
8.1-9	28	17	11	39.3	26	15	11	42.3
9.1-10	19	8	11	57.9	22	9	13	59.1
10.1-11	6	3	3	50	15	7	8	53.3
11.1-12	2	1	1	50	8	3	5	62.5
12.1-13	0	-	-	-	3	0	3	100
13.1-14	0	-	-	-	2	0	2	100

14.1-15	0	-	-	-	1	1	0	0.0
15.1-16	0	-	-	-	0	-	-	-
16.1-17	1	0	1	100	0	-	-	-
17.1-18	0	-	-	-	1	0	1	100

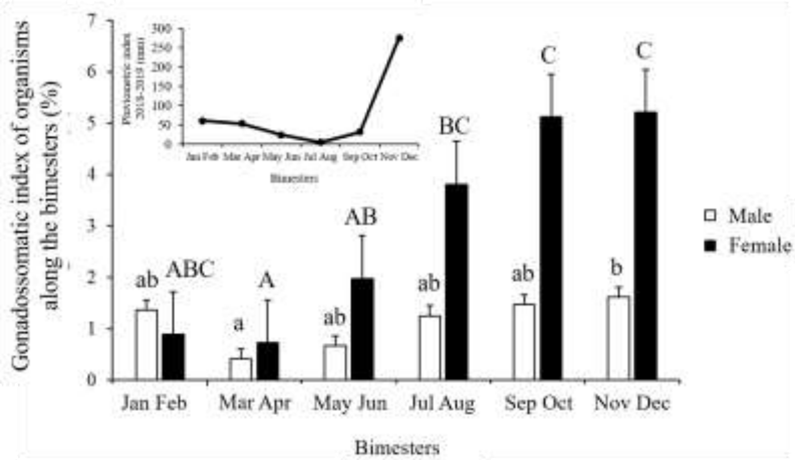
Males had a lower number of gonads at peak maturation (stage 2) in May-Jun and Jul-Aug, with 0% and 40%, respectively. Jan-Feb showed 100% of gonads at peak maturation, being considered the main months of male reproduction. Females had a higher reproduction rate in Sep-Oct and Nov-Dec, with a greater number of gonads at peak maturation (65% and 45%, respectively), being Sep-Oct considered the main months of female reproduction (Figure 3a).

The GSI of males and females were proportional over the months. Both had a GSI peak in Sep-Oct and lower levels of GSI in Mar-Apr. The GSI of the males showed a significant difference only between Mar-Apr and Nov-Dec ($p = 0.02$). For females, there was a significant difference when comparing the months of Nov-Dec with Mar-Apr ($p < 0.001$) and with May-Jun ($p = 0.003$). Sep-Oct also showed a significant difference when compared to Mar-Apr ($p < 0.001$) and May-Jun ($p = 0.002$) (Figure 3b). The Fulton condition factor (K), determinant of body condition or wellbeing of the organism, remained constant in both sexes, with K ranging from 1.3 to 1.6 for males and from 1.2 to 1.6 for females (Figure 3c).

a)



b)



c)

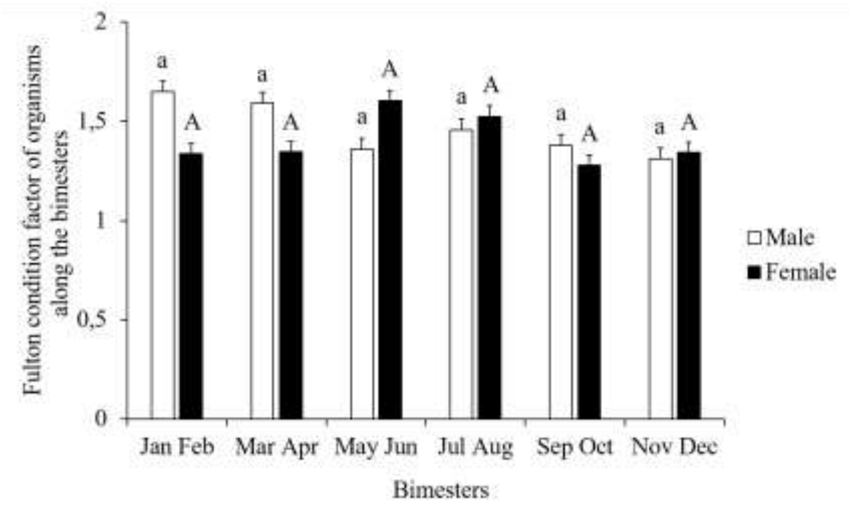


Fig 3 Annual distribution of a) relative frequency of different stages of ovarian and testicular maturation; b) gonadosomatic index and c) Fulton condition factor of *A. lacustris* exposed to the Doce River mining tailings. Lower-case letters indicate a

significant difference between bimonths in relation to males and upper-case letters indicate a significant difference between bimonths and females. Values are represented in mean and standard error

3.3.3. Concentration of metals in the gonads

Both males and females showed higher concentrations of Al and Fe in their gonads, in relation to Cr, Mn, and Pb (Figure 4). Concerning Al, Cr, Fe, and Pb, males bioaccumulated significantly higher concentrations than females. When comparing the concentrations between sexes only Mn showed no significant difference in Sep-Oct. When comparing metal concentrations between bimesters in males and females, separately, Cr and Pb showed no significant difference. Al showed a significant difference for both sexes between May-Jun and Sep-Oct, with $p = 0.006$ for males and $p = 0.016$, for females. Fe and Mn presented a difference only in relation to males between Jul-Aug and Sep-Oct ($p = 0.034$) and Nov-Dec compared to May-Jun and Jul-Aug ($p = 0.044$ and $p = 0.032$), respectively.

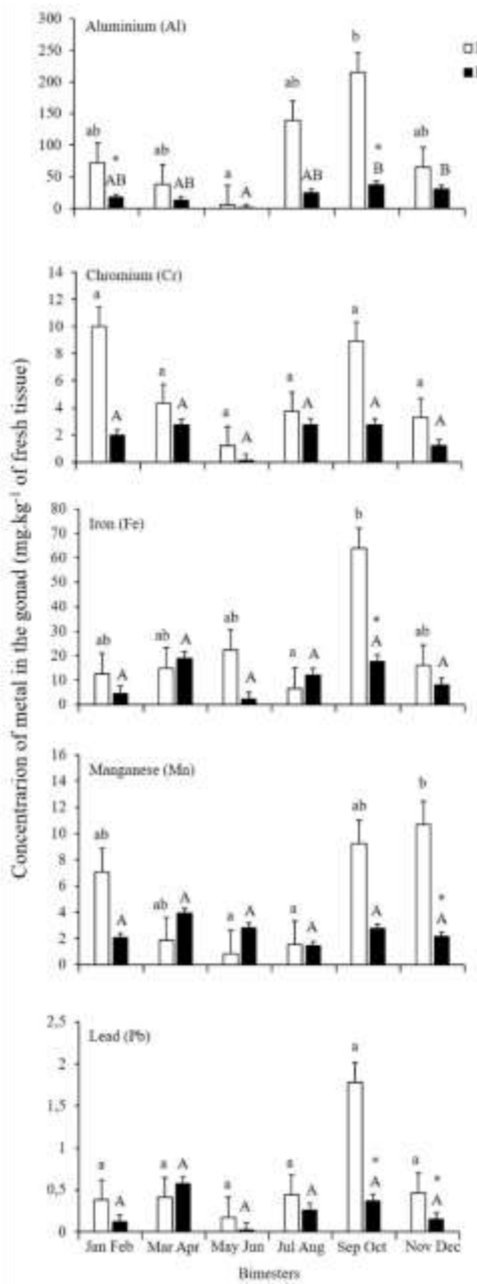


Fig 4 Concentration of metals in the gonads of individuals from *Astyanax lacustris* exposed to mining waste from the Doce River. Values are represented in mean and standard error. Capital letters represent a significant difference in the bioaccumulation of metals in males in relation to periods. The lower-case letters represent a significant difference in the bioaccumulation of metals in females in relation to the periods. Asterisks (*) represent a significant difference in the bioaccumulation of metals between males and females

Al presented a BCF with potential bioaccumulative effects by REACH and TSCA only in Jul-Aug (BCF = 2,167.68). On the other hand, Cr had potentially bioaccumulative BCF in all months, except for May-Jun, with BCF = 120. Jan-Feb months showed highly bioaccumulative effects, with BCF = 10,027.23.

Table 7. Bioconcentration factor (BCF) of *Astyanax lacustris* exposed to the mining tailings from the Doce River water along the bimesters (-) indicates that the formula could not be applied due to water values < LQ.

Sex	Bioconcentration factor				
	Al	Cr	Fe	Mn	Pb
Jan-Feb					
Male	59	10027	12	141	192
Female	14	1956	2	41	111
Mar-Apr					
Male	39	4343	16	62	-
Female	223	5529	205	396	-
May-Jun					
Male	25	120	89	85	-
Female	3	13	9	281	-
Jul-Aug					
Male	2168	3665	424	51	441
Female	655	261	100	48	256
Sep-Out					
Male	743	8907	375	462	-
Female	193	3457	104	137	-
Nov-Dec					
Male	119	3306	14	773	463
Female	70	1220	6	71	868

3.3.4. Histological damage to the gonads

Six types of changes were identified in the fish collected in the Doce River. In males, the greatest amount of damage was found in Jul-Aug. The invasion of immature cells had the highest prevalence among the collected fish. The invasion of immature cells and the detection of desynchronized maturation showed higher prevalence in the months of Jan-Feb and May-Jun (100% both). As for the detection of intersex, we found it present in Jul-Aug and Nov-Dec, with 20% and 16.66% prevalence, respectively, in the collected organisms.

In females, atresia had the highest prevalence among the collected fish, with only Mar-Apr and May-Jun showing a prevalence under 100%. Hyperplasia and membrane detachment had 100% prevalence in Jan-Feb and no prevalence in the months of May-Jun (Table 8).

Table 8. Prevalence (%) of the histopathological changes observed in the *Astyanax lacustris* gonads exposed to the Doce River mining tailings. The importance factor is indicated in parentheses in each change. DC = deleterious changes; IS = Intersex

Organs	Reaction pattern	Alteration	Jan Feb	Mar Apr	May Jun	Jul Aug	Sep Oct	Nov Dec
Testes	DC	Immature cells invading the lumen (2)	100	75	100	100	50	83.3
	DC	Desynchronized growth (3)	100	75	100	20	100	33.3
	IS	Intersex (3)	0	0	0	20	0	16.7
Ovarian	DC	Atresia (3)	100	85.7	0	100	100	100
	DC	Hyperplasia (2)	100	85.7	0	60	90.9	100
	DC	Membrane detachment (1)	100	42.9	0	20	54.5	85.7

On semi-quantitative analyses, both sexes obtained, on average, moderate levels (class 2) of histological damage (Table 8). Pearson correlation ($p < 0.05$), for semi-quantitative analysis, did not present a significant correlation between histological damage and metals in the gonad (Table 9). The average rate of damage to the female gonads on quantitative analysis was 23.41, thus classifying the female gonads as Class 3 of histological damage. Pearson correlation between metals in the gonad and quantitative damage analysis, showed a positive correlation for Al and Fe ($p = 0.04$, CP = 0.37 and $p = 0.03$, CP = 0.41, respectively) (Table 9).

Table 9. Histological damage index (qualitative and quantitative), class and Pearson's correlation (CP) of *Astyanax lacustris* organisms exposed to the Doce River mining tailings. The data are presented as mean and standard error. Significant values are shown in bold.

Sex	Index	Class	N
Qualitative analysis			
Male	16 ± 1.68	2	21
Female	17.24 ± 1.45	2	28
Quantitative analysis			
Female	23.41 ± 2.75	3	28

Pearson correlation ($p < 0.05$)					
Qualitative analysis					
	Al	Cr	Fe	Mn	Pb
Male	CP = 0.09	CP = -0.04	CP = 0.12	CP = 0.14	CP = -0.09
	p = 0.68	p = 0.84	p = 0.68	p = 0.55	p = 0.71
Female	CP = -0.003	CP = 0.15	CP = 0.02	CP = -0.10	CP = 0.20
	p = 0.98	p = 0.42	p = 0.90	p = 0.57	p = 0.27

Pearson correlation ($p < 0.05$)					
Quantitative analysis					
	Al	Cr	**Fe**	Mn	Pb
Female	**CP = 0.37**	CP = 0.08	**CP = 0.41**	CP = -0.12	CP = -0.001
	p = 0.04	p = 0.88	**p = 0.03**	p = 0.81	p = 1.00

3.4. DISCUSSION

3.4.1. Physicochemical and metal analysis in water

Previous studies, such as the one by Escobar (2015) and Gomes et al. (2019), reported that, due to anthropological activities on the banks, the Doce River was already polluted before the disaster. Despite this, Macêdo et al. (2020) found that the metals with the highest concentrations in the Doce River's water 32 months after the disaster were Al and Fe, corroborating the results obtained in the present study. Even four years after the disaster, the results show that Al remains above the maximum limits allowed by Brazilian law (National Environmental Council, Resolution 357/2005) and by USEPA (1998). The results obtained in the analysis of the gonads corroborated, partially, the ones obtained in the water analysis, with Al being the metal with the highest concentration.

3.4.2. Biological indices and sexual maturation

According to Vicentini and Araújo (2003), the sex ratio between individuals is basic information for the reproductive potential, providing important data about the dynamics and population structure of a species. Súarez et al. (2017), studying *A. lacustris* in the Pantanal, Brazil, reported higher proportions of females and reported that the imbalance in the proportion of males and females is unfavorable for natural selection in the environment. Contrary to the findings by Súarez et al. (2017) with *A. lacustris*, the present study revealed a balanced proportion between males and females in the population of the lower Doce River, which is a beneficial factor for the reproduction of this population; furthermore, the onset of sexual maturity is also an important component in the population dynamics of fish (Súarez et al. 2017). When analyzing the data obtained in the present study, we found a latency in gonad maturation when compared to the individuals of other populations of *A. lacustris* (Table 10).

Table 10. Necessary proportional growth (CN in %) for males and females of different species of the *Astyanax* genus from 30-40% of mature individuals (L30 for males and L40 for females) to 50% of mature individuals (L50). TM = maximum population size

Species	Local	Male				Female				Reference
		TM (cm)	L30 (cm)	L50 (cm)	CN (%)	TM (cm)	L40 (cm)	L50 (cm)	CN (%)	
A. lacustris	Doce River, Brazil	17	5	11	35.29	18	7	11	22.22	Present study
A. lacustris	Paraguai River, Brazil	7	2.1	3.1	14.28	7	2.8	3.1	4.28	Súarez et al. (2017)
A. intermedius	E. M. Atlântica Park, Brazil	9	3.6	3.9	3.33	9	4.3	4.4	1.11	Souza et al. (2015)
A. bifasciatus	Iguaçu River, Brazil	12	2.9	3.6	5.83	12	4	4.6	5	Oliveira et al. (2019)
A. henseli	Dos Sinos River, Brazil	14	6.5	6.9	2.86	14	5.5	6	3.57	Dala-Corte e Azevedo (2010)

Studying a smaller population of *A. lacustris* (maximum size = 7 cm for males and females), Súarez et al (2017) reported male individuals with 30% gonadal maturation at 2.1 cm in length and 50% at 3.1 cm in length. The difference between 30 and 50% of matured individuals was only 1 cm (growth = 14.28%). On the other hand, the males in the present study needed to grow 6 cm (35.29% of the maximum size), so that 30% of individuals with gonads passed to 50%. Regarding females, the population of *A. lacustris* in the Pantanal increased 0.3 cm (4.28% of the maximum size) to pass from 40% of individuals with gonads to 50% (Súarez et al., 2017). In the present study, *A. lacustris* females needed to grow 22.22% to increase from 40% of individuals with gonads to 50%. Only one female was collected with a length between 14 and 15 cm, and it did not have evident gonads. Studies carried out by Souza et al (2015), Dala-Corte and Azevedo (2010) and Oliveira et al (2019) with species of the genus *Astyanax* observed a much lower need for proportional growth (1.11-5%). This latency in the formation of gonads is a strong indication of a disruption in the reproductive biology of the population of *A. lacustris* from the lower Doce River and deserves to be the target of future investigations. Previous studies have reported that chronic exposure to metals, such as Al, can cause a delay in the sexual maturation of fish, as well as increased spermatogonia and spermatocytes and a significant decrease in spermatids and spermatozoa (Paschoalini et al., 2019). Another factor observed is that *A. lacustris* individuals from the lower Doce River showed a size greater than 60% of the maximum size for 50% of the population to have gonads (male = 64% and female = 61%). The population of *A. lacustris* of the Paraguay River has 50% of individuals with gonads with 44.28% of the maximum size, for both males and females.

Species of the *Astyanax* genus usually have a seasonal breeding strategy, which increases in the rainy season, between spring and summer (Dala-Corte and Azevedo 2010). The present study corroborates this affirmation since the reproduction peak found was in Sep-Oct for females and Jan-Feb for males, during the rainy season in the region. Despite the higher percentage of gonads with reproductive capacity in the rainy season, the other periods also showed matured gonads in a lower percentage, a fact that indicates multiple reproduction. Souza et al. (2015) suggest that this fragmented reproduction strategy may be associated with the unpredictable nature of the reproduction region's conditions, a reality observed at UHE Mascarenhas due to the high volume of water released.

3.4.3. Concentration of metals in the gonads

Through a study done with *Barbus grypus*, *Barbus sharpeyi* and *Cyprinus carpio*, Alhashemi et al. (2012) concluded that sex is an important factor that can interfere with the bioaccumulation of metals in fish. *C. carpio* males showed greater bioaccumulation of metals when compared to females, corroborating the results obtained in the present study.

According to Passos et al. (2020), the lower Doce River is a region that has frequent resuspension events during the rainfall season (October to March). Due to the high amount of rain, the water flow released by UHE Mascarenhas also increases (in the dry season, the water flow is 183.82 $m^3.s^{-1}$ and in the rainy season, it increases to 387.25 $m^3.s^{-1}$ (ANA, 2018)). Besides that, it is known that the UHE Mascarenhas has a periodic opening of its floodgates twice a day, according to ANA 2018. Those events can make elements, such as metals previously sedimented, available again in the water column. This corroborates the result found in the present study, which showed a higher concentration of Al and Fe in the gonads during the rainfall months. In freshwater fish, Al is known to affect their reproduction, due to a directly proportional reduction in vitellogenesis (Hwang et al. 2000). In addition, acidified water, a result of high concentrations of Al (Hwang et al. 2000 and Correia et al. 2010), is known to impair fish reproduction by affecting fertility, egg viability, spawning success, gonadal development, and production of gametes (Correia et al. 2010). Passos et al. (2020) state that, in addition to Al, Fe also causes deleterious effects in fish exposed to the metal, which can lead to increased mortality and histopathological changes in liver cells. When compared to the results of the present study, it is concluded that, in addition to histopathologies in liver cells, Fe may also be responsible for histopathologies in gonadal cells.

Although Al and Fe showed higher concentrations in the gonads of the organisms, Cr presented a greater bioaccumulative effect in relation to other metals when BCF was calculated. Despite being an essential metal, Bakshi and Panigrahi (2018) revealed that Cr can affect the behavioral, histological, biochemical, genetic, and immunological conditions of organisms, leading to changes in the fish exposed to it.

3.4.4. Histological damage to the gonads

The release of immature cells into the lumen is indicative of a deficiency in spermatogenesis and, possibly, testicular functionality (Agbohessi et al 2015; Da Cuña et al 2011). In the present study was also observed the release of immature cells into the lumen in the testicles of *A. lacustris* in greater quantity (47.36%) when compared to other histological damages found. Intersex was also observed, and despite being found in a smaller quantity, it is of great concern in the reproductive biology of fish since it is considered a signature of exposure to xenobiotics and it can directly impact the development and reproduction of offspring (Prado et al. 2011). Although the genotype of intersex fish had not been determined, the macro and microscopic aspects of their gonads (Prado et al. 2011) indicated that the individuals were male.

Atresia is a degenerative and resorption process that occurs both naturally and under conditions of environmental contamination (Weber et al. 2003). Studies with *Danio rerio*, and *Chalcalburnus tarichi* (Kaptaner and Ünal 2011; Luzio et al. 2016) found a significant number of atretic oocytes when fish were exposed to xenobiotics, corroborating the present study and highlighting that exposure to xenobiotics can delay the development of oocytes, inducing them to death and potentially reducing the individual's reproductive success.

Cardoso et al. (2018) performed an experiment with *Danio rerio* females in order to confirm the applicability of the semi-quantitative method of counting in female gonads. and concluded that, if the objective was to obtain a general classification of maturation or some differences between treatments, the semi-quantitative method is considered appropriate; however, if the objective of the work is to explore in detail the structural components of the gonads (such as primary, cortical, vitellogenic, and previtellogenic oocytes), the recommended method is the quantitative one. The results of the present study are in agreement with this statement since the stages of gonadal maturation of males and females were successfully determined by the semi-quantitative method; nonetheless, when a more detailed analysis of female gonads was made using the quantitative method, the correlation between histological damage and the concentration Al and Fe was significantly positive. Moreover, when the results were classified into levels of histological damage, the semi-quantitative method classified females classified as Class 2 of histological damage with moderate changes, and the quantitative methodology classified females as Class 3 of histological damage with clear alterations in the tissue of the organ. Thus, it is concluded that the method of quantitative determination of histological damage in female gonads from *A. lacustris* is more recommended than the semi-quantitative method.

3.5. CONCLUSIONS

The environmental disaster caused by the rupture of the Fundão dam raised the concentration of metals, such as Al and Fe, in the waters of the affected region, a scenario that persists until today, as observed in the present study and previous ones. The population of *A. lacustris* from the lower Doce River shows obvious deleterious effects on the gonads, namely: 1) latency formation; 2) high concentrations of Al and Fe; and 3) clear histological alterations, such as the presence of atresia, hyperplasia, and the invasion of immature cells into the lumen. In females, there is a positive correlation between the concentration of Al in the gonads and the histological damage index estimated by the quantitative method. Our results allow us to predict that the permanence of high concentrations of metals (ex: Cr, Fe, and Al) in the water of the lower Doce River will continue to subject the population of *A. lacustris* to harmful effects on the gonads. This long-term scenario may further compromise the reproductive success of this population and, consequently, of other species that are at a higher level in the trophic chain.

DECLARATIONS

Ethics approval

The project was carried out with the approval of the animal ethics committee (CEUA/UVV # 563-2018).

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JM contributed to the study conceptualization, the formal analysis, the investigation, methodology, project administration, supervision, visualization and to both writings – original draft and review & editing; The investigation and the Project administration were also performed by DSC and BCT; TMP, AB, CV and SN performed the investigation and LCG contributed with all roles, except for the investigation and the writing of original draft.

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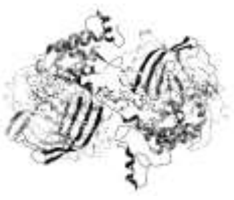
4. CAPÍTULO II – Efeito da sazonalidade nas alterações bioquímicas em *Astyanax lacustris* (Teleostei: Characiformes) do Rio Doce após o colapso da barragem de Fundão, em Mariana, Brasil



Seasonality effects on the metal accumulation and biochemical changes in *Astyanax lacustris* (Teleostei: Characiformes) from Doce River after the collapse of the Fundão dam in Mariana, Brazil

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Abstract

On November 5, 2015, the Fundão dam, located in Mariana, Minas Gerais state, Brazil, collapsed, releasing more than 50 million cubic meters of iron ore tailings mud enriched in metals into the Doce River. This study aimed to evaluate the deleterious effects of the metal accumulation on *Astyanax lacustris* organisms and their biochemical response when exposed to the metals contained in the mining tailings released in the Doce River. The study was carried out in the Baixo Guandu region of the Doce River, Espírito Santo state, Brazil. Samples were collected during the dry and wet seasons in the region. Biochemical alterations were observed in the liver of *A. lacustris*, with clear seasonal effects directly related to the high concentration of Al and Fe in this tissue. Despite this, the bioaccumulation process of metals in the organisms showed seasonal effects only in the gills, as they are the first organ in contact with the contaminated water. The data

generated in this study provide an overview of the health of the ecosystem in this region, highlighting the harmful biochemical and metal accumulation effects on the population of *A. lacustris* affected by the Fundão dam failure.

Keywords: Lambari; contamination; metal; bioconcentration; enzymes.

4.1. INTRODUCTION

On November 5, 2015, the Fundão dam, in the municipality of Mariana, Minas Gerais state, Brazil, suffered a breach and released more than 50 million cubic meters of mining tailings mud into the Doce River (Gomes et al., 2019). According to Bowker (2015), this was the biggest environmental disaster involving mining tailings dams in the world since 1915. The released mud was carried for about 600 km and reached the mouth of the Doce River, located in Regência, in the municipality of Linhares, Espírito Santo state (Davila et al., 2020). The mining tailings mud caused serious environmental impacts, harming the health of the ecosystem and the riverside human populations, also triggering massive mortality of the aquatic biota, the burial of benthic assemblages, and the chronic contamination of the surrounding ecosystems (Gomes et al., 2017).

According to Gomes et al. (2017), the composition of metals in the mining tailings mud released into the Doce River was approximately 57.2% Iron (Fe), 1.3% Aluminum (Al), and trace metals - such as Chromium (Cr), Lead (Pb), and Manganese (Mn) (Hatje et al., 2017). Because the degradation of non-essential metals is slow, exposed organisms accumulate these contaminants throughout their life cycle and may even display higher concentrations in their bodies than those present in the environmental water (Rajeshkumar and Li, 2018). However, each organ plays a different role in the metabolism of metals. The liver, for example, is the organ most commonly affected by contaminants owing to its high blood irrigation and its detoxification role. The gills, on the other hand, are transitional organs with direct contact with the aquatic environment due to their respiratory functions (Passos et al., 2020). Therefore, pollutants are rarely equally distributed throughout all tissues (Merçon et al., 2019).

Exposure to metals can cause an increase in the production of reactive oxygen species (ROS) that cause oxidative stress, which occurs when contaminants modify the balance between antioxidant defenses and ROS (Kim and Kang, 2015). This, in turn, can result in oxidative damage to biomolecules and altered aerobic and energetic metabolism (Jijie et al., 2020). The organisms' defense response triggered by the ROS radicals, produced due to the exposure to metals, is to activate enzymes that intercept and inactivate ROS, such as Catalase (CAT) and Glutathione-S-Transferase (GST) (Davies, 1995). CAT, a phase I enzyme, works by breaking down H_2O_2 into water and oxygen. GST, a phase II biotransformation enzyme that acts in the detoxification of environmental pollutants (Masella et al., 2005), catalyzes the conjugation of reduced glutathione (GSH) with exogenous and endogenous toxic compounds, making them more soluble in water, less toxic, and facilitating their excretion (Lee et al., 2006).

The flow of the Doce River is regulated by a tropical climate and a subequatorial rainfall regime, with peak flow during the wet season (averaging 1,296 m^3/s) and a declining flow throughout the dry season (averaging 525 m^3/s). The accumulated mean annual precipitation varies along the watershed, ranging from 900 mm in the most arid region to 1500 mm in the mountainous region (ANA, 2016). Furthermore, there is high interannual variability in the precipitation regime modulated by events such as the South Atlantic Convergence Zone, which promotes persistent rainy periods over the Doce River basin during the summer (Rudorff et al., 2018).

Here, we tested the hypothesis that populations exposed to the mining tailings released in the Doce River due to the Mariana disaster have a variable response to the metals modulated by the dry and wet seasons. Therefore, the objective of this work was to analyze the metal concentrations and enzymatic alterations in the tissues of *A. lacustris* collected both during the dry and wet seasons.

4.2. METHODOLOGY

4.2.1. Sampling and biometric measurements

The sampling was carried out in the lower Doce River within the area of influence of the Mascarenhas Hydroelectric Power Plant (HPP), Baixo Guandu, ES, Brazil (19°30'04.8”S 40°53'23.2”W) (Figure 2). The plant has a 3.9-km² reservoir and an approximated volume of 21,800,000 m³, being considered the largest hydroelectric plant in the Escelsa system (IGAM, 2010). The climate presents rainfall seasonality, with greater rainfall between the months of October and March, displaying monthly variations ranging from 40 to 133mm and an annual total of 1019mm (Silva et al. 2010). Furthermore, Sales et al. (2018) determined that the driest period is between April and September, with an average monthly rainfall of less than 55 mm.

The project was carried out after approval by the animal ethics committee (CEUA/UVV # 563-2018). Monthly samplings of *A. lacustris* specimens were carried out throughout a one-year period (September – December 2018, January – August 2019) using a ring caster with a 12-mm mesh. A total of 115 individuals were collected: 56 individuals were collected during the Dry season, being 26 males (8.47 ± 0.21 g and 8.87 ± 0.21 cm) and 30 females (9.23 ± 0.24 g and 11.54 ± 0.24cm). A total of 59 individuals were collected during the Wet season, with 28 males (8.23 ± 0.18 g and 8.16 ± 0.18 cm) and 31 females (9.79 ± 0.41 g and 13.44 ± 0.41 cm).

The fish specimens were anesthetized with a Benzocaine solution (0.2 $g.L^{-1}$) and promptly euthanized by cervical section. The sex of individuals was determined through the analysis of the gonads after euthanasia. The liver, gills, and muscle were collected after euthanasia and stored in a -80 °C freezer until further metal and biochemical analyses.

4.2.2. Water sampling and analysis

Additionally, water samples were collected monthly at the same site for physicochemical and metal analyses (n = three per month). The following physicochemical parameters were measured: dissolved oxygen (DO), temperature, and pH using the multiparameter probe Horiba (U53, Tokyo, Japan), while hardness and alkalinity were measured by titration, according to APHA guidelines (2005).

The collected water was preserved in nitric acid ($pH < 2$) until further metal analysis. The metals Al, Cr, Pb, Mn, and Fe were analyzed for corresponding to the five most prevalent metals found in previous analyses of the Doce River water, according to Gomes et al. (2017).

4.2.3. Metals analysis

The concentration of metals in water and tissues were measured. Tissue samples were digested using a solution of ultrapure water, nitric acid, and hydrogen peroxide (5:2:1) in a microwave energy digester (Ethos UP from Millestone). The standard manufacturer protocol for digestion was followed: 30 min at 200 °C and the maximum power of 1000 W. After being digested, the samples were quantified for Al, Cr, Pb, Mn, and Fe in a graphite oven of an atomic absorption spectrophotometer (Thermo ICE3500). Quality assurance and quality control (QA/QC) of the analyses were assessed to monitor and control the reliability of the methodology. A calibration curve was prepared for the quantification of each metal evaluated but Fe and Mn, which used a certified reference material (ERM ® - BB422) (Table 1).

The bioconcentration factor (BCF) of each metal, which links the concentration of metals in water with that in the tissues for each individual, was calculated using the formula $BCF=C_t/C_a$, where C_t is the concentration of a given metal in the tissue and C_a is the concentration of the same metal in the sampled water (Nenciu et al., 2016).

According to the Registration, Evaluation, Authorization, and Restriction of Chemicals, a regulation published by the European Parliament (REACH # 1907/2006), a metal falls within the bioconcentration criteria when the BCF is greater than 2000. For the USEPA (United States Environmental Protection Agency), a metal is considered as a stimulus for potential bioconcentration effects when BCF > 1000. When BCF > 5000, both regulations considered that the metal has a high bioconcentration potential.

Table 1. Percentage of recovery of analytes present in the certified reference material (ERM-BB422), practical limits of quantification (LQ) and detection (LD) ($mg.kg^{-1}$ wet weight), and limits of quantification and detection of the analytes.

Metal	Certified values (mg/kg)	Obtained values (mg/kg)	Recovery (%)	LD (mg/L)	LQ (mg/L)	R^2
Al	-	-	-	0.03	0.09	0.99
Cr	-	-	-	0.01	0.03	0.99
Mn	0.37	0.40	108	0.01	0.03	0.99
Fe	9.4	5.7	61.4	0.05	0.15	0.99
Pb	-	-	-	0.00003	0.0001	0.99

4.2.4. Biochemical analysis

The gill, muscle, and liver tissues were homogenized with phosphate buffer (pH 7.0) and centrifuged at 3030g for 30 min at 4 °C to obtain the supernatant for the evaluation of CAT, GST, and total proteins.

Catalase (CAT)

The method of Aebi (1984) was employed in the analysis of CAT, using the Enzymatic Reading Buffer (TE Buffer) at pH 8.0. The analysis was performed in a spectrophotometer measuring the absorbance at 240 nm for 1 minute. The formula used to calculate final values was Absorption Variance/Protein Value in mg*0.071 (CAT correction factor).

Glutathione-S-transferase (GST)

GST was evaluated according to the method of Habig and Jacob (1981). A phosphate buffer (pH 7.0), 1mM of 1-chloro-2,4-dinitrobenzene (CDNB), and 1mM of GSH were used to determine the GST activity. Its activity was estimated from the absorbance reading in a microplate reader (Molecular Device-Spectra Max 190) at 340 nm. The absolute activity was achieved using the CDNB extinction coefficient as recommended by Habig and Jacob (1981).

Total proteins

The protein content was determined according to the Bradford method (1976). Total protein concentration in the tissues was determined by absorbance in a microplate reader (Molecular Device – Spectra Max 190) at 595 nm. To determine the total proteins, a standard curve with concentrations ranging from 6.25 to 800 mg and intervals of 2x, was prepared. Total protein analyses were required for the final calculation of enzymatic activities.

4.2.5. Statistical analyses

The metal concentration and water quality results were grouped by season (Dry and Wet) and presented as the mean and standard error. To verify if the difference between seasons was significant, the results were logarithmized and tested with the T-Test ($p < 0.05$).

The results of the metal concentration in tissues were grouped by season (Dry, April to September; Wet, October to March) and sex (male or female), and presented as median, maximum, and minimum values. The results were analyzed for normality using the

Shapiro-Wilk test but did not display normal distribution even after logarithm transformation. An ANOVA for non-parametric data was performed, with the Wilcoxon command, which provides p values between variables using the Kruskal-Wallis test ($p < 0.05$).

The correlation between the variables (metal concentration in the tissue and enzymatic activity) was assessed by the Spearman correlation test, which classifies and applies the data in the Pearson formula ($p < 0.05$). All statistical analyses were performed in the SAS Institute Inc© 2021 software.

4.3. RESULTS

4.3.1. Analysis of water: concentration of metals and physicochemical parameters

The results of physicochemical parameters and the concentration of Fe, Mn, Al, Cr, and Pb in water are described in Table 2. Among the water parameters observed, the dissolved oxygen, pH, alkalinity, and conductivity showed no significant difference between seasons ($p > 0.05$). The temperature, hardness, and average rainfall in the region displayed significantly higher averages in the Rainy season. The technical data of the operation of the UHE Mascarenhas showed that water flow and its velocity, both controlled by the opening of the floodgates, and the reservoir quota did not present a significant difference between the two seasons.

Regarding the concentration of the metals, only Pb showed a significant difference between seasons ($p < 0.0001$). Al, Cr, Fe, and Mn did not show significant differences. The concentration of metals in water throughout the sampling period showed the following order: Dry (Al > Fe > Mn > Pb > Cr) and Wet (Fe > Al > Mn > Pb > Cr). The Cr, in both seasons, presented values below the quantification limit (< LQ).

Table 2: Pysicochemical parameters and metal concentration for dry and wet seasons in the Doce River. Data are presented as mean and standard error. < LQ = below the limit of quantification. Asterisks (*) represent a significant difference between seasons as assessed by the T-Test ($p < 0.05$).

Parameters	Season	
	Dry	Wet
[a] Rainfall (mm)	25.50 ± 8.73	88.82 ± 19.21 *
[a] Water flow ($m^3.s^{-1}$)	183.82 ± 37.76	387.25 ± 90.86
[a] Water Speed ($m.s^{-1}$)	0.51 ± 0.06	0.68 ± 0.04
[a] Quota (cm)	107.0 ± 8.15	147.0 ± 16.20
Dissolved Oxygen ($mg.L^{-1}$)	8.3 ± 0.5	7.2 ± 0.2
Temperature (°C)	25.3 ± 0.7	28.8 ± 1.0 *
pH	7.0 ± 0.2	6.3 ± 0.5
Conductivity ($\mu s.cm^{-1}$)	70.0 ± 0.01	70.5 ± 0.01
Hardness ($mg.L^{-1}$)	50.4 ± 9.2	38.7 ± 3.6 *
Alkalinity ($mg.L^{-1}$)	55.6 ± 11.1	43.9 ± 4.0
Al ($mg.L^{-1}$)	0.30 ± 0.14	0.7 ± 0.1
Cr ($mg.L^{-1}$)	< LQ	< LQ
Fe ($mg.L^{-1}$)	0.25 ± 0.09	0.8 ± 0.1
Mn ($mg.L^{-1}$)	0.02 ± 0.008	0.1 ± 0.02
Pb ($mg.L^{-1}$)	0.0001 ± 0.00001	0.001 ± 0.0005 *

[a] Values obtained from the National Water Agency (ANA) (2018)

4.3.2. Analysis of the concentration of metals in tissues

The results of the analysis of metals in the tissues of the collected fish are shown in Table 3. Overall, males showed higher bioconcentration than females. Males collected in the Wet season showed higher Al bioconcentration in the gills and liver than females collected in the same season ($p = 0.023$ and $p = 0.033$, respectively). Likewise, the liver showed higher Fe bioconcentration in males than females collected both during the Dry and Wet seasons ($p = 0.0003$ and $p = 0.025$, respectively).

During the Dry season, the muscle of females and males showed significantly lower bioconcentration of Al, Cr, and Pb than the other analyzed tissues ($p < 0.0001$, $p = 0.003$, and $p = 0.002$, respectively). On the other hand, the liver of males collected in the Dry season showed a significantly higher Fe concentration than other tissues ($p < 0.0001$). Similarly, the liver of males and females collected in the Wet season showed higher concentrations of Cr ($p < 0.0001$), Fe ($p = 0.025$), and Pb ($p < 0.0001$) than other tissues. As in the Dry period, the muscle of males and females collected in the Wet showed significantly lower Al bioconcentration than the other analyzed tissues ($p = 0.049$).

In general, the females' gills showed higher bioconcentrations of Al ($p = 0.0002$), Cr ($p = 0.011$), Mn ($p = 0.025$), and Pb ($p = 0.008$) in the Dry season than in the Wet, as well as the muscle of males when analyzing the concentration of Cr in the tissue ($p = 0.019$). The bioconcentration of Al and Fe in the males' liver and gills, respectively, were the only significant differences, with higher values in the Wet season ($p = 0.049$ and $p = 0.011$, respectively).

Although Al and Fe have shown higher concentrations in the tissues of the organisms, Pb showed higher BCF in all tissues for both sexes during the Dry season, followed by Fe in the liver of both sexes in the same season. During the Wet season, Mn had the highest BCF in the gills and muscles, followed by Pb. On the other hand, Fe was the metal with the highest BCF in the liver of both sexes in the Wet season, also followed by Pb. According to REACH and USEPA, only the metals observed in tissues collected during the Dry season showed bioconcentration potential. Pb showed high bioconcentration potential, with values above 5000 in the gills of males and females, and the liver of males. Fe presented bioconcentration potential, according to REACH, in male livers, while Mn presented bioconcentration potential in male and female gills according to USEPA (Table 4).

Table 3. The concentration of metals in tissues of males and females of *A. lacustris* collected in Doce River during Dry and Wet seasons. The results are described with median, minimum, and maximum values. The asterisks (*) denote a significant difference between seasons. The lowercase letters represent significant differences found between the tissues in the Dry season. The capital letters represent significant differences found between the tissues in the Wet season. Double asterisks (**) represent a significant difference between males and females.

Metals (mg/kg)		Season					
		Dry			Wet		
		Gill	Liver	Muscle	Gill	Liver	Muscle
Male							
Al	Median	14.14 ^{a}	6.71 ^{a}	1.04 ^{b}	11.14 ^{A}	23.73 ^{A*}	2.34 ^{B}
	Min - Max	1.08 - 123.46	0.51 - 185.02	0.32 - 12.33	6.00 - 105.30	0.60 - 449.53	0.57 - 4.66
Cr	Median	0.25 ^{a}	0.38 ^{a}	0.14 ^{a}	0.25 ^{A}	0.56 ^{B}	0.04 ^{A*}
	Min - Max	0.13 - 1.46	0.002 - 41.96	0.02 - 0.55	0.004 - 0.42	0.39 - 1.98	0.002 - 0.24
Fe	Median	9.80 ^{a}	425.26 ^{b}	8.71 ^{a}	70.79 ^{A*}	501.52 ^{B}	3.66 ^{C}
	Min - Max	3.37 - 78.50	60.67 - 2224.51	0.50 - 110.11	0.88 - 244.29	29.99 - 1147.05	0.69 - 14.51
Mn	Median	32.56 ^{a}	1.18 ^{b}	2.26 ^{b}	23.81 ^{A}	2.31 ^{B}	1.17 ^{B}
	Min - Max	2.82 - 94.55	0.33 - 78.22	1.11 - 6.14	6.11 - 80.22	0.65 - 185.37	0.03 - 13.12
Pb	Median	0.14 ^{a}	0.39 ^{a}	0.01 ^{B}	0.08 ^{A}	0.21 ^{B}	0.01 ^{C}
	Min - Max	0.01 - 3.81	0.02 - 6.52	0.001 - 0.43	0.03 - 3.52	0.06 - 1.00	0.004 - 0.04

Female							
Al	Median	25.81 ^{a}	7.37 ^{a}	2.17 ^{b}	6.85 *^{A}**	14.08 ^{A}**	1.29 ^{B}
	Min - Max	5.35 - 130.90	0.59 - 192.28	0.34 - 9.88	1.92 - 53.20	2.00 - 104.27	0.39 - 8.01
Cr	Median	0.71 ^{a}	1.16 ^{a}	0.07 ^{b}**	0.13 ^{A}*	1.15 ^{B}	0.07 ^{A}
	Min - Max	0.02 - 4.18	0.14 - 14.51	0.002 - 0.27	0.02 - 0.78	0.06 - 3.34	0.005 - 0.19
Fe	Median	35.70 ^{ab}	55.93 ^{a}**	8.65 ^{b}	22.79 ^{A}	250.83 ^{B}**	3.98 ^{C}
	Min - Max	2.48 - 372.11	2.67 - 459.89	0.27 - 97.84	2.13 - 223.91	11.65 - 833.24	1.33 - 11.44
Mn	Median	31.69 ^{a}	1.53 ^{b}	2.93 ^{b}	24.51 ^{A}*	1.51 ^{B}	0.92 ^{B}*
	Min - Max	10.20 - 79.71	0.56 - 12.14	0.27 - 13.32	6.82 - 69.90	0.02 - 43.24	0.24 - 12.72
Pb	Median	0.28 ^{a}	0.13 ^{a}	0.01 ^{b}	0.05 ^{A}*	0.18 ^{B}	0.01 ^{C}
	Min - Max	0.005 - 6.05	0.07 - 1.17	0.002 - 0.55	0.01 - 3.82	0.03 - 0.71	0.001 - 0.05

Table 4. Bioconcentration factor (BCF) calculated for *A. lacustris* exposed to the mining tailings present in Doce River water during the Dry and Wet seasons. (-) indicates that the formula could not be applied due to metal concentrations in the water below LQ.

Metals	Bioconcentration Factor					
	Dry			Wet		
	Gill	Liver	Muscle	Gill	Liver	Muscle
Male						
Al	98.8	142.5	11.1	29.3	101.2	3.3
Cr	-	-	-	-	-	-
Fe	103.6	**2440.3**	74.5	105.5	656.1	7.6
Mn	**1591.6**	369.1	122.4	399.8	169.2	36.6
Pb	**6647**	**12621.6**	511.4	280.2	333.0	16.2
Female						
Al	143.2	143.1	9.6	15.6	25.9	2.8
Cr	-	-	-	-	-	-
Fe	243.5	572.9	79.1	63.8	364.4	6.6
Mn	**1751.3**	113.9	189.7	349.0	56.5	28.4
Pb	**13184.0**	**2701**	603.2	234	236.9	16.5

4.3.3. Biochemical analysis

The analysis of CAT activity in the tissues of the females collected in the Wet season revealed a significant difference only in the liver, which showed greater enzymatic activity than the other tissues ($p = 0.0003$). During the Dry season, however, the gills of females displayed lower enzymatic activity than those verified in the liver and muscle ($p = 0.024$ and $p = 0.05$, respectively) (Figure 1B). There was no significant difference relative to the CAT enzymatic activity among the analyzed tissues of males between the Dry and Wet seasons (Figure 1A).

The GST enzymatic activity in the tissues of females collected during the Wet season showed a significant difference only between the liver and muscle, with the gills showing an intermediate activity (p = 0.004). Conversely, no significant difference in GST activity was found between the analyzed tissues from females collected during the Dry season (Figure 1D).

For males, the GST activity in the liver of organisms collected during the Wet season showed significantly higher activity than the gills and muscle (p = 0.034 and p = 0.002, respectively). Finally, the analysis of tissues from males collected during the Dry season showed a difference in GST activity only between the gills and muscle, with the liver displaying an intermediate activity (p = 0.046) (Figure 1C).

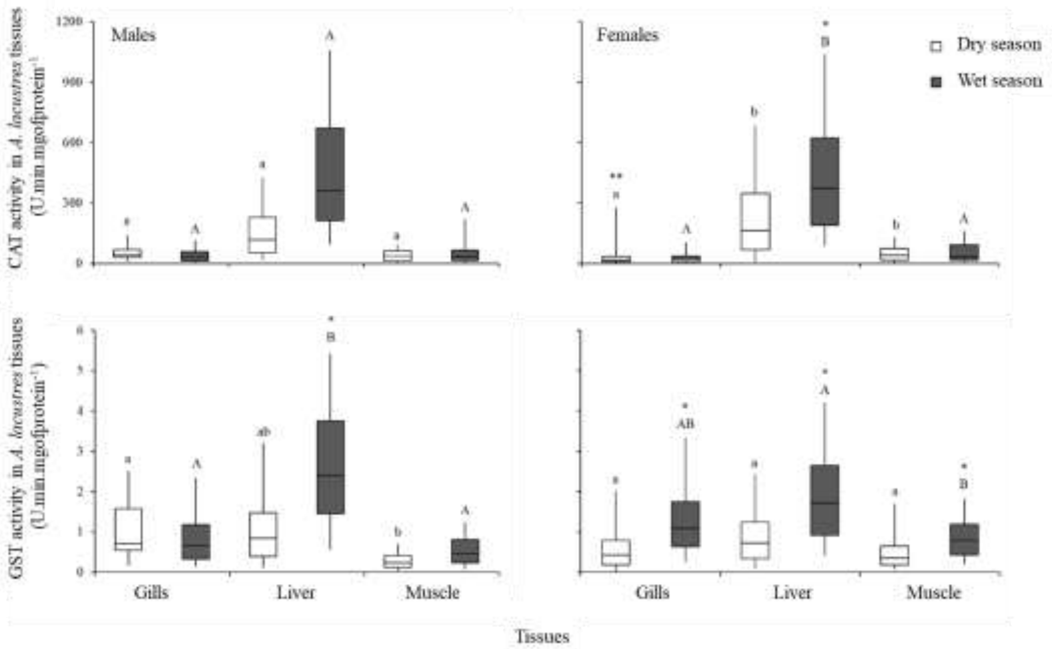

Fig 5 Enzymatic activity in tissues of *A. lacustris* collected in Doce River. A) CAT enzymatic activity in tissues from males; B) CAT enzymatic activity in tissues from females; C) GST activity in tissues from males; D) GST activity in tissues from females. The asterisks (*) denote a significant difference between the Dry and Wet seasons. The double asterisks (**) indicate that a significant difference was found between the sexes. The lowercase letters represent that a significant difference was found between the tissues

during the Dry season. The capital letters represent that a significant difference was found between the tissues during the Wet season.

Spearman correlation results are shown in Table 5. For the Wet season, a positive correlation between the concentration of Al and the activities of CAT and GST was verified in the liver of females (correlation coefficient = 0.900, $p = 0.0002$; and correlation coefficient = 0.854, $p = 0.002$, respectively), while in males such a correlation was only found for the GST activity in the liver (correlation coefficient = 0.900, $p = 0.037$). Regarding Cr, a positive correlation was found only with CAT activity in the females' gills (correlation coefficient = 0.745, $p = 0.013$), while Fe correlated negatively with CAT activity in males (correlation coefficient = -1,000, $p < 0.0001$).

On the other hand, for samples from the Dry season, positive correlations between Al concentration and GST activity in female livers (correlation coefficient = 0.830 and p = 0.003), and CAT activity in male muscle (correlation coefficient of 0.900 and $p = 0.037$) were found. The enzymatic activity of GST in the muscle of females was shown to be positively correlated to the concentration of Cr found in the tissue (correlation coefficient = 0.650 and $p = 0.022$). Males collected during the Dry season showed a strong positive correlation between Fe concentration found in the gills and liver and GST enzymatic activity, with a correlation coefficient of 0.900 ($p = 0.037$) and 0.883 ($p = 0.020$), respectively.

Table 5. Spearman correlation coefficient values, followed by their p values, between the enzymatic activity of CAT and GST and the concentration of the analyzed metals. Correlation values in bold denote $p < 0.05$.

Metals mg/g	Season											
	Dry						Wet					
	Gill		Liver		Muscle		Gill		Liver		Muscle	
	CAT	GST	CAT	GST	CAT	GST	CAT	GST	CAT	GST	CAT	GST
Male												
Al	0.26	0.09	0.62	0.12	**0.90**	0.10	-0.53	-0.02	0.50	**0.90**	-0.16	0.39
	0.62	0.87	0.19	0.82	**0.04**	0.87	0.12	0.96	0.39	**0.04**	0.62	0.21
Cr	-0.40	0.60	0.60	0.87	-0.20	-0.80	----	-----	**1.00**	**-1.00**	-0.10	0.20
	0.60	0.40	0.28	0.05	-0.80	0.20			**<0.0001**	**<0.0001**	0.87	0.75
Fe	-0.20	**0.90**	0.76	**0.88**	0.10	-0.40	-0.27	0.23	**-1.00**	-0.50	-0.16	0.02
	0.75	**0.04**	0.08	**0.02**	0.87	0.50	0.49	0.55	**<0.0001**	0.67	0.62	0.95
Mn	0.03	0.03	0.32	0.32	0.40	-0.60	0.18	0.37	0.70	-0.30	-0.17	0.09
	0.96	0.96	0.68	0.68	0.50	0.28	0.63	0.29	0.19	0.62	0.60	0.78
Pb	0.10	0.20	-0.14	-0.09	**1.00**	**1.00**	-0.60	-0.25	-0.10	-0.80	-0.19	0.40
	0.87	0.75	0.76	0.85	**<0.0001**	**<0.0001**	0.09	0.52	0.87	0.10	0.56	0.20
Female												
Al	-0.11	0.11	0.55	**0.83**	-0.09	0.19	0.33	0.31	**0.90**	**0.85**	0.14	**0.65**

	0.69	0.70	0.10	**0.003**	0.74	0.50	0.35	0.33	**0.0002**	**0.002**	0.70	**0.04**
Cr	-0.12	0.36	0.10	-0.30	0.06	**0.65**	**0.74**	-0.25	0.00	-0.31	0.16	0.09
	0.78	0.38	0.87	0.62	0.85	**0.02**	**0.01**	0.45	1.00	0.46	0.65	-0.80
Fe	0.12	0.17	0.54	0.66	-0.17	0.14	0.07	0.14	-0.02	-0.57	0.24	0.24
	0.68	0.56	0.27	0.16	0.55	0.63	0.86	0.70	0.95	0.18	0.57	0.57
Mn	0.20	0.44	-0.17	-0.26	-0.19	0.11	-0.32	0.53	0.33	0.02	0.05	**0.64**
	0.48	0.11	0.69	0.53	0.49	0.69	0.36	0.07	0.38	0.95	0.88	**0.05**
Pb	-0.24	0.24	-0.03	0.77	0.26	0.14	0.05	0.31	-0.04	-0.08	0.26	0.51
	0.48	0.48	0.96	0.07	0.43	0.67	0.88	0.32	0.91	0.83	0.47	0.13

4.4. DISCUSSION

4.4.1. Analysis of water: concentration of metals and physicochemical parameters

Previous studies have shown an increase in the concentration of metals in the Doce River, mainly Al and Fe, after the disaster of the Fundão dam, in Mariana (Quadra et al., 2019; Macêdo et al., 2020). The results of the present study corroborate such findings. Four years after the collapse of the Fundão dam, the concentration of Al in the water still exceeds the limits established by the Brazilian law (National Council for the Environment - CONAMA, Resolution 357/2005), by the US Environmental Protection Agency (USEPA, 1998), and by the WHO Drinking Water Guidelines (WHO, 2006). On the other hand, Fe does not exceed the limit established by the CONAMA resolution 357/2005. However, the Fe concentration in Doce River in both seasons exceeds the limits described by the WHO (2006). Regarding the USEPA guidelines (1998), Fe exceeds the limit allowed only in the Wet season.

4.4.2. Analysis of the concentration of metals in tissues

The concentration of Cr observed in fish tissues was higher than that detected in water, the latter found to be below the limit of quantification. Cr is an essential metal with significant biological value (La Colla et al., 2017), which may have been the reason for organisms to display higher Cr concentrations in their tissues than the concentration found in the water.

In general, the metal concentration in the liver was higher than in other tissues. This may be associated with its high blood irrigation and its detoxification role against toxic substances (Passos et al., 2020). Conversely, muscle showed significantly lower concentrations than the liver, as it does not have an important role in the biotransformation and bioconcentration of metals (Coppo et al., 2018). However, muscle is an important indicator from the perspective of human health and fish meat consumption (Ali et al., 2018).

It is common to observe a higher concentration of metals in periods of higher rainfall, due to the higher frequency of resuspension events (Silva et al., 2010) that can cause previously sedimented elements to become available in the water column (Passos et al., 2020). However, the present study demonstrates overall similar bioconcentration of metals between both seasons, except in the gills of females, which showed higher bioconcentration in the Dry season. In a comparison made by Pestana et al. (2019) between fish collected downstream and upstream of the hydroelectric dams, it was concluded that with the opening of the hydroelectric dams and a high volume of water being discharged into the river, the exposed fish need more energy to swim in these environments. Kelly et al. (2016) confirm this statement and affirm that the discharge of high volumes of water can cause an increase in the need for fish movement and, consequently, its metabolic demand, such as oxygenation. As previously mentioned, the studied region has a large water flow due to the presence of the Mascarenhas HPP, which holds up to 21,800,000 m^3 of water and works on a cyclic opening operation (twice a day, according to ANA 2018). Thus, it is concluded that fish collected downstream of the dam can be equally exposed to metals available in the water column in both seasons. A possible explanation for the robust support that the results obtained for the gills here give to this reasoning could be that they are the first organ in contact with pollutants present in water, thus being considered an important entry route of metals into organisms (Ali et al., 2019).

The present study showed that males had higher concentrations of Al and Fe than females. In a study by Alhashemi et al. (2012) with the species *Barbus grypus, Barbus sharpeyi*, and *Cyprinus carpio*, females of *Barbus grypus* and *Barbus sharpeyi* bioaccumulate more metal than males. However, the authors observed in that same study that the species *Cyprinus carpio* displayed a higher concentration of metal in males than in females. Given this, it can be inferred that one genus does not necessarily have a greater accumulation of non-essential metals than the other (Bastos et al., 2011; Voigt et al., 2014).

4.4.3. Biochemical analysis

Biotransformation enzymes catalyze the conversion of fat-soluble organic xenobiotics into more easily excretable water-soluble metabolites (Livingstone, 1998). Furthermore, the presence of toxic agents in the aquatic environment can affect the detoxification synthesis of cationic enzymes and increase the concentration of ROS (Sreejai and Jaya 2010). Under normal physiological conditions, the harmful effects of ROS are effectively neutralized by the body's antioxidant defense systems. However, when exposed to excess production of free radicals, the cellular environment is subject to oxidative stress, which can damage cell macromolecules (Biesalski, 2000). According to Jomova et al. (2012), organisms exposed to higher levels of metals, such as Al and Fe, would be more prone to ROS formation. Thus, the enzymatic activity of an organism's tissue is linked to its capacity to capture and accumulate metal.

Each organ has a different function in metal metabolization and, therefore, pollutants are hardly evenly distributed in all tissues (Allen, 1995; Cinier et al., 1999). The results of the present study corroborate this statement, as the enzymatic activity was different between the sampled tissues. The gills, for example, despite being one of the most vital organs for detoxification functions (Goss et al., 1998), showed no significant difference in the enzymatic activities of CAT and GST relative to other tissues, a result that as similarly found for the muscle. This can be associated with a process known as Organotropism. Several studies report that, for most metals, the tissues with the greatest capacity for metal accumulation and detoxification are the liver and kidneys (Pannetier et al. 2016). Within the tissues analyzed in this study, we found support for this statement, as the muscle and the gills were not good oxidative stress indicators.

Although there was no general difference in bioconcentration between seasons, the results showed that the enzymatic activity of CAT and GST in the liver was higher in the Wet season. This may be associated with the strong correlation found between the enzymatic activity of liver samples collected in the Wet season and the concentration of Al in that same tissue. Nunes et al. (2020) associated the liver enzymatic activity of *Sciades herzbergii* with the concentration of metals, such as Al and Fe, and reported a significant increase in CAT activity when associated with Fe. Nunes et al. (2020) concluded that this adaptive biological response to exposure to Fe may be an important factor in preventing irreversible damage to the organism. The enzymatic activity of liver samples collected during the Dry season was also correlated with the concentrations of Al and Fe. However, the enzymatic activity observed in the liver samples was not different from other tissues. This may be associated with excessive production of H_2O_2 radicals due to exposure to metals, especially Fe. Khalil et al. (2020) reported an inhibition of the enzymatic activity of CAT and GST in the liver of *Cyprinus carpio* collected from the Shahpur dam in Pakistan. According to Khalil et al. (2020), the excessive production of H_2O_2 can inhibit both the CAT activity and the GST activity, which, together with the GSH substrate, are rapidly consumed during the electron donation process for the transformation of H_2O_2 into O_2 and H_2O.

4.5. CONCLUSIONS

Previous studies had already established that the concentration of metal in the water of the Doce River increased after the collapse of the dam, mainly Al and Fe. Although the organisms displayed a high concentration of metals in tissues, the influence of seasonality was only observed in the gills, as they are the first organ in contact with the contaminated water. The other analyzed tissues were directly influenced by the volume of water from the Mascarenhas HPP, showing no change in bioconcentration due to seasonality. On the other hand, biochemical changes in the liver showed clear seasonal effects, which were directly related to the high concentration of the main metals found in the tissue, Al and Fe.

Thus, the present study demonstrates that organisms collected during the Dry season showed insufficient biochemical responses to deal with the high production of ROS. Furthermore, it demonstrates a chronic exposure of *A. lacustris* populations to high concentrations of metals, especially Al and Fe, throughout the year.

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