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Jossiele Wesz Leitemperger

PARÂMETROS BIOQUÍMICOS EM GUPPY (*Poecilia vivipara*) E JUNDIÁ (*Rhamdia quelen*) EXPOSTOS AO ZINCO

Santa Maria, RS
2019

Jossiele Wesz Leitemperger

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Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

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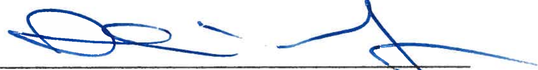

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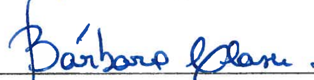

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SANTA MARIA, RS,
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Dedico este trabalho aos meus pais, Jomar e Santa, à minha irmã Jossane, à minha segunda mãe, tia Tereza, ao meu namorado Luis Eduardo, que sempre me apoiaram, me incentivaram, me deram forças e acreditaram em mim.

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RESUMO

PARÂMETROS BIOQUÍMICOS EM GUPPY (*Poecilia vivipara*) E JUNDIÁ (*Rhamdia quelen*) EXPOSTOS AO ZINCO

AUTORA: Jossiele Wesz Leitemperger
ORIENTADORA: Vania Lucia Loro

Atividades antropogênicas, como agricultura, urbanização e mineração, podem aumentar a contaminação do ambiente aquático por metais. O zinco (Zn) é um mineral essencial que está envolvido em muitos processos biológicos. No entanto, este metal pode ter efeitos tóxicos para organismos aquáticos. Sendo assim, o primeiro objetivo deste trabalho foi avaliar a bioacumulação e os possíveis efeitos sobre parâmetros bioquímicos em jundiás (*Rhamdia quelen*) expostos a 0,0; 0,1; 0,25 e 0,5 mg L^{-1} de Zn por 96 h. Como segundo objetivo, avaliou-se os efeitos comportamentais e bioquímicos do guppy (*Poecilia vivipara*) expostos ao Zn (500 µg L^{-1}) em águas com diferente salinidade (25 ppt) ou dureza (120 mg L^{-1} $CaCO_3$). No primeiro experimento, os parâmetros analisados em jundiás foram bioacumulação e a dosagem de substâncias reativas ao ácido tiobarbitúrico medidas através dos níveis de malondialdeído (MDA), proteína carbonil (PC), superóxido dismutase (SOD), catalase (CAT), glutationa S-transferase (GST), tióis não-proteicos (NPSH), ácido ascórbico (AA) em cérebro, brânquias, fígado e músculo. Os níveis de glicogênio, proteína, lactato, amônia e aminoácidos foram medidos em fígado e músculo. A acumulação do Zn foi observada nas brânquias (0,5 mg L^{-1}), intestino e fígado (0,25 e 0,5 mg L^{-1}). Os níveis de MDA aumentaram no cérebro e reduziram no fígado e músculo em todas as concentrações testadas. Houve diminuição da PC em cérebro e aumento dos seus níveis em fígado. A atividade da CAT no fígado foi reduzida em todos os grupos expostos e a atividade da GST diminuiu em cérebro (0,5 mg L^{-1}) e em fígado (0,1; 0,25 e 0,5 mg L^{-1}). Os níveis de NPSH aumentaram no músculo (0,5 mg L^{-1}) e nas brânquias (0,25 e 0,5 mg L^{-1}) e de AA em cérebro (0,5 mg L^{-1}) e brânquias em todas concentrações testadas. A exposição ao Zn também alterou os parâmetros metabólicos, causando diminuição dos níveis de lactato e amônia no músculo, e a diminuição do glicogênio hepático. Houve aumento dos níveis de amônia, aminoácidos e proteína no fígado, e aumento do glicogênio e aminoácidos no tecido muscular. No segundo experimento, os guppies foram divididos em 6 grupos: controle, Zn (500 µg L^{-1}), salinidade (25 ppt), dureza (120 mg L^{-1} $CaCO_3$), salinidade (25 ppt) + Zn (500 µg L^{-1}) e dureza (120 mg L^{-1} $CaCO_3$) + Zn (500 µg L^{-1}). Houve uma diminuição da distância percorrida pelo peixe em água dura e do número de entradas no topo, na dureza e dureza + Zn. A exposição ao Zn, registrou um aumento na velocidade máxima do peixe. As análises bioquímicas foram avaliadas no corpo inteiro do guppy, onde o MDA aumentou, exceto na associação entre a salinidade e o Zn. A PC não foi alterada no grupo salinidade, nos demais grupos, aumentou. O grupo Zn aumentou a quantidade de peróxidos totais, as espécies reativas de oxigênio, a capacidade antioxidante contra radicais peroxil, SOD e NPSH. E diminuiu as atividades da acetilcolinesterase e da Na^+/K^+-ATPase. O índice integrado das respostas dos biomarcadores (IBR) foi calculada para cada parâmetro para auxiliar na interpretação dos resultados e indicou que a água dura contendo Zn teve o maior efeito nos parâmetros bioquímicos dos guppies. Com estes resultados, podemos concluir que o Zn, mesmo em concentrações ambientais relevantes, alterou os parâmetros bioquímicos analisados. Houve acumulação do metal em alguns tecidos de jundiá e alterações em seus parâmetros bioquímicos e metabólicos. O Zn também alterou os parâmetros analisados no guppy, onde nem a salinidade, nem a dureza, foram capazes de proteger o peixe dos danos causados pelo metal.

Palavras-chave: Análise Comportamental. Contaminação Aquática. Guppy. Jundiá. Parâmetros Antioxidantes. Parâmetros Oxidativos. Zinco.

ABSTRACT

BIOCHEMICAL PARAMETERS IN GUPPY (*Poecilia vivipara*) AND SILVER CATFISH (*Rhamdia quelen*) EXPOSED TO ZINC

AUTHOR: Jossiele Wesz Leitemperger
ADVISER: Vania Lucia Loro

Anthropogenic activities such as agriculture, urbanization and mining may increase the contamination of the aquatic environment by metals. Zinc (Zn) is an essential mineral that is involved in many biological processes. However, this metal may have toxic effects on aquatic organisms. Therefore, the first objective of this work was to evaluate the bioaccumulation and the possible effects on biochemical parameters in silver catfish (*Rhamdia quelen*) exposed to 0.0; 0.1; 0.25 and 0.5 mg L^{-1} Zn for 96 h. As a second objective, was evaluated the behavioural and biochemical effects of guppy (*Poecilia vivipara*) exposed to Zn (500 µg L^{-1}) in water with different salinity (25 ppt) or hardness (120 mg L^{-1} $CaCO_3$). In the first experiment, the parameters analyzed in silver catfish were bioaccumulation, determination of thiobarbituric acid reactive substances measured through the malondialdehyde levels (MDA), protein carbonyl (PC), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), non-protein thiols (NPSH), ascorbic acid (AA) in the brain, gill, liver and muscle. The levels of glycogen, protein, lactate, ammonia, and amino acids were measured in liver and muscle. Zn accumulation was observed in gill (0.5 mg L^{-1}), intestine and liver (0.25 and 0.5 mg L^{-1}). MDA levels increased in the brain and decreased in liver and muscle at all concentrations tested. There was a decrease in PC in brain and increase in liver. CAT activity in liver was reduced in all exposed groups and GST activity decreased in brain (0.5 mg L^{-1}) and in liver (0.1, 0.25 and 0.5 mg L^{-1}). NPSH levels increased in muscle (0.5 mg L^{-1}) and in gill (0.25 and 0.5 mg L^{-1}) and AA in brain (0.5 mg L^{-1}) and gill in all concentrations tested. Exposure to Zn also altered metabolic parameters, causing decreased levels of lactate and ammonia in the muscle, and decreased hepatic glycogen. There was increased levels of ammonia, amino acids and protein in the liver, and increased glycogen and amino acids in muscle tissue. In the second experiment, guppies were divided into 6 groups: control, Zn (500 µg L^{-1}), salinity (25 ppt), hardness (120 mg L^{-1} $CaCO_3$), salinity (25 ppt) + Zn (500 µg L^{-1}) and hardness (120 mg L^{-1} $CaCO_3$) + Zn (500 µg L^{-1}). There was a decrease in the distance traveled by fish in hard water and the number of entries into the top region in hardness and hardness + Zn. Exposure to Zn recorded an increase in the maximum speed of the fish. Biochemical analyzes were evaluated in the whole body of the guppy, where MDA increased, except in the association between salinity and Zn. PC was not altered in the salinity group, in the other groups, there was an increased. Zinc group increased the total peroxides, the reactive oxygen species, the antioxidant capacity against peroxyl radicals, SOD and NPSH. And decreased the activities of acetylcholinesterase and Na^+/K^+-ATPase. The integrated biomarker response index (IBR) was calculated for each parameter to aid in the interpretation of the results and indicated that the hard water containing Zn had the greatest effect on the biochemical parameters of the guppies. With these results, we can conclude that Zn, even in relevant environmental concentrations, altered the biochemical parameters analyzed. There was metal accumulation in some tissues of silver catfish and alterations in its biochemical and metabolic parameters. Zinc also altered the parameters analyzed in the guppy, where neither the salinity nor the hardness was able to protect the fish from the damages caused by the metal.

Keywords: Behavioural Analysis. Aquatic Contamination. Guppy. Silver catfish. Antioxidant Parameters. Oxidative Parameters. Zinc.

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1 INTRODUÇÃO

1.1 PROBLEMÁTICA AMBIENTAL DE METAIS

Um dos mais graves problemas ambientais gerados pela intervenção antropogênica sobre o meio natural é a poluição, pois esta prejudica o meio ambiente, inviabiliza o cultivo e o consumo de recursos naturais, provocando desequilíbrios ecológicos e baixando o potencial crescimento dos ecossistemas. No Brasil, o órgão responsável pelas deliberações de toda política nacional do meio ambiente é o CONAMA (Conselho Nacional do Meio Ambiente), criado em 1982 pela Lei 6.938/81. Cabe a este órgão, criar normas e determinar padrões relativos ao controle e à manutenção da qualidade do meio ambiente, com vistas ao uso racional dos recursos ambientais, principalmente os hídricos. O crescimento da populacional, a urbanização, o rápido desenvolvimento econômico e o mau planejamento das áreas urbanas exercem uma crescente pressão sobre os ecossistemas aquáticos, causando vários impactos ambientais, como a liberação de níveis alarmantes de elementos traços no meio ambiente. Algumas fontes naturais contribuem para as concentrações desses metais no ambiente aquático, mas a grande maioria é proveniente de atividades antropogênicas, que aumentam sua mobilização, circulação e liberação (ÁRVAY et al., 2014; AUDRY et al., 2004; CAUSSY et al., 2003; EGGLETON e THOMAS, 2004; GAVRILESCU et al., 2015; NRIAGU e PACYNA, 1988). O aumento da poluição dos oceanos, rios, lagos e reservatórios com produtos químicos orgânicos, agrotóxicos e metais, tornou-se uma preocupação mundial, uma vez que são considerados muito perigosos para invertebrados, peixes e humanos (ULUTURHAN e KUCUKSEZGIN, 2007). A presença de metais em ambientes aquáticos ocorre naturalmente, mas suas concentrações podem aumentar como resultado das descargas de produtos domésticos, efluentes industriais, uso de fertilizantes na agricultura, bem como a queima de combustíveis fósseis (NETO et al., 2008; PAYTAN et al., 2009). Estes metais podem ser fortemente acumulados na água, em sedimentos e em cadeias alimentares aquáticas, resultando em efeitos subletais ou a morte de populações de peixes locais (ALMEIDA et al., 2002; McGEER et al., 2000; XU et al., 2004).

O zinco (Zn) é um metal essencial para o metabolismo dos peixes, obtido a partir da água, alimentos ou sedimentos. Atua como componente estrutural e tem propriedades indispensáveis à vida (EISLER, 1988). Tem importante papel no metabolismo celular, atuando como cofator de uma série de reações enzimáticas. No entanto, a absorção de zinco pode levar a efeitos tóxicos (ATLI e CANLI, 2007; LEITEMPERGER et al., 2016; LORO et al., 2012,

2014). Os peixes também podem bioacumular e biomagnificar grandes quantidades de metais em seus tecidos, já que algumas espécies são geralmente consideradas o topo da cadeia alimentar aquática (MACEDA-VEIGA et al., 2012; MANSOUR e SIDKY, 2002). A bioacumulação é o termo geral que descreve um processo pelo qual substâncias ou compostos químicos são absorvidos pelos organismos ao longo do tempo. O processo pode ocorrer de forma direta, quando as substâncias são assimiladas a partir do meio ambiente (solo, sedimento, água) ou de forma indireta pela ingestão de alimentos que contém essas substâncias. Em ambientes aquáticos, esses processos tendem a ocorrer de forma simultânea. A biomagnificação é um fenômeno que ocorre quando há acúmulo progressivo destas substâncias de um nível trófico para outro, ao longo da cadeia alimentar (Figura 1) (DROUILLARD, 2008; USEPA, 2010; VOUTSAS et al., 2002).

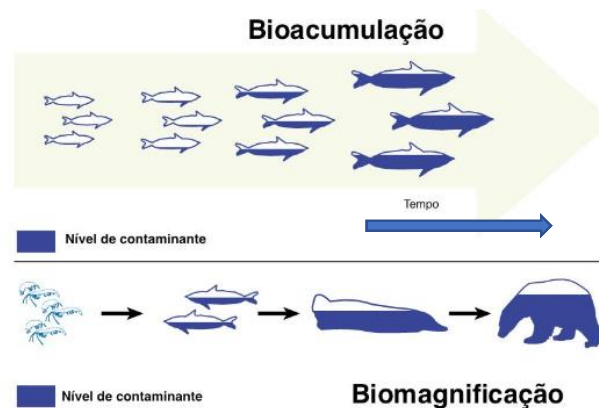

Figura 1. Bioacumulação e biomagnificação na cadeia trófica. (Fonte: Google adaptada pelo autor)

Estudos demostram que o acúmulo de metais nos órgãos dos peixes é dependente da concentração destes na água, do período de exposição e de outros fatores ambientais tais como salinidade, pH, dureza e temperatura (BAYSOY et al., 2012; LORO et al., 2012, 2014; SAGLAM et al., 2013; YI et al., 2011). A salinidade é um dos fatores mais importantes no ecossistema aquático, podendo afetar o crescimento e a sobrevivência dos peixes (LIN et al., 2005), e sua diversidade pode causar uma variedade de respostas fisiológicas ao estresse (CHOI et al., 2008). A alteração da salinidade na água também afeta a disponibilidade do metal e subsequente toxicidade ao competir com íons metálicos pela ligação com as moléculas biológicas (HEATH, 1987).

A dureza pode ser definida como a soma da concentração dos cátions bivalentes, particularmente Ca^{2+} e Mg^{2+} em água. Também é considerada como um dos fatores mais importantes nos sistemas aquáticos que afetam a absorção e excreção de metais. A dureza tem

influência significativa na biodisponibilidade do metal e, portanto, na toxicidade em organismos aquáticos. Estudos mostram que os metais são mais tóxicos em água macia do que em água dura, devido a uma maior disponibilidade (GROSELL et al., 2002; HEATH, 1987; MONSERRAT et al., 2007). Há um consenso de que o principal efeito tóxico é a hipocalcemia, causada pela interferência do Zn^{2+} na captação ativa de cálcio (Ca^{2+}) nas brânquias, ocorrendo um "mimetismo iônico" entre o Zn^{2+} e o Ca^{2+} (BURY et al., 2003). Pode-se prever, assim, que a toxicidade do Zn diminuiria à medida que a salinidade ou dureza aumentasse, especialmente porque a disponibilidade de Zn^{2+} livre diminuiria devido à maior complexidade dos ânions presentes na água do mar.

1.2 ALTERAÇÕES BIOQUÍMICAS CAUSADAS POR METAIS

A exposição a metais em peixes pode alterar o metabolismo de proteínas, carboidratos e parâmetros hematológicos (DE SMET e BLUST, 2001; PRETTO et al., 2014a). Entre as alterações decorrentes da toxicidade destes elementos, as mudanças no metabolismo energético dos organismos podem ser verificadas através da medida dos níveis de glicogênio, glicose e proteína em diferentes órgãos (PRETTO et al., 2014a). O aumento de espécies reativas ao oxigênio (ERO), como peróxido de hidrogênio (H_2O_2), radicais superóxido ($O_2^{\cdot -}$) e radicais hidroxilas (OH), também pode ocorrer após a exposição a metais, levando ao comprometimento do metabolismo oxidativo normal e, finalmente, ao estresse oxidativo (LEITEMPERGER et al., 2016; LORO et al., 2012; LUSHCHAK, 2011). Estes incluem a peroxidação lipídica (LPO), a carbonilação de proteínas, bem como enzimas, que funcionam como mecanismos de defesa, em órgãos de peixes (CAMPANA et al., 2003; FAROMBI et al., 2007; GIODA et al., 2007). O dano aos lipídeos induz o fenômeno conhecido como peroxidação lipídica, que é o resultado da atuação de radicais livres sobre as membranas biológicas, que são ricas em ácidos graxos poli-insaturados. Um dos mais conhecidos produtos da LPO é o malondialdeído (MDA), o qual é produto final da degradação não enzimática de ácidos graxos poli-insaturados e é ensaiado com o ácido tiobarbitúrico e expresso em substâncias reativas ao ácido tiobarbitúrico (TBARS). Altos níveis de MDA elevam a formação de lipoperóxidos e indicam um aumento da LPO (LUSHCHAK e BAGNYUKONA, 2006). As ERO também podem causar prejuízo às proteínas, levando a clivagens das ligações peptídicas, modificações nos resíduos dos aminoácidos, oxidações dos grupos sulfidrila, formação de proteína carbonil (PC), entre outras (LUSHCHAK e BAGNYUKONA, 2006; MÜLLER et al., 2017; SIES et al., 1993). Apesar dos efeitos das ERO, as células possuem mecanismos de defesa para neutralizar os efeitos prejudiciais dos radicais

livres. As enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) são importantes para os mecanismos de proteção celular contra as ERO, assim como a glutationa S-transferase (GST). A SOD é a primeira enzima da linha de defesa, é responsável por catalisar a conversão do ânion superóxido em peróxido de hidrogênio. O peróxido de hidrogênio é degradado em água e oxigênio molecular via CAT e GPx. A GST age em processos de biotransformação e desintoxicação de xenobióticos, transformando compostos tóxicos em formas mais fáceis de serem eliminadas (ABDELAZIM et al., 2018; MÜLLER et al., 2017). Da mesma forma, o sistema de defesa antioxidante não-enzimático atua impedindo reações de auto-oxidação e têm sido vinculados à redução de radicais livres e prevenção de dano oxidativo (MODESTO e MARTINEZ, 2010). Ácido ascórbico (AA) e tióis não-proteicos (NPSH) são importantes no sistema de defesa antioxidante não enzimático, pois protegem os tecidos de peixes contra danos oxidativos ao neutralizar os radicais livres e outros tipos de ERO.

Outro parâmetro utilizado para avaliar a intoxicação por metais é a medida da atividade da enzima acetilcolinesterase (AChE). As colinesterases são enzimas que desempenham importantes papéis na neurotransmissão colinérgica central e periférica, hidrolisando ésteres de colina. Alterações na atividade da AChE em resposta à exposição de peixes a xenobióticos podem afetar a locomoção e o equilíbrio dos mesmos (ARAÚJO et al., 2018; CLASEN et al., 2018; GIODA et al., 2013; LEITEMPERGER et al., 2016). Os mecanismos pelos quais os metais alteram a atividade das colinesterases ainda não são bem conhecidos. Uma das hipóteses é que os metais se ligam aos receptores da acetilcolina nas membranas das células pós-sinápticas, impedindo a ligação da mesma ao receptor. Assim, a acetilcolina se acumularia causando um aumento inicial na expressão da enzima para degradar o excesso do neurotransmissor. Com o tempo, após o efeito prolongado dos metais nos receptores, menos acetilcolina seria produzida, causando uma diminuição progressiva na atividade da enzima que o degrada (BAINY et al., 2006). As diferentes respostas da atividade da AChE podem estar relacionadas com os processos de polarização, despolarização e repolarização das células nervosas causadas por alterações no equilíbrio iônico (MEHTA et al., 2005). As células nervosas conduzem informações umas com as outras através de impulsos elétricos conhecidos como potenciais de ação (POPOVIC e THRASHER, 2004). Na célula, o sódio (Na^+) é o principal ânion extracelular, o potássio (K^+) é o cátion intracelular e o cloro (Cl^-) o principal ânion extracelular (BARNES e GREENEBAUM, 2006). No estado de repouso da célula (polarizada), os canais iônicos permanecem fechados até que ocorra a geração do potencial de ação, provocado por um estímulo. Com a abertura dos canais de Na^+, ocorre a entrada de sódio e a célula torna-se despolarizada, isto é, ativa/excitada. Para haver compensação em função da entrada de alta quantidade de Na^+, ocorre a abertura dos canais

de K^+ e a saída de parte desses íons, tornado a célula repolarizada (NELSON e COX, 2011). As ATPases são enzimas ligadas à membrana responsáveis pelo transporte de íons através destas ajudando na regulação do volume celular, da pressão osmótica, e a permeabilidade da membrana (ATLI e CANLI, 2010; MONSERRAT et al., 2007). O mecanismo chave da toxicidade de metais tem sido relatado como sendo uma disfunção osmorregulatória associado com a inibição da ATPase em brânquias e intestino (Mc GEER e WOOD, 1998). Variações consideráveis (inibição e estimulação) nas atividades da ATPase em peixes expostos a vários metais, incluindo Cd, Cu, Zn e Pb (ATLI e CANLI, 2007). Mudanças nas atividades ou níveis desses parâmetros têm sido utilizados com sucesso como biomarcadores de exposição a contaminantes metálicos (FIRIDIN, 2018; LEITEMPERGER et al., 2016; LORO et al., 2014).

1.3 ÍNDICE INTEGRADO DE RESPOSTAS DE BIOMARCADORES

O índice integrado de respostas biomarcadores (IBR) descrito por Beliaeff e Burgeot (2002) é uma metodologia que integra as respostas de diferentes biomarcadores em um único valor ou gráfico e vem sendo utilizada em estudos de campo e laboratório (MURUSSI et al., 2015; VIEIRA et al., 2016) para facilitar a análise e interpretação dos dados. O resultado de cada análise é primeiramente dividido pelo valor do grupo controle (o grupo controle é dividido por ele mesmo, resultando em valor igual a 1) em seguida, para cada resultado é feito o logaritmo para diminuir a variância. Do resultado do logaritmo (Y_i) é realizado a média (μ) e o desvio padrão (s) de todos os resultados. Então os valores de Y_i são padronizados pela fórmula: $Z_i = (Y_i - \mu)/s$ – e a diferença entre Z_i e Z_0 (controle) é calculada para determinar os valores de A. Os valores de A representam o resultado atribuído para cada biomarcador calculado. O IBR é estimado para cada grupo pela soma dos valores de A resultando em valores de S: $S = A_1+A_2+A_3+...+A_i$. Os resultados são apresentados como gráficos de radar que indica o desvio de todos os biomarcadores em relação ao controle (0). Os valores acima da linha do zero representam a indução dos biomarcadores, enquanto que os valores abaixo de zero representam inibição dos biomarcadores.

1.4 JUNDIÁ E GUPPY COMO ORGANISMOS MODELO PARA ESTUDOS ECOTOXICOLÓGICOS

O jundiá, *Rhamdia quelen*, (Figura 2) é uma espécie de peixe endêmica da parte sul da América do Sul, tem distribuição neotropical sendo encontrado desde o centro da Argentina até o sul do México. No Brasil, está presente na região da Depressão Central do Rio Grande do Sul

(GUEDES, 1980). É uma espécie de grande importância comercial para a pesca, devido seu filé de alta qualidade (CARNEIRO e MIKOS, 2005; BALDISSEROTTO, 2009). É um peixe de água doce, omnívoro com tendência piscívora preferindo crustáceos, insetos, restos de vegetais e detritos orgânicos. Apresenta barbilhões junto à boca, que provavelmente possuem receptores de gosto para ajudar na localização do alimento e na percepção da água (BALDISSEROTTO e NETO, 2004; GOMES et al., 2000). Possui hábito noturno e vive em lagos e poços fundos dos rios, preferindo ambientes de águas mais calmas com fundo de areia e lama, junto às margens e vegetação (GOMES et al., 2000; PERDICES et al., 2002).

Esta espécie tem despertado grande interesse no sul do Brasil devido as suas características como resistência ao manejo, crescimento rápido, boa eficiência alimentar, carne saborosa e sem espinhos intramusculares (BOCHI et al., 2008). Além disso, o jundiá é um peixe que apresenta excelente aceitação pelo mercado consumidor, tanto para pesca quanto para a alimentação, sendo uma espécie com excelentes características para o processamento. No contexto da contaminação ambiental, os peixes teleósteos têm se mostrado bons modelos experimentais devido a suas respostas bioquímicas serem semelhantes a mamíferos e outros vertebrados (SANCHO et al., 2000).

Figura 2. Exemplar de jundiá (*Rhamdia quelen*). (Fonte própria)

O guppy, *Poecilia vivipara*, (Figura 3) é um peixe eurialino brasileiro, é encontrado em corpos de água fresca e ao longo do litoral do Oceano Atlântico (GOMES e MONTEIRO, 2008). São considerados omnívoros, porém, apresentando uma tendência à ingestão de larvas de insetos (ANDRADE et al., 2000). A alta tolerância a extremos ambientais, particularmente temperatura e salinidade (TREXLER, 1989) faz com que *P. vivipara* seja uma das poucas espécies presentes em quase todos ambientes lênticos do sudeste e sul do Brasil (PETRY et al., 2016; TREXLER, 1989). Os poecilideos apresentam como principal característica, o seu modo de reprodução de viviparidade. Além de produzir os gametas femininos, os ovários podem

estocar espermatozoides, servir de sítio para a fertilização e local de desenvolvimento dos embriões até o nascimento. Este peixe tem sido apontado como uma espécie promissora para monitorar a saúde das águas costeiras (INCT-TA, 2012).

Figura 3. Exemplar de guppy (*Poecilia vivipara).* (Fonte: FishBase)

1.5 JUSTIFICATIVA E HIPÓTESE

Os peixes são amplamente utilizados como avaliadores da qualidade de ambientes aquáticos, e algumas espécies de peixes servem como bons bioindicadores de poluição ambiental. Considerando que há poucos estudos relacionando a toxicidade de metais em jundiá e guppy, pretendeu-se observar se as concentrações subletais de Zn (CONAMA, 2005), causam alterações bioquímicas nestas duas espécies.

As hipóteses testadas foram:

- Concentrações ambientais de Zn são capazes de se acumular nos tecidos dos jundiás em 96 h de exposição?

- Estas concentrações são capazes de alterar o metabolismo energético e bioquímico dos jundiás?

- Zinco pode alterar parâmetros comportamentais em guppy?

- A exposição a concentração ambiental relevante de Zn pode causar danos nos parâmetros oxidativos e antioxidantes de guppy?

- A salinidade ou a dureza são capazes de diminuir a toxidade do zinco em guppy?

2 OBJETIVOS

2.1 Objetivo geral

Avaliar os parâmetros bioquímicos de estresse oxidativo e os parâmetros comportamentais em guppy (*Poecilia vivipara*) e parâmetros de estresse oxidativo e metabólicos em jundiá (*Rhamdia quelen*) expostos a concentrações ambientais relevantes de zinco.

2.2 Objetivos específicos

- Avaliar parâmetros comportamentais relacionados ao padrão motor e de locomoção em guppy expostos ao zinco, salinidade e dureza;
- Analisar parâmetros bioquímicos em corpo inteiro de guppy;
- Avaliar a influência da salinidade e da dureza da água na toxicidade do zinco em guppy;
- Verificar a possível acumulação do zinco em diferentes tecidos de jundiá;
- Analisar parâmetros oxidativos, antioxidantes e metabólicos em jundiás expostos ao zinco durante 96 h.

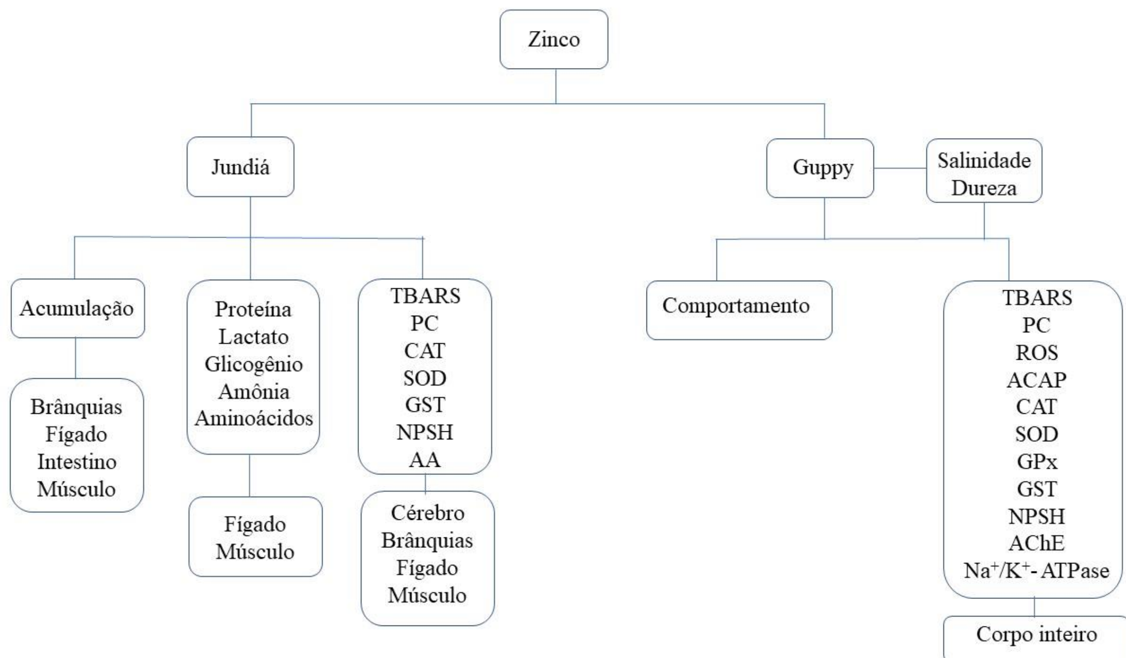

Figura 4. Fluxograma dos objetivos.

3 DESENVOLVIMENTO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo científico e manuscrito. Os itens Resumo, Introdução, Materiais e métodos, Resultados, Discussão e Referências encontram-se no próprio artigo e manuscrito. O artigo e o manuscrito estão dispostos conforme as normas das revistas científicas que foram enviados.

3.1 ARTIGO

Biometals
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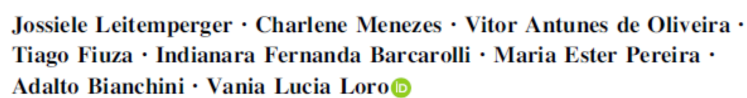
The bioaccumulation of waterborne zinc in tissues of silver catfish (*Rhamdia quelen*) and its effect on biochemical parameters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The bioaccumulation of waterborne zinc in tissues of silver catfish (*Rhamdia quelen*) and its effect on biochemical parameters

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ABSTRACT

Silver catfish (*Rhamdia quelen*) is a fish species with neotropical distribution, and is a potential model organism to study polluted environment. The aim of this study is to analyze the response of silver catfish to environmental concentrations of waterborne zinc (Zn) over 96 h. Significant metal accumulation was seen in gill, intestine and liver tissues. No significant accumulation was seen in muscle tissue. Lipid peroxidation increased in the brain, and decreased in the muscle and liver at all levels of exposure. Zinc exposure led to decreased protein carbonyl levels in the brain and increased levels in the liver. The activity of catalase in the liver was reduced for all exposed groups. Glutathione S-transferase activity decreased in the brain at the highest level of exposure and in the liver at all Zn concentrations tested. Non-protein thiols increased in the muscle and in the gills after exposure. Ascorbic acid levels increased in the brain and in the gills. Exposure to Zn also altered the metabolic parameters, causing decreased lactate and ammonia levels in the muscle, and decreased glycogen in the liver. Zinc exposure increased ammonia and amino acid levels in the liver, and increase glycogen and amino acid levels in muscle tissue. Our results demonstrate that exposure to environmentally relevant concentrations of Zn led to accumulation of metals in the tissues of silver catfish, with significant changes in biochemical parameters.

Keywords Aquatic contamination, environmental concentrations, fish, metal

Introduction

The pollution of freshwater ecosystems through anthropogenic actions such as the use of pesticides is increasing, as is pollution by metals of natural origin. Metals are continuously released into aquatic ecosystems where they pose a serious threat due to their potential toxicity, long persistence, bioaccumulation and biomagnification in the food chain (Eisler 1988). Zinc (Zn) is an essential micronutrient required for proper cellular function. However, excessive waterborne Zn can have detrimental impacts on fish, causing oxidative and antioxidant damage through the inhibition of acetylcholinesterase (AChE) and Na^+/K^+-ATPase and the concomitant increase of muscle protein carbonylation (McGeer et al. 2000; Romani et al. 2003; Leitemperger et al. 2016). Exposure to Zn can cause alterations at biochemical and physiological levels which in turn affect the metabolism of carbohydrates and proteins. These changes could be studied in order to elucidate the mechanism of physiological compensation that organisms develop in response to environmental stressors. Biochemical parameters related to energy metabolism, such as lactic acid and glycogen levels, have been used to assess the metabolic state of fish tissues after exposure to metals (Pretto et al. 2014a,b). The changes observed after exposure to waterborne metals are primarily due to oxidative damage. Several ecotoxicological studies have been carried out to identify and characterize the modulation of antioxidant defenses in experimental models of fish following poisoning with metals or pesticides (Loro et al. 2014; Luschack 2011; McRae et al. 2016). These have provided insight into how pollutants interact with living organisms. High concentrations of Zn in waters can arise through human activities such as irrigation. Brazilian legislation states that the maximum concentration of Zn in fresh water can range from 0.18 mg L^{-1} to 5 mg L^{-1} in waters which are destined for human consumption after treatment (CONAMA 2005).

The silver catfish (*Rhamdia quelen*) was used in this study because it is a native species with significant economic importance for the southern region of Brazil. It is found living freely

in the environment, as well as being a farmed fish, and is a good model for toxicity studies. Previous research using this species as a model for Zn poisoning showed that it is more sensitive to this metal than other fish described in the literature, with an LC_{50} of 8.07 mg L^{-1} (Leitemperger et al. 2016).

The aim of this study is to evaluate the effects of Zn contamination of water on the fish species *R. quelen* through the evaluation of metal accumulation in tissue, oxidative damage, antioxidant and metabolic responses using environmentally-relevant concentrations of Zn.

Materials and Methods

Chemicals

Malondialdehyde (MDA), 2-thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), $ZnSO_4.7H_2O$ and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical reagent grade and purchased from Merck (Rio de Janeiro, Brazil).

Animals

Male and female silver catfish (8.5 ± 1.0 g and 9.0 ± 1.2 cm) were obtained from a fish farm (Santa Maria, Rio Grande do Sul, Brazil). For 10 days, the fish were acclimatized in a tank (250 L) with non-chlorinated water treated with AquaSafe™ (Tetra, VA, USA) under constant aeration and a photoperiod cycle (12 h light/12 h dark). The water conditions were: temperature 24.7 ± 1 °C, pH 7.4 ± 0.2, dissolved oxygen 7.8 ± 0.9 mg L^{-1}, non-ionized ammonia 0.5 ± 0.03 µg L^{-1}, nitrite 0.03 ± 0.01 mg L^{-1}, hardness 28.5 ± 2.2 mg L^{-1} $CaCO_3$ and alkalinity 40.5 ± 3.0 mg L^{-1} $CaCO_3$. The experimental protocol was approved by the Committee on Ethics and Animal Welfare of Federal University of Santa Maria – RS – Brazil under the approval number 117/2013.

Experimental design

Exposure to waterborne zinc

After the acclimatization period, groups of eight fish were transferred to 40 L boxes for exposure to different concentrations of Zn (0.0, 0.1, 0.25 or 0.5 mg L^{-1}) for 96 h. The fish were not fed during the experimental period. The physicochemical characteristics of the water did not differ from those during the acclimatization period. Metal concentration was analyzed every 48 h by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, IL, USA), using Zinc Reference Solution (Fisher Scientific, Nepean, ON) as a standard. The metal concentration in the water did not have to be adjusted (Table 1).

Biochemical Parameters

Sample preparation

At the end of the experimental period (96 h), each of the eight fish in each group were sampled in triplicate. They were anaesthetized with benzocaine hydrochloride (12 mg L^{-1}) and euthanized by punching the spinal cord behind the opercula. Liver, gill, brain and muscle tissue samples were quickly removed, washed with 150 mmol L^{-1} saline solution, packed in Teflon tubes and kept at -80 °C until analysis. Tissue samples were prepared as previously described (Leitemperger et al. 2016).

Determination of zinc levels

Tissue Zn levels were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES – ICPE-9000; Shimadzu Scientific Instruments). Tissue samples were digested with concentrated HNO_3 overnight in a water bath (100 ºC). After digestion, samples were diluted with deionized water and transferred to graduated poly-propylene vials for analysis

by ICP-AES. The analytical standard Zn (Merck®) was used to generate a standard curve and results were expressed as µg g^{-1} of tissue and mg L^{-1} of water (Ineu et al. 2013).

Thiobarbituric acid reactive substance assay

The thiobarbituric acid reactive substances (TBARS) assay was carried out as follows. Liver, gill, brain and muscle tissue homogenates (sample size 100–400 µL) were added to 10% trichloroacetic acid (TCA) and 0.67% TBA, to final sample volumes of 1.0 mL. The reaction mixtures were placed in microcentrifuge tubes, incubated for 30 min at 95 °C and the optical density measured at 532 nm. The TBARS levels were expressed as nmol MDA mg^{-1} of protein (Buege and Aust 1978).

Protein carbonylation assay

For determination of protein carbonylation, soluble protein (1.0 mL) from the tissue samples was reacted with 10 mmol L^{-1} 2,4-dinitrophenylhydrazine (DNPH) in 2N hydrochloric acid. After incubation at room temperature for 1 h in the dark, 0.5 mL of denaturing buffer (150 mmol L^{-1} sodium phosphate buffer, pH 6.8, containing 3.0% SDS), 2.0 mL heptane (99.5%) and 2.0 mL ethanol (99.8%) were added sequentially, the mixture vortexed for 30 s and centrifuged for 15 min. Then, the precipitated protein at the interface was washed twice by resuspension in ethanol and ethyl acetate (1:1 solution) and suspended in 1.0 mL of denaturing buffer. The carbonyl content was assessed by measurement of absorbance at 370 nm. The assay was performed in duplicate, and two blank tubes incubated with 2N HCl without DNPH were included for each sample. The total carbonyl content was calculated using a molar extinction coefficient of 22,000 M cm^{-1} and expressed as nmol carbonyl mg^{-1} of protein (Yan et al. 1995).

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured in liver tissue only, based on inhibition of the radical superoxide reaction with adrenalin (Misra and Fridovich 1972). In this method, SOD present in the sample competes with the detection system for the radical superoxide. One unit of SOD is defined as the amount of enzyme which inhibits the speed of oxidation of adrenaline by 50%. The activity of SOD was determined by measuring the speed of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50 mmol L^{-1}, pH 10) and adrenaline (1 mmol L^{-1}). The activity was expressed as UI SOD mg^{-1} of protein.

Catalase activity

Catalase (CAT) activity was assessed in liver tissue only by measuring the decomposition of hydrogen peroxide (H_2O_2) with ultraviolet spectrophotometry (Nelson and Kiesow 1972). The assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mmol L^{-1}, pH 7.0), 0.05 mL H_2O_2 (0.3 mol L^{-1}) and 0.05 mL tissue homogenate. The change in H_2O_2 concentration aftes 60 s was calculated by measuring the absorbance at 240 nm. Catalase activity was expressed in µmol min^{-1} mg^{-1} of protein.

Glutathione S-transferase (GST) Activity

Glutathione S-transferase (GST) activity was measured in all tissue samples using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate (Habig et al. 1974). The formation of S-(2, 4-Dinitrophenyl)-glutathione was monitored by the increase in absorbance at 340 nm. The activity was expressed as µmol GS-DNB min^{-1} mg^{-1} of protein.

Non-protein thiols and ascorbic acid levels

Non-protein thiols (NPSH) and ascorbic acid levels were determined in all tissues. The preparation of homogenate for the two assays was the same. The tissues were homogenized with 1.5 mL Tris HCl 50 mmol L^{-1} (pH 7.5) followed by centrifugation at 3.000 **g** for 10 min. Aliquots of the supernatants (1.0 mL) were mixed (1:1) with 10 % TCA and then centrifuged. To determine the NPSH content of the tissues, aliquots of the supernatants (500 µL) were added to a phosphate buffer (0.5 mmol L^{-1} pH 6.8, 10 mmol L^{-1} 5,5'-dithio-bis[2-nitrobenzoic acid] [DTNB], 0.5 mmol L^{-1} cysteine) and the color change measured at 412 nm (Ellman et al. 1959). Levels of NPSH were expressed as µmol g^{-1} of tissue. For determination of ascorbic acid content, aliquots of the supernatants (300 µL) were mixed with 2,4-dinitrophenylhydrazine (4.5 mg mL^{-1}), 0.6 mg mL^{-1} thiourea, $CuSO_4$ (0.075 mg mL^{-1}) and TCA 13.3%; and incubated for 3 h at 37° C. Next, 500 µL of H_2SO_4 65% (v/v) was added to the solutions (Roe 1954). Ascorbic acid levels were expressed as µg ascorbic acid g^{-1} of tissue.

Protein concentration

Protein concentration was determined by the Bradford Coomassie Blue method using bovine serum albumin as a standard (Bradford 1976). The absorbance of samples was measured at 595 nm.

Metabolic Parameters

Glycogen levels in the liver and muscle were determined after addition of KOH and ethanol for hydrolysis and precipitation of glycogen respectively (Bidinotto et al. 1997). For protein analysis tissues were hydrolyzed after addition of KOH (1 mL), heating to 100 ºC and centrifugation at 1000 g for 10 min. The total protein level in the supernatant was estimated using bovine serum albumin as standard (Lowry et al. 1951). For lactate and ammonia determination, tissue samples were homogenized by the addition of 10% TCA in a motor driven

Teflon pestle and centrifuged at 1000 g for 10 min to precipitate the proteins. The protein free supernatant was used for determination of lactate and ammonia concentrations (Harrower and Brown 1972; Verdouw et al. 1978). For amino acid quantification, tissues were homogenized with 2 mL of phosphate buffer 20 mmol L^{-1}, pH 7.5, and the homogenates were centrifuged at 1000 g for 10 min (Spies 1957).

Statistical analysis

The normal distribution and homogeneity of variances among groups were tested with the Kolmogorov-Smirnov and Bartlett's tests, respectively. Data presented homogeneous variances, and comparisons between treatments groups were made using one-way ANOVA followed by Tukey's post hoc test and expressed as mean ± standard error. Differences were considered to be significant at a probability level of $P < 0.05$.

Results

Waterborne Zn levels remained constant during the experimental period (Table 1). Bioaccumulation of Zn was measured in gill, liver, intestine and muscle tissues (Figure 1). Significant accumulation in gill tissue was only seen when fish were exposed to 0.5 mg L^{-1} of Zn compared to control and other concentrations tested (Figure 1A). The liver and intestine (Figures 1B and 1C respectively) showed accumulation when exposed to Zn at 0.25 or 0.5 mg L^{-1}. Muscle did not show metal accumulation at any Zn concentration (Figure 1D).

After 96 h of exposure, TBARS levels of brain tissue increased for every treatment group (Figure 2A). In the liver, TBARS levels decreased when Zn concentration was 0.25 or 0.5 mg L^{-1}, with both concentrations producing equivalent results (Figure 2B). All Zn concentrations caused a decrease in the TBARS levels of muscle tissue (Figure 2C). No effect

was seen on the TBARS levels of gill tissue samples treated with any concentration of Zn (Figure 2D).

Protein carbonyl levels in brain tissue samples were reduced in every group that was exposed to Zn. Conversely, liver tissue samples showed an increase in carbonyl levels at 0.25 or 0.5 mg L^{-1} of Zn. In muscle and gill tissue samples, no changes were observed in protein carbonylation at any Zn concentration (Table 2).

The activity of SOD in liver tissue samples showed no variation after exposure to Zn (Figure 3A). However, CAT activity decreased with Zn exposure in a concentration-dependent manner (Figure 3B).

The activity of GST in brain tissue samples decreased following treatment with 0.5 mg L^{-1} Zn. In liver tissue, GST activity decreased in all treatment groups with no difference seen between fish exposed to 0.1 or 0.25 mg L^{-1} Zn. No effect of Zn exposure was seen in muscle or gill GST activities (Table 2).

The NPSH levels of brain and liver tissue samples did not show any changes following any level of Zn exposure. In muscle tissue, an increase was observed after exposure to 0.5 mg L^{-1} Zn. In gill tissue, NPSH increased in the 0.25 and 0.5 mg L^{-1} treatment groups (Table 2). Ascorbic acid levels increased in brain tissue only after exposure to 0.5 mg L^{-1} Zn. No differences were seen in the ascorbic acid levels of liver and muscle tissues of any treatment groups, whereas levels in the gill tissue increased in all exposure groups (Table 2).

The results of metabolic parameter assays are shown in Table 3. The protein levels in liver tissue increased when fish were exposed to levels of 0.25 or 0.5 mg L^{-1} of Zn, with no difference seen at 0.1 mg L^{-1}. No effect was seen on protein levels of muscle tissue in any treatment group. Lactate levels in the liver did not change after exposure to Zn, and in muscle levels only decreased in the fish exposed to 0.25 or 0.5 mg L^{-1} of Zn. Liver glycogen decreased in all groups exposed to Zn, whereas muscle tissue showed a concentration-dependent increase

in glycogen at all Zn concentrations tested. Ammonia levels in the liver increased only when Zn concentrations were 0.5 mg L^{-1}, but decreased in muscle tissues in every exposure group, in a concentration-dependent manner. Amino acid levels increased in liver tissue samples of every treatment group, while in muscle tissues increased levels only occurred at concentrations of 0.25 mg L^{-1} and above.

Discussion

Release of Zn into water supplies can occur as a result of natural erosion or anthropogenic actions such as the use of Zn-containing fertilizers and agrochemicals. Detrimental effects occur when internal concentrations exceed the requirements and Zn detoxification capacity of the organism (Viarengo et al. 1990). The present investigation aimed to measure the effects of 96 h of exposure to sublethal contaminations of Zn in silver catfish, to provide insight into the risks of environmental Zn contamination. The results indicated that significant metal retention occurred in the gills, intestines and livers of fish exposed to waterborne zinc. Accumulation of metal in the gills and liver can be cleared by the organs' elimination processes which occur due to the metabolic and excretory functions of these organs. No metal accumulation was seen in muscle tissue, which is a key result, considering that muscle is the preferential fish tissue for human consumption. In the conditions of this study, it is possible that the exposure time and Zn concentrations tested may have been insufficient to cause accumulation in the muscle tissue. However, other tissues showed accumulation of Zn even low concentrations and within the short time of the experiment. Metals have a tendency to accumulate in the fatty tissues of aquatic organisms and accumulation of Zn is known to affect the reproductive physiology (Rahaman et al. 2012). Other studies have shown similar results to those presented here, indicating a preference of metal to accumulate in the liver or gill tissues compared with muscle tissue (Dural et al. 2007; Yilmaz et al. 2010; La Colla et al. 2018). The

concentrations of Zn that were tested in this study are representative of levels that may be found in the environment.

All concentrations tested led to increased lipid peroxidation in brain tissue, which correlated with reduced GST activity seen in this tissue. The activity of GST is important for the detoxification of lipoperoxides, and the reduced activity could therefore be the cause of the increased brain TBARS levels that were measured. Conversely, liver and muscle tissues showed reduced TBARS levels, which highlights the efficiency of the antioxidant systems of these organs. This hypothesis is supported by the increase of NPSH seen in muscle tissue. The decrease in protein carbonyls in brain tissue could result from the increase of ascorbic acid in this tissue. The increase in protein carbonyls in liver tissue could similarly be explained due to a decrease in the activity of the antioxidant system of this organ, indicated by the reduction of CAT and absence of SOD activity. Exposure to Zn also affected the non-enzyme antioxidants NPSH and ascorbic acid. The unchanged levels of liver NPSH is indicative of Zn accumulation in liver, revealing that even low levels of Zn exposure can disrupt normal liver metabolism.

Changes may occur in the metabolism of carbohydrates and proteins in response to metal exposure. We observed increased levels of protein in the liver of silver catfish at the two highest concentrations of Zn tested, which suggested that a protective mechanism may occur to deal with tissue protein loss through increasing the synthesis of proteins. Tissue protein content depends on a dynamic equilibrium between the rates of synthesis and degradation. Although we only observed minimal differences in muscle protein levels, increased amino acid concentrations and decreased levels of ammonia were observed after exposure to 0.25 or 0.5 mg L^{-1} Zn. Hepatic glycogen depletion was observed upon exposure to Zn, and the changes in muscle lactate levels indicates the stimulation of hepatic gluconeogenesis through the reduction and recycling of muscle lactate. To summarize when silver catfish were exposed to Zn at environmentally relevant concentrations, adjustments in metabolic processes occurred in order

to manage the effects of external stress and maintain internal homeostasis. The changes in protein levels indicated that fish preferentially use glycogen or other carbohydrate sources to maintain bioenergetics during Zn exposure, reducing the use of protein as an energy source.

Conclusion

This study was carried out to provide information on the effect of environmental concentrations of Zn in silver catfish. The results of biochemical analyses revealed that even at low concentrations, Zn had a significant effect on the parameters metabolic and of oxidative damage in silver catfish. The changes as evidenced by the increase of TBARS in the brain, decrease of CAT activity in liver and changes in the metabolism of proteins and carbohydrates. The most important conclusion is that besides bioaccumulation of Zn was seen high levels in the liver, gill and intestine, the lowest levels were recorded in the muscle, meaning that this tissue is safe for human consumption. The concentrations measured in this study were below the recommended limit for fish (30 µg g^{-1}, FAO/WHO 1983, 1989). In addition, these results could provide a useful approach to evaluate the quality of the aquatic ecosystem.

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Tables

Table 1. Zn measurements in water during experimental period

Day	0.0 mg L^{-1}	0.1 mg L^{-1}	0.25 mg L^{-1}	0.5 mg L^{-1}
1	< 0.005	0.115±0.003	0.249±0.002	0.505±0.004
3	< 0.003	0.095±0.002	0.247±0.002	0.496±0.006
5	<0.003	0.090±0.003	0.241±0.003	0.484±0.005

Table 2. Protein Carbonyl, GST, NPSH and Ascorbic acid in brain, liver, muscle and gill of silver catfish after exposure to 0.0; 0.1; 0.25 or 0.5 mg L^{-1} of Zn.

Protein carbonyl	Brain	Liver	Muscle	Gill
0.0 mg L^{-1}	6.94±0.15^{b}	4.49±0.25^{a}	5.60±0.39^{a}	7.08±0.88^{a}
0.1 mg L^{-1}	2.98±0.11^{a}	6.51±0.76^{a}	4.87±0.49^{a}	5.85±0.59^{a}
0.25 mg L^{-1}	2.99±0.17^{a}	9.99±0.91^{b}	4,73±0.69^{a}	6.51±0.66^{a}
0.5 mg L^{-1}	2.98±0.16^{a}	10.75±0.45^{b}	4.10±0.53^{a}	4.48±0.57^{a}
GST	Brain	Liver	Muscle	Gill
0.0 mg L^{-1}	0.16±0.01^{b}	0.38±0.02^{c}	0.16±0.01^{a}	0.28±0.02^{a}
0.1 mg L^{-1}	0.15±0.01^{b}	0.24±0.01^{b}	0.17±0.01^{a}	0.35±0.03^{a}
0.25 mg L^{-1}	0.13±0.01^{b}	0.26±0.03^{b}	0.18±0.004^{a}	0.34±0.02^{a}
0.5 mg L^{-1}	0.09±0,003^{a}	0.17±0.02^{a}	0.18±0.01^{a}	0.34±0.02^{a}
NPSH	Brain	Liver	Muscle	Gill
0.0 mg L^{-1}	0.33±0.02^{a}	0.37±0.04^{a}	0.18±0.003^{a}	0.23±0.02^{a}
0.1 mg L^{-1}	0.34±0.01^{a}	0.46±0.01^{a}	0.21±0.02^{ab}	0.37±0.01^{ab}
0.25 mg L^{-1}	0.34±0.01^{a}	0.46±0.04^{a}	0.22±0.01^{ab}	0.46±0.02^{b}
0.5 mg L^{-1}	0.37±0.04^{a}	0.46±0.02^{a}	0.21±0.002^{b}	0.46±0.005^{b}
Ascorbic acid	Brain	Liver	Muscle	Gill
0.0 mg L^{-1}	4.78±0.04^{a}	2.49±0.05^{a}	1.48±0.02^{a}	2.74±0.10^{a}
0.1 mg L^{-1}	4.94±0.01^{a}	2.60±0.05^{a}	1.33±0.12^{a}	3.34±0.04^{b}
0.25 mg L^{-1}	5.04±0.08^{ab}	2.63±0.05^{a}	1.33±0.05^{a}	3.55±0.06^{b}
0.5 mg L^{-1}	5.36±0.07^{b}	2.64±0.05^{a}	1.33±0.06^{a}	3.56±0.02^{b}

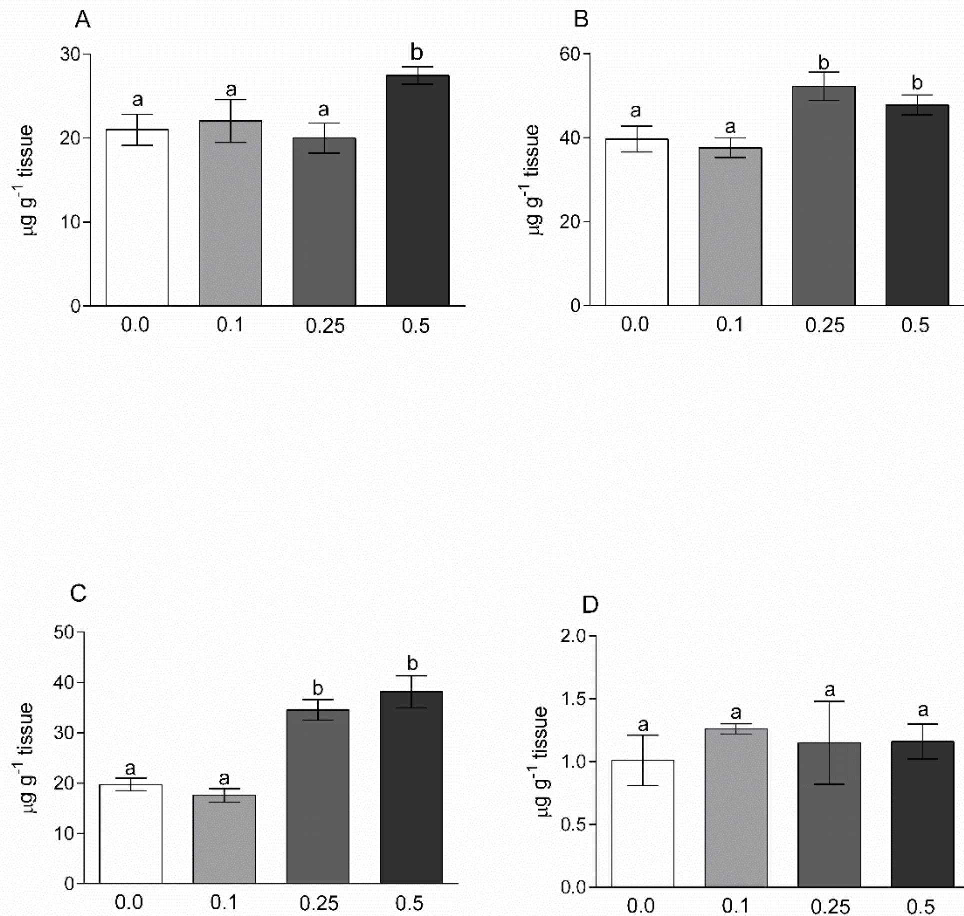
Values are means ± SEM, n = 8 fish/group. Protein carbonyl as nmol carbonyl mg^{-1} of protein, GST as µmol GS-DNB min^{-1} mg^{-1} of protein, NPSH as µmol g^{-1} of tissue and ascorbic acid as µg ascorbic acid g^{-1} of tissue. Different letters indicated significant differences among the groups. $p < 0.05$

Table 3. Protein, lactate, glycogen, ammonia and amino acids in liver and muscle of silver catfish during Zn exposure

Protein (mg g^{-1} tissue)	Liver	Muscle
0.0 mg L^{-1}	121.38±4.17[a]	154.02±2.25[a]
0.1 mg L^{-1}	122.40±3.02[a]	161.16±2.98[a]
0.25 mg L^{-1}	158.10±6.03[b]	163.61±5.08[a]
0.5 mg L^{-1}	190.74±6.20[c]	165.75±2.73[a]
Lactate (µmol g^{-1} tissue)		
0.0 mg L^{-1}	6.38±0.35[a]	6.56±0.20[b]
0.1 mg L^{-1}	6.81±0.56[a]	5.62±0.25[b]
0.25 mg L^{-1}	7.33±0.13[a]	4.39±0.18[a]
0.5 mg L^{-1}	7.34±0.14[a]	4.28±0.05[a]
Glycogen (µmol g^{-1} tissue)		
0.0 mg L^{-1}	146.32±2.75[b]	3.37±0.16[a]
0.1 mg L^{-1}	116.68±0.82[a]	5.75±0.35[b]
0.25 mg L^{-1}	119.19±0.36[a]	6.91±0.17[b]
0.5 mg L^{-1}	119.45±0.56[a]	8.64±0.28[c]
Ammonia (µg g^{-1} tissue)		
0.0 mg L^{-1}	25.12±2.32[a]	5.57±0.12[c]
0.1 mg L^{-1}	30.73±2.02[ab]	3.56±0.23[b]
0.25 mg L^{-1}	32.76±0.30[ab]	3.07±0.13[b]
0.5 mg L^{-1}	37.91±0.81[b]	1.23±0.06[a]
Amino acids (µmol g^{-1} tissue)		
0.0 mg L^{-1}	72.35±2.12[a]	19.25±0.20[a]
0.1 mg L^{-1}	83.45±1.57[b]	20.78±0.37[a]
0.25 mg L^{-1}	83.83±1.81[b]	29.60±1.38[b]
0.5 mg L^{-1}	83.82±1.72[b]	29.70±2.84[b]

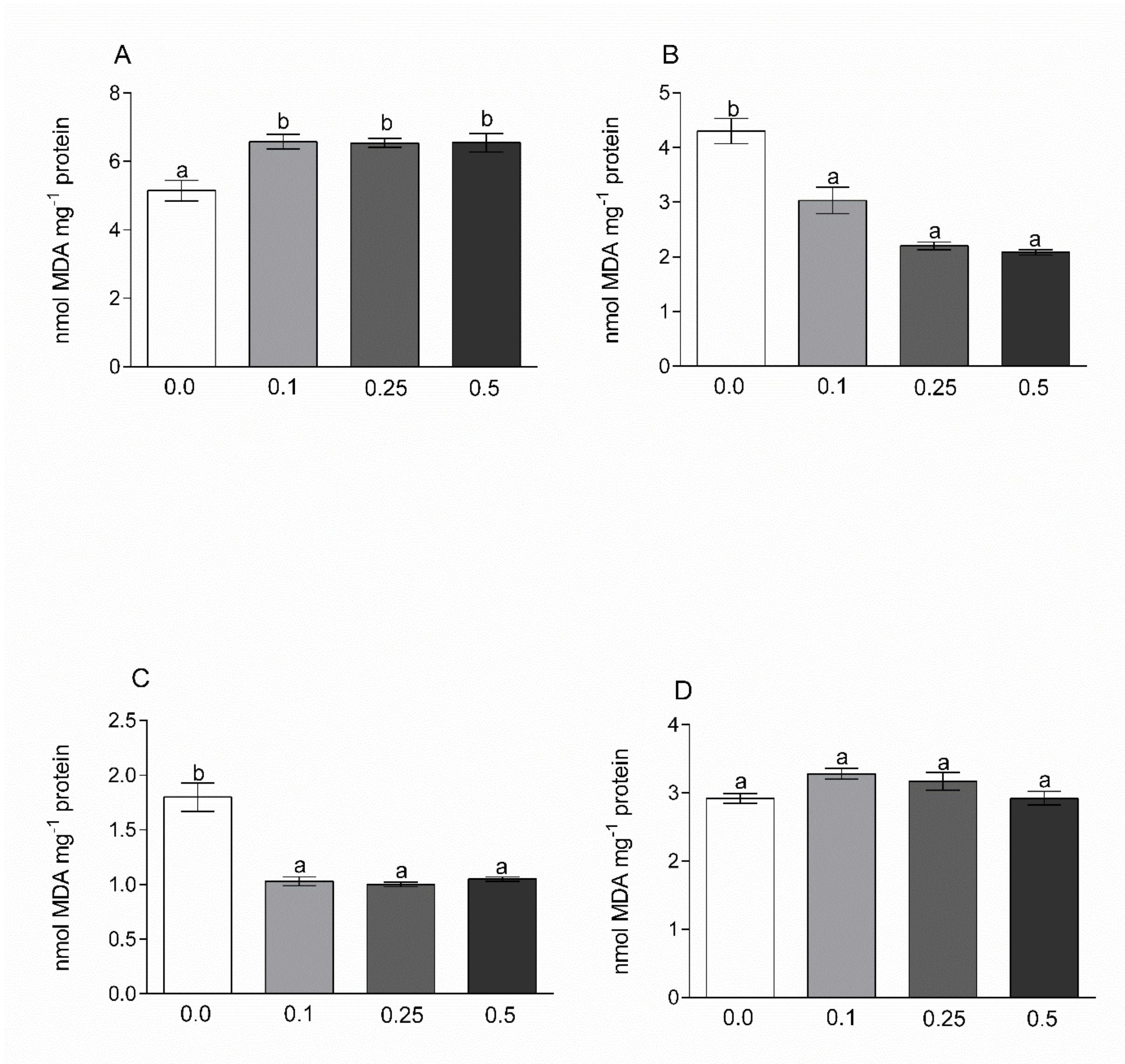
Values are means ± SEM, n = 8 fish/group. Different letters indicated significant differences among the groups. $p < 0.05$

Figure 1. Concentration of Zn in gill (A), liver (B), intestine (C) and muscle (D) of silver catfish after exposure to 0.0; 0.1; 0.25 or 0.5 mg L^{-1} of Zn.



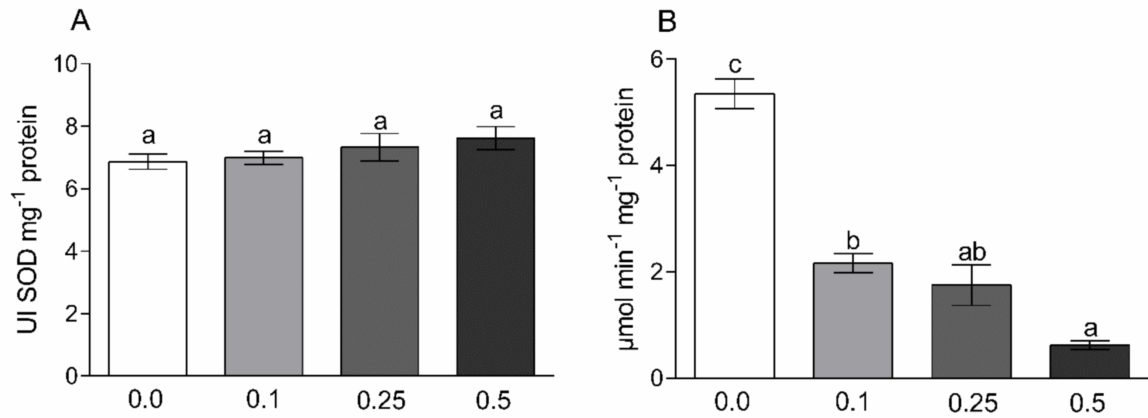
Values are means ± SEM, n = 8 fish/group. Different letters indicated significant differences among the groups. $p < 0.05$

Figure 2. TBARS in brain (A), liver (B), muscle (C) and gill (D) of silver catfish after exposure to 0.0; 0.1; 0.25 or 0.5 mg L^{-1} of Zn.



Values are means ± SEM, n = 8 fish/group. Different letters indicated significant differences among the groups. $p < 0.05$

Figure 3. SOD (A) and CAT (B) activities in liver of silver catfish after exposure to 0.0; 0.1; 0.25 or 0.5 mg L^{-1} of Zn.



Values are means ± SEM, n = 8 fish/group. Different letters indicated significant differences among the groups. $p < 0.05$

3.2 MANUSCRITO

Behavioural and biochemical parameters in guppy (*Poecilia vivipara*) following exposure to waterborne zinc in salt or hard water

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Behavioural and biochemical parameters in guppy (*Poecilia vivipara)* following exposure to waterborne zinc in salt or hard water

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ABSTRACT

Zinc is an essential trace mineral that is involved in many biological processes. In elevated concentrations, this metal may have toxic effects for aquatic organisms. Physicochemical properties of water, such as salinity and hardness, can influence the bioavailability of zinc and, therefore its toxicity in aquatic environments. Therefore, this study aimed investigate the influence of salinity, hardness or/and Zn toxicity on the behaviours and biochemical parameters of the estuarine guppy (*Poecilia vivipara*). The fish were exposed to waterborne zinc (500 µg L^{-1}) in salt water (25 ppt) or hard water (120 mg L^{-1} $CaCO_3$). For behavioural analysis, the locomotive and exploratory parameters of fish in novel environment and light-dark tests were evaluated. We observed that exposure to hard water decreased the distance covered by the fish, and when zinc also present the vertical exploratory behavior decreased. When zinc was tested alone, an increase in the maximum speed of fish was recorded. Activities of antioxidant enzymes, levels of lipid peroxidation, protein carbonylation, total peroxidation and, reactive oxygen species content, antioxidant capacity against peroxyl radicals, non-proteins thiols levels, acetylcholinesterase and Na^+/K^+-ATPase activities were evaluated in the whole fish body. The integrated biomarker response (IBR) was calculated for each parameter to aid in the interpretation of the results and indicated that hard water containing zinc had the greatest effect on the biochemical parameters of the fish. In general, neither salinity nor hardness were effective in protecting the guppy from the biochemical damage caused by exposure to zinc.

Keywords estuarine fish, environmental risk, IBR, hardness, metal, salinity

1. Introduction

Zinc (Zn) is an essential transition metal involved in various biological processes [1]. The concentration of Zn found in the environment can differ. In the south of Brazil, for example, levels can be 10% above the acceptable level, as defined by the Brazilian legislation (5.0 mg L^{-1} for water) [2]. High concentrations of metals can be toxic because they are able to induce oxidative stress by accelerating the generation of highly reactive oxygen species (ROS). If these ROS are not detoxified, they can oxidize proteins, lipids and nucleic acids, leading to damage of various cellular components or even cell death [3]. Antioxidant defence systems help to mitigate ROS damage, and changes in antioxidant systems have been successfully used as biomarkers for exposure to metallic contaminants [3,4].

Salinity and hardness are physicochemical properties of water that influence the toxicity of metals thereby modulating the osmoregulatory and ionoregulatory functions of fish. Salinity is one of the most important factors in the aquatic ecosystem affecting fish growth and survival [5]. Alterations in salinity and hardness can significantly influence metal bioavailability and toxicity towards aquatic organisms, and for this reason some metals are more toxic in soft water than in hard water [6,7]. Therefore, it is important to examine the toxic effects of metals in different waters in order to estimate the consequences of metal contamination on the physiology of fish in fresh water environments.

Various biomarkers have the potential to provide valuable information concerning the biochemical responses of metal contamination. However, their use are limited if measurements cannot be integrated into a system to facilitate data analysis and interpretation. A useful tool for assessing the biological effects and health status of organisms exposed to several chemicals is the Integrate Biomarker Response (IBR), that allows comparison of the effects of different treatments [8, 9, 10, 11]. In the present study, we examined the guppy (*Poecilia vivpara*), a Brazilian euryhaline teleost fish common in both fresh and coastal water bodies along the South

Atlantic Ocean [12]. Studying this species has been highlighted as a promising approach to monitor the health of tropical and sub-temperate coastal waters. The aim of this study was to determine the influence of salinity, hardness and/or Zn toxicity on the behaviour and biochemical parameters of *P. vivipara*.

2. Materials and Methods

2.1. Chemicals

Malondialdehyde (MDA), 2-Thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), $ZnSO_4.7H_2O$ and 2,4-Dinitrophenylhydrazine (DNPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical reagent grade and purchased from Merck (Rio de Janeiro, RJ, Brazil).

2.2. Animals and water preparation

Male fish were obtained from a local supplier (Hobby Aquários, RS, Brazil) and acclimated in 40 L tanks for one week at a maximum density of four fish per litre. The experiments were carried out only male fish, because the females have internal fertilization and could be pregnant, which could interfere in the biochemical analysis. In addition to minimizing possible differences in behavioural parameters. Fish were kept under controlled conditions (photoperiod: 14 h light, 10 h dark; temperature: 25 ºC) and fed daily to satiation with commercial omnivorous fish food. The food was a complex mixture containing minerals, vitamins, animal and vegetable protein, algae, lipids and several probiotics (Alcon Basic, Camboriú, SC, Brazil). Saline water was produced by the addition of Instant Ocean® sea salt (Aquarium Systems Inc., Mentor, OH, USA) to fresh water, considering 25 g L^{-1} to be a salinity of 25 ppt. Hard water was prepared by dissolving 120 mg L^{-1} $CaCO_3$ in the fresh water. Zinc (in the form of $ZnSO_4$) was added to fresh water, salt water or hard water from a stock solution

to reach an environmentally representative concentration of 500 µg L^{-1}. All glassware and exposure tanks were acid washed and rinsed thoroughly with distilled water prior to use. All experiments were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (approval number: 5026261016).

2.3. Experimental design

After the acclimation period, six groups of eight fish were transferred to 20 L tanks for exposure. Fish were incubated in different waters for 96 h under the same conditions (temperature and photoperiod) as during acclimation: Group 1 (Control: tap water), Group 2 (Zn: 500 µg L^{-1}), Group 3 (Salt water: 25 ppt), Group 4 (Hard water: 120 mg L^{-1}), Group 5 [Salt water (25 ppt) + Zn (500 µg L^{-1})] and Group 6 [Hard water (120 mg L^{-1}) + Zn(500 µg L^{-1})]. Fish were not fed during the experimental period. The metal concentration of Zn was analysed every 48 h by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC Scientific, Hampshire, IL, USA). Standards were made using Zinc Reference Solution (Fisher Scientific, Nepean, ON).

2.4. Behavioural parameters

Behavioural analysis was carried out after 72 h in experimental conditions. Fish were returned to the treatment tank after analysis and remained there for a further 24 h, to reach the total 96 h of exposure prior to biochemical analyses.

2.4.1 Novel tank test

The novel tank test was designed to analyse the locomotor and exploratory activity of the guppy when exposed to a novel environment. Each fish was placed individually into a tank

(25 cm long x 15 cm high x 6 cm wide) containing 2 L of water and the behaviour of the fish was recorded during 6 min of exploration. After each trial, the tank was cleaned and the water replaced with new water. More details in Müller et al. [13] and Quadros et al. [14].

2.4.2 Light-dark test

The light-dark apparatus consisted of a glass tank (25 com long x 15 cm high x 10 cm deep) divided into two equally sized dark and lit areas, filled with 2 L home water. The areas were made as fish only perceive black and white. Fish were filmed for 6 min and the following behaviours analysed. Details regarding this assay have been published elsewhere [14, 15, 16].

2.5 Biochemical assays

2.5.1. Sample preparation

At the end of the experimental period (96 h), the fish were anaesthetised with benzocaine hydrochloride (100 mg L^{-1}) and euthanised by decapitation. Whole body samples were quickly collected and macerated with 150 mM saline solution, packed in Teflon tubes and kept at -80 °C to be used for the oxidant and antioxidant parameters assays, as described by Íspir et al. [17]. Whole body samples (100 mg) were homogenised on ice in 1 mL Tris HCl buffer (50 mM, pH 7.4). Homogenised samples were centrifuged at 3 000 × g for 10 min at 4 ºC and the supernatants kept in microtubes at -80 ºC for biochemical assays.

2.5.2 Thiobarbituric acid reactive substance and protein carbonyl assay

Lipid peroxidation was estimated by measurement of Thiobarbituric acid reactive substance (TBARS) production. The reaction of MDA with TBA was measured optically according to the protocol described by Buege and Aust [18]. The absorbance was measured at

532 nm using a microplate reader. Malondialdehyde was used as the standard and results were expressed as nmol MDA mg protein^{-1} [13].

Protein carbonyl determination was carried out using a method described by Yan et al. [19] with modifications. The absorbance was measured at 370 nm using a microplate reader. The total carbonylation was calculated using a molar extinction coefficient of 22 000 M cm^{-1} and expressed as nmol carbonyl mg protein^{-1} [13].

2.5.3 Determination of H_2O_2 content

Hydrogen peroxide content was determined according to the method published by Velikova et al. [20] and adapted for fish. Tissue samples (100 µL) were mixtured with 100 µL of 0.1% (w/v) TCA, was centrifuged at 12 000 × g for 15 min and 50 µL of the supernatant was added to 50 µL of 10 mM potassium phosphate buffer (pH 7.0) and 100 µL of 1 M KI. The absorbance of the supernatant was measured at 390 nm using a microplate reader and the concentration of H_2O_2 calculated from a standard calibration curve that was generated using known concentrations of H_2O_2.

2.5.4 Reactive oxygen species and antioxidant capacity against peroxyl radicals

ROS levels were determined using a spectrofluorimetric method. The oxidation of 2', 7' - Dichlorofluorescein diacetate (H_2DCF-DA) to fluorescent dichlorofluorescein (DCF) as described by Ali et al. [21]. The levels of ROS were expressed as pmol DFC mg protein^{-1} according to Müller et al. [13].

The total antioxidant competence against peroxyl radicals (ACAP) was evaluated through comparison of the measured ROS level of untreated tissue samples with those treated with 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (ABAP) a peroxyl radical generator. More details have been published elsewhere [22].

2.5.5 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was assessed by measuring the inhibition of the radical superoxide reaction in the presence of adrenaline as described by Misra and Fridovich [23]. The activity of SOD was determined by measuring the speed of adrenochrome formation, observed at 480 nm and was measured using a microplate reader and expressed as UI SOD mg protein^{-1} [13].

2.5.6 Catalase activity

Catalase (CAT) activity was assayed using ultraviolet spectrophotometry [24]. The change in H_2O_2 absorbance at 240 nm over 60 s was measured by spectrophotometry. Catalase activity was expressed in µmol min^{-1} mg protein^{-1}.

2.5.7 Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assessed based on the coupled reaction with glutathione reductase [25], by analyzing the rate of NADPH oxidation measured via the absorbance at 340 nm using a microplate reader and expressed as µmol min^{-1} mg protein^{-1} [13].

2.5.8 Glutathione S-transferase activity

Glutathione S-transferase (GST) activity was measured according to the protocol published by Habig et al. [26]. The formation of S-(2, 4-Dinitrophenyl) glutathione was assessed by monitoring the increase in absorbance at 340 nm. The absorbance was measured using a microplate reader and the activity determined and expressed as µmol GS-DNB min^{-1} mg protein^{-1} [13].

2.5.9 Non-protein thiols concentration

Non-protein thiols (NPSH) levels as previously described by Ellman [27] and adapted to a microplate reader [13]. Results were expressed as µmol g^{-1} of tissue.

2.5.10 Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was measured as described by Ellman et al. [28] with some modifications for a microplate reader. The absorbance at 412 nm was measured continuously for 2 min to determine the level of hydrolysed acetylthiocholine – thiocholine (SCh). Activity was expressed as µmol (SCh) min^{-1} mg $protein^{-1}$.

2.5.11 Na^+/K^+-ATPase activity

Whole body Na^+/K^+- ATPase activities were assayed using a modified version of the method described by Bianchini and Castilho [29]. The phosphate concentration in the reaction medium was determined using a modification of the method of Fiske and Subbarow [30]. Enzyme-specific activity was expressed as µmol Pi $hour^{-1}$ mg $protein^{-1}$ [4].

2.5.12 Protein concentration

Total protein concentration was determined by the Bradford Coomassie Blue method [31] using bovine serum albumin as standard. The absorbance of the sample was measured at 595 nm.

2.6 Integrated biomarker response

Using the results obtained from the previous assays, the integrated biomarker response (IBR) was calculated to characterise the effects of different water conditions and/or levels of Zn exposure. The IBR values were calculated by log transformation and represented as star plots [32, 33].

2.7 Statistical analysis

The normal distribution and homogeneity of the data were confirmed by Kolmogorov-Smirnov and Bartlett's testes respectively. Results are presented as mean ± the standard error of mean (SEM). Comparisons between groups were made using two-way analysis of variance (ANOVA) followed by Newman-Keuls test with a significance level $P < 0.05$. Analyses and graphical were performed using GraphPad Prism software version 6.01 (GraphPad Software, CA, USA).

3. Results and Discussion

3.1 Behavioural parameters

In this study, fish were exposed to a sublethal concentration of Zn (500 µg L^{-1}) in tap water, salt water (25 ppt) or hard (120 mg L^{-1} $CaCO_3$) for 96 h. Measurements of waterborne Zn concentration showed that there were no variations in the metal concentration during the experimental period. The concentration studied was environmentally relevant, and so the present study aimed to verify if water salinity or hardness could protect the guppy from Zn toxicity. This species was chosen due to its high capacity to resist high salinity and others environmental changes [34]. The behavioural parameters evaluated in this experiment were analysed using the novel tank and light-dark test. Table 1 presents the results of the novel tank test, which indicate the motor activity and locomotion of the fish. Only fish exposed to hard water showed a significant decrease in the distance travelled compared with the control (CT) and Zn exposed groups. Fish exposed to Zn or hard water alone showed reduced locomotor and AChE activity (Fig.4). This indicates a direct relationship between the movement of the fish and enzyme activity. Analysis of the maximum speed revealed that the Zn-exposed group exhibited increased speeds compared with the CT group. No difference was observed between other treatments, indicating that neither water salinity nor hardness affected the locomotion of

the fish. When the water included Zn, neither salinity nor hardness resulted in significant differences to the CT group. The number of entries into the top region of the tank and time spent in top did not differ between any of the treatment groups. From these results, it can be concluded that none of the treatments affected the vertical exploration, or locomotion of the fish. The high maximum speed of the Zn group compared with the CT group could be related to the inhibition of AChE that was observed in the Zn group, as this enzyme is important for locomotion and neuromuscular transmission. The light-dark test did not reveal any difference between the any of the treatment groups (data not shown), suggesting that the treatments also had no impact on the anxiety-like behaviour of the fish.

3.2 Analysis of molecular damage

Exposure to Zn, salt and hard water alone all led to increased levels of lipid peroxidation as measured by TBARS (Fig. 1). When coupled with salinity, Zn exposure did not affect the lipid peroxidation levels (Fig. 1a), but when Zn was present in hard water the lipid peroxidation increased (Fig. 1b). There was an increase in protein carbonyl levels of Zn, hard water, salinity + Zn, and hard water + Zn groups compared with the CT group (Fig. 1c and 1d). The only treatment that did not show a difference in carbonylation was salt water. The TBARS and carbonyl results indicate that, contrary to the literature [35], water hardness was not sufficient to protect the fish from lipid and protein damages caused by Zn toxicity. This could be due to the fundamental role of Ca^{2+} ions in the regulation of biological membrane permeability in order to prevent diffuse flow and high loss of ions to surrounding water. We observed that Zn increased peroxide levels compared with groups that were not exposed to Zn (Fig. 1e and 1f). When associated with salt or hardness, Zn caused no increase in peroxides levels compared with the CT group. This indicates that both, salinity and hardness were effective in preventing the formation of peroxides.

3.3 Oxidative and antioxidants assays

Levels of reactive oxygen species (ROS) increased in the whole body of fish exposed to Zn (Fig. 2a and 2b). The presence of salt alone and in association with Zn did not affect ROS levels. Similar results were reported by Loro et al. [36], who observed that salt did not affect ROS levels, but led to increased ROS levels in killifish when combined with 500 $\mu g\ L^{-1}$ of Zn^{2+}. In the present study, hard water with or without Zn, had no effect on ROS levels compared with the CT group. Regarding ACAP values (Fig. 2c and 2d), Zn and salt both resulted in significantly increased relative areas, indicating reduced ROS scavenging capacities. When fish were exposed to salt and Zn together, ACAP values remained equivalent to those of the CT groups (Fig. 2c). Hard water with or without Zn had no effect on ACAP levels (Fig. 2d).

The antioxidant defence system is critical in preventing damage caused by free radicals. In this experiment we observed increased SOD activity after exposure to Zn, salt, salt + Zn, hardness or hardness + Zn (Fig. 3a and 3b). This is in agreement with the increase in peroxides observed in the Zn group (Fig. 1). The activity of CAT was not sufficient to eliminate these peroxides, as there was no change following exposure to Zn. The increase in CAT activity in the salt, salt + Zn and hardness + Zn groups could explain the lower amounts of total peroxides observed in these groups (Fig. 3c and 3d). Glutathione peroxidase is an important intracellular enzyme which catalyses the decomposition of H_2O_2 to water. The enzymes GPx and CAT together control the level of peroxides within the cell. However, in this study GPx activity was not found to be affected by Zn exposure alone, although it decreased following exposure to salt, salt + Zn, hardness or hardness + Zn. The present results indicate that CAT, along with other antioxidants such as NPSH or SOD, could be used to prevent peroxide formation or protein oxidation. Our results of water salinity and hardness in association with Zn support this suggestion, as these conditions were able to prevent some Zn-induced toxicity.

Another important enzyme in the defence against metal-generated free radicals is GST. In our experimental model, GST activity decreased in fish exposed to hard water with or without Zn compared with Zn alone (Table 2). This could be a response to combat damage from the increased TBARS and protein carbonyl levels observed in these conditions. Table 2 includes the recorded NPSH levels, which are also part of the non-enzymatic antioxidant defence system and key in the prevention of metal-induced damage through their high affinity for -SH groups. We observed an increase in NPSH levels in the Zn group compared with other groups. In the salt and salt + Zn groups, a decrease in NPSH levels was recorded compared with both the CT and Zn groups. Exposure to hard water resulted in lower levels of NPSH compared with the CT and Zn only groups. When associated with Zn, hard water did not affect the level of NPSH compared with those of the CT group. Although these changes indicate that the antioxidant defence system was activated by Zn exposure, it was not sufficient to combat the damage observed by the increased TBARS and protein carbonyl levels.

Fig. 4 illustrates the inhibition of AChE activity in fish exposed to Zn, salt, salt + Zn, hardness and hardness + Zn. This inhibition can result in excessive stimulation of the cholinergic nerves, and can be explained by the fact that metals such as Zn bind to the functional groups of enzymes, compromising their catalytic activity. Fig. 4 also shows the Na^+/K^+-ATPase activity after exposure to salt or hardness. It is known that exposure to metals increases the epithelial permeability and inhibits ion absorption, reducing Na^+/K^+-ATPase activity and deregulating osmoregulation functions in cells. We observed that Zn inhibited the activity of Na^+/K^+-ATPase in comparison with the CT group. Salt alone did not affect the enzyme activity, however, when combined with Zn an increase in activity was observed. Hardness and hardness with Zn also increase the activity of Na^+/K^+-ATPase.

3.4 Integrated biomarker response

The values of IBR for fish exposed to Zn, salt, hardness, salt + Zn and hardness + Zn were calculated to be 15.13, 15.79, 14.32, 14.25 and 16.73 respectively. The star plots indicate that SOD, H_2O_2 content, ROS, ACAP and protein carbonyl levels were found to increase in fish tissues following Zn exposure, and can be concluded to be representative biomarkers of Zn exposure, along with reduced AChE and Na^+/K^+-ATPase activities.

For the analysis of salt exposure, CAT, ACAP, SOD and TBARS levels were found to be the most representative biomarkers as they showed greater variations to compared with the CT group, along with AChE and GPx activities which decreased after exposure to salt.

Exposure to hard water caused increased levels of protein carbonylation and TBARS, as well as SOD and Na^+/K^+-ATPase activities. Reductions in ROS, ACAP and NPSH levels and GPx, GST and AChE activities were seen after exposure to hard water.

When salt was combined with Zn, increased in protein carbonyl levels, CAT, SOD, GST and Na^+/K^+-ATPase activities were observed, along with reduced levels of NPSH, H_2O_2 and TBARS and, AChE and GPx activities. When hard water was combined with Zn, the activities of AChE, GPx and GST decreased as did the levels of NPSH, ACAP and H_2O_2 compared with the CT group. Increased TBARS and protein carbonyl levels and SOD and Na^+/K^+-ATPase activities were also seen in these conditions (Fig. 5).

4. Conclusion

In conclusion although the protection from Zn toxicity afforded by salt and hard water are incomplete, some important conclusions can be drawn about the mechanism of protection. The presence of salt prevented Zn-induced lipid peroxidation, both salt and hardness were able to prevent peroxides formation caused by Zn exposure and prevented the increase in ROS that was caused by Zn exposure. Together, our results reveal that neither water salinity nor hardness were effective in protecting the guppy from Zn toxicity.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

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Table 1. Novel tank test. Effects of Zn (500 µ L^{-1}) and salt water (25 ppt) (A), and hardness water (120 mg L^{-1} $CaCO_3$) (B) in locomotor and exploratory parameters. Table shows the distance traveled (m), maximum speed (m s^{-1}), entrances in the top and time spent at top.

A	Control	Zn	Salt	Salt + Zn
Distance	3.95±0.5[a]	3.43±0.41[a]	3.94±0.63[a]	4.04±0.07[a]
Maximum speed	0.07±0.005[a]	0.30±0.09[b]	0.40±0.14[ab]	0.15±0.03[ab]
Entrances in the top	25.5±3.68[a]	19.88±4.5[a]	13.37±2.02[a]	19.75±4.41[a]
Time spent at top	146.6±16.5[a]	157.9±21.98[a]	174.3±19.93[a]	150.7±16.20[a]
B	**Control**	**Zn**	**Hardness**	**Hardness + Zn**
Distance	3.95±0.5[a]	3.43±0.41[a]	2.23±0.19[b]	2.81±0.16[ab]
Maximum speed	0.07±0.005[a]	0.30±0.09[b]	0.09±0.02[a]	0.16±0.05[a]
Entrances in the top	25.5±3.68[a]	19.88±4.5[a]	10.87±1.72[b]	12.50±1.68[b]
Time spent at top	146.6±16.5[a]	157.9±21.98[a]	138.3±18.08[a]	129.5±23.47[a]

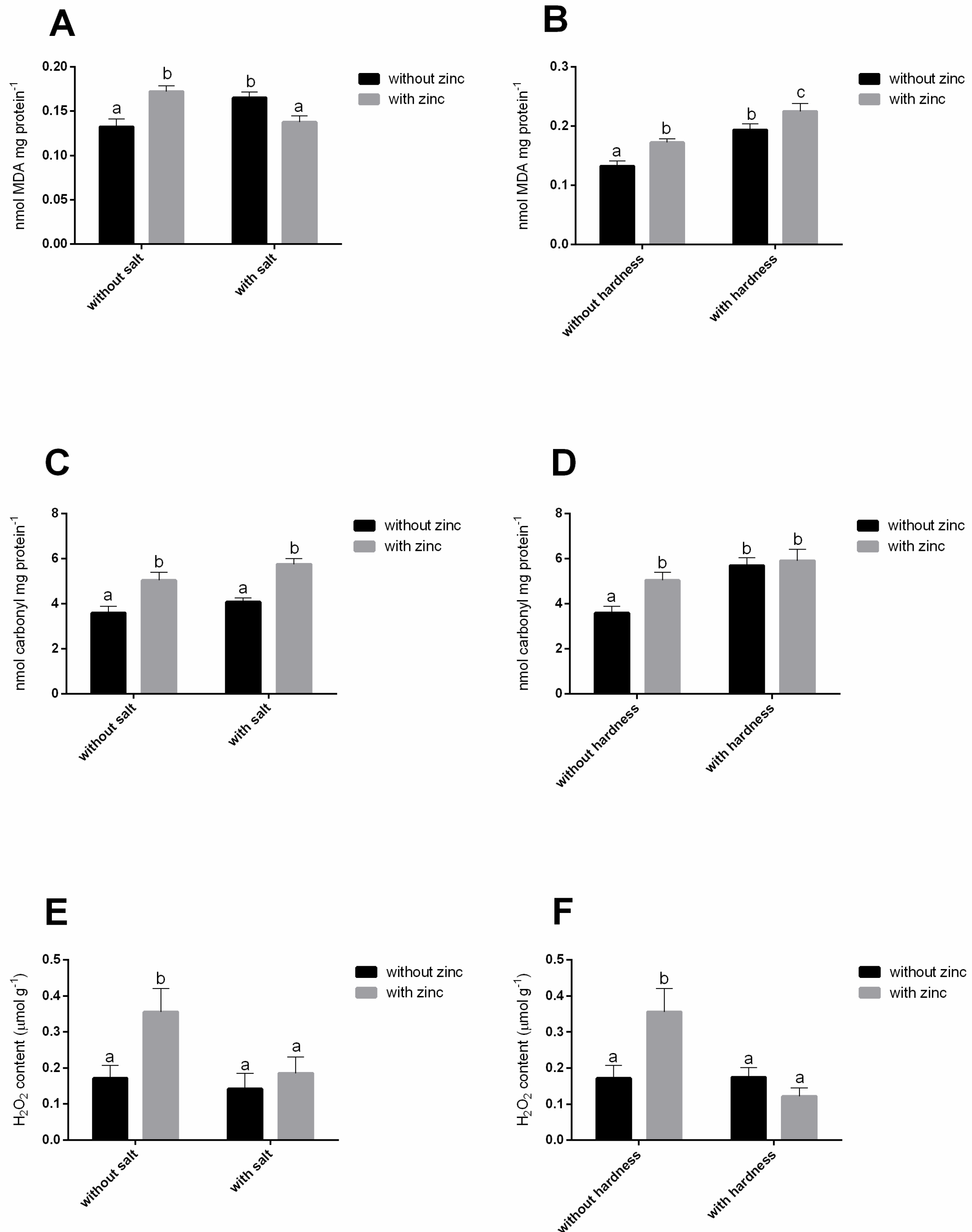
Date are expressed as means ± SEM and analyzed by two-way ANOVA followed by Newman-Keuls post-hoc. Different letters indicated significant differences among the groups ($p < 0.05$, n=8)

Table 2. Effects of Zn (500 µ L^{-1}) and salt water (25 ppt) (A), and hardness (120 mg L^{-1} $CaCO_3$) (B) in GST activity and NPSH levels in whole body of guppy (*Poecilia vivipara*).

A	Control	Zn	Salt	Salt+Zn
GST	0.085±0.005[a]	0.093±0.006[a]	0.092±0.009[a]	0.097±0.008[a]
NPSH	0.799±0.030[b]	0.882±0.020[c]	0.715±0.011[a]	0.682±0.009[a]
B	**Control**	**Zn**	**Hardness**	**Hardness + Zn**
GST	0.085±0.005[ab]	0.093±0.006[b]	0.066±0.005[a]	0.068±0.004[a]
NPSH	0.799±0.030[b]	0.882±0.020[c]	0.695±0.021[a]	0.728±0.022[ab]

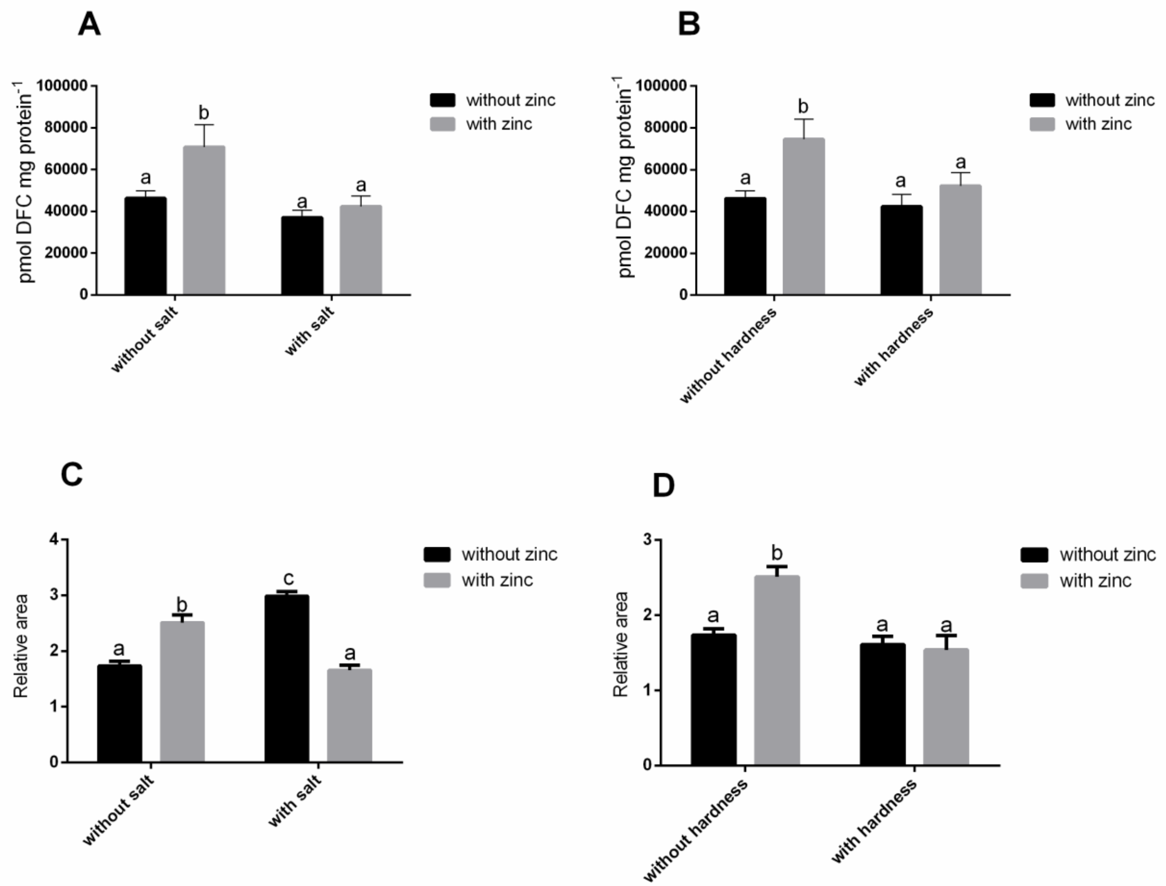
Values are means ± SEM. GST µmol GS-DNB min^{-1} mg $protein^{-1}$, NPSH µmol g^{-1} tissue. Different letters indicated significant differences among the groups ($p < 0.05$, n=8)

Fig. 1 Effects of Zn (500 µg L^{-1}) on lipid peroxidation (TBARS), protein carbonyl and H_2O_2 levels in whole body of the guppy exposed in different water conditions: salt (a, c, e) and hardness (b, d, f), respectively.



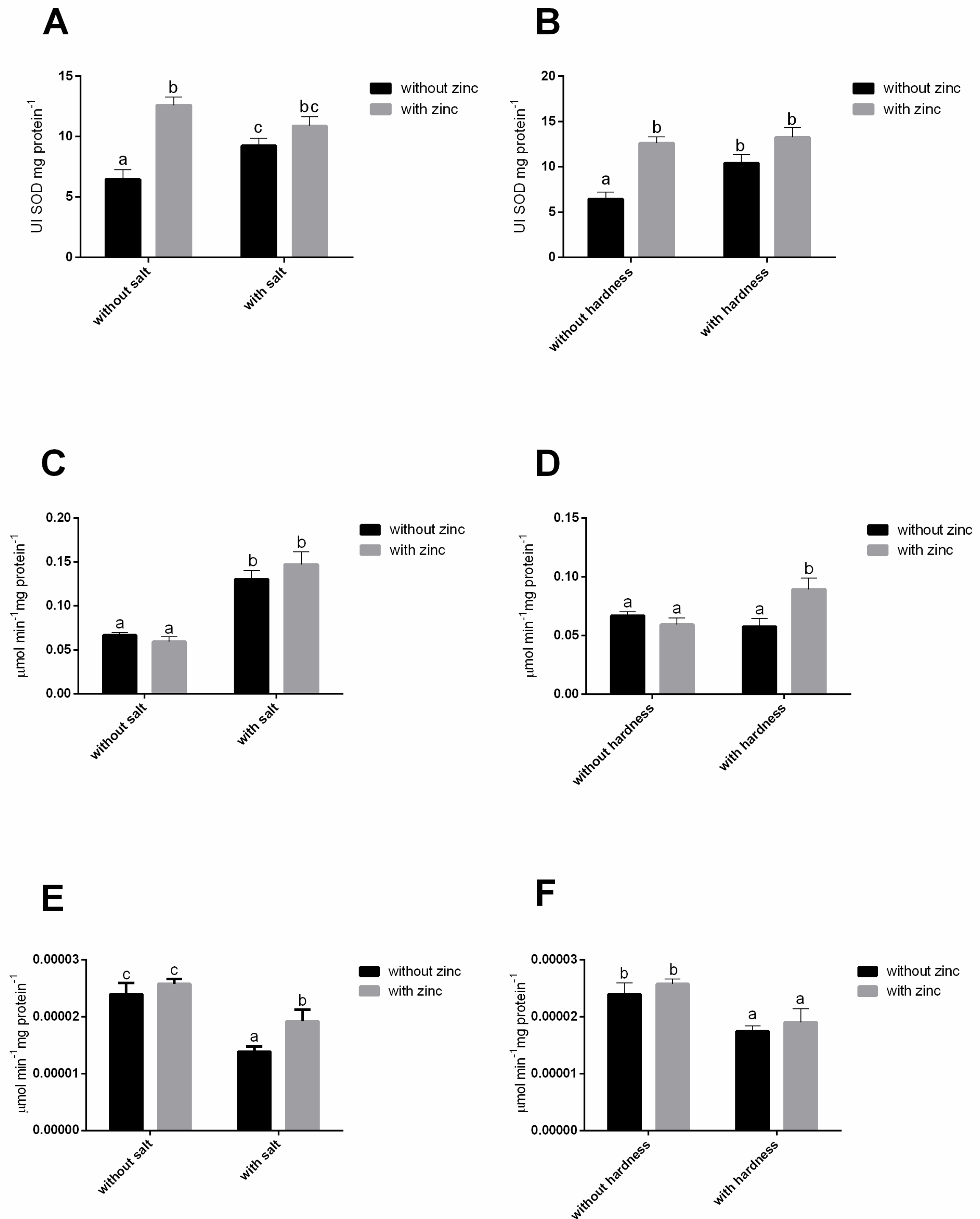
Data are expressed as mean ± SEM and analysed by two-way ANOVA, followed by Newman-Keuls post-hoc. Different letters indicate differences between groups ($p<0.05$, $n = 8$).

Fig. 2 Effects of Zn (500 µg L^{-1}) on ROS and ACAP in whole body of the guppy exposed to different water conditions: salt (a and c) and hardness (b and d), respectively.



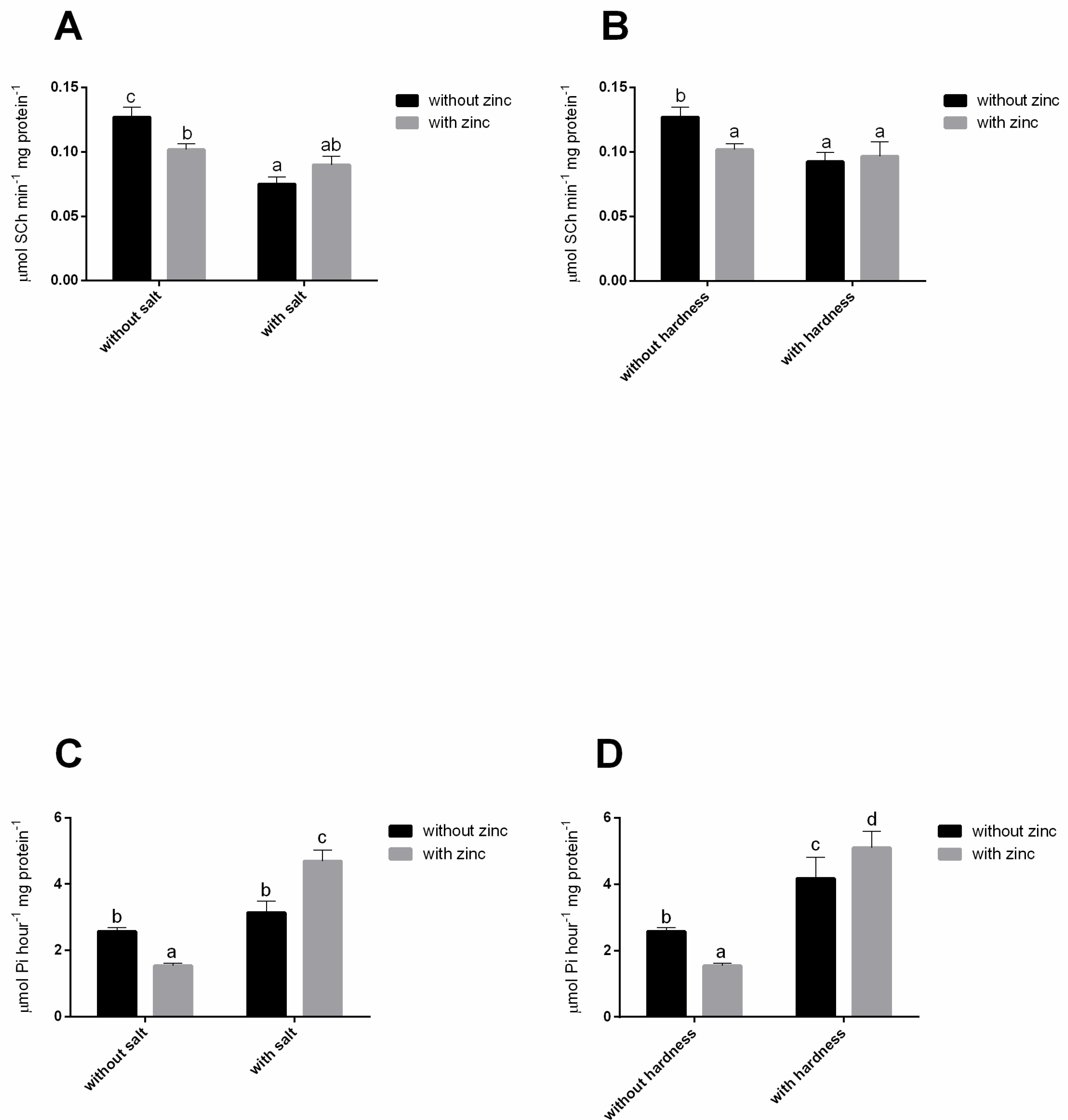
Data are expressed as mean ± SEM and analysed by two-way ANOVA, followed by Newman-Keuls post-hoc. Different letters indicate differences between groups ($p<0.05$, n = 8).

Fig. 3 Effects of Zn (500 µg L^{-1}) on SOD, CAT and GPx activities in whole body of the guppy exposed to different water conditions: salt (a, c, e) and hardness (b, d, f), respectively.



Data are expressed as mean ± SEM and analyzed by two-way ANOVA, followed by Newman-Keuls post-hoc. Different letters indicate differences between groups ($p<0.05$, n = 8).

Fig. 4 Effects of Zn (500 µg L^{-1}) on AChE and Na^+/K^+-ATPase activities in whole body of the guppy exposed to different water conditions: salt (a and c) and hardness (b and d), respectively.



Data are expressed as mean ± SEM and analyzed by two-way ANOVA, followed by Newman-Keuls post-hoc. Different letters indicate differences between groups ($p<0.05$, $n = 8$).

Fig. 5 IBR values for each treatment: zinc (a), salt (b), hardness (c), salt + zinc (d) and hardness + zinc (e). Biomarkers results are represented in relation to the reference control group. The area > 0 reflects induction of the biomarker, and that < 0 indicates reduction of the biomarker.

4 DISCUSSÃO

A poluição dos ecossistemas de água doce através de ações antropogênicas está aumentando, assim como a poluição por metais de origem natural. Metais são continuamente liberados em ambientes aquáticos, onde representam uma série de ameaças devido ao seu potencial de toxicidade, longa persistência, bioacumulação e biomagnificação na cadeia alimentar (EISLER, 1988). O Zn é um metal essencial e desempenha um importante papel em muitos processos bioquímicos, porém torna-se tóxico em altas concentrações, afetando negativamente a taxa de sobrevivência, o metabolismo energético e respostas antioxidantes (CHEN et al., 2016; LEITEMPERGER et al., 2016; LORO et al., 2012; ZHENG, J.L. et al., 2013 a, b). No meio aquático, este metal pode afetar vários organismos invertebrados e vertebrados. Uma atenção especial tem-se dado aos peixes por serem constituintes importantes do ecossistema aquático e fonte de alimentação humana (BURGER, 2008). O jundiá (*Rhamdia quelen*) é uma espécie nativa com ótimo potencial de desenvolvimento e considerado bom modelo de toxicidade, enquanto o guppy (*Poecilia vivipara*) é um peixe estuariano que tem despontado grande interesse por ser um bom indicador de poluição aquática. Há poucos estudos sobre a toxicidade de Zn nessas duas espécies. Sendo assim, este estudo buscou investigar os efeitos de concentrações ambientais relevantes do Zn para estas duas espécies de peixes.

No **artigo** podemos observar que o Zn acumulou em brânquias, fígado e intestino nas maiores concentrações testadas e não acumulou no tecido muscular. Este dado é importante, uma vez que o músculo é o tecido utilizado para o consumo humano. As baixas concentrações de Zn utilizadas e o curto tempo de exposição podem ter sido insuficientes para causar o acúmulo no músculo, já que os metais têm tendência a se acumular em tecidos gordurosos (RAHMAN et al., 2012). Outros trabalhos mostram resultados semelhantes, indicando uma preferência do metal se acumular no fígado ou brânquias em comparação com o tecido muscular (DURAL et al., 2007; LA COLLA et al., 2018; YILMAZ et al., 2010). A exposição ao Zn também causou alterações no metabolismo de carboidratos e proteínas nos jundiás, onde ocorreu uma estimulação da gliconeogênese hepática, observada através da diminuição do glicogênio no fígado e do lactato no músculo (**artigo**). Na literatura há poucos dados avaliando parâmetros metabólicos de peixes expostos a metais, mas respostas semelhantes foram encontradas por Pretto et al. (2014a) em jundiás expostos ao cádmio (Cd) por 7 e 14 dias. Nestes dois casos, os jundiás fizeram ajustes nos seus processos metabólicos para gerenciar os efeitos do estresse externo e manter a homeostase interna. Tanto na exposição ao Zn, quanto na exposição ao Cd, os peixes preferiram usar fontes de glicogênio ou outro carboidrato para

manter a bioenergética. Quando expostos ao cobre (Cu) por 45 dias, os jundiás mostraram respostas opostas ao que observamos, mostrando que houve uma preferência pela via anaeróbica de produção de energia e pelo catabolismo das proteínas do fígado para suprir a demanda energética (PRETTO et al., 2014b).

Os íons metálicos são conhecidos como indutores de estresse oxidativo e podem estimular a produção de ERO por meio de dois mecanismos diferentes. O primeiro está relacionado com a interferência de processos relacionados aos metais e o segundo, com a geração de radicais livres por íons com valência variável (LUSHCHAK, 2011). Em ambos experimentos (**artigo** e **manuscrito**), a exposição ao Zn causou danos oxidativos nos peixes, como alterações nos níveis de TBARS, PC, H_2O_2 e ERO. Resultados semelhantes foram encontrados em outros trabalhos, mostrando que mesmo em concentrações ambientais, o Zn foi capaz de causar alterações nos parâmetros oxidativos de peixes (KAYAN et al., 2015; LORO et al., 2012; PILLET et al., 2019). Devido à competição iônica, a salinidade e a dureza deveriam proteger os peixes da toxicidade de metais (LORO et al., 2012; SAGLAM et al., 2013), fato que não foi totalmente observado no **manuscrito**. Observamos que a salinidade foi eficiente em proteger os peixes apenas dos danos lipídicos causados pelo Zn. Quanto ao dano proteico, nem a salinidade, nem a dureza foram eficazes na proteção dos guppies e a dureza em associação ao Zn parece ter potencializado este dano. A exposição ao Zn aumentou o número de H_2O_2 e de ERO, porém, quando associado à salinidade ou à dureza, não houve diferença em relação ao controle, indicando que ambas foram efetivas na prevenção da formação de H_2O_2 e de ERO.

Analisando os parâmetros antioxidantes, observamos diferentes respostas em jundiás e em guppies após a exposição ao Zn. Jundiás tiveram uma diminuição na atividade da CAT e ausência de resposta da SOD (**artigo**). Já no corpo inteiro de guppy, houve um aumento da atividade da SOD e da CAT, e uma diminuição da GPx. Nos guppies, também verificamos os valores da capacidade antioxidante contra radicais peroxil (ACAP), onde áreas relativas aumentadas nos grupos Zn e salinidade, indicam capacidades reduzidas de eliminação de EROs. O aumento da atividade da SOD no **manuscrito** está de acordo com a maior quantidade de H_2O_2 observadas no grupo Zn. As enzimas CAT e GPx atuam juntas para controlar os níveis de peróxidos dentro das células, porém no **manuscrito**, a atividade da GPx não foi afetada pela exposição ao Zn e diminuiu na associação do Zn com a salinidade e com a dureza. Outra enzima importante na defesa contra os radicais livres gerados por metais é a GST. Em jundiás, a atividade desta enzima diminuiu em cérebro na concentração maior e em fígado em todas concentrações testadas (**artigo**), nos guppies ela diminuiu nos grupos dureza e dureza + Zn

(**manuscrito**). Esta diminuição, em ambos experimentos, poderia ser uma resposta em combater os danos causados pelo aumento dos níveis de TBARS e PC (**artigo** e **manuscrito**). NPSH e AA fazem parte do sistema de defesa antioxidante não enzimático são importantes na prevenção de danos induzidos por metais, uma vez que estes possuem alta afinidade pelos grupos -SH. Observamos um aumento do AA em cérebro e brânquias, e dos NPSH em músculo e brânquias de jundiás expostos ao Zn durante 96 h (**artigo**). No **manuscrito**, houve um aumento dos NPSH em guppies expostos ao Zn e uma diminuição nos grupos expostos à salinidade, dureza e salinidade + Zn. Embora essas alterações indiquem que o sistema de defesa antioxidante foi ativado após a exposição ao Zn, não foi suficiente para combater os danos observados pelos níveis de TBARS e de PC.

A atividade das enzimas AChE e Na^+/K^+-ATPase, a análise comportamental e o IBR foram realizadas apenas no experimento com os guppies (**manuscrito**). Podemos observar que houve uma inibição da atividade da AChE em corpo inteiro de guppy expostos ao Zn, salinidade, dureza, salinidade + Zn e dureza + Zn. Essa inibição pode resultar em uma estimulação excessiva dos nervos colinérgicos e pode ser explicada pelo fato de que metais como o Zn se ligam a grupos funcionais das enzimas, comprometendo sua atividade (BAINY et al., 2006). As ATPases são enzimas ligadas à membrana e são responsáveis pelo transporte de íons, ajudando a regulação do volume celular, da pressão osmótica e da permeabilidade da membrana (ATLI e CANLI, 2011; MONSERRAT et al., 2007). O mecanismo chave da toxicidade de metal tem sido relatado como um comprometimento osmorregulatório associado à inibição das ATPases nos tecidos como brânquias e rins (McGEER e WOOD, 1998). Dados da literatura mostram que há variações consideráveis de inibição ou estimulação da atividade das ATPases em peixes expostos a metais, incluindo Cd, Cu, Zn e Pb em águas duras (ATLI e CANLI, 2007; AY et al., 1999). Sabe-se que a exposição a metais aumenta a permeabilidade epitelial e inibe a absorção de íons, reduzindo a atividade da Na^+/K^+-ATPase e desregulando as funções de osmorregulação nas células. A atividade da Na^+/K^+-ATPase mostrou-se inibida em corpo inteiro de guppy após a exposição ao Zn (**manuscrito**). Esta diminuição pode estar associada ao acúmulo do metal nos tecidos e possíveis alterações estruturais nas enzimas (CANLI e STAGG, 1996; HAQUE et al., 2011; HEATH, 1987). Nos grupos dureza, salinidade + Zn e dureza + Zn, houve um aumento da atividade desta enzima. O aumento da atividade enzimática pode ser resultado de um mecanismo compensatório relacionado com as interferências iônicas (SAGLAM et al., 2013).

Alterações de respostas bioquímicas e fisiológicas também pode afetar parâmetros comportamentais em peixes. Estudos sobre o comportamento de peixes têm sido utilizados para

auxiliar na avaliação de riscos ambientais. Os guppies quando expostos ao Zn, tiveram parâmetros comportamentais avaliados através dos testes de novo tanque e de claro-escuro (**manuscrito**). O teste de novo tanque serve para avaliar a atividade motora e de locomoção. O teste de claro-escuro avalia o possível comportamento de ansiedade de peixes-zebra (KALUEFF et al., 2013; MAXIMINO et al., 2013; QUADROS et al., 2016). A única alteração observada pela exposição ao Zn foi o aumento da velocidade máxima (m/s) dos peixes. Quando em água dura, foi observada uma diminuição da distância percorrida (m) e do número de entradas no topo. A dureza em associação com o Zn também diminuiu o número de entradas no topo. A análise dos parâmetros comportamentais mostrou que os tratamentos não afetaram a exploração vertical ou a locomoção dos peixes. O teste de claro-escuro não apresentou diferenças entre os grupos, sugerindo que as exposições não afetam comportamentos relacionados à ansiedade ou medo nos peixes. Não há estudos avaliando o comportamento de *P.vivipara* após a exposição a metais. Mas para outras espécies de peixes, como por exemplo o peixe zebra, foi observada uma diminuição do comportamento locomotor e prejuízo da memória de curto prazo quando expostos em diferentes dias a $ZnCl_2$ (SARASAMMA et al., 2018). Em outro estudo, o $ZnSO_4$ causou efeitos ansiosos, mas não prolongados no comportamento do peixe zebra, onde foi observada uma diminuição da distância percorrida, do ângulo de giro, da velocidade média e do número de entradas no topo (ABREU et al., 2017). Apesar da inibição da AChE, podemos observar que não houve prejuízo nas funções locomotoras dos guppies expostos ao Zn.

Uma ferramenta útil para avaliar estes efeitos biológicos e o estado de saúde dos organismos expostos, permitindo a comparação dos efeitos de diferentes tratamentos é o IBR (MARINS et al., 2018; MURUSSI et al., 2015; VIEIRA et al., 2016; ZHENG, Q. et al., 2013). Os valores de IBR seguiram a ordem dureza + Zn > salinidade > Zn > dureza > salinidade + Zn. Mostrando que o grupo dureza + Zn foi o que teve mais alterações nos parâmetros analisados, como a diminuição das atividades de AChE, GPx e GST, assim como os níveis de NPSH, ACAP e H_2O_2. Houve um aumento nos níveis de TBARS e PC, da atividade da SOD e da Na^+/K^+-ATPase.

O Zn mostrou efeitos significativos nos parâmetros metabólicos, oxidativos e antioxidantes nos jundiás e guppies.

Uma vez que muitos estudos se utilizam de biomarcadores como ferramentas de avaliação de toxicidade a compostos, o presente estudo avaliou as respostas das duas espécies de peixes para compreender melhor a resposta à toxicidade do Zn. Considerando os biomarcadores de efeito (TBARS e PC), observamos que proteínas e lipídeos foram atacadas

pelos radicais livres. Como marcador de dano oxidativo, TBARS e PC demonstraram diferentes resultados em jundiá e em guppy. Levando em consideração que PC é irreversível e que foi afetada por todos tratamentos, esses resultados demonstram o potencial tóxico do Zn. As observações mais importantes sobre a influência da salinidade ou dureza da água, é que nenhuma delas foi efetiva na proteção do guppy a toxicidade do Zn e mais estudos seriam necessários para confirmar o mecanismo destas respostas.

5 CONCLUSÕES

A partir dos resultados deste estudo, podemos concluir que:

1. O zinco, mesmo em concentrações ambientais relevantes, foi capaz de alterar os parâmetros bioquímicos em guppy (*Poecilia vivipara*) e jundiá (*Rhamdia quelen*):

- Os parâmetros comportamentais no guppy não mostraram muitas alterações, indicando que a exposição ao Zn, à salinidade e/ou dureza não afetaram nem a locomoção, nem a exploração vertical dos peixes;
- Os parâmetros bioquímicos analisados nos guppies foram alterados pela exposição ao Zn, à salinidade e/ou dureza;
- A salinidade e a dureza não foram eficazes em proteger o guppy da toxicidade do Zn;
- Houve acumulação do zinco em brânquias, em intestino e fígado de jundiá;
- No músculo de jundiá não foi observada acumulação do metal, o que pode ser um indicativo de que em baixas concentrações não ocorre contaminação do pescado por zinco;
- O zinco causou dano oxidativo, e alterações nos parâmetros antioxidantes e metabólicos de jundiás;
- Ambas espécies foram bons modelos para o estudo da toxicidade de metais e sua interação com os parâmetros ambientais.

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ANEXO A – RESULTADOS DO TESTE DE CLARO-ESCURO

Light-dark test. Effects of Zn (500 µ L^{-1}) and salt water (25 ppt) (A), and hardness water (120 mg L^{-1} $CaCO_3$) (B) in locomotor and exploratory parameters. Table shows the entries, time (s) and mean visit in dark or light area.

A	Control	Zn	Salt	Salt + Zn
Dark: entries	2.63±0.63[a]	3.25±1.20[a]	6.00±1.97[a]	5.14±1.84[a]
Dark: time (s)	97.6±33.66[a]	29.96±6.6[a]	52.3±17.84[a]	47.24±26.55[a]
Dark: mean visit	33.27±13.57[a]	12.85±3.65[a]	8.38±3.31[a]	18.80±13.73[a]
Light: entries	2.28±0.57[a]	2.14±0.59[a]	7.86±3.03[a]	5.0±1.88[a]
Light: time (s)	196.3±35.32[a]	273.7±6.97[a]	242.9±20.17[a]	252.7±26.55[a]
Light: mean visit	105.6±39.50[a]	162.34±37.7[a]	39.40±10.6[a]	72.23±35.43[a]
B	**Control**	**Zn**	**Hardness**	**Hardness + Zn**
Dark: entries	2.63±0.63[a]	3.25±1.20[a]	4.43±1.18[a]	3.37±0.98[a]
Dark: time (s)	97.6±33.66[a]	29.96±6.60[a]	71.14±18.7[a]	61.70±21.14[a]
Dark: mean visit	33.27±13.57[a]	12.85±3.65[a]	45.80±29.19[a]	39.88±17.92[a]
Light: entries	2.28±0.57[a]	2.14±0.59[a]	4.28±1.03[a]	3.0±0.94[a]
Light: time (s)	196.3±35.32[a]	273.7±6.97[a]	203.9±26.80[a]	211.11±31.12[a]
Light: mean visit	105.6±39.50[a]	162.34±37.7[a]	42.97±9.98[a]	108.6±30.16 [a]

Date are expressed as means ± SEM and analyzed by two-way ANOVA followed by Newman-Keuls post-hoc. Different letters indicated significant differences among the groups ($p < 0.05$, n=8)

ANEXO B – CARTA DE APROVAÇÃO DA COMISSÃO DE ÉTICA

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Parâmetros bioquímicos e comportamentais em guppy (Poecilia vivipara) e jundiá (Rhamdia quelen) expostos a metais em diferentes condições ambientais.", protocolada sob o CEUA nº 5026261016, sob a responsabilidade de **Vania Lucia Loro** *e equipe; Jossiele Wesz Leitemperger; Ana Carolina Lopes Gressler; Bibiana Silveira Moraes; Tiago da Luz Fiuza* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 01/12/2016.

We certify that the proposal "Behavioral and biochemical parameters in guppy (Poecilia vivipara) and Silver catfish (Rhamdia quelen) exposed to metal in different environmental conditions ", utilizing 520 Fishes (males and females), protocol number CEUA 5026261016, under the responsibility of **Vania Lucia Loro** *and team; Jossiele Wesz Leitemperger; Ana Carolina Lopes Gressler; Bibiana Silveira Moraes; Tiago da Luz Fiuza* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 12/01/2016.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 12/2016 a 12/2018 Área: Bioquímica E Biologia Molecular

Origem:	Biotério externo						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	1 a 6 meses	N:	240
Linhagem:	Poecilia vivipara			Peso:	1 a 5 g		
Origem:	Biotério externo						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	1 a 6 meses	N:	280
Linhagem:	Rhamdia quelen			Peso:	10 a 18 g		

Resumo: Com a rápida urbanização e intenso desenvolvimento da indústria e da agricultura, a poluição de ecossistemas aquáticos com metais pesados tornou-se um problema a nível mundial, devido ao seu longo tempo de persistência na natureza, bioacumulação e possível biomagnificação. A exposição de peixes a metais pesados pode resultar em um aumento de espécies reativas ao oxigênio, podendo comprometer o metabolismo oxidativo normal e causar mudanças comportamentais. Metais essenciais, como o zinco (Zn) e o cobre (Cu) desempenham um importante papel nas atividades biológicas destes organismos, e os metais não essenciais, como o cádmio (Cd) e o chumbo (Pb) são elementos muito tóxicos, exercendo uma série de efeitos patológicos em peixes, afetando o crescimento, a reprodução, as funções respiratórias e a osmorregulação. Até mesmo os metais essenciais podem ser tóxicos para as atividades biológicas dos peixes acima de determinadas concentrações. Entre os fatores ambientais que podem afetar a biodisponibilidade de metais, a salinidade, bem como a dureza da água, pode alterar tanto a fisiologia do organismo, bem como a biodisponibilidade e especiação de metais. Com isso, o objetivo deste trabalho será avaliar alterações bioquímicas e comportamentais em guppy (Poecilia vivipara) e jundiá (Rhamdia quelen) expostos a concentrações subletais de cádmio, chumbo, cobre e zinco em água com diferentes salinidades e durezas. Os peixes serão expostos a concentrações subletais, considerando o previsto na resolução do CONAMA (2005). Cada metal será adicionado na água e os peixes permanecerão expostos por 96 horas, de acordo com experimentos prévios (Leitemperger et al., 2016). Passado esse período, será feita análise comportamental, logo após, os peixes serão anestesiados, eutanasiados e serão coletados cérebro, fígado, brânquias, rim, intestino e músculo para posteriores análises. Desta forma, pretende-se observar se as concentrações de metais permitidas pela legislação causam alterações bioquímicas em P.vivipara e R.quelen. Outro aspecto será verificar se a salinidade e/ou a dureza atuam como fator de proteção contra a toxicidade de metais nos tecidos destes organismos, comparando uma espécie que tem facilidade em fazer osmorregulação (P. vivipara) com uma espécie pouco resistente a essas mudanças ambientais (R. quelen). Salienta-se que a espécie Rhamdia quelen possui grande aceitação comercial, e uma vez comprovando-se neste estudo que a salinidade ou dureza da água podem reduzir a toxicidade de metais, podemos futuramente aplicar estes resultados em sistemas de criação com problemas de contaminação por metais na água. CONAMA (Conselho Nacional do Meio Ambiente), 2005. Resolução CONAMA nº357 de 17/03/05. http://www.mma.gov.br/port/conama/legiabre.cfm?codlegi=459. Access in: 18.09.2016. Leitemperger, J., Menezes, C., Santi, A., Murussi, C., Lópes, T., Costa, M., Nogueira, L.S., Loro, V.L. Early biochemical biomarkers for zinc in silver

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catfish (Rhamdia quelen) after acute exposure. Fish Physiology and Biochemistry, v.42 (3), p.1005-1014, 2016.

Local do experimento: Os peixes serão aclimatados e mantidos durante o período experimental no biotério de experimentação do laboratório de fisiologia de peixes sobre responsabilidade do professor Bernardo Baldisserotto e professora Vania Lucia Loro.

Santa Maria, 04 de dezembro de 2016

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

ANEXO C – COMPROVANTE DE SUBMISSÃO DO MANUSCRITO

Molecular Biology Reports

Behavioural and biochemical parameters in guppy (Poecilia vivipara) following exposure to waterborne zinc in salt or hard water

--Manuscript Draft--

Manuscript Number:	MOLE-D-18-02057R1
Full Title:	Behavioural and biochemical parameters in guppy (Poecilia vivipara) following exposure to waterborne zinc in salt or hard water
Article Type:	Original Article
Keywords:	estuarine fish; environmental risk; IBR; hardness; metal; Salinity
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	Conselho Nacional de Desenvolvimento Científico e Tecnológico (309314/2017-8) — Drª Vania Lucia Loro
Abstract:	Zinc is an essential trace mineral that is involved in many biological processes. In elevated concentrations, this metal may have toxic effects for aquatic organisms. Physicochemical properties of water, such as salinity and hardness, can influence the bioavailability of zinc and, therefore its toxicity in aquatic environments. Therefore, this study aimed investigate the influence of salinity, hardness on Zn toxicity on the behaviours and biochemical parameters of the estuarine guppy (Poecilia vivipara). The fish were exposed to waterborne zinc (500 µg L-1) in salt water (25 ppt) or hard water (120 mg L-1 CaCO3). For behavioural analysis, the locomotive and exploratory parameters of fish in novel environment and light-dark tests were evaluated. We observed that exposure to hard water decreased the distance covered by the fish, and when zinc also present the vertical exploratory behavior decreased. When zinc was tested alone, an increase in the maximum speed of fish was recorded. Activities of antioxidant enzymes, levels of lipid peroxidation, protein carbonylation, total peroxidation and, reactive oxygen species content, antioxidant capacity against peroxyl radicals, non-proteins thiols levels, acetylcholinesterase and Na+/K+-ATPase activities were evaluated in the whole fish body. The integrated biomarker response (IBR) was

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	calculated for each parameter to aid in the interpretation of the results and indicated that hard water containing zinc had the greatest effect on the biochemical parameters of the fish. In general, neither salinity nor hardness were totally effective in protecting the guppy from the biochemical damage caused by exposure to zinc.