JHEIMISON JUNIOR DA SILVA ROSA

RESPOSTAS DE MÚLTIPLOS BIOMARCADORES EM *Aegla castro*: UM MODELO ECOTOXICOLÓGICO PARA A AVALIAÇÃO DA QUALIDADE DE RIACHOS

Londrina
2022

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutor.

Orientador: Prof.ª Dr.ª Claudia Bueno dos Reis Martinez.

Londrina
2022

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Londrina, 16 de dezembro de 2022.

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“There are no freshwater Crustacea at all like *Aegla*
anywhere else in the world.”

Waldo Schmitt (1942)

ROSA, Jheimison Junior da Silva. **Respostas de múltiplos biomarcadores em *Aegla castro*:** um modelo ecotoxicológico para a avaliação da qualidade de riachos. 2022. 135 f. Tese (Doutorado em Ciências Biológicas) – Universidade Estadual de Londrina, Londrina, 2022.

RESUMO GERAL

O uso intensivo de insumos agrícolas tem contribuído significativamente para a degradação de rios e riachos, principalmente devido ao aporte de contaminantes na água. O cobre (Cu) é um exemplo de metal essencial que é amplamente empregado na composição de agrotóxicos e fertilizantes e que causa toxicidade a invertebrados aquáticos por meio da bioacumulação, estresse oxidativo e disrupção osmo-iônica. Os caranguejos do gênero *Aegla* são os decápodes dulcícolas mais ameaçados da América do Sul e podem ocorrer em cursos de água contaminados por Cu. Nesse sentido, a análise de múltiplos biomarcadores constitui uma estratégia relevante para diagnosticar precocemente os efeitos da contaminação por Cu em eglídeos, assim como para avaliar o potencial destes animais como modelos biológicos em estudos ecotoxicológicos. Portanto, este trabalho avaliou a resposta de múltiplos parâmetros funcionais (enzimas de osmorregulação e concentração de íons, enzimas antioxidantes, moléculas geradas por dano oxidativo, bioacumulação e composição hemocitária) como potenciais biomarcadores da exposição ao Cu em *Aegla castro*, assim como a aplicabilidade desta espécie como modelo biológico. No capítulo I, a concentração de Cu dissolvido na água do rio Couro foi aferida e vários parâmetros funcionais foram caracterizados em animais coletados diretamente do campo em julho de 2018 e em janeiro de 2019. Na segunda coleta, em janeiro de 2019, um aumento na concentração de Cu dissolvido na água foi observado (0.72 $\mu g\ L^{-1}$) quando comparado à primeira coleta em julho 2018 (0.01 $\mu g\ L^{-1}$); assim como alterações de parâmetros funcionais nos animais coletados em janeiro de 2019, como aumento da concentração de Cu nas brânquias e no músculo. A presença de Cu na água do riacho e a bioacumulação deste metal em tecidos pode ter levado à inatividade de enzimas relacionadas à osmorregulação e a uma disrupção iônica, seguida da ativação de defesas antioxidantes no hepatopâncreas de *A. castro*. Além disto, neste capítulo caracterizou-se pela primeira vez as categorias de hemócitos presentes na hemolinfa de uma espécie de *Aegla*: hemócitos hialinos, semigranulares e granulares. Para o capítulo II foram conduzidas exposições agudas (24 h) em laboratório a 11 $\mu g\ L^{-1}$ de Cu dissolvido (Cu 11) e a água sem adição de Cu (CTR), a fim de avaliar os efeitos isolados deste metal na regulação osmoiônica de *A. castro*. Após 24 h de exposição, os animais apresentaram disrupção osmoiônica, evidenciada pela inibição da atividade da anidrase carbônica (AC) nas brânquias, e um aumento das concentrações de Ca^{2+} e Mg^{2+} na hemolinfa, indicando a ativação de mecanismos que anteciparam o ciclo de muda em animais expostos ao Cu. No capítulo III foi observado um aumento na concentração de Cu e do conteúdo de metalotioneínas nas brânquias, indicando que o acúmulo de Cu levou à ativação de mecanismos de detoxificação neste tecido. No hepatopâncreas foi observado uma diminuição do conteúdo de metalotioneínas, evidenciando um consumo de metalotioneínas no hepatopâncreas após 24 h de exposição ao Cu. Além disso, o número de hemócitos hialinos e totais foi maior nos eglídeos do Cu 11, sugerindo a contagem de hemócitos como um importante biomarcador de resposta imune em eglídeos expostos ao Cu. Não foram observados danos no DNA nos animais expostos ao Cu por meio do ensaio alcalino do cometa. Este estudo demonstrou que *A. castro* é um modelo biológico adequado em estudos ecotoxicológicos, apresentando sensibilidade ao Cu tanto em exposições em campo quanto em laboratório.

Palavras-chave: *Aegla*; bioacumulação; cobre; estresse oxidativo; osmorregulação.

ROSA, Jheimison Junior da Silva. **Multiple biomarker responses in *Aegla castro*:** an ecotoxicological model to the assessment of stream quality. 2022. 135 pp. Thesis (Doctorate's degree in Biological Sciences) – Universidade Estadual de Londrina, Londrina, 2022.

ABSTRACT

The intensive use of agrochemicals has significantly contributed to the degradation of rivers and streams, mainly due to contaminant inputs into the water. Copper (Cu) is an example of essential metal applied in the composition of pesticides and fertilizers which causes toxicity to aquatic invertebrates through bioaccumulation, oxidative stress, and osmoionic disruption. The crabs of the genus *Aegla* are the most endangered freshwater decapods in South America and may occur in Cu-contaminated watercourses. In this sense, the analysis of multiple biomarkers is a relevant approach for early diagnosis of the harmful effects of Cu contamination in aeglids, as well as for assessing the potential of these animals as biological models for ecotoxicological studies. Therefore, this study evaluated the response of multiple functional parameters (osmoregulation enzymes and ion concentration, antioxidant enzymes, molecules originated by oxidative damage, bioaccumulation and hemocyte composition) as potential biomarkers of Cu exposure in *Aegla castro*, and the applicability of this species as a biological model. In chapter I, the Cu dissolved concentration in the water of the Couro stream was measured and several functional parameters were characterized in animals collected directly from the field in July 2018 and January 2019. In the second collection, in January 2019, an increase in dissolved Cu concentration in the water was observed (0.72 μg L^{-1}), when compared to the first collection in July 2018 (0.01 μg L^{-1}); as well as changes in some functional parameters, such as increased Cu concentration in gills and muscle. The presence of Cu in the stream water and the bioaccumulation of this metal in tissues might have led to the inhibition of osmoregulation-related enzymes and an osmo-ionic disruption, followed by the activation of antioxidant hepatopancreatic defenses in *A. castro*. Besides, in this chapter, we characterized for the first time the three hemocyte categories in the hemolymph of *A. castro*: hyaline, semigranular, and granular hemocytes. In chapter II we conducted acute exposures (24 h) in laboratory conditions to 11 μg L^{-1} dissolved Cu (Cu 11 group) and to water without Cu addition (CTR group), to assess the isolated effects of this metal in the osmo-ionic regulation of *A. castro*. After 24 h of exposure, the animals showed osmo-ionic disruption, evidenced by inhibition of the carbonic anhydrase (CA) in gills, and an increase in the Ca^{2+} and Mg^{2+} concentration in the hemolymph, indicating the activation of mechanisms that anticipated the molting cycle in animals exposed to Cu. In chapter III we observed increased Cu concentration and MT content in gills, indicating that Cu accumulation led to the activation of detoxification mechanisms in this tissue. We observed decreased MT content in hepatopancreas, evidencing a consumption of MT after 24 h of Cu exposure. In addition, the number of hyaline and total hemocytes was higher in the animals from the Cu 11, suggesting the hemocytes count as an important biomarker of immune response in aeglids exposed to Cu. We did not observe DNA damage in the animals exposed to Cu using the alkaline comet assay. This study demonstrated that *A. castro* is a suitable biological model in ecotoxicological studies, presenting sensitivity to Cu both in field or laboratory exposures.

Keywords: *Aegla*; bioaccumulation; copper; oxidative stress; osmoregulation.

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LISTA DE ABREVIATURAS E SIGLAS

AC	anidrase carbônica
AChE	acetilcolinesterase
Ca^{2+}	cálcio
Ca^{2+}/H^{+}	trocador Ca^{2+}/H^{+}
$Ca^{2+}/2H^{+}$	trocador $Ca^{2+}/2H^{+}$
$Ca^{2+}/2\text{-}3Na^{+}$	trocador $Ca^{2+}/2\text{-}3Na^{+}$
CAT	catalase
CATP	Ca^{2+}-ATPase
CDNB	1-cloro-2, 4-dinitrobenzeno
CONAMA	Conselho Nacional do Meio Ambiente
Cl^{-}	cloreto
Cu	cobre
Cu^{+}	cuproso
Cu^{2+}	cúprico
DMSO	dimetilsulfóxido

DNPH	dinitrofenilidrazina
DTNB	ácido 5,5'-ditio-bis-(2-nitrobenzoico)
EDTA	ácido etilenodiamino tetra-acético
ERO	espécie reativa de oxigênio
FURG	Universidade Federal do Rio Grande
GPx	glutationa peroxidase
GSH	glutationa reduzida
GSSG	glutationa oxidada
GST	glutationa *S*-transferase
H^+	próton
H_2O_2	peróxido de hidrogênio
HATP	H^+-ATPase
HCO_3^-	bicarbonato
HNO_3	ácido nítrico
K^+	potássio
IBRv2	índice de resposta de biomarcadores versão 2
$L^{\bullet}$	radical lipídio
LH	ácido graxo
LPO	lipoperoxidação
MDA	malondialdeído
MT	proteínas semelhantes a metalotioneínas
Na^+	sódio
Na^+/H^+	trocador Na^+/H^+
NaCl	cloreto de sódio
NADH	nicotinamida adenina dinucleotídeo
NADPH	nicotinamida adenina dinucleotídeo fosfato
NaOH	hidróxido de sódio
NEM	N-etilmaleimida
NKA	Na^+/K^+-ATPase
NKCC	co-transportador $Na^+/K^+/2Cl^-$
NPSH	tiois não-proteicos
$O_2^{\bullet-}$	radical superóxido
O_2	oxigênio

$^{\bullet}OH$	radical hidroxila
OH^{-}	ânion
PCC	conteúdo de proteínas carboniladas
-SH	grupamento tiol
SOD	superóxido dismutase
TBARS	substâncias reativas ao ácido tiobarbitúrico
UEL	Universidade Estadual de Londrina
UFPR	Universidade Federal do Paraná
UFSM	Universidade Federal de Santa Maria

SUMÁRIO

1 APRESENTAÇÃO

Esta tese está estruturada na forma de artigos científicos, seguindo as orientações do Programa de Pós-graduação em Ciências Biológicas da Universidade Estadual de Londrina. Cada um dos capítulos segue as normas dos respectivos periódicos aos quais eles serão submetidos para publicação.

A **INTRODUÇÃO GERAL** contém os principais conceitos necessários para guiar o leitor a compreender os capítulos, assim como contém a justificativa, os objetivos gerais e específicos e as hipóteses do trabalho.

O **Capítulo I** intitulado "Characterization of the hemocytes and parameters related to oxidative stress and osmoregulation of the freshwater anomuran crab *Aegla castro*" busca padronizar a utilização de diversos parâmetros em *A. castro* e recomendá-los como biomarcadores para avaliar os efeitos do cobre em eglídeos. Este capítulo está em preparação para ser submetido no periódico "Ecological Indicators".

O **Capítulo II** intitulado "Short communication: Effects of acute copper exposure on ionic regulation of the freshwater crab *Aegla castro*" foi publicado em 2021 no periódico "Comparative Biochemistry and Physiology – Part C" e aponta os efeitos da exposição ao cobre na osmorregulação em *A. castro*.

O **Capítulo III** intitulado "Multiple biomarker responses in *Aegla castro* exposed to copper: a laboratory approach" apresenta de forma integrada os efeitos de múltiplos biomarcadores em *A. castro* expostas ao cobre, assim como enfatiza a importância de estudos sob uma abordagem experimental em laboratório. Este capítulo está em preparação para ser submetido no periódico "Aquatic Toxicology".

As **Conclusões gerais** são apresentadas na forma de tópicos para a melhor compreensão dos resultados gerais dos manuscritos. As **Considerações finais** da tese apresentam uma discussão integrada dos resultados dos manuscritos e comentários sobre conservação de eglídeos.

Introdução geral

2 INTRODUÇÃO GERAL

2.1 DEGRADAÇÃO E CONTAMINAÇÃO DE ECOSSISTEMAS AQUÁTICOS NA AMÉRICA DO SUL

Os ambientes de água doce da América do Sul são formados por rios, riachos e lagos com altíssima riqueza de espécies e altas taxas de endemismo, representando os ecossistemas aquáticos com as maiores biodiversidades do mundo (Abell, 2002; Abell et al., 2008). No entanto, estes ambientes dulcícolas são também os ecossistemas aquáticos mais vulneráveis do mundo, principalmente devido à pressão antropogênica, advinda do crescimento populacional e urbanização (Torremorell et al., 2021).

A América do Sul apresentou um crescimento populacional de cerca de 1% ao ano no intervalo de 2000 a 2015 (United Nations, 2017), o que acarreta um aumento na demanda de energia, assim como um aumento no crescimento industrial e na produção de alimentos. Por sua vez, isso tem levado a um aumento da demanda por recursos naturais e na entrada de xenobióticos nos cursos de água, causando degradação de habitat e declínio de populações de muitas espécies de invertebrados (Strayer, 2006). Dentre as pressões ambientais que os ecossistemas de água doce sul-americanos têm enfrentado, destacam-se a eutrofização, perturbações hidrológicas, mudanças climáticas, sobre-exploração de recursos, alteração da paisagem pelo desmatamento e usos do solo e aporte de contaminantes devido à agricultura intensiva e mineração (Torremorell et al., 2021).

A liberação de efluentes industriais e agrícolas têm contribuído de maneira decisiva para a poluição e degradação de ambientes dulcícolas. Dentre esses efluentes, os metais constituem uma das principais classes de contaminantes de preocupação global, seja pela sua toxicidade, natureza e persistência, seja pela dificuldade de metabolização nos sistemas biológicos e potencialidade de acúmulo nas cadeias tróficas (Chowdhary et al., 2018; Tang et al., 2017).

Os metais podem atingir os cursos de água a partir de diversas fontes, tais como processos geoquímicos, esgoto doméstico, mineração e liberação de efluentes industriais e agrícolas (Nordberg et al., 2007). Na última década, dois desastres emblemáticos foram responsáveis pelo aporte significativo de diversos metais em ecossistemas aquáticos sul-americanos, os rompimentos das barragens de Fundão (Mariana-MG) em 2015 e da Mina do Feijão (Brumadinho-MG) em 2019, ambas da empresa Vale S.A. Estes dois desastres acarretaram prejuízos inestimáveis à qualidade de habitat de rios e riachos no sudeste do Brasil, causando efeitos subletais da exposição a metais em comunidades aquáticas (Marques

et al., 2022; Siqueira et al., 2022).

Diversos metais podem contaminar os ambientes aquáticos por meio da liberação de fertilizantes e agrotóxicos que contém esses elementos em sua composição, tais como cobre (Cu), zinco (Zn), níquel (Ni), manganês (Mn), cobalto (Co), ferro (Fe) e chumbo (Pb) (Nordberg et al., 2007). O incremento de metais a partir de efluentes agrícolas é particularmente importante no Brasil, já que o país tornou-se o maior consumidor de agrotóxicos do mundo (Albuquerque et al., 2016), assim como o quarto maior consumidor de fertilizantes minerais dentre 120 países (apenas atrás de China, Índia e Estados Unidos), tendo esse recorde sido obtido apenas nos últimos 60 anos da série histórica (Fig. 1) (Gonçalves et al., 2018). Esse aumento vertiginoso no consumo de insumos agrícolas tem sido acompanhado por um aumento igualmente expressivo no aporte de metais no solo e nos ambientes aquáticos (Gonçalves et al., 2018).

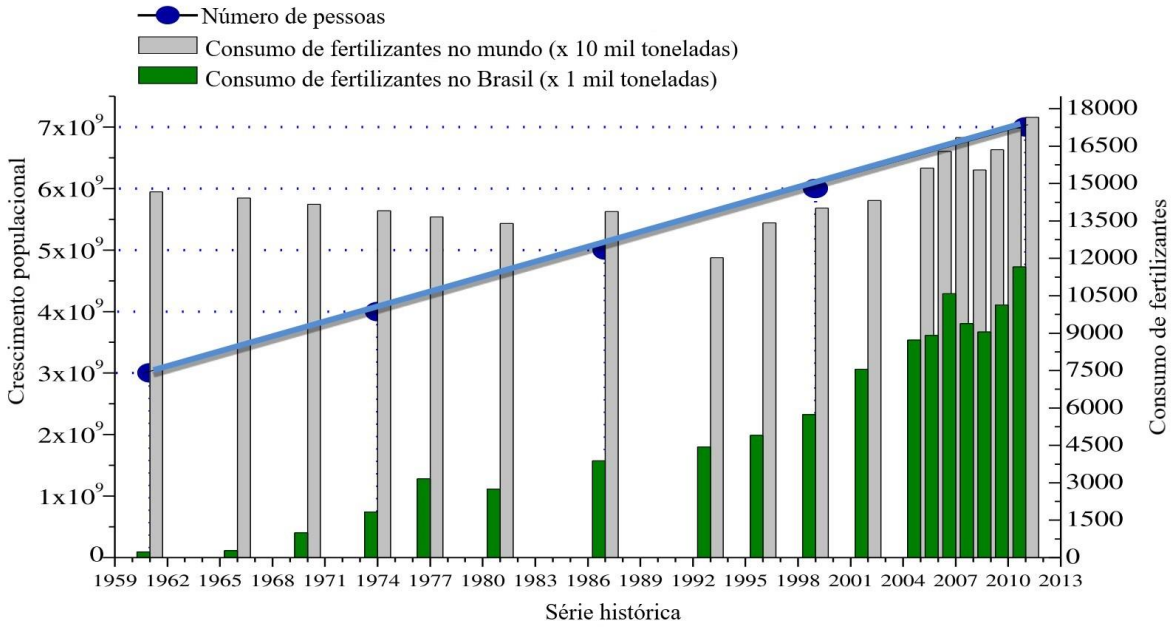

Figura 1. Crescimento populacional e consumo de fertilizantes no Brasil e no mundo.
Fonte: adaptado de Gonçalves et al. (2018).

É importante notar que o percentual de propriedades rurais que fazem uso intensivo de agrotóxicos não é expressivo apenas em estados do Brasil com grandes propriedades rurais (como Mato Grosso e Rondônia), mas também em estados das regiões sul e sudeste do Brasil (como São Paulo, Santa Catarina, Paraná e Rio Grande do Sul), cujas propriedades rurais são de menores dimensões (Fig. 2) (IPEA, 2019). Para além da degradação ambiental intrínseca, a utilização de agrotóxicos pode levar a um aumento da despesa financeira associada a intoxicação aguda de trabalhadores rurais. Londres (2011)

discutiram sobre os custos e benefícios associados à utilização de agrotóxicos em lavouras do estado do Paraná e relataram um aumento de 64% de despesa com o Sistema Único de Saúde para o estado quando o cultivo de milho é baseado na utilização de fertilizantes e agrotóxicos, e em contrapartida, esses custos representariam apenas 8% implementando-se o cultivo livre de agrotóxicos.

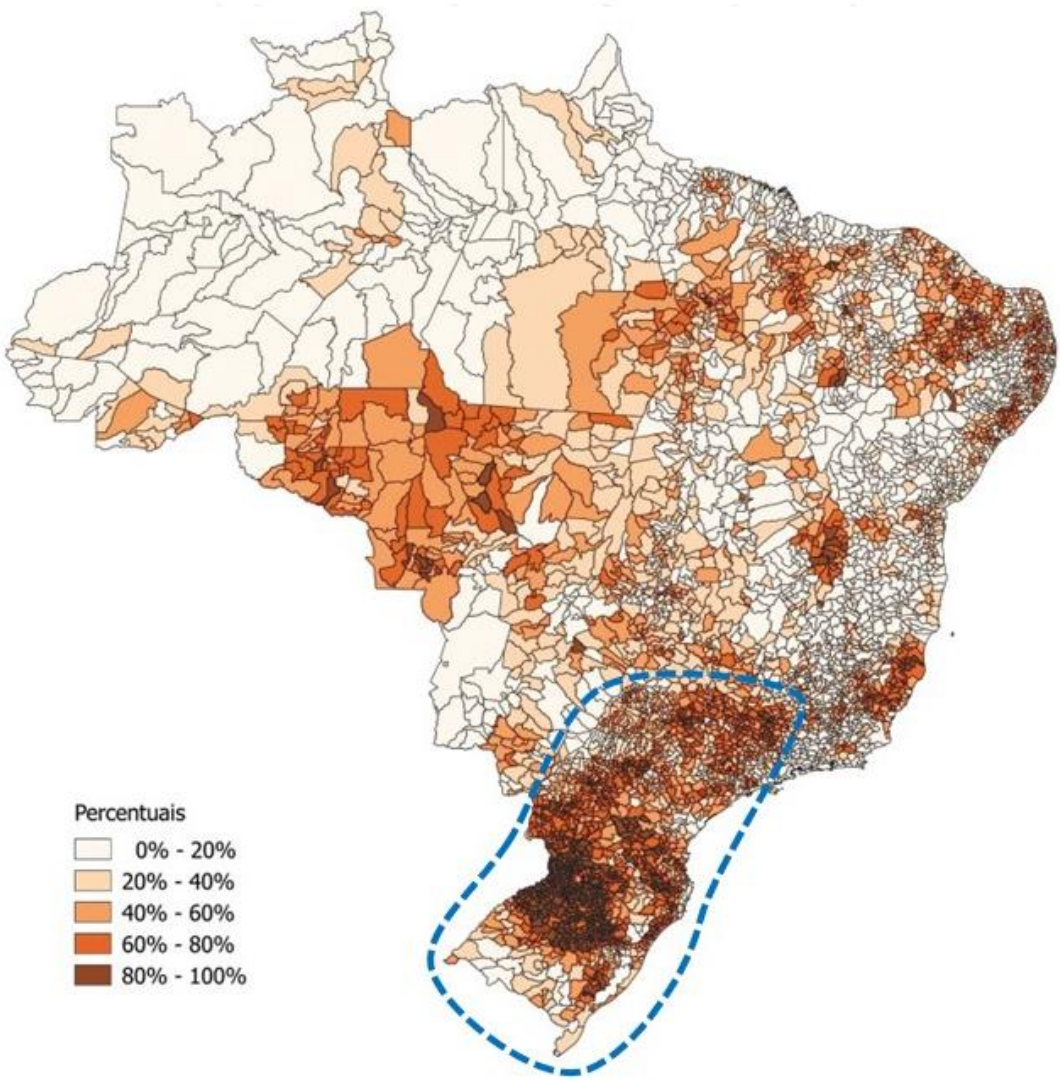

Figura 2. Percentual de propriedades rurais que utilizam agrotóxicos nas lavouras, por município, em 2017. O perímetro tracejado em azul indica os estados com alto percentual de propriedades rurais que usam agrotóxicos nas regiões sul e sudeste do Brasil.
Fonte: adaptado de IPEA (2019).

O cobre (Cu) é um exemplo de metal amplamente aplicado em processos industriais, na fabricação de ligas metálicas, encanamentos, tintas e fiação elétrica, assim como na agricultura, na produção de fertilizantes, algicidas e fungicidas que contém Cu em

sua composição (Li et al., 2020; Simonato et al., 2016). O oxicloreto de cobre, por exemplo, é um princípio ativo de agrotóxicos que esteve entre os 10 ingredientes mais aplicados em lavouras no Brasil, somando sete mil toneladas consumidas em 2017 (IPEA, 2019). Li et al. (2020), em um estudo de monitoramento da contaminação por Cu na China, evidenciaram que o uso intensivo de fertilizantes e agrotóxicos nos últimos 30 anos foi um dos principais fatores responsáveis pelo aumento significativo da concentração de Cu em solos agricultáveis. Os autores enfatizaram que esse consumo intensivo tem favorecido o aporte de Cu em ecossistemas aquáticos por meio de processos de lixiviação de solos contaminados, contribuindo para que o Cu seja considerado atualmente como um contaminante globalmente ubíquo.

No Brasil, o Cu tem sido reportado como um contaminante recorrente tanto na água quanto no sedimento de muitos ambientes aquáticos das regiões sul e sudeste (Bidone et al., 2001; Blume et al., 2010; Costa et al., 2013; Dalzochio et al., 2018; Niencheski et al., 2006; Pestana et al., 1997), incluindo ecossistemas de riachos que abrigam grande diversidade de espécies de macroinvertebrados, como os caranguejos eglídeos, por exemplo (Borges et al., 2022; Faria et al., 2018; IAP, 2012; Rosa and Martinez, 2021).

2.2 EGLÍDEOS: OS DECÁPODES DE ÁGUA DOCE MAIS AMEAÇADOS DA AMÉRICA DO SUL

Os rios e riachos são ecossistemas dulcícolas extremamente. Estes ambientes são emblemáticos do ponto de vista conservacionista, já que apresentam altíssima diversidade de espécies de vertebrados e invertebrados (Malmqvist e Rundle, 2002). No entanto, devido ao fato de que as proximidades dos rios e riachos foram historicamente habitadas por populações humanas e sobre-exploradas ao longo dos anos para a obtenção de recursos e destinação de rejeitos (Malmqvist e Rundle, 2002), a degradação de águas continentais constitui uma das principais ameaças à biodiversidade dulcícola global, principalmente para aquelas espécies altamente endêmicas e com área de distribuição restrita. Nesse sentido, os caranguejos anomuros do gênero *Aegla* Leach, 1820 (Aeglidae Dana, 1852) constituem um exemplo típico de fauna endêmica da região Neotropical que é muito sensível aos efeitos da degradação de habitat e da contaminação aquática (Fig. 3) (Bond-Buckup et al., 2008; Bueno et al., 2016; Santos et al., 2017).

Os eglídeos são crustáceos de água doce notáveis em muitos aspectos. Do ponto de vista evolutivo, todas as espécies de eglídeos pertencem a um único gênero atual (*Aegla*), e a uma única família (Aeglidae), sendo esta família a única dentre os crustáceos anomuros que contém representantes exclusivamente dulcícolas (Bartholomei-Santos et al., 2020). Os ancestrais marinhos dos eglídeos atuais invadiram as águas continentais durante as grandes transgressões marinhas do Cretáceo Inferior há cerca de 75 milhões de anos atrás. Desde então, estas populações tiveram um sucesso formidável na colonização de ambientes dulcícolas e se dispersaram ao longo das bacias hidrográficas durante o soerguimento da Cordilheira dos Andes e durante a formação das drenagens neotropicais (Pérez-Losada et al., 2004). O sucesso na colonização das águas doces se deveu muito aos ajustes na osmolalidade da hemolinfa (~ 400 mOsm kg H_2O^{-1}), o que garantiu uma vantagem evolutiva expressa na economia de energia para hiper-regular em meios diluídos (Bozza et al., 2019; Faria et al., 2011). Por outro lado, quando comparados a outros grupos de invertebrados de água doce, os eglídeos apresentam as maiores osmolalidades dos fluidos corporais, evidenciadas pela presença de cutícula e de sua recente ancestralidade marinha (Bozza et al., 2019).

Figura 3. Vista dorsal de um espécime macho de *Aegla castro*, coletado no rio Couro, Mauá da Serra, Paraná, Brasil.
Fonte: o próprio autor.

Do ponto de vista ecológico, os caranguejos eglídeos são exigentes quanto à qualidade de habitat, habitando águas limpas e bem oxigenadas de riachos, arroios, lagos, lagoas e cavernas (Bond-Buckup e Buckup, 1994). Além disso, eles são elos essenciais nas cadeias tróficas de pequenos riachos, já que contribuem para a ciclagem de nutrientes se alimentando de macrófitas, larvas de insetos, microcrustáceos, matéria orgânica alóctone e microflora do sedimento, bem como servindo de alimento para aves, anfíbios e peixes (Arenas, 1976; Bond-Buckup e Buckup, 1994). Assim, muitos estudos têm considerado estes animais como bioindicadores de qualidade de água e habitat (Bortoluzzi et al., 2007; Correa-Araneda et al., 2010; Trevisan et al., 2009).

Em decorrência do sucesso de adaptação aos ambientes dulcícolas do sul da região Neotropical, *Aegla* tornou-se um gênero muito especioso ao colonizar cursos de água de várias ordens durante a formação das paleo-drenagens da América do Sul e o soerguimento da Cordilheira dos Andes. Cerca de 90 espécies são conhecidas até agora, a maioria delas tendo sido formalmente descrita apenas nos últimos 20 anos (Marçal et al., 2021, 2020; Santos et al., 2017; Santos e Bueno, 2020). No entanto, a crescente degradação de habitats dulcícolas na América do Sul representa a principal ameaça a essa diversidade, fazendo com que pelo menos 70% de todas as espécies conhecidas de eglídeos até 2017 fossem categorizadas em algum grau de ameaça e 20% consideradas criticamente ameaçadas (Santos et al., 2017), de acordo com os critérios da Lista Vermelha de Espécies Ameaçadas (IUCN,

2012). De fato, Bueno et al. (2016) categorizaram 26 das 42 espécies de eglídeos endêmicas no Brasil em pelo menos algum grau de ameaça (criticamente ameaçado, ameaçado e vulnerável) (Fig. 4). Em vista disso, os eglídeos são considerados o grupo de crustáceos mais ameaçado do Brasil (Magris et al., 2010) e os decápodes de água doce mais ameaçados da América do Sul (Bond-Buckup et al., 2008; Boos et al., 2020).

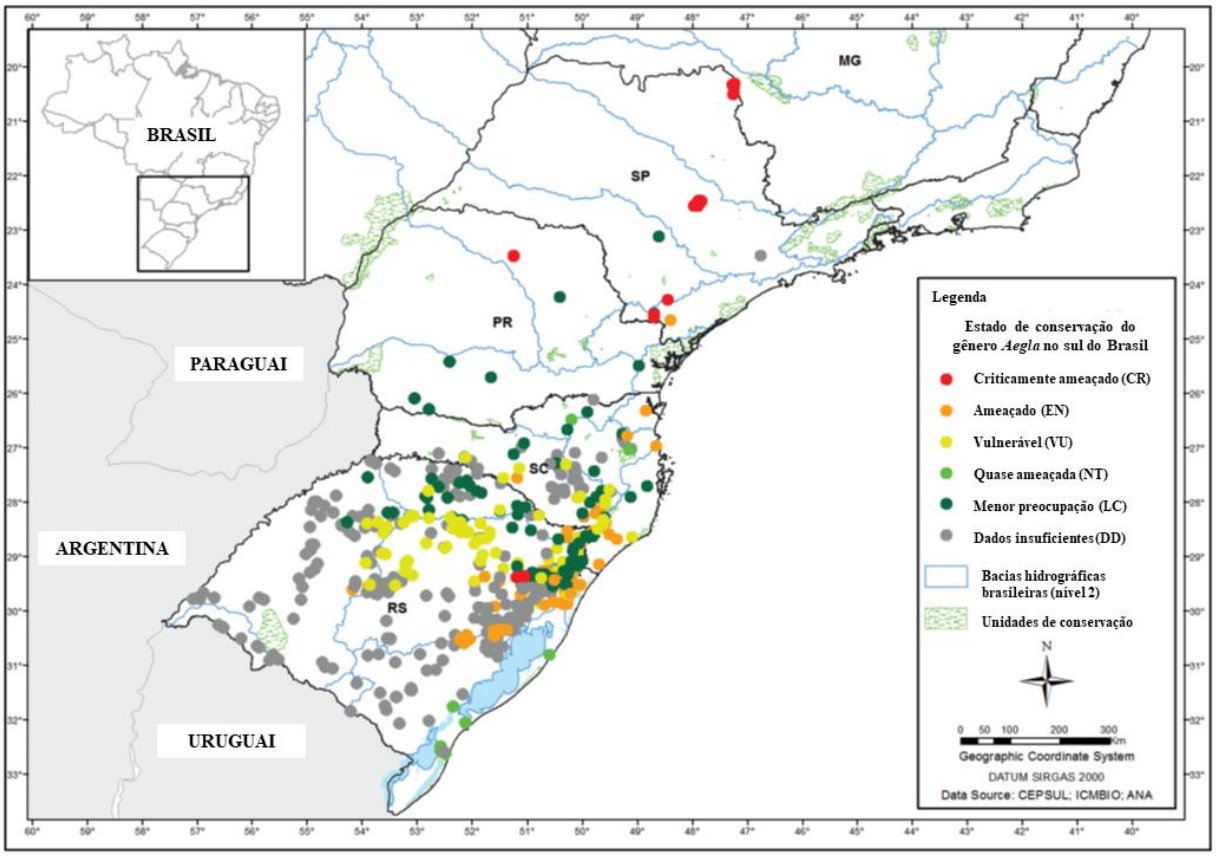

Figura 4. Distribuição e categorias de risco de extinção de espécies de *Aegla* coletadas no Brasil de acordo com Bueno et al. (2016). MG (Minas Gerais), SP (São Paulo), PR (Paraná), SC (Santa Catarina) e RS (Rio Grande do Sul).
Fonte: adaptado de Boos et al. (2020).

Em geral, as fontes de ameaça para a diversidade de eglídeos são advindas das pressões ambientais que as atividades econômicas e a urbanização exercem sobre os habitats dulcícolas onde eglídeos ocorrem. Boos et al. (2020) listaram as fontes de ameaça reportadas para as espécies do Brasil e concluíram que a supressão de vegetação ripária, assoreamento de rios e riachos e a contaminação de corpos de água constituem as três principais causas de perda de habitat para eglídeos (Fig. 5). De fato, vários estudos apontaram a ocorrência de espécies de *Aegla* em áreas contaminadas tanto por agrotóxicos (e. g. Cerezer et al., 2020) quanto por metais (e. g. Faria et al., 2018).

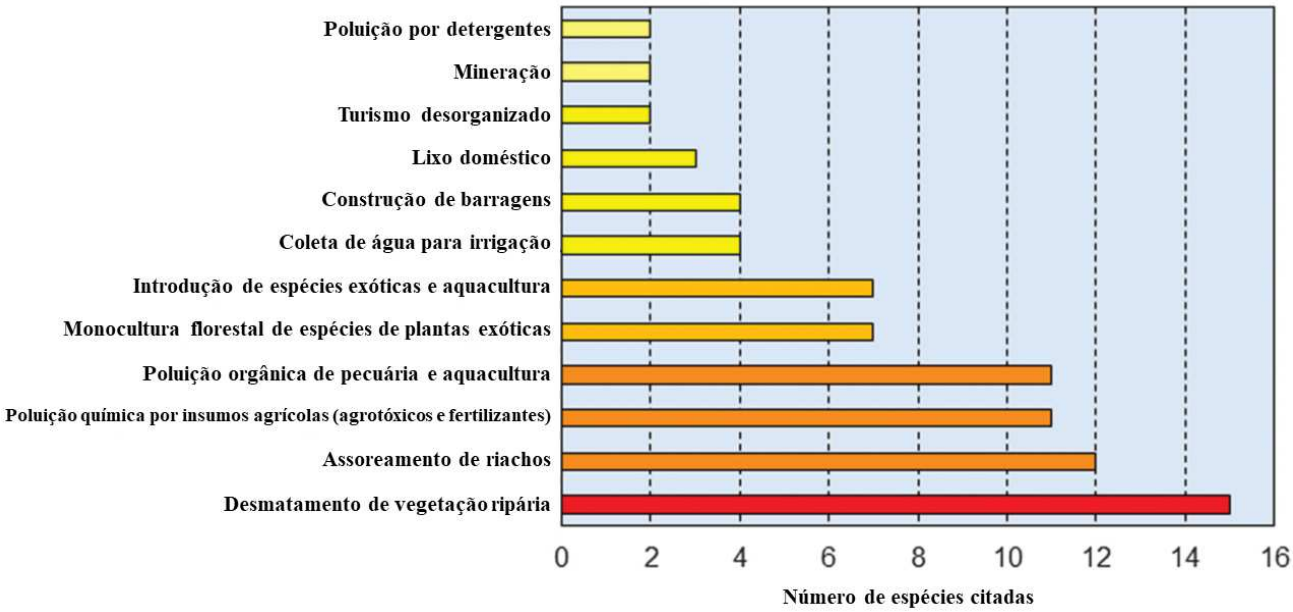

Figura 5. Número de espécies de eglídeos citadas para cada fonte de ameaça identificada nos habitats em que ocorrem populações de eglídeos.
Fonte: adaptado de Boos et al. (2020).

Portanto, embora a presença de eglídeos possa inferir boa qualidade de água e de habitat nos riachos em que eles ocorrem, muitas populações podem estar sujeitas à contaminação por metais e agrotóxicos, assim como apresentar efeitos subletais da exposição a esses xenobióticos. Por exemplo, Borges et al. (2022) apontaram a presença de múltiplos metais nos sedimentos de 14 riachos localizados em três bacias hidrográficas do sul do Brasil onde populações de eglídeos ocorrem e encontraram correlações significativas entre a concentração de metais nos sedimentos e os níveis de biomarcadores de estresse oxidativo; Faria et al. (2018) encontraram pelo menos 10 espécies de *Aegla* que ocorrem em riachos contaminados por Cu e observaram acúmulo de metais no hepatopâncreas desses organismos; e Rosa e Martinez (2021) encontraram concentrações de Cu dissolvido na água em um riacho onde ocorre *Aegla castro* e reportaram efeitos subletais na osmorregulação causados pela exposição aguda ao Cu utilizando testes de toxicidade em laboratório.

2.3 EFEITOS DO CU EM CRUSTÁCEOS DE ÁGUA DOCE

Do ponto de vista fisiológico, o Cu é um micronutriente crucial para crustáceos, uma vez que constitui a matriz do exoesqueleto e compõe a estrutura básica da proteína respiratória hemocianina. Além disso, o Cu pode ser encontrado como cofator em muitas enzimas, como a Cu-Zn superóxido dismutase, anidrase carbônica, citocromo oxidase e monoamino oxidase (Rainbow, 2007). Embora o Cu seja um metal essencial para a manutenção dos sistemas biológicos, efeitos subletais da exposição de organismos aquáticos a altas concentrações de Cu são amplamente reportados na literatura (Bjerregaard e Vislie, 1986; Chen e Lin, 2001; Goodyear e McNeill, 1999; Govindaraju et al., 2013; Lawson et al., 1995; Wei e Yang, 2015; Zhao et al., 2019).

Assim como outros metais, o Cu pode ser absorvido do meio circundante pelos organismos aquáticos através das superfícies permeáveis do corpo (como o epitélio branquial), do exoesqueleto (absorção passiva) ou através da dieta (Fig. 6).

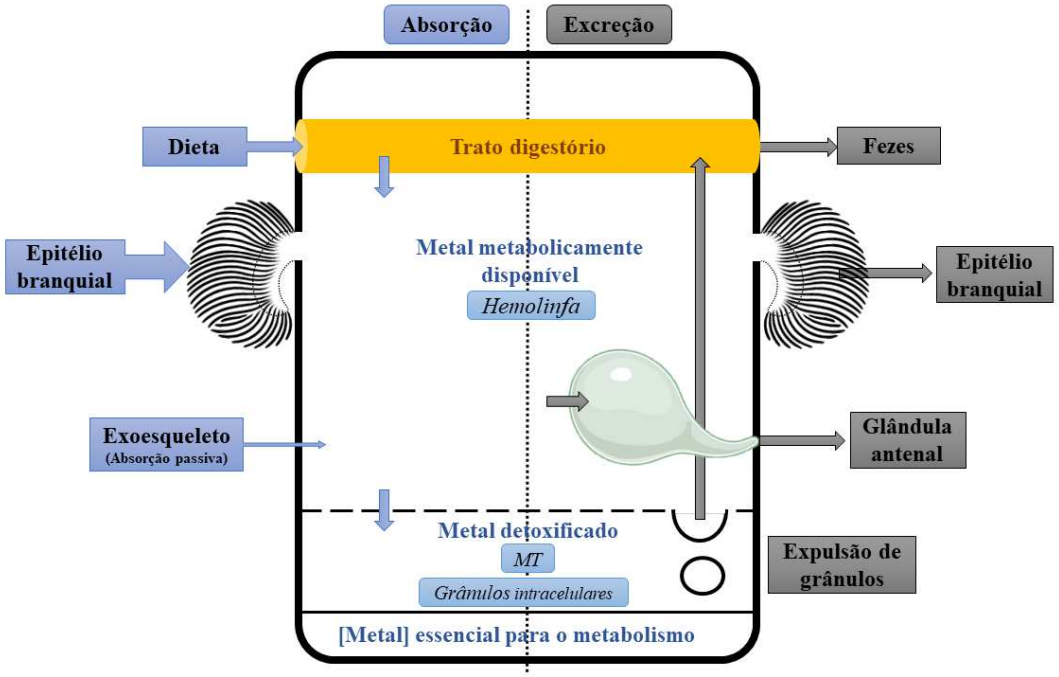

Figura 6. Representação esquemática da homeostase de metais essenciais em crustáceos decápodes. Fonte: adaptado de Rainbow (2002) e Rainbow (2007).

Uma vez absorvido, o metal permanece em uma forma metabolicamente disponível, ou seja, com potencial para se ligar a biomoléculas. Quando o metal absorvido é um metal essencial, como o Cu, essas ligações podem ocorrer para suprir necessidades metabólicas do organismo ou podem desencadear efeitos tóxicos, isto é, quando a

concentração absorvida excede o limiar de concentração essencial requerida para as funções metabólicas. O metal em excesso pode ser excretado, através do trato digestório, das brânquias e das glândulas antenais; e/ou ser acumulado em uma forma detoxificada, por meio de associação a metalotioneínas ou grânulos intracelulares, que podem ser expulsos por exocitose para a hemolinfa, até chegar ao trato digestório (Fig. 6) (Ahearn et al., 2004; Rainbow, 2007; 2002).

Embora metais possam ser absorvidos por várias vias, a absorção primária ocorre por meio do epitélio branquial, uma vez que este órgão constitui a primeira interface do organismo com o meio externo (Freire et al., 2008). Após atingir as brânquias, os metais são difundidos para a hemolinfa e, posteriormente, acumulados no hepatopâncreas e nos músculos (Güner, 2011). Assim, o metal total acumulado no corpo de um crustáceo consiste na fração do metal metabolicamente disponível (incluindo a fração que causa toxicidade a biomoléculas) mais a fração detoxificada que está ligada a metalotioneínas (MT) e/ou no interior de grânulos intracelulares (Rainbow, 2007). As MT são importantes metaloproteínas ricas em grupamentos tiois (–SH) que realizam a homeostase de metais, acumulando-os em uma forma detoxificada (Ahearn et al., 2004; Amiard et al., 2006)

O cobre é um metal que pode estar em dois estados de valência, cúprico (Cu^{2+}) e cuproso (Cu^{+}), e por isso apresenta alto poder redox. Isso faz com que este metal seja uma fonte importante de transferência de elétrons nos sistemas biológicos. Assim, o Cu que não é excretado ou acumulado em uma forma detoxificada, pode estar metabolicamente disponível e pode desencadear a formação de espécies reativas de oxigênio (ERO) por meio da reação de Fenton, favorecendo a formação dos radicais superóxido ($O_2^{\bullet-}$) e hidroxila ($^{\bullet}OH$) (Lushchak, 2011). Para contrabalancear a formação de ERO originadas do próprio metabolismo e as causadas pela exposição a xenobióticos, as células possuem um refinado sistema de defesa antioxidante. As defesas primárias da célula são compostas de antioxidantes não-enzimáticos de baixo peso molecular, como o tripeptídeo glutationa reduzida (GSH); e de enzimas especializadas na eliminação de ERO, como as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa *S*-transferase (GST) (Lushchak, 2011). Quando a taxa de geração de ERO torna-se maior do a taxa de eliminação destas espécies pelas defesas antioxidantes enzimáticas e não-enzimáticas, ocorre a instauração de uma condição chamada de estresse oxidativo (Lushchak, 2011).

A exposição de crustáceos ao Cu pode mobilizar defesas antioxidantes, como aumentar a concentração de GSH (e. g. Zhao et al., 2019), aumentar a atividade das enzimas antioxidantes primárias SOD, CAT, GPx (e. g. Wei e Yang, 2016), e aumentar a

atividade da GST (e. g. Capparelli et al., 2019), que catalisa a conjugação de xenobióticos à GSH. Quando esse sistema de defesa é insuficiente para contrabalancear a formação de ERO e reestabelecer o equilíbrio estacionário formação/eliminação de ERO podem ocorrer danos oxidativos no DNA, proteínas e lipídios (Lushchak, 2011). É importante ressaltar que os danos oxidativos no DNA podem também ocorrer a partir da ligação direta do Cu à estrutura do DNA, desencadeando alterações estruturais e funcionais (Govindaraju et al., 2013). A Figura 7 mostra a influência do Cu na formação de ERO por meio da reação de Fenton, as enzimas antioxidantes que combatem as ERO formadas durante o estado de estresse oxidativo e os potenciais danos oxidativos que podem ocorrer nas macromoléculas (Fig. 7).

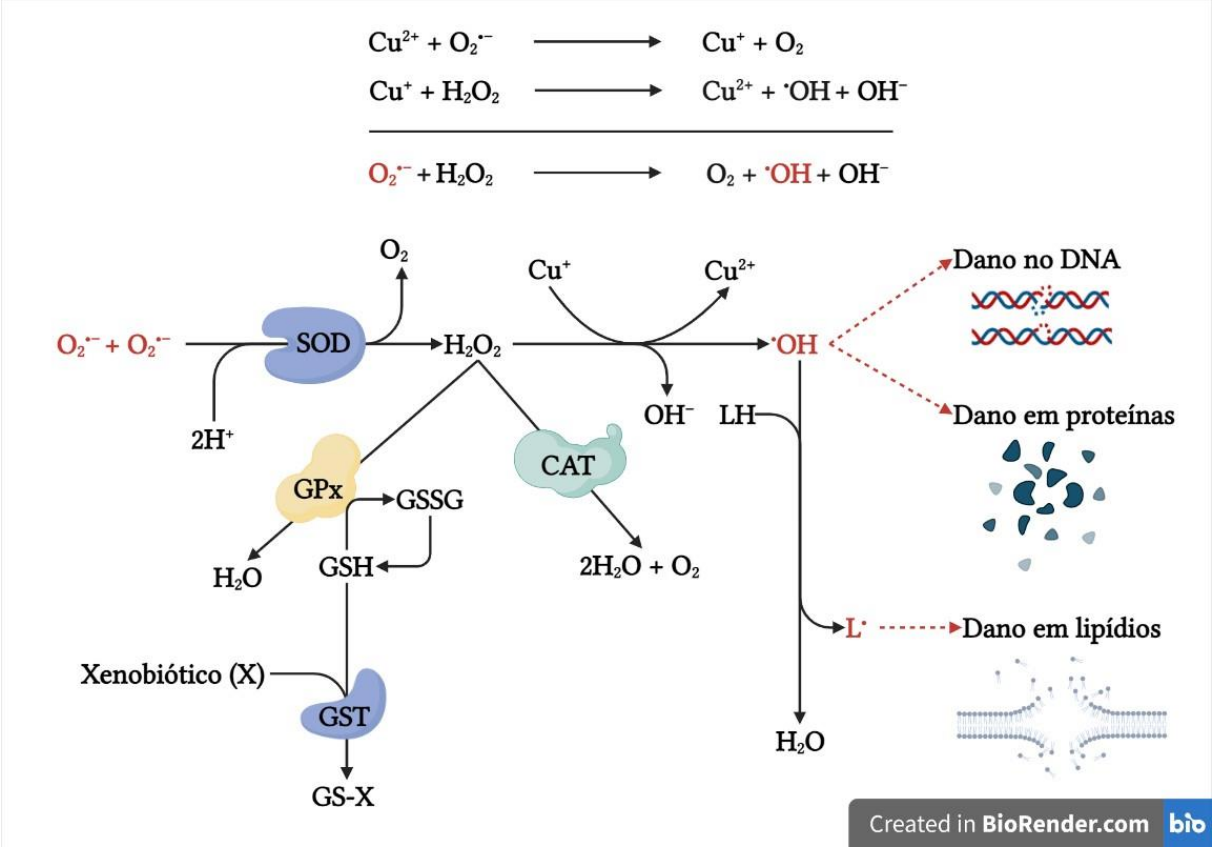

Figura 7. Representação esquemática simplificada do papel do Cu (Cu^{+} e Cu^{2+}) na formação de ERO por meio da reação de Fenton. Vias bioquímicas das enzimas antioxidantes primárias envolvidas na eliminação de ERO e seus subprodutos. Potenciais danos oxidativos advindos da oxidação de macromoléculas por radicais livres. CAT (catalase), Cu^{+} (cuproso), Cu^{2+} (cúprico), GPx (glutationa peroxidase), GSH (glutationa reduzida), GSSG (glutationa oxidada), GST (glutationa *S*-transferase), H^{+} (próton), H_2O_2 (peróxido de hidrogênio), $L^{\bullet}$ (radical lipídio), LH (ácido graxo), $O_2^{\bullet-}$ (radical superóxido), O_2 (oxigênio), $^{\bullet}OH$ (radical hidroxila) e OH^{-} (ânion).
Fonte: baseado em Lushchak (2011) e Lushchak (2015).

Embora o Cu possa ser absorvido através da superfície corporal e pela dieta, a absorção através das brânquias é a mais significativa, uma vez que este órgão constitui

primeira interface do organismo com o meio externo (Freire et al., 2008). Para além dos danos oxidativos causados pela geração descompensada de ERO, o Cu pode prejudicar a regulação osmoiônica e o correto funcionamento das brânquias (Hebel et al., 1997). No epitélio branquial, o Cu pode competir com o Na^+ na membrana apical pelos canais de Na^+ (Grosell et al., 2002) e pelos trocadores Na^+/H^+ (Henry et al., 2012) (Fig. 8). Uma vez absorvido pela célula, o Cu pode se ligar e inibir a atividade da Na^+/K^+-ATPase presente na membrana basolateral (NKA) (e. g. Brooks e Mills, 2003), da H^+-ATPase (HATP) localizada na membrana apical (e. g. Chowdhury et al., 2016) e da anidrase carbônica citosólica (AC) (e. g. Capparelli et al., 2017) (Fig. 8).

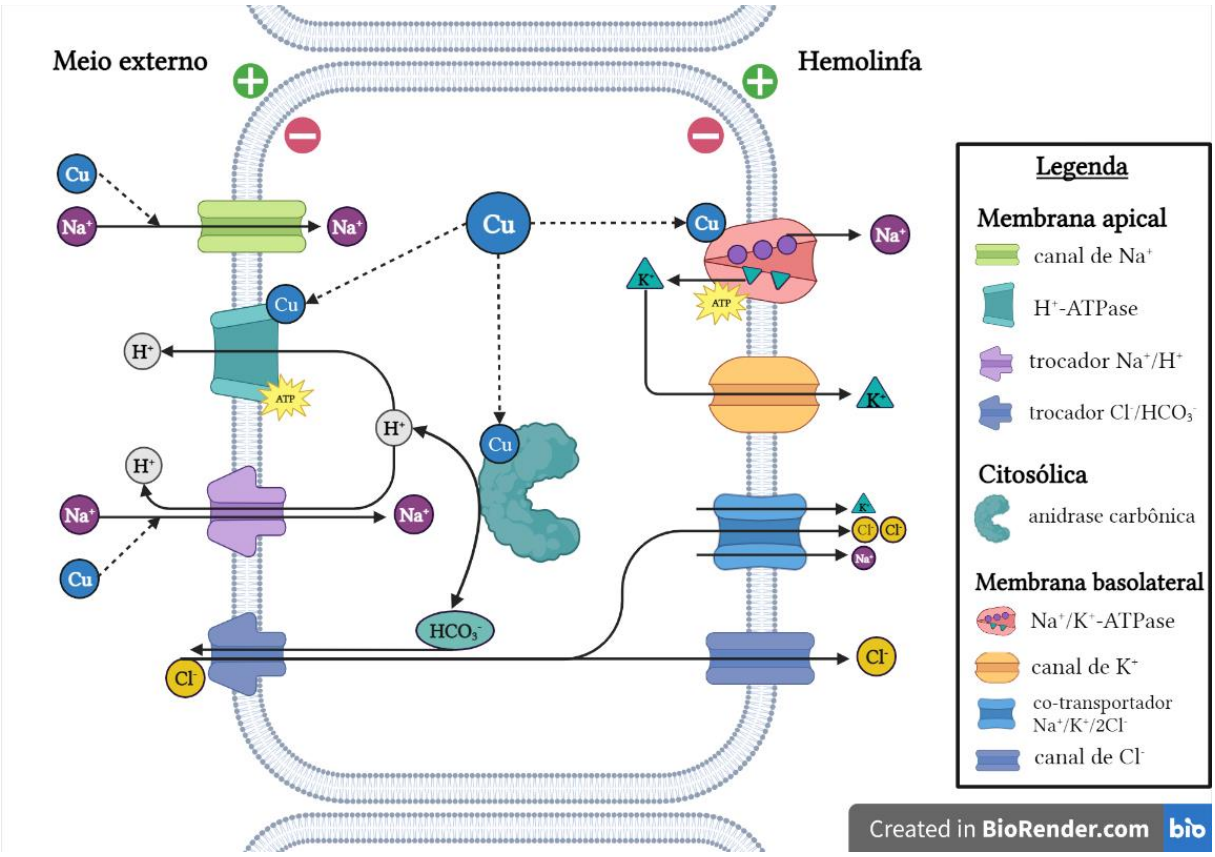

Figura 8. Representação esquemática simplificada das vias de transporte de Na^+, Cl^- e K^+ e os transportadores presentes nas membranas apical e basolateral do epitélio branquial de crustáceos decápodes hiperosmorreguladores fortes, de acordo com Freire et al. (2008) e McNamara e Faria (2012). O trocador Na^+/H^+ na membrana apical e o co-transportador $Na^+/K^+/2Cl^-$ na membrana basolateral foram sugeridos por McNamara e Faria (2020) por estarem presentes no epitélio branquial de eglídeos. As vias de entrada do Cu pelos canais de Na^+ e pelos trocadores Na^+/H^+ na membrana apical foram representadas de acordo com os estudos de Grosell et al. (2002) e Henry et al. (2012). Os símbolos "+" e "-" indicam os potenciais de membrana. Linhas pontilhadas indicam as vias de competição com o Na^+ e os potenciais sítios de ligação do Cu às enzimas relacionadas à osmorregulação.
Fonte: baseado em Freire et al. (2008), Grosell et al. (2002), Henry et al. (2012), McNamara e Faria (2012) e McNamara e Faria (2020).

A inibição da atividade das enzimas NKA, HATP e AC pode causar distúrbios osmorregulatórios, já que elas são essenciais na tomada de Na^+ e Cl^- em crustáceos dulcícolas considerados hiperosmorreguladores fortes. O Na^+ entra por canais de Na^+ a favor de um gradiente eletroquímico, que é gerado pela extrusão de prótons (H^+) para o meio extracelular através da HATP na membrana apical. Por sua vez, a anidrase carbônica citosólica catalisa a hidratação do CO_2 metabólico que fornece H^+ para a HATP e para o trocador Na^+/H^+; assim como também fornece HCO_3^- para o trocador Cl^-/HCO_3^-, levando a absorção de Cl^-. Na membrana basolateral o Na^+ é transportado para a hemolinfa através da NKA, enquanto que o Na^+ e o Cl^- são transportados para a hemolinfa através do co-transportador $Na^+/K^+/2Cl^-$ (NKCC) e dos canais de Cl^- (Freire et al., 2008). É importante notar que, além dos mecanismos reportados por Freire et al. (2008) e McNamara e Faria (2012) para crustáceos hiperosmorreguladores fortes, os trocadores Na^+/H^+ na membrana apical e o co-transportador NKCC também foram representados na Figura 8 por terem sido sugeridos como vias de transporte de Cl^- alternativas das células epiteliais branquiais de eglídeos por McNamara e Faria (2020).

O Cu também pode causar distúrbios na absorção de íons divalentes, tais como o Ca^{2+} e Mg^{2+}, como reportado por Bjerregaard e Vislie (1986) e Chavez-Crooker et al. (2002). Os íons Ca^{2+} e Mg^{2+} desepenham um papel crucial em crustáceos, uma vez que eles constituem a maioria dos componentes inorgânicos do exoesqueleto (Fieber e Lutz, 1985). Embora a homestase de Mg^{2+} esteja envolvida nos mecanismos de regulação do ciclo de muda, é o Ca^{2+} o principal efetor que estimula a síntese de ecdisteroides pelos órgãos Y durante a pré-muda em crustáceos (Fieber e Lutz, 1985).

Crustáceos dulcícolas obtém Ca^{2+} através da dieta ou através da absorção pelas brânquias. Freire et al. (2008) propuseram que a absorção de Ca^{2+} na membrana apical do epitélio branquial é dependente de canais de Ca^{2+} sensíveis a verapamil, de trocadores Ca^{2+}/H^+ sensíveis a amilorida e de trocadores $Ca^{2+}/2H^+$ insensíveis a amilorida (Fig. 9). Depois de absorvido na membrana apical, o Ca^{2+} intracelular pode ser armazenado no retículo endoplasmático, através do transporte ativo de Ca^{2+} *via* Ca^{2+}-ATPases presentes na membrana no retículo endoplasmático (SERCA), como sugerido por Wheatly et al. (2002); ou pode ser transportado para a hemolinfa através de Ca^{2+}-ATPases e trocadores $Ca^{2+}/2–3Na^+$, que são sustentados pelo gradiente eletroquímico gerado pela NKA na membrana basolateral (Freire et al., 2008). Dessa forma, a exposição ao Cu poderia causar prejuízos na absorção de Ca^{2+} tanto pela inibição de Ca^{2+}-ATPases, como reportado em mexilhões dulcícolas (Canli, 2021), quanto pela inibição da NKA na membrana basolateral, diminuindo o gradiente eletroquímico

de Na^+ e a concentração de Ca^{2+} na hemolinfa.

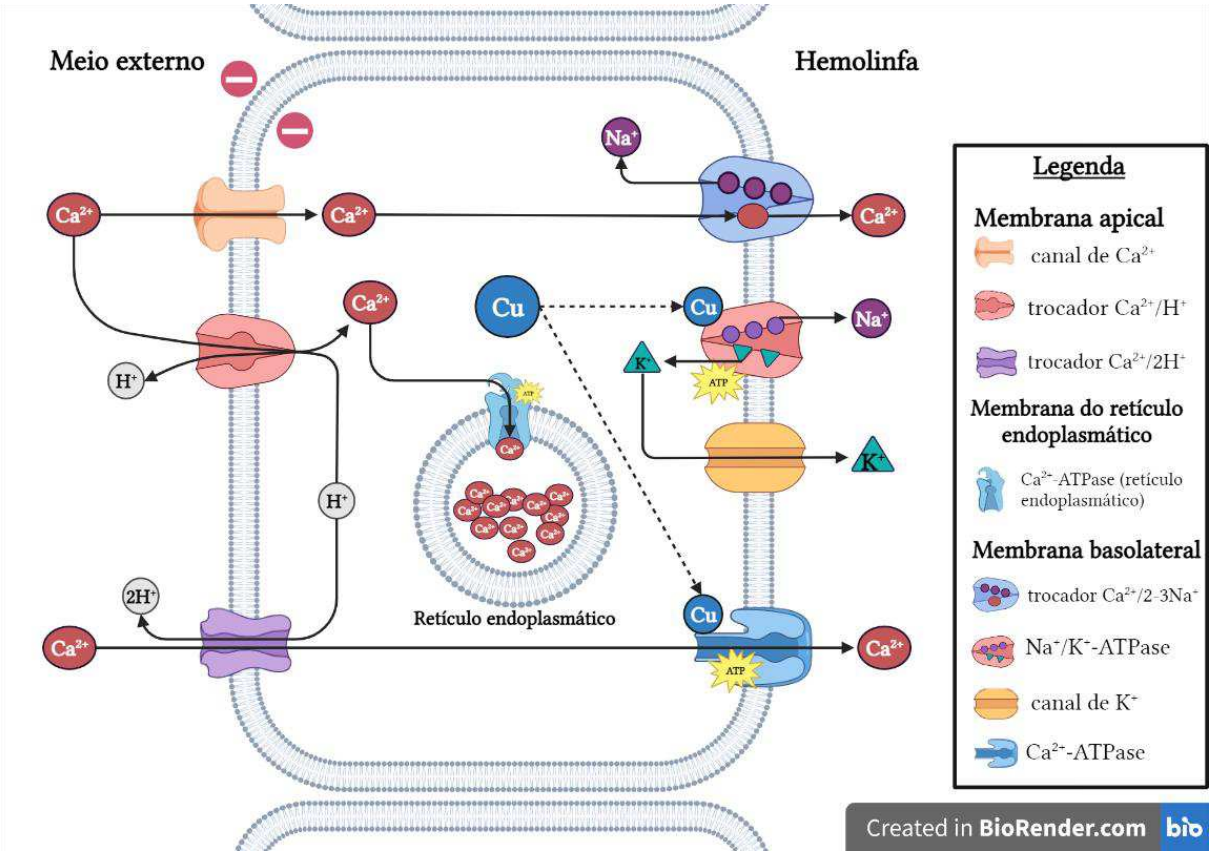

Figura 9. Representação esquemática simplificada das vias de transporte branquial de Ca^{2+} e os transportadores presentes nas membranas apical, do retículo endoplasmático e basolateral do epitélio branquial de crustáceos decápodes hiperosmorreguladores fortes, de acordo com Freire et al. (2008) e Wheatly et al. (2002). A absorção de Ca^{2+} depende do gradiente eletroquímico mantido pela NKA na membrana basolateral, sendo absorvido na membrana apical através de trocadores Ca^{2+}/H^+ e $Ca^{2+}/2H^+$ e canais de Ca^{2+}. O Ca^{2+} intracelular pode ser armazenado no retículo endoplasmático através de Ca^{2+}-ATPases (SERCA) ou ser transportado para a hemolinfa por meio de Ca^{2+}-ATPases ou trocadores $Ca^{2+}/2–3Na^+$ na membrana basolateral. O símbolo "-" indica a polaridade da membrana. A linha pontilhada indica a ligação do Cu para inibir a NKA na membrana basolateral.
Fonte: baseado em Freire et al. (2008) e Wheatly et al. (2002).

Por outro lado, a exposição de crustáceos a metais pode alterar a regulação dos mecanismos de muda em crustáceos, e por sua vez, alterar a homeostase de Ca^{2+} e Mg^{2+} na hemolinfa. Por exemplo, espécimes de *Pennaeus monodon* anteciparam o ciclo de muda após exposição ao Cu (Chen e Lin, 2001); espécimes de *Uca pugnax* iniciaram um novo ciclo de muda como estratégia para depurar concentrações excessivas de cobre, zinco e chumbo (Bergey e Weis, 2007). Portanto, um aumento significativo na concentração de Ca^{2+} e Mg^{2+} na hemolinfa é uma resposta esperada em crustáceos que iniciam um novo ciclo de muda (estágio de pré-muda) (Ahearn et al., 2004; Fieber e Lutz, 1985; Greenaway, 1985). De fato, Rosa e Martinez (2021) também sugeriram que a exposição de *A. castro* ao Cu desencadeou a

ativação de mecanismos de reabsorção de Ca^{2+} e Mg^{2+} na hemolinfa, o que representaria um sinal bioquímico para a antecipação de um novo ciclo de muda.

2.4 BIOMARCADORES EM EGLÍDEOS

Para avaliar os efeitos de xenobióticos em organismos aquáticos os biomarcadores são uma ferramenta essencial no diagnóstico precoce de efeitos tóxicos subletais. Os diferentes tipos de biomarcadores podem fornecer informações sobre vários níveis de organização biológica, apontado alterações ao nível bioquímico, celular, fisiológico ou comportamental. Essas alterações podem ser medidas a partir de amostras biológicas de tecido, fluídos corporais, ou mesmo a partir de extratos de indivíduos completos (Depledge et al., 1995). Portanto, como não existe um único biomarcador perfeito para todas as circunstâncias, uma abordagem integrada com a análise de múltiplos biomarcadores e delineamentos experimentais têm sido recomendada pela literatura para uma compreensão global dos efeitos prejudicais dos xenobióticos em organismos aquáticos (Hagger et al., 2006).

Embora os eglídeos sejam considerados bioindicadores de qualidade de habitat há bastante tempo, estes animais só foram adotados em estudos ecotoxicológicos na última década. Por exemplo, os estudos de Borges et al. (2018) e Faria et al. (2018) avaliaram pela primeira vez parâmetros relacionados ao estresse oxidativo em espécimes de eglídeos coletados diretamente do campo. Posteriormente, sob uma abordagem ecotoxicológica, Cerezer et al. (2020) avaliaram parâmetros relacionados ao estresse oxidativo em espécimes coletados em diversos riachos do sul do Brasil contaminados por diversos agrotóxicos.

Além de estudos de campo, estudos em laboratório também foram conduzidos com eglídeos, com destaque para Rosa e Martinez (2021), que padronizaram pela primeira vez, exposições de eglídeos a contaminantes em condições de laboratório. Os autores avaliaram a resposta de enzimas relacionadas à regulação osmoiônica em *A. castro* exposta a concentrações de Cu dissolvido que podem ser encontradas no ambiente.

2.5 JUSTIFICATIVA

Os caranguejos eglídeos representam um exemplo típico de fauna Neotropical com elevada riqueza de espécies e endemismo, além de exigirem boa qualidade de água e constituírem elos importantes nas cadeias tróficas de riachos de pequena ordem (Bond-Buckup e Buckup, 1994). Por essas características, os eglídeos são considerados bons modelos biológicos para delimitar áreas prioritárias para a conservação na América do Sul (Pérez-Losada et al., 2009; Tumini et al., 2019) e bons bioindicadores de qualidade de água e habitat em ecossistemas límnicos (Correa-Araneda et al., 2010). No entanto, muitas populações ocorrem em riachos localizados em regiões densamente povoadas e/ou sob intensa atividade econômica, fazendo com que estes animais estejam suscetíveis a diversas fontes de degradação de habitat, como desmatamento de vegetação ripária, assoreamento e contaminação da água por efluentes industriais e agrícolas (Santos et al., 2017). Esse panorama preocupante quanto à conservação de eglídeos justifica a adoção de abordagens que permitam identificar precocemente os efeitos prejudiciais (subletais) da degradação de habitat na saúde de eglídeos e que previnam o declínio das populações.

Nesse sentido, estudos ecotoxicológicos sob uma abordagem de múltiplos biomarcadores podem fornecer informações valiosas para a compreensão dos efeitos aos quais as populações de eglídeos podem estar sujeitas nos riachos em que ocorrem, ainda que a sua presença nesses ambientes possa indicar uma boa qualidade de habitat. A quantidade de biomarcadores padronizados em eglídeos até agora é reduzida se compararmos aos biomarcadores já comumente empregados em estudos com peixes, por exemplo. É importante ressaltar que embora peixes e os eglídeos sejam animais que habitam os mesmos ambientes, os biomarcadores mais responsivos em peixes podem não ser os mais apropriados para crustáceos bentônicos, como os eglídeos. Devido às características distintas de nicho ecológico, eglídeos e peixes podem apresentar respostas diferentes a um mesmo contaminante presente no riacho. Por ocuparem o substrato do riacho e se alimentarem da matéria orgânica alóctone e da microflora que cresce no sedimento, os eglídeos podem estar em contato mais direto com os contaminantes e apresentar maior sensibilidade do que outros organismos que ocupam a coluna de água, como muitas espécies de peixes.

Portanto, para além dos objetivos de padronização de biomarcadores, este estudo buscou enfatizar a necessidade de adoção dos eglídeos em estudos ecotoxicológicos utilizando múltiplos biomarcadores, uma vez que estes organismos são menos abordados em estudos de impacto ambiental do que peixes, por exemplo. Além disso, os eglídeos possuem

alta sensibilidade a alterações ambientais e são organismos-chave, seja pela importância ecológica, seja pela urgência de conservação de muitas espécies desse gênero.

2.6 OBJETIVOS

2.6.1 Objetivo geral

Padronizar a utilização de *A. castro* como um modelo biológico em estudos ecotoxicológicos avaliando a resposta de múltiplos parâmetros funcionais como potenciais biomarcadores da exposição ao Cu.

2.6.2 Objetivos específicos

1) Investigar a presença e as concentrações de Cu na água do riacho de referência (rio Couro) e nos tecidos dos espécimes de *A. castro* coletados.
2) Caracterizar parâmetros funcionais relacionados ao sistema de defesa antioxidante, osmorregulação, células envolvidas na resposta imune e integridade do DNA em *A. castro*.
3) Validar biomarcadores em *A. castro*.
4) Padronizar o uso de eglídeos em testes de toxicidade em laboratório.
5) Avaliar a resposta de múltiplos biomarcadores em espécimes de *A. castro* expostas ao Cu dissolvido na água utilizando testes de toxicidade em laboratório.
6) Investigar os biomarcadores mais responsivos em *A. castro* expostas ao Cu.

2.7 HIPÓTESES

1) A exposição de *A. castro* ao Cu, seja em exposições agudas em laboratório seja ao Cu presente na água do riacho, acarretará em acúmulo de Cu nos tecidos, distúrbios na regulação osmoiônica, indução da resposta antioxidante e danos oxidativos em proteínas, lipídios e DNA.
2) Parâmetros funcionais relacionados ao estresse oxidativo, distúrbios osmoiônicos, resposta imunológica e integridade do DNA serão biomarcadores apropriados para avaliar a toxicidade do Cu em *A. castro.*
3) As concentrações permitidas de Cu dissolvido (9 a 13 $\mu g\ L^{-1}$) para águas doces segundo a legislação brasileira vigente (CONAMA, 2005) é segura para eglídeos.
4) *Aegla castro* pode ser usada como um modelo biológico para avaliar a qualidade de riachos.

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Capítulo I

Characterization of the hemocytes and parameters related to oxidative stress and osmoregulation of the freshwater anomuran crab *Aegla castro*

3 CAPÍTULO I

Manuscrito em preparação para ser submetido à revista Ecological Indicators

Characterization of the hemocytes and parameters related to oxidative stress and osmoregulation of the freshwater anomuran crab *Aegla castro*

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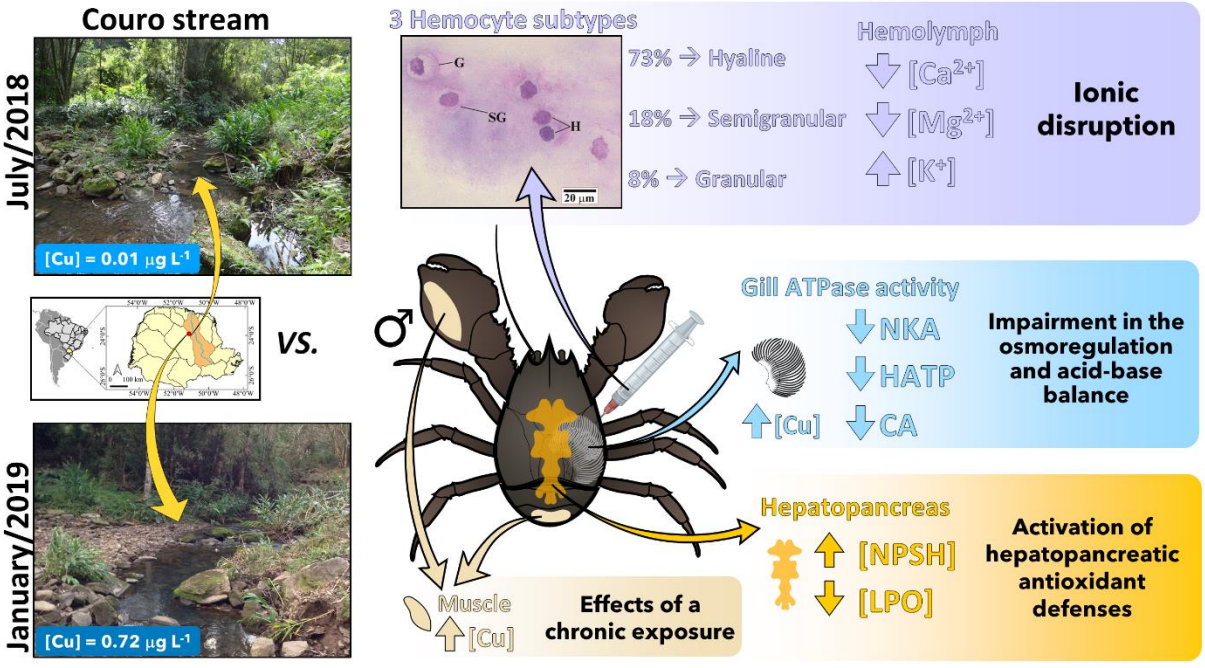
Highlights

1. *Aegla castro* has hyaline, semigranular, and granular hemocytes in its hemolymph
2. Cu in the stream water increased after 6 months to the first collection
3. Aeglids collected in January/2019 increased Cu bioaccumulated in gill and muscle
4. Higher Cu concentration in the stream water impaired osmo-ionic regulation
5. Higher Cu concentration activated hepatopancreatic antioxidant responses

Graphical abstract



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Abstract

Water contamination, deforestation, and habitat loss are the main threats to aeglid biodiversity, making them the most endangered freshwater decapods in South America. Thus, characterization of some biological parameters of these animals is important to support biomonitoring studies using aeglids. Here, we characterized for the first time the hemocytes of an aeglid species, *Aegla castro*. We also analyzed biochemical parameters in intermolt males collected in July 2018 and January 2019. Hemolymph was withdrawn to determine the ion concentration and analyze the hemocyte composition, while gill, hepatopancreas, and muscle were used to determine the Cu concentration and the following parameters: metallothionein (MT), non-protein thiol (NPSH), and protein carbonylation (PCC) contents; catalase (CAT), glutathione *S*-transferase (GST), acetylcholinesterase (AChE), Na^+/K^+-ATPase (NKA), H^+-ATPase (HATP), Ca^{2+}-ATPase (CATP), and carbonic anhydrase (CA) activities; and lipid peroxidation (LPO). Three hemocyte subtypes are present in the hemolymph of *A. castro*: hyaline, semigranular, and granular. We identified an increase in the Cu concentration in the water of the stream in January 2019 when compared to July 2018, followed by increased Cu in gill and muscle; increased NPSH and decreased LPO in hepatopancreas; decreased Ca^{2+} and Mg^{2+} and increased K^+ in the hemolymph; and decreased NKA, HATP, and CA in gill. Multivariate analyses showed that the higher Cu concentration in the stream water in 2019 not only led to Cu accumulation in tissues of *A. castro* but promoted osmo-ionic disruption and the activation of hepatopancreatic antioxidant responses.

Keywords: *Aegla*; biomarker; copper; oxidative stress; osmo-ionic regulation.

3.1 INTRODUCTION

Freshwater anomuran crabs known as “aeglids” are endemic to the Neotropics, inhabiting clear and well-oxygenated watercourses, and playing a crucial role in trophic chains and nutrient cycling along the Southern American watersheds (Bond-Buckup and Buckup, 1994). However, because of the current degradation status of South American freshwater habitats, coupled with the high endemism and fragmented distribution pattern of some species of *Aegla*, aeglids are considered the most endangered freshwater decapods in South America (Bond-Buckup et al., 2008). The main threats to aeglid biodiversity consist of habitat loss and degradation, mainly by the devastation of riparian forests, silting, and water pollution by agriculture, livestock, and aquaculture activities (Boos et al., 2020).

Aeglids have been addressed in a myriad of taxonomic, biological, and ecological studies, since the extensive review of the Aeglidae family published by Bond-Buckup and Buckup (1994). Studies with *Aegla* have focused mainly on species description (e. g. Marçal et al., 2020), geographical distribution patterns (e. g. Bond-Buckup et al., 2008), current taxonomic and conservation status (Santos et al., 2017), morphology (e. g. Marçal et al., 2018), and trophic ecology (Bueno and Bond-Buckup, 2004). Although some authors have already mentioned aeglids as bioindicators of habitat and water quality (Bortoluzzi et al., 2007; Correa-Araneda et al., 2010), these animals have only been highlighted in ecotoxicological studies using biomarkers for the assessment of environmental degradation since 2018. For example, Borges et al. (2018) characterized some oxidative stress biomarkers, such as catalase activity and lipid peroxidation (TBARS) in biological extracts of *Aegla singularis*; and Faria et al. (2018) investigated, under a phylogenetic comparative approach, metal bioaccumulation, metallothionein-like protein content, antioxidant capacity against peroxides, and the glutathione system (GSH-GSSG) in the hepatopancreas of ten aeglid species from Southern and Southeastern Brazil.

Several aeglid species have a restricted area of occurrence, and their geographical distribution area comprises ecosystems historically damaged by human activities (Boos et al., 2020), like the Atlantic Forests and South Brazilian watersheds. In addition, due to the increasing water contamination and loss of aeglid habitat (Santos et al., 2017), biomonitoring studies focusing on these animals as biological models are of urgent concern to better understand whether and how the populations are being impacted by habitat contamination. For example, Faria et al. (2018) reported that at least ten aeglid species occur in streams with Cu-contaminated sediments. Therefore, the study of biomarkers using aeglids as biological

models represents a relevant approach in biomonitoring studies for early identification of the sublethal effects of water contamination before ecological damage, such as population decline and an increased risk of extinction, can occur.

Apart from biomarkers at the biochemical level, some cellular parameters have been used in crustaceans to evaluate health status, such as the density of the circulating hemocytes in the hemolymph (Battison et al., 2003; Zhou et al., 2016). Crustacean hemolymph is usually composed of three general categories of circulating hemocytes: the hyaline hemocytes, the semigranular hemocytes, and the granular hemocytes. These hemocyte categories are traditionally categorized based on morphological characteristics, such as size and number of cytoplasmic granules; and morphometric characteristics, such as the nucleocytoplasmic ratio, that is, the relationship between the maximum nucleus diameter and the maximum cell diameter (N/C ratio) (Clare and Lumb, 1994; Hose et al., 1990; Söderhäll, 1992). Crustacean hemocytes are responsible for the immune response, playing a major role in the phagocytosis, encapsulation, and lysis of foreign cells (Ratcliffe et al., 1985). In addition, earlier studies on crustacean immunity reported that each hemocyte subtype presents a functional specificity (Söderhäll, 1992) and that the composition of hemocytes in terms of the relative proportion of each subtype can vary among species (Martin et al., 1991).

Here, we aim to perform the morphological characterization of the hemocyte subtypes as well as their relative abundance in the hemolymph of *Aegla castro* Schmitt, 1942, by performing total and differential hemocyte counts, for the first time in an aeglid species. In addition, we measured Cu accumulation in tissues and Cu concentration in the water of the Couro Stream and characterized parameters related to oxidative stress, oxidative damage, neurotoxicity, and ionic regulation in animals from two field collections, evaluating the applicability of these parameters as biomarkers in *A. castro*.

3.2 MATERIAL AND METHODS

3.2.1 Field collections

Two field collections of males of *A. castro* Schmitt, 1942, in the intermolt stage, were carried out in the Couro Stream (23º57'15''S, 51º07'00''W), Tibagi River Basin, Southern Brazil (Fig. 1), one in the winter of 2018 (July 2018) and another in the summer of 2019 (January 2019). The Couro Stream is a first-order watercourse, with no apparent pollution sources, with riparian forest along some stretches of its extension. Despite this, the Couro Stream is within an agricultural matrix, with sparse private properties and near a highway (Fig. 1). The bottom substrate is mainly composed of rocks in the rapids and muddy sediment in the backwaters, with leaf litter deposited on the bottom and between rocks in the rapids.

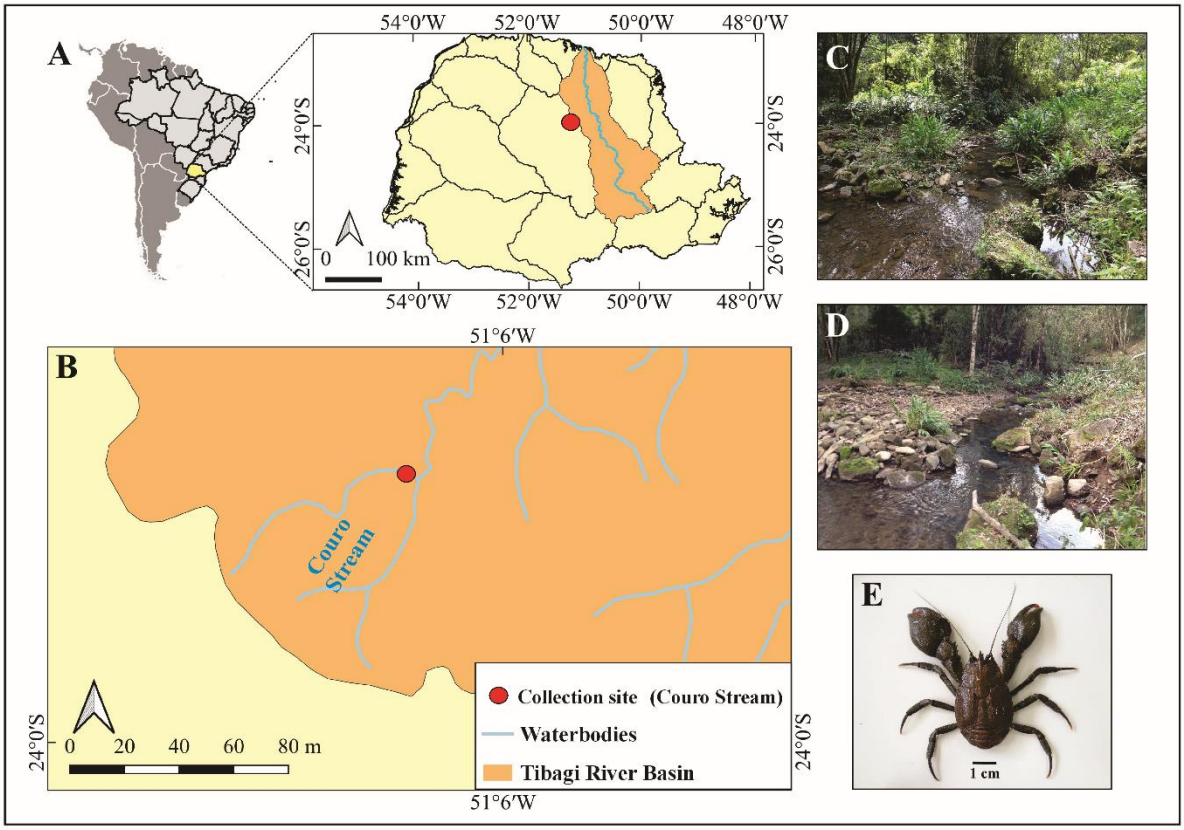

Fig. 1 – Field collection site of specimens of *Aegla castro* (A-B), in the Couro Stream, Tibagi River Basin, Southern Brazil, in July/2018 (C) and January/2019 (D). Dorsal view of a male specimen of *Aegla castro* (E).

Aeglids were captured along a stretch up to 100 m long (Fig. 1). While a researcher turned over the rocks and leaf litter upstream another positioned a trawl (length 150 cm *vs.*

width 100; mesh 1 mm) downstream of the rapids so that the animals were carried by the current flow to the trawl.

The abiotic parameters of temperature (°C), pH, dissolved oxygen (mg O_2^{-1}), turbidity (NTU), conductivity (μS cm^{-1}), and total dissolved solids (g L^{-1}) were measured in the stream water using a multiparameter reader (Horiba U-52). The EDTA titrimetric method was used to measure the hardness (mg L^{-1} $CaCO_3$). Unfiltered and filtered (0.45-μm mesh filter, Millipore Millex HV/PVDF) water samples were collected, and immediately fixed with HNO_3 (65%). The samples were kept at 4 °C in the laboratory, until the determination of the ion concentrations and total (Cu T) and dissolved (Cu D) Cu concentrations. The animals were transported to the laboratory (~ 90 min) in thermic boxes containing 15 L of stream water, not exceeding the density of 2 individuals per liter of water. Hiding places made of plastic were offered to the animals to mitigate aggressive behavior between males during transportation.

3.2.2 Cu and ionic concentrations in the water and the hemolymph

Cu T and Cu D concentrations were measured in a graphite furnace atomic absorption spectroscope (AAnalyst700, PerkinElmer), with a detection limit of 0.014 μg L^{-1} Cu. Ion concentrations were determined only from unfiltered water samples. Na^+ and K^+ concentrations were measured by flame photometry (Digimed DM-62), and Ca^{2+} and Mg^{2+} by atomic absorption spectroscopy (AAnalyst700, PerkinElmer).

3.2.3 Tissue sampling

In the laboratory, the animals were transferred to 30 L boxes filled with water at constant temperature and aeration until the tissue sampling, not exceeding 12 h after the field collection. No mortality was recorded during this period. The crabs were cryo-anesthetized, weighed, and measured (July 2018: n = 20; 2.02 ± 0.10 g wet body mass; 15.29 ± 0.28 mm carapace length without rostrum (CLwR); January 2019: n = 30; 4.01 ± 0.31 g wet body mass; 18.49 ± 0.54 mm carapace length without rostrum (CLwR); mean ± SD). Hemolymph aliquots were withdrawn from the arthrodial membrane of the chelipeds with an insulin syringe. Aliquots of 5 μL were used to make smears (in duplicate) to analyze the hemocyte

composition, and 100 μL were immediately diluted (1:50) in ultrapure water and stored at 4 °C until the analysis of ion concentrations. Gill, hepatopancreas, and muscle from the cheliped and abdomen were dissected and were used for the determination of Cu content and biochemical parameters. After dissection, tissues were kept at -80 °C until analysis.

3.2.4 Hemocyte characterization

Hemolymph smears were kept at room temperature for 24 h before staining. The slides with the smears were then fixed in methanol (20 min) and prepared using two staining methods: 10 smears stained with Giemsa and 10 smears stained with Fast Panoptic. Giemsa stain was diluted (1:10) in Sørensen buffer (40 mM KH_2PO_4, 60 mM $NaHPO_4$, pH 7.0), and the slides were stained (for 20 min) and kept at room temperature for 24 h, before being mounted with Permount®. The staining method using Fast Panoptic was performed by dipping the slides for 5 s in the three commercial solutions of rapid differential staining (LaborClin®). After 24 h of drying at room temperature, the slides were mounted with Permount®. The slides were analyzed under a light microscope (ZEISS Primo Star) coupled to a camera (ZEISS AxioCaM ERc 5s) using the image software ZEN Blue Edition (v. 2.3).

3.2.4.1 Characterization of hemocyte subtypes

Hemocytes were morphologically characterized into subtypes according to Battison et al. (2003) and Clare and Lumb (1994). These classifications are based mainly on the nucleocytoplasmic (N/C) ratio and cytoplasmic granule content (size and number). We measured the maximum cell diameter (C) and the maximum nucleus diameter (N) and calculated the nucleocytoplasmic ratio (N/C). Morphometric data are presented in μm as mean ± standard error.

3.2.4.2 Total (THC) and differential (DHC) hemocyte counts

The first 300 hemocytes observed in each of the 20 slides were counted. Differential hemocyte count (DHC) is a relative proportion of each hemocyte subtype that composes the

hemolymph of *A. castro.* DHC was assessed by counting the number of hemocytes belonging to each subtype in relation to the total hemocytes counted (THC) in each slide.

3.2.5 Cu bioaccumulation in tissues

Gill, hepatopancreas, and muscle were completely dried before digestion in ultrapure nitric acid (5 N) at 60 °C for 48 h, according to Roda et al. (2020). Afterward, digests were analyzed in a graphite furnace atomic absorption spectroscope (AAnalyst700, PerkinElmer), using reference solutions (Specsol, Brazil) as standards. Results are expressed as mg Cu g dry weight^{-1}.

3.2.6 Oxidative stress parameters

Hepatopancreas was homogenized (1:8 m/v) in buffer (0.05 M potassium phosphate, 0.5 mM EDTA, 10 μM PMSF, pH 7.2) and centrifuged (16,000 × g, 20 min, at 4 °C) following Borges et al. (2018). To compare biochemical parameters (NPSH, MT, CAT, GST, and PCC) between tissues (hepatopancreas *vs.* gill), the anterior gills were homogenized (1:8 m/v) in the same way as the hepatopancreas.

3.2.6.1 Antioxidants

Non-protein thiol content (NPSH) was determined according to Beutler et al. (1963). The supernatant was used in the reaction between –SH groups and the substrate 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and the formation of thiolate (TNB) was quantified in a microplate reader (Victor3™, Perkin Elmer) at 412 nm. NPSH content was expressed as μmol –SH g protein^{-1}.

Catalase activity (CAT) was determined by adding a solution of hydrogen peroxide (0.03% H_2O_2) to the sample and by measuring the decrease in the absorbance in a microplate reader (SpectraMax Plus 384) at 240 nm. CAT activity was expressed as μmol H_2O_2 min^{-1} mg protein^{-1}.

The activity of glutathione *S*-transferase (GST) was determined according to Gagné

(2014) by measuring the complexation rate of reduced glutathione (GSH) with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), in a microplate reader (Victor3™, Perkin Elmer) at 340 nm, for 10 min. GST activity was expressed as nmol CDNB conjugated min^{-1} mg $protein^{-1}$.

3.2.6.2 Oxidative damage

The thiobarbituric reactive substances (TBARS) assay described by Camejo et al. (1998) was used to determine (fluorescence reading: ex/em 535/590 nm) the lipid peroxidation (LPO) by measuring the fluorescence of MDA (a thiobarbituric reactive substance) formed from peroxidized lipids in a microplate reader (Victor3™, Perkin Elmer). The LPO was expressed as nmol MDA mg $protein^{-1}$.

The protein carbonylation content (PCC) was assessed following Levine et al. (1994), by measuring (360 nm) the content of dini-trophenylhydrazones after the reaction with 2,4-dinitrophenylhydrazine (10 mM of 2,4-dinitrophenylhydrazine in 2 M HCl) and guanidine hydrochloride (6 M). Results are expressed as nmol carbonyl mg $protein^{-1}$.

3.2.7 Metallothionein-like protein content (MT)

The MT content was measured following Viarengo et al. (1997), with modifications. Hepatopancreas were homogenized (1:3 m/v) in buffer (0.5 M sucrose, 26 mM Tris, 0.5 mM phenylmethylsulfonyl fluoride, and 1.3 mM β-mercaptoethanol) and centrifuged (18,000 × g, 45 min, at 4 °C). The supernatant was treated with ethanol/acid chloroform solution, and the purified metalloprotein fraction (–SH groups) was measured in a microplate reader at 412 nm (Victor3™, Perkin Elmer). We used reduced glutathione (GSH) as standard and expressed the MT content in nmol –SH mg $protein^{-1}$.

3.2.8 Neurotoxicity

Muscle samples from chelipeds and abdomen were thawed, weighed, and homogenized (1:4 w/v) in buffer (0.1 M potassium phosphate, pH 7.5) and centrifuged (16,000 × g, 20 min, at 4 °C) to evaluate the acetylcholinesterase (AChE) activity, according to Ellman et al. (1961), with modifications. The substrate acetylcholine iodide (9 mM) and the

color reagent DTNB (0.5 mM) were added to the supernatant containing AChE in the sample, and the activity of AChE was measured at 415 nm. The results are expressed as nmol DTNB min^{-1} mg $protein^{-1}$.

3.2.9 Ionic regulation

Posterior gill samples were thawed, weighed, and homogenized with an ultrasonic sonicator (1:5 w/v) in SEID buffer (150 mM sucrose, 50 mM imidazole, 10 mM EDTA, 12 mM sodium deoxycholate (pH 7.5)), and centrifuged (20 min, 16,060 × g, at 4 °C). The total protein content of the supernatant was adjusted to 1 mg mL^{-1}. Na^+/K^+-ATPase (NKA) and H^+-ATPase (HATP) activities were measured in a simultaneous assay following Gibbs and Somero (1989), with modifications described in Tesser et al. (2020). A reactive solution (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM KCN, 1 mM ATP, 0.2 mM NADH, 3 U mL^{-1} pyruvate kinase, 2 U mL^{-1} lactate dehydrogenase, 0.1 mM fructose-1-6-diphosphate, 2 mM phosphoenolpyruvate, pH 9.0) with 2 mM ouabain (specific NKA inhibitor) or with 2 mM N-Ethyl-d_5-maleimide (specific HATP inhibitor) was used to determine the activities of NKA and HATP, respectively. The reactive solution without inhibitors was used to measure the total activity of the ATPases. The decrease in the absorbance of NADH was measured every minute for 15 min at 340 nm (Victor3, PerkinElmer). The specific activities of NKA and HATP were calculated by subtracting the results obtained in the wells without inhibitors (total activity of the ATPases) from the wells with ouabain or NEM, respectively. Results are expressed in μmol ADP mg $protein^{-1}$ h^{-1}.

Ca^{2+}-ATPase activity (CATP) was measured following Vijayavel et al. (2007), with modifications. The basal concentration of inorganic phosphate (P_i) and the activity of CATP in the samples (5 μL) were measured by incubating the samples (30 °C for 30 min) in 100 μL of a reactive solution (189 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris, 5 mM $CaCl_2$, 2 mM ouabain, pH 7.6) without ATP and with 3 mM ATP, respectively. The reaction was then stopped on ice for 10 min; and the Ames reagent (1:6 v/v 10% ascorbic acid:0.42 % ammonium molybdate in 0.5 mM H_2SO_4) was added to the samples. The formation of P_i was measured at 620 nm after 10 min in a microplate reader (ELX 800, Bio-Tek Instruments). To quantify the CATP activity a phosphate standard curve (0.08–0.65 mM) was used. The results are expressed as μmol Pi mg $protein^{-1}$ min^{-1}.

We followed Vitale et al. (1999) to measure the activity of the carbonic anhydrase (CA). An aliquot of 50 μL of the sample was added to CO_2-saturated distilled water (2.0 – 2.5

°C), and the decrease in pH was quantified every 4 s for 20 s with a pH meter (Jenway 3510). The catalyzed reaction (CR) rate was generated from the linear relationship between the formation of H^+ (acidification of the medium) as a function of time. The non-catalyzed reaction (NCR) rate was measured every four samples by the decrease in pH when the supernatant was not added to the assay (blank). The specific reaction rate of CA (SCA) was calculated as follows: SCA = [CR/NCR – 1]/mg protein. Results are expressed as UAC mg $protein^{-1}$ min^{-1}.

All biochemical biomarkers were normalized by total protein content and quantified (at 595 nm) in a previously reserved homogenate aliquot, according to Bradford (1976).

3.2.10 Statistical analysis

Data for morphometric and biochemical parameter are expressed as mean ± standard error (SE). The normality (Shapiro-Wilk test) and homoscedasticity (Levene test) of all data were verified. The morphometric analyses (maximum cell and nucleus diameters and the N/C ratio) and the hemocyte counts (DHC) were compared (Hyaline *vs.* Semigranular *vs.* Granular) by an analysis of variance (ANOVA) or Kruskal-Wallis test, followed by Tukey or Dunn's *posthoc* multiple comparisons procedures, when necessary, according to the data distribution. Biochemical parameters of each collection (July 2018 *vs.* January 2019) were compared using the Student's *t*-test or Mann-Whitney test, according to the data distribution. All statistical analyses were performed in the R environment using the packages *car*, *RVAideMemoire*, *rstatix*, and *psych*. The significance level adopted for all tests was 0.05.

The parameters were analyzed with a Principal Component Analysis (PCA) and a correlation matrix was created to investigate the variables that explained most of the variation and also the correlations among variables. We used the function *facto_summarize* of the *factoextra* R package to plot a color scale of the contribution of each variable for the explanation of the whole data variability for the first (Dim1) and second (Dim2) components concomitantly. PCA was performed in an R environment using the packages *FactoMineR*, *factoextra*, and *ggplot2*.

3.3 RESULTS

3.3.1 Environmental parameters

Physical and chemical parameters of the water measured at the Couro Stream in the field collections of July 2018 and January 2019 are presented in Table 1. We observed an increase in the concentrations of both Cu T and Cu D (~ 53% and ~ 98%, respectively) in the water of the Couro Stream in January 2019 when compared to July 2018 (Table 1).

Table 1. Abiotic variables, ion concentration, and total and dissolved copper concentrations (Cu T and Cu D, respectively) in the water of field collections 1 (July 2018) and 2 (January 2019) in the Couro Stream. Data are presented as mean followed by the sample number and range between parenthesis.

Abiotic variable	July 2018	January 2019
Temperature (°C)	15.2 (n=6; 15.1–15.3)	20.3 (n=6; 20.3–20.4)
pH	6.7 (n=6; 6.6–6.8)	8.0 (n=6; 7.9–8.1)
Dissolved oxygen (mg O_2^{-1})	8.4 (n=6; 15.1–15.3)	8.2 (n=6; 8.0–8.5)
Turbidity (NTU)	51.6 (n=6; 45.2–56.4)	40.7 (n=6; 28.5–53.7)
Conductivity (µS cm^{-1})	61.2 (n=6; 61.0–62.0)	58.0 (n=6; 58.0–59.0)
Total dissolved solids (g L^{-1})	0.06 (n=6; 0.05–0.06)	0.04 (n=6; 0.04–0.04)
Hardness mg L^{-1} $CaCO_3$	14.8 (n=6; 10.1–20.2)	20.9 (n=6; 20.2–22.2)
Na^+ (mM)	0.11 (n=6; 0.10–0.13)	0.08 (n=6; 0.07–0.08)
K^+ (mM)	0.02 (n=6; 0.02–0.02)	0.07 (n=6; 0.07–0.08)
Cu T (µg L^{-1})	0.43 (n=6; 0.10–0.76)	0.92 (n=6; 0.78–1.13)
Cu D (µg L^{-1})	0.01 (n=6; <LD–0.01)	0.72 (n=6; 0.60–0.89)

3.3.2 Circulating hemocytes

We described the hemocytes based only on the analysis of hemolymph samples of aeglids collected in January 2019. Under light microscopy, we identified three morphologically distinctive hemocyte subtypes in the hemolymph of *A. castro*, using both Giemsa (Fig. 2A–B and 2E–F) and Fast Panoptic (Fig. 2C–D) preparations and categorized them into three subtypes: hyaline hemocytes (H), semigranular hemocytes (SG), and granular hemocytes (G).

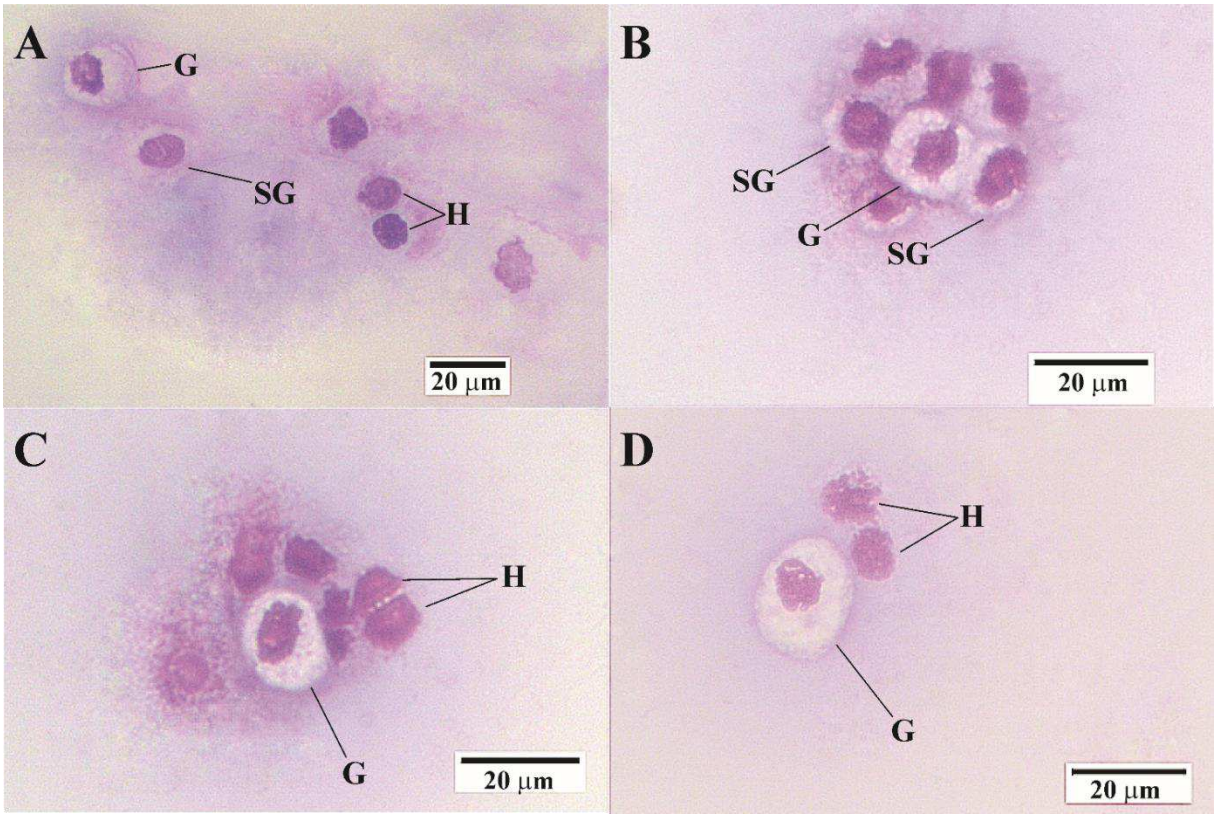

Fig. 2 – Characterization of the hemocyte subtypes of the hemolymph of *Aegla castro* collected from the Couro Stream in January 2019, stained with Giemsa preparation (A-D). Three subtype populations of hemocytes are evident: hyaline hemocytes (H), semigranular hemocytes (SG), and granular hemocytes (G).

Hyaline hemocytes are rounded cells with the nucleus stained purple, in which the nucleus occupies a large part of the total volume of the cell and the cytoplasmic granules are not evident (e.g. Fig. 2A); semigranular hemocytes are rounded cells with the nucleus stained purple, in which an unstained cytoplasm with few granules is visible and the nucleus does not occupy the total volume of the cell (Fig. 2B and 2D); and granular hemocytes are larger rounded cells with the nucleus stained dark violet, with abundant granules (more visible using the Fast Panoptic preparation) in the unstained large cytoplasm, in which the nucleus occupies a small portion of the total volume of the cell (e.g. Fig. 2B, 2D, and 2F).

We performed two measurements (maximum cell diameter and maximum nucleus diameter) in a total of 180 hemocytes (60 of each subtype), subjected to two staining methods (Giemsa and Fast Panoptic). The three hemocyte subtypes were also morphometrically distinctive from each other (Table 2). Hyaline hemocytes presented the lowest maximum cell diameter when compared to the semigranular and granular hemocytes. In contrast, granular hemocytes presented the highest ($p < 0.050$) maximum cell diameter, under the two staining methods, when compared to the hyaline and semigranular hemocytes. Considering the

maximum nucleus diameter, the three hemocyte subtypes did not present statistical differences from each other (Table 2).

Table 2. Hemocyte morphometry and hemocyte counts of *Aegla castro* from field collection 2 (January 2019) in the Couro Stream, using Giemsa and Fast Panoptic preparations. The hemocyte morphometry was based on 180 hemocytes (60 of each subtype). Hemocyte counts of the three evident hemocyte subtypes (hyaline, semigranular, and granular) were based on the analysis of 20 slides, two slides from each animal (n = 20). Differential (DHC) hemocyte count is reported as a relative proportion of the quantity of each hemocyte subtype to the total (THC) hemocyte count (reported as absolute values).

	Parameters	Hemocyte morphometry			Hemocyte counts	
		Maximum cellular diameter (μm)	Maximum nuclear diameter (μm)	N/C ratio (%)	Differential (%)	Total (THC)
Hemocyte subtypes	Hyaline	10.51±0.27 [a]	7.83±0.26 [a]	0.75±0.01 [a]	0.73±0.02 [a]	4040
	Semigranular	13.93±0.30 [b]	8.11±0.27 [a]	0.58±0.02 [b]	0.18±0.02 [b]	1020
	Granular	17.06±0.37 [c]	8.21±0.24 [a]	0.49±0.01 [c]	0.08±0.01 [c]	468
	Comparisons (ANOVA)	$p<0.050$	$p=0.342$	$p<0.001$	$p<0.001$	5528

Results are mean ± SE. Superscript letters (a, b, and c) indicate statistical differences (ANOVA) among the hemocyte subtypes (hyaline *vs.* semigranular *vs.* granular).

The nucleocytoplasmic (N/C) ratio was different among the three hemocyte subtypes: hyaline hemocytes presented the highest N/C ratio (N/C > 0.70), followed by the median N/C ratio of the semigranular hemocytes (0.50 < N/C < 0.70), and the lowest N/C ratio of the granular hemocytes (N/C < 0.50) (Table 2).

The differential (DHC) and total (THC) hemocyte counts were performed based on the three distinctive hemocyte subtypes found in the hemolymph of *A. castro*, using two staining methods (Giemsa and Fast Panoptic). A total of 5528 hemocytes were counted in this study (Table 2). Hyaline hemocytes were the most abundant subtype and the hemocyte subtype with the highest relative proportion (73% of the hemolymph cell composition), followed by the semigranular (18%) and granular hemocytes (8%) (Table 2).

3.3.3 Cu bioaccumulation in tissues

When comparing the Cu concentrations in the tissues of *A. castro* collected in the Couro Stream both in July 2018 and January 2019, we observed a significant increase in the Cu bioaccumulation in gills ($p < 0.001$) and muscle ($p < 0.001$) of aeglids collected in January 2019 (Fig. 3); however, this bioaccumulation pattern was not observed in the hepatopancreas.

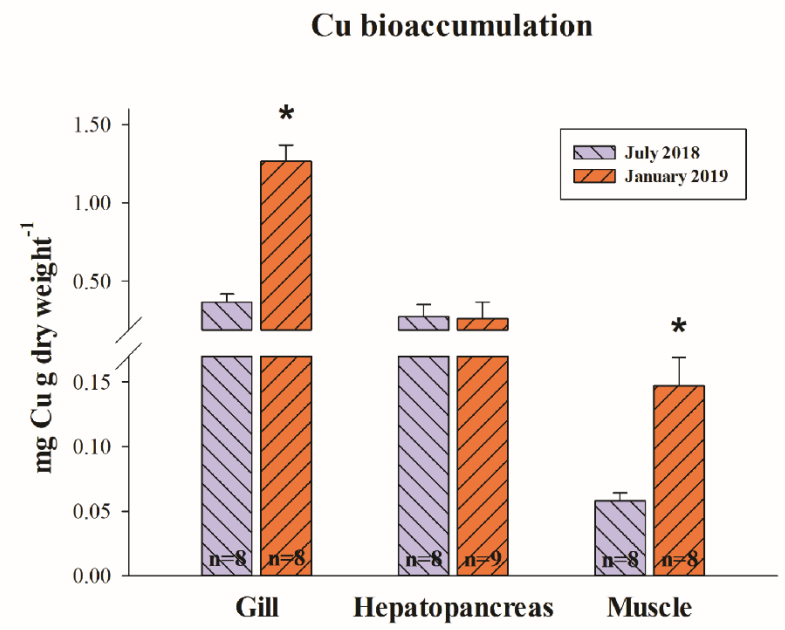

Fig. 3 – Copper bioaccumulation in gill, hepatopancreas, and muscle of *Aegla castro* from the two field collections in the Couro Stream (July 2018 and January 2019). Data are reported as mean values ± standard error. Asterisks indicate statistical differences between the parameters of animals collected in July 2018 *vs.* January 2019. Sample sizes (n) are indicated on the chart.

3.3.4 Oxidative stress and oxidative damage parameters

We observed a higher concentration of NPSH ($p < 0.001$) and lower LPO ($p = 0.005$) in the hepatopancreas of *A. castro* collected in January 2019 when compared to the animals from July 2018 (Figs. 4A and 4D). However, we did not observe any alteration in the CAT and GST activities, PCC, and MT content in the hepatopancreas (Figs. 4B–C and 4E–F). In the same way, no alteration was observed in the AChE activity in the muscle ($t(14) = 0.389$, $p = 0.703$) of *A. castro* when comparing the two field collections.

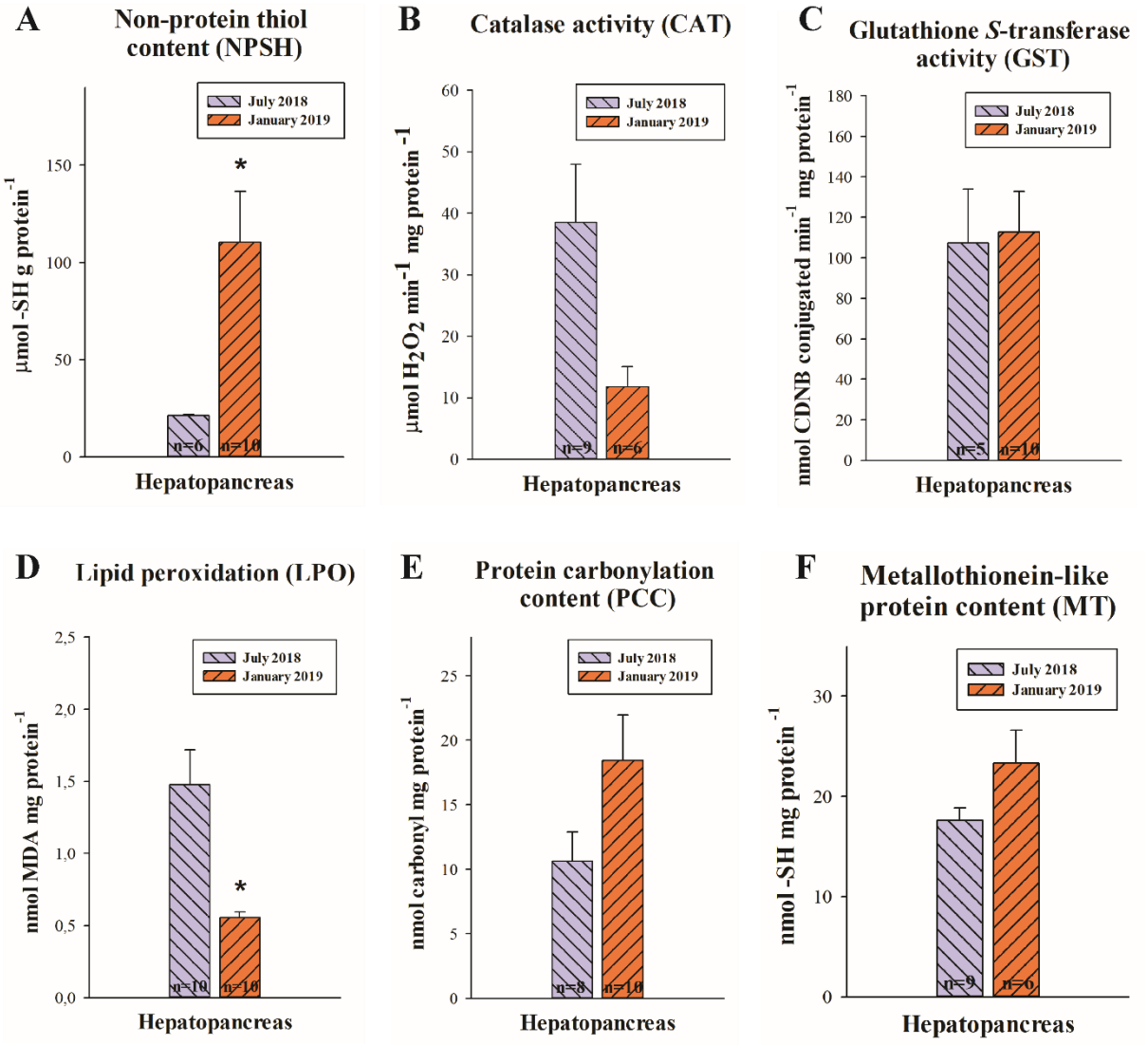

Fig. 4 – Non-protein thiol content (NPSH), catalase activity (CAT), glutathione *S*-transferase activity (GST), lipid peroxidation (LPO), protein carbonylation content (PCC), and metallothionein-like protein content (MT) in the hepatopancreas (A-F) of *Aegla castro* from the two field collections in the Couro Stream (July 2018 and January 2019). Data are reported as mean values ± standard error. Asterisks indicate statistical differences between the parameters of animals collected in July 2018 *vs.* January 2019. Sample sizes (n) are indicated on the chart.

When comparing the parameters of the gill *vs.* hepatopancreas, we used samples of the aeglids collected in July 2018. We found that the MT content in the hepatopancreas was higher (17.60±1.25; 9) than that found in the gill (3.70±0.48; 6) (W=0; $p<0.001$), but we did not observe any alteration concerning the parameters NPSH, CAT, GST, and PCC when comparing gill *vs.* hepatopancreas.

3.3.5 Ions in the hemolymph

The aeglids collected in January 2019 presented a higher concentration of K^+ (p = 0.003) and a lower concentration of Ca^{2+} (p = 0.002) and Mg^{2+} (p = 0.010) in their hemolymph when compared to those collected in July 2018. The concentration of Na^+ in the hemolymph did not alter between the two field collections (Fig. 6).

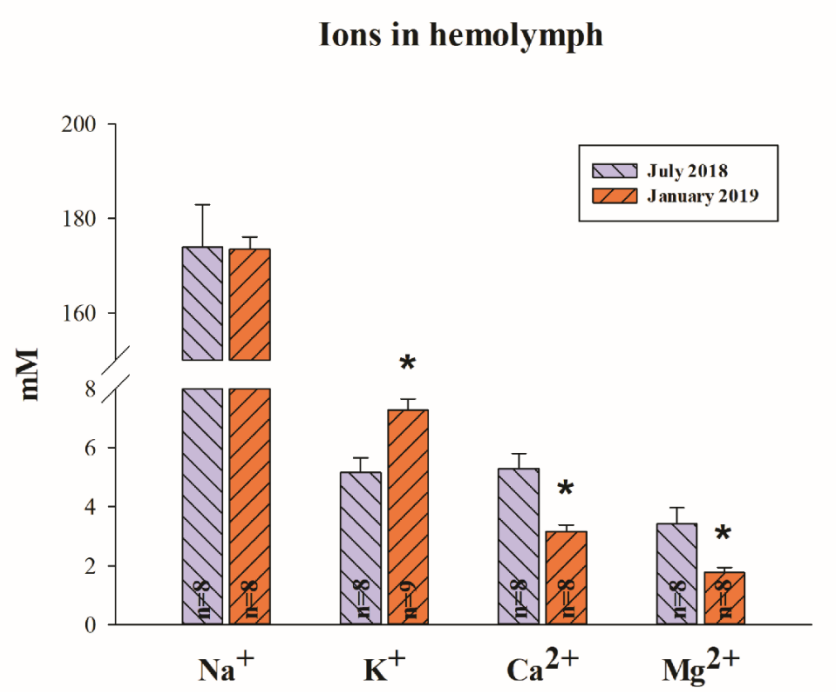

Fig. 6 – Concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} in the hemolymph in posterior gills of *Aegla castro* from the two field collections in the Couro Stream (July 2018 and January 2019). Data are reported as mean values ± standard error. Asterisks indicate statistical differences between the parameters of animals collected in July 2018 *vs.* January 2019. Sample sizes (n) are indicated on the chart.

3.3.6 Biochemical parameters in the gills (NKA, HATP, CATP, and CA)

Concerning the enzymes involved in osmoregulation, we observed inhibition in the activity of NKA (p < 0.001), HATP (p < 0.001), and CA (p = 0.024) in the gills of *A. castro* collected in January 2019 when compared to those collected in July 2018 (Figs. 7A–B and 7D). We did not observe any alteration in the activity of CATP in the gill of *A. castro* when comparing the two field collections (Fig. 7C).

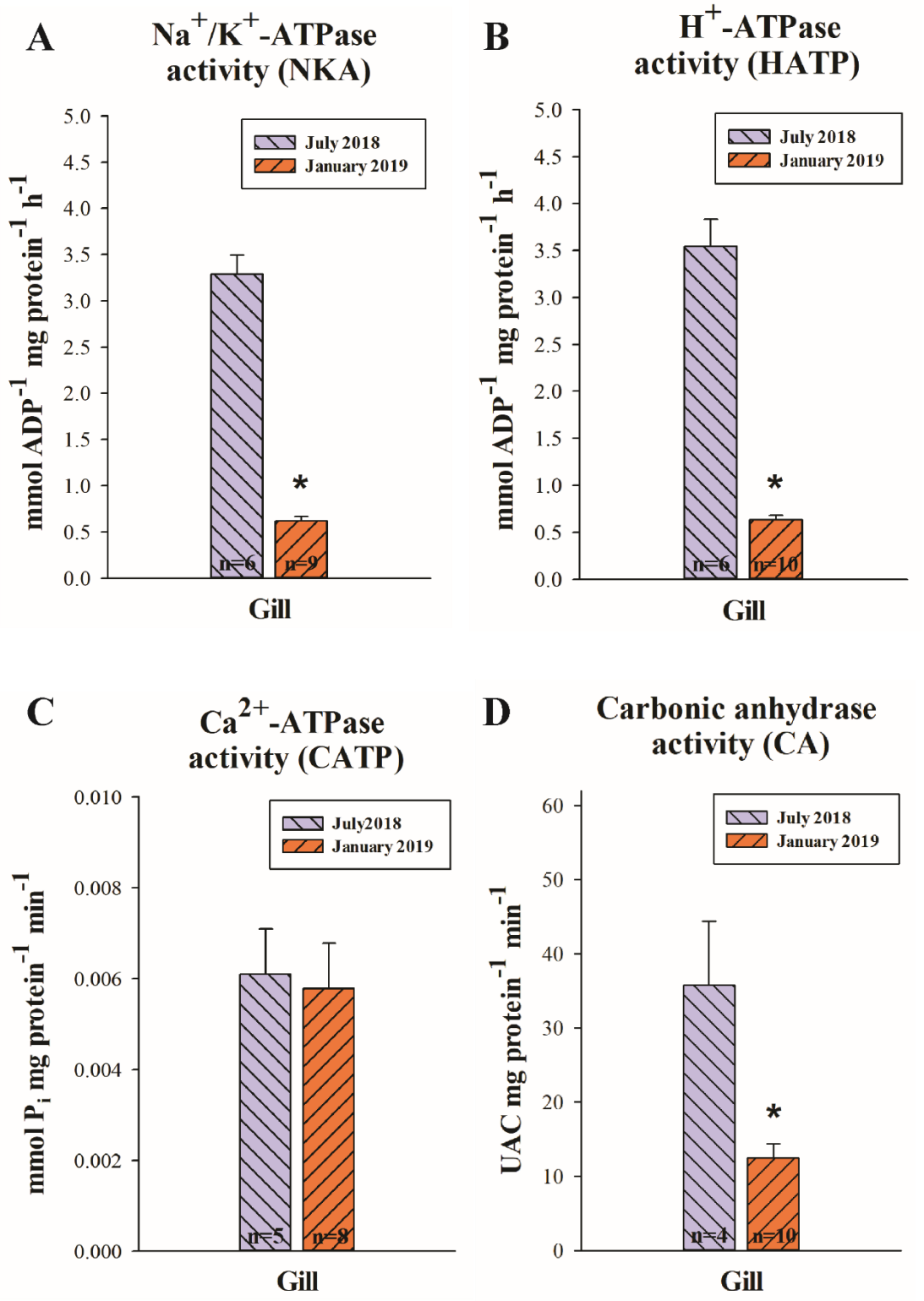

Fig. 7 – Na^+/K^+-ATPase (NKA), H^+-ATPase (HATP), Ca^{2+}-ATPase (CATP), and carbonic anhydrase (CA) activities in posterior gills (A-D) of *Aegla castro* from the two field collections in the Couro Stream (July 2018 and January 2019). Data are reported as mean values ± standard error. Asterisks indicate statistical differences between the parameters of animals collected in July 2018 *vs.* January 2019. Sample sizes (n) are indicated on the chart.

3.3.7 Principal component analysis (PCA)

The PCA showed that 55% of the data variability could be explained by the first (Dim1 = 36.7%) and second (Dim2 = 18%) components (Fig. 8). The first component (PC1) is strongly associated with the parameters Na^+/K^+-ATPase (NKA_gill), H^+-ATPase (HATP_gill), and Cu bioaccumulation (Cu_gill) in gills, and Ca^{2+} concentration (Ca_hemo) in the hemolymph. This component may represent "Cu bioaccumulation in the gill and the osmo-ionic regulation" (Fig. 8).

On the other hand, the second component was more associated with the parameters carbonic anhydrase (CA_gill) in the gill, and protein carbonylation content (PCC_hep) and metallothionein-like protein content (MT_hep) in the hepatopancreas. This component can be interpreted as "oxidative damage in proteins and hepatopancreatic antioxidant response" (Fig. 8).

From the correlation matrix, we found that the parameters Ca^{2+} concentration (Ca_hemo) *vs.* Mg^{2+} concentration (Mg_hemo) *vs.* NKA_gill *vs.* HATP_gill were all positively correlated; as well as the parameters K^+ concentration (K_hemo) *vs.* MT_hep *vs.* PCC_hep. The Cu bioaccumulation in gill (Cu_gill) *vs.* Cu bioaccumulation in muscle (Cu_mus) were positively correlated, as well as Cu_mus *vs.* MT_hep and Cu_mus *vs.* PCC_hep. The Cu_gill *vs.* non-protein thiol content (NPSH_hep) in the hepatopancreas were positively correlated.

Negative correlations were found among Cu_gill *vs.* HATP_gill, Cu_gill *vs.* NKA_gill, and Cu_gill *vs.* catalase activity (CAT_hep) in the hepatopancreas; as well as the Cu_mus *vs.* HATP_gill, Cu_mus *vs.* NKA_gill, and Cu_mus *vs.* CAT_hep. The variables Cu_gill *vs.* Ca_gill, Cu_gill *vs.* Mg_gill, and Cu_gill *vs.* lipid peroxidation (LPO_hep) in hepatopancreas were also negatively correlated among the respective comparisons.

Concerning the contribution of variables to explain the data variability for the first (Dim1) and second (Dim2) components together, the variables with the highest contributions were NKA_gill (9.5%), HATP_gill (9.3%), PCC_hep (8.4%), MT_hep (8.0%), and Cu_gill (7.1%); while the variable with the lowest contribution was Cu_hep (0.02%) (Fig 8).

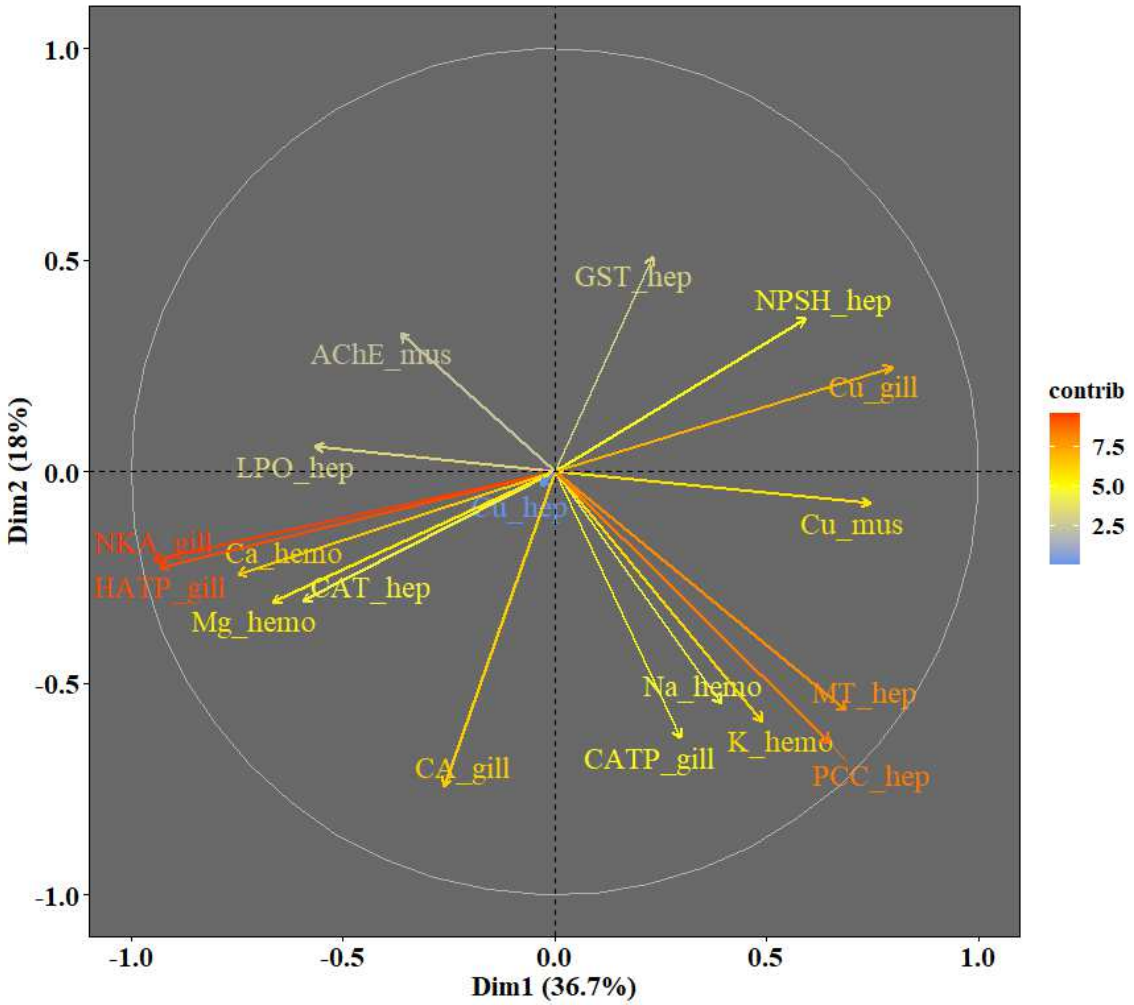

Fig. 8 – Principal component analysis (PCA) of the parameters measured in *Aegla castro* from the two field collections in the Couro Stream (July 2018 and January 2019). Vectors represent the parameters analyzed in each tissue and are denoted by the parameter abbreviations followed by the tissue abbreviations: gill (_gill), hemolymph (_hemo), hepatopancreas (_hep), and muscle (_mus). Different colors of the vectors are related to the contribution of each variable to the whole data variability ("contrib"), where a red vector is more influential and a blue one less influential. Acetylcholinesterase activity (AChE), Ca^{2+}-ATPase activity (CATP), Ca^{2+} concentration (Ca), carbonic anhydrase activity (CA), catalase activity (CAT), copper bioaccumulation (Cu), glutathione *S*-transferase activity (GST), K^{+} concentration (K), lipid peroxidation (LPO), metallothionein-like proteins content (MT), Mg^{2+} concentration (Mg), Na^{+} concentration (Na), Na^{+}/K^{+}-ATPase activity (NKA), non-protein thiol content (NPSH), protein carbonylation content (PCC), and H^{+}-ATPase activity (HATP).

3.4 DISCUSSION

Although still scarce in the literature, ecotoxicological studies using aeglid species as biological models have demonstrated that the investigation of multiple biomarkers constitutes an important early-warming tool to (i) identify whether aeglid populations are subjected to the sublethal effects of environmental stressors that impair habitat quality; and (ii) assess the risk of decline of some aeglid populations in the face of habitat degradation and water contamination caused by both metals and pesticides (Borges et al., 2022; Cerezer et al., 2020). Thus, the characterization of a series of suitable biomarkers and the use of aeglids as biological models becomes essential for early diagnosis of the harmful effects of habitat degradation in low-order streams, especially considering the high degree of threat of extinction and the urgency for conservation of many aeglid species.

In the present study, we collected specimens of *A. castro* from the Couro Stream on two different occasions: winter (July 2018) and summer (January 2019). We characterized a series of biochemical parameters related to oxidative stress, oxidative damage, neurotoxicity, and ionic regulation in the animals of these two field collections. We studied the hemocytes of *A. castro* only from the collection performed in January 2019. We characterized the hemocytes of *A. castro* for the first time, and, to the best of our knowledge, this is the first study to characterize the hemocytes of an *Aegla* species. Therefore, both the biochemical parameters and the morphological characterization of hemocytes in *A. castro* make a valuable contribution to increasing the knowledge of aeglid physiology, encouraging this immune parameter as a new biomarker in ecotoxicological studies with species of *Aegla*.

Circulating hemocytes are important components of crustacean immunity, since hemocytes are immunocompetent cells (Ray et al., 2015). The main competencies of these cells described in the literature include: (i) the agglutination of foreign particles and phagocytosis of pathogens, (ii) the formation of blood clots through the release of coagulant factors, (iii) the formation of nodules for trapping microorganisms, and (iv) the encapsulation processes (Söderhäll, 1992). Hemocytes have distinct specificities and exert different roles in crustacean immunity, allowing the categorization of these cells into subtypes. Hyaline, semigranular, and granular hemocytes are the three most reported categories of circulating hemocytes in crustaceans and are based mainly on morphological characteristics, such as size and number of cytoplasmic granules (Clare and Lumb, 1994; Hose et al., 1990; Söderhäll, 1992). Because the functional characterization of each hemocyte subtype is still a much-debated subject, Bouallegui (2021) reviewed and updated several aspects of the crustacean

immune system, with a focus on freshwater crayfishes, and summarized the main functions of each hemocyte subtype in crustaceans as follows: the hyaline hemocytes are the typical phagocytic cells; the semigranular hemocytes are responsible for encapsulation, infiltration processes, and also phagocytosis; and the granular hemocytes are involved in storing and releasing active molecules in degranulation processes and encapsulation.

In *A. castro* we also observed the same three hemocyte subtypes commonly found in crustaceans: hemocytes without evident cytoplasmic granules (hyaline) and hemocytes with evident cytoplasmic granules (semigranular and granular). As we analyzed the permanent slides in a light microscope we observed evident granules only in the semigranular and granular hemocytes, but Clare and Lumb (1994) argued that at the ultrastructural level all these three hemocyte subtypes could be observed to contain cytoplasmic granules. For example, Martin et al. (1991), using transmission electron microscopy, reported the presence of small electro-dense particles in the cytoplasm of hyaline hemocytes, which the authors suggested as being the stock of transglutaminase, an essential protein for coagulation. Regarding the granular hemocytes, some authors suggested that the cells with abundant and evident granules are responsible for the source of prophenoloxidase (ProPO) (Battison et al., 2003), an immune response-related enzyme system that encloses pathogens in melanized spots to prevent their development in the hemocoel (Svoboda et al., 2017).

Considering the morphometry parameters, we found significant differences in the maximum cell diameter among the three hemocyte subtypes, as found in the lobsters studied by Hose et al. (1990), in which the granular hemocytes were the larger hemocytes in the hemolymph when compared to the hyaline and semigranular hemocytes. With respect to the nucleus, the measurements of the maximum nucleus diameter remained constant in the three hemocyte subtypes but differed in terms of the nucleocytoplasmic ratio (N/C). This reinforces that the N/C ratio is an important proportional measure to illustrate the relative cell volume that is occupied by the nucleus. Thus, the higher the N/C ratio, the lower the cell volume occupied by the nucleus. The N/C ratio found in *A. castro* was higher in the hyaline and lower in the granular hemocytes. This same pattern was observed in *Loxorhynchus grandis* and *Panulirus interruptus*, but not in *Homarus americanus*, in which the granular and hyaline hemocytes present the highest and lowest N/C ratio, respectively (Hose et al., 1990).

Although the abundance of hemocyte subtypes was also considered a valuable immunological parameter to evaluate the sublethal effects of contaminants in aquatic invertebrates (Ray et al., 2015), it is important to mention that the relative proportion of hemocytes of each subtype can vary among species (Martin et al., 1991). For example, the

hyaline hemocytes were the most abundant subtype in the hemolymph of *A. castro*, similar to those found in the green crab *Carcinus aestuarii* (Qyli et al., 2020), but different from those found in *Procambarus clarkii* (Ding et al., 2012). Therefore, identification of the hemocyte subtypes, as well as the study of their abundance in the hemolymph through the performance of total and differential hemocyte counts, constituted the first step to establishing the hemocytes as a potential biomarker for the assessment of health status in *A. castro*.

Concerning the chemical abiotic parameters of the water, the values showed expected oscillations for each of the two seasons. Seasonal variations in the water abiotic parameters of small watercourses where aeglids occur are common and were also reported in several streams studied by Cerezer et al. (2020) in Southern Brazil. However, we identified a notable increase (~ 98%) in the dissolved Cu concentration in the water of the Couro Stream from the first collection (July 2018) when compared to six months later, in the summer field collection (January 2019). In the same stream, Rosa and Martinez (2021) reported higher values of Cu in May 2019 (0.80 and 0.67 μg L^{-1} total and dissolved Cu, respectively). Interestingly, we identified a progressive increase in both total (1.93 μg L^{-1}) and dissolved (1.05 μg L^{-1}) Cu concentration in the Couro Stream almost a year later (February 2020), in a new field collection (data not published). Therefore, considering that the Couro Stream is within an agricultural matrix and that many pesticides and fertilizers commonly applied in agriculture can have Cu in their composition (Li et al., 2020), this could indicate that *A. castro* may be subjected to the sublethal effects of chronic Cu exposure in the Couro Stream, even though the Cu concentrations measured in the water are within the limits permitted by the Brazilian guidelines (9 to 13 μg L^{-1} dissolved Cu) (Conama, 2005). Indeed, after measuring the Cu concentration in the tissues of *A. castro*, we observed higher content of Cu in the gill and muscle of the aeglids collected in January 2019 when compared to those collected in July 2018.

Despite Cu being an essential micronutrient at low concentrations, since it constitutes the structure of several enzymes, the hemocyanin, and the exoskeleton matrix of crustaceans (Rainbow, 2007), at high concentrations it becomes a ubiquitous contaminant of urgent concern in ecosystems worldwide, mainly due to its large application and release into aquatic environments (Li et al., 2020), being found in sediment and water of several rivers and streams of Southern Brazil (Borges et al., 2022; Faria et al., 2018; Rosa and Martinez, 2021).

After uptake by the water-permeable surfaces (gills, gut, and general body surface), Cu is used in essential metabolic needs, and the excess Cu must be excreted and/or detoxified and

bioaccumulated in a less toxic form in the tissues (Ahearn et al., 2004; Rainbow, 2002). As the gill epithelia constitute the primary interface to the external medium (Freire et al., 2008), the gills constitute the major entry to contaminants in the crustacean body (Güner, 2011).

The crustacean hepatopancreas is a typical metal storage tissue and plays a crucial role in metal homeostasis, by associating these toxicants with metallothioneins, non-protein thiols, and metal-containing granules, which are found in the hepatopancreatic epithelial cells of several invertebrates (Ahearn et al., 2004). These intracellular granules contain several metals, such as Cu, that can be complexed with sulfur or phosphorous (Henry et al., 2012). These granules can be excreted into the circulation through organismic excretory mechanisms performed both by the gills and antennal glands (Ahearn et al., 2004). This mechanism may explain the absence of an increase in the bioaccumulation in the hepatopancreas of *A. castro* from one collection to another, since this organ could be constantly performing excretory mechanisms under longer Cu exposure times (Ahearn et al., 2004). In contrast, bioaccumulation in the muscle of *A. castro* is expected during a long exposure period, since this organ is the last to be affected by metal contamination in crustaceans (Güner, 2011), as reported for *P. clarkii* exposed to Cu (Zhao et al., 2019).

Cu exposure can also trigger increasing ROS and consequent oxidative stress in aquatic animals. The ROS formation is counterbalanced by the antioxidant systems of the cell, maintaining a steady-state ROS concentration (Lushchak, 2011). The main non-enzymatic antioxidant defense of the cell is reduced glutathione (GSH), a non-protein thiol (NPSH) which is very abundant in the hepatopancreas. GSH can both directly bind Cu as soon it enters the cell (Ahearn et al., 2004) and act as a ROS scavenger after Cu-induced toxicity (Lushchak, 2011). In this study, we observed higher concentrations of NPSH in the hepatopancreas of the aeglids collected in January 2019. This significant induction evidences the protective role of NPSH in preventing oxidative damage in the hepatopancreas, as observed in the significantly lower concentration of LPO measured in the aeglids from January 2019. Moreover, the induction of NPSH may have supported the demands of Cu detoxification in the hepatopancreas without an increase in the MT content in this organ; as well as contributing to the prevention of oxidative stress and disturbance of the activities of catalase and glutathione *S*-transferase, since the NPSH functions as ROS scavenger (Lushchak, 2011).

In addition to ROS induction, Cu may cause severe impairment in gill functioning related to osmo-ionic disruptions (Hebel et al., 1997). In contact with the gill epithelia, Cu may compete with other cations for binding to the apical Na^+ channels or intracellular

enzymes, like the cytosolic CA and the basolateral transmembrane NKA. The direct binding of Cu to these enzymes can alter their catalytic function, with consequent failure of osmoregulatory mechanisms (Grosell et al., 2002). Here, we observed inhibition of NKA, HATP, and CA in the aeglids in January 2019, when the Couro Stream presented a higher Cu concentration when compared to July 2018. However, the inhibition of NKA did not lead to a disruption of gill Na^+ uptake regulation in *A. castro*. Indeed, it is well-known that Na^+ homeostasis is linked to NaCl homeostasis. The mechanisms of NaCl uptake in strong, hyperosmoregulating crustaceans—like aeglids—are dependent on the HATP promoting the electrogenic gradient (supported by H^+ provided by CA), which sustains the Na^+ influx through apical Na^+ channels; as well as the Cl^- influx, through apical Cl^-/HCO_3^- antiporters (supplied by HCO_3^- provided by CA) (Freire et al., 2008). The apical NaCl transport from the external medium to the hemolymph is, in turn, maintained by the electrochemical gradient generated by both the NKA located in the gill basolateral membrane and the cytosolic CA (Freire et al., 2008). Although disruption of Na^+ uptake regulation and a decrease in the hemolymph Na^+ concentration are expected responses when NKA and CA are inhibited (Grosell et al., 2002), other pathways can be related to the hemolymph Na^+ homeostasis in aeglids, like the exchanger $Na^+/H^+(NH4^+)$ in the apical membrane, performing Na^+ uptake to the intracellular medium; and the sodium-potassium two-chloride (NKCC) symporters in the basal membrane, performing the transport of Na^+, K^+, and Cl^- to the hemolymph (McNamara and Faria, 2020). In fact, Rosa and Martinez (2021) argued that the presence of other pathways maintaining the Na^+ uptake regulation would support the Na^+ homeostasis even after CA inhibition triggered by an acute Cu exposure. Therefore, we suggest that even though we observed inhibition of both NKA and CA, the Na^+ uptake regulation in *A. castro* may have been supported by other membrane transporters present in aeglid gills. Concerning the increased K^+ concentration in the hemolymph of *A. castro* collected in January 2019, we suggest that inhibition of the basolateral NKA would lead to a decrease in the K^+ influx in the gill cells (from the hemolymph to intracellular medium) and an increase in the K^+ efflux (from the intracellular medium to the hemolymph) through the basolateral K^+ channels, down its concentration gradient.

The Cu toxicity mechanisms related to the apical HATP are much less widely investigated than those related to the basolateral NKA. Cu has high affinity for the thiol groups and may directly bind to cysteine residues of NKA, interfering in a critical activation site (Li et al., 1996). Cu inhibitory effects on HATP were suggested by Grosell (2012), but more recently Chowdhury et al. (2016) observed, experimentally, inhibition in HATP in the

gills of the rainbow trout *Oncorhynchus mykiss* exposed to several Cu concentrations (0, 15, 25, 100 μg L^{-1} dissolved Cu) in the presence of Ca^{2+} (3.0 mmol L^{-1}) in the exposure medium. These authors suggested that the HATP is a new enzymatic target for Cu toxicity, since Cu may also bind to the cysteine residues in a specific catalytic site of HATP. Accordingly, our results showed a decrease in the activity of HATP in the aeglids collected in January 2019, and, to our knowledge, this is the first study to report the effects of Cu in the HATP in a species of *Aegla*.

Concerning the decreases in Ca^{2+} and Mg^{2+} concentrations in the hemolymph of aeglids collected in January 2019, we believe that the downregulation of these two cations is also related to the inhibition of the basolateral NKA. The transport pathways of Ca^{2+} are well investigated in crustaceans (e. g. Wheatly, 1999). Freire et al. (2008) summarized the mechanisms for the apical-to-basolateral transcellular Ca^{2+} transport in crustaceans, indicating that the apical Ca^{2+} transport in the gill involves the (i) amiloride-sensitive Ca^{2+}/H^{+} exchanger (unique to crustaceans), (ii) amiloride-insensitive electroneutral $Ca^{2+}/2H^{+}$ exchanger, and (iii) verapamil-sensitive Ca^{2+} channel; while the basolateral Ca^{2+} transport to the hemolymph involves the Ca^{2+}-ATPase and the $Ca^{2+}/2–3Na^{+}$ exchanger (both dependent on the Na^{+} transmembrane gradient, energized by the basolateral NKA). Therefore, as Ca^{2+} uptake regulation seems to be involved with Na^{+} homeostasis (Freire et al., 2008), the inhibition of the basolateral NKA observed in *A. castro* collected in January 2019 may have decreased the intracellular Na^{+} concentration required to maintain the correct functioning of the basolateral $Ca^{2+}/2–3Na^{+}$ exchangers, reducing Ca^{2+} transport to the hemolymph.

The PCA and the correlation matrix revealed that the parameters of *A. castro* collected in the Couro Stream between July 2018 and January 2019 were affected in two main ways: (i) osmo-ionic regulation and Cu homeostasis and (ii) hepatopancreatic antioxidant response. The variables Na^{+}/K^{+}-ATPase (NKA_gill) and H^{+}-ATPase (HATP_gill) in the gill, and Ca^{2+} (Ca_hemo) and Mg^{2+} (Mg_hemo) concentrations in the hemolymph were all positively correlated and explained most of the variation in the first component (PC1), suggesting the involvement of osmoregulation enzymes in the Ca^{2+} homeostasis in *A. castro*. Moreover, the negative correlations found when comparing NKA_gill, HATP_gill, Ca_hemo, and Mg_hemo with Cu concentration in the gill (Cu_gill) and muscle (Cu_mus) evidenced that the osmo-ionic regulation was impaired by Cu bioaccumulation in *A. castro*, so an increase in the Cu concentration in tissues may trigger an osmo-ionic disruption. Even though the CAT activity was not statistically different between the two field collections, PCA revealed that the variable CAT activity in the hepatopancreas (CAT_hep) was also influenced by Cu exposure

due to the negative correlations with both Cu_gill and Cu_mus.

Another response to Cu bioaccumulation is related to the hepatopancreatic antioxidant response, reflected in the induction of NPSH in the hepatopancreas (NPSH_hep). PCA revealed a positive correlation between the variables Cu_gill *vs.* NPSH_hep and a negative correlation between Cu_gill and lipid peroxidation in the hepatopancreas (LPO_hep), indicating that NPSH represents an important antioxidant response to Cu bioaccumulation in the gill, and also in the hepatopancreas, evidenced by the decreased lipid peroxidation observed in this tissue.

Although the MT content was not statistically different between the two field collections, PCA revealed that the variable MT_hep had a high contribution to the whole data variability and a positive correlation with Cu_mus, suggesting that high Cu concentrations in both gill and muscle may increase the NPSH and MT contents in the hepatopancreas. In addition, the oxidative damage in proteins (PCC_hep) was positively correlated with the variable MT_hep; and Cu_mus was positively correlated with both PCC_hep and MT_hep, suggesting that Cu bioaccumulation could lead to MT induction as a response to the protein carbonylation in the hepatopancreas caused by Cu stress.

According to the contribution of the variables to the whole data variability of the PC1 and PC2, we suggest that the increase in Cu concentration in the Couro stream caused the Cu bioaccumulation in tissues and affected the osmo-ionic regulation in *A. castro* in the field collection of January 2019 when compared to the collection in July 2018. In addition, the PCA suggested that Cu bioaccumulation could activate the hepatopancreatic antioxidant responses (MT and NPSH induction) to counterbalance the oxidative damage in proteins (PCC) caused by ROS formation.

Carbonic anhydrase activity was already reported to be inhibited by acute Cu exposure in *A. castro* (Rosa and Martinez, 2021). Here, we observed the same response in CA activity in the gill of *A. castro*, under chronic Cu exposure. However, the variable CA_gill was not significantly correlated with any other variable but had an expressive contribution to explain the whole data variability of the two principal components together in the PCA. Therefore, we believe that CA is an important enzyme of *A. castro* that is influenced by Cu, under both acute and chronic exposures.

3.5 CONCLUSION

We characterized for the first time the morphometry and the composition of hemocytes in an aeglid species, *A. castro*, which represents a first step towards the usage of this parameter as a new biomarker of immune response in aeglids. In addition, we analyzed a series of biochemical parameters in *A. castro*, which were very responsive to an increase in the dissolved Cu concentration observed in the water of the Couro Stream (January 2019), even in the short interval of six months since the first collection in July 2018.

Even though in this study we did not intentionally expose aeglids to Cu, we strongly believe that the higher Cu bioaccumulation in the gill and muscle of *A. castro* collected in January 2019 was triggered by a contamination event that favored Cu input in the Couro Stream, exposing the aeglids to increased Cu concentrations. Furthermore, this environmental Cu input and its consequent bioaccumulation in tissues may have contributed to the sublethal effects observed by the activation of hepatopancreatic antioxidant defenses, like NPSH induction; the ionic disruption in the gill, evidenced by the extracellular K^+ increase; and, ultimately, impairment in the osmoregulation and acid-base balance, with decreases in the activity of the enzymes NKA, HATP, and CA. The decrease in the Ca^{2+} and Mg^{2+} concentrations may be the result of the disruption of the osmoregulation enzymes which maintain the electrochemical gradient underpinning the Ca^{2+} uptake regulation. Moreover, the principal component analysis showed that Cu bioaccumulation in tissues tends to induce MT and NPSH in the hepatopancreas in response to the oxidative damage in proteins triggered by Cu stress.

Thus, we consider that the parameters analyzed here proved to be suitable biomarkers to assess the sublethal effects of Cu contamination in *A. castro*. Furthermore, we believe that the adoption of aeglids as biological models and the characterization of multiple biomarkers for the evaluation of the effects of xenobiotics can guide early decision-making in conservation studies concerning aeglids, especially in regions under high anthropogenic influence and contamination risk.

3.6 Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Capítulo II

Short communication: Effects of acute copper exposure on ionic regulation of the freshwater crab *Aegla castro*

4 CAPÍTULO II

4.1 ARTIGO PUBLICADO NA REVISTA COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY, PART C

Short communication: Effects of acute copper exposure on ionic regulation of the freshwater crab *Aegla castro*

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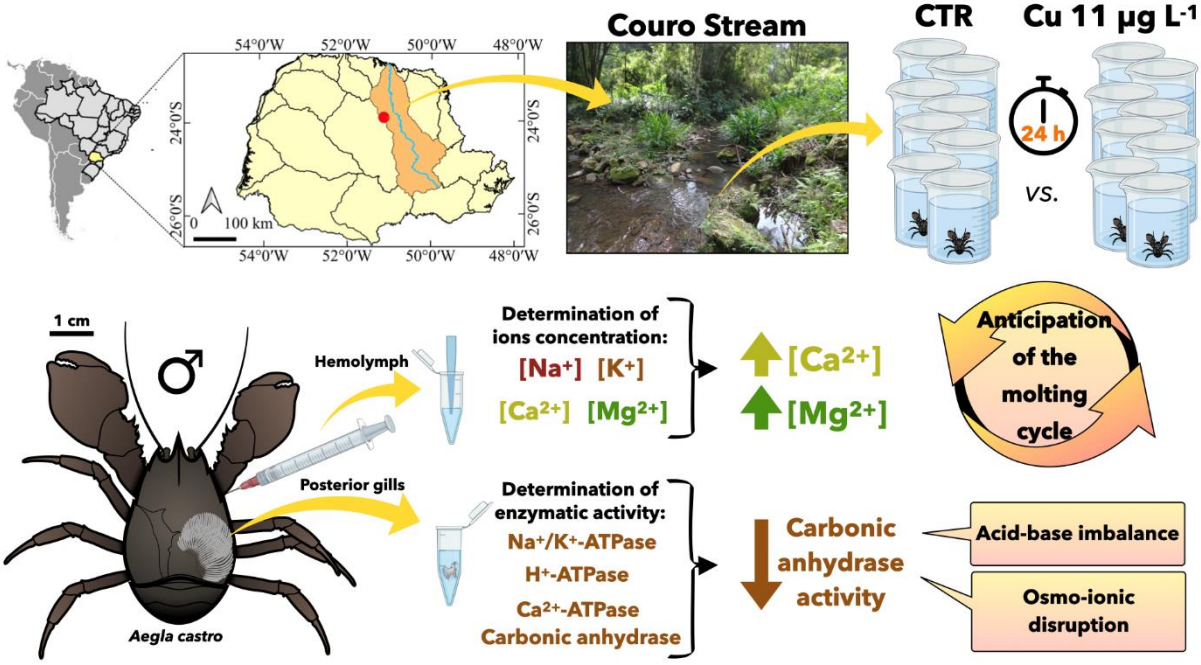
Highlights

1. Freshwater anomuran crab *Aegla castro* was exposed to Cu (11 μg L^{-1}) for 24 h
2. Acute Cu exposure increased hemolymph Ca^{2+} and Mg^{2+} concentrations
3. Cu exposure may have triggered the start of a new molting cycle
4. Cu inhibited gill activity of carbonic anhydrase
5. Cu did not affect the hemolymph Na^{+} and K^{+} concentrations

Graphical abstract



Abstract

Aeglids are unique freshwater decapods whose habitats are being impacted by metallic compounds, such as copper (Cu). Thus, we investigated the effects of acute Cu exposure on ionic regulation of *Aegla castro*. For this, male specimens in intermolt were collected from a reference stream and acclimated for 5 days in laboratory. After which, crabs were exposed to 11 μg L^{-1} Cu (Cu11) or only to water (CTR) for 24 h. Hemolymph samples were withdrawn for the determination of Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations and the posterior gills removed for the analysis of Na^+/K^+-ATPase, Ca^{2+}-ATPase, H^+-ATPase, and carbonic anhydrase (CA) activities. Increased Ca^{2+} and Mg^{2+} hemolymph concentrations were observed in animals from Cu11, when compared with CTR group. In addition, decreased activity of CA was observed in animals exposed to Cu. In the current study, alterations in Ca^{2+} and Mg^{2+}concentrations probably indicate that animals activated exoskeleton reabsorption mechanisms, characteristic of the premolt. Therefore, increased Ca^{2+} and Mg^{2+} concentrations in hemolymph may indicate that a biochemical signal associated with the molting cycle was triggered by Cu exposure. Despite the known harmful effects of Cu on osmoregulatory enzymes, here we observed decreased activity only in CA. However, decreased activity of CA could trigger both acid-base imbalance and ionic disruption, since CA provides H^+ and HCO_3^- for intracellular pH maintenance, and underpins Na^+ and Cl^- for ionic regulation. Therefore, understanding how aeglids respond to metal contamination in laboratory conditions is crucial to assess their potential as an alternative biological model for aquatic ecotoxicology.

Keywords: aeglid; carbonic anhydrase; ion transport; metal exposure; molting; osmoregulation.

4.2 INTRODUCTION

In the last few years, the crabs of the genus *Aegla* Leach, 1820 (Anomura, Aeglidae) have been increasingly addressed in physiological and ecotoxicological studies (Faria et al., 2011; Freire et al., 2013; Faria et al., 2018; Bozza et al., 2019; Cerezer et al., 2020). Indeed, some aspects illustrate why these remarkable freshwater anomurans have received additional attention in applied studies, for instance, the increasing number of new species descriptions (26) in the last two decades (Santos et al., 2017), the ecological relevance and wide variety of freshwater habitats that aeglids are adapted to (Bond-Buckup and Buckup, 1994; Bueno et al., 2016), and the adequacy of aeglids as bioindicators of environmental quality under a biomarker approach (Faria et al., 2018; Cerezer et al., 2020). On the other hand, the fragmentation of riparian forest, degradation of limnic habitats by siltation, and contamination of water bodies by pesticides and fertilizers represent the three main anthropogenic threats to aeglid diversity (Boos et al., 2020), raising concern and demonstrating the urgency of addressing these freshwater decapods in ecotoxicological studies.

Aegla castro Schmitt, 1942 — target species of the present study — is endemic to Southern Brazil (Bond-Buckup and Buckup, 1994). Regardless of the slightly interspecific differences on the ecological niche, aeglids constitute an important group of freshwater crustaceans from an ecological perspective since they require environments with well-oxygenated waters and play a key role in the cycling of nutrients, becoming bioindicators of habitat quality (Bond-Buckup and Buckup, 1994). More recently, Almeida et al. (2021) investigated the trophic ecology of *A. castro* and characterized it as an omnivorous generalist and opportunist species.

Despite *A. castro* being considered a species of “least concern (LC)”, approximately 70% of species of *Aegla* are under some degree of threat according to the criteria established by the IUCN (Santos et al., 2017) since many species are being threatened by habitat loss due to the metal contamination (Gonçalves et al., 2018; Bueno et al., 2016). Cu is one of the most common pollutants found in all aquatic environments (Wei and Yang, 2016), being widely applied in industry, electric wires, water pipes, metal mixtures, and boat paints (Simonato et al., 2016). Cu also constitutes agricultural fertilizers and pesticides, mainly in the fungicidal composition (Gonçalves et al., 2018; Li et al., 2020), leading this metal to be leached or even discharged into headwater streams within agricultural or urban areas (Simonato et al., 2016). Indeed, Faria et al. (2018) found Cu concentrations in the sediment taken from different streams in Southern and in Southeastern Brazil where ten aeglid species were collected,

including *A. castro*. Under a phylogenetic perspective, the authors investigated metal accumulation and antioxidant defense system and emphasized the importance of a systematic approach for future monitoring studies with aeglids.

Although Cu is an essential micronutrient at low concentrations, at high concentrations Cu has been extensively linked to adverse effects in ionic balance and inhibitory effects on enzymes involved in osmoregulation in fish (e. g. Malhotra et al., 2020) and crustacean species (Hansen et al., 1992; Hebel et al., 1999; Brooks and Mills, 2003; Capparelli et al., 2020), being considered an osmoregulatory toxicant (Grosell et al., 2002; Bianchini et al., 2004).

In this sense, to our knowledge, we investigated for the first time the effects of acute Cu exposure in an aeglid species, by assessing the effects of this metal on ionic regulation of specimens of *A. castro* kept under controlled laboratory conditions. Thus, as Cu is widely reported as an osmoregulatory toxicant (Grosell et al., 2002; Bianchini et al., 2004), we hypothesize that acute Cu exposure will disrupt the ion regulation and inhibit the enzymes involved in osmoregulation in the freshwater crab *A. castro*. Although field studies have been carried out with other aeglid species, like *Aegla longirostri* (Cerezer et al., 2020), biomarker data from laboratory toxicity tests are still scanty in the literature.

4.3 MATERIAL AND METHODS

For this, male specimens of *A. castro* in the intermolt stage were collected from the Couro Stream (23º57'15''S, 51º07'00''W), Tibagi River Basin, Southern Brazil in May 2019. This stream has no apparent pollution sources, with riparian forest along some stretches of its extension. Non-filtered and filtered (0.45-µm mesh filter, Millipore Millex HV/PVDF) water samples from the collection site were taken for determination of total (Cu T) and dissolved Cu (Cu D) concentrations, and the concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+}. We also collected 100 L of stream water to be used in the laboratory for the acclimation of the crabs. The aeglids were transported to the laboratory (~90 min) in boxes containing 15 L of stream water and plastic artificial hiding places.

Acclimation was performed for five days in a 100-L plastic tank containing hiding places and stream water with constant aeration, filtration, and a 12/12 h light/dark photoperiod. Animals were fed every day with flocked fish feed, except on the day of the experiment. Partial water renewal of 25% was performed every 24 h with dechlorinated tap water at the same temperature as the water of the acclimation tank. Physical and chemical variables were monitored (Horiba U-52) during the acclimation.

Thereafter, the crabs were randomly divided into two groups: the control group (CTR), which was kept under controlled conditions with no addition of Cu in the exposure medium; and the waterborne Cu group (Cu 11), which was exposed to 11 µg L^{-1} dissolved Cu. The exposures were carried out in 2-L beakers containing 1.5 L of dechlorinated tap water with Cu (Cu 11 group), or without Cu (CTR group), with constant aeration and temperature control; both groups were exposed for 24 h. Physical and chemical parameters were monitored at 0 h and 24 h.

Non-filtered and filtered water samples from two beakers of each group were taken at 0 h and 24 h to determine the Cu T and Cu D concentrations. Samples were immediately acidified with HNO_3 and kept at 4 °C until the analysis. The Cu T and Cu D concentrations were determined using a graphite furnace atomic absorption spectroscopy (AAnalyst700, PerkinElmer), with a detection limit of 0.014 µg L^{-1}. Ion concentrations in the water were measured in non-filtered samples, both from the field and the exposure media (0 h and 24 h). Na^+ and K^+ concentrations were determined by flame photometry (Digimed DM-62) and Ca^{2+} and Mg^{2+} concentrations by atomic absorption spectroscopy (AAnalyst700, PerkinElmer).

After 24 h of exposure, the crabs were cryo-anesthetized, weighed, and measured (CTR group: n = 8; 2.43 ± 1.03 g; 14.13 ± 2.03 mm carapace length; and Cu 11 group: n = 8;

2.69 ± 1.35 g; 16.50 ± 2.19 mm carapace length; mean ± SD). Hemolymph aliquots were withdrawn from the arthrodial membrane of chelipeds for the determination of ion concentrations using the same methodology described above for the ions measured in water samples. Subsequently, the gills were dissected and stored in SEI buffer (150 mM sucrose, 50 mM imidazole, 10 mM EDTA) at –80 °C until the analysis.

The gills were thawed, weighed (0.095 ± 0.010 g, mean ± SE), and homogenized (1:5 w/v) in SEI buffer with 12 mM sodium deoxycholate (pH 7.5), using an ultrasonic sonicator. Next, the homogenates were centrifuged (20 min, 16060×g, 4 °C) and the supernatant was used to determine the activities of carbonic anhydrase (CA), Na^+/K^+-ATPase (NKA), V-H^+-ATPase (HATP), and Ca^{2+}-ATPase (CaATP). The total protein content was measured at 595 nm, according to Bradford (1976).

To assay the CA activity we followed Vitale et al. (1999), with some adaptations in reaction medium and centrifugation. An aliquot of the homogenate was added to CO_2-saturated distilled water (2.0 – 2.5 °C) and the acidification of the medium was quantified every 4 s for 20 s with a pH meter (Jenway 3510). The linear relationship of the decrease in pH as a function of time generated a slope of the curve, the catalyzed reaction (CR) rate. The non-catalyzed reaction (NCR) rate was obtained by measuring the decrease in pH when the sample was not added to the assay. Thus, the specific reaction rate of CA (SCA) was calculated as follows: SCA = [CR/NCR – 1]/mg protein.

Na^+/K^+-ATPase (NKA) and V-H^+-ATPase (HATP) activities were measured in a simultaneous assay following Gibbs and Somero (1989) and adapted to a microplate reader (Tesser et al., 2020). The samples were adjusted to a protein concentration of 1 mg mL^{-1}. The reactive solutions (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM KCN, 1 mM ATP, 0.2 mM NADH, U mL^{-1} pyruvate kinase, 2 U mL^{-1} lactate dehydrogenase, 0.1 mM fructose-1-6-diphosphate, 2 mM phosphoenolpyruvate, pH 9.0) containing 2 mM ouabain or 2 mM N-Ethyl-d_5-maleimide (NEM) were made to determine the activities of NKA and HATP, respectively. The total activity of the ATPases was measured without adding inhibitors to the medium. The absorbance was measured every minute for 15 min at 340 nm (Victor3, PerkinElmer). The specific activities of NKA and HATP were calculated by the difference between the total activity of the ATPases and the medium with ouabain and NEM, respectively. Enzyme activities were expressed in μmol ADP mg $protein^{-1}$ h^{-1}.

CaATP activity was assayed according to Vijayavel et al. (2007), with modifications. Samples were incubated at 30 °C for 30 min in reactive solution (189 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris, 5 mM $CaCl_2$, 2 mM ouabain, pH 7.6) with 3 mM ATP or without ATP

added, to assess the reaction of CaATP and the basal concentration of inorganic phosphate (Pi), respectively. After incubation, the microplate was placed on ice for 10 min to stop the reaction; and then, a staining solution (1:6 v/v 10% ascorbic acid:0.42% ammonium molybdate in 0.5 mM H_2SO_4) was added to the plate (Ames, 1966). The formation of Pi was measured at 620 nm after 10 min in a microplate reader. A phosphate standard curve (0.08–0.65 mM) was used to quantify the CaATP activity. The results were expressed as μmol Pi mg protein^{-1} min^{-1}.

Normality (Shapiro-Wilk test) and homoscedasticity (Levene test) of the biomarker results were verified. The results obtained for Cu exposure groups (Cu 11) were compared to their respective control groups (CTR) using the Student's t-test or Mann-Whitney test, according to data distribution. Non-parametric data are illustrated as boxplots. All statistical analyses were performed in the R environment (R Development Core Team, 2020), and the significance level adopted was 0.05.

4.4 RESULTS AND DISCUSSION

Physical and chemical parameters of the water measured during the exposures at 0 h and 24 h are presented in Table 1. Although not statistically tested, we found mean values of turbidity lower (~ 50%), and conductivity higher (~ 90%) during the experiments when compared to the collection site (Table 1).

Table 1. Abiotic variables, ions concentration, and total and dissolved copper concentrations (Cu T and Cu D, respectively) in the water of the collection site (Couro Stream), the laboratory acclimation tank, and the beakers where the two groups (CTR and Cu 11) were exposed. The CTR group was exposed to dechlorinated water with no addition of Cu in the exposure medium; and the Cu 11 group was exposed to dechlorinated water with addition of 11 μg L^{-1} Cu. The data from the CTR and Cu 11 groups were measured at 0 h and 24 h. Results are reported as mean values.

Abiotic variable	Collection site	Acclimation	CTR		Cu 11	
			0 h	24 h	0 h	24 h
Temperature (°C)	15.6	16.6	17.3	16.1	16.4	16.0
pH	7.7	7.6	7.6	7.8	6.8	7.5
Dissolved oxygen (mg O_2^{-1})	8.5	8.3	7.3	7.8	7.6	7.3
Turbidity (NTU)	23.0	9.3	13.4	12.8	5.9	8.8
Conductivity (μS cm^{-1})	61.0	192.0	114.5	121.5	112.5	112.5
Oxidation-reduction potential (ORP mv^{-1})	359	363	358	317	399	344
Total dissolved solids (g L^{-1})	0.04	0.12	0.07	0.08	0.07	0.07
Hardness (mg L^{-1} $CaCO_3$)	21	—	38	—	34	—
Na^+ (mM)	0.139	—	0.222	0.226	0.218	0.226
K^+ (mM)	0.043	—	0.023	0.033	0.020	0.022
Ca^{2+} (mM)	0.084	—	0.074	0.083	0.069	0.071
Mg^{2+} (mM)	0.058	—	0.066	0.067	0.065	0.066
Cu T (μg L^{-1})	0.803	—	3.251	3.732	15.245	12.180
Cu D (μg L^{-1})	0.670	—	3.055	3.478	11.035	11.035

The dashes indicate variables not measured. The data of ion concentration in the water, Cu T, and Cu D were obtained from non-filtered water samples.

These differences can be related to the physicochemical characteristics of stream water when compared to the dechlorinated tap water used in the exposure media. Also, the mean values of Na^+ concentrations in the water of the exposure media were nearly double those found in the collection site. The turbidity of stream water may have been higher due to the greater amount of suspended sediments, and the conductivity in the exposure media may have

been lower due to the higher Na^+ concentration of the exposure media when compared to the stream water (Table 1).

The Cu T and Cu D from the CTR group had similar values, both at 0 h and 24 h (Table 1). In the Cu 11 group, the mean Cu D concentration corresponded to 81.5% of the mean Cu T concentration. In addition, as the mean Cu D concentration deviated only 0.32% from the expected one, the concentration of 11 μg L^{-1} dissolved Cu was used. This dissolved Cu concentration is within the limits set by the Brazilian guidelines for freshwater (Conama, 2005). Cu-contaminated watercourses have been progressively found in Southern Brazil watersheds. Specifically, concerning the Tibagi River Basin, a study conducted on water quality monitoring pointed out dissolved Cu concentrations above the limits in all the sampling points along the Tibagi River (IAP, 2012). From April 2010 to December 2011 up to 207 μg L^{-1} dissolved Cu was found in the water (IAP, 2012), approximately 23-fold the permitted concentration (Conama, 2005). Furthermore, Cu has also been found in the sediments of small streams from Southeastern and Southern Brazil, where at least 10 species of *Aegla* are recorded (Faria et al., 2018). In this same study, Cu accumulation was reported in the hepatopancreas tissue of several species of *Aegla*, including *A. castro*, indicating that aeglids not only experience contact with Cu in their habitats but can accumulate this metal, being susceptible to the sublethal effects of Cu exposure. Therefore, dissolved Cu concentrations above 11 μg L^{-1} can be found in the environment, even in the streams belonging to the same watershed where *A. castro* occurs.

Crab survival was 100% during the experiment in both treatment groups. Aeglids exposed to Cu 11 presented no alterations in the concentrations of Na^+ and K^+ in the hemolymph when compared to CTR group (Fig. 1A–B). Although adverse effects caused by Cu exposure could promote the disruption of gill Na^+ uptake regulation, mainly associated with the NKA inhibition (Grosell et al., 2002; Brooks and Mills, 2003), here we did not observe any alteration in the activity of NKA and HATP in *A. castro,* after Cu exposure (Fig. 1C–D). This seems to be reasonable since the maintenance of the NKA and HATP contributes for the homeostasis of Na^+ and K^+ in the hemolymph (Freire et al., 2008).

Indeed, the maintenance of the Na^+, K^+, and Cl^- equilibrium is crucial in hyperosmoregulating, hololimnetic crustaceans, like aeglids, since these animals experience a very dilute external medium and constantly have to employ active, transbranchial NaCl absorption to compensate for the passive ions loss to the environment (Freire et al., 2008; McNamara and Faria, 2012).

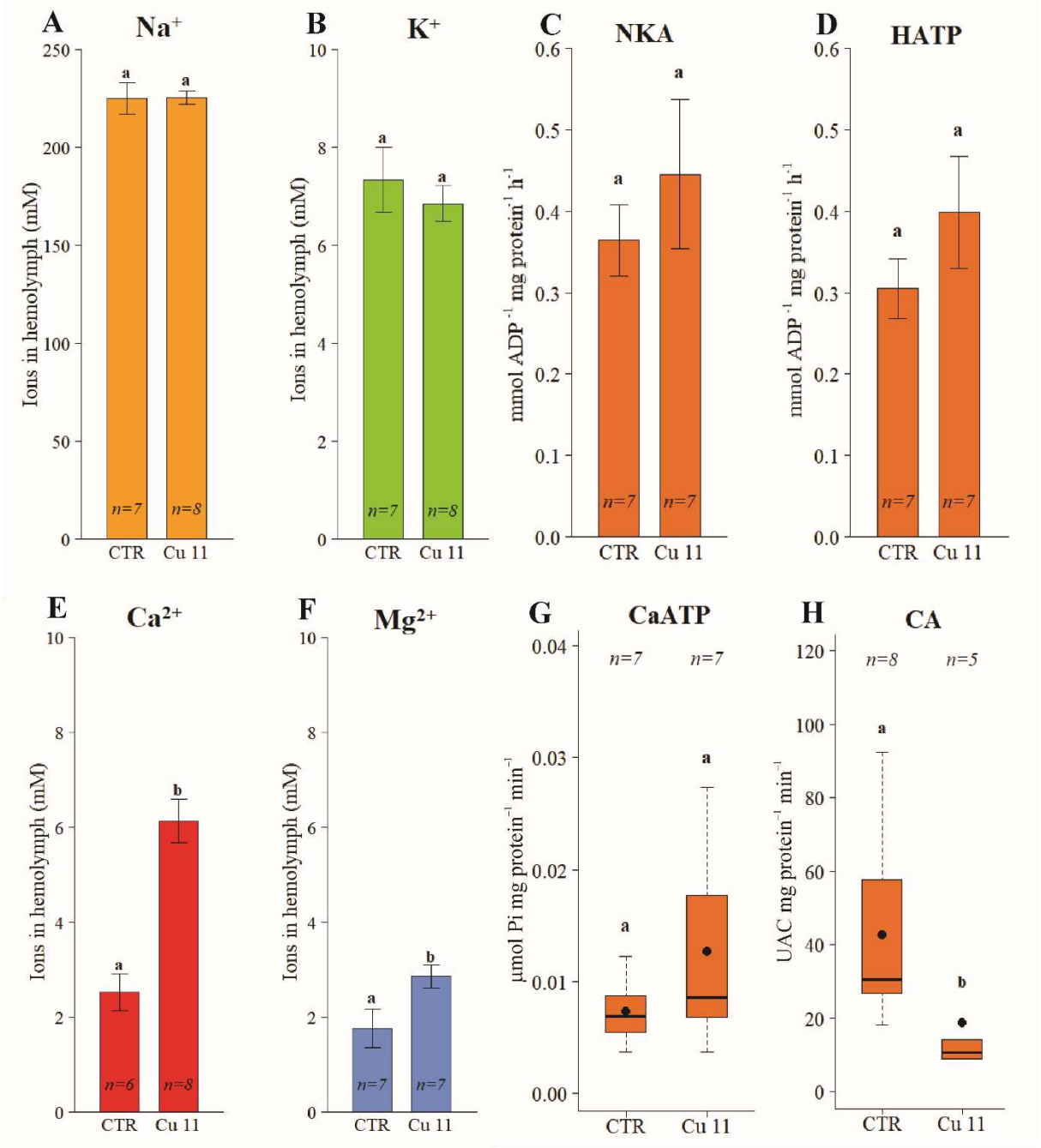


Fig. 1 – Concentrations of Na^+ (A), K^+ (B), Ca^{2+} (E), and Mg^{2+} (F) in the hemolymph, and activities of Na^+/K^+-ATPase (C), V-H^+-ATPase (D), carbonic anhydrase (G), and Ca^{2+}-ATPase (H) in posterior gills of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) or exposed to waterborne Cu (addition of 11 µg L^{-1}dissolved Cu), at 0 h and 24 h. Data are expressed as mean ± SE. Different letters indicate statistical differences when compared to the respective control group ($p < 0.05$). Sample sizes (n) are indicated on the chart. Boxplots express non-parametric data.

The HATP has a pivotal role on Na^+ uptake, from the external medium to the cytosol and then to the hemolymph. The H^+ (provided by CA) is actively discharged by HATP into the subcuticular space, hyperpolarizing the apical membrane, promoting the Na^+ influx through apical Na^+ channels down its electrochemical gradient. Active Na^+ transport from the

cytosol into the hemolymph is, in turn, maintained by the NKA located in the gill basolateral membrane (Freire et al., 2008). On the other hand, the Cl^- uptake from the external medium is favored by the ion exchange through the apical Cl^-/HCO_3^- antiporters (supplied by HCO_3^- provided by CA), and then the passive transport to the hemolymph through basolateral Cl^- channels (Freire et al., 2008).

However, the concentrations of Ca^{2+} and Mg^{2+}in the hemolymph after Cu exposure were 58.8% and 38.3% higher when compared to their respective CTR groups (Ca^{2+}: $t(12)$=-5.7230, p<0.001; Mg^{2+}: $t(12)$=-2.1388, p=0.0211) (Fig. 1E–F). These increases could indicate that the crabs exposed to Cu activated reabsorption mechanisms from the exoskeleton by mobilizing Ca^{2+} and Mg^{2+} through the integumentary epithelium and depositing them into the hemolymph in the form of carbonates of calcium and magnesium (Greenaway, 1985; Ahearn et al., 2004). Different storage strategies of these carbonated deposits are reported for crustaceans, such as in the form of gastroliths; calcium phosphate granules in hepatopancreas; or even as microspherules in the hemolymph, giving the milky aspect to the decapods hemolymph in premolt (Greenaway, 1985). Indeed, at least for Ca^{2+}, massive cation fluxes across epithelia (Ahearn et al., 2004), as well as a conspicuous peak in the hemolymph Ca^{2+} concentration are widely reported for both terrestrial, marine, brackish, and freshwater crustacean species in the late premolt (Greenaway, 1985). Also, the high concentrations of Ca^{2+} and Mg^{2+} found in the hemolymph of *A. castro* exposed to Cu appear to be in the same order of magnitude as those found in other crustacean species in premolt (Li and Cheng, 2012). This conspicuous increment in the concentrations of Ca^{2+} and Mg^{2+} can represent a biochemical signal that is associated with the start of new molting cycles in many crustaceans during premolt, mainly due to the massive storage of Ca^{2+} and Mg^{2+} into the hemolymph. Furthermore, a significant shortening of the time to the first molt as well as a decreased growth rate and molting frequency has been reported for the tiger shrimp *Penaeus monodon* exposed to Cu (Chen and Lin, 2001).

In addition, we observed a growing trend in the activity of CaATP (Fig. 1G), which although not statistically different from the respective control, may indicate an increase in the Ca^{2+} flux towards the hemolymph. Thus, as Cu was reported to disrupt the Ca^{2+} homeostasis in the lobster *Homarus americanus* (Chavez-Crooker et al., 2002), coupled with the fact that the molting process was indicated to be a way of depurating metals (Bergey and Weis, 2007), acute Cu exposure contributed to the imbalance of Ca^{2+} and Mg^{2+} in the hemolymph of *A. castro*, which may represent a biochemical signal associated with the start of molting cycle.

On the other hand, we identified a decrease of 55.8% in the CA activity (W=34,

p=0.0451) in posterior gills of *A. castro* after Cu exposure (Fig. 1H). Similarly, decreased gill CA activity was reported in *Minuca rapax* exposed to both dietary and waterborne Cu (Capparelli et al., 2017; 2020); as well as inhibition of the posterior gills CA of *Chasmagnathus granulata* exposed to waterborne Cu (Vitale et al., 1999). These effects are expected since Cu enters the gill cells, and readily binds to CA (DiTusa et al., 2001), triggering, in turn, reduced branchial Na^+ and Cl^- transport due to depletion of the exchangeable cellular supplies required in these fluxes (Grosell et al., 2002).

Although CA is primarily a cytosolic enzyme responsible for the reversible hydration/dehydration reactions of CO_2, it has been widely stated that acid-base balance is coupled with NaCl homeostasis through the supply of cellular substrates (H^+ and HCO_3^-) in crustacean gill epithelia (Grosell et al., 2002; Freire et al., 2008; McNamara and Faria, 2012). On the other hand, Wang et al. (1998) reported that Cu exposure can lead to an acid-base imbalance even when the Cu concentration was not enough to provoke marked osmoregulatory disturbances. Likewise, Grosell et al. (2002) suggested that CA is another component of branchial Na^+ transport that could be affected by Cu exposure before the basolateral NKA. Thus, the decreased CA activity found in our study might reveal that the 24 h exposure time was not sufficient to disturb Na^+ and K^+ homeostasis in the hemolymph; however, Cu could trigger an initial process of ionic disturbance through the decreased activity of CA, even though no alteration was observed in the activities of the HATP and NKA after 24 h of Cu exposure. Moreover, McNamara and Faria (2020), studying the gill tissue of *Aegla franca* reported that other pathways of hemolymph Na^+ homeostasis may be present in aeglids, such as the $Na^+/H^+(NH_4^+)$ exchangers in the apical membrane, supporting the influx of Na^+ to the intracellular medium; while Cl^- channels and sodium-potassium two-chloride symporters (NKCC) could be located in the basolateral membrane, also providing the transport of Cl^-, K^+, and Na^+ to the hemolymph.

Our hypothesis was partially accepted, as acute Cu exposure disrupted the Ca^{2+} and Mg^{2+}, but not the Na^+ and K^+ homeostasis, as well as inhibited only the activity of carbonic anhydrase among all the other enzymes analyzed involved in osmoregulation. Therefore, these results could indicate that both the concentrations of Ca^{2+} and Mg^{2+} in the hemolymph and the gill activity of carbonic anhydrase may be considered suitable biomarkers for the evaluation of Cu sublethal effects in *A. castro* exposed to 11 μg L^{-1} dissolved Cu, a concentration that is within the limits set by the Brazilian guidelines for freshwater (Conama, 2005).

In summary, this study is the first step towards enhancing our understanding of Cu

exposure effects on aeglids. Metal exposure studies with *Aegla* are essential to comprehend how these unique freshwater decapods cope with environmental contamination as well as their potential as an alternative biological model in ecotoxicology. Further experimental investigations regarding other aeglid species and exposure times could clarify our knowledge of how *Aegla* faces habitat contamination by Cu.

4.5 Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Capítulo III

Multiple biomarker responses in *Aegla castro* exposed to copper: a laboratory approach

5 CAPÍTULO III

Manuscrito em preparação para ser submetido à revista Aquatic Toxicology

Multiple biomarker responses in *Aegla castro* exposed to copper: a laboratory approach

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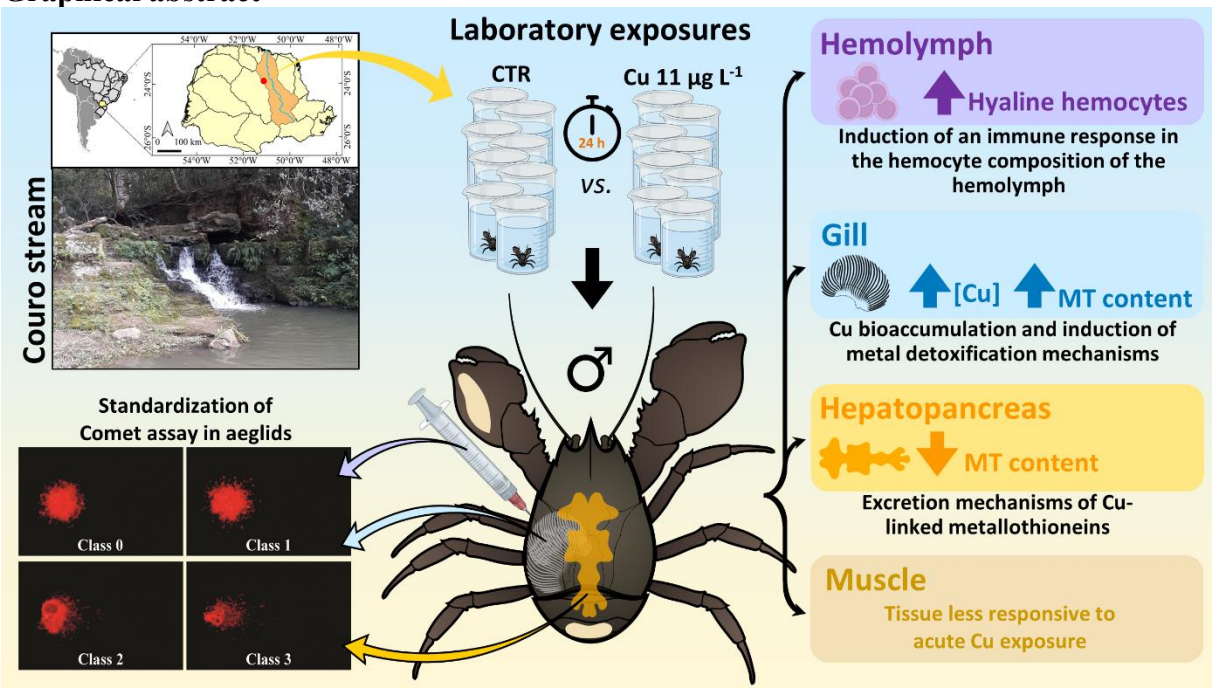
Highlights

1. *Aegla castro* accumulated Cu in gills after 24 h of acute exposure
2. Cu exposure activated detoxification mechanisms in gills and hepatopancreas
3. Cu provoked an immune response related to the increase in hyaline hemocytes
4. Hemocyte counts is a potential biomarker for biomonitoring studies using aeglids
5. This study assessed DNA damages for the first time in an aeglid species

Graphical abstract



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Abstract

Aeglids are considered bioindicators of habitat quality and some populations occur in areas contaminated by Cu. Although some biomarkers have already been determined in aeglids collected in the field, data from laboratory exposures are scarce. To our knowledge, no studies have investigated oxidative stress biomarkers in aeglids exposed to metals in the laboratory, or performed the comet assay and hemocyte counts under a biomarker perspective. Thus, we investigated the effects of acute Cu exposure in biomarkers of *Aegla castro* and propose their use in future studies with aeglids. For this, male specimens in intermolt were collected from a reference stream and acclimated for 6 days in the laboratory. The crabs were then exposed to 11 μg L^{-1} dissolved Cu (Cu 11) or only to water (CTR), for 24 h. Gill and hepatopancreas samples were used to determine Cu accumulation, DNA damage, and metallothionein content (MT), while hemolymph samples were used to determine Cu accumulation, DNA damage, and hemocyte counts. Muscle samples were used to determine Cu accumulation and acetylcholinesterase activity (AChE). Non-protein thiol content (NPSH), catalase (CAT), and glutathione *S*-transferase activities (GST), lipoperoxidation (LPO) and protein carbonylation content (PCC) were measured only in the hepatopancreas. Biomarker data were explored with a principal component analysis (PCA). *Aegla castro* exposed to Cu presented accumulated metal in gills and activated detoxification mechanisms, through increased MT content in the gill, as well as presenting an immune response, evidenced by an increase in hyaline hemocytes. Therefore, gill and hemocytes appear to have a protective role in preventing the transport and bioavailability of Cu through the body. We also observed MT content decreased in the hepatopancreas of animals exposed to Cu, suggesting its consumption. The PCA results suggest that Cu may cause oxidative damage in the hepatopancreas of *A. castro* at higher exposure times.

Keywords: *Aegla*; bioaccumulation; comet assay; hemocytes; immune response; metallothioneins.

5.1 INTRODUCTION

Aeglids are unique freshwater crustaceans that inhabit continental waters in southern South America, and which have been considered bioindicators of water and habitat quality, especially due to their ecologically key role in freshwater ecosystems (Bueno et al., 2016). They frequently occur in clear and well-oxygenated waters of headwater streams, small creeks, rivers, lakes, lagoons, and caves from the Neotropics. They are essential for the maintenance of trophic chains and contribute as important shredders in the decomposition processes of leaf litter and nutrient cycling (Bond-Buckup and Buckup, 1994; Bueno et al., 2016).

However, some aeglid species are highly endemic and exhibit a fragmented distribution pattern, occurring in densely populated areas (Gonçalves et al., 2018). Thus, some populations occur in anthropogenic areas severely impacted by changes in land-use, such as exposed soil, agriculture, and pasture as well as habitat degradation like water-flow alterations for the construction of irrigation systems, canals, and dams (Boos et al., 2020). Furthermore, water contamination by pesticides and metals from intensive agriculture or even from effluent discharges of industrial activities and domestic sewage are among the most common threats to habitat loss for aeglids (Santos et al., 2017). For instance, Borges et al. (2022) found multiple metals, including Cu, in the bioavailable fraction of sediments from 14 streams within 3 hydrographic basins in Southern Brazil where aeglid populations occur, and observed a correlation between the concentration of metals in the sediment and the levels of oxidative stress biomarkers in biological extracts of aeglids.

At low concentrations, Cu is an essential micronutrient. It is involved in multiple enzymatic processes and constitutes the structure of a variety of enzymes, such as copper-zinc superoxide dismutase, carbonic anhydrase, cytochrome oxidase, and monoamine oxidase. Cu is also essential in oxygen transport, constituting the hemocyanin and exoskeleton matrix of crustaceans (Rainbow, 2007). On the other hand, Cu is applied in a myriad of industrial processes, being used in metal mixtures, water pipes, boat paints, and electric wires, as well as in the composition of fertilizers, pesticides, and fungicides (Li et al., 2020; Simonato et al., 2016). As a consequence of its extensive employment, Cu can be leached or even discharged into headwater streams from agricultural or urban areas (Simonato et al., 2016). Not surprisingly, several watercourses from southern Brazil have been contaminated by Cu (Faria et al., 2018), including the distribution area of *A. castro* in the Tibagi River basin (IAP, 2012).

High Cu concentrations by both dietary and waterborne exposures have been well

demonstrated to increase the generation of reactive oxygen species (ROS), leading to oxidative stress in aquatic organisms (Lushchak, 2011). In crustaceans, when the concentration of an essential metal increases above the required physiological thresholds and overcomes the detoxification and excretion capacities it can trigger metal-induced toxicity (Rainbow, 2002) through the production of ROS by multifactorial mechanisms, including Fenton-like reactions (Lushchak, 2011). When there is an imbalance between the generation of ROS and the elimination of these molecules by the antioxidant defense systems of the cell, there is a condition of oxidative stress. This stress can cause damage to biomolecules, such as alterations in thiol status, increased lipid peroxidation, protein and nucleic acid oxidation, and depletion in the antioxidant defense systems as a whole (Lushchak, 2011). Apart from the ROS-induced stress, Cu can also directly bind to DNA, triggering functional and structural alterations in the DNA molecule (Govindaraju et al., 2013).

To evaluate the biological responses caused by a toxicant, the use of biomarkers is a reliable approach to determine "biochemical, cellular, physiological or behavioral variations that can be measured in tissue or body fluids or at the level of whole organisms and provide evidence of exposure to and/or effects of one or more chemical pollutants" (Depledge et al., 1995). They are useful early-warning tools for the detection of adverse effects caused by environmental toxicants at suborganismal levels of biological organization, by predicting the potential damage of these substances on an ecological scale (Depledge et al., 1995). In this context, some biochemical biomarkers have already been determined for aeglid species in ecotoxicological studies, such as oxidative stress, metal exposure, and neurotoxic biomarkers (Borges et al., 2018; Cerezer et al., 2020; Faria et al., 2018). However, to our knowledge, no study has yet determined any genotoxic biomarkers in an aeglid species.

For the evaluation of genotoxicity, the comet assay under alkaline conditions is one of the most sensitive, reliable, and low-cost methods for DNA damage assessment in environmental toxicology and biomonitoring studies, being applicable in a myriad of invertebrate and vertebrate taxa, as recorded by Gajski et al. (2019). The comet assay allows the detection of alkali labile sites and double- and single-strand breaks in the DNA molecule, which enables the visualization of undamaged, supercoiled DNA in a "head" of the comet as well as the detection of a "comet tail", where the DNA is more damaged and less condensed (Collins, 2004). Among the crustaceans, the comet assay was applied to several freshwater species, for example, *Daphnia magna*, *Astacus leptodactylus*, and *Macrobrachium rosenbergii* (Gajski et al., 2019). Concerning the cell types used for the comet assay, the hemocytes of the hemolymph are the most commonly used for the evaluation of sublethal

effects caused by various water pollutants in crustaceans (Gajski et al., 2019). Hemocytes are multifunctional cells responsible for the cellular-mediated responses of innate immunity, being essential for defense against pathogens and environmental contaminants (Ray et al., 2015). The homeostasis of hemocyte density is an important immunological parameter to evaluate the effects of xenobiotics in crustaceans (Ray et al., 2015).

In the last twenty years, the sources of threats to aeglid biodiversity have become a central concern in the conservation of the freshwater fauna of South American inland waters (Santos et al., 2017). The main challenge for aeglid conservation is related to the myriad of environmental degradation sources that can lead to habitat loss for aeglids, including suppression of riparian forests, silting of streams, and chemical pollution from agriculture, livestock, and aquaculture (Boos et al., 2020). Indeed, as aeglids are very demanding organisms regarding habitat and water quality, conservation efforts for these animals are also closely related to the conservation of their habitats (Boos et al., 2020). Thus, identifying environmental stressors and their potentially toxic effects is essential to support biomonitoring studies and outline conservation strategies focusing on aeglids, especially those that occur in densely populated and highly contaminated areas in Neotropical ecosystems.

Therefore, to encourage the application of aeglids in biomonitoring studies using biomarkers, as well as to better comprehend the isolated effects of metal contamination on aeglids, we investigated the effects of acute Cu exposure on a set of biomarkers of *Aegla castro* Schmitt, 1942, under a laboratory perspective. In addition, to evaluate the genotoxicity of Cu exposure, we performed the comet assay using an aeglid species for the first time. We also analyzed the effects of Cu exposure on the composition of hemocytes—an important parameter in the crustacean immune system—and investigated the total and differential hemocyte counts as potential biomarkers of Cu exposure in aeglids. To obtain an integrated view of the effects of Cu exposure on the biomarker data from the different tissues analyzed we performed a principal component analysis and calculated an integrated biomarker response index (Sanchez et al., 2013).

5.2 MATERIAL AND METHODS

5.2.1 Animal collection and maintenance

Male individuals of *A. castro* Schmitt, 1942, in the intermolt stage were collected from the Couro Stream (23º57'15''S, 51º07'00''W), Tibagi River Basin, Southern Brazil (Fig. 1) in May 2019. The Couro Stream is a first-order watercourse with no apparent pollution sources, with riparian forests along some stretches of its extension. The bottom substrate is primarily composed of rocks in the rapids and muddy sediment in the backwaters, with leaf litter deposited on the bottom and between rocks in the rapids. Animals were captured by positioning a trawl (length 150 cm *vs.* width 100; mesh 1 mm) downstream of the rapids, while a researcher turned over the rocks and leaf litter upstream so that the animals were carried by the current flow to the trawl.

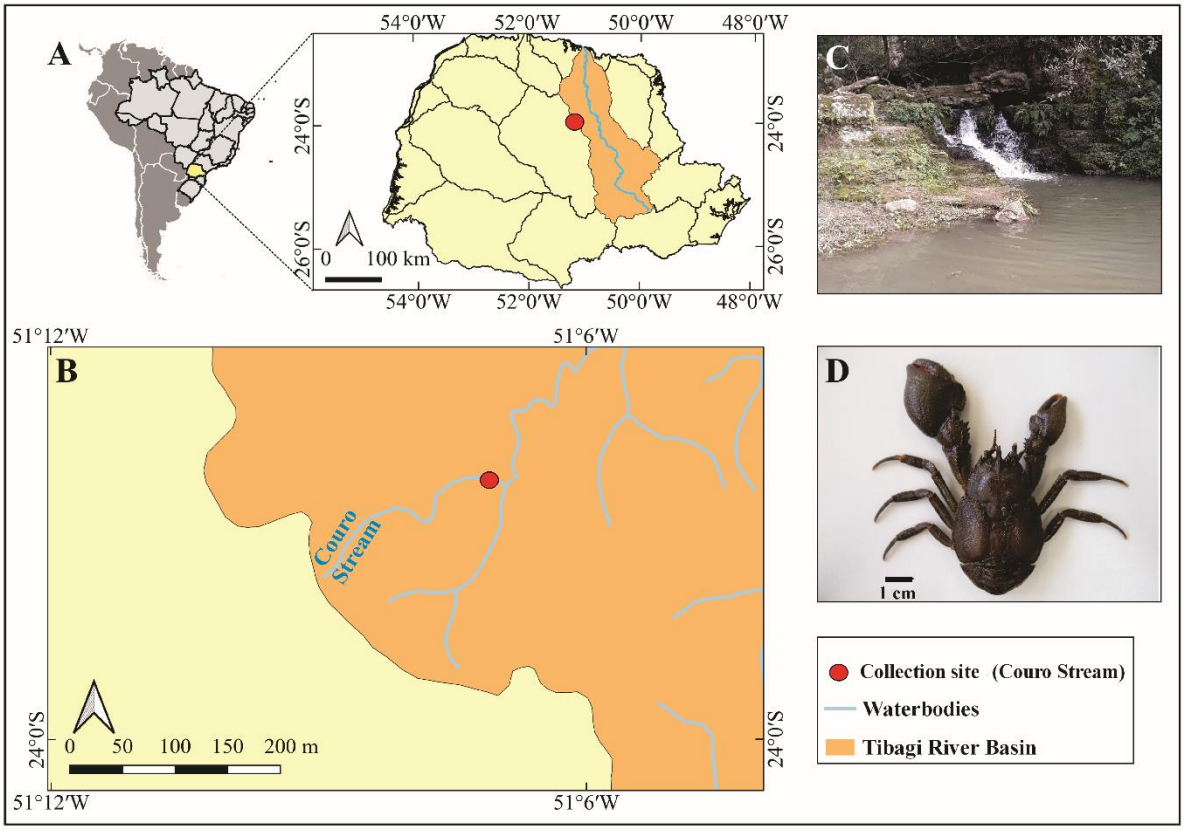

Fig. 1 – Collection site of specimens of *Aegla castro* (A-B), Couro Stream (C), Tibagi River Basin, Southern Brazil. Dorsal view of a male specimen of *Aegla castro* (D).

Unfiltered and filtered (0.45-µm mesh filter, Millipore Millex HV/PVDF) water samples from the collection site were taken for posterior determination of total (Cu T) and dissolved Cu (Cu D) concentrations, respectively. Immediately after, the water samples were

fixed with HNO_3 (65%). For laboratory acclimation of the crustaceans, we collected 100 L of stream water to be used in the water renewals. Abiotic parameters of the water, such as temperature (°C), pH, dissolved oxygen (mg O_2^{-1}), turbidity (NTU), conductivity (μS cm^{-1}), and total dissolved solids (g L^{-1}) were measured at the sampling sites using a multiparameter reader (Horiba U-52). Water hardness (mg L^{-1} $CaCO_3$) was measured by the EDTA titrimetric method.

We transported the aeglids to the laboratory (~ 90 min) in boxes with 15 L of stream water, not exceeding the density of 2 individuals per liter of water. We also offered plastic artificial hiding places to avoid aggressive behavior between males. In the laboratory, the animals were acclimated in a 100-L plastic tank containing hiding places and stream water with a constant temperature (17 ºC), aeration, filtration, and a 12/12 h light/dark photoperiod, for 6 days. The acclimation room was kept at 18 ºC during animal maintenance and experiments. Every 24 h a 25% partial renewal was performed with dechlorinated tap water at the same temperature as the water of the acclimation tank. Animals were fed every day with flocked fish feed (450 g protein kg^{-1}), except on the day of the experiment. Abiotic parameters were measured every day during the acclimation period.

5.2.2 Exposure media and experimental delineation

We prepared two stock solutions (5 and 1 g L^{-1} dissolved Cu) with copper chloride to obtain the final exposure solution of 11 μg L^{-1} of dissolved Cu. This concentration is between the limits of dissolved Cu permitted in watercourses (9 and 13 μg L^{-1} dissolved Cu) by the current Brazilian guidelines (Conama, 2005).

As aeglids are relatively small animals, to ensure sufficient tissue for all the biomarkers proposed, we conducted repeated experiments, one immediately after the other, under the same laboratory conditions. For each experiment, the crabs were randomly distributed into two groups of 8 individuals: the control group (CTR) which was kept under controlled conditions with no addition of Cu in the exposure medium; and the waterborne Cu group (Cu 11) which was exposed to 11 μg L^{-1} dissolved Cu. Each crab was individually exposed in 2-L beakers containing 1.5 L of dechlorinated tap water with Cu (Cu 11 group), or without Cu (CTR group), with constant aeration and controlled temperature; both groups were exposed for 24 h. Abiotic parameters were monitored at 0 h and 24 h.

5.2.3 Cu and ionic concentrations in the water

Unfiltered and filtered water samples from two beakers of each experimental group were taken at 0 h and 24 h to determine the Cu T and Cu D concentrations, respectively. The samples were immediately fixed with HNO_3 (65%) and kept at 4 °C until the analysis. The Cu T and Cu D concentrations were determined through graphite furnace atomic absorption spectroscopy (AAnalyst700, PerkinElmer), with a detection limit of 0.014 μg L^{-1} Cu. Ion concentrations in the water were measured in unfiltered samples, both from the field and the exposure media (0 h and 24 h). Na^+ and K^+ concentrations were determined by flame photometry (Digimed DM-62) and Ca^{2+} and Mg^{2+}concentrations by atomic absorption spectroscopy (AAnalyst700, PerkinElmer).

5.2.4 Tissue sampling

Mortality was recorded after 24 h and the crabs were cryo-anesthetized, weighed, and measured. The hemolymph samples were withdrawn from the arthrodial membrane of the chelipeds with an insulin syringe, while gill, hepatopancreas, and muscle from chelipeds and abdomen were excised for the analyses of biomarkers and bioaccumulation in tissues.

5.2.5 Cu bioaccumulation in tissues

Hemolymph aliquots (50 μL) were diluted in ultrapure nitric acid 5 N (1:5 v/v) and placed in a drying oven at 60 °C for 48 h. Gill, hepatopancreas, and muscle samples were completely dried before being digested, according to Roda et al. (2020). Cu concentrations in the digested tissues were determined through a graphite furnace atomic absorption spectroscopy (AAnalyst700, PerkinElmer), using reference solutions (Specsol, Brazil) as standards. Results were expressed as mg Cu g dry $weight^{-1}$.

5.2.6 Differential hemocyte count (DHC)

To evaluate the total abundance of hemocytes in the hemolymph, immediately after

sampling each animal we made smears with 5 μL of hemolymph per slide. Slides were kept at room temperature for drying for 24 h and then fixed in methanol for 20 min. Subsequently, the slides were prepared with the Giemsa stain (10% v/v) diluted in Sørensen buffer (40 mM KH2PO4, 60 mM NaHPO4, pH 7.0), and kept at room temperature for 24 h. Permanent slides were made with Permount™ Mounting Medium. The cytological analyses were performed under a light microscope (ZEISS Primo Star) coupled to a camera (ZEISS AxioCam Erc 5s). For the differential hemocyte count, the three categories of hemocytes usually found in crustaceans (Hose et al., 1990) were considered: the hyaline hemocytes (HYA), the semigranular hemocytes (SEM), and the granular hemocytes (GRA). These categories are based on the size and number of cytoplasmic granules, and the nucleocytoplasmic ratio (N/C).

5.2.7 DNA damage

We performed the alkaline comet assay with hemocytes from the hemolymph, and with gill and hepatopancreas cells in suspension, according to Singh et al. (1988), with modifications described in Alvim and Martinez (2019). Hemolymph aliquots (100 μL) were withdrawn and immediately added to an anticoagulant solution (1:1 v/v) (50 mM glucose, 4 mM sodium citrate, 10 mM sodium chloride, pH 7.4). The cell suspension was centrifuged (200 × g, 10 min, at 4 °C) to concentrate the hemocytes at the bottom of the tube. The supernatant was then discarded and the hemocyte concentrate was resuspended with low melting point agarose (0.5%), placed on slides pre-coated with normal melting point agarose (1%), covered with coverslips, and kept refrigerated (4 °C) for 50 min. Gill and hepatopancreas samples were placed in fetal bovine serum and mechanically dissociated (protected from light) by sectioning the tissue with surgical scissors for 1 minute without interruption. The homogenates (10 μL) were filtered (30 mm mesh size) and resuspended, and the slides were mounted and kept under refrigeration similar to the hemolymph slides.

The coverslips were removed from the slides, which were subjected to the following steps: (a) cell lysis: 18 h at 4 °C, protected from light, in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); (b) DNA unwinding: 20 min, in the dark, in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); (c) electrophoresis: 20 min, 300 mA, 25 V, 1 V cm^{-1}; and (d) neutralization: three washes for 5 min each in buffer (0.4 M Tris, pH 7.5). Slides were then fixed with absolute ethanol for 10 min and kept under refrigeration until the cytological analysis; they were stained with GelRed

and blindly analyzed under a Leica microscope (DM 2500), adapted for fluorescence at 400 X magnification.

The sample unit was defined as follows: from each crustacean tissue (gill, hemolymph, and hepatopancreas) we made smears in duplicate (to ensure the minimum number of 100 nucleoids per tissue, per individual) for each individual sampled in both treatments (CTR and Cu 11). DNA damage was visually quantified in 100 randomly selected cells and not overlapped by the length of the tail formed by migrating DNA fragments and classified into four comet classes (from 0 to 3), according to Alvim and Martinez (2019). The damage score was determined by the number of cells in each comet class multiplied by the damage class (0 to 3), resulting in a damage score ranging from 0 (undamaged) to 300 (all cells with maximum observable damage). Mean damage scores were obtained for each experimental group (CTR *vs.* Cu 11, for each tissue analyzed).

5.2.8 Metallothionein-like protein content (MT)

Gill, hepatopancreas, and muscle were homogenized (1:2 for gill and muscle, and 1:3 for hepatopancreas, m/v) in buffer (0.5 M sucrose, 26 mM Tris, 0.5 mM phenylmethylsulfonyl fluoride, and 1.3 mM β-mercaptoethanol), and centrifuged (18000 × g, 45 min, at 4 °C); and, after ethanol/acid chloroform fractionation, the homogenates were used to determine sulfhydryl group (–SH) content, at 412 nm, according to Viarengo et al. (1997). Reduced glutathione (GSH) was used as standard, and the MT content was expressed in nmol –SH mg protein^{-1}.

5.2.9 Oxidative stress biomarkers

Hepatopancreas was used to determine the content of the non-protein thiols (NPSH), the activities of catalase (CAT) and glutathione *S*-transferase (GST), and lipoperoxidation (LPO) and protein carbonylation content (PCC). Samples were homogenized (1:8 m/v) in buffer (0.05 M potassium phosphate, 0.5 mM EDTA, 10 μM PMSF, pH 7.2) and centrifuged (16000 × g, 20 min, at 4 °C) according to Borges et al. (2018). To evaluate the acetylcholinesterase activity, muscle samples were prepared with a specific homogenization buffer (0.1 M potassium phosphate, pH 7.5) and centrifuged (16000 × g, 20 min, at 4 °C).

Both the supernatants were stored at -80 °C until the biomarker assays were performed.

5.2.9.1 Antioxidants

Non-protein thiol content (NPSH) was determined according to Beutler et al. (1963), which consists of a reaction between –SH groups and the substrate 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), resulting in the formation of thiolate (TNB), quantified at 412 nm. The NPSH content was expressed as μmol –SH g protein^{-1}.

The catalase activity (CAT) was measured by the decrease in the absorbance of hydrogen peroxide (H_2O_2) at 240 nm (Beutler, 1975). The CAT activity was expressed as μmol H_2O_2 min^{-1} mg protein^{-1}.

The glutathione *S*-transferase activity (GST) was determined by measuring (at 340 nm) the complexation rate of reduced glutathione (GSH) with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), for 10 min (Keen et al., 1976). The GST activity was expressed as nmol CDNB conjugated min^{-1} mg protein^{-1}.

5.2.9.2 Oxidative damage

Lipid peroxidation (LPO) was determined using the thiobarbituric reactive substances (TBARS) fluorescence assay by measuring (ex/em 535/590 nm) the concentration of malonaldehyde (MDA, a thiobarbituric reactive substance) formed from peroxidized lipids (Camejo et al., 1998). The LPO was expressed as nmol MDA mg protein^{-1}.

Oxidative damage in proteins was analyzed by quantifying (360 nm) the protein carbonylation content (PCC), according to Levine et al. (1994), by measuring the formation of dini- trophenylhydrazones in the presence of 2,4-dinitrophenylhydrazine (DNPH). Results were expressed as nmol carbonyl mg protein^{-1}.

5.2.10Neurotoxic biomarker

Muscle tissue was homogenized (1:4 m/v) in phosphate buffer (0.1 M) to determine the activity of acetylcholinesterase (AChE), following the method described by Ellman et al. (1961). The AChE activity was measured at 415 nm in a microplate reader, using the substrate

acetylcholine iodide (9 mM) and the color reagent DTNB (0.5 mM). The results were expressed as nmol DTNB min^{-1} mg $protein^{-1}$.

All biochemical biomarkers were normalized by total protein content and quantified (at 595 nm) in a previously reserved homogenate aliquot, according to Bradford (1976). Biomarker results are expressed as mean ± standard error.

5.2.11 Integrated Biomarker Response Index version 2 (IBRv2)

The IBRv2 was calculated for each tissue to summarize the responses of the biomarkers in a single index (Sanchez et al., 2013), showing us which tissue and biomarkers were more influenced by Cu exposure. The principle of the IBRv2 is the reference deviation between a disturbed state and an undisturbed state, assigned here as the Cu 11 group and the CTR group, respectively. As there was only one Cu exposure treatment in this study, we considered the CTR group as the baseline value (T_0). The ratio between the mean value from each biomarker from the Cu exposure group (Cu 11) and the baseline value T_0 (CTR) was log-transformed (Y_i), and a general mean (μ) was obtained from the Y_i values of a given biomarker measured from each tissue. The Y_i values were applied to the formula: $Z_i = (Y_i - \mu)$, where the difference between Z_i and Z_0 (T_0) was used to calculate the index (A). The standard deviation of the general mean (μ), as suggested by Sanchez et al. (2013) was not applied to the index, since the μ value was calculated from only two values of Y_i. The sum of the A absolute values calculated for each biomarker generated an integrated response index (IBRv2) for each tissue analyzed, representing the A values in a star plot where the area above 0 reflects biomarker induction and the area below 0 indicates biomarker inhibition.

5.2.12 Statistical analysis

The normality (Shapiro-Wilk test) and homoscedasticity (Levene test) of the biomarker results were verified. The Cu exposure groups were compared to their respective control groups (CTR *vs.* Cu 11) using the Student's t-test or Mann-Whitney test, according to data distribution. In addition, a two-way analysis of variance (ANOVA) was performed to evaluate the interaction of the treatment groups (CTR and Cu 11) and tissues (gill, hemolymph, hepatopancreas, and muscle) on the MT and Cu bioaccumulation; and treatment

groups (CTR and Cu 11) and hemocyte subtypes (HYA, SEM, and GRA) on the DHC. To perform the multiple comparisons we used the Bonferroni multiple-comparison correction. Statistical analyses were carried out in the R environment (R Development Core Team, 2020). The significance level adopted was 0.05.

Biomarker data were explored with a Principal Component Analysis (PCA) and a correlation matrix was performed to investigate which variables contributed to explaining the data variability and the correlations among variables. The contribution of each variable for the explanation of the whole data variability was concomitantly calculated both for the first (Dim1) and second (Dim2) components, using the function *facto_summarize* of the *factoextra* package. The magnitude of the contribution was then plotted by adopting a color scale on the vectors. All the PCA analyses were made in R environment using the packages *FactoMineR*, *factoextra*, and *ggplot2*.

5.3 RESULTS

5.3.1 Biological data

Crabs from the CTR (15.1±0.5 mm carapace length without rostrum (CLwR); 2.3±0.2 g wet weight; n=16) and the Cu 11 (17.4±0.4 mm CLwR; 3.4±0.5 g wet weight; n=16) presented no mortality at the end of 24 h of exposure.

5.3.2 Abiotic parameters

Physical and chemical parameters of the water measured at the Couro Stream (Fig. 1), during the acclimation, and during the exposures at 0 h and 24 h are presented in Table 1.

Table 1. Abiotic variables, ion concentration, and total and dissolved copper concentrations (Cu T and Cu D, respectively) in the water of the collection site (Couro Stream) in Autumn 2019, the laboratory acclimation tank, and the beakers where the two groups (CTR and Cu 11) of specimens of aeglids were exposed. The CTR group was exposed to dechlorinated water with no addition of Cu in the exposure medium, and the Cu 11 group was exposed to dechlorinated water with the addition of 11 µg L^{-1} Cu. The data from the CTR and Cu 11 groups were measured at 0 h and 24 h, except for hardness. Results are reported as mean values.

Abiotic variable	Field	Acclimation	CTR		Cu 11	
			0 h	24 h	0 h	24 h
Temperature (°C)	15.6	16.9	14.0	16.6	14.4	16.5
pH	7.7	7.7	6.4	7.1	7.1	6.7
Dissolved oxygen (mg O_2^{-1})	9.1	8.0	8.9	8.0	8.0	8.4
Turbidity (NTU)	23.0	9.6	2.95	35.80	3.65	29.35
Conductivity (µS cm^{-1})	61.0	200.0	142.0	146.0	141.0	148.0
Total dissolved solids (g L^{-1})	0.04	0.10	0.09	0.09	0.09	0.10
Hardness (mg L^{-1} $CaCO_3$)	20.2	37.4	—	38.4	—	36.4
Na^+ (mM)	0.139	—	0.370	0.411	0.376	0.409
K^+ (mM)	0.043	—	0.022	0.026	0.022	0.024
Ca^{2+} (mM)	0.084	—	0.064	0.077	0.076	0.075
Mg^{2+} (mM)	0.058	—	0.068	0.084	0.080	0.080
Cu T (µg L^{-1})	0.80	—	3.29	3.26	12.94	8.75
Cu D (µg L^{-1})	0.67	—	2.81	2.52	11.22	7.16

The dashes indicate variables not measured. The data on ion concentration in the water and Cu T were obtained from non-filtered water samples.

Temperature and dissolved oxygen (two very important variables for the occurrence of aeglids) were kept similar to those found in the collection site during the acclimation and exposure periods. Although not statistically tested, the turbidity was lower (~ 60%) and conductivity higher (~ 230%) in the acclimation when compared to the collection site (Table 1). However, we found an increase (~ 400%) in turbidity in the exposure media after 24 h, in both CTR and Cu 11 groups (Table 1). The Na^+ concentration in the water was also higher (~ 180%) during the experiments (CTR and Cu 11) when compared to the collection site (Table 1).

5.3.3 Cu concentrations in the water and tissues

The Cu T and Cu D from the CTR group had similar values at 0 h (Table 1). In the Cu 11 group, the mean Cu D concentration corresponded to 85% of the mean Cu T concentration. In addition, as the mean values measured for Cu D at 0 h were close to the expected nominal concentration, the value of 11 μg L^{-1} dissolved Cu was used. This dissolved Cu concentration is within the limits set by the Brazilian guidelines (Conama, 2005). We identified a decrease in both Cu T (~ 32%) and Cu D (~ 36%) concentrations in the exposure media after 24 h of Cu exposure.

Cu bioaccumulation was significantly higher in the gill (p = 0.009), but not in the hemolymph, hepatopancreas, and muscle in the aeglids from Cu 11 when compared to their respective CTR groups (Fig. 2).

Two-way ANOVA revealed that the Cu bioaccumulation was affected by the treatment group [$F(1, 50) = 12.08$, $p = 0.001$)] and by the interaction between the treatment group and tissue [$F(3, 50) = 3.84$, $p = 0.015$)]. The gill of the animals from the Cu 11 group showed the highest Cu bioaccumulation among the tissues analyzed (Fig. 2).

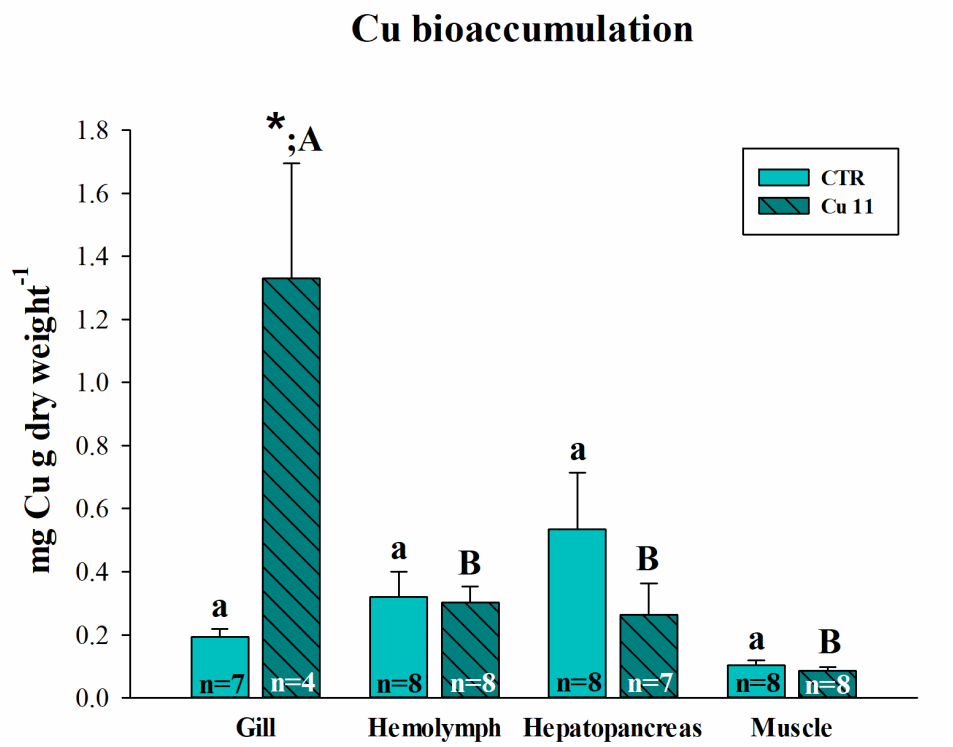

Fig. 2 – Copper bioaccumulation in gill, hemolymph, hepatopancreas, and muscle of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) or exposed to waterborne Cu (addition of 11 µg L^{-1} dissolved Cu). Asterisks indicate statistical differences between the Cu 11 group when compared to its respective CTR group. Lowercase letters indicate statistical differences when comparing the tissues of the animals from the CTR group; uppercase letters indicate statistical differences when comparing the tissues of the animals from the Cu 11 group. Results are expressed as mean ± standard error. Sample sizes (n) are indicated on the chart.

5.3.4 Differential hemocyte count (DHC)

We observed three hemocyte categories in *A. castro*: hyaline (HYA), semigranular (SEM), and granular (GRA) (Fig. 3A). An increase in the number of both HYA (p = 0.034) and THC (p = 0.020) in the aeglids exposed to Cu was observed when compared to their respective CTR groups, but we did not find any alteration in the number of SEM and GRA after Cu exposure (Fig. 3B).

No interaction between the treatment group and hemocyte category was found by two-way ANOVA. However, when comparing the number of hemocytes among the three categories (HYA *vs.* SEM *vs.* GRA) we found that HYA was more abundant than SEM and GRA in the aeglids from both the CTR and Cu 11 groups (Fig. 3B).

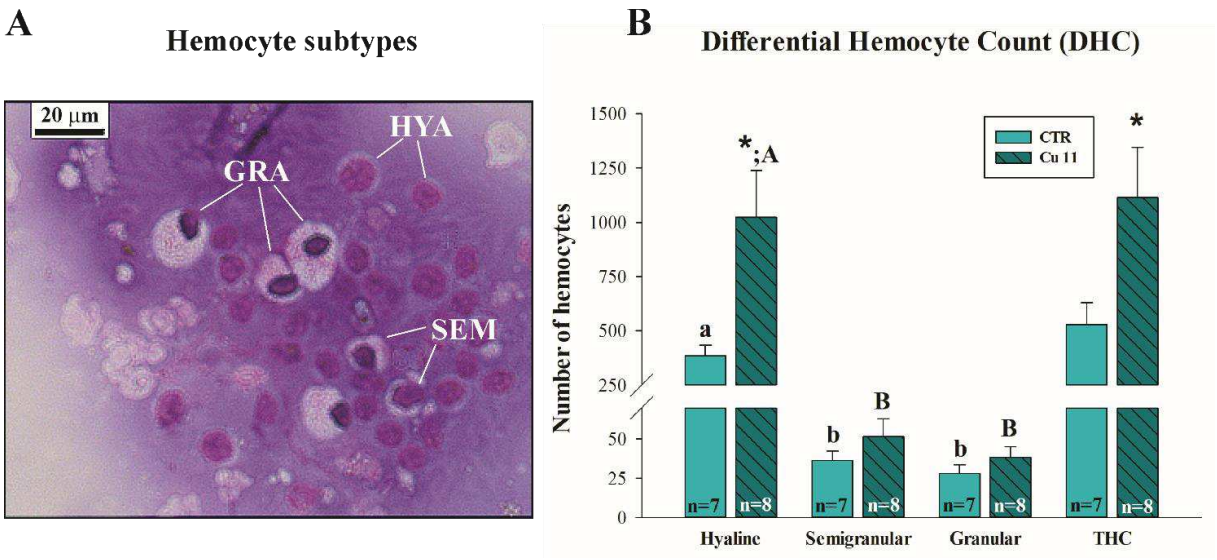

Fig. 3 – The three hemocyte categories found in *Aegla castro*: hyaline (HYA), semigranular (SEM), and granular (GRA) (A). Differential Hemocyte Count (DHC) of the total number of hemocytes in the hemolymph of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) or exposed to waterborne Cu (addition of 11 µg L^{-1} dissolved Cu) (B). Asterisks indicate statistical differences between the Cu 11 group when compared to its respective CTR group. Lowercase letters indicate statistical differences when comparing the hemocyte categories of the animals from the CTR group; uppercase letters indicate statistical differences when comparing the hemocyte categories of the animals from the Cu 11 group. THC represents the sum of all hemocytes in the hemolymph in each experimental group (CTR and Cu 11), regardless of the hemocyte category. Results are expressed as mean ± standard error. Sample sizes (n) are indicated on the chart.

5.3.5 DNA damage

Regarding the DNA damage analysis, the comet assay was successfully performed in gill, hemolymph, and hepatopancreas (Fig. 4A–B). However, the majority of the samples of hemolymph from the aeglids exposed to Cu coagulated, impairing the preparation of the slides and making comparisons impossible for this experimental group due to the small sample size (n=2; see Fig. 4A). For gill and hepatopancreas, we did not observe any alterations concerning DNA damage in aeglids exposed to Cu after 24 h when compared to the CTR groups (Fig. 4A).

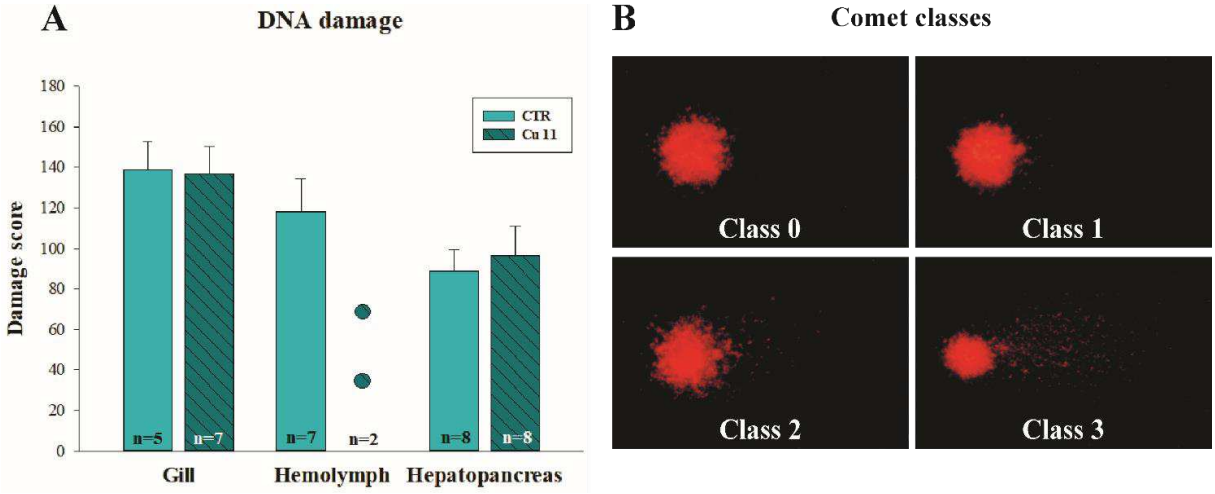

Fig. 4 – DNA damage in gill, hemocytes, and hepatopancreas (A) of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) or exposed to waterborne Cu (addition of 11 μg L^{-1} dissolved Cu). The four different classes of comets (classes 1, 2, 3, and 4) considered in the DNA damage score (B). Asterisks indicate statistical differences between the Cu 11 group when compared to its respective CTR group. Results are expressed as mean ± standard error. Sample sizes (n) are indicated on the chart.

5.3.6 Metallothionein-like protein content (MT)

We identified an increase in the MT content in the gill (p = 0.015) and a decrease in the MET content in the hepatopancreas (p = 0.006) of the aeglids exposed to Cu when compared to their respective CTR groups, but we did not find any alteration in the MT content in muscle after 24 h of Cu exposure (Fig. 5).

Two-way ANOVA revealed that the MT content was affected by the treatment group [$F(1, 37) = 8.57$, $p = 0.005$)], by the tissue [$F(2, 37) = 19.29$, $p < 0.001$)], and by the interaction between the treatment group and tissue [$F(2, 37) = 9.56$, $p < 0.001$)]. When comparing the MT content between the tissues (gill *vs.* hepatopancreas *vs.* muscle), by each treatment group (CTR and Cu 11), we identified a higher MT content in the hepatopancreas than those found in the gill ($p < 0.001$) and muscle ($p < 0.001$) of aeglids from the CTR group (Fig. 5). In contrast, the aeglids of the Cu 11 group presented higher MT content in the gill (p = 0.005) and hepatopancreas (p = 0.033) when compared to the content found in the muscle of the Cu 11 group (Fig. 5).

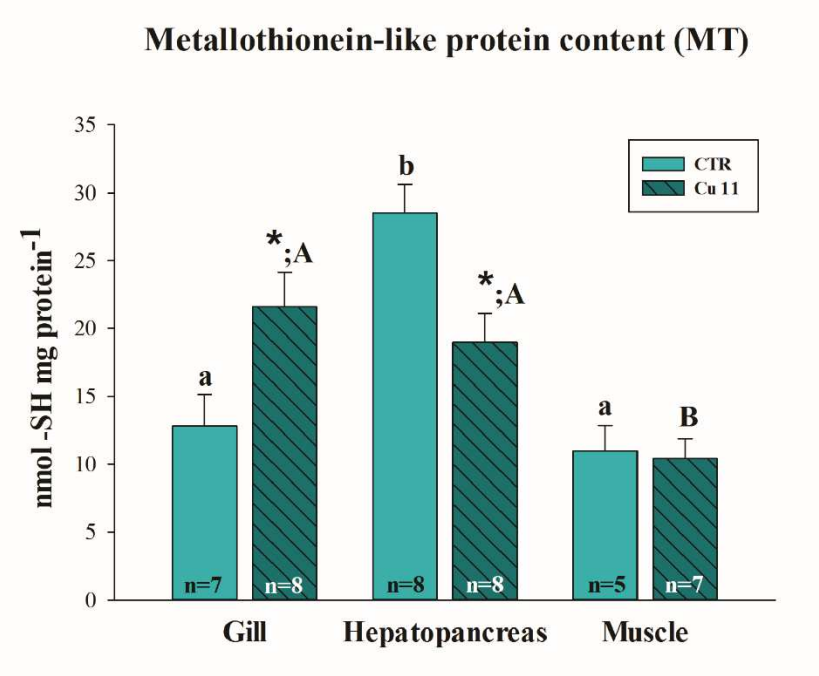

Fig. 5 – Metallothionein-like protein content (MT) in the gill, hepatopancreas, and muscle of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) or exposed to waterborne Cu (addition of 11 μg L^{-1} dissolved Cu). Asterisks indicate statistical differences between the Cu 11 group when compared to its respective CTR group. The different letters indicate statistical differences in the metallothionein content when comparing the three tissues (gill *vs.* hepatopancreas *vs.* muscle), in the animals of both the CTR and Cu 11 group. Results are expressed as mean ± standard error. Sample sizes (n) are indicated on the chart.

5.3.7 Antioxidants and oxidative damage biomarkers

There were no significant differences in the content of the NPSH, in the activities of CAT and GST, and the LPO and PCC in the hepatopancreas of aeglids which underwent 24 h of Cu exposure when compared to their respective CTR groups (Fig. 6A–E).

5.3.8 Neurotoxic biomarker

In the same way, we did not observe any alteration in the activity of AChE in the muscle of aeglids from Cu 11 when compared to the CTR group ($t(14) = -0.986$, $p = 0.341$).

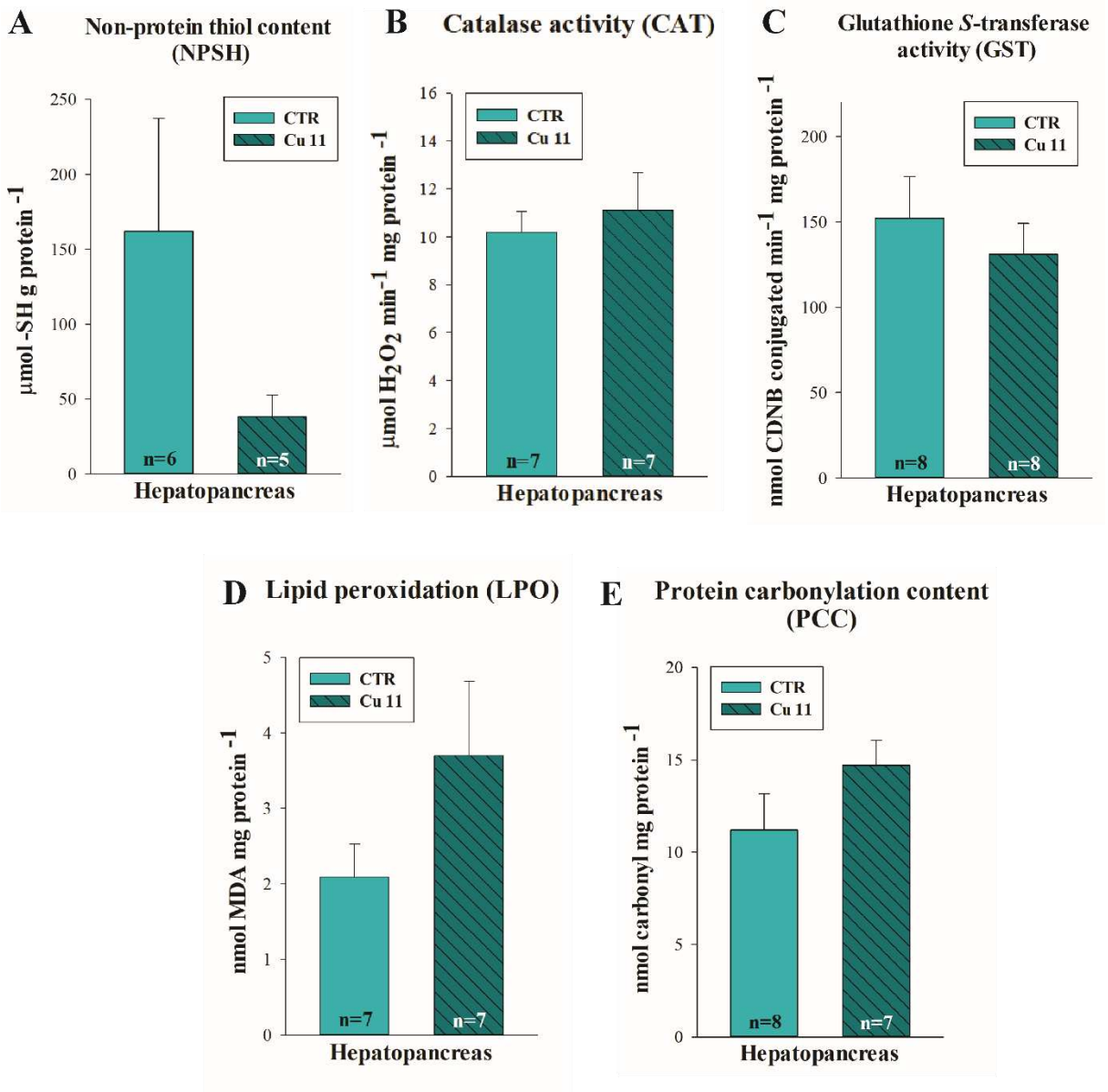

Fig. 6 – Non-protein thiol content (NPSH), catalase activity (CAT), glutathione *S*-transferase activity (GST), lipid peroxidation (LPO), and protein carbonylation content (PCC) in hepatopancreas (A-E) of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) or exposed to waterborne Cu (addition of 11 µg L^{-1} dissolved Cu). Asterisks indicate statistical differences between the Cu 11 group when compared to its respective CTR group. Results are expressed as mean ± standard error. Sample sizes (n) are indicated on the chart.

5.3.9 Principal Component Analysis (PCA)

The PCA performed with the biomarkers pointed out that 47% of the data variability could be explained by the first (Dim1 = 24.5%) and second (Dim2 = 22.3%) components (Fig. 7). The first principal component is strongly associated with PCC_hep (-0.45) and MET_gill (-0.36). This component can be interpreted as being primarily a measurement of "the

protective role of MT against Cu and the oxidative damage in proteins of the hepatopancreas". The second component is strongly associated with HYA (-0.36) and Cu_hep (0.36). This component can be interpreted as being a measurement of "the response of hemocytes of the hemolymph and Cu homeostasis in the hepatopancreas" (Fig. 7).

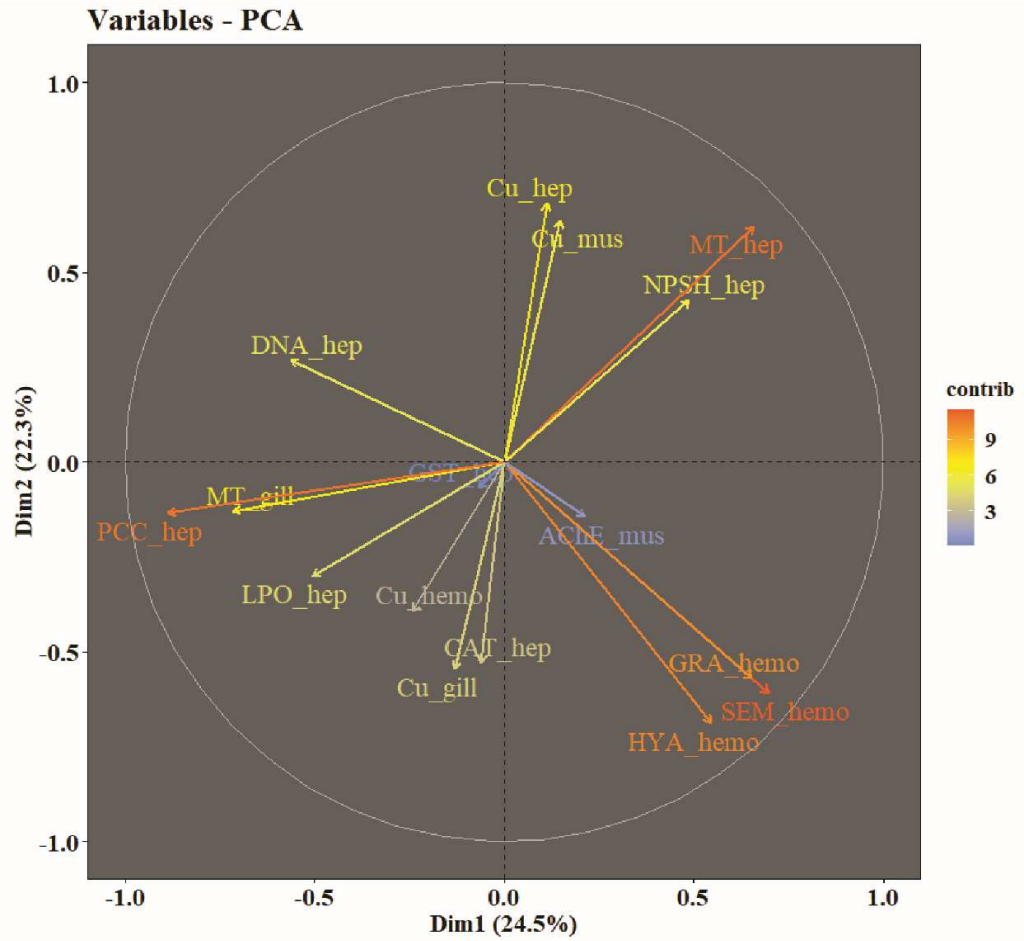

Fig. 7 – Principal component analysis (PCA) of the biomarkers measured in gill (_gill), hemolymph (_hem), hepatopancreas (_hep), and muscle (_mus) of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) and those exposed to waterborne Cu (addition of 11 µg L^{-1} dissolved Cu). Vectors represent the variables (biomarkers analyzed in each tissue or the number of hemocytes counted in each category). Different colors of the vectors are related to the contribution of each variable to the whole data variability ("contrib"), where a red vector is more influential and a blue one less influential. Acetylcholinesterase activity (AChE), catalase activity (CAT), copper bioaccumulation (Cu), DNA damage (DNA), glutathione *S*-transferase activity (GST), lipoperoxidation (LPO), metallothionein-like protein content (MT), non-protein thiol content (NPSH), protein carbonylation content (PCC), and the total number of hyaline (HYA), semigranular (SEM), and granular (GRA) hemocytes.

The correlation matrix showed that HYA *vs.* SEM *vs.* GRA were all positively correlated with each other. The variables MET_hep *vs.* NPSH_hep, MET_hep *vs.* Cu_hep, PCC_hep *vs.* COM_hep, and PCC_hep *vs.* LPO_hep also presented significant positive correlations between the respective comparisons. In contrast, the comparisons between the

variables PCC_hep *vs.* MET_hep and PCC_hep *vs.* NPSH_hep presented significant negative correlations.

The variables that most contributed to the explanation of the whole data variability for the first (Dim1) and second (Dim2) components together were SEM (11.5%), MET_hep (10.9%), PCC_hep (10.7%), HYA (10.3%), and GRA (10.0%), while the variable with the lowest contribution was GST_hep (1.2%) (Fig. 7).

5.3.10 Integrated Biomarker Response Index version 2 (IBRv2)

Cu bioaccumulation (Cu) was the most responsive biomarker in the gill tissue (Fig. 8A), while the number of hyaline hemocytes (HYA) was the most responsive biomarker in the hemolymph (Fig. 8B). Lipoperoxidation (LPO) was the most responsive biomarker in the hepatopancreas (Fig. 8C), while the activity of the acetylcholinesterase (AChE) was most responsive in the muscle (Fig. 8D).

The least responsive biomarkers were DNA damage (DNA) in the hemolymph, non-protein thiol content (NPSH) in the hepatopancreas, and Cu bioaccumulation (Cu) in the muscle (Fig. 8B–D).

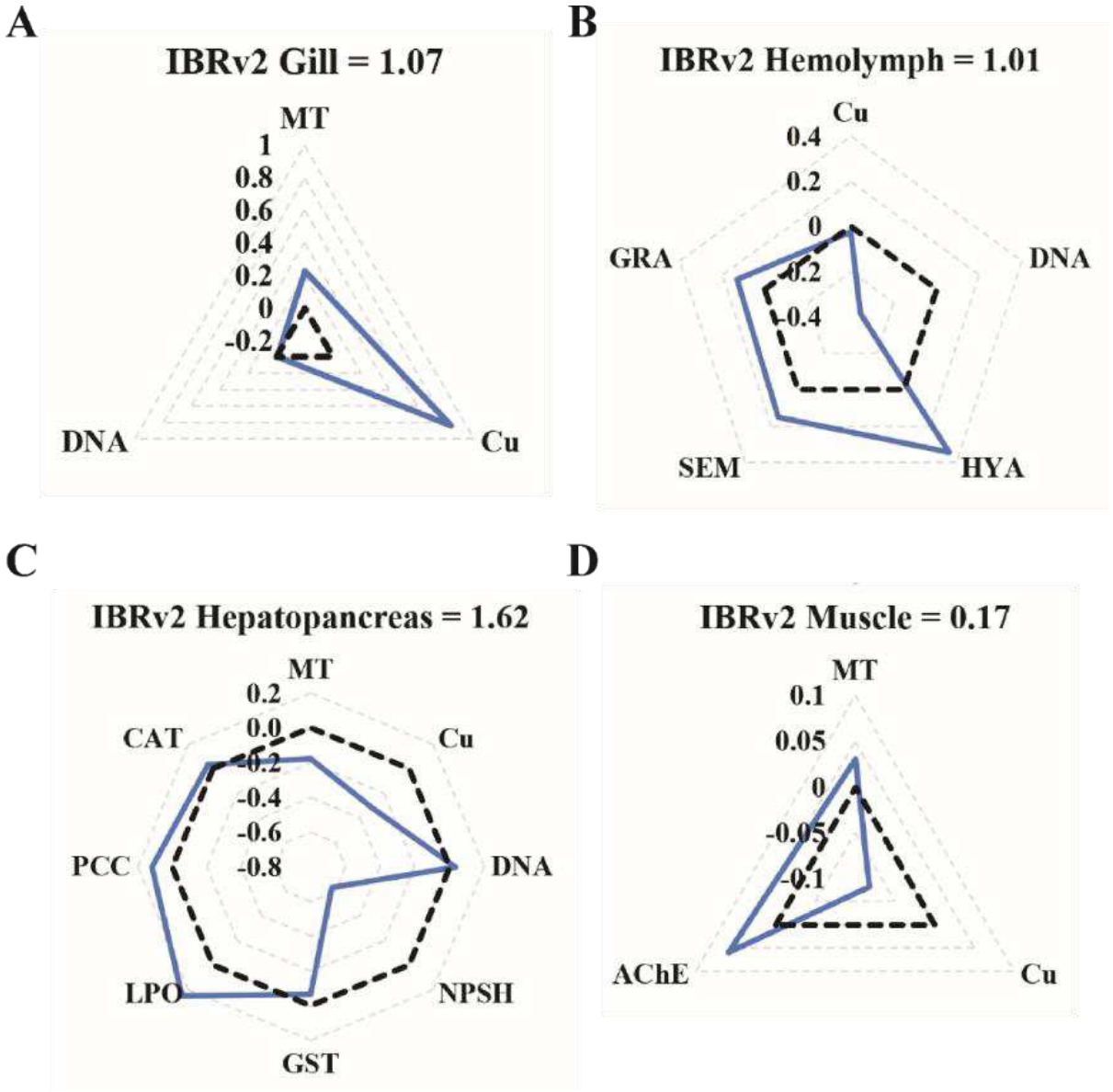

Fig. 8 – Integrated biomarker response index (IBRv2) in the biomarkers acetylcholinesterase activity (AChE), catalase activity (CAT), copper bioaccumulation (Cu), DNA damage (DNA), glutathione *S*-transferase activity (GST), lipoperoxidation (LPO), metallothionein-like protein content (MT), non-protein thiol content (NPSH), protein carbonylation content (PCC), and the total number of hyaline (HYA), semigranular (SEM), and granular (GRA) hemocytes, in the gill (A), hemolymph (B), hepatopancreas (C), and muscle (D) of *Aegla castro*. Biomarker results of the Cu 11 group (continuous blue line) are represented in relation to the baseline value (T_0), assigned here as the CTR group (black dashed line). The area above 0 reflects the induction of the biomarker and below 0 indicates a reduction of the biomarker.

5.4 DISCUSSION

Aeglids constitute an interesting group of macroinvertebrates that are suitable for use in ecotoxicological studies for several reasons, such as, (i) the recognition of these crustaceans as potential bioindicators for assessing habitat quality (e. g. Correa-Araneda et al., 2010); (ii) the employment of *Aegla* as an indicator taxon in genetic diversity studies that determine priority areas for conservation of freshwater ecosystems from South America (e. g. Gonçalves et al., 2018); (iii) the increasing degree of threat of some species due to the habitat loss by water contamination and land-use changes over the past two decades (e. g. Santos et al., 2017), and (iv) the assignment of *Aegla* as the most severely threatened genus among South American freshwater decapods (Bond-Buckup et al., 2008). All of these factors contribute to justifying the recent growth in the literature on ecotoxicological studies under a biomarker approach, focusing on the sublethal effects of habitat alteration or contamination in aeglid species.

Field and laboratory studies have already been delineated using aeglid species. For example, Cerezer et al. (2020) conducted field toxicity tests with *Aegla longirostri* in streams contaminated with pesticides, and Rosa and Martinez (2021) performed laboratory exposures under controlled conditions and investigated for the first time the isolated effects of acute Cu exposure on the enzymes involved in osmoregulation in the gill and on ionic regulation in the hemolymph of *A. castro*.

Field investigations are reliable because they allow the exposure of organisms under natural environmental conditions, being environmentally relevant (USEPA, 1994). However, this approach does not favor the establishment of clear cause-effect relationships between the selected contaminants and the biological responses in the field, since the researcher does not control all the variables under which an *in situ* toxicity test occurs (USEPA, 1994). Therefore, considering that both field and laboratory studies are necessary for an integrative science of extrapolating artificial laboratory results to a more complex natural system (Chapman, 1995), coupled with the fact that the effects of Cu exposure in aeglids are not well known, we delineated a laboratory study to investigate the isolated effects of Cu contamination on several biomarkers as well as to aid standardization of the repeatability of laboratory maintenance and experimentation using aeglids.

Considering the field or *in situ* studies using aeglids, some authors presented distinctive experimental delineations. Borges et al. (2018) characterized lipoperoxidation (TBARS) and catalase activity from biological extracts of males and females of *Aegla*

singularis. The authors advised the use of females due to the absence of seasonal variation in oxidative stress biomarkers, in contrast to that observed in males (Borges et al., 2018). Thus, Borges et al. (2022) used only females for the determination of oxidative stress biomarkers in aeglids from streams with metal-contaminated sediments from three hydrographic basins. On the other hand, Cerezer et al. (2020) used only males in intermolt for the determination of several biochemical biomarkers in *Aegla longirostri*. The authors mentioned the existence of chemical differences between the hepatopancreas compounds of males and females, and a typical fasting period occurring before the molting stage in aeglids. In addition, Cerezer et al. (2020) did not use biological extracts of the whole animal but dissected the tissues and analyzed them individually.

In the same way, here we adopted only males for the experimentation, and we used the different tissues separately for the determination of biomarkers. Furthermore, we conducted two repeated experiments, one after the other, aiming to ensure enough tissue for the biomarker analyses. We did not observe mortality in any of the two experiments. This suggests that the experimental delineation adopted here was able to ensure the survival of the animals in laboratory maintenance conditions for the days of acclimation as well as during the exposures, both in the CTR and Cu 11 groups.

Water temperature and dissolved oxygen are essential abiotic variables that drive aeglid occurrence since they inhabit fast-flowing, low-order streams (Bond-Buckup and Buckup, 1994). In the laboratory exposures, we aimed to keep these parameters similar to those found in the field, however, some parameters were slightly different due to the physicochemical characteristics of the water from the stream and the laboratory. For example, the turbidity during the acclimation was lower than that found in the stream due to the lower amounts of suspended particles in the water used for the partial renewals. Likewise, the higher conductivity values in the water in the acclimation and exposure media when compared to that found in the field may be explained by the higher concentrations of Na^+ in the water furnished in the laboratory. Interestingly, we observed the highest turbidity values only at 24 h of experimentation, in both CTR and Cu 11 groups. This may be the result of the detachment of the microflora and dirt adhered to the carapace that influenced the values of turbidity at the end of the exposure time.

Cu could represent a potential threat to aeglid populations since it is currently present in all aquatic environments. The contamination of soil, watercourses, and sediments has increased in recent years, mainly due to anthropogenic sources such as smelting activities, mining, sewage discharge, and the application of Cu-based fertilizers, algaecides, and

fungicides (Li et al., 2020). Consequently, several studies have reported rivers and streams from Southern Brazil with both sediment (e. g. Borges et al., 2022; Faria et al., 2018) and water (e. g. Dalzochio et al., 2018; Piassão et al., 2019) contaminated by Cu, including watercourses where aeglids are reported to occur.

In general, when a metal comes into contact with a crustacean, it can be absorbed both from the food (e.g. gut epithelium) and the water, through permeable body surfaces (e.g. gill epithelium) or water ingestion (Ahearn et al., 2004). Once absorbed, an essential metal is metabolically available and may bind to a variety of proteins and other biomolecules, where it supplies an essential metabolic need in the animal. When there is an excess of the required levels of the metabolically available form of the metal it must be excreted and/or detoxified (Güner, 2011; Rainbow, 2002). Metal excretion in crustaceans relies on a physiological regulatory mechanism that maintains an equilibrium between the metal uptake rates from the environment and the metal excretion rates through the gill and antennal glands (Ahearn et al., 2004). Metallothioneins are cysteine-rich proteins of low molecular weight with a high affinity for metals. Because of their high content of thiol groups (–SH), metallothioneins play a key role in the homeostatic control of essential metals like Cu and Zn (Amiard et al., 2006), as well as performing the sequestration of metals and storing them in a detoxified form when intracellular metal concentrations exceed the metabolic demands (Ahearn et al., 2004).

Apart from the excretion processes, the excess metal can be detoxified by accumulation in intracellular vacuolar granules containing sulfur or phosphorous, by the formation of extracellular granules, or *via* metallothioneins (Ahearn et al., 2004). Metal bioaccumulation in crustaceans is a combination of the metal in a metabolically available form, including those that induce toxic effects on biomolecules, plus the metal that has been detoxified through binding with metallothioneins and/or the formation of granules (Rainbow, 2007).

In this study, the exposure of *A. castro* to 11 μg L^{-1} dissolved Cu resulted in metal bioaccumulation in the gill tissue, even though the concentration of dissolved Cu used in this laboratory study is within the Brazilian guidelines for permitted limits of dissolved Cu in freshwaters (9 to 13 μg L^{-1}) (Conama, 2005). Cu accumulation in the gill is expected since this is the first interface between the internal and external media in crustaceans, being in direct contact with the toxic substances from a polluted environment (Ahearn et al., 2004). Moreover, the gill is the major entry site during metal exposure, since this epithelium is a very permeable surface with high gas exchange and ion uptake rate (Freire et al., 2008). High Cu accumulation was also observed in the gill tissue of *Procambarus clarkii* exposed to dissolved

Cu (0.03, 0.30, and 3.00 mg L^{-1}), but a significant accumulation in the hepatopancreas tissue was only reported under the higher concentrations (0.30, and 3.00 mg L^{-1}) and after 7 days of Cu exposure (Zhao et al., 2019).

Besides the gills, we did not observe Cu accumulation in the hepatopancreas of *A. castro* exposed to Cu, or in the hemolymph and muscle, after 24 h. Similarly, Wang et al. (2022) did not observe Cu accumulation in the hepatopancreas of the freshwater shrimp *Macrobrachium nipponense* after 96 h of exposure to 0.05 mg L^{-1} dissolved Cu. Regarding muscle, this tissue is usually the last to bioaccumulate metals in the crustacean body (Güner, 2011), as also reported for *P. clarkii* exposed to Cu (Zhao et al., 2019). Thus, although the hepatopancreas is the typical metal storage and detoxification organ in crustaceans (Ahearn et al., 2004; Güner, 2011), hepatopancreatic cells appear to play a major role in metal detoxification after longer exposure times and higher Cu concentrations (Zhao et al., 2019). In addition, as the gills of decapods are one of the pathways for metal excretion (Rainbow, 2007) the gills of *A. castro* were able to cope with Cu homeostasis, for at least up to 24 h, before the metal was transported and accumulated in the hepatopancreas.

MT content of aeglids from the CTR group was naturally higher in the hepatopancreas when compared to the gill and muscle, mainly due to the known role of the hepatopancreas in metal storage, excretion, and detoxification in crustaceans (Güner, 2011). However, we observed an increase in the MT content in the gill and a decrease in the MT content in the hepatopancreas in response to Cu exposure. The induction of MT is widely reported in the literature when vertebrates and invertebrates are exposed to essential and non-essential metals (Amiard et al., 2006). Therefore, the marked increase in MT content of *A. castro* may be a response to Cu accumulation in the gills.

On the other hand, our results suggested that the decrease in the MT content in the hepatopancreas of aeglids exposed to Cu may be related to the excretion of Cu-linked metallothioneins, since one of the metal excretion pathways in crustaceans is related to intracellular sequestration mechanisms *via* metallothioneins and its elimination through the lysosomal endomembrane system (Ahearn et al., 2004), making the MT content values lower than those found in the CTR group. This may justify the absence of Cu accumulation in the hepatopancreas, since this organ plays a key role in metal excretion mechanisms to the circulation, allowing Cu excretion to the environment through both gills and antennal glands (Ahearn et al., 2004). The response of MT (both in the gill and hepatopancreas) was effective in detoxifying Cu, preventing the spread of Cu throughout the body and the bioaccumulation in other tissues (hepatopancreas and muscle), at least at the end of 24 h of exposure.

In crustaceans, after the metal has been absorbed by the gills or the permeable surfaces it can be transported to other organs of the body *via* the hemolymph linked to the respiratory protein hemocyanin (Güner, 2011). This characteristic was reported to facilitate the process of detoxification, transportation, and distribution to other tissues in a less toxic form of Cu concentrations above metabolic needs (Rtal et al., 1996). *Procambarus clarkii* and *M. nipponense*, both exposed to Cu, presented a significant increase in the concentration of hemocyanin in the hemolymph (Wang et al., 2022). Apart from hemocyanin, circulating hemocytes are also important components of the crustacean hemolymph, that are recognized to sequestrate metals and transport them to detoxification organs, such as the hepatopancreas (Ahearn et al., 2004). Because the hemocytes of crustaceans are the immunocompetent cells, the homeostasis of hemocyte density is an important immunological parameter (Ray et al., 2015), and the total and differential hemocyte counts were pointed out as reliable biomarkers of the immune system health status of aquatic invertebrates (Klobučar et al., 2012; Qyli et al., 2020; Wang et al., 2022; Wei and Yang, 2016). In *A. castro*, we observed the three hemocyte subtypes frequently documented in crustaceans: hyaline, semigranular, and granular hemocytes. Hyaline hemocytes were the most abundant subtype in the hemolymph of *A. castro* (similarly to those found in the green crab *Carcinus aestuarii* (Qyli et al., 2020)), in aeglids from both the CTR and Cu 11 groups.

Environmental contaminants were reported to induce immunomodulation in the density of circulating hemocytes in aquatic invertebrates (Ray et al., 2015). In *C. aestuarii*, the total number of hemocytes decreased in animals exposed to Cu (Qyli et al., 2020), while the total number of hemocytes and the phagocytic activity increased in *M. nipponense* exposed to 0.05 and 0.10 mg L^{-1} dissolved Cu (Wang et al., 2022). The authors argued that this characteristic increase in the total number of hemocytes may be the result of enhanced cell proliferation or even hemocyte migration from the hematopoietic tissue to the hemolymph; and that this hemocyte recruitment represents an overcompensation mechanism for the loss of hemocyte homeostasis caused by Cu, improving the ability to restore homeostasis quickly after an eventual injury.

Both pathogens and water contaminants can enhance hemocyte release from the hematopoietic tissue to the hemolymph as an immune response, with hyaline hemocytes being the typical emergency release cell type (Bouallegui, 2021). Therefore, in this study, acute Cu exposure for 24 h may have triggered an immune challenge in *A. castro*, evidenced by the increased number of both total and hyaline hemocytes in the Cu 11 when compared to their respective CTR groups, leading to modulation in cell composition through the hyaline

hemocyte migration from the hematopoietic tissue to the hemolymph to cope with Cu-induced stress. In this sense, as circulating hemocytes can sequestrate metals (Ahearn et al., 2004), the increased recruitment of hyaline hemocytes to the hemolymph may have contributed to reducing the bioavailability of Cu in the hemolymph of *A. castro*.

To the best of our knowledge, this study performed the alkaline comet assay with an aeglid species for the first time, reporting the gill, hepatopancreas, and hemolymph as suitable tissues for the analyses of DNA damage with aeglids. However, the hemolymph samples from the Cu 11 group clotted very quickly during the assay, precluding the analysis of DNA damage in the hemolymph of the aeglids exposed to Cu due to the low sample number. Interestingly, the hemolymph samples from the CTR group did not present this response. Hemolymph clotting is closely related to the invertebrate innate immune system (Hose et al., 1990), with hyaline being the main hemocyte subtype that initiates the plasm clotting by cell lysis and releases cytoplasmic clotting factors into the hemolymph. Therefore, we suggest that Cu exposure may have induced an immune response in the hemocyte composition of the hemolymph, represented by an increase in the number of total and hyaline hemocytes, which, in turn, contributed to the clot formation observed in the hemolymph samples from the Cu 11 group during the comet assay.

Cu can cause DNA strand breaks both by directly binding and generating ROS *via* Fenton reactions, leading to structural and functional modifications in the DNA molecule (Govindaraju et al., 2013). These damages are sensitively detected by the comet assay, which makes it possible to identify increased DNA migration from the nucleoid toward the anode in damaged cells. DNA damage in crustaceans exposed to metals was reported by several authors (e. g. Gajski et al., 2019; Klobučar et al., 2012). Although we observed an increase in the concentration of Cu in the gills of *A. castro*, there was no significant increase in DNA damage in this tissue. Similarly, Roda et al. (2020) did not find DNA damage in the gills and blood cells of the neotropical teleost *Prochilodus lineatus* exposed to 10 μg L^{-1} of dissolved Cu for 24 h. These authors suggested that the absence of DNA damage in the gills may be related to the repair mechanisms present in this tissue. Indeed, DNA strand breaks are well-documented to be repairable by excision repair mechanisms of the cell (Collins, 2004; Lee and Steinert, 2003). Therefore, the absence of DNA damage in the gill of *A. castro* may be related to the activity of DNA repair and Cu excretion mechanisms as well as to the protective role of MT, by decreasing the bioavailability of Cu in the gills; while the absence of DNA damage in hepatopancreas may be due to both the role of MT in the Cu sequestration and the role of hemocytes in the immune response, by removing Cu from the hemolymph and

transferring to sites of excretion before reaching the hepatopancreas.

Like other metals, Cu is a well-known inducer of reactive oxygen species. To counterbalance the increment of ROS caused by metal exposure, aquatic animals have a complex antioxidant system consisting of both low and high molecular mass antioxidants. These defenses maintain a balance between ROS generation and elimination, called steady-state ROS level. When the antioxidant system is not efficient to counterbalance the enhanced ROS, oxidative stress occurs (Lushchak, 2011). Non-protein thiols and catalase are both important cellular antioxidant defenses that have been investigated in field studies with aeglid species (Borges et al., 2022; Cerezer et al., 2020). *Procambarus clarkii* under Cu exposure (0.03 and 0.3 mg L^{-1} dissolved Cu) for 7 days showed increased levels of NPSH and decreased activity of CAT in the hepatopancreas (Zhao et al., 2019). However, exposure of *P. clarkii* to 0.75 mg L^{-1} dissolved Cu for 24 h did not induce alteration in the CAT activity in the gills (Wei and Yang, 2015). Here, we did not observe a significant alteration in NPSH content and CAT activity after Cu exposure. Although we did not directly determine the concentration of reactive oxygen species, this consistency of the NPSH content and CAT activity in the hepatopancreas of the aeglids from the CTR and Cu 11 groups suggests that there was no significant increase in ROS generation that could overwhelm the antioxidant defenses and trigger an oxidative stress condition in this tissue, at least up to 24 h of exposure. This may be due to the protective role of the gills of *A. castro* by both excreting Cu and decreasing its bioavailability through high levels of metallothioneins that prevented the occurrence of oxidative stress and oxidative damage in the hepatopancreas. Guo et al. (2017) observed increased expression of the genes coding for metallothioneins in the hemocytes of *L. vannamei* exposed to 1.0 and 5.0 mg L^{-1} dissolved Cu, in a dose-dependent and time-dependent manner; and pointed out that MT plays an important role in the protection against oxidative stress driven by Cu.

In general, ROS that is not removed by antioxidant defenses, like GSH and CAT, can enhance the risk of deleterious alterations in polyunsaturated lipids and proteins. Lipid peroxidation and protein carbonylation are the main effects of oxidative stress in aquatic animals (Lushchak, 2011). Here, we suggest that the absence of oxidative stress (no alteration in NPSH and CAT) in the hepatopancreas could explain the lack of oxidative damage in lipids and proteins in *A. castro*. The same reasoning can be applied to the absence of alteration in the activities of GST in the hepatopancreas and its role in preventing oxidative damage, since GST contributes to the elimination of the secondary metabolite products of lipid peroxidation during oxidative stress (Hayes et al., 2005). No alteration in GST of the hepatopancreas of *P.*

clarkii was reported after 7 days of Cu exposure (Zhao et al., 2019). Even though the increased ROS caused by Cu exposure is reported to cause oxidative damage in crustaceans (Wang et al., 2022), Wei and Yang (2016) reported no effects on lipids and proteins in the hemolymph of *P. clarkii* after exposure to 0.5 mg L^{-1} dissolved Cu. Acetylcholinesterase was also reported to be affected by metal exposure, such as Hg, Cd, Pb, and Cu (Devi and Fingerman, 1995; Roda et al., 2020). In *A. castro* we did not observe any alteration in AChE in the muscle, probably due to both the fact that muscle is typically the last tissue to be affected and accumulate Cu in crustaceans (Güner, 2011), due to the high accumulation of Cu in the gills by the metallothioneins, and by the protective role of the hepatopancreas in detoxifying Cu.

Even though the concentration of Cu in this study is within the permitted limits according to Brazilian guidelines, the exposure of aeglids to 11 μg L^{-1} dissolved Cu, even for 24h, resulted in sublethal effects, emphasizing the sensitivity of aeglids to metal contamination, as previously evidenced by the inhibition of carbonic anhydrase and ionic imbalance of *A. castro* after exposure to this same Cu dissolved concentration (Rosa and Martinez, 2021). On the other hand, the absence of significant effects on the oxidative stress and oxidative damage biomarkers revealed the ability of *A. castro* to deal with Cu contamination quickly, performing an immune response already in the hemolymph, and metal detoxification and excretion mechanisms in the gill and hepatopancreas, involving metallothioneins, before oxidative damages were triggered. In fact, *Aegla castro* did not present significant impairment in the antioxidant defenses or significant oxidative damage in DNA, proteins, and lipids, but, considering the results of the principal component analysis and the significant positive correlations found among the oxidative damage biomarkers (DNA damage, PCC, and LPO), we strongly believe that higher exposure times and/or concentrations of Cu could lead to additional sublethal effects in *A castro*. For example, although we did not observe significant protein carbonylation, the protein carbonylation content in the hepatopancreas (PCC_hep) and the metallothionein-like protein content in the gill (MT_gill) were the variables that explained most of the data variability of the first component, and the PCC_hep presented a high contribution (10.7%) to the explanation of the whole data variability for the first (Dim1) and second (Dim2) components together. In addition, the negative significant correlation between PCC_hep *vs.* metallothionein-like protein content in the hepatopancreas (MET_hep) and PCC_hep *vs.* non-protein thiol content in the hepatopancreas (NPSH_hep) indicates that the antioxidant response performed by the non-protein thiols and the detoxification by metallothioneins prevent the increase in protein

carbonylation in the hepatopancreas. Our data support the protective role of MT in the detoxification of Cu in the gill (by increased MT content) and hepatopancreas (by decreased MT content); as well as indicating that NPSH and MT are essential to prevent the potential oxidative damage caused by Cu in the proteins of the hepatopancreas.

From another point of view, Cu exposure appears to have triggered immunomodulation in *A. castro*, evidenced by the significantly increased recruitment of hyaline hemocytes to the hemolymph. However, the second component of the PCA revealed that all three categories of hemocytes (HYA, SEM, and GRA) were positively correlated with each other as were variables with high contributions to the explanation of the whole data variability (10.3%, 11.5%, and 10.0%, respectively). This indicates that Cu led to a combined immune response in *A. castro* by increasing the number of hemocytes in the hemolymph as a whole, as observed by the significant increase in the total hemocyte count (THC) of aeglids from the Cu 11 group.

The PCA revealed that the responses of *A. castro* exposed to Cu corroborated the essential role of the hepatopancreas in the metal sequestration and detoxification reported for crustaceans (Ahearn et al., 2004; Güner, 2011). The variable Cu bioaccumulation in the hepatopancreas (Cu_hep) was strongly associated with the second component, even though we did not observe significant differences between the CTR and Cu 11 groups. In addition, the variable Cu_hep was positively correlated with MET_hep, indicating that Cu detoxification in the hepatopancreas was performed mainly through the consumption of metallothioneins by binding Cu to –SH groups and accumulating the metal in a less toxic form (Ahearn et al., 2004).

The IBRv2 calculated with the available data in this study did not allow comparisons between the tissues analyzed (e.g. gill *vs.* hemolymph *vs.* hepatopancreas *vs.* muscle), mainly because the IBRv2 index is defined as a sum of all the indices of each biomarker separately, and we did not analyze the same set of biomarkers in all the tissues sampled (Fig. 8). However, this does not preclude the radar charts from showing the biomarkers that proved most responsive in each tissue. The biomarkers most responsive in the gill and hemolymph were Cu accumulation and the number of hyaline hemocytes, respectively, agreeing with the statistical differences found between the CTR and Cu 11 groups. However, the radar chart showed LPO and NPSH as the most and less responsive biomarkers in the hepatopancreas, respectively. This may be due to the wide range of mean values of LPO and NPSH between the CTR and Cu 11 groups, which, although not statistically different, could indicate a trend towards an increase in LPO and a decrease in NPSH in the hepatopancreas of *A. castro*

exposed to Cu. Regarding muscle tissue, the most responsive biomarker was AChE, since the muscle does not play a crucial role in metal detoxification and is typically the last tissue to be affected by metal contamination (Güner, 2011).

5.5 Conclusion

This study investigated a suite of biomarkers of *A. castro*, including bioaccumulation, oxidative stress, oxidative damage, and neurotoxic and genotoxic biomarkers, under a laboratory approach, and was pioneering in performing the comet assay using an aeglid species, as well as in proposing the abundance of hemocytes in the hemolymph as an alternative biomarker in aeglids exposed to Cu. The acute exposure of *A. castro* to Cu affected the metal homeostasis (evidenced by Cu bioaccumulation in the gills), activated detoxification mechanisms (due to the increased MT content in the gills and decreased MT content in the hepatopancreas), and provoked a marked immune response (by the increased recruitment of hemocytes to the hemolymph). However, based on the multivariate analyses, we believe that Cu has the potential to cause oxidative damage in the hepatopancreas of *A. castro* when exposing aeglids at higher concentrations or longer exposures. Thus, further experimental studies can contribute to better understanding of how aeglids deal with metal contamination. Furthermore, experimental studies focusing on other contaminants can throw light on how aeglids deal with the increasing environmental contamination in headwater streams and how suitable these freshwater crustaceans could be as biological models in biomonitoring studies from South America.

5.6 Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conclusão geral

6 CONCLUSÃO GERAL

- *Aegla castro* mostrou-se um modelo biológico adequado para ser usado em estudos ecotoxicológicos devido a dois fatores principais: a (i) possibilidade de manutenção e experimentação em laboratório e a (ii) sensibilidade de seus parâmetros funcionais, tanto a partir de animais coletados direto do campo, quanto de animais expostos ao Cu em laboratório.
- O riacho de referência (rio Couro) apresentou um aumento progressivo nas concentrações de Cu na água desde julho de 2018 (ver Apêndice 1), indicando que a população de *A. castro* estava sujeita aos efeitos desse metal na água.
- Eglídeos podem ocorrem em riachos contaminado por Cu e podem apresentar efeitos subletais desta contaminação antes que seja observado declínio populacional.
- *Aegla castro* apresentou três subtipos de hemócitos na hemolinfa: hialino, semigranular e granular.
- O aumento na concentração de Cu na água do riacho provocou a bioacumulação de Cu nos tecidos.
- A exposição de *A. castro* ao Cu desencadeou um prejuízo da regulação osmoiônica e no equilíbrio ácido-base, tanto em exposições agudas em laboratório, quanto em animais amostrados diretamente do campo expostos a água contaminada por Cu, o que confirmou a toxicidade osmorregulatória do Cu reportada na literatura (Bianchini et al., 2004).
- O ajuste na densidade e na proporção relativa de hemócitos da hemolinfa sugere que a exposição aguda ao Cu pode ter desencadeado uma reposta imunológica em *A. castro*.
- A exposição aguda ao Cu parece desencadear mecanismos de antecipação do ciclo de muda, evidenciados pelo aumento da concentração de Ca^{2+} e Mg^{2+} na hemolinfa, como forma de depuração do excesso de metal.
- Em exposições agudas, a detoxificação através de metalotioneínas parece representar uma estratégia primária para a homeostase de Cu em eglídeos, antes mesmo que alterações em enzimas relacionadas à resposta antioxidante primária sejam observadas.
- O sistema de defesas antioxidantes em *A. castro* é capaz de atenuar danos oxidativos causados pelo Cu até 24 h de exposição, mas análises multivariadas sugeriram que danos oxidativos e uma resposta antioxidante hepatopancreática pode ser observada em tempos de exposição superiores.

- Em geral, os biomarcadores mais responsivos em espécimes de *A. castro* expostas ao Cu foram aqueles relacionados à regulação osmoiônica, como concentração de íons e atividade da AC, NKA e HATP; à detoxificação de metal, como a concentração de MT; e ao ajuste da densidade e proporção relativa de hemócitos da hemolinfa. Por outro lado, os biomarcadores menos responsivos foram as atividades das enzimas GST e AChE.
- Em vista do Cu ser um conhecido contaminante prejudicial às enzimas de osmorregulação e a presença deste contaminate no riacho ter afetado a osmorregulação em *Aegla castro* mesmo em concentrações menores do que as estabelecidas pela legislação vigente, é necessário uma reformulação Resolução Conama 357/2005 e a reavaliação das concentrações seguras de Cu para as águas doces.

Considerações finais

7 CONSIDERAÇÕES FINAIS

Esta tese buscou evidenciar a importância da adoção de caranguejos eglídeos em estudos ecotoxicológicos—utilizando *Aegla castro* como modelo biológico—levando em consideração alguns fatores determinantes para recomendar esses crustáceos como modelos de estudo, tais como (i) o papel ecológico chave que os eglídeos desempenham no equilíbrio das cadeias tróficas de ambientes límnicos da América do Sul; (ii) a crescente degradação de habitats típicos que esses animais ocupam causado pelas atividades antropogênicas (iii); o elevado grau de ameaça em que muitas espécies se encontram; e (iv) a escassez de estudos para a avaliação das condições de saúde em caranguejos eglídeos. De fato, em meados de 2017, quando este trabalho começou a ser delineado, não havia na literatura estudos que investigassem espécies de *Aegla* sob um ponto de vista ecotoxicológico, tampouco sob uma abordagem de biomarcadores.

Em contrapartida, há pelo menos 30 anos os eglídeos são conhecidos na literatura (desde o trabalho de descrição da família Aeglidae em 1994) como ótimos organismos bioindicadores de qualidade de habitat em ecossistemas límnicos, uma vez que eles exigem habitats bem preservados com vegetação ripária estruturada, água bem oxigenada e de boa qualidade para a sua ocorrência. De forma geral, esses animais se adaptaram com muito êxito aos ambientes límnicos ao longo da formação das paleo-drenagens Neotropicais, principalmente se considerarmos que *Aegla* é o único gênero dentre todos os gêneros de caranguejos anomuros que é exclusivamente adaptado à água doce. Além disso, o elevado número de espécies descritas para o gênero *Aegla* (cerca de 90 espécies) e a ampla distribuição reportada ao longo das bacias hidrográficas do sul da América do Sul reforça o sucesso da adaptação, dispersão e especiação desses crustáceos nos ambientes dulcícolas.

Embora seja observada uma elevada riqueza de espécies de eglídeos, também é aspecto preocupante o fato de algumas espécies de *Aegla* estarem sob alto grau de ameaça de extinção e de que outras, que sequer são conhecidas pela ciência, podem sofrer declínio em suas populações antes mesmo de serem formalmente descritas. Em vista disso, alguns estudos elencaram ecorregiões (Pérez-Losada et al., 2009) e áreas prioritárias dentro de ecorregiões (Gonçalves et al., 2018) para implementar estratégias que busquem a conservação do maior número possível de espécies de *Aegla*, baseando-se em informações sobre filogenia, riqueza e endemicidade de eglídeos.

Portanto, a justificativa inicial que motivou esta tese—e que foi sendo corroborada conforme os estudos ecotoxicológicos com eglídeos foram publicados (p. ex. Faria et al., 2018; Borges et al., 2018; Cerezer et al., 2020)—foi a necessidade de se aplicar abordagens de diagnóstico precoce para avaliar a saúde dos eglídeos frente à exposição destes animais a contaminantes ambientais. Então algumas perguntas como "*A presença de populações de eglídeos por si só denota boa qualidade de habitat*?", "*Se eglídeos são bioindicadores, até que ponto a presença deles no ambiente pode dar informações sobre a qualidade do riacho?*", "*Por que algumas populações de eglídeos ainda são encontradas em rios e riachos que não apresentam condições de habitats desejáveis para animais tão sensíveis como eglídeos?*" e "*Será que essas populações que ocorrem em riachos degradados estão sujeitas a efeitos subletais de algum contaminante ambiental?*" surgiram no início desta tese. Estas questões ajudaram a justificar a adoção de uma espécie de eglídeo (*A. castro*) que ocorria em um riacho com alta densidade de indivíduos desde 2012 como modelo biológico em um estudo ecotoxicológico, utilizando diversos biomarcadores para avaliar os efeitos subletais causados por contaminantes nesta espécie e inferir sobre a qualidade do ambiente.

A utilização de biomarcadores em *A. castro* foi uma estratégia muito acertada para detectar a progressiva degradação do riacho de referência (rio Couro) desde a primeira coleta deste estudo em julho de 2018 até a última coleta em fevereiro de 2020 (observar tabela contida no Apêndice 1). O capítulo I apontou claramente um aumento da concentração de Cu dissolvido na água do riacho em um intervalo de 6 meses, desde a primeira até a segunda coleta em janeiro de 2019 (Apêndice 1). Esse aumento pode não ter sido tão expressivo em termos numéricos, mas foi muito relevante em termos biológicos para a população de eglídeos, que tiveram que enfrentar esse aumento de Cu no ambiente. Os indivíduos apresentaram respostas enzimáticas na segunda coleta (janeiro de 2019) que refletiram efeitos típicos da contaminação por Cu, como diminuição da atividade das principais enzimas relacionadas à osmorregulação em crustáceos (NKA, HATP e AC) e dificuldade de regulação iônica de Ca^{2+} (que depende do gradiente gerado pela NKA). Além disso, os indivíduos apresentaram um aumento na concentração de Cu na brânquia (a principal interface do organismo com o meio externo) e no músculo (o último órgão a apresentar concentrações de metal acumulado, indicando efeitos de exposição crônica). No entanto, pode ser que embora tenham sido observados efeitos característicos da contaminação por Cu, a água do riacho pudesse estar contaminada também com outros xenobióticos que influenciaram na resposta dos biomarcadores analisados.

Para investigar os efeitos do Cu isolado na resposta de biomarcadores em *A. castro*, os capítulos II e III apresentaram pela primeira vez eglídeos expostos a um contaminante dissolvido na água em condições de laboratório. No capítulo II, desequilíbrio ácido-base e disrupção iônica foram novamente reportados como efeitos da exposição aguda de *A. castro* ao Cu, evidenciados pela inibição da AC e aumento das concentrações de Ca^{2+} e Mg^{2+} na hemolinfa. Essa resposta de aumento de Ca^{2+} e Mg^{2+} na hemolinfa pode ter sido distinta da resposta observada em campo devido à concentração de Cu utilizada nos experimentos (11 μg L^{-1}), que pode ter desencadeado uma antecipação do ciclo de muda como uma estratégia de depuração do excesso de metal. Por outro lado, o capítulo III mostrou que a exposição aguda ao Cu ativou mecanismos de detoxificação de metais, como o aumento da concentração de metalotioneínas na brânquia, por exemplo. Portanto, este estudo mostrou que concentrações de Cu permitidas pela legislação brasileira para corpos de água doce (entre 9 e 13 μg L^{-1}) podem causar efeitos subletais em populações de eglídeos.

Este estudo também caracterizou novos biomarcadores em eglídeos, como a composição (capítulo I) e a contagem de hemócitos na hemolinfa (capítulo III) e a análise de danos no DNA, por meio do ensaio do cometa (capítulo III). Exposições de eglídeos em laboratório apresentaram-se como valiosas abordagens para se estudar os efeitos do Cu em *A. castro*, principalmente devido à sensibilidade dos parâmetros analisados e à aplicabilidade deles como potenciais biomarcadores. No entanto, para determinar biomarcadores em eglídeos a quantidade de tecido disponível pode se tornar um fator limitante para o número de biomarcadores a serem analisados.

Pelo menos dois estudos já utilizaram indivíduos de *A. castro* do riacho de referência (rio Couro) anteriormente: um estudo de estrutura populacional de *A. castro* (Marçal et al., 2017) e outro sobre fauna de simbiontes de *A. castro* (Rosa et al., 2018). Em ambos os estudos, as coletas foram feitas entre 2012 e 2013, obtendo em média 50 indivíduos a cada 30 minutos de esforço amostral. Ao longo dos meses de julho de 2018, janeiro de 2019 e maio de 2019 o rio Couro apresentava boa estrutura de hábitat, com vegetação ripária considerável e qualidade de água apropriada para a manutenção de eglídeos (observar imagens contidas no Apêndice 2), porém o esforço amostral necessário para coletar 50 indivíduos no presente estudo foi bem maior do que o reportado na literatura.

O rio Couro é um pequeno riacho de baixa ordem, afluente do rio Preto. Ambos os cursos de água abrigavam populações de eglídeos. No entanto, sobre a confluência

destes dois cursos de água passa a Rodovia do Café (BR-376), que teve obras de duplicação da via iniciadas em 2021 (observar mapa e imagens do Apêndice 3). Após o início das obras para a duplicação da rodovia, foram observadas em uma coleta de campo (junho de 2021) profundas alterações da estrutura do rio Couro, tais como (i) alteração da estrutura do leito do riacho à jusante da rodovia, (ii) alteração do curso natural pela construção de uma nova ponte, (iii) diminuição do fluxo de água pelo represamento à montante da rodovia, (iv) desmatamento de vegetação ripária, (v) erosão das margens, (vi) assoreamento, (vii) contaminação por cimento e rejeitos plásticos à jusante (observar imagens contidas no Apêndice 4). Nesta coleta realizada em junho de 2021 não foram encontrados espécimes de *A. castro* no rio Couro depois de pelo menos 2 horas de esforço amostral, tanto à montante quanto à jusante da rodovia.

Em suma, os resultados desta tese contribuíram para aumentar o conhecimento acerca de como os eglídeos respondem à contaminação por metais, tanto no ambiente quanto em exposições agudas em laboratório. Além disso, vários parâmetros funcionais de *A. castro* foram bastante responsivos e podem ser utilizados como biomarcadores em futuros estudos ecotoxicológicos que adotem outras espécies de *Aegla* como modelos biológicos. Por exemplo, as enzimas HATP, NKA e AC, a concentração de metalotioneínas e a densidade total de hemócitos foram biomarcadores muito responsivos à contaminação por Cu e podem fornecer indícios precoces de toxicidade causada pelo Cu em eglídeos.

Apesar dos eglídeos normalmente ocorrerem em ambientes livres de contaminação, este estudo apontou que as populações de *A. castro* do rio Couro podem estar sujeitas aos efeitos subletais da contaminação por Cu, indicando que algumas populações de eglídeos são capazes de resistir a essa contaminação antes do seu declínio populacional. Por fim, a partir de observações em campo, este estudo apontou uma profunda degradação de habitat para eglídeos decorrente de uma obra pública, que pode ter contribuído significativamente para o declínio populacional de *A. castro* no rio Couro.

Apêndices

8 APÊNDICES

8.1 APÊNDICE 1 – CONCENTRAÇÕES DE CU NA ÁGUA DO RIO COURO

Concentrações de Cu total (Cu T) e dissolvido (Cu D) no riacho de referência (rio Couro), de julho de 2018 a fevereiro de 2020. Os dados são apresentados como média seguida do número amostral e amplitude, entre parênteses.

Concentração de Cu	Julho de 2018	Janeiro de 2019	Maio de 2019	Fevereiro de 2020
Cu T ($\mu g\ L^{-1}$)	0,43 (n=3; 0.10–0.76)	0,92 (n=3; 0.78–1.13)	0,83 (n=1)	1,93 (n=1)
Cu D ($\mu g\ L^{-1}$)	0,01 (n=3; <LD–0.01)	0,72 (n=3; 0.60–0.89)	0,67 (n=1)	1,05 (n=1)

Os dados de fevereiro de 2020 são autorais, mas fazem parte de outro estudo não comtemplado pela tese. Cu T (concentração de cobre total), Cu D (concentração de cobre dissolvido).

8.2 APÊNDICE 2 – RIO COURO EM JULHO DE 2018, JANEIRO DE 2019 E MAIO DE 2019.

Fotos do riacho de referência (rio Couro) em julho de 2018, janeiro de 2019 e maio de 2019. Pesquisadores realizando coleta de espécimes, amostras de água e aferindo parâmetros abióticos.

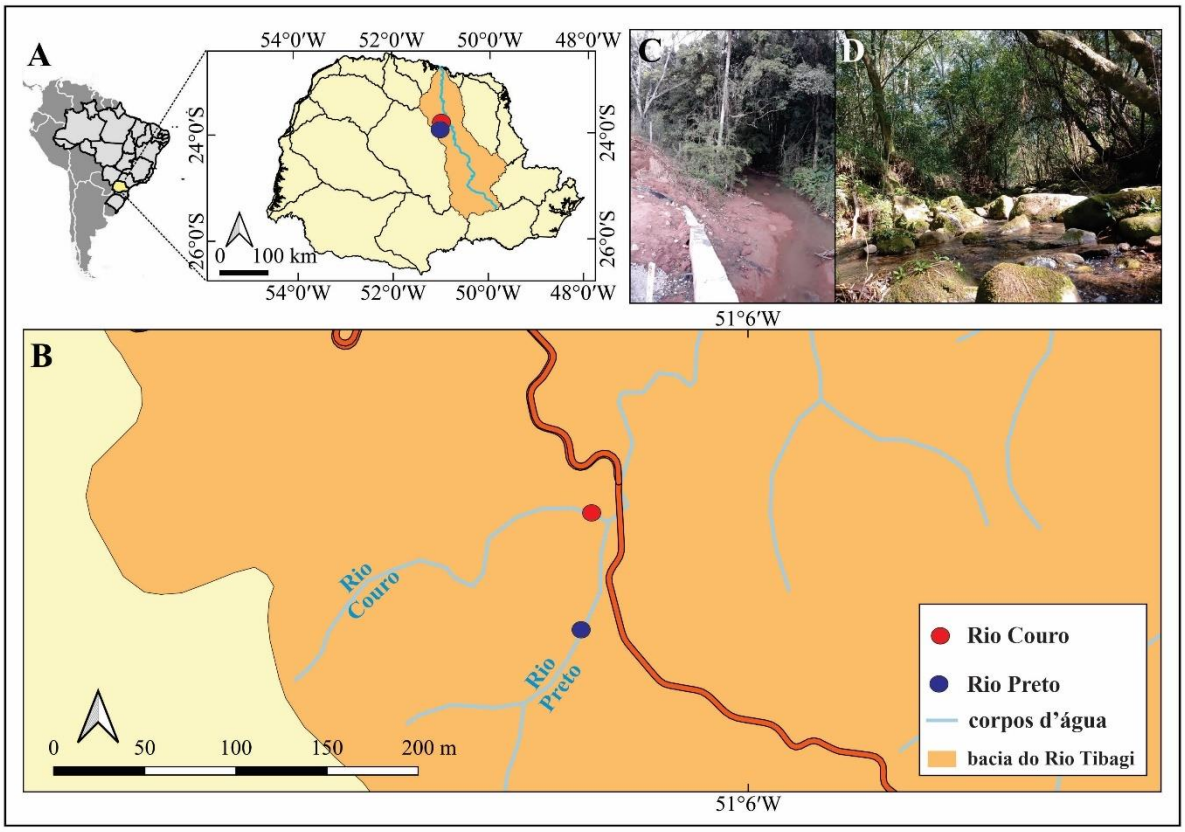
Julho de 2018

Janeiro de 2019

Maio de 2019

8.3 APÊNDICE 3 – LOCALIZAÇÃO DOS RIOS COURO E PRETO, BACIA DO RIO TIBAGI.

Localização do rio Couro e rio Preto (A–B) na bacia do rio Tibagi. Trecho à jusante do rio Couro após o início da duplicação da Rodovia do Café (BR-376, km 2), em junho de 2021 (C). Rio preto em junho de 2021, à montante da área de duplicação da rodovia (D).



8.4 APÊNDICE 4 – ÁREA DE DUPLICAÇÃO DA RODOVIA DO CAFÉ (BR-376, KM 2).

Fotos do riacho de referência (rio Couro) após o início da duplicação da rodovia (à montante e à jusante) em junho de 2021. Detalhe para represamento e erosão à montante; erosão e assoreamento à jusante.

Área de duplicação da BR-376, km 2

Junho de 2021 (à montante da rodovia)

Junho de 2021 (à jusante da rodovia)

Anexos

9 ANEXOS

9.1 Artigo Publicado na revista Comparative Biochemistry and Physiology, Part C

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Short communication

Short communication: Effects of acute copper exposure on ionic regulation of the freshwater crab *Aegla castro*

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ABSTRACT

Aeglids are unique freshwater decapods whose habitats are being impacted by metallic compounds, such as copper (Cu). Thus, we investigated the effects of acute Cu exposure on ionic regulation of *Aegla castro*. For this, male specimens in intermolt were collected from a reference stream and acclimated for 5 days in laboratory. After which, crabs were exposed to 11 μg L^{-1} Cu (Cu11) or only to water (CTR) for 24 h. Hemolymph samples were withdrawn for the determination of Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations and the posterior gills removed for the analysis of Na^+/K^+-ATPase, Ca^{2+}-ATPase, H^+-ATPase, and carbonic anhydrase (CA) activities. Increased Ca^{2+} and Mg^{2+} hemolymph concentrations were observed in animals from Cu11, when compared with CTR group. In addition, decreased activity of CA was observed in animals exposed to Cu. In the current study, alterations in Ca^{2+} and Mg^{2+}concentrations probably indicate that animals activated exoskeleton reabsorption mechanisms, characteristic of the premolt. Therefore, increased Ca^{2+} and Mg^{2+} concentrations in hemolymph may indicate that a biochemical signal associated with the molting cycle was triggered by Cu exposure. Despite the known harmful effects of Cu on osmoregulatory enzymes, here we observed decreased activity only in CA. However, decreased activity of CA could trigger both acid-base imbalance and ionic disruption, since CA provides H^+ and HCO_3^- for intracellular pH maintenance, and underpins Na^+ and Cl^- for ionic regulation. Therefore, understanding how aeglids respond to metal contamination in laboratory conditions is crucial to assess their potential as an alternative biological model for aquatic ecotoxicology.

1. Introduction

In the last few years, the crabs of the genus *Aegla* Leach, 1820 (Anomura, Aeglidae) have been increasingly addressed in physiological and ecotoxicological studies (de Faria et al., 2011; Freire et al., 2013; Faria et al., 2018; Bozza et al., 2019; Cerezer et al., 2020). Indeed, some aspects illustrate why these remarkable freshwater anomurans have received additional attention in applied studies, for instance, the increasing number of new species descriptions (26) in the last two decades (Santos et al., 2017), the ecological relevance and wide variety of freshwater habitats that aeglids are adapted to (Bond-Buckup and Buckup, 1994; Bueno et al., 2016), and the adequacy of aeglids as bioindicators of environmental quality under a biomarker approach (Faria et al., 2018; Cerezer et al., 2020). On the other hand, the fragmentation of riparian forest, degradation of limnic habitats by siltation, and contamination of water bodies by pesticides and fertilizers represent the three main anthropogenic threats to aeglid diversity (Boos et al., 2020), raising concern and demonstrating the urgency of addressing these freshwater decapods in ecotoxicological studies.

Aegla castro Schmitt, 1942 — target species of the present study — is endemic to Southern Brazil (Bond-Buckup and Buckup, 1994). Regardless of the slightly interspecific differences on the ecological niche, aeglids constitute an important group of freshwater crustaceans from an ecological perspective since they require environments with well-oxygenated waters and play a key role in the cycling of nutrients, becoming bioindicators of habitat quality (Bond-Buckup and Buckup, 1994). More recently, de Almeida et al. (2021) investigated the trophic ecology of *A. castro* and characterized it as an omnivorous generalist and opportunist species.

Despite *A. castro* being considered a species of "least concern (LC)", approximately 70% of species of *Aegla* are under some degree of threat according to the criteria established by the IUCN (Santos et al., 2017) since many species are being threatened by habitat loss due to the metal contamination (Gonçalves et al., 2018; Bueno et al., 2016). Cu is one of

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the most common pollutants found in all aquatic environments (Wei and Yang, 2016), being widely applied in industry, electric wires, water pipes, metal mixtures, and boat paints (Simonato et al., 2016). Cu also constitutes agricultural fertilizers and pesticides, mainly in the fungicidal composition (Gonçalves et al., 2018; Li et al., 2020), leading this metal to be leached or even discharged into headwater streams within agricultural or urban areas (Simonato et al., 2016). Indeed, Faria et al. (2018) found Cu concentrations in the sediment taken from different streams in Southern and in Southeastern Brazil where ten aeglid species were collected, including *A. castro*. Under a phylogenetic perspective, the authors investigated metal accumulation and antioxidant defense system and emphasized the importance of a systematic approach for future monitoring studies with aeglids.

Although Cu is an essential micronutrient at low concentrations, at high concentrations Cu has been extensively linked to adverse effects in ionic balance and inhibitory effects on enzymes involved in osmoregulation in fish (e.g. Malhotra et al., 2020) and crustacean species (Hansen et al., 1992; Hebel et al., 1999; Brooks and Mills, 2003; Capparelli et al., 2020), being considered an osmoregulatory toxicant (Grosell et al., 2002; Bianchini et al., 2004).

In this sense, to our knowledge, we investigated for the first time the effects of acute Cu exposure in an aeglid species, by assessing the effects of this metal on ionic regulation of specimens of *A. castro* kept under controlled laboratory conditions. Thus, as Cu is widely reported as an osmoregulatory toxicant (Grosell et al., 2002; Bianchini et al., 2004), we hypothesize that acute Cu exposure will disrupt the ion regulation and inhibit the enzymes involved in osmoregulation in the freshwater crab *A. castro*. Although *in situ* tests have been carried out with other aeglid species, like *Aegla longirostri* (Cerezer et al., 2020), biomarker data from laboratory toxicity tests are still scanty in the literature.

2. Material and methods

For this, male specimens of *A. castro* in the intermolt stage were collected from the Couro Stream (23°57′15″S, 51°07′00″W), Tibagi River Basin, Southern Brazil in May 2019. This stream has no apparent pollution sources, with riparian forest along some stretches of its extension. Non-filtered and filtered (0.45-μm mesh filter, Millipore Millex HV/PVDF) water samples from the collection site were taken for determination of total (Cu T) and dissolved Cu (Cu D) concentrations, and the concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+}. We also collected 100 L of stream water to be used in the laboratory for the acclimation of the crabs. The aeglids were transported to the laboratory (~90 min) in boxes containing 15 L of stream water and plastic artificial hiding places.

Acclimation was performed for five days in a 100-L plastic tank containing hiding places and stream water with constant aeration, filtration, and a 12/12 h light/dark photoperiod. Animals were fed every day with flocked fish feed, except on the day of the experiment. Partial water renewal of 25% was performed every 24 h with dechlorinated tap water at the same temperature as the water of the acclimation tank. Physical and chemical variables were monitored (Horiba U-52) during the acclimation.

Thereafter, the crabs were randomly divided into two groups: the control group (CTR), which was kept under controlled conditions with no addition of Cu in the exposure medium; and the waterborne Cu group (Cu 11), which was exposed to 11 μg L^{-1} dissolved Cu. The exposures were carried out in 2-L beakers containing 1.5 L of dechlorinated tap water with Cu (Cu 11 group), or without Cu (CTR group), with constant aeration and temperature control; both groups were exposed for 24 h. Physical and chemical parameters were monitored at 0 h and 24 h.

Non-filtered and filtered water samples from two beakers of each group were taken at 0 h and 24 h to determine the Cu T and Cu D concentrations. Samples were immediately acidified with HNO_3 and kept at 4 °C until the analysis. The Cu T and Cu D concentrations were determined using a graphite furnace atomic absorption spectroscopy (AAnalyst700, PerkinElmer), with a detection limit of 0.0014 μg L^{-1}. Ion concentrations in the water were measured in non-filtered samples, both from the field and the exposure media (0 h and 24 h). Na^+ and K^+ concentrations were determined by flame photometry (Digimed DM-62) and Ca^{2+} and Mg^{2+} concentrations by atomic absorption spectroscopy (AAnalyst700, PerkinElmer).

After 24 h of exposure, the crabs were cryo-anesthetized, weighed, and measured (CTR group: $n = 8$; 2.43 ± 1.03 g; 14.13 ± 2.03 mm carapace length; and Cu 11 group: n = 8; 2.69 ± 1.35 g; 16.50 ± 2.19 mm carapace length; mean ± SD). Hemolymph aliquots were withdrawn from the arthrodial membrane of chelipeds for the determination of ion concentrations using the same methodology described above for the ions measured in water samples. Subsequently, the gills were dissected and stored in SEI buffer (150 mM sucrose, 50 mM imidazole, 10 mM EDTA) at −80 °C until the analysis.

The gills were thawed, weighed (0.095 ± 0.010 g, mean ± SE), and homogenized (1:5 *w/v*) in SEI buffer with 12 mM sodium deoxycholate (pH 7.5), using an ultrasonic sonicator. Next, the homogenates were centrifuged (20 min, 16,060 ×g, 4 °C) and the supernatant was used to determine the activities of carbonic anhydrase (CA), Na^+/K^+-ATPase (NKA), V-H^+-ATPase (HATP), and Ca^{2+}-ATPase (CaATP). The total protein content was measured at 595 nm, according to Bradford (1976).

To assay the CA activity we followed Vitale et al. (1999), with some adaptations in reaction medium and centrifugation. An aliquot of the homogenate was added to CO_2-saturated distilled water (2.0–2.5 °C) and the acidification of the medium was quantified every 4 s for 20 s with a pH meter (Jenway 3510). The linear relationship of the decrease in pH as a function of time generated a slope of the curve, the catalyzed reaction (CR) rate. The non-catalyzed reaction (NCR) rate was obtained by measuring the decrease in pH when the sample was not added to the assay. Thus, the specific reaction rate of CA (SCA) was calculated as follows: SCA = [CR/NCR − 1]/mg protein.

Na^+/K^+-ATPase (NKA) and V-H^+-ATPase (HATP) activities were measured in a simultaneous assay following Gibbs and Somero (1989) and adapted to a microplate reader (Tesser et al., 2020). The samples were adjusted to a protein concentration of 1 mg mL^{-1}. The reactive solutions (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM KCN, 1 mM ATP, 0.2 mM NADH, 3 U mL^{-1} pyruvate kinase, 2 U mL^{-1} lactate dehydrogenase, 0.1 mM fructose-1-6-diphosphate, 2 mM phosphoenolpyruvate, pH 9.0) containing 2 mM ouabain or 2 mM N-Ethyl-d_5-maleimide (NEM) were made to determine the activities of NKA and HATP, respectively. The total activity of the ATPases was measured without adding inhibitors to the medium. The absorbance was measured every minute for 15 min at 340 nm (Victor3, PerkinElmer). The specific activities of NKA and HATP were calculated by the difference between the total activity of the ATPases and the medium with ouabain and NEM, respectively. Enzyme activities were expressed in μmol ADP mg $protein^{-1}$ h^{-1}.

CaATP activity was assayed according to Vijayavel et al. (2007), with modifications. Samples were incubated at 30 °C for 30 min in reactive solution (189 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris, 5 mM $CaCl_2$, 2 mM ouabain, pH 7.6) with 3 mM ATP or without ATP added, to assess the reaction of CaATP and the basal concentration of inorganic phosphate (Pi), respectively. After incubation, the microplate was placed on ice for 10 min to stop the reaction; and then, a staining solution (1:6 v/v 10% ascorbic acid:0.42% ammonium molybdate in 0.5 mM H_2SO_4) was added to the plate (Ames, 1966). The formation of Pi was measured at 620 nm after 10 min in a microplate reader. A phosphate standard curve (0.08–0.65 mM) was used to quantify the CaATP activity. The results were expressed as μmol Pi mg $protein^{-1}$ min^{-1}.

Normality (Shapiro-Wilk test) and homoscedasticity (Levene test) of the biomarker results were verified. The results obtained for Cu exposure groups (Cu 11) were compared to their respective control groups (CTR) using the Student's *t*-test or Mann-Whitney test, according to data distribution. Non-parametric data are illustrated as boxplots. All statistical analyses were performed in the R environment (R Development Core Team, 2020), and the significance level adopted was 0.05.

3. Results and discussion

Physical and chemical parameters of the water measured during the exposures at 0 h and 24 h are presented in Table 1. Although not statistically tested, we found mean values of turbidity lower (~50%), and conductivity higher (~90%) during the experiments when compared to the collection site (Table 1). These differences can be related to the physicochemical characteristics of stream water when compared to the dechlorinated tap water used in the exposure media. Also, the mean values of Na^+ concentrations in the water of the exposure media were nearly double those found in the collection site. The turbidity of stream water may have been higher due to the greater amount of suspended sediments, and the conductivity in the exposure media may have been lower due to the higher Na^+ concentration of the exposure media when compared to the stream water (Table 1).

The Cu T and Cu D from the CTR group had similar values, both at 0 h and 24 h (Table 1). In the Cu 11 group, the mean Cu D concentration corresponded to 81.5% of the mean Cu T concentration. In addition, as the mean Cu D concentration deviated only 0.32% from the expected one, the concentration of 11 μg L^{-1} dissolved Cu was used. This dissolved Cu concentration is within the limits set by the Brazilian guidelines for freshwater (Conama, 2005).

Cu-contaminated watercourses have been progressively found in Southern Brazil watersheds. Specifically, concerning the Tibagi River Basin, a study conducted on water quality monitoring pointed out dissolved Cu concentrations above the limits in all the sampling points along the Tibagi River (IAP, 2012). From April 2010 to December 2011 up to 207 μg L^{-1} dissolved Cu was found in the water (IAP, 2012), approximately 23-fold the permitted concentration (Conama, 2005). Furthermore, Cu has also been found in the sediments of small streams from Southeastern and Southern Brazil, where at least 10 species of *Aegla* are recorded (Faria et al., 2018). In this same study, Cu accumulation was reported in the hepatopancreas tissue of several species of *Aegla*, including *A. castro*, indicating that aeglids not only experience contact with Cu in their habitats but can accumulate this metal, being susceptible to the sublethal effects of Cu exposure. Therefore, dissolved Cu concentrations above 11 μg L^{-1} can be found in the environment, even in the streams belonging to the same watershed where *A. castro* occurs.

Crab survival was 100% during the experiment in both treatment groups. Aeglids exposed to Cu 11 presented no alterations in the concentrations of Na^+ and K^+ in the hemolymph when compared to CTR group (Fig. 1A–B). Although adverse effects caused by Cu exposure could promote the disruption of gill Na^+ uptake regulation, mainly associated with the NKA inhibition (Grosell et al., 2002; Brooks and Mills, 2003), here we did not observe any alteration in the activity of NKA and HATP in *A. castro,* after Cu exposure (Fig. 1C–D). This seems to be reasonable since the maintenance of the NKA and HATP contributes for the homeostasis of Na^+ and K^+ in the hemolymph (Freire et al., 2008).

Indeed, the maintenance of the Na^+, K^+, and Cl^- equilibrium is crucial in hyperosmoregulating, hololimnetic crustaceans, like aeglids, since these animals experience a very dilute external medium and constantly have to employ active, transbranchial NaCl absorption to compensate for the passive ions loss to the environment (Freire et al., 2008; McNamara and Faria, 2012). The HATP has a pivotal role on Na^+ uptake, from the external medium to the cytosol and then to the hemolymph. The H^+ (provided by CA) is actively discharged by HATP into the subcuticular space, hyperpolarizing the apical membrane, promoting the Na^+ influx through apical Na^+ channels down its electrochemical gradient. Active Na^+ transport from the cytosol into the hemolymph is, in turn, maintained by the NKA located in the gill basolateral membrane (Freire et al., 2008). On the other hand, the Cl^- uptake from the external medium is favored by the ion exchange through the apical Cl^-/HCO_3^- antiporters (supplied by HCO_3^- provided by CA), and then the passive transport to the hemolymph through basolateral Cl^- channels (Freire et al., 2008).

However, the concentrations of Ca^{2+} and Mg^{2+}in the hemolymph after Cu exposure were 58.8% and 38.3% higher when compared to their respective CTR groups (Ca^{2+}: $t(12) = -5.7230$, $p < 0.001$; Mg^{2+}: $t(12) = -2.1388$, $p = 0.0211$) (Fig. 1E–F). These increases could indicate that the crabs exposed to Cu activated reabsorption mechanisms from the exoskeleton by mobilizing Ca^{2+} and Mg^{2+} through the integumentary epithelium and depositing them into the hemolymph in the form of carbonates of calcium and magnesium (Greenaway, 1985; Ahearn et al., 2004). Different storage strategies of these carbonated deposits are reported for crustaceans, such as in the form of gastroliths; calcium phosphate granules in hepatopancreas; or even as microspherules in the hemolymph, giving the milky aspect to the decapods hemolymph in premolt (Greenaway, 1985). Indeed, at least for Ca^{2+}, massive cation fluxes across epithelia (Ahearn et al., 2004), as well as a conspicuous peak in the hemolymph Ca^{2+} concentration are widely reported for both terrestrial, marine, brackish, and freshwater crustacean species in the late premolt (Greenaway, 1985). Also, the high concentrations of Ca^{2+} and Mg^{2+} found in the hemolymph of *A. castro* exposed to Cu appear to be in the same order of magnitude as those found in other crustacean species in premolt (Li and Cheng, 2012). This conspicuous increment in the concentrations of Ca^{2+} and Mg^{2+} can represent a biochemical signal

Table 1
Abiotic variables, ions concentration, and total and dissolved copper concentrations (Cu T and Cu D, respectively) in the water of the collection site (Couro Stream), the laboratory acclimation tank, and the beakers where the two groups (CTR and Cu 11) were exposed. The CTR group was exposed to dechlorinated water with no addition of Cu in the exposure medium; and the Cu 11 group was exposed to dechlorinated water with addition of 11 μg L^{-1} Cu. The data from the CTR and Cu 11 groups were measured at 0 h and 24 h. Results are reported as mean values.

Abiotic variable	Collection site	Acclimation	CTR		Cu 11	
			0 h	24 h	0 h	24 h
Temperature (°C)	15.6	16.6	17.3	16.1	16.4	16.0
pH	7.7	7.6	7.6	7.8	6.8	7.5
Dissolved oxygen (mg O_2^{-1})	8.5	8.3	7.3	7.8	7.6	7.3
Turbidity (NTU)	23.0	9.3	13.4	12.8	5.9	8.8
Conductivity (μS cm^{-1})	61.0	192.0	114.5	121.5	112.5	112.5
Oxidation-reduction potential (ORP mv^{-1})	359	363	358	317	399	344
Total dissolved solids (g L^{-1})	0.04	0.12	0.07	0.08	0.07	0.07
Hardness mg L^{-1} $CaCO_3$	21	–	38	–	34	–
Na^+ (mM)	0.139	–	0.222	0.226	0.218	0.226
K^+ (mM)	0.043	–	0.023	0.033	0.020	0.022
Ca^{2+} (mM)	0.084	–	0.074	0.083	0.069	0.071
Mg^{2+} (mM)	0.058	–	0.066	0.067	0.065	0.066
Cu T (μg L^{-1})	0.803	–	3.251	3.732	15.245	12.180
Cu D (μg L^{-1})	0.670	–	3.055	3.478	11.035	11.035

The dashes indicate variables not measured. The data of ion concentration in the water, Cu T, and Cu D were obtained from non-filtered water samples.

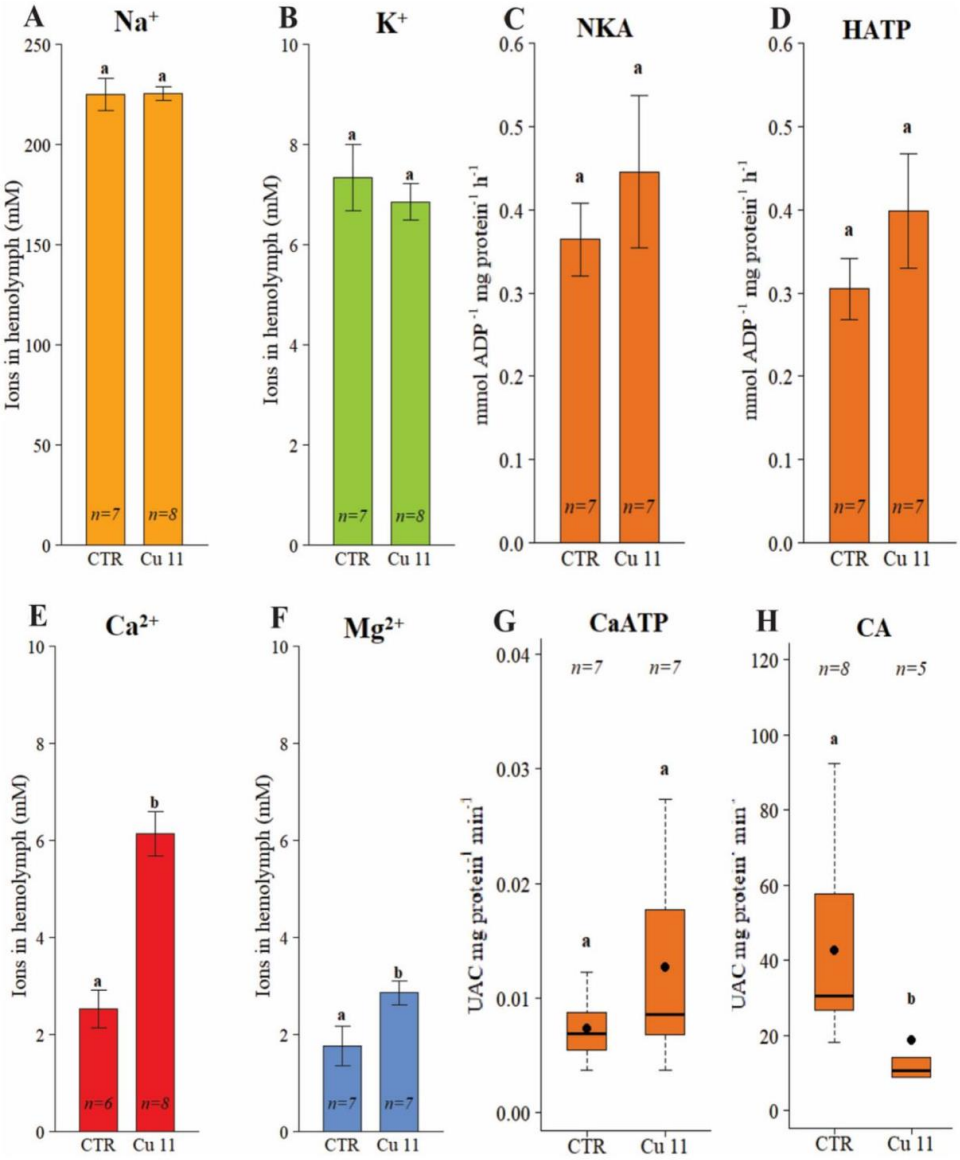

Fig. 1. Concentrations of Na^+ (A), K^+ (B), Ca^{2+} (E), and Mg^{2+} (F) in the hemolymph, and activities of Na^+/K^+-ATPase (C), V-H^+-ATPase (D), carbonic anhydrase (G), and Ca^{2+}-ATPase (H) in posterior gills of *Aegla castro* kept under the control condition (no addition of Cu in the exposure medium) or exposed to waterborne Cu (addition of 11 μg L^{-1}dissolved Cu), at 0 h and 24 h. Data are expressed as mean ± SE. Different letters indicate statistical differences when compared to the respective control group ($p < 0.05$). Sample sizes (n) are indicated on the chart. Boxplots express non-parametric data.

that is associated with the start of new molting cycles in many crustaceans during premolt, mainly due to the massive storage of Ca^{2+} and Mg^{2+} into the hemolymph. Furthermore, a significant shortening of the time to the first molt as well as a decreased growth rate and molting frequency has been reported for the tiger shrimp *Penaeus monodon* exposed to Cu (Chen and Lin, 2001).

In addition, we observed a growing trend in the activity of CaATP (Fig. 1G), which although not statistically different from the respective control, may indicate an increase in the Ca^{2+} flux towards the hemolymph. Thus, as Cu was reported to disrupt the Ca^{2+} homeostasis in the lobster *Homarus americanus* (Chavez-Crooker et al., 2002), coupled with the fact that the molting process was indicated to be a way of depurating metals (Bergey and Weis, 2007), acute Cu exposure contributed to the imbalance of Ca^{2+} and Mg^{2+} in the hemolymph of *A. castro*, which may represent a biochemical signal associated with the start of molting cycle.

On the other hand, we identified a decrease of 55.8% in the CA activity ($W = 34$, $p = 0.0451$) in posterior gills of *A. castro* after Cu exposure (Fig. 1H). Similarly, decreased gill CA activity was reported in *Minuca rapax* exposed to both dietary and waterborne Cu (Capparelli et al., 2017, 2020); as well as inhibition of the posterior gills CA of *Chasmagnathus granulata* exposed to waterborne Cu (Vitale et al., 1999). These effects are expected since Cu enters the gill cells, and readily binds to CA (DiTusa et al., 2001), triggering, in turn, reduced branchial Na^+ and Cl^- transport due to depletion of the exchangeable cellular supplies required in these fluxes (Grosell et al., 2002).

Although CA is primarily a cytosolic enzyme responsible for the

reversible hydration/dehydration reactions of CO_2, it has been widely stated that acid-base balance is coupled with NaCl homeostasis through the supply of cellular substrates (H^+ and HCO_3^-) in crustacean gill epithelia (Grosell et al., 2002; Freire et al., 2008; McNamara and Faria, 2012). On the other hand, Wang et al. (1998) reported that Cu exposure can lead to an acid-base imbalance even when the Cu concentration was not enough to provoke marked osmoregulatory disturbances. Likewise, Grosell et al. (2002) suggested that CA is another component of branchial Na^+ transport that could be affected by Cu exposure before the basolateral NKA. Thus, the decreased CA activity found in our study might reveal that the 24 h exposure time was not sufficient to disturb Na^+ and K^+ homeostasis in the hemolymph; however, Cu could trigger an initial process of ionic disturbance through the decreased activity of CA, even though no alteration was observed in the activities of the HATP and NKA after 24 h of Cu exposure. Moreover, McNamara and Faria (2020), studying the gill tissue of *Aegla franca* reported that other pathways of hemolymph Na^+ homeostasis may be present in aeglids, such as the $Na^+/H^+(NH_4^+)$ exchangers in the apical membrane, supporting the influx of Na^+ to the intracellular medium; while Cl^- channels and sodium-potassium two-chloride symporters (NKCC) could be located in the basolateral membrane, also providing the transport of Cl^-, K^+, and Na^+ to the hemolymph.

Our hypothesis was partially accepted, as acute Cu exposure disrupted the Ca^{2+} and Mg^{2+}, but not the Na^+ and K^+ homeostasis, as well as inhibited only the activity of carbonic anhydrase among all the other enzymes analyzed involved in osmoregulation. Therefore, these results could indicate that both the concentrations of Ca^{2+} and Mg^{2+} in the hemolymph and the gill activity of carbonic anhydrase may be considered suitable biomarkers for the evaluation of Cu sublethal effects in *A. castro* exposed to 11 μg L^{-1} dissolved Cu, a concentration that is within the limits set by the Brazilian guidelines for freshwater (Conama, 2005).

In summary, this study is the first step towards enhancing our understanding of Cu exposure effects on aeglids. Metal exposure studies with *Aegla* are essential to comprehend how these unique freshwater decapods cope with environmental contamination as well as their potential as an alternative biological model in ecotoxicology. Further experimental investigations regarding other aeglid species and exposure times could clarify our knowledge of how *Aegla* faces habitat contamination by Cu.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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