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TESE DE DOUTORADO

Metabolismo energético e estado oxidativo no peixe *Poecilia vivipara*: efeitos da combinação entre aumento de temperatura e exposição ao cobre

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Do not go gentle into that good night

Do not go gentle into that good night,
Old age should burn and rave at close of day;
Rage, rage against the dying of the light.

Though wise men at their end know dark is right,
Because their words had forked no lightning they
Do not go gentle into that good night.

Good men, the last wave by, crying how bright
Their frail deeds might have danced in a green bay,
Rage, rage against the dying of the light.

Wild men who caught and sang the sun in flight,
And learn, too late, they grieved it on its way,
Do not go gentle into that good night.

Grave men, near death, who see with blinding sight
Blind eyes could blaze like meteors and be gay,
Rage, rage against the dying of the light.

And you, my father, there on the sad height,
Curse, bless, me now with your fierce tears, I pray.
Do not go gentle into that good night.
***Rage, rage against the dying of the light**.*

Dylan Thomas (1952)
"In Country Sleep, And Other Poems"

Sumário

Resumo geral

Atualmente, a poluição ambiental vem aumentando em grande proporção e seus efeitos sobre o ambiente tem se tornado uma grande preocupação. O cobre se destaca entre os poluentes químicos. Este metal essencial é importante para processos como estruturação de enzimas, resposta ao estresse oxidativo, sinalização de hormônios esteroides, respiração mitocondrial e osmorregulação. Apesar disto, é toxico quando encontrado em altas concentrações. Os efeitos tóxicos já descritos para o cobre incluem alterações na expressão e atividade de enzimas, permeabilidade de membrana, proliferação celular e apoptose. O ambiente aquático é um dos alvos da poluição por cobre, portanto, animais aquáticos, como os peixes, estão sob risco de contaminação por este metal. Vários estudos vêm esclarecendo os efeitos tóxicos deste metal no peixe *Poecilia vivipara*, um potencial modelo para ecotoxicologia neotropical. Os efeitos já descritos para *P. vivipara* incluem bioacumulação, alterações na capacidade de produção energética e no estado oxidativo. No entanto, pouco se sabe sobre os efeitos interativos da temperatura e da exposição ao cobre em animais neotropicais, apesar da crescente preocupação em relação aos efeitos de alterações climáticas globais sobre a biota. Neste contexto, um dos grandes desafios científicos da atualidade se refere ao melhor entendimento das possíveis relações entre alterações climáticas globais e estressores locais, como a poluição por metais. Sendo assim, o objetivo desta tese foi avaliar o efeito combinado da temperatura de aclimatação e da exposição ao cobre no perfil de acumulação deste metal (fígado e brânquias), no estado oxidativo (capacidade antioxidante total e dano oxidativo), no desempenho térmico individual (CTMax) e metabolismo energético (atividade da piruvato quinase, lactato desidrogenase, citrato sintase e cadeia transportadora de elétrons) no peixe *P. vivipara*. Para tal, os indivíduos foram aclimatados por três semanas a duas temperaturas (20 e 28ºC) e posteriormente expostos ao cobre (controle, 9 e 20 µg/L) por 96h. Os resultados demonstram que os animais aclimatados à maior temperatura exibiram maior acumulação de cobre e foram os únicos que tiveram sua CTMax reduzida. Além disto, os peixes mantidos em 28ºC foram os únicos a apresentar redução na capacidade antioxidante total após exposição a este metal, fato que contribuiu para o aumento de dano oxidativo no fígado visto nestes animais. Portanto, fica evidente que a exposição ao cobre em alta temperatura levou à maior acumulação tecidual do cobre e dano oxidativo hepático, explicando a diminuição observada na CTMax. Interessantemente, estes resultados não estão relacionados à grandes ajustes em termos da atividade de enzimas do metabolismo energético. Logo, ajustes energéticos para fins de aclimatação à temperatura elevada não parecem estar relacionados ao aumento de toxidade do cobre visto neste estudo. Portanto, conclui-se que em contextos ambientais de temperatura elevada, como frente ao processo de aquecimento global ou em períodos de verão, um aumento considerável na toxicidade do cobre deve ser esperado. É demonstrado ainda que este processo está intimamente relacionado a alterações no estado oxidativo hepático.

Palavras-chave: Metais Traço, Ecotoxicologia, Aquecimento Global, Estresse Oxidativo, Atividade Enzimática.

Abstract

Environmental pollution is growing in unprecedent levels and has been raising concerns nowadays. Within this context, copper is highlighted as a major organic pollutant. This essential metal is important for many biological processes such as enzymatic structure, oxidative stress response, hormonal signaling, mitochondrial respiration and osmoregulation. Even though, this metal can be toxic in elevated concentrations. Copper toxic effects are related to alteration in enzymatic expression and activity, membrane permeability, cellular proliferation and apoptosis. Aquatic environments are the mostly impacted by copper contamination, therefore, aquatic animals such as fishes, are endangered. Many studies have been evaluating the toxic effects of copper exposure in the fish *Poecilia vivipara*, a potential model for neotropical ecotoxicology. The already described effects are related to bioaccumulation, energy production and oxidative status. Nonetheless, little is known regarding interactive effects of temperature and copper exposure in neotropical animals, even with the growing concerns regarding the impacts of global warming. Within this context, one of the major challenges nowadays is to understand how global climate changes may affect local impacts, such as metal pollution. Therefore, the objective of this thesis was to evaluate the combined impact of acclimation temperature and copper exposure in tissue accumulation (liver and gills), oxidative status (total antioxidant capacity and oxidative damage), individual thermal performance (CTMax) and energy metabolism (activity of pyruvate kinase, lactate dehydrogenase, citrate synthase and the electron transport chain system) in the fish *P. vivipara*. For such, animals were acclimated for three weeks in two temperatures (20 and 28ºC) and then exposed for 96h to two copper concentrations (control, 9 and 20 µg/L). Results shows that fish kept at the higher temperature accumulated more metal following cu exposure. Moreover, these were the only animals to exhibit CTMax reduction. In addition, fish acclimated to elevated temperature and exposed to copper displayed a reduction in hepatic total antioxidant capacity and elevated oxidative damage in this tissue. Therefore, it is clear that copper exposure in elevated temperature leads to elevated metal accumulation and hepatic oxidative damage, both resulting in the observed reduction in CTMax. Interestingly, these results are not related with major changes in the energy metabolism, at least in terms of enzymatic activity adjustments. Therefore, energy adjustments related to elevated temperature acclimation does not seem to explain the increase in copper toxicity observed in this thesis. Finally, it is concluded that within the environmental contexts of elevated temperature, such as those predicted for global warming or expected in summer times, a considerable increase in copper toxicity should be expected. Also, it is demonstrated that this process is intimately related to hepatic oxidative status imbalance.

Keywords: Trace metals, Ecotoxicology, Global Warming, Oxidative Stress, Enzymatic Activity.

1. Introdução geral

O desenvolvimento da civilização humana caminha lado a lado com a exploração extrativista de recursos naturais, os quais são usados como matéria prima para a fabricação de produtos com valor agregado (Mazzuco, 2008). Este eixo produtivo tem sido o grande motor da nossa sociedade, principalmente após os períodos da segunda revolução industrial e segunda guerra mundial, gerando riqueza, empregos e fomentando o rápido florescimento de metrópoles densamente populadas e altamente interconectadas. Não menos importante, o período mencionado também foi marcadamente impactado pelo repentino surgimento de conglomerados industriais massivos (IPCC, 2014). Como resultado deste rápido, intenso e muitas vezes descontrolado desenvolvimento, a civilização humana, juntamente com todas as outras formas de vida que habitam este planeta, precisam lidar com os impactos causados pela degradação ambiental, considerada hoje como um dos problemas mais sérios que afligem nosso globo (IPCC, 2014).

Os processos de contaminação ambiental ocorrem quando elementos, moléculas ou compostos são introduzidos em ambientes aos quais não seriam naturalmente encontrados, ou então passam a ocorrer em concentrações mais altas que o esperado (Mazzuco, 2008). O cobre, por exemplo, é um metal comumente encontrado na crosta terrestre que é naturalmente redistribuído para outros ecossistemas, como o aquático, por processos de erosão e lixiviação (Santore et al 2000). Apesar disto, atividades antrópicas como a mineração, indústria portuária, rompimento de barragens de contenção de rejeitos e esgoto acabam por contribuir com descargas excessivas deste metal no ambiente,

fazendo com que o cobre acabe sendo encontrado em concentrações muito além daquelas naturalmente esperadas (Mazzuco, 2008).

O cobre é um elemento de transição que pode ser encontrado no ambiente em quatro formas químicas distintas: elementar (Cu^0), cuprosa (Cu^{1+}), íon cúprico (Cu^{2+}) e íon trivalente (Cu^{3+}), sendo a forma cúprica a mais reativa. O Cu^{2+} reage preferencialmente com ligantes inorgânicos via oxigênio, como H_2O_2, OH^-, CO_3^- e SO_4^{2-}, ou como compostos orgânicos através de grupos fenólicos e carboxílicos (Barceloux, 1999). Sendo assim, o cobre pode ser absorvido por plantas e animais e cumprir papéis bioquímicos e fisiológicos. De fato, este metal é considerado como essencial à vida, pois participa de diversos processos fisiológicos importantes, como a estruturação de proteínas reguladoras da homeostase celular (Knight et al 1994), regulação da resposta ao estresse oxidativo (Leary et al 2009), sinalização de hormônios esteroides (Dang et al 2000) e respiração mitocondrial (Lauer et al 2012).

Por ser um metal essencial, as concentrações circulantes e celulares do cobre são ativamente reguladas pela ação conjunta e ordenada de diversos tecidos. Este processo pode ser dividido em absorção, transporte, armazenamento e excreção (La Fontaine et al 2010). A absorção deste metal pode ser feita pelo intestino, a partir de vias tróficas, ou então por epitélios de troca gasosa e excreção, como brânquias e pele de alguns animais (Markossian, e Kurganov, 2003). Para que esta absorção possa ocorrer, o cobre precisa estar em sua forma ionizada monovalente (Cu^+) (Dancis et al 1994), a qual é reconhecida pelo transportador de alta afinidade ao cobre (CTR1) e capturada. Apesar disto, o cobre também pode entrar nas células a partir do transportador de metais divalentes 1, ou então por canais de Na^+ (Wood et al 2011). Após

absorvido, este metal é sequestrado por proteínas chaperonas e entregue para outros componentes celulares. Por exemplo, a proteína antioxidante 1 (Atx1) é responsável por entregar o cobre para Cu-ATPases (ATP7A e ATP7B) responsáveis pelo transporte intracelular deste metal (Markossian, e Kurganov, 2003). Semelhantemente, outro grupo de proteínas chamadas “chaperonas de cobre” (CCS) funcionam como carreadoras deste elemento para a superóxido dismutase (SOD), uma importante enzima do sistema antioxidante (Markossian, e Kurganov, 2003). Existem ainda as proteínas carregadoras de cobre para a citocromo c oxidase (Cox17, Sco1, Sco2, and Cox11), um dos complexos proteicos que compõem a cadeia transportadora de elétrons presente nas mitocôndrias (Markossian, e Kurganov, 2003).

Já dentro das células, as concentrações de cobre são mantidas em níveis regulados pelas Cu-ATPases ATP7A e ATP7B (Markossian, e Kurganov, 2003). Em concentrações normais, estas proteínas são responsáveis por carrear este metal até o complexo de Golgi, onde o cobre será armazenado e utilizado na formação das chamadas Cu-proteínas (Mercer et al 2003; Minghetti et al 2010). Apesar disto, quando este metal se encontra em elevadas concentrações, as Cu-ATPases ATP7A e ATP7B são direcionadas para a membrana plasmática por onde este metal é expelido (Minghetti et al 2010). Com exceção dos tecidos pertencentes ao trato gastrointestinal, o cobre é excretado para o sangue e transportado até o intestino. Em caso de excesso deste metal, o cobre é compartimentalizado no intestino e excretado para a luz deste tecido, sendo descartado junto com as fezes (Minghetti et al 2010).

Apesar de suas funções essenciais, o cobre pode provocar toxicidade quando encontrado em elevadas concentrações ambientais. O mecanismo de toxicidade mais classicamente estabelecido para este metal está relacionado a alterações no equilíbrio iônico e osmótico em organismos aquáticos, visto que o cobre pode levar a uma perturbação na regulação dos níveis de sódio (Na^+) e cloreto (Cl^-), acarretando em disfunções na excreção de amônia e na regulação ácido-base (Zimmer et al 2012). Existem hoje algumas hipóteses para explicar estes resultados. Primeiramente, acredita-se que o cobre pode inibir a transcrição e atividade da Na^+/K^+-ATPase, acarretando nos resultados descritos, apesar disto, sabe-se também que este metal pode inibir a atividade da anidrase carbônica, o que pode também levar a disfunções citadas (Zimmer et al 2012). Frente a noção de que o cobre pode ser tóxico em concentrações elevadas, a legislação Brasileira estipula os níveis máximos deste metal em 9 e 5 µg/L em água doce e salgada, respectivamente (CONAMA 357).

Além disto, o cobre pode participar de reações Fenton e induzir a formação direta da espécie radicalar hidroxila ($HO^{\bullet}$), ou então induzir indiretamente a formação deste composto a partir de reações do tipo Haber-Weiss (fig.1), contribuindo significativamente para a indução de estresse oxidativo (Guaratini et al 2007). O cobre pode ainda participar no processo de geração de danos oxidativos pela inibição de enzimas participantes do sistema antioxidante celular (Guaratini et al 2007), potencializado os danos causados por espécies reativas de oxigênio (ROS) naturalmente produzidas pelo metabolismo celular (Guaratini et al 2007).

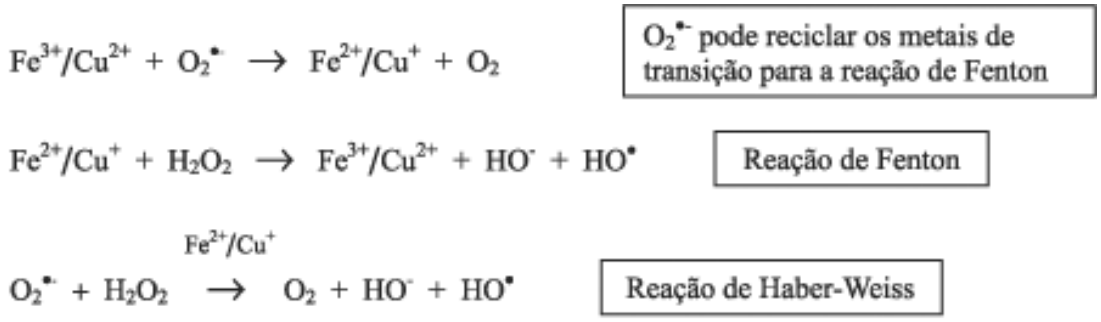

Figura 1 – Participação direta e indireta do cobre na formação da espécie radicalar hidroxila (adaptado de Guaratini et al 2007).

As ERO são moléculas altamente reativas que interagem com componentes celulares e acarretam danos oxidativos (Pamplona e Constantini 2011). A formação destes compostos ocorre majoritariamente em função da produção mitocondrial de ATP de forma aeróbica. Em condições ideais, o O_2 sofre uma redução tetravalente nesta organela, recebendo 4 elétrons para a formação de água, processo essencial para o correto funcionamento da cadeia transportadora de elétrons e fosforilação oxidativa (Barbosa et al 2010). Esta redução é catalisada pela citocromo oxidase, afim de limitar a produção de ERO, apesar disto, cerca de 5% do O_2 metabolizado pelas mitocôndrias acaba por ser oxidado de forma univalente para a formação do radical superóxido ($O_2^{\bullet}$) (Barbosa et al 2010). A partir deste momento, uma serie de intricadas reações podem culminar com a formação de diversas outras ERO, como $OH^{\bullet}$ e o peróxido de hidrogênio (H_2O_2), ou até espécies reativas de nitrogênio, como o peroxinitrito (Pamplona e Constantini 2011).

Frente ao risco representado pelas ERO, os sistemas celulares possuem ferramentas biológicas para combater estes perigosos compostos, são os

chamados sistemas de defesa antioxidante enzimático e não enzimático (Pamplona e Constantini 2011). No primeiro caso, as enzimas que compõem este sistema são: superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (Grd). A primeira destas enzimas a agir é a SOD, responsável pela transformação do $O_2^{\bullet}$ em H_2O_2. Posteriormente, as enzimas CAT e GPx catalisam a transformação do H_2O_2 em H_2O e O_2. Cabe ressaltar que neste processo a GPx acaba por converter a glutationa reduzida (GSH), um importante antioxidante não enzimático, para sua forma oxidada (GSSG). Por fim, a Grd é a enzima responsável pela recuperação de GSH a partir de GSSG (Fig.2). Apesar deste elegante sistema enzimático, o $O_2^{\bullet}$ produzido na mitocôndria pode participar do sistema de reações Fenton/Haber Weiss (Fig.1) e culminar na produção de $HO^{\bullet}$, uma espécie radicalar que não é neutralizada por nenhuma das enzimas citadas e é considerada como extremante reativa (Pamplona e Constantini 2011).

Frente a isto, a importância do sistema de defesa antioxidante não enzimático fica evidente, visto ser o único capaz de neutralizar o $HO^{\bullet}$ (Pamplona e Constantini 2011). Esta outra linha de defesa é baseada em pequenas moléculas produzidas pelas células ou obtidas pela alimentação que podem funcionar tanto em ambientes hidrofílicos como lipofílicos. As principais moléculas antioxidantes não enzimáticas endógenas que podem ser citadas são a GSH, as tiorredoxinas e o ascorbato. Já as principais moléculas antioxidantes não enzimáticas que só podem ser adquiridas pela alimentação são os carotenoides e tocoferóis (para uma minuciosa revisão deste tópico: Pamplona e Constantini 2011).

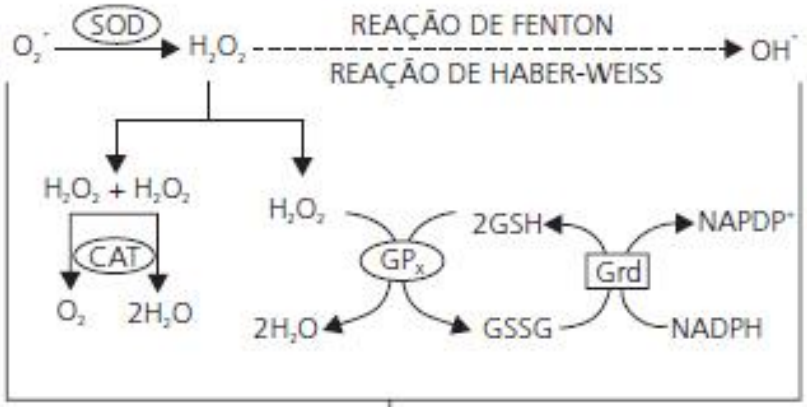

Figura 2 – Sistema antioxidante enzimático (adaptado de Barbosa et al 2010).

É interessante observar o quão diverso e complexo o sistema antioxidante é. Este fato acaba por dificultar estudos que objetivam a compreensão da forma como estressores ambientais podem interferir neste conjunto de processos. Por exemplo, um cientista que tenha por objetivo avaliar o sistema de defesa antioxidante terá que primeiramente determinar se irá avaliar o sistema enzimático em termos da expressão de seus genes, da concentração de suas proteínas ou da atividade de suas enzimas (Amado et al 2009). Além disto, algum tipo de avaliação do sistema não enzimático deverá ser realizada, o que exigirá, ainda, a determinação da concentração e do estado redox de mais uma série de outros compostos. Frente a esta dificuldade, muitos pesquisadores optam pela avaliação da capacidade antioxidante total nos tecidos alvo. Esta interessante análise pode ser entendida como a determinação da resultante redutora do órgão em questão, ou seja, estes métodos permitem que o somatório das capacidades antioxidantes enzimáticas e não enzimáticas sejam determinadas através de um valor único, de forma rápida e simples (Amado et al 2009). Certamente, este grau de sintetização também tem seus lados negativos, visto que fica difícil

determinar precisamente qual é o mecanismo fisiológico que está por trás do resultado em questão (Amado et al 2009).

Além de seus efeitos sobre o sistema de defesa antioxidante, o cobre pode também causar impactos no metabolismo energético das células através de dois mecanismos distintos. Primeiramente, este metal pode impactar os processos de produção de energia pela inibição de enzimas chave deste processo. Por exemplo, Anni et al (2019) demonstrou que *Poecilia vivipara* exposta por 345 dias ao cobre, nas concentrações de 5 e 9 µg/L teve a enzima lactato desidrogenase (LDH) inibida em fígado e brânquias e a enzima piruvato quinase (PK) inibida em fígado. De forma semelhante, o peixe estuarino *Pomatoschistus microps* exposto à 25 µg/L de cobre por 4 dias demonstrou inibição da LDH muscular (Vieira et al 2009) e o peixe dourado *Carassius auratus gibelio* demonstrou inibição desta enzima nas brânquias após exposição por três dias à 100 µg/L de cobre (Teodorescu et al 2012). Por outro lado, este metal pode também gerar um aumento no metabolismo enérgico como resposta ao seu custoso processo de detoxificação hepática (Anni et al 2019).

O metabolismo energético pode ser caracterizado como a produção de energia (ATP) a partir de nutrientes absorvidos do ambiente ou adquiridos através da alimentação, e pode se dar por vias aeróbicas ou anaeróbicas (Navarro e Boveris, 2007). O primeiro passo na cadeia produtiva de energia se dá no processo chamado glicólise, que consiste em dez reações enzimaticamente catalisadas no citoplasma que visam a oxidação da glicose e produção de piruvato, formado na última etapa da via glicolítica pela ação da PK. Além disto, a glicólise também é responsável pela formação de ATP e da coenzima reduzida NADH (Nelson e Cox 2000; Navarro e Boveris, 2007) (Fig.3).

Após a glicólise, o piruvato pode seguir dois caminhos metabólicos distintos. No caso de privação de oxigênio, esta molécula pode ser reduzida à lactato pela ação da LDH no processo chamado fermentação láctica, que culmina na regeneração de NAD^+ que pode ser novamente utilizado na glicólise para formação de mais moléculas de ATP (Nelson e Cox 2000) (Fig.4). Na condição de presença de oxigênio, o piruvato é encaminhado para a dentro da mitocôndria, onde o chamado ciclo de Krebs, ou ciclo dos ácidos tricarboxílicos, irá ocorrer. Nesta via metabólica, o piruvato é primeiramente quebrado em acetil-CoA e posteriormente transformado à citrato pela ação da citrato sintetase (CS). Uma série de outras reações ocorrem após este processo inicial, culminando na produção das coenzimas reduzidas NADH e $FADH_2$ (Navarro e Boveris, 2007) (Fig.5).

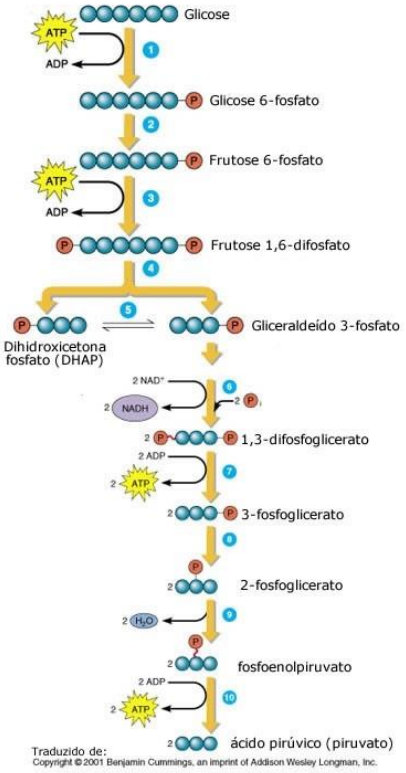

Figura 3 – Esquema demonstrando as etapas da via metabólica da glicólise (traduzido de Benjamin Cummings, 2001).

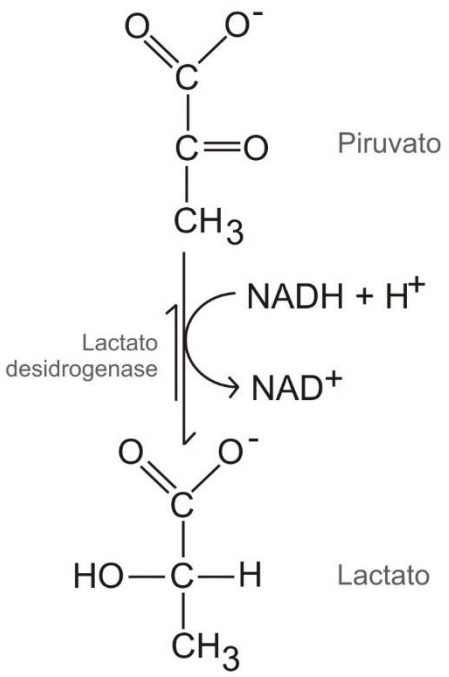

Figura 4 – Esquema demonstrando as etapas da via metabólica da fermentação láctica (traduzido de Benjamin Cummings, 2001).

As coenzimas reduzidas produzidas no ciclo de Krebs são utilizadas pela mitocôndria para a produção de ATP (Nelson e Cox 2000). Este processo depende de uma série de complexos proteicos (I, II, III e IV) presentes na membrana interna da mitocôndria, o que se atribui o nome de cadeia transportadora de elétrons (ETS) (Navarro e Boveris, 2007). Parte destes complexos (complexos I e II) são responsáveis por oxidar as coenzimas reduzidas NADH e $FADH_2$. Os elétrons liberados no processo são transferidos pelos complexos III e IV para o oxigênio, culminado na formação de água. O transporte destes elétrons pela ETS faz com que os complexos I, III e IV bombeiem prótons da matriz mitocondrial para o espaço intermembranas, gerando uma energia potencial armazenada na forma de um gradiente eletroquímico (Navarro e Boveris, 2007). Este gradiente é dissipado pela ATP sintase, que permite a passagem destes prótons de volta para a matriz

mitocondrial. Desta forma, a energia potencial eletroquímica é transformada em energia cinética, possibilitando a conjugação de ADP mais fosfato orgânico para a síntese de ATP, no processo chamado fosforilação oxidativa (Devenish et al 2000) (Fig.6).

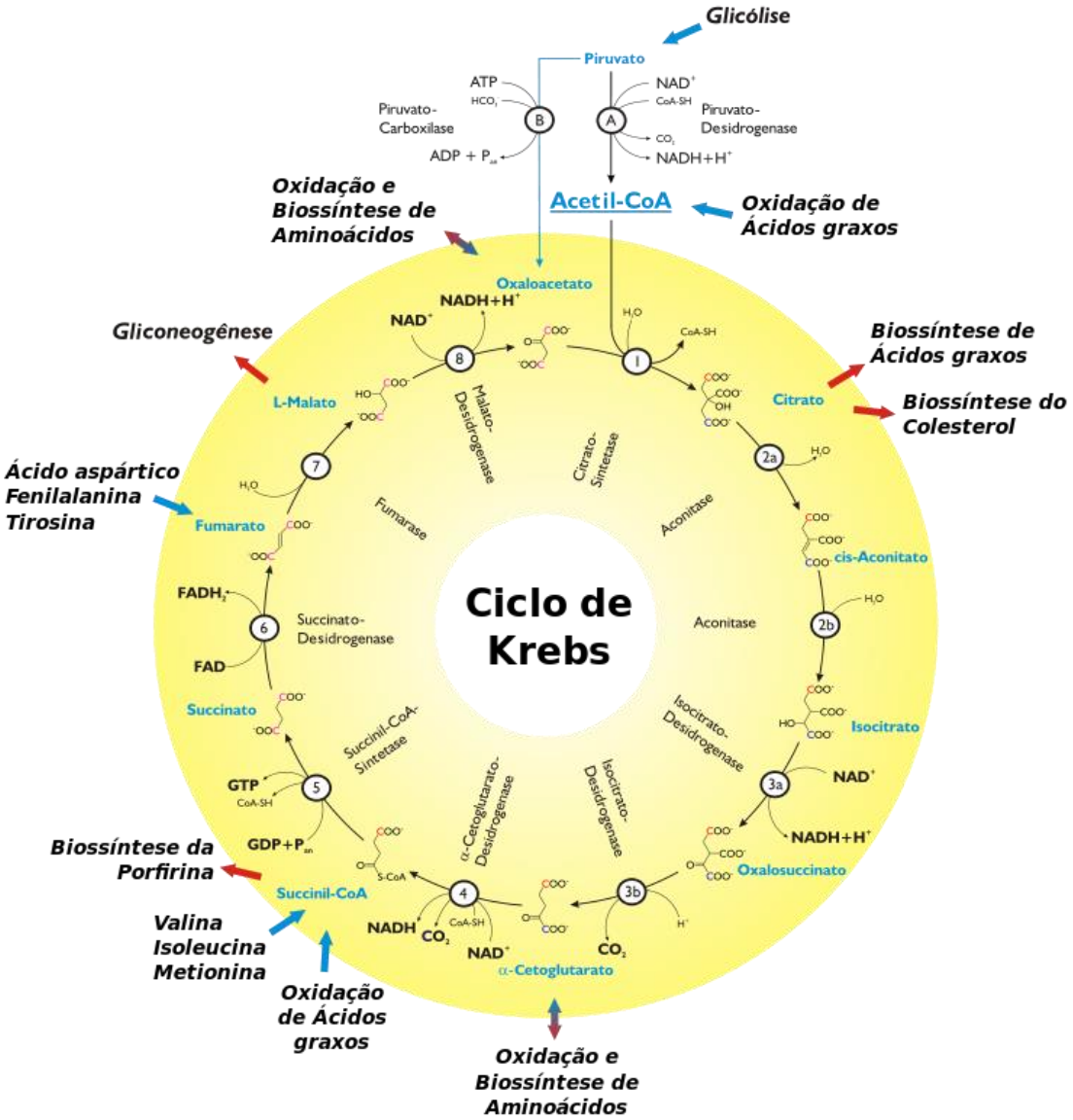

Figura 5 – Esquema demonstrando as etapas da via metabólica do ciclo de Krebs. Adaptado de Nelson e Cox (2008).

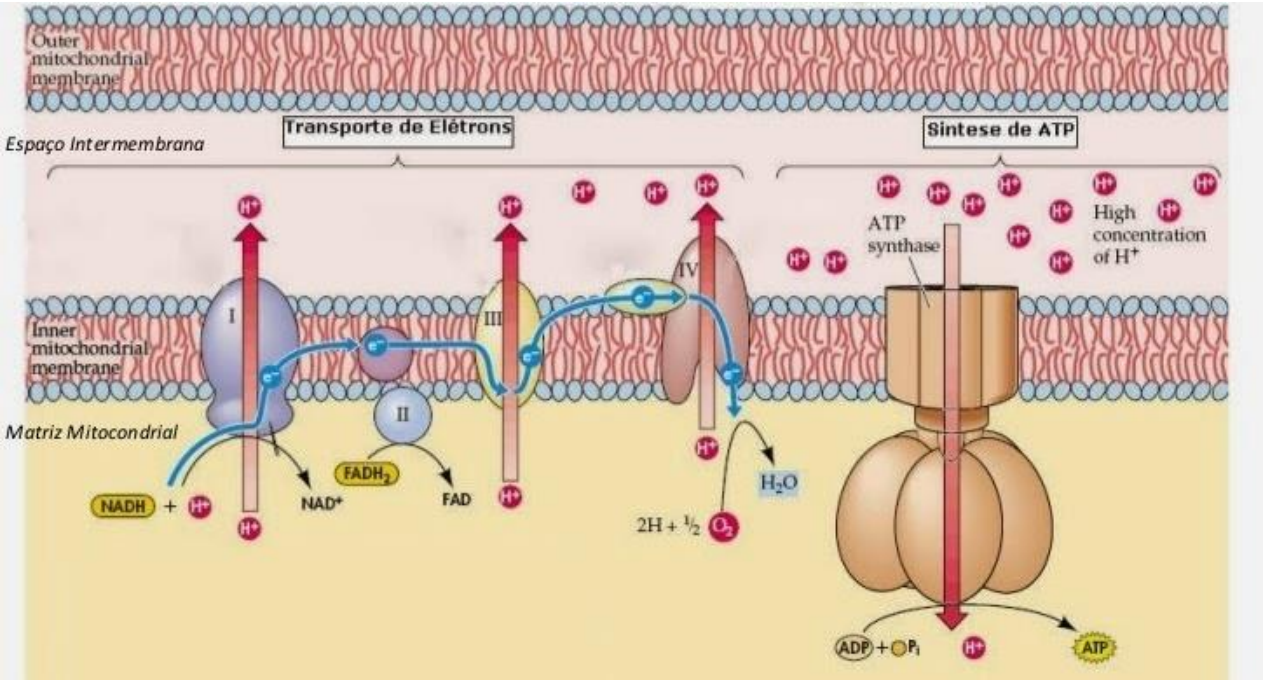

Figura 6 - Esquema demonstrando os complexos proteicos que constituem a cadeia transportadora de elétrons e a fosforilação oxidativa. Adaptado de Nelson e Cox (2008).

Os impactos provocados pelo rápido e intenso crescimento da sociedade humana não são vistos apenas em processos de contaminação ambiental. Por exemplo, a intensa liberação antrópica de gases como o CO_2, o metano, o óxido nitrito, os fluorocarbonos, o hexafluoreto de enxofre e os hidrofluorocarbonetos vem a décadas expandindo o efeito estufa natural do nosso planeta, culminando em um insidioso processo de aquecimento global (IPCC, 2014; Zebral et al 2019a). De fato, as projeções de aquecimento global médio feitas pelo *Intergovernmental Panel on Climate Change* para o fim do século 21 são de 2,6 a até 4,8 ºC (IPCC, 2014). Este dado tem gerado profundas preocupações na comunidade científica (Cook et al 2016), pois sabe-se que alterações térmicas desta magnitude podem provocar profundos impactos ambientais, econômicos

e sociais (IPCC, 2014). Somado a isto, ainda não conhecemos bem quais são os possíveis efeitos interativos que podem emergir entre o estresse térmico e outros estressores ambientais, como a poluição (Sokolova and Lannig, 2008).

De fato, os impactos negativos da elevação térmica em animais podem ser muito semelhantes a aqueles vistos para a poluição ambiental (Sokolova and Lannig, 2008; Zebral et al 2019a). Por exemplo, o aumento de temperatura pode levar a profundas alterações no metabolismo energético (Hochachka and Somero, 2002; Cherkasov et al 2006) como o objetivo de atender a elevada demanda energética resultante de um contexto térmico mais elevado (Sokolova and Lannig, 2008). Grande parte deste ajuste está relacionado ao aumento na produção aeróbica de energia via mitocôndria, processo conhecidamente responsável pela geração de ERO que contribuem grandemente para o estresse oxidativo nas células (Sokolova and Lannig, 2008). Interessantemente, sabe-se que o aumento de temperatura pode provocar alterações no estado oxidativo (Madeira et al 2013; Fonseca et al 2017) e induzir estresse (Madeira et al 2013; Wang et al 2018) e dano oxidativo (Zafalon-Silva et al 2017) em organismos aquáticos. Frente ao exposto, fica evidente que a elevação de temperatura e o cobre estão conectados pelos efeitos prejudiciais que podem causar à organismos aquáticos. Em resposta a isto, a literatura científica tem se demonstrado preocupada em relação à possíveis interações aditivas ou sinérgicas entre estes dois estressores (Sokolova and Lannig, 2008). Em outras palavras, a pergunta é: será que a coocorrência do estresse térmico provocado pelo aquecimento global irá potencializar os já tão prejudiciais efeitos causados pela contaminação ambiental?

O peixe *P. vivipara* (Fig.7), popularmente conhecido como barrigudinho ou killifish, é uma espécie pertencente à família Poeciliidae que se distribui ao longo de toda a costa brasileira, sendo facilmente encontrada nas margens de lagoas, rios e canais (Santos et al 2011). Assim como todos os poecilídeos, esta espécie possui uma estratégia reprodutiva vivípara, onde as fêmeas carregam seus embriões em uma estrutura semelhante a uma placenta até a eclosão. Estes animais possuem forte dimorfismo sexual, sendo as fêmeas maiores e menos coloridas. A fecundação nesta espécie é interna e é realizada por uma estrutura derivada da nadadeira anal chamada de gonopódio (Meredith et al 2011). *P. vivipara* apresenta diversas características que a torna uma espécie interessante para ser usada como modelo em experimentação animal. Por exemplo, *P. vivipara* consegue tolerar uma ampla faixa de salinidade e temperatura, além de ser também bem tolerante a condições de hipóxia. Além disto, estes animais podem ser facilmente mantidos e reproduzidos em cativeiro (Paulo et al 2012).

Figura 7 – Exemplar macho do peixe *Poecilia vivipara* em detalhe.

De fato, esta espécie vem sendo utilizada como organismo modelo em diferentes estudos com contaminantes químicos ambientais, incluindo o cobre, no âmbito do Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática (INCT-TA) (www.inct-ta.furg.br). Por exemplo, Zimmer et al (2012) avaliou o efeito da exposição por 96h ao cobre em *P. vivipara*, em água doce e salgada, e constatou uma inibição transiente na excreção de amônia sincronizada com uma também transiente inibição na atividade da enzima anidrase carbônica. Além disto, Harayashiki et al (2013) demonstrou que quando o peixe *P. vivipara* foi exposto a concentrações de até 0,7 mg/L de Roundup, diminuições na permeabilidade e integridade de membrana, na funcionalidade da mitocôndria, na integridade de DNA e na motilidade de esperma desta espécie foram observados. Semelhantemente, Machado et al (2013) constatou que *P. vivipara* exposto a concentrações de até 20 µg/L de cobre teve aumento na produção de ERO, de dano oxidativo, na atividade da CAT e da GST e uma redução na capacidade antioxidante total, sendo todos estes resultados encontrados em fígado, brânquias e músculo.

Ainda, uma série de 5 artigos demonstrou efeitos tóxicos do cobre (5 e 9 µg/L) após exposição crônica por 345 dias em *P. vivipara*. No primeiro destes trabalhos, Zebral et al (2018) mostrou que estes animais apresentaram desregulação endócrina do eixo somatotrópico, culminando em diminuição de crescimento. Após isto, foi demonstrado também que *P. vivipara* teve um aumento na acumulação tecidual de cobre e alteração na expressão das proteínas transportadoras (*atp7b*) deste metal (Anni et al 2019a). Na sequência deste trabalho, Anni et al (2019b) mostrou que este animal sofreu também uma inibição da LDH branquial e muscular, e na PK hepática. Além disto, foi mostrado

também uma elevação na atividade da CS hepática. Por fim, Zebral et al (2019) mostrou que machos de *P. vivipara* apresentaram uma diminuição em diversos parâmetros usados para a avaliação da qualidade espermática em peixes, evidenciando que exposição crônica ao cobre pode impactar a reprodução da espécie.

Frente ao exposto acima, o intuito desta tese foi de entender como a aclimatação em uma temperatura considerada elevada para a região dos Pampas (extremo sul do Brasil) poderia afetar a toxicidade do cobre para o peixe *P. vivipara*. Para tal, aclimatamos estes animais por 3 semanas a uma temperatura considerada normal (média anual para a região: 22 ºC) e uma temperatura considerada alta (média de verão somada à projeção de aquecimento global mais pessimista do IPCC: 28 ºC) e posteriormente expusemos os peixes por 96h a concentrações ambientalmente relevantes de cobre (9 e 20 µg/L). A primeira linha de avaliação realizada foi relacionada a determinação do estado oxidativo destes animais em fígado e braquiais, avaliados pela capacidade antioxidante total e dano oxidativo, assim como a determinação da acumulação de cobre nestes tecidos. Por fim, o *fitness* dos indivíduos foi avaliado pela determinação de seus limites térmicos (método da temperatura crítica máxima [CTMax]). Estes resultados compõem o artigo apresentado no capítulo 1, já publicado no periódico Aquatic Toxicology.

O seguimento dado a esta tese foi relacionado à avaliação do metabolismo energético nos animais experimentais. Nossa hipótese era de que a aclimatação em temperatura elevada iria demandar ajustes cinéticos neste processo que poderiam estar relacionados à um aumento na produção de ERO e estresse oxidativo. Para tal, avaliamos a atividade de enzimas pertencentes as

vias da glicólise (PK e LDH), Ciclo de Krebs (CS) e cadeia transportadora de elétrons (ETS) em fígado, brânquias e músculo. Os resultados deste segundo trabalho podem ser encontrados no capitulo 2 desta tese, formado por um manuscrito em revisão no periódico Chemosphere.

2. Objetivos

2.1. Objetivo geral

O objetivo geral desta tese foi avaliar se a aclimatação à temperatura elevada poderia levar a um aumento da toxicidade do cobre, usando o peixe *Poecilia vivipara* como animal modelo. Objetivamos também a compreensão de possíveis mecanismos fisiológicos relacionados ao processo citado.

2.2. Objetivos específicos

1) Avaliar a interação entre estresse térmico e exposição ao cobre na acumulação deste metal em fígado e brânquias de *P. vivipara*;

2) Estudar a interação entre estresse térmico e exposição ao cobre no estado oxidativo de *P. vivipara*, através da avaliação da capacidade antioxidante total (TAC e ACAP) e de dano oxidativo (TBARS) em fígado e brânquias;

3) Avaliar a interação entre estresse térmico e exposição ao cobre na tolerância térmica máxima de *P. vivipara*, determinada pelo método da temperatura crítica máxima (CTMax);

4) Estudar a interação entre estresse térmico e exposição ao cobre no Metabolismo energético de *P. vivipara*, avaliado em termos da

atividade de enzimas pertencentes às vias da glicólise (piruvato quinase e lactato desidrogenase), ciclo de Krebs (citrato sintase) e cadeia transportadora de elétrons (ETS) em fígado, brânquias e musculo.

3. Capitulo I

Este capítulo é representado pelo artigo "**Waterborne copper is more toxic to the killifish *Poecilia vivipara* in elevated temperatures: Linking oxidative stress in the liver with reduced organismal thermal performance**" já publicado no prestigiado periódico Aquatic Toxicology (DOI: https://doi.org/10.1016/j.aquatox.2019.02.005). O trabalho pode ser encontrado a partir da página seguinte.

Waterborne copper is more toxic to the killifish *Poecilia vivipara* in elevated temperatures: linking oxidative stress in the liver with reduced organismal thermal performance

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Abstract

In this study, we measured the interactive effect of temperature (22ºC and 28ºC) and waterborne copper (Cu) contamination (9µg/L and 20µg/L) on the killifish *Poecilia vivipara*. Endpoints analyzed included parameters involved in Cu-accumulation, antioxidant capacity (antioxidant capacity against peroxyl radicals [ACAP] and total antioxidant capacity [TAC]), oxidative damage (lipid peroxidation [LPO]) and upper thermal tolerance (critical thermal maximum [CTMax]). Results show that Cu hepatic accumulation was elevated in 28ºC in comparison to 22ºC in both exposure groups. For gills, this was true only in 20µg/L. Moreover, hepatic and brachial accumulation were concentration-dependent in both acclimation temperatures. Additionally, Hepatic ACAP and TAC were elevated in animals acclimated to 28ºC and only the animals kept at this temperature had reduced ACAP and TAC levels facing metal exposure (9 and 20µg/L). Similarly, the combination of elevated temperature and Cu exposure raised hepatic LPO levels. Finally, animals acclimated to 28ºC had higher CTMax levels in comparison to fish acclimated to 22ºC both in control and exposed animals, however, CTMax of contaminated fish were only reduced in comparison to control in animals kept at 28ºC. Concluding, we show that the physiological mechanism besides the potentiating effect of elevated temperature in Cu toxicity is related to higher hepatic and branchial metal accumulation and elevated oxidative stress in the liver, outlined by reduced antioxidant capacity and elevated oxidative damage. We also show that these outcomes lead to compromised organismal performance, characterized by reduced CTMax. Finally, it is concluded that Cu exposure in warmer periods of the year or within global warming predictions may be more hazardous to fish populations.

Keywords: Trace Metals; Ecotoxicology; Critical Thermal Maximum; Biomarker; Antioxidant system; Metal accumulation; Oxidative damage

Highlights

- *P. vivipara* acclimated to 28ºC and exposed to Cu showed elevated hepatic and branchial metal accumulation.

- Acclimation to elevated temperature in association to metal exposure reduced hepatic ACAP and TAC in *P. vivipara*.

- *P. vivipara* acclimated to 28ºC and exposed to Cu showed elevated hepatic LPO.

- Acclimation to elevated temperature in association to metal exposure reduced CTMax of *P. vivipara*.

- Exposure to Cu at elevated temperature leads to elevated oxidative stress and reduced organismal performance.

1. Introduction

Copper (Cu) is a trace metal commonly found in nature. It is an essential micronutrient to many organisms, acting as an enzymatic co-factor (Yu-Ting Lim et al. 2015). Also, this metal is needed for the correct functioning of many physiological traits, such as the mitochondrial respiratory chain (Mercer and Llanos 2003), the antioxidant system (Bopp et al. 2008; Leary et al. 2009) and the maintenance of enzymatic activity (Knight et al. 1994). Despite its natural occurrence in the nature, environmental contamination may cause elevation of waterborne Cu concentration. When present at elevated levels, this metal may accumulate in tissues (Machado et al. 2013; Silva et al. 2014) and produce toxic effects (Sokolova and Lannig 2008; Martins and Bianchini 2008) such as oxidative status imbalance (Machado et al. 2013; Ransberry et al. 2015; Braz-Mota et al. 2017), disturbances in energy metabolism (Lauer et al. 2012; Satyaparameshwar et al. 2006; Carvalho and Fernandes, 2008) and ionic and osmotic dysfunction (Craig et al. 2010).

Brazilian legislation stipulates maximum waterborne Cu concentrations of 5µg/L in saltwater and 9µg/L in freshwater (CONAMA, 2005). Even so, concentrations as high as 20µg/L has already been found in Brazilian estuarine zones (Barbosa 2012). These concentrations are usually considered low, even so, Cu toxic effects at these levels have already been shown. Zebral et al. (2018) showed that the killifish *P. vivipara* chronically exposed to 9µg/L Cu had reduced growth. Similarly, Machado et al. (2013) demonstrated that this fish species acutely exposed to the same metal concentration had elevated frequency of erythrocytic nuclear abnormalities and oxidative damage in the liver. Moreover, it

has already been demonstrated that exposure to 20µg/L Cu may inhibit ammonia excretion in *P. vivipara* (Zimmer et al. 2012). Similarly, exposure to 50µg/L may disrupt the oxidative status (liver and gills) of the killifish *Fundulus heteroclitus* (Ransberry et al. 2015). Therefore, Cu concentrations ranging from 9µg/L to 20µg/L may elicit sub-lethal toxicity for fish.

Not so different, temperature can also deeply affect fish physiology and survival (Brett, 1971). The reason for such intense effects relies on the fact that fish are ectotherms and, therefore, internal body temperature is similar to ambient water temperature (Somero, 2010). As consequence, physiological trait fitness is known to follow a bell-shaped curve, been elevated when ambient temperature is similar to optimal (Kellogg and Gift 1983; Handeland et al. 2008; Pang et al. 2010; Hoffmann et al. 2012). Despite that, ambient temperatures that are distant from optimal may lead to poor performance (Hoffmann et al. 2012). From this perspective, the notion that earth is passing through a warming process has been arousing deep concerns in the scientific community (Cook et al. 2016). The most recent projections for the elevation of mean global temperature made by the Intergovernmental Panel on Climate Change (IPCC) is of 2.6°C to 4.8°C by the end of 21st century (IPCC 2014). Mean water temperature of Rio Grande do Sul state (southern Brazil) is approximately 22°C, but considering IPCC projections, water temperature could get as high as 28°C in warmers periods of the year (INMET EMBRAPA; Garcia et al. 2004; IPCC 2014).

It is not difficult to understand why so many scientists are concerned about global warming. The empirical data showing that elevated temperature can be stressful to animals is vast (Calderon-Aguilera et al. 2012; Madeira et al. 2013; Gomiero and Viarengo 2014; Lee et al. 2014; Klein et al. 2016; Pang et al. 2016;

Zafalon-Silva et al. 2017; Fonseca et al. 2017; Hughes et al. 2018). Interestingly, heat stress may affect physiological traits in a similar manner to waterborne Cu exposure (Sokolova and Lannig 2008). For example, it is known that thermal stress leads to disturbances in the energy metabolism (Pörtner 2001; Pörtner 2002; Hochachka and Somero 2002; Cherkasov et al. 2006), alteration in the oxidative status (Madeira et al. 2013; Klein et al. 2016; Fonseca et al. 2017) and elevation of oxidative stress (Wang et al. 2018). Therefore, if thermal stress and Cu exposure are connected by the physiological mechanisms that they may affect, one can infer that the co-occurrence of these two stressors may lead to potentiated hazardous effects (Sokolova and Lannig 2008). In fact, many studies have already demonstrated that elevated temperature enhances Cu toxicity in terms of lethality (Smith and Heath, 1979; Nussey et al., 1996; Carvalho and Fernandes, 2006; Furuta et al., 2008), but the mechanism of this process is poorly understood. Even so, a few studies shed light upon the matter. For example, Braz-Mota et al. (2017) showed that the Amazonian fish *Hoplosternum littorale* exposed to elevated Cu concentrations (50 and 500µg/L) in association with high temperature displayed reduced survival time and raised oxidative stress. Also, Ravinder et al. (2015) demonstrated that the interaction of warm temperature (20ºC) and Cu exposure (20µg/L) altered the mitochondria functioning in the rainbow trout *Oncorhynchus mykiss*. With no doubt, these two studies were important advances to the field, nevertheless, a link between biochemical and non-lethal organismal endpoints is still lacking.

In light of the background described above, the objective of this study was to better understand the mechanisms related to the temperature-dependent elevation of Cu toxicity. To accomplish that, the interaction between two

acclimation temperatures (22ºC and 28ºC) and exposure to two waterborne Cu concentrations (9µg/L and 20µg/L) was evaluated in the killifish *P. vivipara*. We assessed physiological endpoints related to metal accumulation and oxidative status, and one organismal endpoint related to individual upper thermal tolerance. This fish species was indicated as a good experimental model by the Brazilian National Institute of Aquatic Toxicology (INCT-TA 2012) and has been successfully used in many ecotoxicological studies (Zimmer et al. 2012; Harayashiki et al. 2013; Machado et al. 2013; Machado et al. 2014; Silva et al. 2014; Torreiro-Melo et al. 2015; Zebral et al. 2018). This viviparous fish species can be easily found in the Atlantic coast of South America, occupying both freshwater and saltwater environments (Froese and Pauly, 2011). Females are usually bigger than males and less colorful. This hardy species can be easily maintained and reproduced under captivity (Froese and Pauly, 2011).

2. Materials and Methods

2.1. Animal collection and rearing

The *Poecilia vivipara* individuals used in this experiment (weight (g) = 0.33±0.13; length (cm) = 3.01±0.35) were obtained from the local stock of Universidade Federal de Rio Grande (FURG) aquatic bioterium. Fish were maintained in 20L plastic tanks containing continuously aerated water at fixed room temperature (25°C) and photoperiod (12h light: 12h dark cycle) being daily fed *ad libitum* with a commercial diet (Alcon Basic MEP 200 Complex; 45% crude protein, 5% lipids, 2% calcium, 0.7% phosphorus and 10% humidity). Animals

were transferred to the aquatic laboratory located at UFPel (Universidade Federal de Pelotas), where the experiments were performed. Animals were acclimated to laboratory conditions for 3 weeks in 38L aquariums filled with freshwater. Following acclimation period, animals were distributed among treatment groups, as described in experimental design section. During acclimation and experimental period, animals were maintained under constant aeration and controlled temperature (22ºC or 28ºC) and photoperiod (12h:12h). During acclimation period, fish were daily fed *ad libitum* with a commercial diet (Alcon Basic MEP 200 Complex; 45% crude protein, 5% lipids, 2% calcium, 0.7% phosphorus and 10% humidity). Water temperature, pH, NH3, NO_2^- and NO_3^- (Nutrafin test, Hagen, Mansfield, MA, USA) were daily measured.

2.2. Experimental design

Following acclimation period, animals were distributed among experimental units which consisted in the combination of two acclimation temperatures (22ºC or 28ºC) and three Cu exposure conditions (control, 9µg/L or 20µg/L). A concentrated Cu stock solution was prepared by addition of $CuCl_2$ to distilled water. Contamination of exposure media was performed by the dilution of the concentrated Cu stock solution in clear water at the experimental unit. Animals were transferred to experimental units following one day of tank contamination. Fish were exposed to the described conditions for 96h and were not fed during this time. Following exposure period, CTMax was individually obtained as described below. For CTMax validation, animals were returned to experimental condition and survival was observed for the following 24h. Fish

mortality was not observed. Following that, animals were anesthetized with a cold benzocaine solution and euthanized by spinal cord sectioning. Liver and gills were collected. Tissues were immediately stored in ultrafreezer (-70ºC) until accomplishment of the biochemical analysis described below.

2.3. Cu determination in water and biological samples

Filtered (0.45µm filter) and non-filtered water samples were daily taken for determination of dissolved and total Cu concentration in experimental media, respectively. Water samples were acidified with 65% HNO_3 (1% final concentration; SupraPur, Merck, USA). Cu concentration were also measured in liver and gills samples. In order to that, tissues were weighed wet, completely dried until constant weight and completely digested with 65% HNO_3 (SupraPur, Merck, Darmstadt, Germany) at 60°C for 24h. High purity deionized water (resistivity of 18.2 MΩ/cm) was employed to dilute samples and standard solutions. A standard (Cu) stock solution at 1 g/L (Merck, Darmstadt, Germany) was employed to prepare the standard solutions. Cu concentration in biological and water samples were analyzed using the Atomic Absorption Spectrometry with Graphite Furnace (HR-CS GF AAS, Analytic Jena, Germany). Data related to tissue metal accumulation are expressed using wet weight. The detection and quantification limits of this method was 0.017 and 0.050 µg, respectively. The quality assurance and quality control procedures for Cu quantification in water and biological samples were based on regular analysis of blanks and spiked matrices. Also, certified reference material (TORT-3 - Lobster Hepatopancreas; DORM-4 – Fish Protein; and SLRS-6 for water Reference Material for Trace

Metals; [National Research Council Canada, Ottawa, ON, Canada]) were analyzed as an additional quality assurance protocol. Certified reference material was processed following the same procedures adopted for sample analysis. Quality control procedures showed good agreement with the certified values, with recoveries ranging from 93.5 to 98.6%. All the quality control procedures were performed in triplicate.

2.4. Determination of total antioxidant capacity against peroxyl radicals (ACAP)

Total antioxidant capacity against peroxyl radicals (ACAP) was evaluated in *P. vivipara* liver and gills using the fluorimetric method (excitation: 485nm; emission: 520nm) described by Amado et al. (2009). In brief, samples were homogenized (1:5w/v) in homogenization buffer containing Tris-HCl (100mM, pH 7.75), EDTA (2mM) and $MgCl_2$ (5mM) and centrifuged (13,000g) at 4°C for 10min. The resulting supernatant was collected and total protein was adjusted to 1g/L and used for the analysis. A reaction buffer containing 30mM HEPES, 200mM KCl and 1mM MgCl2 (pH 7.2) was also used. The ACAP assay is based on the formation of ROS, in the form of peroxyl radicals, generated by the thermal decomposition of 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP, Sigma-Aldrich, USA). Similarly, the substrate 2′,7′- dichlorodihydrofluorescein diacetate (H2DCF-DA, Molecular Probes, USA) is also added to reaction solution. This compound is cleaved by esterases present in sample homogenates to form the non-fluorescent compound 2′,7′ dichloro- flurescein (H2DCF). The peroxyl radicals formed by ABAP further oxidizes H2DCF to the fluorescent compound

dichloroflurescein (DCF). Fluorescence measurements (excitation: 485nm; emission: 530nm) were made every 5 minutes for up to 40 minutes at 37°C using a fluorometer (Victor 2, Perkin Elmer, Waltham, MA, USA). ACAP was determined by the relative difference in ROS production of samples in the presence and absence of ABAP. Data were expressed as 1/relative area. Therefore, area differences are positively correlated with ACAP levels.

2.5. Determination of total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) was evaluated in *P. vivipara* liver and gills samples using the "OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit" (Cell Biolabs Inc., San Diego, CA, USA). As stated by the manufacturer, this kit is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. Net absorbance values of antioxidants are compared with a known uric acid standard curve. Absorbance values are proportional to the sample's total reductive capacity. This essay detects all classes of antioxidants, including thiols, with marginal radical interference. Absorbance readings were made in a microplate reader (ELx-800, Biotek, Winooski, VT, USA). Data were normalized by sample homogenates total protein content and expressed as mM copper reducing equivalents/mg protein.

2.6. Determination of lipid peroxidation (LPO)

Oxidative damage, measure as lipid peroxidation (LPO), was assessed in *P. vivipara* liver and gills according to the fluorimetric method described by Oakes and Van Der Kraak (2003). The 2-thiobarbituric acid reactive substances (TBARS) method evaluates the reaction between malondialdehyde (MDA), which is produced by lipids peroxidation, and thiobarbituric acid (TBA). The florescence produced by this reaction was measured (excitation: 515nm; emission: 553nm) using a fluorometer (Victor 2, Perkin Elmer,Waltham, MA, USA). The concentration of TBARS was calculated employing a standard curve built with tetramethoxypropane (TMP; Sigma-Aldrich, USA). Data were normalized by homogenates total protein and expressed as nmol MDA/mg protein.

2.7. Determination of critical thermal maximum (CTMax)

Following exposure period, animals CTMax was assessed. For this, fish were individually transferred to a 6L aquarium containing clean water (no metal addition) at the respective acclimation temperature (22ºC or 28ºC) as showed in Lutterschmidt and Hutchison (1997). Each evaluation lasted approximately half an hour, therefore, exposure to clear water during CTMax assessment are not expected to affect Cu toxicity. The temperature of the water present in the aquarium was elevated by an aquarium heater (100W) at the mean heating rate of 0.34±0.01ºC/min and was assessed by a digital thermometer (0.01°C). Fish were constantly observed and the experiment was finalized when animals displayed absence of the righting response (endpoint). At this point, the temperature was annotated as the animal's CTMax and the fish was transferred to its respective treatment condition (combination of acclimation temperature and

Cu exposure or acclimation temperature alone). The CTMax was validated by fish survival in the following 24h. In order to obtain homogeneous heating rates, water was constantly aerated. With the objective to minimize methodological bias, the observer could not see the water temperature that fish were experiencing neither know if the animal was from exposition or control group.

2.8. Statistical analysis

Data are expressed as mean ± standard error. Differences among treatment groups were assessed using analysis of variance (two-way ANOVA) or covariance (two-way ANCOVA, fish weight as covariance). Parametric assumptions were assessed. Residuals were evaluated to test if the experimental data had a normal distribution and if the variances were homogeneous using the Kolmogorov–Smirnov and Levene's tests, respectively. The independency of observations was also assessed by the Durbin-Watson test. If parametric assumptions were not met, data were mathematically (exponential) transformed and assumptions were tested again. Experimental groups differences were assessed by the Duncan *post hoc* test. In all cases, the significance level adopted was 95% ($\alpha = 0.05$). All statistical analyses were performed using the SigmaPlot 12.0 software (Systat, San Jose, CA, USA).

3. Results

3.1. Water parameters and Cu concentration in experimental media

The water parameters in experimental units were equal among groups. pH values for aquariums held at 22°C were 7.10 ± 0.06; 7.20 ± 0.03 and 7.20 ± 0.00 for control, 9µg/L and 20µg/L groups, respectively. pH values for aquariums held at 28°C were 7.20 ± 0.03; 7.20 ± 0.00 and 7.20 ± 0.03 for control, 9µg/L and 20µg/L groups, respectively. Nitrogen compounds were always below indicated safe levels for fish maintenance (NH_3<0.25 mg/L; NO_2^-<0.1 mg/L; NO_3^-<1.5 mg/L). Water temperature in control, 9µg/L and 20µg/L groups held at 22°C were 21.79 ± 0.15; 21.76 ± 0.11 and 22.02 ± 0.07, respectively. Water temperature in control, 9µg/L and 20µg/L groups held at 28°C were 28.15 ± 0.03; 28.00 ± 0.30 and 28.00 ± 0.15, respectively. Measured total and dissolved Cu concentrations in experimental media were similar to nominal values in both exposure groups (9µg/L and 20µg/L) evaluated (Tab.1).

Table 1 – Determined total and dissolved Cu concentration in control, 9µg/L and 20µg/L experimental media within each acclimation temperature (22ºC and 28ºC) (n=3).

Acclimation temperature	Nominal concentration	Measured concentration (µg/L)	
		Total	Dissolved
22°C	Control	0.73 ± 0.47	0.35 ± 0.28
	9µg/L	8.12 ± 0.93	5.51 ± 0.65
	20µg/L	19.57 ± 0.85	14.53 ± 0.45
28°C	Control	0.01 ± 0.06	0.08 ± 0.07
	9µg/L	7.88 ± 0.07	6.45 ± 0.81
	20µg/L	19.29 ± 1.23	15.69 ± 1.70

3.2. Cu accumulation

Significantly interactions between Cu concentration and acclimation temperature were seen for metal accumulation in the liver ($F_{2, 26}$=15.02, p<0.001) (Fig.1A). In this case, fish held at 28°C and exposed to 9µg/L or 20 µg/L had elevated levels of Cu content in comparison to animals held at 22°C. Conversely, acclimation temperature had no effect in hepatic Cu content of control animals (Fig.1A). Moreover, in comparison to control animals, fish exposed to 9µg/L and 20µg/L Cu had elevated levels of this metal accumulated in the liver for both acclimation temperatures tested. Similarly, animals exposed to 20µg/L also had elevated levels of hepatic Cu content in comparison to animals exposed to 9µg/L, in both acclimation temperatures (Fig.1A).

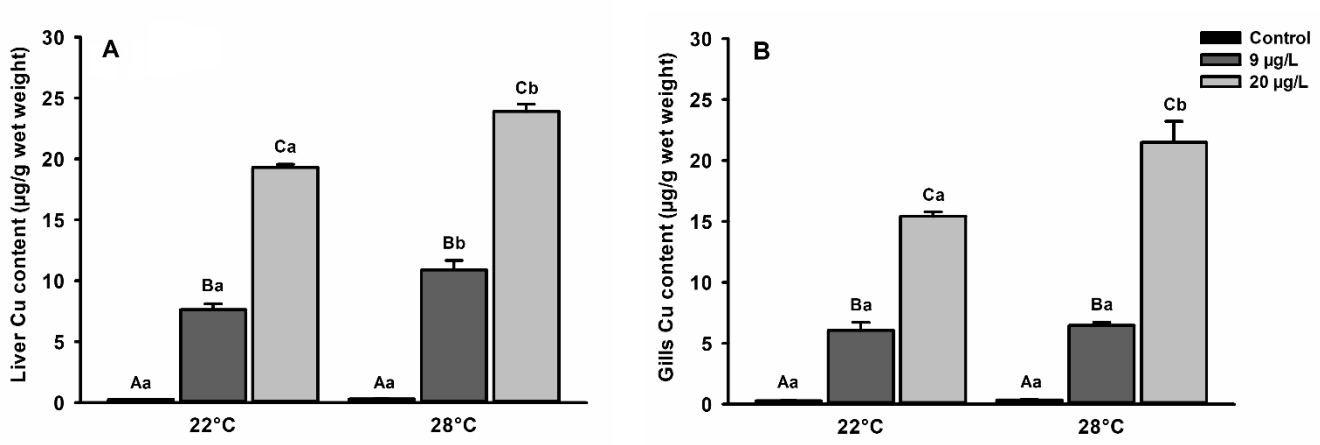

Figure 1 – Cu accumulation in the liver (A) and gills (B) of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences between copper concentrations within each acclimation temperature. Lowercase letters represent significant differences of each copper concentration between acclimation temperatures (two-way ANOVA; p<0.05; n= 5-6).

Similar results were observed for metal accumulation in the gills, where significant interaction was observed ($F_{2, 27}$=8.03, p<0.001) (Fig.1B). In comparison to control animals, fish exposed to 9µg/L and 20µg/L had elevated branchial levels of Cu in both acclimation temperatures tested. Also, acclimation temperature affected only the group exposed to 20µg/L, given that animals held at 28°C accumulated more Cu in the gills than animals held at 22°C. Moreover, animals exposed to 20µg/L had elevated branchial levels of Cu than animals exposed to 9µg/L, in both acclimation temperatures (Fig.1B).

3.3. Total antioxidant capacity against peroxyl radicals (ACAP)

A significant interaction between Cu concentration and acclimation temperature was observed for ACAP levels in the liver ($F_{2, 27}$=3.88, p<0.05). In this case, animals held at 28°C at control condition or exposed to 9µg/L had elevated ACAP levels in comparison to animals held at 22°C (Fig.2A). Conversely, acclimation temperature did not affect hepatic ACAP levels of animals exposed to 20µg/L (Fig.2A). Moreover, for fish kept at 28°C, Cu affected ACAP levels in a concentration-dependent manner, given that animals exposed to 20µg/L had this parameter reduced in comparison to fish exposed to 9µg/L. Moreover, both Cu concentrations decreased ACAP levels in comparison to control animals (Fig.2A). Conversely, for fish kept at 22°C, no significant differences were seen between exposed and control animals (Fig.2A). In the case of branchial ACAP levels, no significant interaction was seen ($F_{2, 21}$=0.16, p>0.05) (Fig.3A).

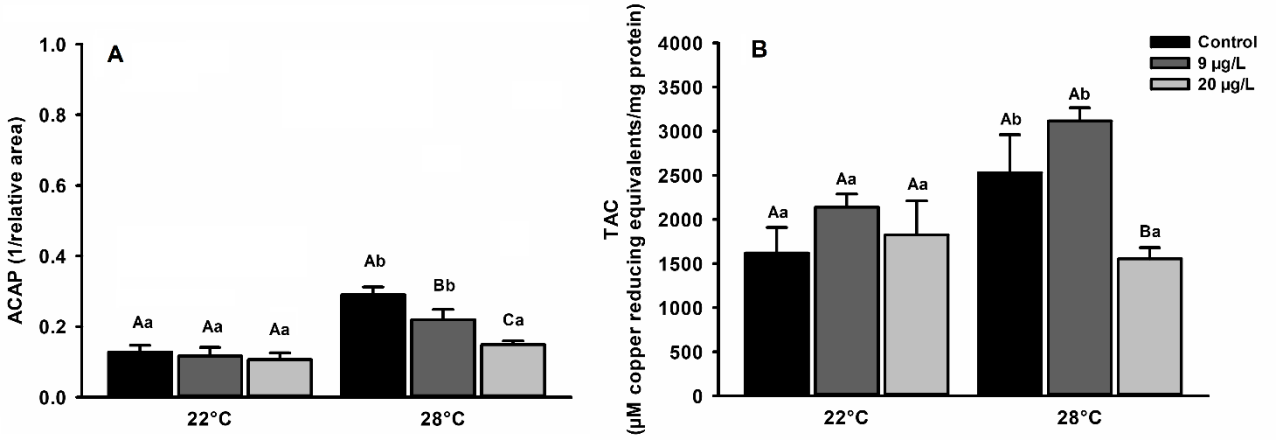

Figure 2 – Antioxidant capacity measured as ACAP (A) and TAC (B) in the liver of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences between copper concentrations within each acclimation temperature. Lowercase letters represent significant differences of each copper concentration between acclimation temperatures (two-way ANOVA; $p<0.05$; n=5-6).

3.4. Total antioxidant capacity (TAC)

Significant interaction between Cu concentration and acclimation temperature was observed for hepatic TAC levels ($F_{2, 26}=3.44$, $p<0.05$). In this case, animals held at 28°C in control conditions or exposed to 9µg/L had elevated TAC levels when compared to animals kept at 22°C. Conversely, acclimation temperature had no effect in fish exposed to 20µg/L (Fig.2B). Moreover, for fish kept at 28°C, exposure to 20µg/L reduced TAC levels in comparison to control and 9µg/L groups (Fig.2B). Differently, exposure to Cu did not have an impact in hepatic TAC levels of fish held at 22°C (Fig.3B). Similarly, acclimation

temperature neither Cu exposure affected the TAC levels in the gills. Therefore, no significant interaction was observed ($F_{2, 27}$=0.56, p>0.05) (Fig.3B).

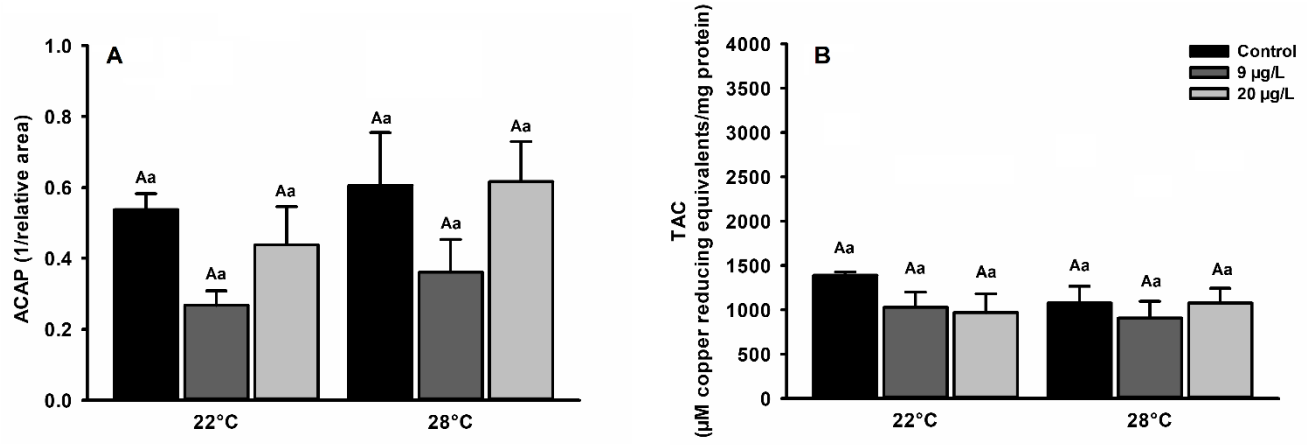

Figure 3 – Antioxidant capacity measured as ACAP (A) and TAC (B) in the gills of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences between copper concentrations within each acclimation temperature. Lowercase letters represent significant differences of each copper concentration between acclimation temperatures (two-way ANOVA; p<0.05; n=5-6).

3.5. Lipid peroxidation (LPO)

Significant interactions between Cu concentration and acclimation temperature was observed for hepatic LPO levels ($F_{2, 25}$=5.36, p<0.05) (Fig.4A). In this case, acclimation temperature only affected hepatic LPO level of exposed animals, given that fish held at 28°C and exposed to 9µg/L or 20µg/L had elevated levels of this parameter in comparison to animals held at 22°C.

Conversely, acclimation temperature did not affect hepatic LPO levels of control fish (Fig.4A). Moreover, for fish held at 28°C, exposure to 9µg/L or 20µg/L elevated LPO levels in comparison to control condition. Despite that, no significant differences were observed among exposure groups (Fig.4A). In the case of animals held at 22°C, effect of Cu exposure was not observed (Fig.4A). Similarly, acclimation temperature neither Cu exposure affected LPO levels in the gills and no significant interaction was observed ($F_{2, 27}$=1.19, p>0.05) (Fig.4B).

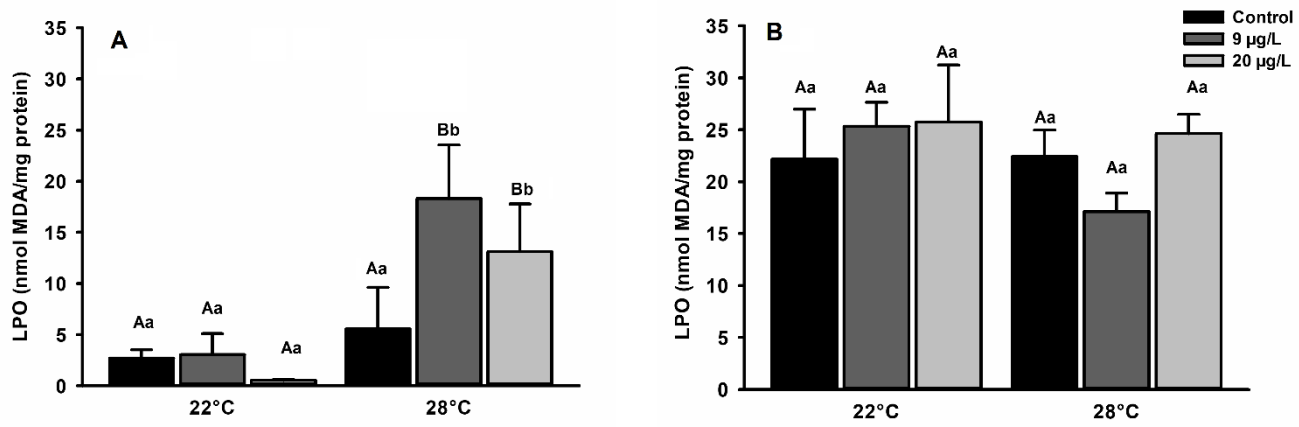

Figure 4 – Lipid peroxidation (LPO) in the liver (A) and gills (B) of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences between copper concentrations within each acclimation temperature. Lowercase letters represent significant differences of each copper concentration between acclimation temperatures (two-way ANOVA; p<0.05; n=5-6).

3.6. Critical thermal maximum (CTMax)

Significant interactions between Cu concentration and acclimation temperature was observed, given that metal exposure only affected the CTMax of animals held at 28°C ($F_{2, 54}$=3.70, p<0.05) (Fig.5). In this case, exposure to 9µg/L or 20µg/L Cu reduced fish CTMax in comparison to control animals. Despite that, no significant differences were observed between the two exposure groups (Fig.5). Significant effects of acclimation temperature were also observed, given that fish held at 28°C had a higher CTMax in comparison to animals kept at 22°C, both in control and exposed groups (Fig.5).

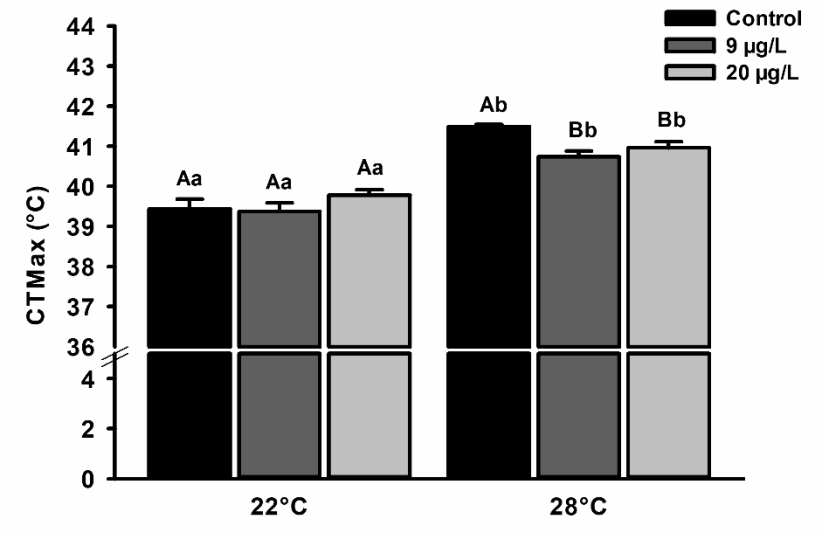

Figure 5 - CTMax of *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences between copper concentrations within each acclimation temperature. Lowercase letters represent significant differences of each copper concentration between acclimation temperatures (two-way ANCOVA; p<0.05; n=10-12 weight as covariate).

4. Discussion

4.1. Metal accumulation

We show in the present study that exposure to Cu lead to metal accumulation in liver and gills. Similar results have also been found by Silva et al. (2014), which demonstrated that *P. vivipara* acclimated to freshwater and exposed to 20µg/L Cu had elevated levels of this metal in the same tissues. Moreover, the present work also showed that animals acclimated to elevated temperature accumulated more metal. This interesting result can be explained by the direct effect of increased temperature in the elevation of Cu bioavailability, caused by higher proportion of metal free ions and solubility (Worms et al. 2006). Furthermore, elevated temperature may also indirectly affect fish predisposition to accumulate Cu. As ectotherms, fish body temperature is extremely similar to ambient water temperature (Guderley 2004). As consequence, temperature elevation leads to heighten metabolic state (in species that do not aestivate) and oxygen demand (Bagnyukova et al. 2007), which is attended by ventilation rate uprisen (Willmer et al. 2000; Pörtner 2001; Pörtner 2002). Therefore, in higher temperatures, fish have more branchial contact with contaminated water, leading to elevated tissue Cu accumulation.

4.2. The interaction between waterborne Cu and thermal stress enhances oxidative stress in the liver

In the present work, we show that acclimation to 28ºC elevated the hepatic levels of ACAP and TAC in comparison to fish acclimated to 22ºC. Similar results

have also been found by other authors. Madeira (et al. 2013) showed that several fish species had elevated muscular levels of catalase and glutathione S-transferase activities following heat stress. Similarly, the fish yellow perch acclimated to elevated temperature had higher levels of superoxide dismutase activity (Grasset et al. 2016). Interestingly, we could only observe raised ACAP and TAC levels in control fish and animals exposed to 9µg/L Cu, whereas exposure to 20µg/L Cu inhibited the temperature-dependent elevation in this parameter. This is a concerning result, given that enhanced antioxidant capacity is a physiological adjustment made by fish to cope with the enlarged ROS production resulted from elevated temperature (Lushchak and Bagnyukova 2006; Lushchak 2011), in order to avoid oxidative damage (Toyokun 1999; Lushchak 2011). This is an important result to be considered, given that waterborne Cu concentrations similar to 20µg/L are often found in the environment. In this sense, Cu contamination may reduce fish capacity to deal with oxidative stress related to global warming. Likewise, we also show that fish acclimated to 28ºC and exposed to Cu had reduced ACAP and TAC hepatic levels in comparison to control animals, nonetheless, this was not observed for fish held at 22ºC. This result show that the interaction between elevated temperature and Cu exposure overwhelm *P.vivipara* antioxidant system, leading to depletion of total antioxidant capacity. Similar results have also been found by Braz-mota et al. (2016). These authors demonstrated that the fish *Hoplosternum littorale* acclimated to 34ºC and exposed to 50µg/L Cu had elevated hepatic ROS production and reduced hepatic total antioxidant capacity (measured as ACAP) in comparison to control animals acclimated to 34ºC or 28ºC.

It was observed in our study that exposure to 9µg/L and 20µg/L Cu enlarged LPO levels in the liver of fish kept at the higher temperature when compared to control animals. This is an interesting result, given that it was in this experimental group that some of the highest TAC and ACAP hepatic levels were observed. Therefore, it is clear that the temperature-dependent rise in antioxidant system of animals kept at 28ºC was not sufficient to avoid Cu-induced oxidative stress in the liver. Furthermore, it is important to note that hepatic LPO levels were also affected by acclimation temperature, given that animals kept at 28ºC and exposed to both Cu concentrations showed increase in this parameter in comparison to animals maintained at 22ºC. Despite that, hepatic LPO levels of control fish were not affected by acclimation temperature. This exciting result indicates that the physiological adjustments made by control animals to deal with increased temperature, in terms of hepatic antioxidant system, were sufficient to avoid oxidative damage. Contrarily, exposed animals could not avoid oxidative damage facing thermal stress.

Taken together, it is clear that the interaction between elevated temperature and Cu exposure leads to oxidative status imbalance with hepatic oxidative damage as consequence. Interestingly, two other studies have reached similar conclusions (Braz-mota et al. 2016; Grasset et al. 2016). Therefore, we argue that oxidative imbalance caused by the association of metal exposure and elevated temperature is a physiological mechanism of toxicity that may be shared by a broad range of fish species, but more studies are needed in order to confirm this hypothesis.

We also evaluated the interaction effects between acclimation temperature and Cu exposure in *P. vivipara* gills. Of all biomarkers evaluated, we could only

observe significant differences in tissue accumulation. Ransberry et al. (2015) had similar results. In this study, it was demonstrated that the killifish *Fundulus heteroclitus* acclimated to freshwater and exposed to Cu (50µg/L and 200µg/L) had elevated metal accumulation in the gills. Despite that, branchial antioxidant enzymes activity (CAT and SOD) and carbonylated protein concentration were unaltered by metal exposure (50µg/L Cu). Taken together, these results demonstrate that killifish gills are less responsive than the liver, at least in terms of oxidative status.

4.3. Deleterious effect at the organismal level: reduction of critical thermal maximum (CTMax)

We show that exposure to Cu only reduced the CTMax of animals acclimated to the higher temperature tested. However, fish acclimated to 28°C had larger CTMax than animals acclimated to 22°C, either for control or exposed fish. At first, this result may seem contradictory, but it is widely known that animals kept for sufficient time at high temperature exhibits CTMax elevation (Layne and Claussen 1982; Lutterschmidt and Hutchison 1997; Ribeiro et al. 2012; Bilyk et al. 2012). Consequently, this otherwise paradoxical result actually shows that fish acclimated to elevated temperature were able to cope with metal exposure and rise its thermal limits, nevertheless, the degree of such response was not performed at the same extension of control fish. This is a concerning result, knowing that thermal limit plasticity is consider to be an important adaptive mechanism of animals to deal with circadian and seasonal environmental changes (Somero 2005; Lagerspetz 2006; Hoffmann et al. 2012) and that thermal

limit constraint is thought to be one of the main causes of species extinction facing global warming (Hoffmann et al. 2012). Moreover, given that CTMax has been considered as a reliable way of measuring the individual performance of aquatic (Becker and Genoway 1979; Lutterschmidt and Hutchison 1997) and terrestrial (Ribeiro et al. et al. 2012) animals, the results presented in our study indicate that metal exposure at 28ºC constrained not only fish ability to deal with thermal stress, but also reduced the overall fitness of the animals.

In accordance to our results, Cu-dependent reduction in fish CTMax has also been demonstrated by Lydy and Wissing (1988). These authors showed that the fishes *Etheostoma flabellare* and *Etheostoma nigrum* exposed to elevated concentrations of this metal (50µg/L-292 µg/L) had reduced CTMax. Other studies also demonstrated that metal exposure can reduce this parameter (Paladino and Spotila 1978; Becker and Wolford 1980; Poulton et al. 1989; Rosas and Ramirez 1993). As far as we known, these are the only studies that assessed fish CTMax in an ecotoxicological context. Despite that, this parameter has been extensively used in field (Layne and et al. 1987; Wood et al. 2016) and laboratory ecological studies (Sherman and Levitis 2003; Denisse et al. 2012; Madeira et al. 2013). Therefore, we argue that CTMax has been neglected by ecotoxicologists despite been a very practical and almost inexpensive methodology that could be used as an organismal pollution biomarker, as demonstrated by the present and previous studies. Therefore, we encourage other authors to start using CTMax as a pollution biomarker in field and laboratory ecotoxicological studies.

5. Conclusion

Findings reported in the present study shows that acclimation to elevated temperature rises Cu toxicity to the killifish *P. vivipara*. It is demonstrated that the physiological mechanism besides such effect is related to augmented metal accumulation and oxidative stress, characterized by diminished total antioxidant capacity and elevated oxidative damage. It is also demonstrated that this effect is tissue-specific and is more evident in the liver. Moreover, the present study also showed that Cu exposure constrained fish ability to rise its thermal limits facing acclimation to elevated temperature. This is of particular importance, considering that fish have to tolerate daily and seasonal thermal stress imposed by the environment. Also, constrained thermal limits are considered to be a major cause of species extinction facing global warming. Therefore, it is concluded that Cu exposure in warmer periods of the year or expected in global warming projections may cause disruption of oxidative status and lead to reduced thermal tolerance of fish, jeopardizing its populations. Finally, we would like to stress the fact that the Cu concentrations and the acclimation temperatures tested here are ecologically relevant and expected to occur in the environment.

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4. Capitulo II

O segundo capítulo desta tese é representado pelo manuscrito "**Combining elevated temperature with waterborne copper: impacts on the energy metabolism of the killifish *Poecilia vivipara***" em revisão no periódico Chemosphere. A versão submetida do trabalho pode ser encontrada a partir da página seguinte.

Combining elevated temperature with waterborne copper: impacts on the energy metabolism of the killifish *Poecilia vivipara*

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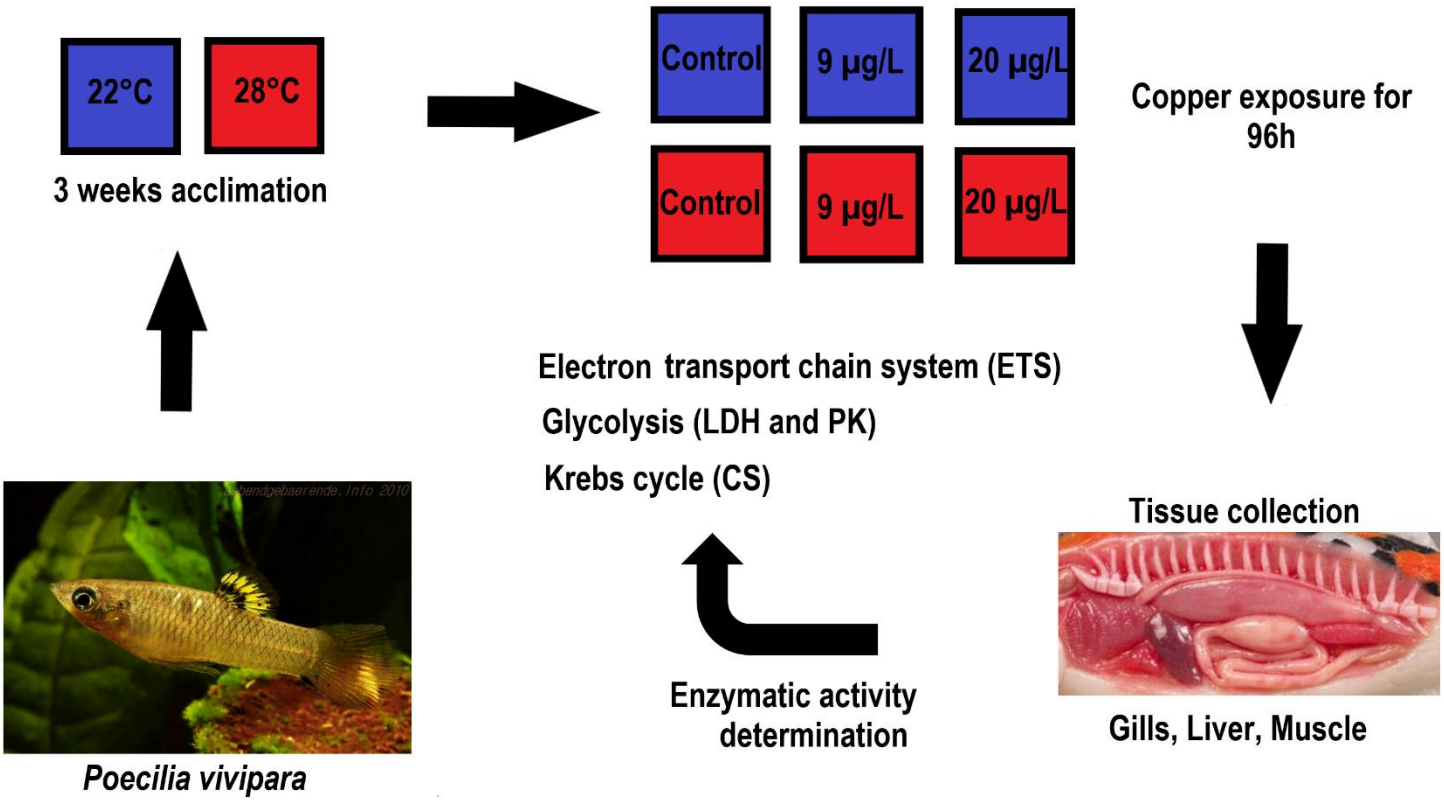
Abstract

We have previously demonstrated in a companion work that acclimation to 28ºC potentiated waterborne copper (Cu) toxic effects in *Poecilia vivipara* through oxidative stress-related processes. In the present study, we hypothesized that these results were related to kinetic metabolic adjustments in enzymes from aerobic and anaerobic pathways. To test this, *P. vivipara* was acclimated to two temperatures (22ºC or 28ºC) for three weeks and then exposed to Cu (control, 9 or 20µg/L) for 96h. The activity of enzymes from glycolysis (pyruvate kinase [PK] and lactate dehydrogenase [LDH]), Krebs cycle (citrate synthase [CS]) and the electron transport chain system (ETS) were assessed in gills, liver and muscle. Interactive effects were only seen for hepatic LDH activity, as both metal exposure and heat stress, combined or not, inhibited this enzyme, showing a suppression in anaerobic pathways. Conversely, a Cu main effect was present in the liver, expressed as an elevation in ETS activity, showing an enhancement in hepatic aerobic metabolism likely related with the very energy-demanding process of metal detoxification. Moreover, this study shows that *P. vivipara* has a remarkable ability to compensate heat stress in terms of energy metabolism, as we could not observe acclimation temperature effects for most of the cases. Nonetheless, a tissue-dependent effect of elevated temperature was observed, as we could observe an inhibition in muscular CS activity. Finally, it is concluded that kinetic adjustments in terms of the energy metabolism are not related with the temperature-dependent elevation of Cu toxicity in *P. vivipara* as we previously hypothesized.

Keywords: Trace Metals; Ecotoxicology; Enzymatic activity; Biomarker; Heat stress; Energy metabolism

Graphical abstract



Highlights

- Interaction of 28ºC and Cu inhibited hepatic anaerobic metabolism (LDH inhibition)

- Cu raised hepatic aerobic metabolism, as ETS activity was elevated (main effect)

- 28ºC inhibited muscular aerobic metabolism, as CS activity was reduced (main effect)

- All the other enzymes were not affected in gills, liver and muscle

- *P. vivipara* can efficiently compensate for thermal stress in terms of enzymatic rate

1. Introduction

The first industrial revolution markedly changed human society. Following this period, agrarian settlements rapidly transformed into highly populated cities and large industrial complexes. Accompanying this process, environmental contamination was on the way to become an issue. At the present time, pollution is considered to be one of the most serious problem that humanity and other life forms have to face (Muralikrishna and Manickam, 2017). As an answer, ecotoxicology has emerged as an important scientific field aiming to identify, track and reduce the insidious impact of contaminants and pollutants. One example of this are copper ions (Cu). This metal is an essential micronutrient required by all life forms, playing important roles in cellular homeostasis (Belyaeva et al 2011). Despite that, Cu can exert toxicity if present in elevated concentrations, especially in aquatic environments. For example, Cu can induce oxidative imbalance (Ransberry et al 2015; Braz-Mota et al 2017; Zebral et al 2019a), disrupt the endocrine system (Zebral et al 2018) and inhibit enzymatic activity, leading to disturbances in ionic and osmotic regulation (Craig et al 2010), and energy metabolism dysfunction (Fonseca at al 2019; Anni et al 2019a; Anni et al 2019b).

In light of the above mentioned, Brazilian legislation permits maximum concentrations of 5 and 9 µg/L of this metal in fresh and saltwater environments, respectively (CONAMA, 2005), but concentrations as high as 20 µg/L Cu has already been found in Brazilian estuaries (Barbosa, 2012). Despite been considered as low, Cu at concentrations of 5 and 9 µg/L has already been shown to be toxic for fishes. For example, the killifish *P. vivipara* exposed for 345 days to 9 µg/L Cu had reduced growth and endocrine disruption (Zebral et al 2018),

elevated metal accumulation in diverse tissues (Anni et al 2019a), altered energy metabolism (Anni et al 2019b) and reproductive alterations (Zebral et al 2019b). Similarly, the same experimental model also exposed to 9 µg/L Cu, but for 96h, had elevated frequency of erythrocytic nuclear abnormalities and disrupted hepatic oxidative status (Machado et al 2013). Similarly, Ransberry et al (2015) showed that the killifish *Fundulus heteroclitus* exposed to 50 µg/L and 200 µg/L Cu had elevated metal accumulation and alterations in the antioxidant system in gills and liver. So, even though permitted, Cu concentrations ranging from 5 to 9 µg/L, or unpermitted concentrations close to 20 µg/L, are known to exert sub-lethal toxicity in fishes.

The rapid human society development following industrial revolution also lead to an unprecedent elevation in the emission of greenhouse gases, such as CO2, methane and nitrous oxide (Zebral et al 2019c). These three gases are major contributors to the anthropogenic-related expansion of earth's greenhouse effect and consequent heating that is driving global warming (Meinshausen et al 2009; Cook et al 2016). In fact, the Intergovernmental Panel on Climate Change (IPCC) projected an increment of 2.6°C to 4.8°C by the end of this century (IPCC 2014). With that in mind, aquatic environments of Rio Grande do Sul, located at southern Brazil, may shift from 22ºC (mean annual temperature) to closely 28ºC in summer periods. The scientific community face these facts with deep concern, as is it widely known that thermal stress can induce major impacts in animals' physiology (Hochachka and Somero 2002; Zafalon-Silva et al 2017; Fonseca et al 2017; Wang et al 2018). Interestingly, the negative impacts of elevated temperature are similar to those elicited by Cu exposure. For example, heat stress may induce oxidative stress (Madeira et al. 2013; Fonseca et al 2017;

Wang et al 2018) and energy disturbances (Hochachka and Somero 2002; Cherkasov et al. 2006). With that in mind, it has been hypothesized that global warming could act as a potentiator of the insidious effect exerted by environmental pollution (Sokolova and Lannig 2008). Interestingly, some studies have been confirming this hypothesis. For example, we have showed in a companion work that acclimation to elevated temperature potentiated the toxic effects of Cu in terms of metal accumulation and hepatic oxidative stress in *P. vivipara*, leading to reduced organismal fitness (assessed by CTMax) (Zebral et al 2019b). Similarly, Braz-mota et al (2016) showed that the fish *Hoplosternum littorale* acclimated to elevated temperature and exposed to 50 and 500 µg/L had raised oxidative stress and reduced survival. Moreover, Sappal et al (2015) demonstrated that the trout *Oncorhynchus mykiss* exposed to 20 µg/L Cu in elevated temperature had altered mitochondria functioning. These studies represent a nice bulk of evidence showing that global warming is in fact very likely to potentiate Cu toxic effects, but the unveiling of the physiological mechanisms besides this is still lacking.

Therefore, the main objective of the present work was to test the hypothesis that metabolic adjustments were related to the potentiated effect of heat stress in Cu toxicity previously showed by ourselves (Zebral et al 2019a), Braz-mota et al (2016) and Sappal et al (2015). In order to do that, two acclimation temperatures (22^{o}C and 28^{o}C) and two waterborne Cu concentrations (9µg/L and 20µg/L) were combined, and possible interactive effects were assessed in the killifish *P. vivipara*. We assessed the rate of enzymes from glycolysis (pyruvate kinase [PK] and lactate dehydrogenase [LDH]), Krebs cycle (citrate synthase [CS]) and the electron transport chain system (ETS) in gills, liver and muscle. *P.*

vivipara is a poecilid that can be found in lakes, rivers and brackish environments throughout the Atlantic coast of South America (Froese and Pauly, 2011). This hardy species can be easily breed and kept under diverse laboratory conditions and can be used as an efficient experimental animal (INCT-TA 2012). Indeed, *P. vivipara* has already been applied in diverse studies aiming to evaluate pollution hazardous effects (Harayashiki et al. 2013; Machado et al. 2013; Zebral et al. 2018; Anni et al 2019a; Anni et al 2019b; Zebral 2019a; Zebral 2019b).

2. Materials and Methods

2.1. Experimental animals

The animals used in this work (weight (g) = 0.33±0.13; length (cm) = 3.01±0.35) were kindly donated by the Instituto de Ciências Biológicas (ICB) located at Universidade Federal de Rio Grande (FURG). At this location, fish were kept in plastic aquariums (20L) at constant temperature (25°C), continuously aerated water and photoperiod of 12h light: 12h dark. Animals were fed *ad libitum* with commercial fish ration (Alcon Basic). Following donation, fish were transferred to the aquatic laboratory of Universidade Federal de Pelotas (UFPel), where the experiment was conducted. In this new location, fish were acclimated during 3 weeks in aquariums filled with 38L of freshwater. Following that, animals were attributed to each Cu treatment that will be described in the experimental design section. In fish acclimation and Cu exposure periods, animals were kept at the constant temperatures of 22ºC or 28ºC, in continually aerated freshwater, under the photoperiod of 12h light: 12h dark. Fish were fed

as stated above. Water parameters such as pH, temperature and nitrogen compounds (NO_3, NO_2 and NH_3) were measured daily.

2.2. Experimental design

At the end of acclimation period (3 weeks), animals from each temperature group were divided among three Cu exposure conditions (control, 9 µg/L or 20 µg/L). Contamination of exposure media was performed by dilution of a concentrated Cu stock solution (prepared with $CuCl_2$) in clean water directly at the experimental unit. Fish were only transferred to experimental aquariums following one day of tank contamination. Animals were maintained in the described conditions for 96h, without being fed. Following exposure period, animals were humanly euthanized by spinal cord sectioning after being anesthetized with cold benzocaine. Finally, liver, gills and muscle were immediately collected and rapidly stored in ultrafreezer (-80ºC) until accomplishment of the enzymatic analysis described below.

2.3. Biochemical analysis

2.3.1. Sample preparation

Following collection, tissues were homogenized in 150 ul imidazole buffer (50mM, pH 7.8) with 0.1mM PMSF and centrifuged for 20 min (10,000g, 4ºC). The obtained supernatant was used as enzymatic source in the procedures described below. For all cases, analyses were performed by spectroscopy in a

microplate reader (ELx808IU, BioTek Instruments, Inc, Winooski, VT, USA) as described by Lallier and Walsh (1991) and Lannig et al (2003). Final enzymatic activity was normalized by each sample total protein content (Bradford Reagent, Sigma, St. Louis, MO, USA) and expressed as enzyme units (U) /mg total protein.

2.3.2. Citrate synthase assessment

CS activity was assessed as stated by Lallier and Walsh (1991). In this method, the consumption of DTNB is quantified by absorbance elevation at 412 nm. Assay was performed in DTNB (0.1mM); oxaloacetate (0.5mM) and acetylcoenzyme A (0.3mM). The volume of homogenate used was 5 ul for all evaluated tissues.

2.3.3. Electron transport system assessment

The assessment of ETS activity was adapted from Lannig et al (2003). Briefly, this method quantifies the reduction of iodonitrotetrazolium chloride by the progressive increase in absorbance at 490 nm. Enzymatic assay was performed with Triton X-100 (0.20 %), sodium phosphate at pH 8.5 (0.1 M), NADH (0.85 mM), NADPH (125µM) and iodonitrotetrazolium chloride (2 mM). The volume of homogenate used was 30 ul for all analyzed tissues.

2.3.4. Lactate dehydrogenase assessment

LDH activity assessment was performed accordingly to Lallier and Walsh (1991). This assay is based in absorbance reduction at 340 nm following NADH oxidation. Reactions were performed with pyruvate (2mM), NADH (0.12mM) and imidazole at pH 7.4 (0.2M). The volume of homogenate used was 10 ul for gills and liver, and 1 ul for muscle.

2.3.5. Pyruvate kinase assessment

PK activity assessment was performed as described by Lallier and Walsh (1991). This method is based in NADH oxidation and consequent absorbance reduction at 340 nm. Reactions were performed with L-LDH (20 units); ADP (2.5mM); $MgCl_2$ (10mM); KCl (30mM); NADH (0.12mM) and phosphoenolpyruvate (0.5mM). The volume of homogenate used was 5 ul for all analyzed tissues.

2.4. Cu determination in water samples

In order to determine Cu concentration in exposure media, water samples were daily collected. Filtered (0.45µm) samples were used for assessment of dissolved concentration and non-filtered samples were used for determination of total metal concentration. Immediately after collection, samples were acidified to 1% final concentration (SupraPur, Merck, USA). Standard solutions and samples were diluted with deionized water (high purity; 18.2 MΩ/cm resistivity). Standard solutions were prepared with a stock solution at 1 g/L (Merck, Darmstadt,

Germany). Analyses were performed with the Atomic Absorption Spectrometry with Graphite Furnace methodology (HR-CS GF AAS, Analytic Jena, Germany).

2.5. Statistical analysis

The numerical values showed in this study were expressed as mean ± standard error. For the accomplishment of this study, the factorial analysis of variance (two-way ANOVA) was used in order to assess possibly differences between experimental treatments. In order to test the data normal distribution behavior and variance homogeny, residuals were evaluated using the Kolmogorov–Smirnov and Levene's tests, respectively. Observation independency was also assessed by the Durbin-Watson test. Whenever parametric assumptions were not met, data were log-transformed and assumptions were re-tested. Statistical differences were assessed by the Fisher *post hoc* test. When no interactions were observed, main effects were discussed (Quinn and Keough, 2002). The level of statistical significance adopted in this study was 95% ($\alpha = 0.05$).

3. Results

3.1. Water parameters and determined metal concentrations

The water parameters in experimental units were similar among groups. For aquariums held at 22°C, pH values were 7.13 ± 0.12; 7.23 ± 0.06 and 7.21 ± 0.02 for control, 9 µg/L and 20 µg/L groups, respectively. For aquariums held at

28°C, pH values were 7.23 ± 0.06; 7.23 ± 0.06 and 7.21 ± 0.01 for control, 9µg/L and 20µg/L groups, respectively. In the case of water temperature, values were 22.16 ± 0.29; 22.30 ± 0.76 and 22.33 ± 0.28, for control, 9µg/L and 20µg/L groups held at 22°C, respectively. Water temperature in control, 9 µg/L and 20 µg/L aquariums held at 28°C were 28.83 ± 0.28; 28.50 ± 0.50 and 28.83 ± 0.29, respectively. The Cu concentration in experimental media is expressed in Tab.1.

Table 1 – Determined total and dissolved Cu concentration in control, 9µg/L and 20µg/L experimental media within each acclimation temperature (22ºC and 28ºC) at the beginning of the experiment (n=3).

Acclimation temperature	Nominal concentration	Measured concentration (µg/L)	
		Total	Dissolved
22°C	Control	0.3±0.2	0.2±0.2
	9µg/L	8.4±0.5	5.9±0.6
	20µg/L	18.5±0.4	16.3±0.4
28°C	Control	0.3±0.0	0.2±0.0
	9µg/L	8.4±0.3	7.2±0.8
	20µg/L	18.3±0.7	16.7±1.7

3.2. Enzymatic activity in the gills

Significantly interactions between the tested factors were not observed for any enzyme evaluated in the gills (Figs.1.C; 1.F; 2.C and 2.F). Similarly, acclimation temperature and metal exposure main effects were not observed in this tissue (Figs.1.A; 1.B; 2.D and 2.E).

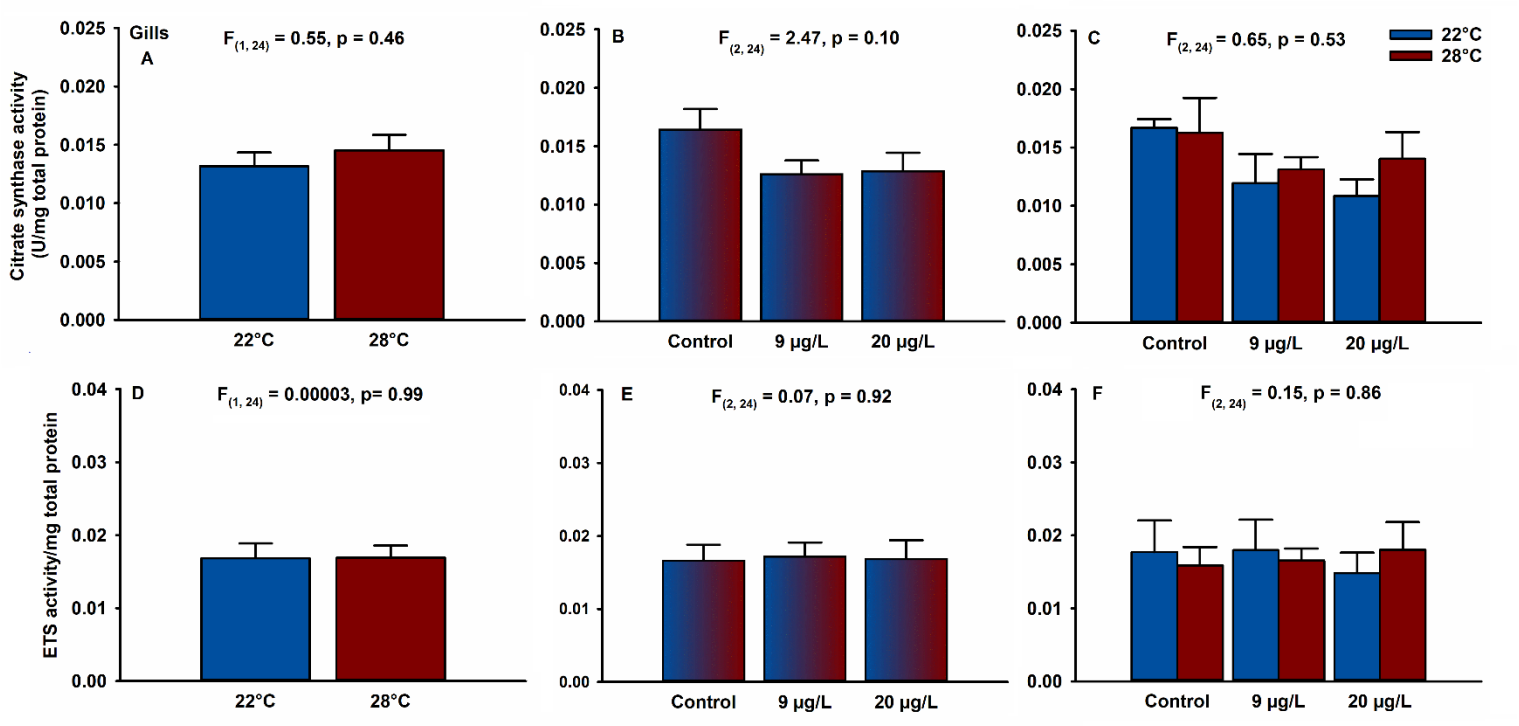


Figure 1 – Citrate synthase (A, B, C) and electron transport chain system (D, E, F) activity in gills of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22°C and 28°C) and control group. A and D) acclimation temperature main effect. B and E) Cu exposure main effect. C and F) Interactive effect. Data are expressed as mean ± standard error. No significant differences were observed (two-way ANOVA; p>0.05).

3.3. Enzymatic activity in the liver

No significant interactions were seen for CS, ETS and PK activities in the liver (Figs.3.C; 3.F and 4.F). Conversely, acclimation temperature and Cu exposure had a significant interaction in LDH activity, as all experimental groups were reduced in comparison to the control group (no metal addition) kept at 22ºC (Fig.4.C).

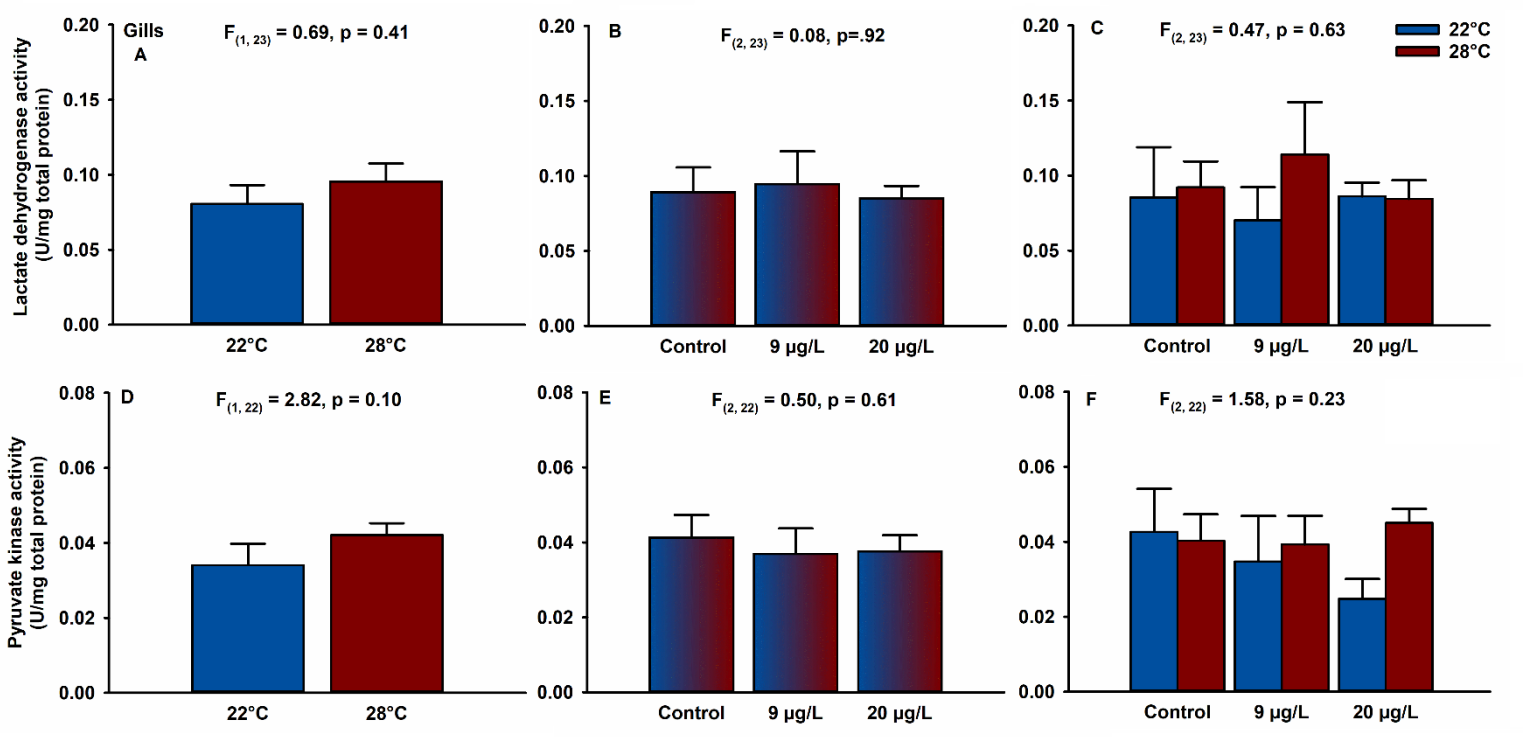


Figure 2 – Lactate dehydrogenase (A, B, C) and pyruvate kinase (D, E, F) activity in gills of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. A and D) acclimation temperature main effect. B and E) Cu exposure main effect. C and F) Interactive effect. Data are expressed as mean ± standard error. No significant differences were observed (two-way ANOVA; p>0.05).

A main effect of metal exposure was observed for the case of ETS hepatic activity, as both exposure concentrations elevated this endpoint in comparison to control group. Despite that, differences between metal groups were not observed (Fig.3.E). In opposition to that, acclimation temperature main effects were not observed for hepatic ETS activity (Fig. 3.D). Similarly, main effects of acclimation temperature and Cu concentration were not observed for CS (Figs. 3.A and 3.B) and PK activities (Figs. 4.D and 4.E). Finally, it was possible to observe main effects of both factors tested in LDH hepatic activity (Figs. 4.A and 4.B), but these results will not be considered, as a strong interaction was also observed (Quinn and Keough, 2002).

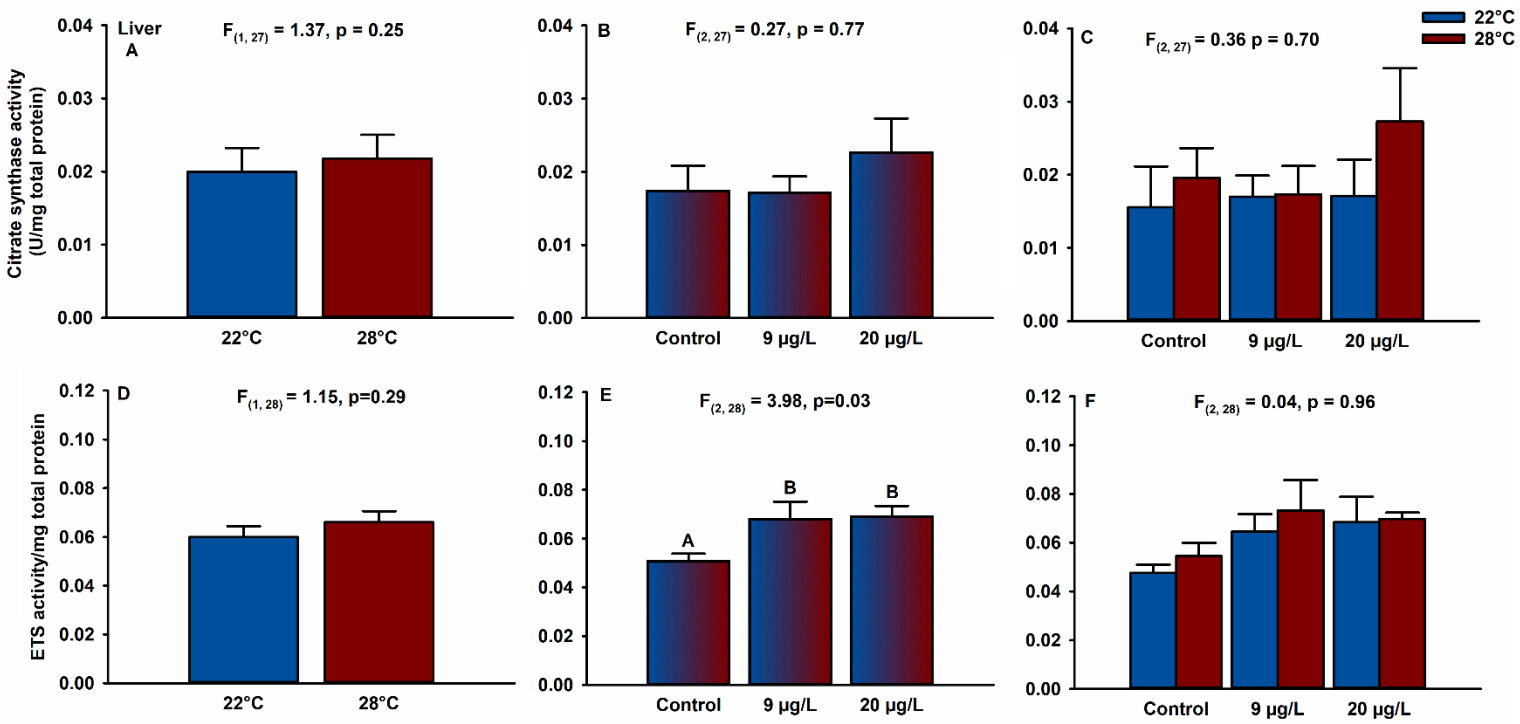


Figure 3 – Citrate synthase (A, B, C) and electron transport chain system (D, E, F) activity in liver of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control

group. A and D) acclimation temperature main effect. B and E) Cu exposure main effect. C and F) Interactive effect. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences (two-way ANOVA; $p<0.05$).

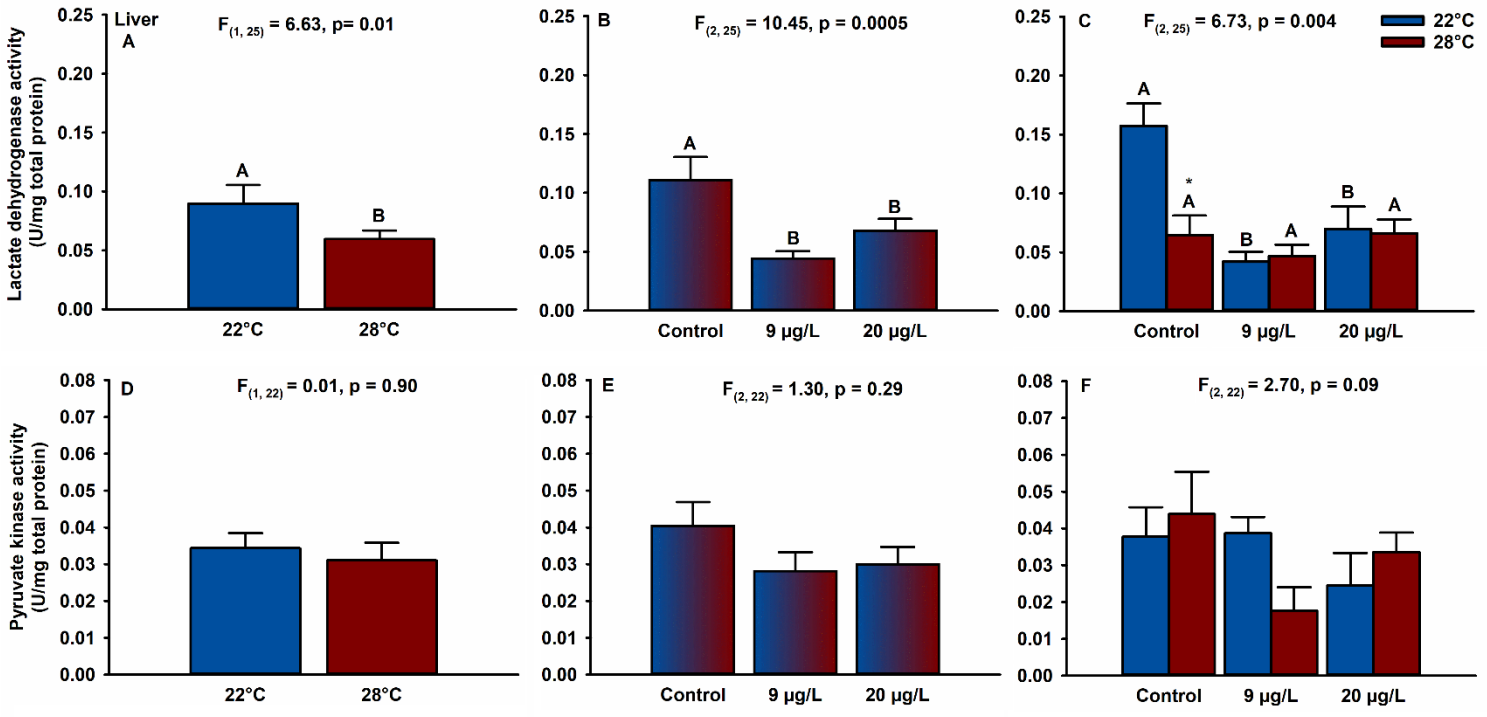


Figure 4 – Lactate dehydrogenase (A, B, C) and pyruvate kinase (D, E, F) activity in liver of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. A and D) acclimation temperature main effect. B and E) Cu exposure main effect. C and F) Interactive effect. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences between copper concentrations within each acclimation temperature. The asterisk represents significant differences of each copper concentration between acclimation temperatures (two-way ANOVA; $p<0.05$).

3.4. Enzymatic activity in the muscle

Significant interactions were not observed for any enzyme assessed in the muscle (Figs. 5.C; 5.F; 6.C and 6.F). Similarly, main effects were not observed for ETS (Figs. 5.D and 5.E), LDH (Figs. 6.A and 6.B) and PK (Figs. 6.D and 6.E) muscular activities. Also, main effects of metal exposure were not observed for CS activity in this tissue (Fig. 5.B). Conversely, it was possible to observe an acclimation temperature main effect, as the elevated temperature reduced this endpoint in comparison to 22ºC group (Fig. 5.A).

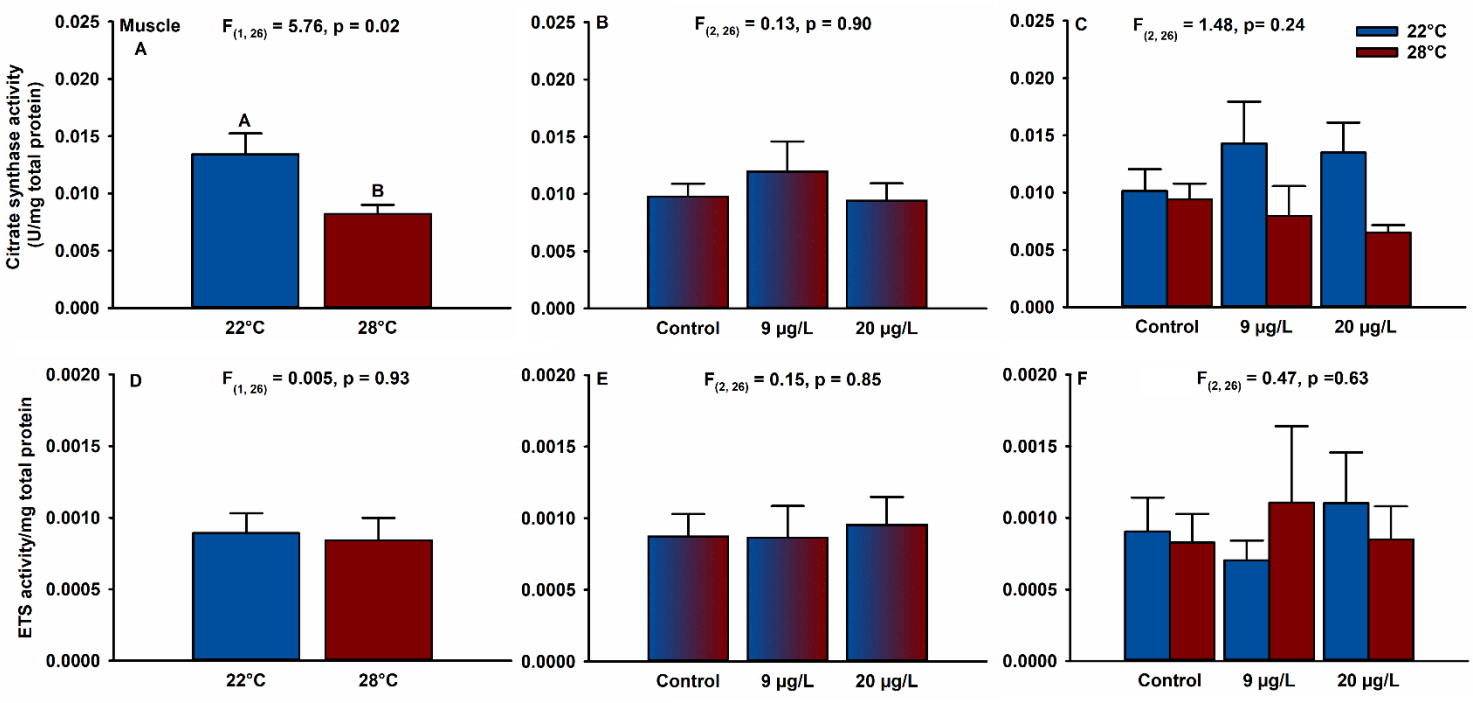


Figure 5 – Citrate synthase (A, B, C) and electron transport chain system (D, E, F) activity in muscle of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control

group. A and D) acclimation temperature main effect. B and E) Cu exposure main effect. C and F) Interactive effect. Data are expressed as mean ± standard error. Distinct uppercase letters represent significant differences (two-way ANOVA; $p<0.05$).

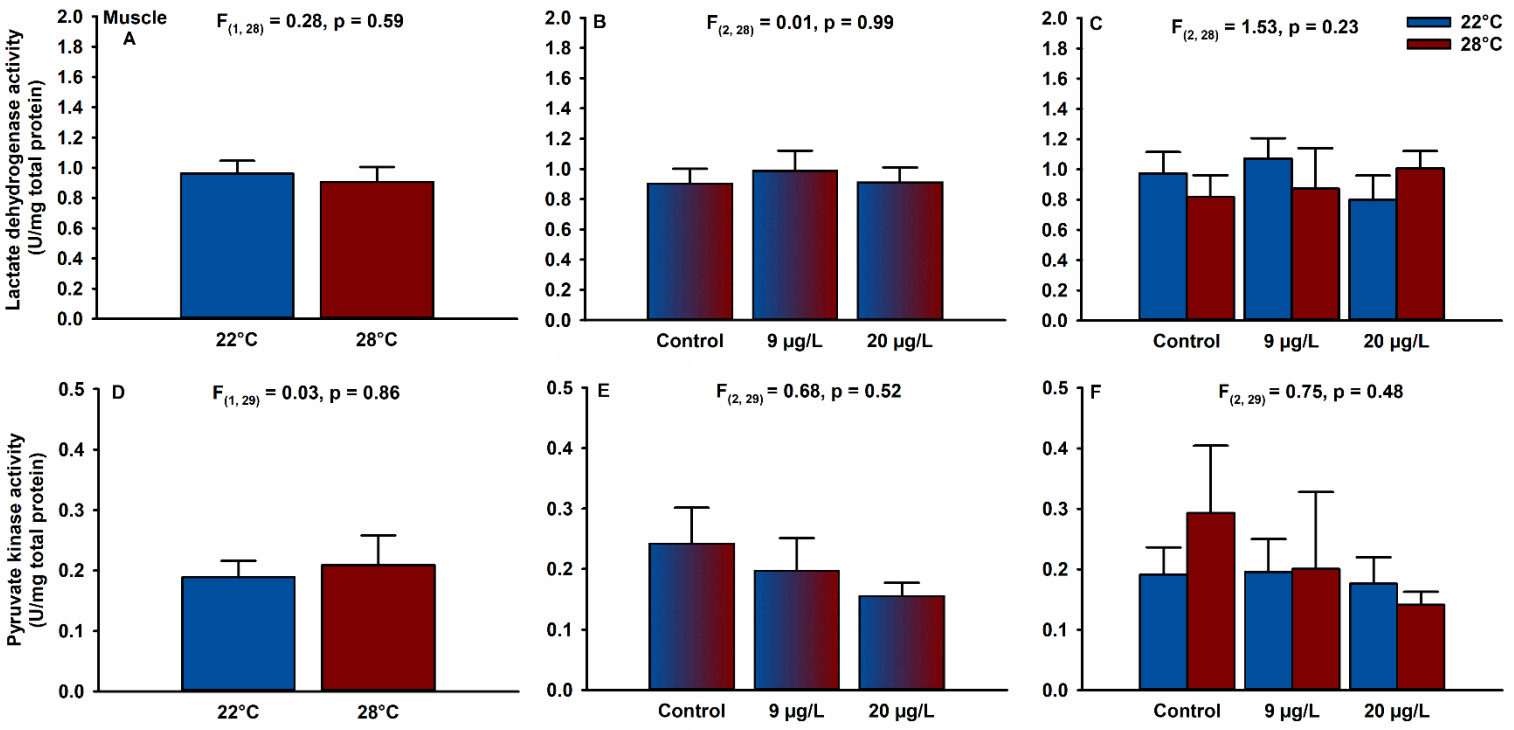


Figure 6 – Lactate dehydrogenase (A, B, C) and pyruvate kinase (D, E, F) activity in muscle of the killifish *Poecilia vivipara* exposed to copper (9µg/L and 20µg/L) in two distinct acclimation temperatures (22ºC and 28ºC) and control group. A and D) acclimation temperature main effect. B and E) Cu exposure main effect. C and F) Interactive effect. Data are expressed as mean ± standard error. No significant differences were observed (two-way ANOVA; $p>0.05$).

4. Discussion

4.1. Why should we look at the energy metabolism?

We have previously demonstrated in a companion paper that elevated temperature (28°C) associated with Cu exposure induced combined negative effects in *P. vivipara.* These impacts were related to reduced total antioxidant capacity and elevated oxidative damage in the liver, both leading to reduced organismal fitness in terms of upper thermal tolerance (Zebral et al 2019a). Interestingly, Braz-mota et al (2016) found similar results, as the fish *Hoplosternum littorale* acclimated to high temperature (34°C) and exposed to waterborne Cu (50 µg/L) also showed elevated production of reactive oxygen species (ROS) and reduced total antioxidant capacity in the same tissue. In light of that, we tested the hypothesis that acclimation to elevated temperature would demand energy metabolism adjustments that could be related to the elevated ROS production and oxidative stress shown in these previous studies, as it is widely known that these toxic molecules are majorly produced in the energy metabolism, especially in aerobic pathways.

4.2. Combined effects were only observed for hepatic LDH: evidence of suppressed anaerobic metabolism

In regard to our hypothesis, we were expecting that Cu exposure and elevated temperature would have interactive effects in the activity of the tested enzymes, especially those related to hepatic aerobic metabolism, but contrarily to this initial thought, we could only observe interactive effects for LDH in the liver,

as elevated temperature and metal exposure both markedly reduced this enzyme activity with similar intensity. It is interesting to note that this inhibition was not potentiated when these stressors occurred together, as one could have expected. In this regard, it is possible to observe that this interactive effect was actually specifically related to control animals kept at 22ºC, as this was the only experimental group that did not experienced an inhibition in this enzyme. This bimodal behavior of LDH demonstrates a suppressed anaerobic energy production in the liver when *P. vivipara* faced the stressful conditions of heat stress and Cu exposure, both alone or combined.

Similar suppressive effect of Cu exposure in LDH has already been shown by other studies, for example, Anni et al (2019b) demonstrated that *P. vivipara* exposed for 345 days to this metal (5 µg/L and 9 µg/L) showed inhibited LDH activity in gills and muscle. Similarly, Antognelli et al (2003) found that the fish *Sparus auratus* also had this enzyme inhibited in liver and muscle following exposure to Cu concentrations ranging from 100 to 500 µg/L. Similarly, the estuarine fish *Pomatoschistus microps* showed reduced muscular LDH activity when exposed to 25 µg/L Cu for 96h (Vieira et al 2009) and the goldfish *Carassius auratus gibelio* exposed to 100 µg/L for 3 days had reduced activity of this enzyme in the gills. Indeed, it is widely known that LDH is exceptionally sensitive to Cu presence, being inhibited both by ROS-related processes or not (Pamp et al 2005).

On the other hand, the effect of temperature on LDH is not as reproducible. For example, Jesus et al (2018) showed that elevated temperature (+3ºC) raised this enzyme muscular activity in the fish *Squalius carolitertii*, but the same degree of heat stress inhibited LDH activity in *Squalius torgalensis*. Similarly, four months

acclimation to 28ºC reduced whole-body activity of this enzyme in comparison to animals kept at 24ºC in the killifish *Nothobranchius furzeri* (Philippe et al 2019). For the case of the fish *Symphysodon aequifasciatus*, 30 days acclimation at 31ºC increased whole-body activity of this enzyme in comparison to animals kept at 28ºC (younis et al 2018). Similarly, Younis (2015) showed that long-term (14 weeks) acclimation to warmer temperatures (increments of 4; 8; 12 and 16ºC) enhanced LDH activity in the fish *Oreochromis aureus* white muscle and liver. Conversely, when the coral *Mussismilia harttii* was acclimated to 26.6 or 27.3°C, it was possible to observe LDH Inhibition in comparison to animals kept at 25ºC (Fonseca at al 2019).

4.3. Cu raised ETS activity in the liver: evidence of an enhanced aerobic metabolism due to detoxification

Moving forward, it is interesting to note that the suppressive effect observed in hepatic anaerobic metabolism was partially counterbalanced as both Cu exposure concentrations led to elevated ETS activity in this tissue, demonstrating an enhanced aerobic metabolism. This reflects the metabolic adjustments made by the liver in order to cope with the highly energy-demanding process of metal detoxification (Kuo et al 2006; Uren Webster et al 2014; Silva et al 2014). Interestingly, comparable results were obtained when *P. vivipara* was chronically exposed (345 days) to similar Cu concentrations (5 and 9 µg/L). Anni et al (2019b) showed that this fish species had elevated CS activity in the liver and up-regulated *atp5a1* mRNA expression in the same tissue, further supporting the idea that Cu exposure enhances hepatic aerobic metabolism. Conversely, we

could not observe any main effect of metal exposure in hepatic CS activity. This interesting result shows that the many aspects of the hepatic aerobic metabolism responds to Cu in a time-dependent manner, as ETS-related processes seems to be affected earlier than those related to Krebs Cycle. In accordance, Anni et al (2019b) showed that exposure to Cu for 28 days was not enough to elicit any effects in *P. vivipara* hepatic CS activity but, unfortunately, ETS activity was not assessed.

4.4. Poecilia vivipara shows a tissue-specific pattern of temperature compensation on enzymes related to energy metabolism

In light of the results obtained in this study, we reached at the unexpected conclusion that an increment of 6ºC in the acclimation temperature, performed throughout 3 weeks, was not enough to produce major alterations in most of the analyses performed. One can find these results quite paradoxical at first sight, as it is widely known that temperature has major implications for biochemical reactions and, due to Arrhenius equation, an increment in enzymatic activity might have been expected. Despite that, as brilliantly stated by Barcroft (1934) ‘‘...nature has learned so to exploit the biochemical situation as to escape from the tyranny of a single application of the Arrhenius equation.’’ In other words, animals can activate physiological mechanisms aiming to surpass the direct physical influence of temperature over biochemical reactions, what is coined as temperature compensation. With that in mind, Precht (1958) proposed 5 different patterns for this process. In the first of them, called overcompensation, animals reduce their enzymatic activities facing thermal stress. The second pattern is

called perfect compensation and occurs when animals are able to maintain the same enzymatic rate regardless of thermal conditions. The third, partial compensation, and fourth, no compensation, are very similar and occur when animals are not capable of elevating enzymatic activities in colder conditions. Finally, in the last case, called inverse compensation, animals reduce their enzymatic activities below to what would be expected as a sole effect of colder regimes. As we consider Precht (1958) classifications, it is clear that *P. vivipara* has a remarkable ability to perfectly compensate the effects of heat stress for most of the enzymes assessed, specially PK, as no acclimation temperature effect was observed. These results are probably related to the elevated capacity of acclimatization and acclimation showed by this species. Indeed, *P. vivipara* is known to be a very eurythermic and euryhaline fish, fact that allows this species to display a massive distribution area that extends for practically the whole South American Atlantic coast (Froese and Pauly, 2011).

In spite of the elevated thermal compensation ability of *P. vivipara*, we could still observe a clear tissue-specific pattern in terms of activity modulation for some of the assessed enzymes. For example, in the case of hepatic LDH and muscular CS, *P. vivipara* actually overcompensated the effect of thermal stress, as these enzymes were inhibited in animals acclimated to 28^{o}C. These interesting results shows that muscle and liver responded to heat stress in opposite directions. In the case of the liver, *P. vivipara* partially suppressed anaerobic pathways, conversely, in the muscle this effect was actually seen in an aerobic pathway. It is still early to say if this result has any functional role in the process of *P. vivipara* acclimation to elevated temperature, but this topic certainly deserves further evaluation.

4.5. Energy metabolism adjustments does not seem to participate in the temperature-dependent elevation of Cu toxicity

As already discussed above, we refuted our hypothesis that energy metabolism kinetic adjustments following acclimation to elevated temperature were related to the potentiated Cu toxic effect observed by ourselves (Zebral et al 2019a) and Braz-mota et al (2016). This is likely related to the fact that *P. vivipara* showed to be exceptionally efficient in compensating the effects of acclimation temperature in terms of enzymatic activity, moreover, whenever an effect was present, it was directed towards an enzymatic inhibition. Nonetheless, we still believe that for the case of species that displays a poor temperature compensation, the energy metabolism may still be an important source of ROS facing heat stress. Certainly, it would be interesting to replicate this experiment using a stenothermic species known to weakly compensate for elevated thermal regimes.

5. Conclusion

This work hypothesis was that the already stablished effect of elevated temperature as a potentiator of Cu toxicity was related to metabolic adjustments in terms of metabolic enzyme activity. Contrary to our initial thoughts, we could only observe interactive effects of acclimation temperature and Cu exposure for hepatic LDH activity, as both metal exposure and heat stress, combined or not, inhibited this enzyme. The fact that no additive or synergistic effect was observed

points out to the fact that this enzyme is regulated in a bimodal fashion, one expressed at normal conditions and the other expressed at stressed conditions, when *P. vivipara* displayed a suppression in anaerobic pathways. Moreover, the present study also showed that exposure to both Cu concentrations (9 and 20 µg/L) enhanced the aerobic metabolism in the liver, expressed as an elevation in ETS activity. We conclude that this represents a metabolic adjustment made in to deal with the very energy-demanding process of metal detoxification. Also, findings reported in this study shows that *Poecilia vivipara* has a remarkable ability to compensate the effects of heat stress in terms of the rate of enzymes from the energy metabolism, as for most of the cases we could not observe an effect of acclimation temperature. Finally, it is concluded that kinetic adjustments in terms of the energy metabolism are not related with the temperature-dependent elevation of Cu toxicity in *P. vivipara* as we expected.

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5. Discussão geral

5.1. Hipóteses iniciais e breve recapitulação dos resultados

A ideia inicial desta tese era de que a aclimatação em temperatura elevada iria potencializar a toxicidade do cobre no peixe *P. vivipara*. Para testar esta hipótese, aclimatamos este animal à duas temperaturas, uma considerada normal (22 ºC) e a outra considerada alta (28 ºC), por três semanas e depois expomos estes peixes por 96h a três condições de cobre: controle, o limite máximo permitido pela legislação brasileira (9 µg/L) e uma concentração não permitida, mas frequentemente encontrada em ambientes aquáticos impactados (20 µg/L).

Posteriormente à realização do experimento, nossa primeira linha de avaliação foi a determinação do desempenho individual dos animais experimentais. Escolhemos o método da temperatura crítica máxima (CTMax) para a obtenção deste dado, visto a praticidade e poder de explicação deste *endpoint*. Após a determinação da CTMax, foi possível observar uma significativa interação entre a temperatura de aclimatação e a exposição ao cobre, sendo que este metal diminuiu a tolerância térmica máxima apenas dos animais mantidos na maior temperatura (28 ºC). A partir deste momento, ficou evidente que os peixes cultivados sob estresse térmico haviam sido mais afetados pela exposição ao cobre, o próximo passo seria entender os processos fisiológicos envolvidos neste primeiro resultado.

Nossa segunda linha de ação foi focada no entendimento de como os dois fatores testados iriam afetar o os padrões de acumulação tecidual do cobre e o estado oxidativo de *P. vivipara*. Neste caso, foi possível observar interação significativa para todos os *endpoints* avaliados. Especificamente em relação à acumulação tecidual de cobre, em ambas as temperaturas avaliadas a concentração deste metal em fígado e brânquias foi diretamente proporcional à quantidade do contaminante na água, sendo a presença de cobre mais intensa nos animais mantidos em 28 ºC. Já no caso dos biomarcadores relacionados ao estado oxidativo, só foi possível observar efeito da exposição ao cobre no fígado dos animais aclimados à maior temperatura. Interessantemente, foi possível observar uma redução nos biomarcadores relacionados à capacidade antioxidante total (ACAP e TAC) e um aumento no biomarcador relacionado ao dano oxidativo (TBARS). Portanto, sob a luz deste conjunto de dados, fica evidente que o cobre em temperatura é acumulado com maior intensidade em fígado e brânquias e também leva à restrição da capacidade antioxidante hepática de *P. vivipara*, culminado em elevação de dano oxidativo neste tecido.

O seguimento dado para nosso estudo envolveu a avaliação do metabolismo energético nos animais experimentais. A hipótese deste segundo trabalho era que a aclimatação em temperatura elevada iria exigir ajustes enzimáticos no metabolismo energético, principalmente do sistema aeróbico hepático, que levariam ao aumento na produção de ROS, ajudando a explicar os resultados que foram apresentados acima. Portanto, avaliamos a atividade de enzimas pertencentes às vias da glicólise (piruvato quinase e lactato desidrogenase), ciclo de Krebs (citrato sintase) e cadeia transportadora de elétrons (ETS) em fígado, brânquias e músculo de *P. vivipara* expostos às

condições experimentais descritas. Ao analisar os resultados, foi possível observar que a combinação entre os fatores avaliados teve pouco impacto sobre a atividade das enzimas testadas, mostrando que *P. vivipara* tem uma capacidade de compensação térmica muito elevada e que, contrariamente à hipótese inicialmente formulada, ajustes em termos destas enzimas não estão fortemente envolvidos nos resultados que foram obtidos no primeiro trabalho.

5.2. Por onde seguir agora? A procura de um mecanismo de ação continua

Analisando todos os dados gerados no contexto desta tese, ainda não é possível indicar com clareza qual é o mecanismo fisiológico relacionado ao aumento de estresse oxidativo gerado pelo cobre frente à exposição em temperatura elevada, resultado claramente demonstrado pelo nosso grupo no artigo I e pelo trabalho de Braz-mota et al (2017). Como demonstramos no capítulo II, este processo não parece estar intimamente relacionado a alterações na atividade de enzimas do metabolismo energético, conhecidamente um dos grandes responsáveis pela produção de ROS intracelular. Sendo assim, hipotetizamos que o aumento de dano oxidativo visto no nosso primeiro trabalho pode estar relacionado não necessariamente a uma maior produção de ROS, mas possivelmente a uma inibição em algum aspecto do sistema antioxidante, como por exemplo a inativação de enzimas antioxidantes, alterações na sinalização redox ou até mesmo uma menor produção de algum composto do sistema antioxidante não enzimático.

Outra linha de avaliação que pode ser seguida é a procura de algum mecanismo de produção de ROS independente àqueles avaliados no capítulo II, mas que possa ter contribuído para os resultados observados no primeiro artigo. Esta parece ser uma hipótese mais improvável e difícil de ser testada, mas não deve deixar de ser citada. Apesar disto, acreditamos que a primeira hipótese apresentada nesta seção merece ser mais profundamente avaliada. Por fim, todas estas interessantes questões serão abordadas em um futuro próximo, já além do contexto desta tese.

6. Conclusão geral

Nesta tese mostramos que o aumento de temperatura pode funcionar como um potencializador dos efeitos nocivos provocados pela exposição aguda ao cobre no peixe *Poecilia vivipara*. Este processo está diretamente envolvido com o aumento na acumulação deste metal e alterações no estado oxidativo hepático, expressados como uma inibição da capacidade antioxidante total e elevação de dano oxidativo. Mostramos ainda que, como resultado de todas estas alterações, o desempenho individual de *P. vivipara* foi comprometido, já que uma diminuição na tolerância térmica máxima (CTMax) foi observada. Este dado é de suma importância, visto que as alterações climáticas globais irão afetar espécies com limites térmicos estreitos de forma mais intensa, inclusive podendo causar processos de extinção massivos. Além disto, é mostrado que este desbalanço oxidativo não é causado por alterações em termos do metabolismo energético, como previamente hipotetizado pela literatura. Por fim, concluímos que em contextos ambientais de temperatura elevada, como frente

ao processo de aquecimento global ou em períodos de verão, um aumento considerável na toxicidade do cobre, e potencialmente de outros metais, deve ser esperado. Estes resultados deveriam ser considerados, inclusive, em políticas públicas de monitoramento e redução de impactos ambientais causados por impactos antrópicos em ambientes aquáticos.

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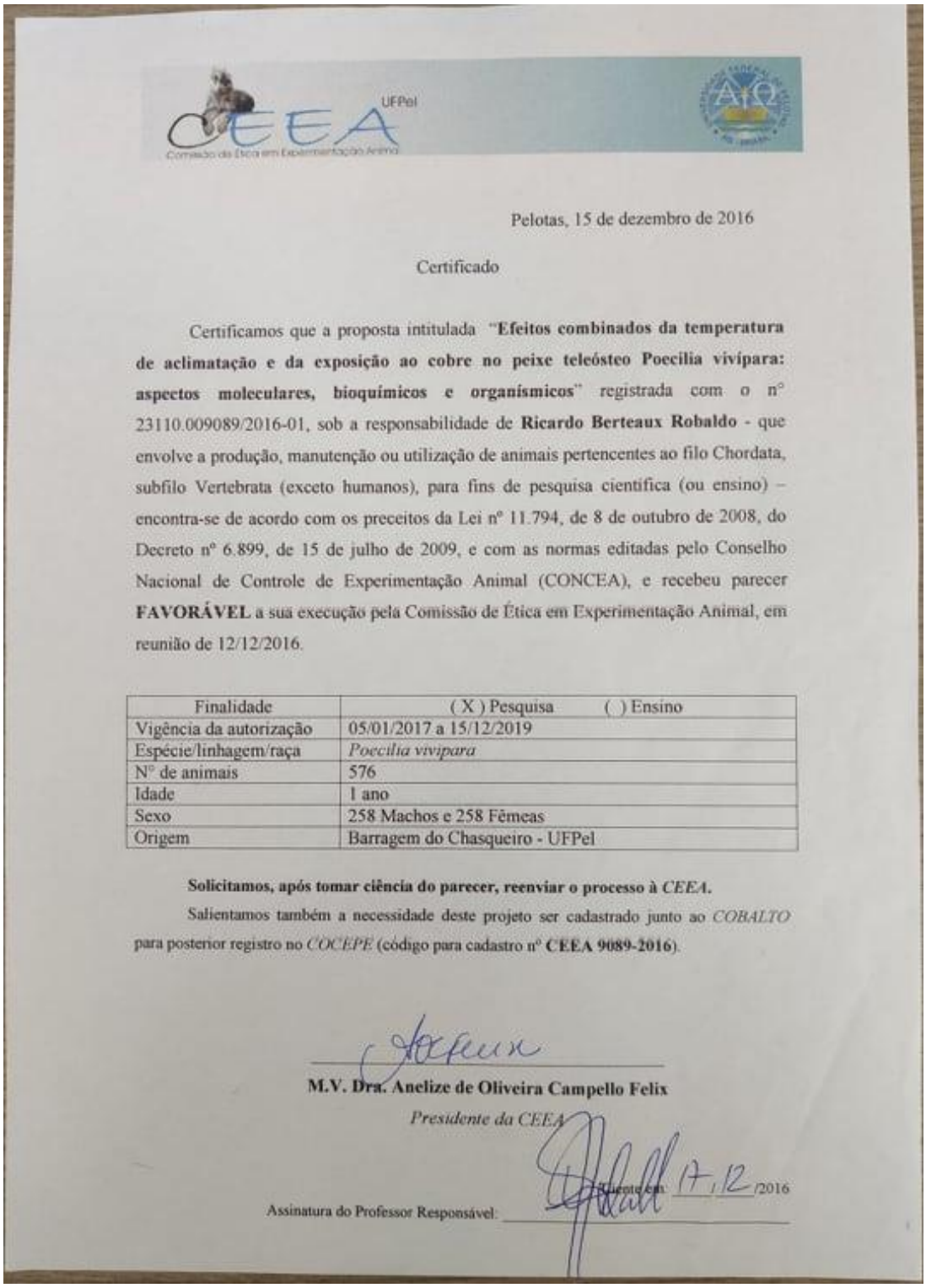
Anexo 1



UFPel

CEEA

Comissão de Ética em Experimentação Animal

Pelotas, 15 de dezembro de 2016

Certificado

Certificamos que a proposta intitulada "**Efeitos combinados da temperatura de aclimatação e da exposição ao cobre no peixe teleósteo Poecilia vivípara: aspectos moleculares, bioquímicos e organísmicos**" registrada com o nº 23110.009089/2016-01, sob a responsabilidade de **Ricardo Berteaux Robaldo** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 12/12/2016.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	05/01/2017 a 15/12/2019
Espécie/linhagem/raça	*Poecilia vivipara*
N° de animais	576
Idade	1 ano
Sexo	258 Machos e 258 Fêmeas
Origem	Barragem do Chasqueiro - UFPel

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº **CEEA 9089-2016**).

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Assinatura do Professor Responsável: _____ Ciente em 17/12/2016

Anexo 1 – Licença do comitê de ética em experimentação animal concedida pela UFPel para a realização dos experimentos desta tese.