UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

FISIOLOGIA ANIMAL COMPARADA

EFEITOS DA EXPOSIÇÃO CRÔNICA AO COBRE EM *Poecilia vivipara* (Bloch e Schneider, 1801) ACLIMATADOS À ÁGUA SALGADA

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Tese apresentada ao Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada da Universidade Federal do Rio Grande, como requisito parcial para a obtenção do Título de Doutor.

Rio Grande, 2015

A ausência de evidências não
significa evidência de ausência.

Carl Sagan

Agradecimentos

O pleno desenvolvimento desse trabalho contou com a cooperação de muitas pessoas, às quais eu gostaria de agradecer:

Ao Professor Adalto Bianchini, pela oportunidade, pela confiança depositada em mim, pelos inúmeros ensinamentos, e, por fim, pela amizade cultivada nestes anos.

Ao Sidnei Afonso, pela dedicação e compromisso durante todo o desenvolvimento dos experimentos.

À Isabel Abril, por toda troca de informações e ajuda nos procedimentos de biologia molecular.

À Mariana Lauer, pelos ensinamentos em bioquímica e auxilio nas atividades de laboratório.

À Marianna Jorge, por toda ajuda e atenção prestadas às análises de laboratório.

À toda equipe do Grupo de Pesquisa em Toxicologia Aquática, pelos ensinamentos, conselhos, discussões produtivas e momentos de descontração.

A todos os professores do PPGCF-FAC, com quem muito aprendi.

A todos os técnicos e funcionários do Instituto de Ciências Biológicas (ICB), pela ajuda sempre que necessária.

A todos os colegas da graduação e da pós-graduação, pelas produtivas conversas e momentos de descontração.

Às agências de fomento, que viabilizaram a realização do trabalho: à CAPES, pela bolsa de doutorado, bem como ao CNPq, através do Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática (INCT-TA) e ao IDRC (Canadá), pelo auxílio financeiro.

Ao Instituto de Ciências Biológicas (ICB) da FURG, por conceder toda infraestrutura necessária para realização desse trabalho.

À minha esposa Thais Rubira e filha Lívia, pela ajuda, companheirismo, paciência e compreensão nos momentos em que mais precisei.

À minha mãe Rita, tia Fátima e avó Doroti, que mesmo à distância, sempre me apoiaram em todos os momentos.

A todos, muito obrigado!

Sumário

Lista de siglas e abreviaturas

ANOVA – análise de variância

ATP - adenosina trifosfato

Atox1 – proteína antioxidante 1

ATP7A – proteína tipo-P de transporte de cobre alfa

ATP7B - proteína tipo-P de transporte de cobre beta

BW – peso úmido final

cDNA – DNA complementar

CCS – cobre chaperona para superóxido dismutase

CcO – citocromo c oxidase

CF – fator de condição

CS – citrato sintase

CONAMA – Conselho Nacional do Meio Ambiente

Cox17 - cobre chaperona para citocromo c oxidase

CTR1 - transportador de alta afinidade ao cobre

Cu - cobre

DTM1 – transportador de metais divalentes 1

FC – conversão alimentar aparente

INCT-TA – Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática

LDH – lactato desidrogenase

NADH – dinucleótido de nicotinamida e adenina reduzido

OC – consumo de oxigênio

P.A. – para análise

PCR - reação em cadeia da polimerase

pH - potencial hidrogeniônico

PK – piruvato quinase

RNA – ácido ribonucléico

RNAse – ribonuclease

S – sobrevivência

SEM – erro padrão da média

SGR – taxa de crescimento específico

SOD1 – superóxido dismutase 1

TGN – complexo de Golgi

WG – ganho de peso

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RESUMO

A acumulação e os efeitos do cobre (Cu) foram analisados após exposição crônica ao metal em filhotes de *Poecilia vivipara* aclimatados à água salgada. Em um primeiro experimento, filhotes recém-nascidos (<24 h) foram mantidos em condição controle (sem adição de Cu na água) ou expostos a diferentes concentrações de Cu (5, 9 e 20 µg/L) por 28 dias. O experimento foi realizado utilizando-se um sistema estático com renovação total do meio a cada 24 h. Os peixes foram mantidos em aquários de vidro contendo água salgada (salinidade 24), sob condições controladas de temperatura (28°C) e fotoperíodo (12 h claro:12 h escuro), aeração constante e alimentação diária *ad libitum* com ração comercial. Após tratamento, o consumo corporal de oxigênio foi medido e os peixes foram eutanasiados, medidos e pesados para o cálculo de índices zootécnicos (sobrevivência, taxa de crescimento específico, conversão alimentar aparente e fator de condição). A seguir, os peixes foram preservados inteiros para análise da acumulação de Cu, expressão de genes codificadores de proteínas transportadoras de Cu (*CTR1* e *ATP7B*) e atividade de enzimas do metabolismo energético (piruvato quinase, lactato desidrogenase e citrato sintase). Em um segundo experimento, filhotes recém-nascidos foram mantidos em condição controle ou expostos às concentrações de Cu por 345 dias. Os peixes foram mantidos em aquários de vidro nas mesmas condições utilizadas no primeiro experimento. Após tratamento, os peixes foram eutanasiados, medidos e pesados para cálculo de índices zootécnicos (taxa de crescimento específico e ganho de peso), bem como preservados inteiros para medida da acumulação corporal de Cu ou tiveram seus tecidos dissecados para análise da acumulação tecidual (brânquias, fígado, intestino e músculo) de Cu, expressão tecidual (brânquias, fígado e intestino) do *CTR1* e da *ATP7B*, bem como da atividade tecidual (brânquias, fígado e músculo) de enzimas envolvidas no metabolismo energético. Os resultados mostram que houve acumulação de Cu (corporal ou tecidual) nos peixes de ambos os experimentos, sendo esta dependente da concentração do metal no meio experimental e do tempo de exposição ao metal. Além disso, houve indução corporal da expressão do *CTR1* e da *ATP7B* após exposição ao Cu por 28 dias, bem como da *ATP7B* após a exposição ao Cu por 345 dias. Após exposição ao Cu por 28 dias, não houve efeito do Cu no crescimento dos peixes, porém houve um aumento no consumo corporal de oxigênio dos peixes expostos a 9 e 20 µg/L Cu. Após 345 dias de exposição, foi observado 100% de mortalidade dos peixes expostos a 20 µg/L Cu, bem como redução no crescimento de machos expostos a 9 µg/L Cu e de fêmeas expostas a 5 e 9 µg/L Cu. Além disso, foi observado aumento na atividade da citrato sintase no fígado dos peixes expostos a 9 µg/L Cu. Estes resultados indicam que *P. vivipara* acumula o Cu em nível tecidual/corporal, sendo esta dependente da concentração de Cu na água salgada e do tempo de exposição ao metal. Eles sugerem ainda que a acumulação de Cu está associada a um aumento da expressão dos genes que codificam proteínas transportadoras de Cu (CTR1 e ATP7B) em tecidos envolvidos na homeostasia do metal. Mostram ainda que o Cu acumulado provoca efeito letal após exposição a uma concentração excessiva do metal (20 µg/L) e efeito subletal (redução no crescimento) após exposição aos atuais critérios brasileiros de qualidade para o Cu em água doce (9 µg/L) ou salgada (5 µg/L). Por fim, o efeito crônico do Cu no crescimento de *P. vivipara* parece estar relacionado a um aumento na demanda energética dependente do metabolismo aeróbico e/ou uma perturbação na função respiratória mitocondrial.

Palavras-chave: acumulação, cobre, exposição crônica, crescimento, metabolismo energético, transportadores de cobre.

ABSTRACT

In the present study, we evaluated the accumulation and effects of copper (Cu) after chronic exposure to the metal in puppies of the guppy *Poecilia vivipara* acclimated to salt water. In a first experiment, newborn (<24 h) fish were kept under control condition (no Cu addition) or exposed to different Cu concentrations (5, 9 and 20 μg/L) for 28 days. Treatments were performed in triplicate using a static system with complete renewal of the experimental media every 24 h. Fish were kept in 1-L glass aquaria with sea water (24 ppt salinity) under controlled conditions (temperature: 28°C; photoperiod: 12 h light: 12 h dark cycle). Experimental media were continuously aerated and fish were daily fed *ad libitum* with commercial feed. After treatment, whole-body oxygen consumption was measured and fish were euthanized, measured (mm) and weighed (g) for calculation of zootechnical indexes (survival, specific growth rate, apparent feeding conversion and condition factor). They were then preserved entirely for analysis of whole-body Cu accumulation, expression of genes encoding Cu-transporting proteins (*CTR1* and *ATP7B*) and activity of enzymes involved in energy metabolism (pyruvate kinase, lactate dehydrogenase and citrate synthase). In a second experiment, newborn (<24 h) fish were kept under control condition or exposed to the Cu concentrations for 345 days. They were maintained in 10-L glass aquaria under the same controlled experimental conditions described above for the 28-days experiment. After exposure, fish were euthanized, measured (mm) and weighed (g) for calculation of zootechnical indexes (specific growth rate and weight gain), as well as preserved entirely for measurement of whole-body Cu accumulation or dissected for analysis of Cu accumulation in tissues (gills, liver, gut and muscle), expression of genes encoding for Cu-transporting proteins in tissues (gills, liver and gut) and activity of enzymes involved in energy metabolism in tissues (gills, liver and muscle). Whole-body and tissue Cu accumulation were observed in fish exposed for 28 days and 345 days, respectively. In both cases, Cu accumulation was dependent on the concentration of the metal in the experimental medium. Also, there was an induction of *CTR1* and *ATP7B* gene expression in whole-body and tissues of fish exposed for 28 days and 345 days, respectively. No significant effect on growth of fish exposed for 28 days was observed. However, there was an increase in whole-body oxygen consumption in fish exposed to 9 and 20 μg/L Cu. In the 345-days experiment, 100% mortality of fish was observed at 20 μg/L Cu. In addition, a reduction in growth was observed in male guppies exposed to 9 μg/L Cu and female guppies exposed to 5 and 9 μg/L Cu. Furthermore, an increase in citrate synthase activity was observed in liver of guppies exposed to 9 μg/L Cu. These findings indicate that puppies of *P. vivipara* accumulate Cu at the whole-body/tissue level. This accumulation is dependent on the Cu concentration in salt water and associated with an increase in the expression of genes encoding for Cu-transporting proteins such as CTR1 and ATP7B in tissues involved in Cu homeostasis (gills, liver and gut). Also, they show that Cu is lethal to fish chronically exposed to an excessive concentration of the metal (20 μg/L) or induces sublethal effect (growth reduction) when fish are chronically exposed to the current Brazilian criteria for Cu in fresh (9 μg/L) and sea water (5 μg/L). Finally, findings reported in the present study indicate that the observed effect on growth may be related to an increased demand for energy produced via aerobic metabolism and/or a disruption in mitochondrial respiratory function induced by chronic exposure of fish to waterborne Cu.

Keywords: Accumulation, copper, chronic exposure, Cu-transporting proteins, energy metabolism, growth.

1. INTRODUÇÃO

1.1 Poluição dos ambientes aquáticos

A poluição dos ecossistemas aquáticos, por diversas classes de poluentes orgânicos e inorgânicos, tem assumido sérias proporções nos últimos anos. O crescimento da população humana e as instalações de grandes centros urbanos próximos aos corpos de água têm contribuído significativamente para o aumento da poluição aquática (Laws, 2000). Dentre os diferentes tipos de poluentes, os metais têm recebido relevante atenção, devido à sua elevada toxicidade e comportamento de acumulação nos organismos (Brown e Welton, 2008; Sánchez, 2008).

Os metais compreendem uma classe de poluentes inorgânicos que são lançados nos ambientes aquáticos a partir de diferentes fontes, sendo que atividades antrópicas, como ocupação urbana, agricultura, pecuária, indústria e mineração, têm sido consideradas como causadoras de grande impacto (Agarwal, 2009). De forma geral, o cobre, zinco, mercúrio, cádmio, chumbo, níquel e o cromo são os principais metais encontrados hoje nos ambientes aquáticos, sendo que estes podem causar toxicidade quando presentes em níveis elevados (Agarwal, 2009).

1.2 Cobre

O cobre (Cu) é considerado um dos mais importantes recursos naturais existentes, não somente pelo seu valor intrínseco, mas também por seus muitos usos e aplicações. Dentre estes, destaca-se a utilização deste metal no fornecimento de energia elétrica, produção de aparelhos eletrônicos, construção civil, indústrias farmacêutica, automobilística e naval, bem como na produção de alimentos (CDA, 2015).

Após ser liberado nos corpos de águas (doce ou salgada), o Cu pode permanecer dissolvido na água em sua forma iônica ou pode se ligar a outras moléculas orgânicas ou inorgânicas. Além disso, este metal pode ser adsorvido por partículas, que, por sua vez, podem permanecer em suspensão ou se sedimentar (Bjorklund e Morrison, 1997; Guthrie et al., 2005). A maior fração do Cu dissolvido está complexada à matéria orgânica dissolvida, tanto em água doce quanto salgada (Guthrie et al., 2005; Buck et al., 2007).

Em relação ao seu papel biológico, o Cu é um metal essencial de transição redox-ativo, que pode apresentar duas valências comuns, Cu (I) e Cu (II). Isto permite

que este metal tenha facilmente alterações no seu estado de oxidação e possa doar e receber elétrons. Devido a essas características, o Cu atua como elemento estrutural de proteínas regulatórias da homeostase celular (Knight et al., 1994). Além disso, este metal exerce importante papel nos processos de respiração mitocondrial, resposta ao estresse oxidativo, sinalização hormonal, funções neurológicas, transporte de oxigênio em alguns moluscos e artrópodes, coagulação sanguínea e indução da formação de vasos sanguíneos (Linder e Hazegh-Azam, 1996; Dang et al., 2000; Bopp et al., 2008; Kim et al., 2008; Leary et al., 2009; Eyckmans et, al., 2010; Belyaeva et al., 2011). Contudo, em concentrações elevadas, o Cu se torna tóxico aos organismos, podendo comprometer seriamente o funcionamento celular.

1.3 Mecanismos celulares de transporte do cobre

Em vertebrados, diferentes mecanismos estão envolvidos na homeostasia do Cu. Estudos mostram a participação de diferentes órgãos na absorção, armazenamento e excreção do metal (Linder e Hazegh-Azam, 1996; Puig e Thiele, 2002; La Fontaine et al., 2010). Em mamíferos terrestres, a absorção do Cu ocorre principalmente pela dieta, através do epitélio intestinal. Nesse caso, o excesso do metal vai para o fígado e é secretado com a bile no canal alimentar, onde grande parte é reabsorvida pelos enterócitos e o restante é eliminado nas fezes. Assim, o fígado é considerado o principal órgão regulador da homeostasia do cobre. Ele exerce importante papel nos processos de detoxificação e reciclagem do metal (Festa e Thiele, 2011).

O Cu entra nas células principalmente através do Transportador de Alta Afinidade ao Cobre (CTR1) e em seguida se liga às proteínas chaperonas Atox1, CCS e Cox17. Essas proteínas garantem a chegada do Cu aos seus respectivos destinos nas células e contribuem para evitar o sequestro deste metal, o qual é mediado pelos mecanismos de detoxificação. Assim, o Cu é transportado para as Cu-ATPases (*ATP7A/B*), a Cu/Zn superoxido dismutase (SOD1) e citocromo c oxidase (CcO) (Mufti et al., 2007; Festa e Thiele, 2011) (Fig. 1).

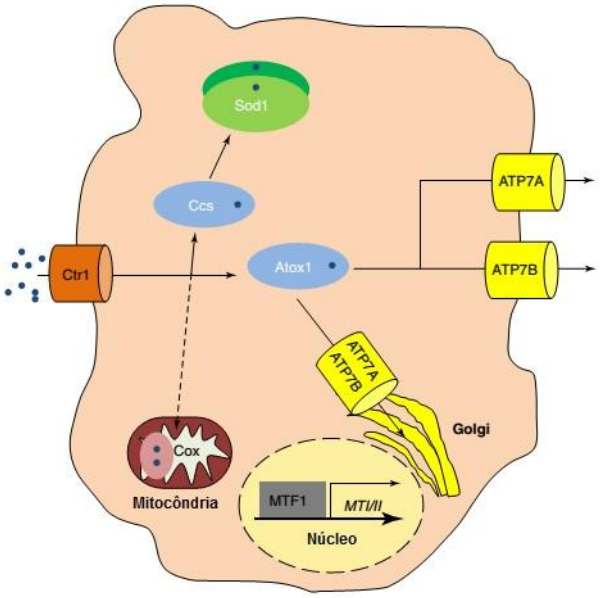

Figura 1. Modelo esquemático do transporte de cobre (Cu) em uma célula de mamífero. Adaptado de Festa e Thiele, 2011.

No ambiente aquático, a espécie atômica mais estável e, portanto, mais comum de Cu é o íon Cu^{2+} (Krot et al., 2005; Buck et al., 2007). Portanto, as proteínas de transporte do Cu operam com uma química de geometria trigonal planar, que se liga ao íon Cu^{+} (Finney e O'Halloran, 2003). Portanto, o Cu^{+} é a espécie atômica requerida pelas estruturas de captação e as proteínas de transporte das células. Dessa maneira, para que os processos de entrada e transporte interno do Cu ocorram com sucesso, o Cu^{2+} tem de ser reduzido a Cu^{+} por uma ou mais redutases endógenas presentes na superfície celular (Dancis et al., 1994).

Em peixes, o Cu pode ser absorvido através das brânquias e trato gastrointestinal. Como mencionado anteriormente, a entrada do Cu nas células ocorre principalmente através do CTR1, mas esta também pode acontecer através do Transportador de Metais Divalentes 1 (DMT1) e dos canais de Na^{+} (Puig e Thiele, 2002; Wood et al., 2011). Os transportadores CTR1 e DTM1 são favorecidos pela camada ácida presente na superfície celular e que é formada pela extrusão de íons H^{+} por meio da bomba protônica e outros secretores de íons ácidos. Alguns estudos evidenciam que o CTR1 é responsável pelo transporte específico do Cu reduzido (Cu^{+}), enquanto o DTM1 atua no transporte dos íons Cu^{2+} e também de íons de outros metais, como por exemplo, o Fe^{2+} (Wood et al., 2001; Grosell e Wood, 2002). Quanto aos canais de Na^{+}, o Cu pode entrar na célula através destes quando as concentrações de Na^{+} no meio são baixas (Wood et al., 2011) (Fig. 2).

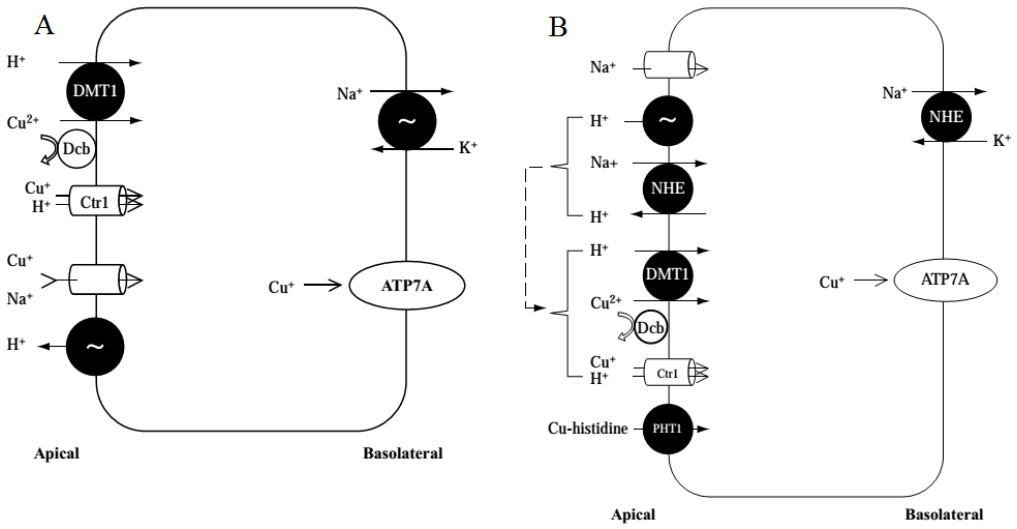

Figura 2. Esquema sugerido para as vias de entrada do cobre (Cu) nas células branquiais (A) e intestinais (B) de peixe de água doce. Adaptado de Grosell e Wood (2002).

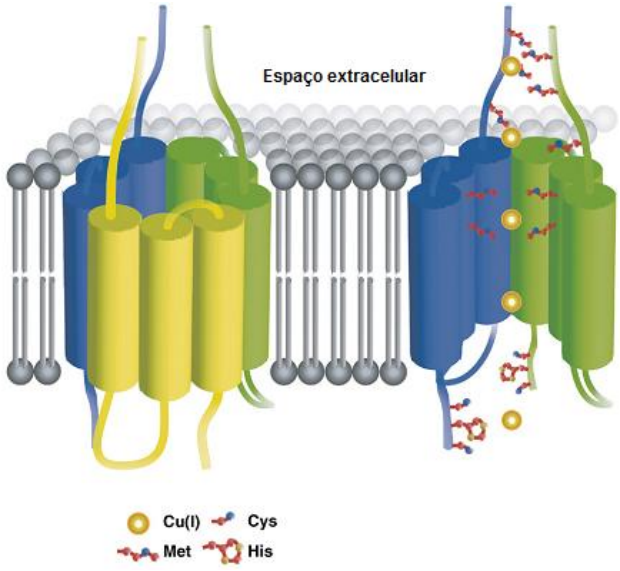

Figura 3. Interpretação do modelo estrutural e funcional do transportador de alta afinidade ao cobre (*CTR1*). Adaptado de Nose et al. (2006).

1.3.1 Transportador de alta afinidade ao cobre - CTR1

O CTR1 é uma proteína transmembrana altamente conservada (Fig. 3), que medeia a internalização especifica de íons Cu^{+} a partir do meio extracelular (Linder e Hazegh-Azam, 1996; Grosell e Wood, 2002 Puig e Thiele, 2002; Sharp, 2003; Mackenzie et al., 2004; Wood et al., 2011). Este transportador é composto por três domínios que formam uma simetria radial homotrimérica para a entrada do Cu

(Mackenzie et al., 2004; Nose et al., 2006). O processo de transporte deste metal para o meio intracelular ocorre independentemente do uso de energia (ATP). Contudo, outros fatores como pH e concentração extracelular de K^+ podem influenciar na atividade do CTR1 (Lee et al., 2002).

Alguns estudos têm demonstrado a influência direta da exposição ao Cu sobre a expressão do *CTR1* em peixes. Craig et al. (2009) observaram um aumento na expressão do *CTR1* no intestino e no fígado do peixe zebra (*Danio rerio*) após exposição aguda ao Cu dissolvido na água. Minghetti et al. (2008) observaram que o *CTR1* pode ser expresso em níveis diferentes no intestino, rim e fígado do peixe *Sparus aurata*, de acordo com a via de exposição ao Cu (dieta ou dissolvido na água). Por sua vez, Silva et al. (2014) observaram uma diminuição na expressão do *CTR1* nas brânquias de indivíduos adultos do peixe *Poecilia vivipara* após exposição aguda (96 h) ao Cu dissolvido na água.

1.3.2 Cu-ATPases

As Cu-ATPases são proteínas responsáveis pelo transporte ativo do Cu no meio intracelular (Fig. 4). A ATP7A e a ATP7B são exemplos de importantes Cu-ATPases envolvidas na homeostasia do Cu (Lutsenko et al., 2007; 2008).

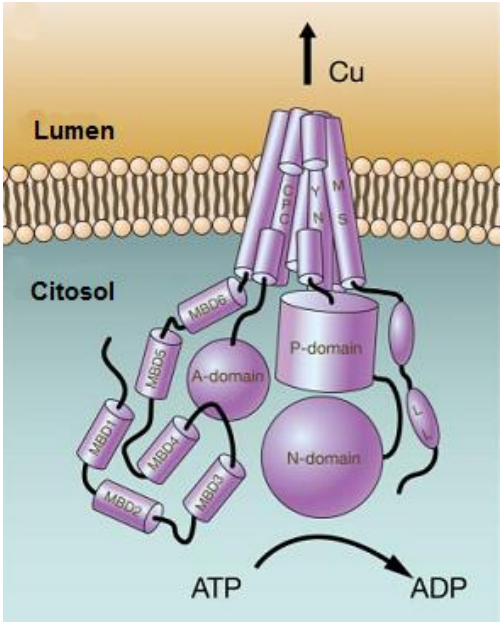

Figura 4. Modelo esquemático da topologia de membrana e organização de domínios da Cu-ATPase. Adaptado de Lutsenko et al. (2007).

As ATP7A e ATP7B são moléculas chave que atuam no processo de distribuição e regulação intracelular do Cu em vertebrados (La Fontaine e Mercer, 2007; Minghetti et al., 2010; La Fontaine et al., 2010). Em mamíferos, a ATP7A e a ATP7B desempenham papel fundamental em diferentes processos fisiológicos. A figura 5 representa um modelo esquemático da participação dessas Cu-ATPases nesses processos (Lutsenko et al., 2007; La Fontaine et al., 2010).

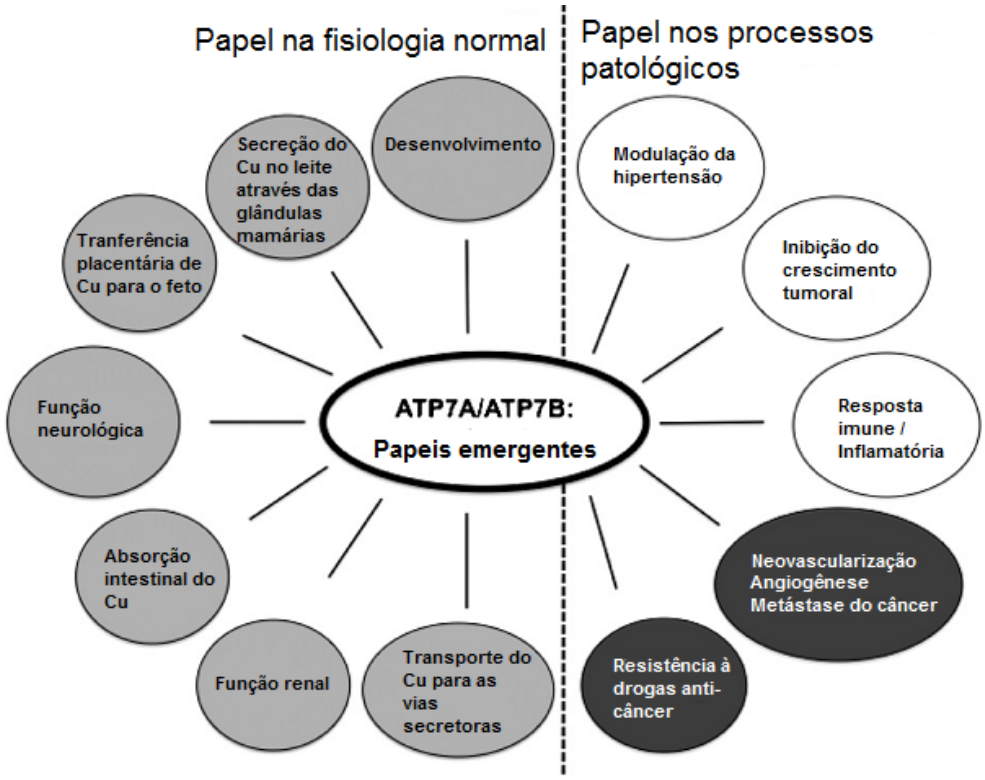

Figura 5. Representação esquemática da participação da ATP7A e ATP7B nos processos fisiológicos normais e patológicos em mamíferos. Adaptado de La Fontaine et al. (2010).

Deficiências no controle da homeostasia do Cu em humanos podem resultar no surgimento de doenças. A doença de Menkes (Fig. 6), causada por mutações no gene *ATP7A*, resulta na deficiência periférica de Cu, a qual é provocada pela falha na mobilização de Cu da dieta a partir de epitélio intestinal para o sangue. Por sua vez, a doença de Wilson (Fig. 6), proveniente de mutações no gene *ATP7B*, e sua consequente inativação, levam ao acúmulo de Cu nos tecidos hepático e neuronal. (Linder e Hazegh-Azam, 1996; Lutsenko et al., 2008; Festa e Thiele, 2011).

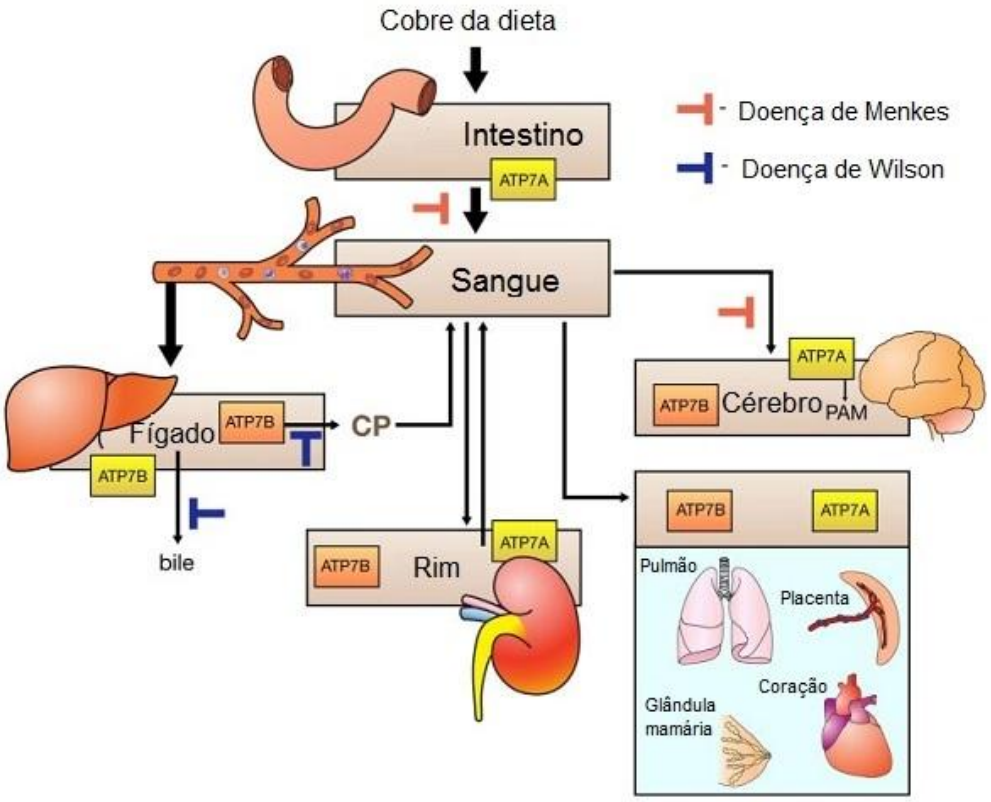

Figura 6. Esquema simplificado da distribuição do cobre no corpo humano e ocorrência das doenças de Menkes e de Wilson. Adaptado de Lutsenko et al. (2007).

Em níveis celulares normais de Cu, as Cu-ATPases residem no complexo de Golgi. Todavia, o excesso de Cu induz o tráfego de ATP7A e ATP7B para a membrana plasmática e o compartimento secretor vesicular, respectivamente, auxiliando assim na excreção do metal. A ATP7A é expressa na maioria dos tecidos, mas está ausente ou expressa em níveis baixos no fígado, onde é abundante a expressão da ATP7B (Fig. 7) (Mercer et al., 2003; Minghetti et al., 2010).

Nos peixes, a expressão de proteínas envolvidas na homeostasia do Cu é regulada em nível transcricional pela exposição ao Cu (Minghetti et al., 2010; 2011). Além disso, Silva et al., (2014) observaram uma relação direta entre a concentração de Cu e o nível da transcrição dos genes *CTR1* e *ATP7B* em diferentes tecidos do peixe *Poecilia vivipara*, após a exposição aguda (96 h) ao Cu.

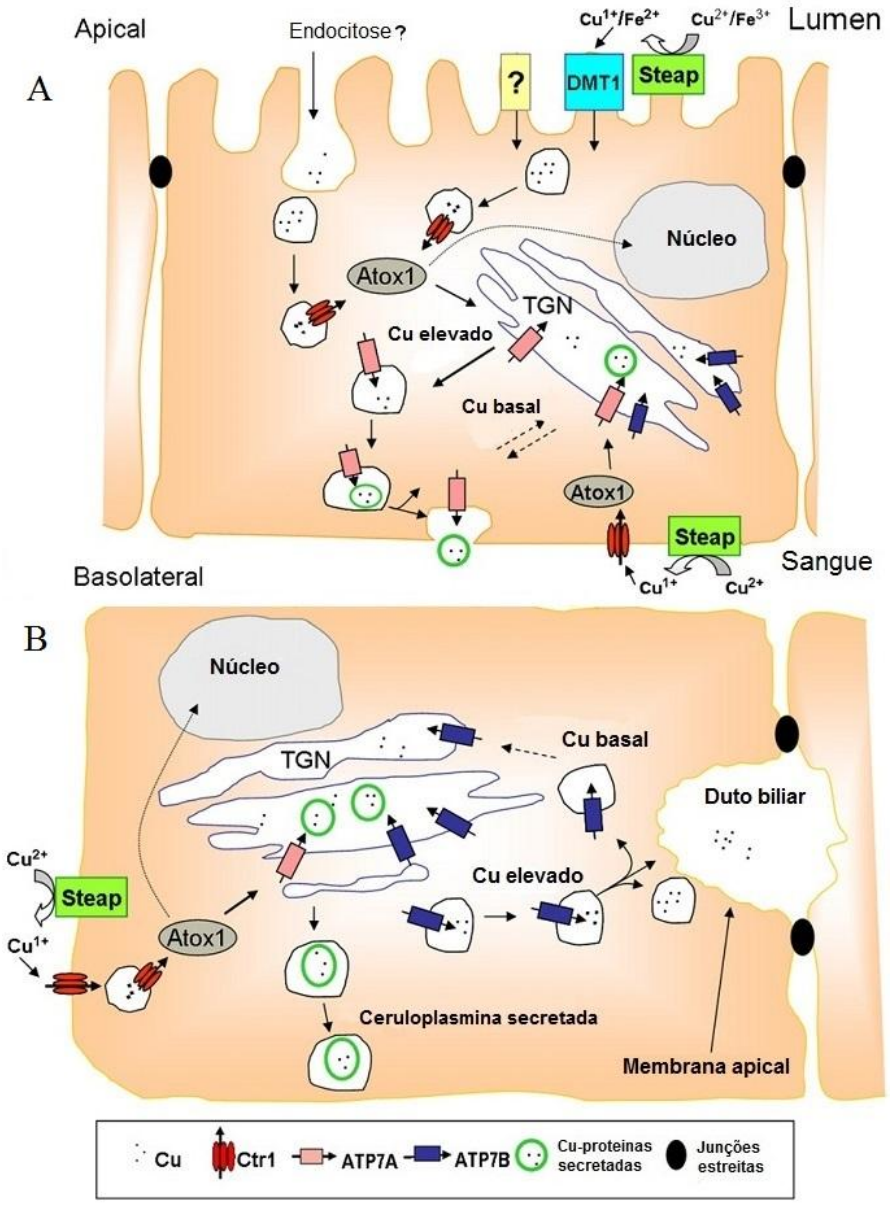

Figura 7. Modelo proposto de transportadores de cobre (CTR1, ATP7A e ATP7B) e sua localização intracelular no enterócito (A) e hepatócito (B) de peixe de água salgada. Adaptado de Minghetti et al. (2010).

1.4 Cobre e metabolismo energético

Para suprir a demanda necessária de energia dos processos envolvidos no transporte do Cu em peixes expostos a este metal, o glicogênio/glicose é mobilizado do fígado (Wood et al., 2011). Cabe salientar que, dentro das células, cada via metabólica é continuamente e finamente regulada, a fim de manter o adequado funcionamento celular (Nelson e Cox, 2008).

No metabolismo de carboidratos, a glicose é considerada o principal substrato

utilizado na geração de energia nas células animais. A via glicolítica (glicólise), processo que quebra uma molécula de glicose (seis carbonos) em duas moléculas de piruvato (três carbonos), ocorre em dez etapas. Nas primeiras cinco etapas a glicose é fosforilada e convertida a gliceraldeído 3-fosfato. Nas últimas cinco etapas ocorre a conversão oxidativa do gliceraldeído 3-fosfato em piruvato, com a formação de ATP e NADH (Nelson e Cox, 2008).

Cada etapa da via glicolítica tem sua reação catalisada por uma enzima específica. A piruvato quinase e a lactato desidrogenase desempenham um importante papel na sequência terminal da via glicolítica. A piruvato quinase catalisa a conversão do fosfoenolpiruvato em piruvato, que, em condições anaeróbicas, é convertido a lactato em reação catalisada pela lactato desidrogenase (Fig. 8A). Em condições aeróbicas, o piruvato é convertido a acetil-CoA, que por sua vez pode ser convertido a citrato, em reação catalisada pela citrato sintase no início do ciclo de Krebs (Fig. 8B) (Nelson e Cox, 2008).

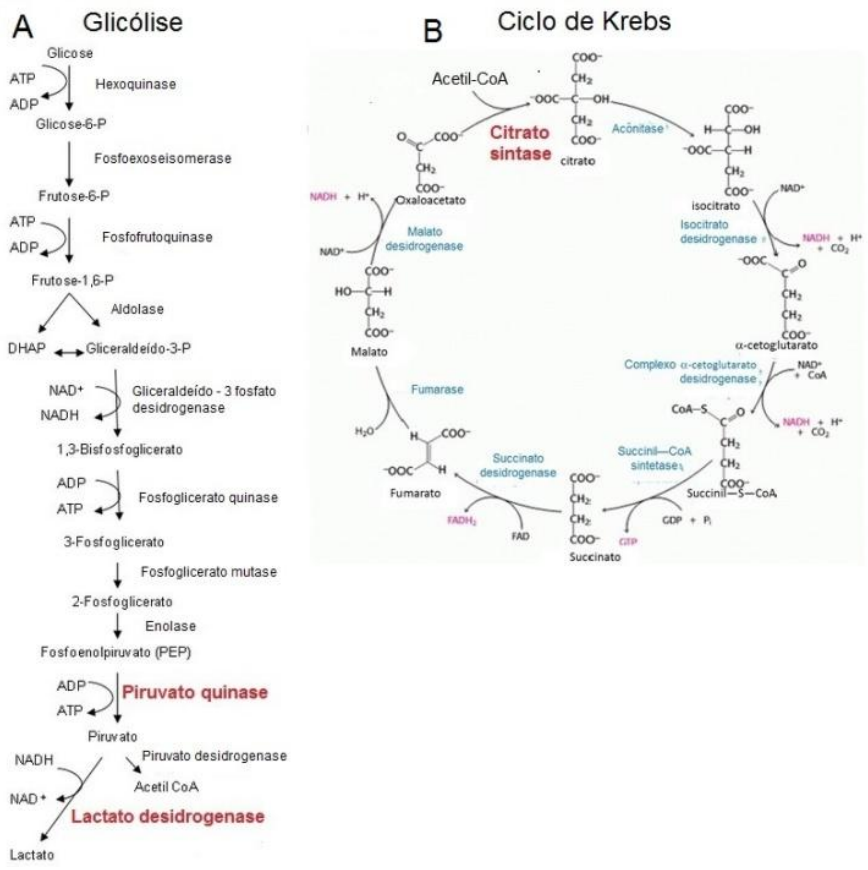

Figura 8. Representação da piruvato quinase e lactato desidrogenase no processo de glicólise (A) e da citrato sintase no Ciclo de Krebs (B). Adaptado de Nelson e Cox (2008).

Alguns estudos têm demonstrado o efeito do Cu sobre determinadas enzimas envolvidas no metabolismo energético de peixes. Liu et al. (2010) observaram um aumento na atividade da piruvato quinase e lactato desidrogenase hepáticas de *Synechogobius hasta*, de acordo com o aumento das concentrações de Cu dissolvido na água salgada (salinidade 28). Carvalho e Fernandes (2008) observaram uma diminuição na atividade da hexoquinase, fosfofrutoquinase, piruvato quinase e lactato desidrogenase hepáticas de *Prochilodus lineatus* após exposição aguda (96 h) ao Cu em água doce. Garceau et al. (2010), não observaram diferença significativa na atividade da citrato sintase hepática e muscular de *Carassius auratus* aclimatado a água doce, após exposição *in vitro* ao cobre.

Todavia, grande parte dos estudos relacionados aos efeitos do Cu sobre enzimas do metabolismo energético são desenvolvidos em peixes de água doce e após exposição aguda ao metal. Sendo assim, ainda há uma lacuna no conhecimento do efeito da exposição crônica ao Cu sobre a atividade dessas enzimas em peixes aclimatados à água salgada (Balavenkatasubbaiah et al., 1984; Couture e Kumar, 2003; Carvalho e Fernandes, 2008; Garceau et al., 2010; Liu et al., 2010 Lapointe et al., 2011)

1.5 Toxicidade do cobre em peixes

O Cu pode apresentar diferentes mecanismos de toxicidade, como alterações na permeabilidade de membrana, síntese de proteínas e atividade de enzimas, bem como indução de apoptose e proliferação celular (Lundebye et al., 1999; Smith et al., 2001; Feng et al., 2003; Krumschnabel et al., 2005; García et al., 2007; Monteiro et al., 2009; Liu et al., 2010; Eyckmans et al., 2010).

Além das alterações acima citadas, outros efeitos fisiológicos são observados em peixes após a exposição ao Cu. Neste caso, o metal pode se acumular em diferentes tecidos, como fígado, rim, brânquias, músculo e sangue, e causar disfunções no consumo de oxigênio, desenvolvimento embrionário, crescimento, reprodução, atividade de enzimas antioxidantes, resposta imunológica, e até mesmo mortalidade (Mazon e Fernandes, 1999; McGeer et al., 2000; Clearwater et al., 2002; Kamunde et al., 2002; Handy et al., 2003; James et al., 2003; Mackenzie et al., 2004; Lugo et al., 2006; Martins e Bianchini, 2008; Findik e Çiçek, 2011; Liu et al., 2011; Das e Gupta, 2013; Machado et al., 2013). Em nível molecular, o Cu pode modular a expressão de genes de proteínas envolvidas no transporte e secreção de metais e na prevenção do

estresse oxidativo (Minghetti et al., 2008; Minghetti et al., 2010; 2011; Chen et al., 2011; Silva et al., 2014).

Contudo, como mencionado anteriormente, grande parte da literatura referente aos efeitos fisiológicos do Cu em peixes está relacionada a estudos de exposição aguda a esse metal. O conhecimento dos efeitos fisiológicos e ajustes metabólicos relacionados a longos períodos de exposição ao Cu ainda é limitado, havendo uma necessidade crescente de mais estudos que investiguem os efeitos crônicos deste metal (Handy, 2003).

1.6 Modelo experimental: *Poecilia vivipara*

Os peixes são considerados importantes modelos para estudos de toxicidade de contaminantes aquáticos. Esse grande grupo de vertebrados apresenta diversos mecanismos fisiológicos muito semelhantes aos de mamíferos, além de serem relativamente de baixo custo para obtenção e manutenção em cativeiro. Outra vantagem é que, por constituírem um grupo altamente diversificado, apresentam boa tolerância a variações nas condições ambientais. Além disso, nos peixes a absorção de contaminantes pode ocorrer via braquial e/ou gastrointestinal (Kelly et al., 1998; Carvan et al., 2007; Dong et al., 2014).

O teleósteo *Poecilia vivipara* (Fig. 9), conhecido popularmente como "Barrigudinho" ou "Guarú", é uma espécie nativa da América do Sul pertencente à família Poeciliidae, sendo um dos peixes mais comuns de ocorrência nos lagos, rios e lagoas costeiras no Brasil (Neves e Monteiro, 2003; Gomes Jr. e Monteiro, 2008; Santos et al., 2011; Zanette, 2013). Os espécimes de *P. vivipara* são considerados onívoros, alimentando-se de invertebrados, detritos, algas e plantas. Os indivíduos apresentam dimorfismo sexual acentuado. As fêmeas são geralmente maiores que os machos, sendo estes últimos de coloração mais evidente (Amaral et al., 2001; Araújo et al, 2009).

Todos os peixes poecilídeos são caracterizados pela presença de um gonopódio (raio da nadadeira anal modificado) e fertilização interna, sendo que todas as espécies são vivíparas, à exceção daquelas do gênero *Tomeurus* (Meredith et al., 2011). A espécie *P. vivipara* é considerada um modelo em potencial para estudos de toxicidade de contaminantes aquáticos (Mattos et al., 2010; Ferreira et al., 2012; Paulo et al., 2012; Zimmer et al., 2012; Machado et al., 2013; Harayashiki et al., 2013; Silva et al., 2014), devido à sua ampla distribuição geográfica (Fig. 10), tolerância a variações ambientais,

facilidade de manutenção e reprodução em cativeiro, e viviparidade. De fato, esta espécie tem sido utilizada como organismo-teste em diferentes estudos com metais conduzidos pelo Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática (INCT-TA) (www.inct-ta.furg.br).

Figura 9. Exemplares do peixe *Poecilia vivipara.* Fonte: < www.fishbase.org.>.

Figura 10. Distribuição geográfica de *Poecilia vivipara* (●). Adaptado de Zanette (2013).

2. OBJETIVOS

2.1 Geral

- Analisar a acumulação e os efeitos da exposição crônica ao cobre (Cu) presente na água em filhotes de *Poecilia vivipara* aclimatados à água salgada.

2.2 Específicos

- Quantificar a acumulação corporal e tecidual do Cu em filhotes de *P. vivipara*.
- Determinar o efeito do cobre sobre a expressão de genes associados à acumulação do metal (*CTR1* e *ATP7B*) em filhotes de *P. vivipara*.
- Avaliar o efeito do Cu no crescimento de filhotes de *P. vivipara*.
- Determinar o efeito do Cu no consumo de oxigênio de filhotes de *P. vivipara*.
- Analisar o efeito do Cu na atividade de enzimas do metabolismo energético (citrato sintase, lactato desidrogenase e piruvato quinase) de filhotes de *P. vivipara*.

3. METODOLOGIA

3.1 Obtenção dos peixes

Machos e fêmeas de *P. vivipara* foram coletados no Arroio do Gelo, na Praia do Cassino (32º10'52''S; 52º8'52''O; Rio Grande, RS, Brasil), com auxílio de um puçá. Após a coleta, os peixes foram acondicionados em caixas plásticas (50 L) e levados ao biotério aquático do Instituto de Ciências Biológicas (ICB) da Universidade Federal do Rio Grande - FURG. Os animais foram mantidos sob condições controladas de salinidade (24), temperatura (28ºC), fotoperíodo (12 h claro:12 h escuro) e aeração constante. Os peixes foram alimentados duas vezes ao dia com ração comercial (Alcon Basic – MP200). As fêmeas prenhas foram transferidas para caixas parideiras até o nascimento dos filhotes, os quais foram separados ao nascer para posterior realização dos experimentos, conforme descrito a seguir. As condições de manutenção dos peixes e os procedimentos experimentais foram previamente aprovados pelo Comitê de Ética em Uso Animal da FURG - CEUA/FURG (permissão # P014/2012).

3.2 Exposição ao cobre - 28 dias

Filhotes recém-nascidos (<24 h de idade; 0,0062 ± 0,0001 g de peso corporal; 8,0 ± 0,10 mm de comprimento total) de adultos não expostos ao Cu, foram mantidos em condições controle (sem adição de Cu na água) ou expostos ao metal por 28 dias. As concentrações de Cu testadas (5, 9 e 20 µg/L) foram selecionadas considerando-se os padrões de qualidade de água estabelecidos pela Resolução CONAMA 357 de 2005. Assim, foram testadas as concentrações de 5 e 9 µg/L Cu, correspondentes aos padrões de qualidade para águas salgada e doce, respectivamente. Além disso, foi testada a concentração de 20 µg/L Cu, visando simular uma situação de contaminação ambiental acima dos níveis da atual legislação ambiental brasileira. Cada tratamento foi realizado em triplicata, usando um sistema estático com renovação total do meio experimental a cada 24 h. Para tal, foram utilizados aquários de vidro (1 L) contendo água salgada (salinidade 24), mantida sob aeração constante. A temperatura (28ºC) e o fotoperíodo (12 h claro: 12 h escuro) da sala foram fixados. Os peixes foram alimentados duas vezes ao dia com a mesma ração comercial utilizada no período de aclimatação. O período experimental foi de 28 dias.

Ao fim do período experimental, os peixes foram mantidos em jejum de 12 h e foi realizada a medida do consumo de oxigênio corporal, utilizando-se um respirômetro estático. Após, os peixes foram eutanasiados através da técnica de espinhalamento (secção da medula espinhal dorsal), medidos (mm) e pesados (g), para posterior cálculo dos índices zootécnicos. Imediatamente após a biometria, os peixes foram individualmente separados e congelados inteiros (-80ºC) para posterior análise da acumulação corporal de cobre e dos parâmetros bioquímicos e moleculares, conforme descrito abaixo.

3.3 Exposição ao cobre - 345 dias

Filhotes recém-nascidos (<24 h de idade; 0,0063 ± 0,0001 g de peso corporal; 7,1 ± 0,13 mm de comprimento total) de adultos não expostos ao Cu foram expostos ao metal em três tratamentos experimentais, utilizando-se as mesmas concentrações de Cu testadas no Experimento 1, descrito acima. Cada tratamento foi realizado em triplicata, usando um sistema estático com renovação parcial (70%) do meio experimental a cada 7 dias. Neste caso, foram utilizados aquários de vidro (10 L) contendo água salgada (salinidade 24), mantida sob constante aeração. A temperatura (28ºC) e o fotoperíodo (12 h claro: 12 h escuro) da sala foram fixados. Os peixes foram alimentados *ad libitumm* diariamente com a mesma ração comercial utilizada no período de aclimatação. O período experimental foi de 345 dias.

Ao fim do período experimental, os peixes foram eutanasiados com benzocaína, medidos (mm) e pesados (g). Imediatamente após a biometria, os peixes foram dissecados e os tecidos (brânquias, fígado, intestino e músculo) coletados e congelados para posterior análise da acumulação de Cu, bem como dos parâmetros bioquímicos e moleculares.

3.4. Parâmetros analisados

3.4.1 Sobrevivência e parâmetros zootécnicos

A sobrevivência (S) foi calculada para os peixes de ambos os experimentos. A taxa de crescimento específico (SGR), o ganho de peso (WG), a conversão alimentar aparente (FC) e o fator de condição (CF) foram calculados para os peixes expostos em

curto prazo (28 dias). Para os peixes do experimento de longo prazo (345 dias), foram calculados a taxa de crescimento específico (SGR) e o ganho de peso (WG). Os índices zootécnicos foram calculados utilizando-se as seguintes fórmulas:

S = (nf/ni) x 100, onde nf é o número de peixes ao fim do experimento e ni é o número de peixes no início do experimento;

SGR = [(ln pf–ln pi)/t] x 100, onde pf é o peso final (g), pi é o peso inicial (g), t é o tempo do experimento em dias;

FC = AO/GP, onde AO é a quantidade de alimento oferecido (g) e GP é o ganho de peso (g);

CF = (p/c3) x 100, onde p é o peso (g) e c é o comprimento (cm);

WG = [(pf - pi) / pi] x 100, onde pf é o peso final (g) e pi é o peso inicial (g).

3.4.2 Acumulação de cobre

Nos peixes expostos em curto prazo, a acumulação de Cu foi avaliada no indivíduo inteiro, enquanto que nos peixes expostos em longo prazo, o conteúdo de Cu foi medido nos tecidos (brânquias, fígado, intestino e músculo). As amostras de ambos os experimentos foram pesadas (peso úmido) e secas em estufa a 50°C, até determinação do peso constante (peso seco). As amostras secas foram completamente digeridas em ácido nítrico (HNO_3) 65% PA e o conteúdo de Cu foi determinado em espectrofotômetro de absorção atômica no modo chama (AAS, Avanta 932 Plus, GBC, Hampshire, IL, USA). Para calibração do equipamento, foram utilizadas soluções padrões certificadas (Tritisol, Merck), conforme procedimentos anteriormente descritos (Martins e Bianchini, 2008; Lopes et al., 2011;. Carvalho et al., 2013;. Machado et al., 2013;. Silva et al., 2014). Os dados foram expressos em μg/g de tecido seco.

3.4.3 Expressão gênica

A expressão de genes codificantes de proteínas transportadoras de Cu foi analisada nos peixes inteiros expostos por 28 dias e nos tecidos (brânquias, fígado e intestino) dos peixes expostos por 345 dias. Após a coleta, as amostras de ambos os experimentos foram imediatamente acondicionadas em RNAlater (Ambion), mantidas a 4°C por 24 h e então armazenadas a -80°C, de acordo com as instruções do fabricante.

O RNA total foi extraído com QIAzol (Quiagen) e transcrito reversamente utilizando-se o "High Capacity cDNA Reverse Transcription kit" (Applied Biosystems), primers oligo-dT e inibidor de RNAse (Applied Biosystems). Foi utilizada PCR em tempo real (qPCR; 7300 Real-Time PCR System; Applied Biosystems) para quantificar a expressão relativa dos genes *CTR1* e *ATP7B*, utilizando-se a mistura reagente "GoTaq qPCR Master Mix" (Promega, Madison, WI, EUA).

Os primers específicos para os genes analisados foram os mesmos utilizados por Silva et al. (2014). Para ambos os genes, a expressão foi analisada em duplicata utilizando-se o seguinte protocolo: 50°C durante 2 min, 95°C durante 2 min, 45 ciclos a 95°C durante 15 s e 60°C durante 30 s. A análise da curva de fusão foi realizada sobre os produtos da PCR no final de cada ciclo, para assegurar que um determinado produto foi amplificado.

Os resultados obtidos foram normalizados utilizando os genes *EF1α* e *β-Actina* como normalizadores (Silva et al., 2014). Os valores de expressão total dos genes *CTR1* e *ATP7B* nas amostras dos peixes inteiros da exposição de curto prazo (28 dias) e de tecidos (brânquias, fígado e intestino) dos peixes da exposição de longo prazo (345 dias) foram analisados através do método $E^{-\Delta ct}$ ($E^{-(\text{gene alvo} - \text{normalizador})}$) (Schmittgen e Livak, 2008). A média dos valores de Ct dos genes normalizadores *EF1α* e *β-actina* foi utilizada no cálculo do nível de transcrição relativa para o gene alvo.

3.4.4 Consumo de oxigênio corporal

O consumo de oxigênio corporal foi medido nos peixes expostos em curto prazo (28 dias). Para medir o consumo de oxigênio corporal, os peixes foram submetidos previamente a um jejum de 12 h. O consumo individual de oxigênio foi medido utilizando-se um respirômetro estático. A concentração inicial de oxigênio dissolvido foi medida com o auxilio de um oxímetro digital (YSI modelo 55, Hexis, Ohio, EUA). Posteriormente, a aeração da água foi interrompida e a superfície do tanque foi coberta com material plástico transparente, a fim de minimizar a difusão do oxigênio do ar para a água. Após 30 min., a concentração final de oxigênio dissolvido foi medida novamente. Todas as medições foram realizadas em níveis de saturação de oxigênio > 70%. A taxa de consumo de oxigênio (CO) foi calculada usando a seguinte fórmula: CO = [(Oi – Of) x V] / B / T, onde Oi e Of são as concentrações iniciais e finais de oxigênio (mg/L O_2), respectivamente, V é o volume do tanque (L), B é a biomassa (g) e T é o

intervalo de tempo entre medições (h) (Cunha et al., 2009). Os dados foram expressos em mg O_2/g/h.

3.4.5 Atividade enzimática

A atividade das enzimas envolvidas no metabolismo energético foi medida nos peixes inteiros expostos em curto prazo (28 dias) e nos tecidos (brânquias, fígado e músculo) dos peixes expostos em longo prazo (345 dias). Em ambos os casos, as amostras foram homogeneizadas em solução tampão (pH 7,8) contendo imidazol (50 mmol/L) e PMSF (0,1 mmol/L). Os homogeneizados foram centrifugados a 10,000 g e 4ºC (Micro 22R HettichZentrifugen, Global Medical Instrumentation, Ramsey, Minnesota, EUA) por 20 min. Os sobrenadantes foram usados diretamente nos ensaios enzimáticos ou diluídos em solução tampão de homogeneização. A atividade da piruvato quinase, lactato desidrogenase e citrato sintase foi medida por espectrofotometria (Lallier e Walsh, 1991; Lauer et al., 2012), utilizando-se uma leitora de microplacas (ELX 808 Universal Microplate Reader/Bio-Teck Instruments - Winooski, Vermont, USA). Os dados foram expressos em U/mg proteínas.

3.5 Análise estatística dos dados

Os resultados foram expressos como média ± erro padrão da média. Os resultados de expressão gênica foram analisados através do método comparativo Ct (Pfaffl et al., 2002; Schmittgen e Livak, 2008). Os dados foram submetidos à análise de variância (ANOVA) de uma via, seguida do teste de Tukey. Os pressupostos da ANOVA (homogeneidade de variâncias e normalidade dos dados) foram previamente verificados. Em todos os casos, o nível de significância adotado foi de 95% ($\alpha = 0,05$).

4. RESULTADOS

Os resultados do presente estudo estão apresentados sob a forma de dois manuscritos, os quais serão submetidos para publicação à revista *AQUATIC TOXICOLOGY* (fator de impacto: 3.45 - 2015):

Manuscrito 1: Role of transporting proteins CTR1 and ATP7B on copper accumulation in newborn puppies of the viviparous fish *Poecilia vivipara* (Bloch and Schneider, 1801) acclimated to salt water

Manuscrito 2: Effects of chronic exposure to copper on growth and energy metabolism in newborn puppies of the viviparous fish *Poecilia vivipara* (Bloch and Schneider, 1801) acclimated to salt water

Role of transporting proteins CTR1 and ATP7B on copper accumulation in newborn puppies of the viviparous fish *Poecilia vivipara* (Bloch and Schneider, 1801) acclimated to salt water

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Abstract

The involvement of transporting proteins on copper (Cu) bioaccumulation was evaluated in puppies of the viviparous fish *Poecilia vivipara* acclimated to salt water (24 ppt) after chronic exposure to environmentally relevant concentrations of waterborne Cu. Newborn (<24-h old) fish (0.020 ± 0.001 g wet body mass; 10.49 ± 0.30 mm body length) were maintained under control condition (no Cu addition into the water) or exposed to different waterborne Cu concentrations (nominally: 5, 9 and 20 µg/L) for 28 and 345 days. After 28 days of exposure, whole fish were collected and stored for analysis of Cu accumulation and expression of genes encoding for the high affinity Cu-transporter (*CTR1*) and the P-type Cu-ATPase (*ATP7B*). After 345 days of exposure, whole fish were collected for analysis of Cu accumulation. Also, additional fish were collected and had their tissues dissected and stored for analyses of Cu accumulation (gills, liver, gut and muscle) and *CTR1* and *ATP7B* expression (gills, liver and gut). After 28 days of exposure to Cu, no fish mortality was observed. No fish survival was observed after exposure to 20 µg/L Cu for 345 days. Whole-body Cu accumulation was dependent on waterborne Cu concentration, being significantly higher in fish exposed for to 20 µg/L Cu for 28 days and fish exposed to 9 µg/L Cu for 345 days when compared to control fish. Furthermore, tissue Cu accumulation was also dependent on waterborne Cu concentration, being significantly higher in gills, liver and gut of fish exposed to 9 µg/L for 345 days than in control fish. However, no significant Cu accumulation was observed in fish muscle. In fish exposed for 28 days, whole-body *CTR1* expression was induced in fish exposed to 9 µg/L Cu. Also, whole-body *ATP7B* expression was induced in fish exposed to 9 and 20 µg/L Cu. In fish exposed for 345 days, no significant change in *CTR1* expression was observed in all tissues analyzed (gills, liver and gut). However, *ATP7B* expression was induced in gills and gut of fish exposed to 5 µg/L Cu and liver of fish exposed to 9 µg/L Cu. These findings indicate that Cu accumulation in *P. vivipara* (whole fish or tissues) is dependent on waterborne Cu concentration and exposure time in salt water (24 ppt). Furthermore, they suggest that Cu accumulation is associated with the expression of genes encoding for Cu-transporting proteins (CTR1 and ATP7B) in key tissues involved in Cu uptake, storage and excretion.

Keywords: accumulation, ATP7B, CTR1, chronic exposure, copper, gene expression

Introduction

Pollution of aquatic ecosystems with organic and inorganic contaminants is of increasing concern (Laws, 2000). Among the different types of pollutants, heavy metals have received significant attention due to their accumulation and high toxicity in aquatic organisms (Brown and Welton, 2008; Sánchez, 2008).

Copper (Cu) is an essential redox-active metal, which acts as structural element of regulatory proteins involved in cellular homeostasis. This metal plays an important role in physiological processes such as respiration, oxidative stress response and hormonal signaling (Knight et al., 1994; Dang et al., 2000; Bopp et al., 2008; Leary et al., 2009; Eyckmans et al., 2010; Belyaeva et al., 2011). However, Cu at elevated concentrations is toxic to organisms, causing serious cellular injuries.

In fish, Cu may induce different effects, including changes in membrane permeability, protein synthesis, enzyme activity, apoptosis, cell proliferation and dysfunctions in embryonic development, growth, reproduction and immune response (Mazon and Fernandes, 1999; Smith et al., 2001; Feng et al., 2003; James et al., 2003; Krumschnabel et al., 2005; Lugo et al., 2006; García et al., 2007; Martins and Bianchini, 2008; Monteiro et al., 2009; Liu et al., 2010; Eyckmans et al., 2010; Findik and Çiçek, 2011; Liu et al., 2011).

In vertebrates, several mechanisms are involved to ensure Cu homeostasis. The high affinity Cu-transporter (CTR1) and P-type Cu-ATPases (ATP7A and ATP7B) are important proteins involved in Cu transport across membranes and inside the cells, respectively (Lutsenko et al., 2007). In fish, the expression of proteins involved in Cu homeostasis is induced by Cu exposure and regulated at the transcriptional level (Minghetti et al., 2010; 2011). In a recent study, Silva et al., (2014) observed a direct relationship between tissue Cu content and the transcriptional level of the *CTR1* and *ATP7B* genes after acute (96 h) exposure to Cu in the viviparous fish *Poecilia vivipara*.

The guppy *P. vivipara* is one of the most common fish in lakes, rivers and coastal lagoons in Brazil (Neves and Monteiro, 2003; Gomes and Monteiro, 2008; Santos et al., 2011). Due to its wide geographic distribution, high tolerance to changes in environmental conditions and easy handling and breeding in captivity, *P. vivipara* is considered a potential model for toxicological studies with chemical contaminants (Mattos et al., 2010; Paulo et al., 2012; Zimmer et al., 2012; Machado et al., 2013; Harayashiki et al., 2013; Silva et al., 2014). In the present study, we have analyzed the

level of Cu accumulation and the effects of this metal on the expression of genes encoding for the CTR1 and ATP7B transporting proteins in newborn puppies of *P. vivipara* after chronic exposure to environmentally relevant concentrations of waterborne Cu.

Material and Methods

Fish rearing

Male and female *P. vivipara* were grown in the hatchery of the Institute of Biological Sciences of the Federal University of Rio Grande - FURG. Couples were separated and maintained in 20-L plastic tanks containing salt water at salinity 24 ppt under constant aeration. Water temperature (25°C) and photoperiod (12 h light: 12 h dark cycle) were fixed. Fish were fed twice daily with a commercial diet (Alcon Basic; 45% crude protein, 5% lipids, 2% calcium, 0.7% phosphorus and 10% humidity) until apparent satiation. Gravid females were transferred to breeding boxes. After birth, newborn puppies (<24 h) were separated and subjected to Cu exposure. All experimental procedures were previously approved by the Ethics Committee for Animal Use of the University (CEUA/FURG; permit # P014/2012).

Experimental design

In a first experiment, newborn (<24-h old) puppies (6.2 ± 0.1 mg; 8.0 ± 0.1 mm) were kept under control condition (no Cu addition into the water) or exposed to different Cu concentrations (nominally: 5, 9 and 20 µg/L) for 28 days. These concentrations were obtained by dilution of Cu standard solution prepared with $CuCl_2$ (Vetec Química Fina, São Paulo, Brazil). They were selected considering the current Brazilian water quality criteria for Cu in sea water (5 µg/L) and fresh water (9 µg/L). The concentration of 20 µg/L was selected as a non-conforming condition (CONAMA, 2005). Each treatment was performed in triplicate. Experimental media were completely renewed every day. Fish density was always < 1 g/L. Fish were maintained in 1-L glass aquaria with salt water at salinity 24 ppt, pH 7.66 ± 0.21, continuously aerated (oxygen saturation >90%). Water temperature (28°C) and photoperiod (12 h light:12 h dark cycle) were fixed. Fish were fed daily until apparent satiation with the same commercial diet used during the fish rearing period. After exposure, fish were collected, quickly rinsed in control sea water (24 ppt salinity), kept on ice, killed by sectioning of the

spinal cord and immediately stored in ultrafreezer (-80ºC) or RNAlater (Ambion) for further analysis of Cu accumulation and gene expression, respectively. Samples for gene expression were held at 4°C for 24 h, and then stored in ultrafreezer (-80°C) until analysis.

In a second experiment, newborn (<24-h old) puppies (wet body mass: 6.3 ± 0. 1 mg; standard body length: 7.16 ± 0.13 mm) were kept under control condition or exposed to the different Cu concentrations (nominally: 5, 9 and 20 µg/L) for 345 days. Fish were maintained in 10-L glass aquaria under the same conditions described for the first experiment. However, the experimental media were completely renewed every week. Fish were fed daily until apparent satiation with the same commercial diet used during the fish rearing period. After exposure, whole fish were collected, quickly rinsed in control salt water (salinity 24 ppt) and immediately stored in ultrafreezer (-80^{o}C) or in RNAlater for further analysis of Cu accumulation and gene expression, respectively. Samples for gene expression were held at 4°C for 24 h, and then stored in ultrafreezer (-80°C) until analysis. Also, additional fish were kept on ice, killed by sectioning of the spinal cord and had their tissues (gill, liver, gut and muscle) dissected and immediately stored as described for whole fish samples.

Whole-body and tissue Cu concentration

Whole-body Cu concentration was measured in fish of the first experiment. Whole-body and tissue (gills, liver, gut and muscle) Cu concentrations were measured in fish of the second experiment. Samples of both experiments were randomly collected, weighed (wet weight) and dried (50ºC) until constant weight (dry weight). Dried samples were completely digested in 65% nitric acid. Cu concentration was determined by atomic absorption spectrophotometry in the flame mode (AAS, Avanta 932 Plus, GBC, Hampshire, IL, USA), following procedures previously described (Martins and Bianchini, 2008; Lopes et al., 2011; Carvalho et al., 2013; Machado et al., 2013; Silva et al., 2014). Cu concentrations were calculated based on a standard curve built with certified standards (Tritisol-Merck). Data were expressed as µg/g dry tissue.

Gene expression analysis

Gene expression was determined in whole fish samples of the first experiment and in whole fish and tissues (gill, liver and gut) samples of the second experiment. Total RNA was extracted with QIAzol (Quiagen) and reversely transcribed with the

High Capacity cDNA Reverse Transcription kit (Applied Biosystems), oligo-dT primers and RNAse inhibitor (Applied Biosystems). Real time PCR (qPCR; 7300 Real-Time PCR System; Applied Biosystems) was used to quantify the relative expression of *CTR1* and *ATP7b* genes using the GoTaq qPCR Master Mix (Promega, Madison, WI). The gene-specifc primers used were those previously described by Silva et al. (2014). For both genes, expression was analyzed in duplicate using the following protocol: 50°C for 2 min, 95°C for 2 min, 45 cycles at 95°C for 15 s, and 60°C for 30 s. Melting curve analysis was performed on the PCR products at the end of each PCR run to ensure that a single product was amplified. The obtained results were normalized using EF1α and β-Actin as housekeeping genes (Silva et al., 2014). The relative values for the total gene expression of *CTR1* and *ATP7B* in whole fish and tissues samples were analyzed by the $E^{-\Delta ct}$ ($E^{-(\text{target gene} - \text{housekeeping gene})}$) method (Schmittgen and Livak, 2008). The average of the housekeeping genes (EF1α and β-Actin) Ct values was used in the calculation of the relative transcriptional level for the target gene.

Data presentation and statistical analysis

Data are expressed as means ± standard error. For each experiment, mean values among treatments were compared using analysis of variance (ANOVA) followed by the Tukey test (parametric data) or the Kruskal-Wallis ANOVA followed by the Multiple Comparisons of Mean Ranks test (non-parametric data). In all cases, the significance level adopted was 95% ($\alpha = 0.05$). Analyses were performed using the software Statistica 7.0 (StatSoft, Tulsa, OK, USA).

Results

For both experiments (28 and 345 days), there was no significant difference between total and dissolved Cu concentrations in each treatment. However, total or dissolved Cu concentrations significantly increased among treatments. For the first experiment, total Cu concentrations corresponded to 1.8 ± 0.3, 7.1 ± 0.5, 10.9 ±1.3 and 21.0 ± 5.6 µg/L for the nominal concentrations of 0, 5, 9 and 20 µg/L, respectively. Dissolved Cu concentrations corresponded to 1.7 ± 0.4, 7.2 ± 0.8, 10.1 ± 1.2 and 16.9 ± 1.6 µg/L, respectively. For the second experiment, total Cu concentrations corresponded to 1.8 ± 0.4, 7.1 ± 0.5, 10.9 ±1.3 and 21.0 ± 5.6 µg/L for the nominal concentrations of

0, 5, 9 and 20 µg/L, respectively. Dissolved Cu concentrations corresponded to 1.3 ± 0.2, 7.2 ± 0.8, 10.1 ± 1.2 and 16.9 ± 1.6 µg/L, respectively.

In the first experiment (28 days), fish survival was not affected by Cu exposure. In the second experiment (345 days), 100% mortality was observed within the first 7 weeks of fish exposure to 20 µg/L Cu. After 28 days of exposure, whole-body Cu concentration was higher in fish exposed to 20 µg/L Cu than in control fish and those exposed to 5 µg/L Cu. After 345 days of exposure, whole-body Cu concentration was higher in fish exposed to 9 µg/L Cu than in control fish and those exposed to 5 µg/L Cu (Fig. 1). Tissue Cu accumulation was also analyzed in fish exposed for 345 days. For gills and gut, Cu concentration was higher in fish exposed to 9 µg/L Cu than in fish kept under control condition. In turn, Cu concentration was higher in liver of fish exposed to 9 µg/L Cu than in liver of control fish and those exposed to 5 µg/L Cu. On the other hand, muscle Cu concentration was not affected by Cu exposure (Table 1).

Whole-body expression of *CTR1* and *ATP7B* was affected by exposure of fish to Cu for 28 days. *CTR1* was up-regulated in fish exposed to 9 µg/L Cu respect of control fish. In turn, *ATP7B* was up-regulated in fish exposed to 9 and 20 µg/L Cu respect of those kept under control condition (Fig. 2). In tissues (gill, liver and gut), *CTR1* expression was not affected by fish exposure to Cu for 345 days. However, *ATP7B* was up-regulated in gill and gut of fish exposed to 5 µg/L Cu, as well as in liver and gut of fish exposed to 9 µg/L Cu (Fig. 3).

Discussion

In the present study we provide information on the effects of chronic Cu exposure on Cu accumulation and gene expression of Cu-transporting proteins in puppies of the viviparous guppy *P. vivipara*. At the best of our knowledge, this is the first paper to report findings on the Cu effects in a saltwater fish after an extremely long time of exposure (345 days) to the waterborne metal. Furthermore, exposures were performed using neonates fish (<24 days after born).

Whole-body Cu accumulation in newborn guppies was dependent on the time of exposure and Cu concentration in salt water, being significantly higher in fish exposed to 20 µg/L Cu for 28 days and to 9 µg/L Cu for 345 days. Indeed, a concentration- and time-dependent whole-body Cu accumulation is a very common feature observed in teleost fish after exposure to waterborne Cu (Ay et al., 1999; Mazon and Fernandes,

1999; McGeer et al, 2000a,b; Taylor et al., 2000; Grosell et al., 2004; James et al., 2008). However, recent studies with adult *P. vivipara* (Machado et al., 2013; Silva et al., 2014) reported no significant whole-body Cu accumulation after exposure to the same Cu concentrations used in the present study. However, it is important to note that these studies were performed for only 96 h. Therefore, the difference observed between the present study and the previous ones is clearly associated with the time of exposure to Cu, which was much longer in the present study. Also, these findings indicate that the guppy *P. vivipara* shows an efficient mechanism to maintain the whole-body Cu homeostasis when exposed for a short period of time (96 h) to environmentally relevant concentrations of waterborne Cu. However, it loses this ability when exposed to these Cu concentrations for longer periods of time (28 or 345 days).

Whole-body and tissue concentrations of essential metals like Cu tend to be highly regulated when compared to those of non-essential metals (Pereira et al., 2009; Wood et al., 2011). Elevated Cu burden was already reported in the gut of adult guppies *P. vivipara* after acute exposure (96 h) to the same waterborne Cu concentrations used in the present study (Machado et al., 2013; Silva et al., 2014). In fish exposed to Cu for 345 days, tissue Cu concentration followed the sequence: liver > gut > gill > muscle. Furthermore, a concentration-dependent Cu accumulation was observed in liver, gut and gills, with significantly increased values being observed in gills and gut of fish exposed to 9 µg/L Cu, as well as in liver of fish exposed to 5 and 9 µg/L. Several studies have reported the involvement and importance of these tissues in the maintenance of Cu homeostasis in teleost fish (Grosell et al., 2004; Minghetti et al., 2010; Silva et al., 2014). In fact, gill and gut are organs with epithelial surface directly in contact with the Cu containing environment, thus playing an important role in Cu uptake. In turn, liver is a tissue mainly involved in Cu storage, detoxification and excretion in fish (Grosell et al., 1997; McGeer et al., 2000b; Grosell et al., 2002; Minghetti et al., 2010).

In aquatic animals, regulation of Cu homeostasis is dependent on metal uptake, storage and excretion (Depledge and Rainbow, 1990; Clearwater et al., 2002; Wood et al., 2011). In turn, Cu uptake is dependent on the regulation of membrane permeability and the amount of permeable membrane to body size over which Cu can be absorbed (Wood et al., 2011). In the present study, we evaluated the involvement of the Cu-transporting proteins CTR1 and ATP7B in Cu accumulation in the viviparous guppy *P. vivipara*, through analysis of the expression of genes encoding for these proteins (*CTR1* and *ATP7B*).

Expression of both *CTR1* and *ATP7B* was affected by exposure of newborn *P. vivipara* to environmentally relevant concentrations of waterborne Cu for 28 or 345 days. As observed for Cu accumulation, whole-body transcriptional level of *CTR1* and *ATP7B* in *P. vivipara* was dependent on waterborne Cu concentration, being significantly up-regulated in fish exposed to 9 µg/L Cu. However, it is worth to mention that *ATP7B* expression was also up-regulated in fish exposed to 20 µg/L while *CTR1* expression was recovered back to the control level. In addition, no significant change in *CTR1* expression was observed in all tissues analyzed (gills, liver and gut) after fish exposure to waterborne Cu for 345 days. On the other hand, the transcriptional level of *ATP7B* was up-regulated in all tissues analyzed of fish exposed to 5 and/or 9 µg/L Cu.

Findings described above for *CTR1* expression in *P. vivipara* can be explained considering the fact that the high-affinity Cu transporter 1 (CTR1) is a highly conserved transmembrane protein that mediates the internalization specifically of Cu ions from the extracellular medium (Linder and Hazegh-Azam, 1996; Grosell and Wood, 2002; Bury et al., 2003; Sharp, 2003; Mackenzie et al., 2004; Wood et al., 2011). Therefore, the lack of response in the whole-body *CTR1* expression in fish exposed to a high concentration of waterborne Cu (20 µg/L) for 28 days, as well as in key tissues of fish exposed to environmentally relevant Cu concentrations (5 and 9 µg/L) for 345 days could be part of a negative feedback mechanism in attempt to limit the excessive Cu uptake from the dissolved phase in salt water (Minghetti et al., 2008; Silva et al., 2014). This statement is based on the fact that fish exposed to 20 µg/L Cu for 28 days showed a significant whole-body Cu accumulation and those exposed to 5 and/or 9 µg/L Cu for 345 days had significant accumulation of Cu in key tissues involved in Cu uptake, storage, detoxification and excretion (gills, liver and gut). In addition, these findings indicate that whole-body Cu accumulation in fish exposed to an elevated Cu concentration (20 µg/L) for a short period of time (28 days) or tissue Cu accumulation in fish chronically exposed (345 days) to a relatively low concentration of Cu (9 µg/L) seems not to be associated to CTR1, but an alternative mechanism of metal uptake (Lee et al., 2002).

In turn, the generalized up-regulation of *ATP7B* expression observed in whole body (28-days exposure) and tissues (345-days exposure) of *P. vivipara* can be explained considering the fact that the Cu-transporting ATPase (ATP7B) is a key molecule involved in the process of regulation and maintenance of Cu homeostasis inside the fish cells (Minghetti et al., 2010; La Fontaine and Mercer, 2007; La Fontaine

et al., 2010). In fact, the expression of the gene encoding for this protein (*ATP7B*) was shown to be regulated at the transcriptional level after fish exposure to Cu (Minghetti et al., 2010; Silva et al., 2014). Therefore, the up-regulation in whole-body *ATP7B* expression observed in *P. vivipara* exposed to 20 µg/L Cu for 28 days, as well as in key tissues of *P. vivipara* exposed to 9 µg/L Cu for 345 days could be an attempt to deal with the excessive amount of Cu accumulated in *P. vivipara* maintained under those experimental conditions.

Previous studies also reported that *CTR1* and *ATP7B* expression is regulated at the transcriptional level after fish exposure to Cu. As observed in the present study, they also showed that these genes are differentially expressed in each particular tissue (Minghetti et al., 2008; Minghetti et al., 2010; Silva et al., 2014). However, it is important to stress the different profiles of *CTR1* and *ATP7B* expression according to the time of exposure to waterborne Cu. Silva et al. (2014) showed a down-regulation of *CTR1* expression in gills of the guppy *P. vivipara* acclimated to salt water and exposed to 5 µg/L Cu for a short period of time (96 h). In the present study, we have observed a whole-body up-regulation of this gene after *P. vivipara* exposure to Cu for 28 days, a longer period of exposure than that used by Silva et al. (2014). Also, Minghetti et al. (2008) reported an induction of *CTR1* expression in gut and kidney of the sea bream *S. aurata* after exposure to waterborne Cu for 30 days. Finally, a lack of response in tissue *CTR1* expression was observed after a much longer time of exposure (345 days) of *P. vivipara* to environmentally relevant concentrations of waterborne Cu.

Regarding *ATP7B*, this gene showed a different profile of expression as a function of the exposure time to waterborne Cu when compared that observed for *CTR1*. Silva et al. (2014) reported a down-regulation of the expression in the gut of fish exposed to 9 and 20 µg/L Cu for a short period of time (96 h). In turn, we have observed an up-regulation of the whole-body *ATP7B* expression after exposure of *P. vivipara* to Cu for 28 days, as well as an up-regulation of *ATP7B* expression in tissues of *P. vivipara*, including the gut, after fish exposure to 9 µg/L Cu for 345 days.

In summary, our findings show that the viviparous guppy *P. vivipara* is not able to maintain whole-body Cu homeostasis when exposed to 20 µg/L Cu for 28 days or to 9 µg/L for 345 days in salt water (salinity 24 ppt). Also, they clearly indicate that Cu accumulation and expression of genes encoding for Cu-transporting proteins (CRT1 and ATP7B) in the guppy *P. vivipara* is dependent on both Cu concentration and the exposure time to the metal. In addition, they provide evidence of the involvement of

CTR1 and ATP7B in the accumulation of Cu at whole body or tissue level in the guppy *P. vivipara* after chronic exposure to environmentally relevant concentrations of waterborne Cu. Finally, our data together with other reported in the literature for *P. vivipara* suggest that *CTR1* and *ATP7B* have different profiles of expression along the chronic exposure of fish to waterborne Cu in salt water. The profile of expression showed by *CTR1* suggests an adaptive response of the encoded protein (high-affinity Cu transporter 1) in attempt to limit the excessive uptake of Cu from the dissolved phase at elevated concentrations of waterborne Cu in salt water. In turn, the profile of expression showed by *ATP7B* suggests an important role of the encoded protein (Cu-transporting ATPase) in the intracellular distribution and fate of Cu in cells of key tissues (gills, liver and gut) involved in Cu homeostasis in the viviparous guppy *P. vivipara*.

Acknowledgements

This research was financially supported by the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq, Brasília, DF, Brazil) and "Coordenação de Apoio ao Pessoal de Ensino Superior" (CAPES, Brasília, DF, Brazil). A. Bianchini is a research fellow from the Brazilian CNPq (Proc. #304430/2009-9) and is supported by the International Research Chair Program from the International Development Research Center (DRC; Ottawa, ON, Canada).

References

Ay, Ö., Kalay, M., Tamer, L., Canli, M. 1999. Copper and lead accumulation in tissues of a freshwater fish *Tilapia zillii* and its effects on the branchial Na,K-ATPase activity. Bull. Environ. Contam. Toxicol. 62, 160-168.

Belyaeva, E.A, Korotkov, S.M., Saris, N-E. 2011. In vitro modulation of heavy metal-induced rat liver mitochondria dysfunction: a comparison of copper and mercury with cadmium. J. Trace Elem. Med. Biol. 25, 63-73.

Bopp, S.K., Abicht, H.K., Knauer, K. 2008. Copper-induced oxidative stress in rainbow trout gill cells. Aquat. Toxicol 86, 197-204.

Brown, S.E., Welton, W.C. 2008. Heavy metal pollution. Nova Science Publishers, Hauppauge, NY, USA. 381 p.

Bury, N.R. 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. 206, 11-23.

Carvalho, P.C., Bugoni, L., McGill, R.A.R., Bianchini, A. 2013. Metal and selenium concentrations in blood and feathers of petrels of the genus *Procellaria*. Environ. Toxicol. Chem. 32, 1641-1648.

Clearwater, S.J., Farag A.M., Meyer J.S. 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp. Biochem. Physiol. Part C 132, 269-313.

CONAMA - Conselho Nacional do Meio Ambiente. 2005. Resolução CONAMA nº 357. http://www.mma.gov.br/port/conama.

Dang, Z.C., Flik, G., Ducouret, B., Hogstrand, C., Wendelaar Bonga, S.E., Lock, R.A. 2000. Effects of copper on cortisol receptor and metallothionein expression in gills of *Oncorhynchus mykiss*. Aquat. Toxicol. 51, 45-54.

Depledge, M.H., Rainbow, P.S. 1990. Models of regulation and accumulation of trace-metals in marine-invertebrates. Comp. Biochem. Physiol. Part C 97, 1-7.

Eyckmans, M., Tudorache, C., Darras, V.M., Blust, R., De Boeck, G. 2010. Hormonal and ion regulatory response in three freshwater fish species following waterborne copper exposure. Comp. Biochem. Physiol. Part C 152, 270-278.

Feng, Q., Boone, A.N., Vijayan, M.M. 2003. Copper impact on heat shock protein 70 expression and apoptosis in rainbow trout hepatocytes. Comp. Biochem. Physiol. Part C 135, 345-355.

Findik, Ö., Çiçek, E. 2011. Metal concentrations in two bioindicator fish species, *Merlangius merlangus*, *Mullus barbatus*, captured from the West Black Sea coasts (Bartin) of Turkey. Bull. Environ. Contam. Toxicol. 87, 399-403.

García, N., Martínez-Abundis, E., Pavón, N., Correa, F., Chávez, E. 2007. Copper induces permeability transition through its interaction with the adenine nucleotide translocase. Cell Biol. Intern. 31, 893-899.

Gomes Jr., J.L., Monteiro, L.R. 2008. Morphological divergence patterns among populations of *Poecilia vivipara* (Teleostei Poeciliidae): test of an ecomorphological paradigm. Biol. J. Linnean Soc. 93, 799-812.

Grosell, M.H., Hogstrand, C., Wood, C.M. 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 38, 257-276.

Grosell, M., McDonald, M.D., Walsh, P.J., Wood, C.M. 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. Aquat. Toxicol. 68, 263-275.

Grosell, M., Wood, C. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 1188, 1179-1188.

Harayashiki, C.A.Y., Varela, A.S., Machado, A.A.D.S., Cabrera, L.D.C., Primel, E.G., Bianchini, A., Corcini, C.D. 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquat. Toxicol. 142-143, 176-84.

James, R., Sampath, K. 2008. Effects of copper toxicity on growth, reproduction and metal accumulation in chosen ornamental fishes. Ecohydrol. Hydrobiol. 8, 89-97.

James, R., Sampath, K., Edward, D.S. 2003. Copper toxicity on growth and reproductive potential in an ornamental fish, *Xiphophorus helleri*. Asian Fisher. Sci. 16, 317-326.

Knight, S.A., Tamai, K.T., Kosman, D.J., Thiele, D.J. 1994. Identification and analysis of a *Saccharomyces cerevisiae* copper homeostasis gene encoding a homeodomain protein. Mol. Cell. Biol. 14, 7792-804.

Krumschnabel, G., Manzl, C., Berger, C., Hofer, B. 2005. Oxidative stress, mitochondrial permeability transition, and cell death in Cu-exposed trout hepatocytes. Toxicol. Appl. Pharmacol. 209, 62-73.

La Fontaine, S., Ackland, M.L., Mercer, J.F.B. 2010. Mammalian copper-transporting P-type ATPases, ATP7A and ATP7B: emerging roles. Intern. J. Biochem. Cell Biol. 42, 206-209.

La Fontaine, S., Mercer, J.F.B. 2007. Trafficking of the copper-ATPases, ATP7A and ATP7B: role in copper homeostasis. Arch. Biochem. Biophys. 463, 149-167.

Laws, E.A. 2000. Aquatic Pollution: An Introductory Text. John Wiley & Sons, New York, 672 p.

Leary, S.C., Winge, D.R., Cobine, P.A. 2009. "Pulling the plug" on cellular copper: the role of mitochondria in copper export. Biochim. Biophys. Acta. 1793, 146-153.

Lee, J., Petris, M.J., Thiele, D.J. 2002. Characterization of mouse embryonic cells deficient in the CTR1 high affinity copper transporter. Identification of a CTR1-independent copper transport system. J. Biol. Chem. 277, 40253-40259.

Linder, M.C., Hazegh-Azam, M. 1996. Copper biochemistry and molecular biology. Amer. J. Clin. Nutr. 63, 797S-811S.

Liu, F., Ni, H.-G., Chen, F., Luo, Z.-X., Shen, H., Liu, L. 2011. Metal accumulation in the tissues of grass carps (*Ctenopharyngodon idellus*) from fresh water around a copper mine in Southeast China. Environ. Monit. Assess. 184, 4289-4299.

Liu, X.J., Luo, Z., Xiong, B.X., Liu, X., Zhao, Y.H., Hu, G.F. 2010. Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. Ecotox. Environ. Safe. 73, 1286-1291.

Lopes, T.M., Barcarolli, I.F., Oliveira, C.B., Souza, M.M., Bianchini, A. 2011. Mechanisms of copper accumulation in isolated mantle cells of the marine clam *Mesodesma mactroides*. Environ. Toxicol. Chem. 30, 1586-1592.

Lugo, R.S., Nathalí, G., Villalobos de B, L.B., Mairin, L. 2006. Immunological response of the freshwater fish *Colossoma macropomum* as a biomarker of copper exposure. Bull. Environ. Contam. Toxicol. 77, 925-930.

Lutsenko, S., Barnes, N.L., Bartee, M.Y., Dmitriev, A.O.Y. 2007. Function and regulation of human copper-transporting ATPases. Physiol. Rev. 87, 1011-1046.

Machado, A.A.S., Hoff, M.L.M., Klein, R.D., Cardozo, J.G., Giacomin, M.M., Pinho, G.L.L., Bianchini, A. 2013. Biomarkers of waterborne copper exposure in the guppy *Poecilia vivipara* acclimated to salt water. Aquat. Toxicol. 138-139, 60-69.

Mackenzie, N.C., Brito, M., Reyes, A.E., Allende, M.L. 2004. Cloning, expression pattern and essentiality of the high-affinity copper transporter 1 (CTR1) gene in zebrafish. Gene 328, 113–120.

Martins, S.E., Bianchini, A. 2008. Copper accumulation and toxicity in the Plata pompano *Trachinotus marginatus* Cuvier 1832 (Teleostei, Carangidae). Pan Amer. J. Aquat. Sci. 1832, 384-390.

Mattos, J.J., Siebert, M.N., Luchmann, K.H., Granucci, N., Dorrington, T., Stoco, P.H., Grisard, E.C., Bainy, A.C.D. 2010. Differential gene expression in *Poecilia vivipara* exposed to diesel oil water accommodated fraction. Mar. Environ. Res. 69, S31-S33.

Mazon, A.F., Fernandes, M.N. 1999. Toxicity and differential tissue accumulation of copper in the tropical freshwater fish, *Prochilodus scrofa* (Prochilodontidae). Bull. Environ. Contam. Toxicol. 63, 797-804.

McGeer, J., Szebedinszky, C. 2000a. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout 2: tissue specific metal accumulation. Aquat. Toxicol. 50, 245-256.

McGeer, J., Szebedinszky, C., McDonald, D., Wood, C. 2000b. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231-243.

Minghetti, M., Leaver, M.J., Carpenè, E., George, S.G. 2008. Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in

tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper. Comp. Biochem. Physiol. Part C 147, 450-459.

Minghetti, M., Leaver, M.J., George, S.G. 2010. Multiple Cu-ATPase genes are differentially expressed and transcriptionally regulated by Cu exposure in sea bream, *Sparus aurata*. Aquat. Toxicol. 97, 23-33.

Minghetti, M., Leaver, M.J., Taggart, J.B., Casadei, E., Auslander, M., Tom, M., George, S.G. 2011. Copper induces Cu-ATPase ATP7A mRNA in a fish cell line, SAF1. Comp. Biochem. Physiol. Part C 154, 93-99.

Monteiro, S.M., Dos Santos, N.M., Calejo, M., Fontainhas-Fernandes, A., Sousa, M. 2009. Copper toxicity in gills of the teleost fish, *Oreochromis niloticus*: effects in apoptosis induction and cell proliferation. Aquat. Toxicol. 94, 219-228.

Neves, F.M., Monteiro, L.R. 2003. Body shape and size divergence among populations of *Poecilia vivipara* in coastal lagoons of south-eastern Brazil. J. Fish Biol. 63, 928-941.

Paulo, D.V., Fontes, F.M., Flores-Lopes, F. 2012. Histopathological alterations observed in the liver of *Poecilia vivipara* (Cyprinodontiformes: Poeciliidae) as a tool for the environmental quality assessment of the Cachoeira River, BA. Braz. J. Biol. 72, 131-140.

Pereira, P., de Pablo, H., Vale, C., Pacheco, M. 2009. Combined use of environmental data and biomarkers in fish (*Liza aurata*) inhabiting a eutrophic and metal-contaminated coastal system – Gills reflect environmental contamination. Mar. Environ. Res. 69, 53-62.

Sánchez, M.L. 2008. Causes and effects of heavy metal pollution. Nova Science Publishers, Hauppauge, NY, USA. 369 p.

Santos, E.G.N., Cunha, R.A., Santos, C.P. 2011. Behavioral responses of *Poecilia vivipara* (Osteichthyies: Cyprinodontiformes) to experimental infections of *Acanthocollaritrema umbilicatum* (Digenea: Cryptogonimidae). Exp. Parasitol. 127, 522-526.

Schmittgen, T.D., Livak, K.J. 2008. Analyzing real-time PCR data by the comparative C_t method. Nature Protocols 3, 1101-1108.

Sharp, P.A. 2003. CTR1 and its role in body copper homeostasis. Int. J. Biochem. Cell Biol. 35, 288-291.

Silva, E.S., Abril, S.I.M., Zanette, J., Bianchini, A. 2014. Salinity-dependent copper accumulation in the guppy *Poecilia vivipara* is associated with *CTR1* and *ATP7B* transcriptional regulation. Aquat. Toxicol. 152, 300-307.

Smith, R.W, Blaney, S.C., Dowling, K., Sturm, A., Jönsson, M., Houlihan, D.F. 2001. Protein synthesis costs could account for the tissue-specific effects of sub-lethal copper on protein synthesis in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 53, 265-277.

Taylor, L., McGeer, J. 2000. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators. Environ. Toxicol. Chem. 19, 2298-2308.

Wood, C.M., Farrell, A.P., Brauner, C.J. 2011. Fish physiology: Homeostasis and toxicology of essential metals. Fish Physiology, Vol. 31A, Academic Press, London, UK.

Zimmer, A.M., Barcarolli, I.F., Wood, C.M., Bianchini, A. 2012. Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. Aquat. Toxicol. 122-123, 172-180.

Table 1. Copper concentration (µg Cu/g dry weight) in tissues of newborn puppies of the guppy *Poecilia vivipara* exposed to waterborne copper for 345 days. Data are expressed as mean ± SEM (n = 5-6). Different letters indicate significant difference among treatments for the same tissue ($p<0.05$).

Tissue	Nominal copper concentration (µg/L)		
	Control	5	9
Gill	4.7 ± 1.5^{a}	16.5 ± 3.5^{ab}	21.2 ± 4.6^{b}
Liver	11.3 ± 3.3^{a}	62.5 ± 17.5^{a}	202.4 ± 32.5^{b}
Gut	3.2 ± 0.8^{a}	22.7 ± 5.5^{ab}	42.1 ± 9.2^{b}
Muscle	2.0 ± 0.2^{a}	1.8 ± 0.6^{a}	2.7 ± 0.7^{a}

Figure Legends

Figure 1. Whole-body copper concentration in newborn guppies *Poecilia vivipara* exposed to waterborne copper for 28 and 345 days. Data are expressed as mean ± standard error (n = 5). Different small and capital letters indicate significant different mean values among treatments for fish exposed for 28 and 345 days, respectively ($p<0.05$).

Figure 2. Transcriptional level of genes encoding for the high-affinity Cu transporter (*CTR1*) and Cu-transporting ATPase (*ATP7B*) in whole-body of newborn guppies *Poecilia vivipara* exposed to waterborne copper for 28 days. Data are expressed as mean ± SEM (n = 8-9). Different small and capital letters indicate significant different mean values among treatments for *CTR1* and *ATP7B*, respectively ($p<0.05$).

Figure 3. Transcriptional level of genes encoding for the high-affinity Cu transporter (*CTR1*) and Cu-transporting ATPase (*ATP7B*) in gill (A), liver (B) and gut (C) of newborn guppies *Poecilia vivipara* exposed to waterborne copper for 345 days. Data are expressed as mean ± SEM (n = 6-10). Different small and capital letters indicate significant different mean values among treatments for *CTR1* and *ATP7B*, respectively ($p<0.05$).

Figure 1

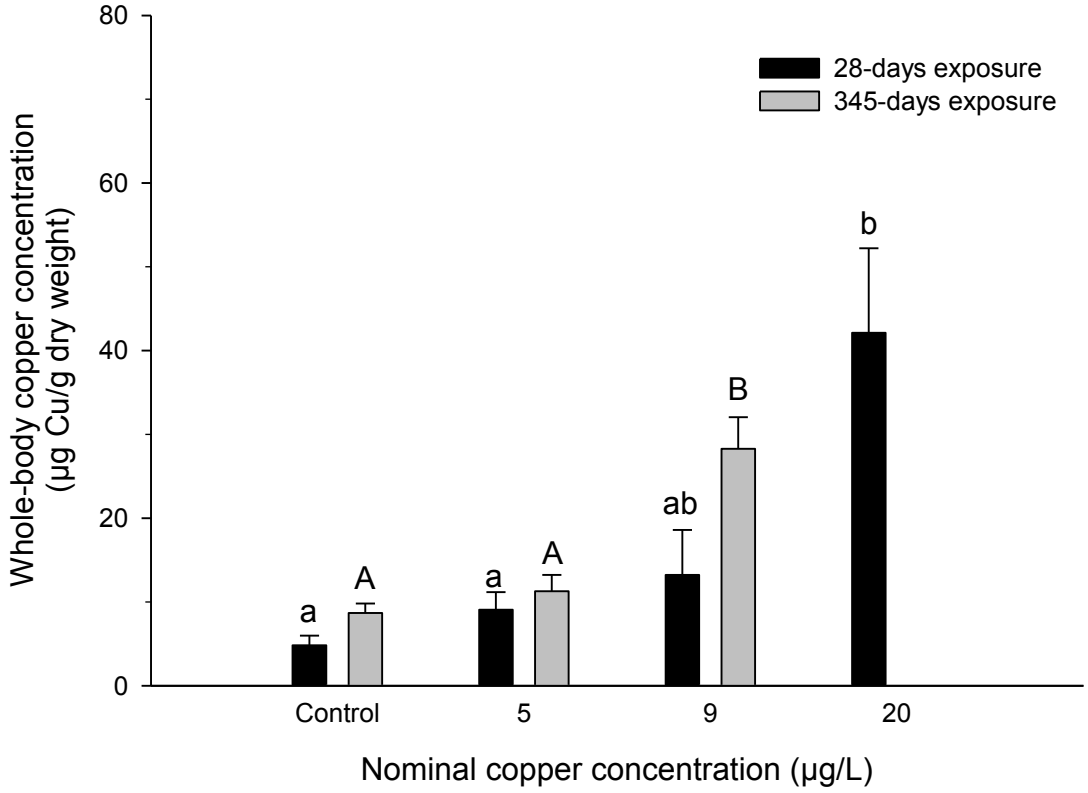

80
60
40
20
0
Whole-body copper concentration
(µg Cu/g dry weight)
28-days exposure
345-days exposure
a
A
a
A
ab
B
b
Control
5
9
20
Nominal copper concentration (µg/L)

Figure 2

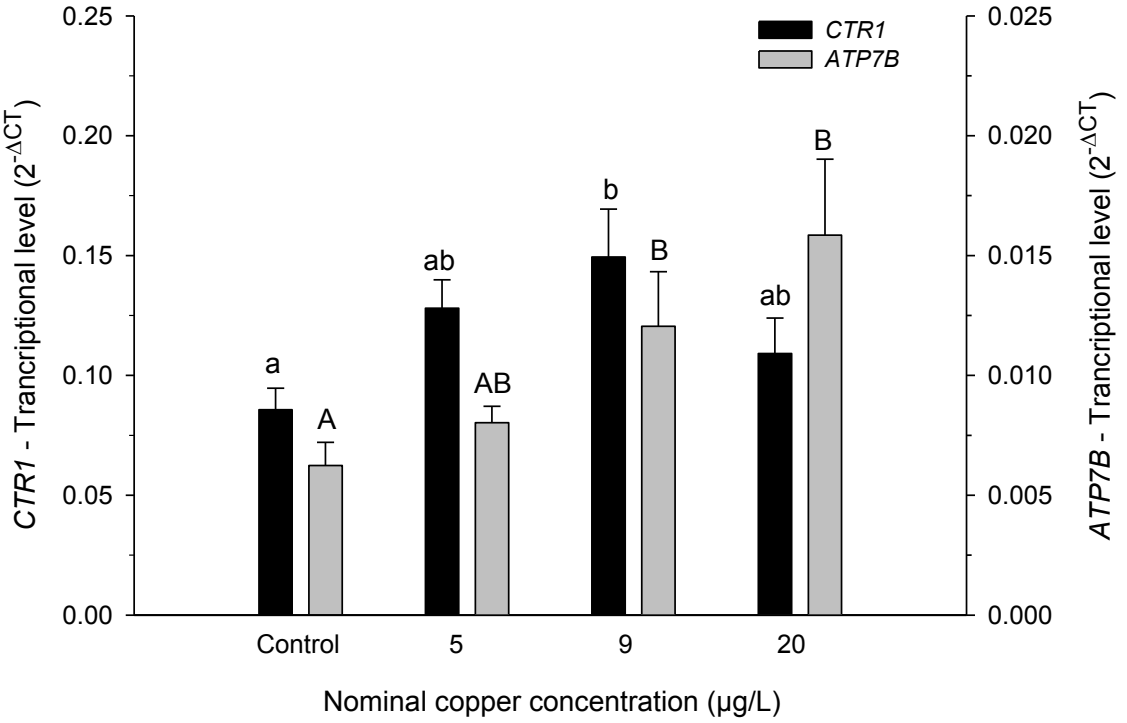
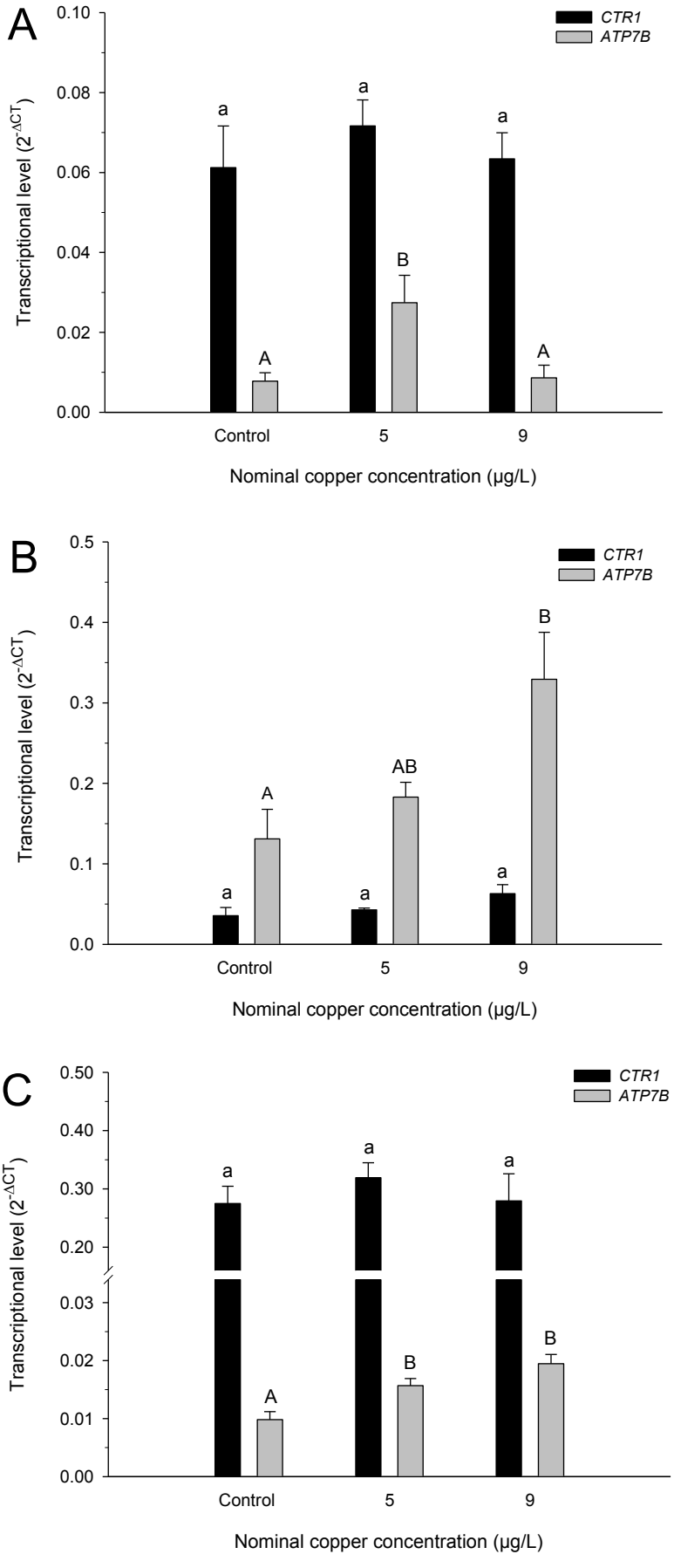

CTR1
ATP7B
0.25
0.20
0.15
0.10
0.05
0.00
0.025
0.020
0.015
0.010
0.005
0.000
CTR1 - Trancriptional level (2-ΔCT)
ATP7B - Trancriptional level (2-ΔCT)
a
A
ab
AB
b
B
ab
B
Control
5
9
20
Nominal copper concentration (µg/L)

Figure 3


A
B
C
CTR1
ATP7B
Transcriptional level ($2^{-\Delta CT}$)
Nominal copper concentration (µg/L)
Control
5
9
a
A
B
AB

Chronic effects of copper on growth and energy metabolism in newborn puppies of the viviparous fish *Poecilia vivipara* (Bloch and Schneider, 1801) in salt water

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Abstract

In a previous study, we have shown that copper (Cu) is significantly accumulated in whole body of newborn puppies of the viviparous guppy *Poecilia vivipara* after exposure to environmentally relevant concentrations of waterborne Cu for 28 days (20 µg/L Cu) and 345 days (9 µg/L Cu). Also, Cu accumulation was observed in key tissues (gills, liver and gut) of guppies exposed to 9 µg/L Cu for 345 days. Therefore, the aim of the present study was to analyze the effects of Cu accumulation on growth and energy metabolism of newborn puppies of *P. vivipara* acclimated to salt water. In a first experiment, newborn puppies were kept under control condition (no Cu addition in salt water) or exposed to different concentrations of waterborne Cu (nominally: 5, 9 and 20 µg/L) for 28 days. After exposure, whole-body oxygen consumption was measured and fish were killed, measured, weighed and stored for further biochemical analysis. In a second experiment, newborn puppies were kept under control condition or exposed to the same Cu concentrations used in the first experiment for 345 days. After exposure, fish were killed, measured, weighed and had their tissues (gills, liver and muscle) dissected and stored for further biochemical analysis. In samples from both experiments (whole fish and tissues), biochemical parameters analyzed included pyruvate kinase (PK), lactate dehydrogenase (LDH) and citrate synthase (CS) activity. After 28 days of exposure, no significant effect of Cu was observed on fish survival, growth and enzyme activity. However, a significant increase in whole-body oxygen consumption was observed in guppies exposed to 9 and 20 µg/L Cu. After 345 days of exposure, no fish survival was observed at 20 µg/L Cu. In addition, growth was significantly reduced in male guppies exposed to 9 µg/L Cu and female guppies exposed to 5 and 9 µg/L Cu. Furthermore, a significant increase in CS activity was observed in liver of guppies exposed to 9 µg/L Cu. Therefore, findings reported in the present study provide evidence that chronic exposure to environmentally relevant concentrations of Cu in salt water affects survival and growth of newborn puppies of the viviparous guppy *P. vivipara*. Also, they suggest that the chronic Cu effect on fish growth is associated with an increased energy demand to deal with Cu homeostasis and/or an impairment in the mitochondrial respiratory chain.

Keywords: copper, chronic exposure, growth, guppy, metabolism, salt water

Introduction

Copper (Cu) is considered one of the most important metal in nature. It is employed in electricity supply, electronics factoring, food production, as well as building, pharmaceutical, automotive and naval industry (CDA, 2015). Therefore, human activities have been considered of great impact on the contamination of aquatic environments with Cu. Regarding the biological role, Cu is considered as an essential metal. It can be easily subjected to changes in its oxidation state and donate or accept electrons. Therefore, Cu acts as a structural element of regulatory proteins involved in cellular homeostasis (Linder and Hazegh-Azam, 1996; Wikström and Verkhovsky, 2007; Kim et al., 2008). However, it can be toxic to organisms at elevated concentrations, inducing impairment in cell functions (Pena et al., 1999).

In fish, toxic effects of Cu have been widely studied. Indeed, Cu exposure was shown to affect fish growth, oxygen consumption, embryonic development and reproduction. At biochemical and molecular levels, Cu can affect membrane permeability, protein synthesis and enzyme activity, as well as the expression of genes encoding for proteins involved in cellular transport of Cu (De Boeck et al., 1995; McGeer et al., 2000; Smith et al., 2001; Clearwater et al., 2002; Kamunde et al., 2002; Handy, 2003; Mackenzie et al., 2004; De Boeck et al., 2006; García et al., 2007; Kunwar et al., 2009; Chen et al, 2011; Das and Gupta, 2013; Machado et al., 2013; Silva et al., 2014).

In vertebrates, glycogen/glucose is mobilized from the liver in order to supply the energy demand associated with processes involved in Cu homeostasis (Strydom et al., 2006; Wood et al., 2011). In this context, pyruvate kinase (PK) and lactate dehydrogenase (LDH) are key enzymes at the end of the glycolytic pathway. Also, citrate synthase (CS) is another important enzyme in the process of energy generation. Under aerobic condition, this enzyme catalyzes the reaction of acetyl-CoA conversion into citrate at the beginning of the Krebs' cycle (Nelson and Cox, 2008). Some studies have shown that Cu can affect the activity of certain enzymes involved in energy metabolism in fish. However, most of these studies are performed with freshwater species. Indeed, there is still a gap in the knowledge on the chronic Cu effect on energy metabolism of saltwater fish (Carvalho and Fernandes, 2008; Garceau et al., 2010; Liu et al., 2010).

The guppy *Poecilia vivipara* (Cyprinodontiformes: Poeciliidae) is native from South America, being widely distributed in lakes, rivers and coastal lagoons along the Southern Atlantic coast, especially in Brazil (Neves and Monteiro, 2003; Gomes Jr. and Monteiro, 2008; Santos et al., 2011; Zanette, 2013). It is a viviparous fish, with internal fertilization (Amaral et al., 2001; Meredith et al., 2011), and has been suggested as a model fish for ecotoxicological studies (Ferreira et al., 2012; Paulo et al., 2012; Zimmer et al., 2012; Harayashiki et al., 2013; Machado et al., 2013; Silva et al., 2014). Therefore, the aim of the present study was to analyze the effects of chronic exposure to relevant concentrations of waterborne Cu on growth, oxygen consumption and activity of enzymes involved in energy metabolism of newborn *P. vivipara* acclimated to salt water.

Material and Methods

Fish rearing and acclimation

Adult male and female *P. vivipara* were grown at the animal care room of the Institute of Biological Sciences of the Federal University of Rio Grande (Rio Grande, RS, southern Brazil). Fish couples were separated and maintained in 20-L plastic tanks containing salt water (salinity 24 ppt) continuously aerated. Water temperature (25°C) and photoperiod (12 h light: 12 h dark cycle) were fixed. Fish were fed twice daily with a commercial fish diet (Alcon Basic MEP 200 Complex; 45% crude protein, 5% lipids, 2% calcium, 0.7% phosphorus and 10% humidity) until apparent satiation. Gravid females were transferred to breeding boxes until puppies were born. Newborn fish were then collected for subsequent exposure to waterborne Cu. All rearing conditions, as well as the experimental procedures described below were previously approved by the Ethics Committee for Animal Use of the University (CEUA/FURG; permit # P014/2012).

Exposure to copper for 28 days

Newborn puppies (<24 h after birth) of the guppy *P. vivipara* (6.3 ± 0.1 mg; 7.1 ± 0.1 mm) were maintained under control condition (no Cu addition in salt water) or exposed to relevant concentrations of waterborne Cu (5, 9 and 20 μg/L) for 28 days. These Cu concentrations were selected to bracket the current water quality criteria for dissolved Cu in salt water (5 μg/L) and fresh water (9 μg/L) established by the Brazilian National Council for Environment (CONAMA, 2005). Each treatment was performed in

triplicate using a static system with complete renew of the experimental media every 24 h. Fish density was always kept <1 g/L. During the experimental period, fish were maintained in 1-L glass aquaria containing salt water at salinity 24 ppt and pH 7.66 ± 0.21. Water temperature (28°C) and photoperiod (12 h ligh:12 h dark) were fixed. Dissolved oxygen saturation in salt water was always >90%. Fish were fed daily with a commercial diet (Alcon BASIC; 45% crude protein, 5% lipids, 2% calcium, 0.7% phosphorus and 10% humidity) until apparent satiation.

After exposure, fish were collected, weighted (wet body weight) and had their whole-body oxygen consumption measured. Fish where fasted for 12 h prior to oxygen consumption measurements. Whole-body oxygen consumption of each fish was measured using a static respirometer, as described by Cunha et al. (2009). Oxygen consumption rate ($M\dot{O}_2$) was calculated using the following formula: $M\dot{O}_2 = [(O_i-O_f)xV]/B/T$, where O_i and O_f are the initial and final oxygen concentrations in salt water (mg O_2/L), respectively, V is the tank volume (L), B is the fish biomass (g), and T is the time interval between measurements (h).

Additional fish were collected, weighed (wet body weight), measured (standard body length), placed on ice, killed by sectioning of the spinal cord, and stored (whole fish) in ultrafreezer (-80ºC) for further analysis of enzyme activity. Whole fish samples were homogenized in a buffer solution containing 50 mM imidazole (pH 7.8) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 10,000 g (Micro 22R HettichZentrifugen, Global Medical Instrumentation, Ramsey, Minnesota, USA) for 20 min at 4ºC. Supernatants were used as enzyme source. PK, LDH and CS activity was measured according to Lallier and Walsh (1991). Absorbance samples readings were measured using a microplate reader (ELx 808 Universal Microplate Reader, Bio-Teck Instruments, Winooski, Vermont, USA).

Survival (S), body weight gain (WG), specific growth rate (SGR), feed conversion (FC) and condition factor (CF) were calculated using the following formulas: $S = (n_f/n_i)x100$, where n_f and n_i is the number of fish at the end and the beginning of the exposure period, respectively; $SGR = [(\ln W_f-\ln W_i)/T]x100$, where W_f is the final body weight (g), W_i is the initial body weight (g) and T is the exposure period (days); $WG = [(W_f-W_i)/W_i]x100$, where W_f and W_i is the final and initial body weight (g), respectively; FC = FO/WG, where FO is the amount of feed offered (g) and WG is the body weight gain (g); $CF = (W/L^3)x100$, where W is the wet body weight (g) and L is the standard body length (mm).

Exposure to copper for 345 days

Newborn puppies (<24 h after birth) of the guppy *P. vivipara* (6.3 ± 0.1 mg; 7.2 ± 0.1 mm) were maintained under control condition (no Cu addition in salt water) or exposed to the environmentally relevant concentrations of waterborne Cu (5, 9 and 20 μg/L). During the experimental period, fish were maintained in 10-L glass aquaria with salt water at salinity 24 ppt continuously aerated. Water temperature (28°C) and photoperiod (12 h light: 12 h dark) were fixed. Dissolved oxygen saturation in salt water was always >90%. The experimental media were completely renewed every week. Fish were fed daily until apparent satiation with the same commercial diet used in the exposure for 28 days. After 345 days of exposure, fish were collected, weighted (wet body weight), measured (standard body length), placed on ice, killed by sectioning of the spinal cord, and had their tissues (gill, liver and muscle) dissected and stored in ultrafreezer (-80ºC) for further analysis of tissue enzyme (PK, LDH and CS) activity, as described above for fish exposed for 28 days. Also, survival (S), body weight gain (WG), and specific growth rate (SGR) were calculated as described above for fish exposed for 28 days.

Data presentation and statistical analysis

For all parameters analyzed, data were expressed as mean ± standard error. Mean values among treatments were compared using one-way analysis of variance (ANOVA) followed by the Tukey test. ANOVA assumptions (data normality and homogeneity of variances) were previously checked. In all cases, the significance level adopted was 95% ($\alpha = 0.05$). Statistical analyses were performed using the software Statistica 7.0 (StatSoft, Tulsa, OK, USA).

Results

Fish survival and growth

After 28 days of exposure, 100% of fish survival was observed in all treatments. Also, no significant effect of Cu was observed for all growth parameters analyzed: wet body weight, weight gain, specific growth rate, feed conversion and condition factor (Table 1). However, 100% mortality was observed within the first seven weeks of experiment when fish were exposed to 20 μg/L Cu for 345 days (Table 2). Also, fish

growth was affected by the longer exposure to environmentally relevant concentrations of waterborne Cu. Male guppies exposed to 9 µg/L Cu showed a lower wet body weight than those maintained under control condition or exposed to 5 µg/L Cu. Female guppies exposed to 5 and 9 µg/L Cu showed a lower wet body weight than control fish. In both male and female guppies, weight gain and specific growth rate were not affected by waterborne Cu exposure (Table 2).

Whole-body oxygen consumption and enzyme activity

After 28 days of exposure, whole-body oxygen consumption was dependent on Cu concentration in salt water. Guppies exposed to 9 and 20 µg/L Cu showed whole-body oxygen consumption higher than those maintained under control condition (Fig. 2). Also, whole-body activity of PK, LDH and CS was not significantly affected by fish exposure to Cu (Table 3). Furthermore, tissue (gill, liver and muscle) PK and LDH activity was not significantly affected by fish exposure to Cu for 345 days. Also, gill and muscle CS activity was not affected by Cu exposure. However, CS activity was higher in liver of fish exposed to 9 µg/L Cu than in liver of control fish (Fig. 3).

Discussion

Survival of puppies of the guppy *P. vivipara* was not affected after 28 days of maintenance under the control condition (no Cu addition in salt water) or exposure to environmentally relevant concentrations of waterborne Cu (5-20 µg/L). Indeed, 100% fish survival was observed in all treatments. After 345 days of exposure, survival was affected, 100% mortality was observed in fish exposed to 20 µg/L Cu. However, it is important to note that survival of fish maintained under control condition or exposed to 5 and 9 µg/L Cu was < 42%.

The mortality rate observed in fish exposed for 345 days, even in the control condition, could be related to the greater sensitivity of fish at the initial life stages to environmental stressing factors, where mortality levels occur more intensely in the first weeks of the experimental period (Dahlberg, 1979; Fortier and Leggett, 1985; Sahin and Üstünda, 2003; Reglero et al., 2014). Therefore, stressing conditions associated with captivity and handling during an experiment of such long period of time (345 days), as the one performed in the present study, could have affected fish survival. In turn, the total mortality observed in fish exposed to 20 µg/L Cu for 345 days indicates that Cu

toxicity in salt water is dependent on both concentration of waterborne Cu and exposure time. The observed lethal effect after the long term exposure to Cu is likely resulting from a significant metal body burden. In fact, we have reported significant whole-body and tissue (gills, liver and gut) Cu accumulation in puppies of *P. vivipara* exposed to 9 µg/L Cu for 345 days under the same experimental conditions employed in the present study (Anni et al., submitted).

In addition to survival, fish growth was also analyzed as a parameter to evaluate the potential chronic effects of waterborne Cu in puppies of the guppy *P. vivipara*. Changes in body weight, specific growth rate, feed conversion and condition factor are generally employed as indicators of growth performance in teleost fish. In the present study, no significant difference was observed among treatments for all these parameters after *P. vivipara* exposure to environmentally relevant concentrations of waterborne Cu. However, a significantly reduced wet body weight was observed in male guppies exposed to 5 µg/L Cu and in female guppies exposed to 5 and 9 µg/L Cu for 345 days. As for fish survival, Cu effect on fish growth in salt water is dependent on the concentration of waterborne Cu, exposure time and fish gender. This finding is in agreement with previous data reported in the literature for teleost fish. As reported in the present study, a decrease in fish growth is generally observed with increasing waterborne Cu concentration (Buckley et al., 1982; Marr et al., 1996; Ali et al., 2003; James et al., 2008; Liu et al., 2010; Nekoubin et al., 2012; Heydarnejad et al., 2013).

From an environmental perspective, it is important to note the absence of effect on survival and growth of puppies of the guppy *P. vivipara* exposed to concentrations of waterborne Cu up to 20 µg/L for 28 days. Based on the current water quality criteria for dissolved Cu in brackish (5 µg/L) and fresh water (9 µg/L), puppies of *P. vivipara* would be protected by Brazilian environmental regulation when taking mortality and growth as chronic toxicity endpoints. However, the situation would be different when a long-term chronic exposure is considered. This statement is based on the fact that body weight of female guppies was significantly affected after exposure to 5 µg/L Cu for 345 days, while male guppies had their body weight reduced after exposure to 9 µg/L for 345 days. Therefore, female puppies of *P. vivipara* are more sensitive to chronic exposure to Cu than male puppies.

From a physiological/biochemical perspective, chronic exposure to Cu induced an increased aerobic metabolism, measured indirectly through the whole-body oxygen consumption, in puppies of the guppy *P. vivipara* exposed to environmentally relevant

concentrations of waterborne Cu for 28 days. Indeed, the observed effect was dependent on the Cu concentration in salt water, being significant in fish exposed to 9 and 20 µg/L Cu. In fact, previous studies have reported the direct effect of Cu on oxygen consumption in teleost fish. In general, they show that Cu exposure induces a reduction in oxygen consumption, in opposition to the effect observed in the present study. However, the decreased oxygen consumption reported in previous studies could be associated with morphological/histological damage caused to gill tissue after exposure to excessive concentrations of waterborne Cu concentrations, thus impairing the oxygen uptake by the gills and its subsequent consumption by fish tissues (O'Hara, 1971; De Boeck et al., 1995; Hassan, 2011; Das and Gupta, 2013).

In turn, the increased whole-body oxygen consumption observed in puppies of the guppy *P. vivipara* exposed to 9 and 20 µg/L Cu for 28 days could be explained considering a Cu-induced impairment of the mitochondrial function, such as inhibition of electron transport chain and/or stimulation of proton leak, resulting in inadequate use oxygen for energy production (Ciapaite et al., 2009; Belyaeva et al., 2011; Belyaeva et al., 2012; Lauer et al., 2012). However, another reasonable explanation could be an augmented metabolic demand to maintain Cu homeostasis (De Boeck et al., 2006). This idea is supported by the fact that an increased Cu accumulation was observed in whole-body of puppies of the guppy *P. vivipara* exposed to 9 and 20 µg/L Cu for 28 days and in tissues (gills, liver and gut) of puppies exposed to 9 µg/L Cu for 345 days, under the same experimental conditions employed in the present study. Indeed, the increased whole-body Cu burden was shown to be paralleled by an augmented whole-body expression of the gene encoding for the P-type Cu-ATPase (*ATP7B*) in puppies exposed to 9 and 20 µg/L Cu for 28 days. Also, the increased tissue Cu burden was reported to be paralleled by a higher *ATP7B* expression in tissues of puppies exposed to 5 µg/L Cu (gills and gut) or 9 µg/L Cu (liver and gut) for 345 days (Anni et al., submitted).

The hypothesis of an increased metabolism induced by Cu exposure could be evaluated based on the activity of metabolic enzymes analyzed. Whole-body PK, LDH and CS activity was not affected by chronic exposure to waterborne Cu for 28 days. A lack of LDH and CS response was also reported in muscle of fathead minnows *Pimephales promelas* after exposure to waterborne Cu for 28 days (Lapointe et al., 2011). These findings suggest that aerobic metabolism is unchanged after fish exposure to waterborne Cu for 28 days. This statement is based on the fact that PK and LDH are key-enzymes playing an essential role in the terminal sequence of glycolytic pathway.

Indeed, PK is responsible for the conversion of phosphoenolpyruvate into pyruvate, while LDH is involved in the conversion of pyruvate into lactate. In turn, CS is a mitochondrial enzyme performing an essential role in the Krebs' cycle, catalyzing the conversion of acetyl-coenzyme A into citrate. In fact, the activity of this enzyme is a reliable indicator of the mitochondrial aerobic capacity (Nelson and Cox, 2008). Therefore, the observed lack of response in the whole-body activity of metabolic enzymes is evidence that the increased whole-body oxygen consumption found in puppies of the guppy *P. vivipara* exposed to Cu for 28 days would not be associated with a Cu-induced increase in aerobic metabolism. Alternatively, it could be related to a Cu-induced impairment in mitochondrial respiratory chain.

As reported for guppies exposed to Cu for 28 days, no significant change in PK and LDH activity was observed in tissues (gills, liver and muscle) of guppies exposed to waterborne Cu for 345 days. As discussed above, this finding suggests that glycolytic activity was also unchanged in tissues of *P. vivipara* exposed to Cu for 345 days. In addition, CS activity was not affected in gills and muscle of puppies exposed to Cu for 345 days. This finding is also evidence that the aerobic metabolism was unchanged in these tissues after chronic exposure to waterborne Cu. However, a significant 2.2-fold increase in CS activity was observed in liver of guppies exposed to 9 μg/L Cu for 345 days. As discussed above, CS activity is a good indicator of the aerobic activity. Therefore, the observed increase in CS activity suggests a higher aerobic activity in the liver of guppies exposed to Cu for 345 days. This response is in alignment with the increased whole-body oxygen consumption observed in guppies exposed to Cu for 28 days. Unfortunately, whole-body or tissue oxygen consumption measurements were not performed in guppies exposed to Cu for 345 days.

Taken together, physiological and biochemical data suggest that an impairment in the mitochondrial respiratory activity is occurring in guppies exposed to Cu for 28 days. However, this impairment would be joined by an increased aerobic metabolism to deal with Cu accumulation when guppies are exposed to waterborne Cu for a much longer period of time (345 days).

In summary, data reported in the present study show evidence that chronic exposure to environmentally relevant concentrations of Cu in salt water affects growth of newborn puppies of the viviparous guppy *P. vivipara*. In this case, female guppies are more sensitive to chronic Cu exposure than male guppies. Also, our findings suggest that the chronic effect of Cu on fish growth is associated with an increased aerobic

metabolism, especially after a long-term (345 days) chronic exposure to environmentally relevant concentrations of waterborne Cu. Furthermore, they indicate that this increased aerobic metabolism is not associated with a higher growth rate, but likely related to a higher energy demand to deal with Cu homeostasis and/or a disruption in the mitochondrial respiratory function.

Acknowledgements

This research was financially supported by the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq, Brasília, DF, Brazil) and "Coordenação de Apoio ao Pessoal de Ensino Superior" (CAPES, Brasília, DF, Brazil). A. Bianchini is a research fellow from the Brazilian CNPq (Proc. #304430/2009-9) and is supported by the International Research Chair Program from the International Development Research Center (DRC; Ottawa, ON, Canada).

References

Ali, A., Al-Ogaily, S.M., Al-Asgah, N.A., Gropp, J. 2003. Effect of sublethal concentrations of copper on the growth performance of *Oreochromis niloticus*. J. Appl. Ichthyol. 19, 183-188.

Amaral, M.D., Bonecker, A.C.T., Ortiz, C.H.D. 2001. Activity determination of $Na^{+}K^{+}$-ATPase and Mg^{++}-ATPase enzymes in the gill of *Poecilia vivipara* (Osteichthyes, Cyprinodontiformes) in different salinities. Braz. Arch. Biol. Technol. 44, 1-6.

Anni, I.S.A., Afonso, S.B., Jorge, M.B., Abril, S.I.M., Bianchini, A. 2015. Role of transporting proteins CTR1 and ATP7B on copper accumulation in newborn puppies of the viviparous fish *Poecilia vivipara* (Bloch and Schneider, 1801) acclimated to salt water. Aquat. Toxicol. In preparation.

Belyaeva, E.A, Korotkov, S.M., Saris, N-E. 2011. In vitro modulation of heavy metal-induced rat liver mitochondria dysfunction: a comparison of copper and mercury with cadmium. J. Trace Elem. Med. Biol. 25, 63-73.

Belyaeva, E.A, Sokolova, T.V, Emelyanova, L.V, Zakharova, I.O. 2012. Mitochondrial electron transport chain in heavy metal-induced neurotoxicity: effects of cadmium, mercury, and copper. Scient. World J. 136063.

Buckley, J., Roch, M., Mccarter, J. 1982. Chronic exposure of coho salmon to sublethal concentrations of copper - I. Effect on growth, on accumulation and distribution of copper, and on copper tolerance. Comp. Biochem. Physiol. Part C 72, 15-19.

Carvalho, C.D.S., Fernandes, M.N. 2008. Effect of copper on liver key enzymes of anaerobic glucose metabolism from freshwater tropical fish *Prochilodus lineatus*. Comp. Biochem. Physiol. Part A 151, 437-442.

CDA - Copper Development Association Inc. 2015. Applications. Accessible at: <http://www.copper.org/applications/>.

Chen, H-R., Yang, H-C., Hsieh, D.J-Y., Zijuan, L., Tsai, K-J. 2011. Zebrafish *sod1* and *sp1* expression are modulated by the copper ATPase gene *atp7a* in response to intracellular copper status. Chem. Biol. Interact. 189, 192-197.

Ciapaite, J., Nauciene, Z., Baniene, R., Wagner, M. J., Krab, K., Mildaziene, V. 2009. Modular kinetic analysis reveals differences in Cd^{2+} and Cu^{2+} ion-induced impairment of oxidative phosphorylation in liver. FEBS J. 276, 3656-3668.

Clearwater, S.J., Farag, A.M., Meyer, J.S. 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. Comp. Biochem. Physiol. Part C 132, 269-313.

CONAMA - Conselho Nacional do Meio Ambiente. 2005. Resolução CONAMA nº 357. Accessible at: < http://www.mma.gov.br/port/conama>.

Cunha, V.L., Rodrigues, R.V., Okamoto, M.H., Sampaio, L.A. 2009. Consumo de oxigênio pós-prandial de juvenis de pampos *Trachinotus marginatus*. Ciência Rural 39, 1257-1259.

Dahlberg, M.D. 1979. A review of survival rates of fish eggs and larvae in relation to impact assessments. Mar. Fisher. Rev. 41, 1-12.

Das, S., Gupta, A. 2013. Accumulation of copper in different tissues and changes in oxygen consumption rate in Indian flying barb, *Esomus danricus* (Hamilton-Buchanan) exposed to sub-lethal concentrations of copper. Jordan J. Biol. Sci. 6, 21-24.

De Boeck, G., Smet, H. De, Blust, R. 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. Aquat. Toxicol. 32, 127-141.

De Boeck, G., Van Der Ven, K., Hattink, J., Blust, R. 2006. Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. Aquat. Toxicol. 80, 92-100.

Ferreira, R.S., Monserrat, J.M., Ferreira, J.L.R., Kalb, A.C., Stegeman, J., Bainy, A.C. D., Zanette, J. 2012. Biomarkers of organic contamination in the South American fish *Poecilia vivipara* and *Jeninsia multidentata*. J. Toxicol. Environ. Health 75A, 1-11.

Fortier, L., Leggett, W. 1985. A drift study of larval fish survival. Mar. Ecol. Progr. Ser. 25, 245-257.

Garceau, N., Pichaud, N., Couture, P. 2010. Inhibition of goldfish mitochondrial metabolism by in vitro exposure to Cd, Cu and Ni. Aquat. Toxicol. 98, 107-112.

García, N., Martínez-Abundis, E., Pavón, N., Correa, F., Chávez, E. 2007. Copper induces permeability transition through its interaction with the adenine nucleotide translocase. Cell Biol. Intern. 31, 893-899.

Gomes Jr., J.L., Monteiro, L.R. 2008. Morphological divergence patterns among populations of *Poecilia vivipara* (Teleostei Poeciliidae): test of an ecomorphological paradigm. Biol. J. Linnean Soc. 93, 799-812.

Handy, R.D. 2003. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comp. Biochem. Physiol. Part A 135, 23-38.

Harayashiki, C.A.Y., Varela, A.S., Machado, A.A.D.S., Cabrera, L.D.C., Primel, E.G., Bianchini, A., Corcini, C.D. 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquat. Toxicol. 142-143, 176-184.

Hassan, B.K. 2011. The effect of copper and cadmium on oxygen consumption of the juvenile common carp, *Cyprinus carpio* (L.). Mesopot. J. Mar. Sci. 26, 25-34.

Eydarnejad, M.S., Khosravian-Hemami, M., Nematollahi, A., Rahnama, S. 2013. Effects of copper at sublethal concentrations on growth and biochemical parameters in rainbow trout (*Oncorhynchus mykiss*). Internat. Rev. Hydrobiol. 98, 71-79.

James, R., Sampath, K., Jothilakshmi, S., Vasudhevan, I., Thangarathinam, R. 2008. Effects of copper toxicity on growth, reproduction and metal accumulation in chosen ornamental fishes. Ecohydrol. Hydrobiol. 8, 89-97.

Kamunde, C., Grosell, M., Higgs, D., Wood, C.M. 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. J. Exper. Biol. 205, 279-290.

Kim, B.E., Nevitt, T., Thiele, D.J. 2008. Mechanisms for copper acquisition, distribution and regulation. Nature Chem. Biol. 4, 176-185.

Kunwar, P.S., Tudorache, C., Eyckmans, M., Blust, R., De Boeck, G. 2009. Influence of food ration, copper exposure and exercise on the energy metabolism of common carp (*Cyprinus carpio*). Comp. Biochem. Physiol. Part C, 149, 113-119.

Lallier, F.H., Walsh, P.J. 1991. Metabolic potential in tissues of the blue crab, *Callinectes sapidus*. Bull. Mar. Sci. 48, 665-669.

Lapointe, D., Pierron, F., Couture, P. 2011. Individual and combined effects of heat stress and aqueous or dietary copper exposure in fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 104(1-2), 80-85.

Lauer, M.M., Oliveira, C.B. de, Yano, N.L.I., Bianchini, A. 2012. Copper effects on key metabolic enzymes and mitochondrial membrane potential in gills of the estuarine crab *Neohelice granulata* at different salinities. Comp. Biochem. Physiol. Part C 156, 140-147.

Linder, M.C., Hazegh-Azam, M. 1996. Copper biochemistry and molecular biology. Amer. J. Clin. Nutr. 63, 797S-811S.

Liu, X.J., Luo, Z., Xiong, B.X., Liu, X., Zhao, Y.H., Hu, G.F. 2010. Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. Ecotoxicol. Environ. Safe. 73, 1286-1291.

Machado, A.A.D.S., Hoff, M.L.M., Klein, R.D., Cardozo, J.G., Giacomin, M.M., Pinho, G.L.L., Bianchini, A. 2013. Biomarkers of waterborne copper exposure in the guppy *Poecilia vivipara* acclimated to salt water. Aquat. Toxicol. 138-139, 60-69.

Mackenzie, N.C., Brito, M., Reyes, A.E., Allende, M.L. 2004. Cloning, expression pattern and essentiality of the high-affinity copper transporter 1 (ctr1) gene in zebrafish. Gene 328, 113-120.

Marr, J.C.A., Lipton, J., Cacela, D., Hansen, J.A., Bergman, H.L., Meyer, J.S., Hogstrand, C. 1996. Relationship between copper exposure duration, tissue copper concentration, and rainbow trout growth. Aquat. Toxicol. 36, 17-30.

McGeer, J., Szebedinszky, C., McDonald, D., Wood, C. 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231-243.

Meredith, R.W., Pires, M.N., Reznick, D.N., Springer, M.S. 2011. Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation

in *Poecilia* (Poeciliidae: Cyprinodontiformes). Mol. Phylogenet. Evol. 59, 148-157.

Nekoubin, H., Hatefi, S., Gharedaashi, E. 2012. Effect of sublethal doses of copper on growth performance and survival rate of grass carp (*Ctenopharyngodon idella*). Amer. Eur. J. Toxicol. Sci. 4, 138-142.

Nelson, D.L.; Cox, M.M. 2008. Lehninger Principles of Biochemistry**.** 5th ed., W.H. Freeman, New York, 1100 p.

Neves, F.M., Monteiro, L.R. 2003. Body shape and size divergence among populations of *Poecilia vivipara* in coastal lagoons of south-eastern Brazil. J. Fish Biol. 63, 928-941.

O'Hara, J. 1971. Alterations in oxygen consumption by bluegills exposed to sublethal treatment with copper. Water Res. 5, 321-327.

Paulo, D.V., Fontes, F.M., Flores-Lopes, F. 2012. Histopathological alterations observed in the liver of *Poecilia vivipara* (Cyprinodontiformes: Poeciliidae) as a tool for the environmental quality assessment of the Cachoeira River, BA. Braz. J. Biol. 72, 131-140.

Pena, M.M.O., Lee, J., Thiele, D.J. 1999. A delicate balance: homeostatic control of copper uptake and distribution. J. Nutr. 129, 1251-1260.

Reglero, P., Ortega, A., Blanco, E., Ø. Fiksen, T., Viguri, F. J., De La Gándara, F., Folkvord, A. 2014. Size-related differences in growth and survival in piscivorous fish larvae fed different prey types. Aquaculture 433, 94-101.

Sahin, T., Üstünda, C. 2003. Effect of different rearing systems on survival rate of hatchery reared black sea turbot, *Scophthalmus maximus*. Turkish J. Fisher. Aquat. Sci. 27, 25-27.

Santos, E.G.N., Cunha, R.A. Santos, C.P. 2011. Behavioral responses of *Poecilia vivipara* (Osteichthyies: Cyprinodontiformes) to experimental infections of *Acanthocollaritrema umbilicatum* (Digenea: Cryptogonimidae). Exper. Parasitol. 127, 522-526.

Silva, E.S., Abril, S.I.M., Zanette, J., Bianchini, A. 2014. Salinity-dependent copper accumulation in the guppy *Poecilia vivipara* is associated with CTR1 and ATP7B transcriptional regulation. Aquat. Toxicol. 152, 300-307.

Smith, R.W., Blaney, S.C., Dowling, K., Sturm, A., Jönsson, M., Houlihan, D. F. 2001. Protein synthesis costs could account for the tissue-specific effects of sub-lethal

copper on protein synthesis in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 53, 265-277.

Strydom, C., Robinson, C., Pretorius, E., Whitcutt, J.M., Marx, J., Bornman, M.S. 2006. The effect of selected metals on the central metabolic pathways in biology: A review. Water SA. 32, 543-554.

Wikström, M., Verkhovsky, M.I. 2007. Mechanism and energetics of proton translocation by the respiratory heme-copper oxidases. Biochim. Biophys. Acta 1767, 1200–1214.

Wood, C.M., Farrell, A.P., Brauner, C.J. 2011. Fish Physiology: Homeostasis and Toxicology of Essential Metals. Fish Physiology, Vol. 31A, Academic Press, 520p.

Zanette, J. 2013. Genes and Proteins Related with Biotransformation in Tropical Fishes. In: Almeida, E. A., Ribeiro, C. A. O. Pollution and Fish Health in Tropical Ecosystems. CRC Press. chapter 2, 15-34.

Zimmer, A.M., Barcarolli, I.F., Wood, C.M., Bianchini, A. 2012. Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. Aquat. Toxicol. 122-123, 172-180.

Table 1. Survival (S), wet body weight (BW), weight gain (WG), specific growth rate (SGR), feed conversion (FC) and condition factor (CF) of puppies of the guppy *Poecilia vivipara* exposed to waterborne copper for 28 days in salt water (salinity 24 ppt). Data are expressed as mean ± standard error (n = 30). For all parameters, no significant difference was observed among treatments ($p>0.05$).

Parameter	Nominal copper concentration (µg/L)			
	Control	5	9	20
S (%)	100	100	100	100
BW (g)	0.020 ± 0.001	0.024 ± 0.001	0.021 ± 0.001	0.019 ± 0.001
WG (%)	217.7 ± 16.3	225.0 ± 19.1	242.2 ± 18.1	200.0 ± 14.0
SGR (%/day)	4.5 ± 0.5	4.6 ± 0.6	4.7 ± 0.5	4.3 ± 0.4
FC	4.6 ± 1.0	4.7 ± 1.3	4.6 ± 1.2	5.0 ± 1.1
CF	1.8 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	1.9 ± 0.1

Table 2. Survival (S), wet body weight (BW), weight gain (WG) and specific growth rate (SGR) of puppies of the guppy *Poecilia vivipara* exposed to waterborne copper for 345 days in salt water (salinity 24 ppt). Data are expressed as mean ± standard error (n = 7-30). Different letters represent significant different mean values among treatments for each parameter ($p<0.05$). * indicates significant difference between male and female fishes for each parameter ($p<0.05$).

Parameter	Sex	Nominal copper concentration (µg/L)		
		Control	5	9
S (%)	Male + Female	39.1	40.6	42.0
BW (g)	Male	0.33 ± 0.02^{a}*	0.34 ± 0.01^{a}*	0.25 ± 0.01^{b}*
	Female	1.15 ± 0.04^{a}	0.84 ± 0.09^{b}	0.60 ± 0.09^{b}
WG (%)	Male	$7{,}028 \pm 1{,}507^{a}$*	$7{,}794 \pm 1{,}205^{a}$*	$5{,}999 \pm 850^{a}$*
	Female	$18{,}643 \pm 1{,}690^{a}$	$15{,}702 \pm 2{,}509^{a}$	$16{,}061 \pm 3{,}207^{a}$
SGR (%/day)	Male	1.24 ± 0.02^{a}*	1.24 ± 0.04^{a}*	1.14 ± 0.04^{a}*
	Female	1.50 ± 0.03^{a}	1.43 ± 003^{a}	1.38 ± 0.03^{a}

Table 3. Whole-body pyruvate kinase (PK), lactate dehydrogenase (LDH) and citrate synthase (CS) activity in of puppies of the guppy *Poecilia vivipara* exposed to waterborne copper for 28 days. Data are expressed as mean ± standard error (n = 6). For all parameters, no significant difference was observed among treatments ($p>0.05$).

Parameter	Nominal copper concentration (µg/L)			
	Control	5	9	20
PK (U/mg proteins)	0.24 ± 0.03	0.22 ± 0.02	0.25 ± 0.06	0.34 ± 0.08
LDH (U/mg proteins)	0.51 ± 0.06	0.47 ± 0.07	0.45 ± 0.08	0.80 ± 0.22
CS (U/mg proteins)	0.11 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.10 ± 0.01

Figure Legends

Figure 1. Whole-body oxygen consumption in puppies of the guppy *Poecilia vivipara* kept under control condition (no copper addition in the water) or exposed to waterborne copper for 28 days. Data are expressed as mean ± standard error (n = 6). Different letters represent significant different mean values among treatments ($p<0.05$).

Figure 2. Pyruvate kinase (A), lactate dehydrogenase (B) and citrate synthase (C) activity in tissues of puppies of the guppy *Poecilia vivipara* kept under control condition (no copper addition in the water) or exposed to waterborne copper for 345 days. Data are expressed as mean ± standard error (n = 5). Different letters represent significant different mean values among treatments for each tissue ($p<0.05$).

Figure 1

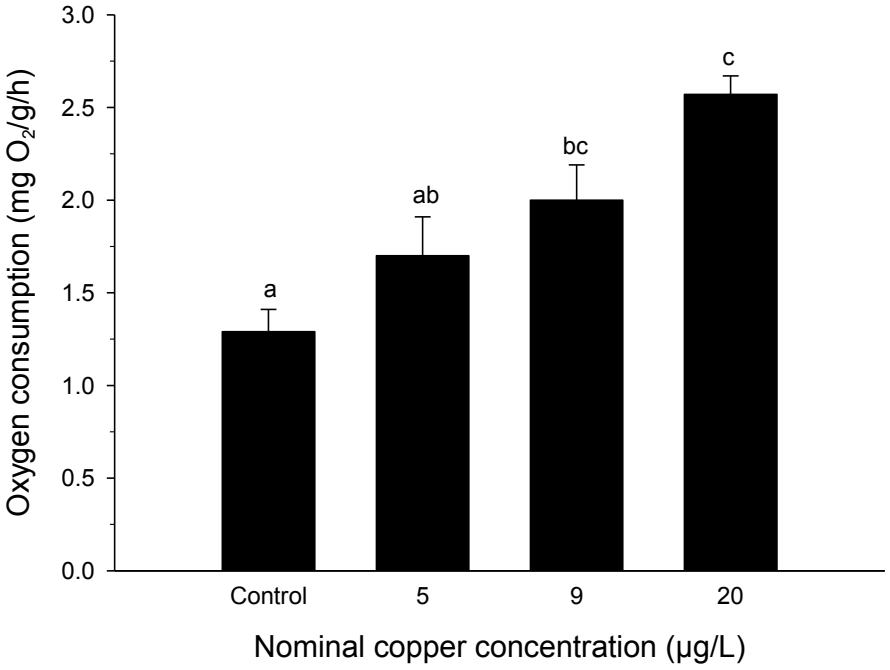
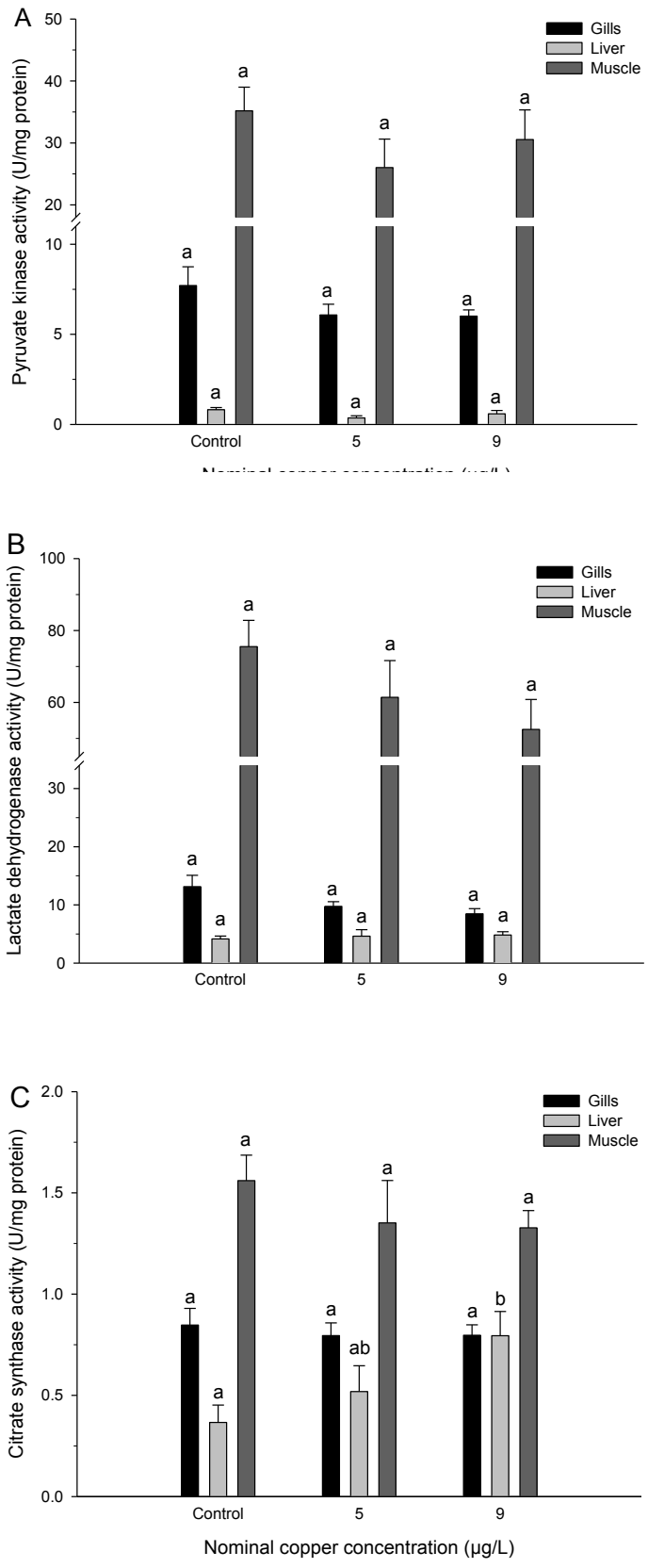

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Oxygen consumption (mg O_2/g/h)
a
ab
bc
c
Control
5
9
20
Nominal copper concentration (µg/L)

Figure 2


A
Pyruvate kinase activity (U/mg protein)
Gills
Liver
Muscle
Control
5
9
B
Lactate dehydrogenase activity (U/mg protein)
C
Citrate synthase activity (U/mg protein)
Nominal copper concentration (µg/L)

5. DISCUSSÃO GERAL

O presente estudo investigou os efeitos da exposição crônica a concentrações ambientalmente relevantes de Cu (0, 5, 9 e 20 µg/L) em filhotes recém-nascidos de *Poecilia vivipara* aclimatados à água salgada. Após 28 dias de exposição, foi observada uma acumulação corporal do metal nos peixes expostos a 20 µg/L Cu. Porém, nenhuma mortalidade foi observada. Por sua vez, foi observada uma mortalidade total dos peixes expostos a 20 µg/L Cu por 345 dias. Neste caso, os peixes expostos a 9 µg/L Cu apresentaram acumulação corporal e tecidual (brânquias, fígado e intestino) de Cu. Em um estudo prévio com adultos de *P. vivipara* aclimatados à água salgada, não foi observada acumulação de Cu em nível corporal ou tecidual (brânquias e fígado) após exposição aguda (96 h) ao cobre às mesmas concentrações de Cu e nas mesmas condições experimentais utilizadas no presente estudo (Silva et al., 2014). Em conjunto, estes resultados indicam que a acumulação de Cu nos tecidos de *P. vivipara* é dependente da concentração de Cu na água salgada e do tempo de exposição ao metal. Alguns estudos têm relatados resultados similares para outras espécies de peixes (AY et al., 1999; MAZON e FERNANDES, 1999; MCGEER et al., 2000; TAYLOR e MCGEER, 2000; GROSELL et al., 2004; JAMES et al., 2008).

Os resultados discutidos acima também sugerem que *P. vivipara* apresenta boa capacidade de lidar com a exposição aguda ao Cu em concentrações ambientalmente relevantes na água salgada. Contudo, esta espécie de peixe demonstra dificuldades de manter a homeostasia celular de Cu após exposição crônica a concentrações ambientalmente relevantes do metal na água salgada. A acumulação de Cu observada nos tecidos envolvidos com a homeostasia do metal (brânquias, fígado e intestino), em especial no fígado, nos peixes expostos a 9 µg/L Cu por 345 dias, combinada com a mortalidade de 100% dos peixes observada após exposição a 20 µg/L Cu por 345 dias, reforça a ideia de que a exposição crônica ao Cu em água salgada pode causar efeitos deletérios a filhotes de *P. vivipara* (HANDY, 2003).

A expressão dos genes que codificam o transportador de alta afinidade ao Cu (*CTR1*) e a Cu-ATPase tipo-P (*ATP7B*) foi diretamente influenciada pela exposição crônica ao Cu. Foi observada uma indução na expressão corporal do *CTR1* nos peixes expostos a 9 µg/L Cu e do *ATP7B* nos peixes expostos a 9 e 20 µg/L Cu por 28 dias. Por outro lado, não foi observada alteração significativa na expressão tecidual (brânquias, fígado e intestino) do *CTR1* após exposição dos peixes ao Cu por 345 dias.

Já a expressão do *ATP7B* foi induzida nas brânquias, fígado e intestino dos peixes expostos ao Cu por 345 dias. O CTR1 é uma proteína transmembrana altamente conservada, que medeia especificamente a internalização de íons de Cu a partir do meio extracelular (LINDER e HAZEGH-AZAM, 1996; GROSELL e WOOD, 2002; SHARP, 2003; MACKENZIE et al., 2004; WOOD et al., 2011). Como relatado no presente estudo, alguns estudos têm demonstrado o efeito direto da exposição ao Cu sobre a expressão do *CTR1* em peixes (MINGHETTI et al., 2008, MINGHETTI et al., 2010). Em estudo com a mesma espécie, Silva et al., (2014) observaram uma inibição na expressão do *CTR1* e do *ATP7B* nas brânquias de indivíduos adultos após exposição aguda (96 h) às mesmas concentrações de Cu utilizadas no presente estudo. Estes resultados indicam que, assim como a acumulação do Cu, o perfil de expressão do *CTR1* e do *ATP7B* é dependente da concentração de Cu na água salgada e do tempo de exposição ao metal.

Quanto aos possíveis efeitos do Cu acumulado em nível corporal, foi observado no presente estudo um aumento do consumo de oxigênio corporal nos peixes expostos a 9 e 20 µg/L Cu por 28 dias. No entanto, geralmente é observada uma diminuição no consumo de oxigênio de peixes após a exposição ao Cu. Todavia, esse fato pode estar relacionado à exposição aguda a concentrações excessivas de Cu dissolvido, a qual pode causar danos morfológicos e/ou histológicos no tecido branquial, reduzindo assim a captação de oxigênio e sua consequente utilização no metabolismo aeróbico (O'HARA, 1971; DE BOECK et al., 1995; HASSAN, 2011; DAS e GUPTA, 2013).

O consumo de oxigênio é uma medida indireta do metabolismo, sendo que alguns estudos têm demonstrado o efeito da exposição aguda ao Cu sobre a atividade de enzimas-chaves do metabolismo energético em peixes de água doce (COUTURE e KUMAR, 2003; CARVALHO e FERNANDES, 2008; GARCEAU et al., 2010; LIU et al., 2010; LAPOINTE et al., 2011). Por outro lado, pouco ainda se sabe a respeito dos efeitos da exposição crônica ao Cu sobre a atividade dessas enzimas em peixes aclimatados à água salgada. Assim, o aumento no consumo de oxigênio corporal observado no presente estudo nos peixes expostos ao Cu por 28 dias pode estar relacionado a uma maior demanda do metabolismo energético para manutenção da homeostase corporal do Cu, o que poderia ser indicado através de um aumento na atividade de enzimas-chaves envolvidas no metabolismo energético. Todavia, as atividades da piruvato quinase e da lactato desidrogenase, enzimas associadas ao metabolismo de carboidratos, não apresentaram variação significativa entre os

tratamentos nos peixes expostos ao Cu por 28 dias. Além disso, não foi observado efeito da exposição ao Cu sobre a atividade destas enzimas em tecidos (brânquias, fígado e músculo) dos peixes expostos ao Cu por 345 dias. Estes resultados sugerem que a atividade da via glicolítica foi mantida nos peixes expostos cronicamente ao Cu.

Assim como observado para a piruvato quinase e a lactato desidrogenase, não foi observada variação significativa na atividade da citrato sintase nas brânquias e músculo dos peixes expostos ao Cu por 345 dias. No entanto, foi observado um aumento significativo da atividade da citrato sintase no fígado dos peixes expostos a 9 μg/L Cu por 345 dias. Cabe lembrar que a citrato sintase é uma enzima-chave do ciclo de Krebs e tem papel importante no processo de geração de energia no metabolismo aeróbico. Portanto, o aumento do consumo de oxigênio corporal observado nos peixes expostos ao Cu por 28 dias poderia estar associado ao aumento na atividade da citrato sintase hepática observado em *P. vivipara* após exposição ao Cu por 345 dias. Porém, infelizmente o consumo corporal e/ou tecidual de oxigênio não foi avaliado nos peixes expostos ao Cu por 345 dias. Por sua vez, este aumento na atividade da citrato sintase parece estar relacionado a uma maior demanda energética para detoxificação do Cu no fígado e manutenção dos níveis tecidual e corporal do metal.

6. CONCLUSÃO FINAL

Os resultados relatados no presente estudo indicam que *P. vivipara* consegue manter os níveis corporais ou teciduais de Cu por longo prazo (345 dias) quando exposto a concentrações ambientalmente relevantes do metal na água salgada, sendo que esta capacidade está associada à regulação da transcrição de genes envolvidos na expressão de proteínas transportadoras de Cu, tais como o CTR1 e a ATP7B. Além disso, eles demonstram que *P. vivipara* acumula Cu após exposição crônica a concentrações excessivas de Cu (9 e 20 μg/L Cu), o que causa efeitos sub-letais (aumento do metabolismo aeróbico e redução no crescimento), podendo causar até mortalidade.

REFERÊNCIAS BIBLIOGRÁFICAS

AGARWAL, S. K. 2009. Heavy Metal Pollution. APH Publishing. 270 p.

AMARAL, M. D., BONECKER, A. C. T., ORTIZ, C. H. D. 2001. Activity determination of Na+K+-ATPase and Mg++-ATPase enzymes in the gill of *Poecilia vivipara* (Osteichthyes, Cyprinodontiformes) in different salinities. *Brazilian Archives of Biology and Technology*, *44*(1), 1–6. DOI: 10.1590/S1516-89132001000100001

ARAÚJO, F. G., PEIXOTO, M. G., PINTO, B. C. T., TEIXEIRA, T. P. 2009. Distribution of guppies *Poecilia reticulata* (Peters, 1860) and *Phalloceros caudimaculatus* (Hensel, 1868) along a polluted stretch of the Paraíba do Sul River, Brazil. *Brazilian Journal of Biology*, *69*(1), 41–48. DOI:10.1590/S1519-69842009000100005

AY, Ö., KALAY, M., TAMER, L., CANLI, M. 1999. Copper and Lead Accumulation in Tissues of a Freshwater Fish Tilapia zillii and its Effects on the Branchial Na,K-ATPase Activity. *Bulletin of Environmental Contamination and Toxicology*, 62, 160–168. DOI: 10.1007/s001289900855

BALAVENKATASUBBAIAH, M., RANI, A U., GEETHANJALI, K., PURUSHOTHAM, K. R., RAMAMURTHI, R. 1984. Effect of cupric chloride on oxidative metabolism in the freshwater teleost, *Tilapia mossambica*. *Ecotoxicology and Environmental Safety*, *8*, 289–293. DOI:10.1016/0147-6513(84)90033-2

BELYAEVA, E. A, KOROTKOV, S. M., SARIS, N-E. 2011. In vitro modulation of heavy metal-induced rat liver mitochondria dysfunction: a comparison of copper and mercury with cadmium. *Journal of Trace Elements in Medicine and Biology*, 25 (1), 63-73. DOI: 10.1016/j.jtemb.2010.10.007

BJORKLUND, L. B., MORRISON, G. M. 1997. Determination of copper speciation in freshwater samples through SPE-spectrophotometry, *Analytica Chimica Acta*, 343 (3), 259-266. DOI:10.1016/S0003-2670(96)00599-5

BOPP, S. K., ABICHT, H. K., KNAUER, K. 2008. Copper-induced oxidative stress in rainbow trout gill cells. *Aquatic Toxicology*, 86, 197-204. DOI: 10.1016/j.aquatox.2007.10.014

BROWN, S. E., WELTON, W. C. 2008. Heavy Metal Pollution. Nova Science Publishers, Hauppauge, NY, USA. 381 p.

BUCK, K. N., ROSS, J. R. M., RUSSELL FLEGAL, A., AND BRULAND, K. W. 2007. A review of total dissolved copper and its chemical speciation in San Francisco Bay, California, *Environmental Research*, 105 (1), 5-19. DOI:10.1016/j.envres.2006.07.006

CARVALHO, C. D. S., FERNANDES, M. N. 2008. Effect of copper on liver key enzymes of anaerobic glucose metabolism from freshwater tropical fish *Prochilodus lineatus*. *Comparative Biochemistry and Physiology, Part A, 151*, 437–442. DOI:10.1016/j.cbpa.2007.04.016

CARVALHO, P. C., BUGONI, L., MCGILL, R. A. R., BIANCHINI, A., 2013. Metal and selenium concentrations in blood and feathers of petrels of the genus *Procellaria*. *Environmental. Toxicology. Chemistry*. 32, 1641–1648. DOI: 10.1002/etc.2204

CARVAN, M. J., GALLAGHER, E. P., GOKSØYR, A., HAHN, M. E., LARSSON, D. G. J. 2007. Fish models in toxicology. *Zebrafish*, *4*(1), 9–20. DOI: 10.1089/zeb.2006.9998

CDA - COPPER DEVELOPMENT ASSOCIATION INC. 2015. Applications. Disponível em: < http://www.copper.org/applications/>

CHEN, A. H.-R., YANG, H.-C., HSIEHA, D.S J.-Y., ZIJUAN, L., TSAI, K.-J. 2011. Zebrafish *sod1* and *sp1* expression are modulated by the copper ATPase gene *atp7a* in response to intracellular copper status. *Chemico-Biological Interactions*, 189, 192–197. DOI: 10.1016/j.cbi.2010.12.003

CLEARWATER, S. J., FARAG, A. M., MEYER, J. S. 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. *Comparative Biochemistry and Physiology. Part C*, *132*(3), 269–313. DOI: 10.1016/S1532-0456(02)00078-9

CONAMA - CONSELHO NACIONAL DO MEIO AMBIENTE. 2005. Resolução Conama nº 357. Disponível em:< http://www.mma.gov.br/port/conama>

COUTURE, P., KUMAR, P. R. 2003. Impairment of metabolic capacities in copper and cadmium contaminated wild yellow perch (*Perca flavescens*). *Aquatic Toxicology*, *64*(1), 107–120. DOI:10.1016/S0166-445X(03)00028-6

CRAIG, P. M., GALUS, M., WOOD, C. M., MCCLELLAND, G. B. 2009. Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (*Danio rerio*). *American Journal of Physiology—Regulatory Integrative and Comparative Physiology* 296 (2), 362–R373. DOI: 10.1152/ajpregu.90581.2008

CUNHA, V. L., RODRIGUES, R. V., OKAMOTO, M. H., SAMPAIO, L. A., 2009. Consumo de oxigênio pós-prandial de juvenis de pampos *Trachinotus marginatus*. *Ciência Rural*. 39, 1257-1259.

DANCIS, A., YUAN, D. S., HAILE, D., ASKWITH, C., EIDE, D. J., MOEHLE, C., KAPLAN, J., KLAUSNER, R. D. 1994. Molecular characterization of a copper transport protein in *S. cerevisiae*: An unexpected role for copper in iron transport. *Cell*, 76, 393-402. DOI:10.1016/0092-8674(94)90345-X

DANG, Z. C., FLIK, G., DUCOURET, B., HOGSTRAND, C., WENDELAAR BONGA, S. E., LOCK, R. A. 2000. Effects of copper on cortisol receptor and

metallothionein expression in gills of *Oncorhynchus mykiss*. *Aquatic toxicology* 51(1), 45-54. DOI: doi:10.1016/S0166-445X(00)00102-8

DAS, S., GUPTA, A. 2013. Accumulation of Copper in Different Tissues and Changes in Oxygen Consumption Rate in Indian Flying Barb, *Esomus danricus* (Hamilton-Buchanan) Exposed to Sub-lethal Concentrations of Copper. *Jordan Journal of Biological Sciences*, *6*(1), 21–24.

DE BOECK, G., SMET, H. DE, BLUST, R. 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. *Aquatic Toxicology*, *32*, 127–141.

DONG, S., KANG, M., WU, X., YE, T. 2014. Development of a promising fish model (*Oryzias melastigma*) for assessing multiple responses to stresses in the marine environment. *BioMed Research International*, 17 p. DOI: 10.1155/2014/563131

EYCKMANS, M., TUDORACHE, C., DARRAS, V. M., BLUST, R., DE BOECK, G. 2010. Hormonal and ion regulatory response in three freshwater fish species following waterborne copper exposure. *Comparative Biochemistry and Physiology Part C*, 152, 270-278. DOI: 10.1016/j.cbpc.2010.05.002

FENG, Q., BOONE, A. N., VIJAYAN, M. M. 2003. Copper impact on heat shock protein 70 expression and apoptosis in rainbow trout hepatocytes. *Comparative Biochemistry and Physiology Part C, 135*(3), 345-355. DOI: 10.1016/S1532-0456(03)00137-6

FERREIRA, R.S., MONSERRAT, J.M., FERREIRA, J.L.R., KALB, A.C., STEGEMAN, J., BAINY, A.C.D., ZANETTE, J. 2012. Biomarkers of organic contamination in the South American fish *Poecilia vivipara* and *Jeninsia multidentata*. *Journal of Toxicology and Environmental Health* 75A, 1–11. DOI: 10.1080/15287394.2012.697813

FESTA, R.A.; THIELE, D.J. 2011. Copper: An essential metal in biology. *Current. Biology*. 8, 877–883. DOI: 10.1016/j.cub.2011.09.040

FINDIK, Ö., ÇIÇEK, E. 2011. Metal Concentrations in Two Bioindicator Fish Species, *Merlangius merlangus*, *Mullus barbatus*, Captured from the West Black Sea Coasts (Bartin) of Turkey. *Bulletin of Environmental Contamination and Toxicology*, 87(4), 399-403 DOI: 10.1007/s00128-011-0373-1

FINNEY, L. A., O'HALLORAN, T. V. 2003. Transition Metal Speciation in the Cell: Insights from the Chemistry of Metal Ion Receptors, *Science*, 300 (5621), 931-936. DOI: 10.1126/science.1085049

GARCEAU, N., PICHAUD, N., COUTURE, P. 2010. Inhibition of goldfish mitochondrial metabolism by in vitro exposure to Cd, Cu and Ni. *Aquatic Toxicology, 98*(2), 107–112. DOI: 10.1016/j.aquatox.2010.01.020

GARCÍA, N., MARTÍNEZ-ABUNDIS, E., PAVÓN, N., CORREA, F., CHÁVEZ, E. 2007. Copper induces permeability transition through its interaction with the adenine nucleotide translocase. *Cell Biology International*, 31(9), 893-899. DOI: 10.1016/j.cellbi.2007.02.003

GOMES JR., J. L., MONTEIRO, L. R. 2008. Morphological divergence patterns among populations of *Poecilia vivipara* (Teleostei Poeciliidae): test of an ecomorphological paradigm. *Biological Journal of the Linnean Society*, 93, 799-812. DOI: 10.1111/j.1095-8312.2007.00945.x

GROSELL, M., MCDONALD, M. D. WALSH, P. J. WOOD, C. M. 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. *Aquatic Toxicology*, 68, 263–275. DOI: 10.1016/j.aquatox.2004.03.006

GROSELL, M., WOOD, C. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. *Journal of Experimental Biology*, 1188, 1179–1188.

GUTHRIE, J. W., HASSAN, N. M., SALAM, M. S. A., FASFOUS, I. I., MURIMBOH, C. A., MURIMBOH, J., CHAKRABARTI, C. L., AND GREGOIRE, D. C. 2005. Complexation of Ni, Cu, Zn, and Cd by DOC in some metal-impacted freshwater lakes: a comparison of approaches using electrochemical determination of free-metal-ion and labile complexes and a computer speciation model, WHAM V and VI. *Analytica Chimica Acta*, 528 (2), 205-218. DOI:10.1016/j.aca.2004.10.003

HANDY, R. D. 2003. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? *Comparative Biochemistry and Physiology, Part A*, 135(1), 23-38. DOI: 10.1016/S1095-6433(03)00018-7

HARAYASHIKI, C. A. Y., VARELA, A. S., MACHADO, A. A. D. S., CABRERA, L. D. C., PRIMEL, E. G., BIANCHINI, A., CORCINI, C. D. 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquatic Toxicology, 142-143, 176–84. DOI:10.1016/j.aquatox.2013.08.006

HASSAN, B. K. 2011. The effect of copper and cadmium on oxygen consumption of the juvenile common carp, *Cyprinus carpio* (L.). *Mesopotamian Journal of Marine Science*, 26(1), 25–34.

JAMES, R., SAMPATH, K. JOTHILAKSHMI, S., VASUDHEVAN, I., THANGARATHINAM, R. 2008. Effects of copper toxicity on growth, reproduction and metal accumulation in chosen ornamental fishes. *Ecohydrology and Hydrobiology*. DOI:10.2478/v10104-009-0007-y

JAMES, R., SAMPATH, K., EDWARD, D. S. 2003. Copper Toxicity on Growth and Reproductive Potential in an Ornamental Fish, *Xiphophorus helleri*. *Asian Fisheries Science*, 16, 317-326.

KAMUNDE, C., GROSELL, M., HIGGS, D., WOOD, C. M. 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between

dietary and waterborne copper uptake. *The Journal of Experimental Biology*, 205(Pt 2), 279–90.

KELLY, S. A., HAVRILLA, C. M., BRADY, T. C., ABRAMO, K. H., LEVIN, E. D. 1998. Oxidative stress in toxicology: Established mammalian and emerging piscine model systems. *Environmental Health Perspectives*, *106*(7), 375–384. DOI: 10.1289/ehp.98106375

KIM, B. E., NEVITT, T., THIELE, D. J. 2008, Mechanisms for copper acquisition, distribution and regulation, *Nature Chemical Biology*, 4(3), 176-185. DOI: 10.1038/nchembio.72.

KNIGHT, S. A., TAMAI, K. T., KOSMAN, D. J., THIELE, D. J. 1994. Identification and analysis of a *Saccharomyces cerevisiae* copper homeostasis gene encoding a homeodomain protein. *Molecular and Cellular Biology*, *14*(12), 7792-804. DOI: 10.1128/MCB.14.12.7792

KROT, K. A., DE NAMOR, A. F. D., GUILAR-CORNEJO, A., NOLAN, K. B. 2005, Speciation, stability constants and structures of complexes of copper(II), nickel(II), silver(I) and mercury (II) with PAMAM dendrimer and related tetraamide ligands. *Inorganica Chimica Acta*, 358 (12), 3497-3505. DOI:10.1016/j.ica.2005.05.001

KRUMSCHNABEL, G., MANZL, C., BERGER, C., HOFER, B. 2005. Oxidative stress, mitochondrial permeability transition, and cell death in Cu-exposed trout hepatocytes. *Toxicology and Applied Pharmacology*, *209*(1), 62-73. DOI: 10.1016/j.taap.2005.03.016

LA FONTAINE, S., ACKLAND, M. L., MERCER, J. F. B. 2010. Mammalian copper-transporting P-type ATPases, ATP7A and ATP7B: emerging roles. *The International Journal of Biochemistry & Cell Biology*, 42(2), 206–9. DOI:10.1016/j.biocel.2009.11.007

LA FONTAINE, S., MERCER, J. F. B. 2007. Trafficking of the copper-ATPases, ATP7A and ATP7B: role in copper homeostasis. *Archives of Biochemistry and Biophysics,* 463(2), 149–67. DOI:10.1016/j.abb.2007.04.021

LALLIER, F. H., WALSH, P. J. 1991. Metabolic potential in tissues of the blue crab, *Callinectes sapidus*. *Bulletin of Marine Science*. 48(3), 665-669.

LAPOINTE, D., PIERRON, F., COUTURE, P. 2011. Individual and combined effects of heat stress and aqueous or dietary copper exposure in fathead minnows (*Pimephales promelas*). *Aquatic Toxicology. 104*(1-2), 80–85. DOI:10.1016/j.aquatox.2011.02.022

LAUER, M. M., OLIVEIRA, C. B. DE, YANO, N. L. I., BIANCHINI, A. 2012. Copper effects on key metabolic enzymes and mitochondrial membrane potential in gills of the estuarine crab *Neohelice granulata* at different salinities. *Comparative Biochemistry and Physiology, Part C*, *156*(3-4), 140–147. DOI: 10.1016/0147-6513(84)90033-2

LAWS, E., A. 2000. Aquatic Pollution: An Introductory Text. Wiley. 3 edition. 672 p.

LEARY, S. C., WINGE, D. R., COBINE, P. A. 2009. "Pulling the plug" on cellular copper: the role of mitochondria in copper export. *Biochimica et Biophysica Acta*. *1793*(1), 146-153. DOI: 10.1016/j.bbamcr.2008.05.002

LEE, J., PETRIS, M. J., AND THIELE, D. J. 2002, Characterization of Mouse Embryonic Cells Deficient in the Ctr1 High Affinity Copper Transporter. Identification of a Ctr1- independent copper transport system. *Journal of Biological Chemistry*, 277, (43), 40253-40259. DOI: 10.1074/jbc.M208002200

LINDER, M. C., HAZEGH-AZAM, M., 1996. Copper biochemistry and molecular biology. American Journal of Clinical Nutrition, 63, 797S–811S

LIU, F., NI, H.-G., CHEN, F., LUO, Z.-X., SHEN, H., LIU, L. 2011. Metal accumulation in the tissues of grass carps (*Ctenopharyngodon idellus*) from fresh water around a copper mine in Southeast China. *Environmental Monitoring and Assessment*. DOI: 10.1007/s10661-011-2264-7.

LIU, X. J., LUO, Z., XIONG, B. X., LIU, X., ZHAO, Y. H., HU, G. F. 2010. Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. *Ecotoxicology and Environmental Safety*, 73(6), 1286-1291. DOI: 10.1016/j.ecoenv.2010.06.019

LOPES, T. M., BARCAROLLI, I. F., OLIVEIRA, C. B., SOUZA, M. M., BIANCHINI, A. 2011. Mechanisms of copper accumulation in isolated mantle cells of the marine clam *Mesodesma mactroides*. *Environmental. Toxicology. Chemistry*. 30, 1586–1592. DOI: 10.1002/etc.527

LUGO, R. S., NATHALÍ, G., VILLALOBOS DE B, L. B., MAIRIN, L. 2006. Immunological response of the freshwater fish *Colossoma macropomum* as a biomarker of copper exposure. *Bulletin of Environmental Contamination and Toxicology*, 77(6), 925-930. DOI: 10.1007/s00128-006-1232-3

LUNDEBYE, A. K., BERNTSSEN, M. H. G., WENDELAAR BONGA, S. E., MAAGE, A. 1999. Biochemical and physiological responses in Atlantic salmon (*Salmo salar*) following dietary exposure to copper and cadmium. *Marine Pollution Bulletin*, 39, 137–144. DOI:10.1016/S0025-326X(98)00208-2

LUTSENKO, S., BARNES, N. L., BARTEE, M. Y., DMITRIEV, A. O. Y., 2007. Function and regulation of human copper-transporting ATPases. *Physiological Reviews*., 87, 1011–1046. DOI: 10.1152/physrev.00004.2006

LUTSENKO, S., GUPTA, A., BURKHEAD, J., ZUZEL, V. 2008. Cellular multitasking: the dual role of human Cu-ATPases in cofactor delivery and intracellular copper balance. *Archives of Biochemistry and Biophysic*, 476, 22–32. DOI: 10.1016/j.abb.2008.05.005

MACHADO, A. A. D. S., HOFF, M. L. M., KLEIN, R. D., CARDOZO, J. G., GIACOMIN, M. M., PINHO, G. L. L., BIANCHINI, A. 2013. Biomarkers of

waterborne copper exposure in the guppy *Poecilia vivipara* acclimated to salt water. *Aquatic Toxicology. 138-139*(2013), 60–9. DOI:10.1016/j.aquatox.2013.04.009

MACKENZIE, N. C., BRITO, M., REYES, A. E., ALLENDE, M. L. 2004. Cloning, expression pattern and essentiality of the high-affinity copper transporter 1 (ctr1) gene in zebrafish. *Gene*, 328, 113–120. DOI:10.1016/j.gene.2003.11.019

MARTINS, S. E., BIANCHINI, A. 2008. Copper accumulation and toxicity in the Plata pompano *Trachinotus marginatus* Cuvier 1832 (Teleostei , Carangidae). *Journal of Aquatic Sciences*, 1832, 384-390.

MATTOS, J. J., SIEBERT, M. N., LUCHMANN, K. H., GRANUCCI, N., DORRINGTON, T., STOCO, P. H., GRISARD, E. C., BAINY, A. C. D. 2010. Differential gene expression in *Poecilia vivipara* exposed to diesel oil water accommodated fraction. Marine Environmental Research. 69, S31–S33 DOI: 10.1016/j.marenvres.2009.11.002

MAZON, A F., FERNANDES, M. N. 1999. Toxicity and differential tissue accumulation of copper in the tropical freshwater fish, *Prochilodus scrofa* (Prochilodontidae). *Bulletin of Environmental Contamination and Toxicology*, 63(6), 797-804. DOI: 10.1007/s001289901049

MCGEER, J., SZEBEDINSZKY, C., MCDONALD, D., WOOD, C. 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. *Aquatic Toxicology*, 50, 231–243. DOI: 10.1016/S0166-445X(99)00105-8

MERCER, J. F., BARNES, N., STEVENSON, J., STRAUSAK, D., AND LLANOS, R. M. 2003. Copper- induced trafficking of the Cu-ATPases: a key mechanism for copper homeostasis. *Biometals* 16, 175–184. DOI: 10.1023/A:1020719016675

MEREDITH, R. W., PIRES, M. N., REZNICK, D. N., SPRINGER, M. S. 2011. Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation in *Poecilia* (Poeciliidae: Cyprinodontiformes). *Molecular Phylogenetics and Evolution,* 59, 148–157. DOI:10.1016/j.ympev.2011.01.014

MINGHETTI, M., LEAVER, M. J., CARPENÈ, E., GEORGE, S. G. 2008. Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper. *Comparative Biochemistry and Physiology, Part C*, 147(4), 450-459. DOI:10.1016/j.cbpc.2008.01.014

MINGHETTI, M., LEAVER, M. J., GEORGE, S. G. 2010. Multiple Cu-ATPase genes are differentially expressed and transcriptionally regulated by Cu exposure in sea bream, *Sparus aurata*. *AquaticToxicology,* 97(1), 23-33. DOI: 10.1016/j.aquatox.2009.11.017

MINGHETTI, M., LEAVER, M. J., TAGGART, J. B., CASADEI, E., AUSLANDER, M., TOM, M. 2011. Copper induces Cu-ATPase ATP7A mRNA in a fish cell line,

SAF1. *Comparative Biochemistry and Physiology Part C,* 154, 93-99. DOI:10.1016/j.cbpc.2011.03.010

MONTEIRO, S. M., DOS SANTOS, N. M., CALEJO, M., FONTAINHAS-FERNANDES, A., SOUSA, M. 2009. Copper toxicity in gills of the teleost fish, *Oreochromis niloticus*: effects in apoptosis induction and cell proliferation. *Aquatic Toxicology*, 94, 219-228. DOI: 10.1016/j.aquatox.2009.07.008

MUFTI, A. R., BURSTEINB, E., DUCKETTA, C. S. 2007. XIAP: Cell death regulation meets copper homeostasis. *Archives of Biochemistry and Biophysics*, 463, 2, 168–174. DOI: 10.1016/j.abb.2007.01.033

NELSON, D. L.; COX, M. M. 2008. Lehninger Principles of Biochemistry**.** 5th ed. W. H. Freeman: New York, 1100p.

NEVES, F. M., MONTEIRO, L. R. 2003. Body shape and size divergence among populations of *Poecilia vivipara* in coastal lagoons of south-eastern Brazil. *Journal of Fish Biology* 63, 928–94. DOI: 10.1046/j.1095-8649.2003.00199.x

NOSE, Y., REES, E. M., THIELE, D. J. 2006. Structure of the Ctr1 copper trans'PORE'ter reveals novel architecture. *Trends in Biochemical Sciences*, 31(11) 604-607. DOI: 10.1016/j.tibs.2006.09.003

O'HARA, J. 1971. Alterations in oxygen consumption by bluegills exposed to sublethal treatment with copper. *Water Research*. DOI:10.1016/0043-1354(71)90177-1

PAULO, D. V., FONTES, F. M. ,FLORES-LOPES, F. 2012.Histopathological alterations observed in the liver of *Poecilia vivipara* (Cyprinodontiformes: Poeciliidae) as a tool for the environmental quality assessment of the Cachoeira River, BA. *Brazilian Journal of Biology* 72(1), 131-140.

PFAFFL, M. W., HORGAN, G. W., DEMPFLE, L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, 30(9): e36. DOI: 10.1093/nar/30.9.e36

PUIG, S., THIELE, D. J. 2002. Molecular mechanisms of copper uptake and distribution. *Current Opinion in Chemical Biology*, 6(2), 171-180.

SÁNCHEZ, M. L. 2008. Causes and effects of heavy metal pollution. Nova Science Publishers, Hauppauge, NY, USA. 392 p.

SANTOS, E. G. N., CUNHA, R. A. SANTOS, C. P. 2011. Behavioral responses of *Poecilia vivipara* (Osteichthyies: Cyprinodontiformes) to experimental infections of *Acanthocollaritrema umbilicatum* (Digenea:Cryptogonimidae). *Experimental Parasitology*, 127 522–526. DOI: 10.1016/j.exppara.2010.10.018

SCHMITTGEN, T. D., LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative C_t method. *Nature Protocols*, 3(6), 1101–1108. DOI: 10.1038/nprot.2008.73

SHARP, P. A. 2003. Ctr1 and its role in body copper homeostasis. *The International Journal of Biochemistry & Cell Biology*, 35(3), 288–291. DOI:10.1016/S1357-2725(02)00134-6

SILVA, E. S., ABRIL, S. I. M., ZANETTE, J., BIANCHINI, A. 2014. Salinity-dependent copper accumulation in the guppy *Poecilia vivipara* is associated with *CTR1* and *ATP7B* transcriptional regulation. *Aquatic Toxicology*, 152, 300–7. DOI:10.1016/j.aquatox.2014.04.024

SMITH, R. W., BLANEY, S. C., DOWLING, K., STURM, A., JÖNSSON, M., HOULIHAN, D. F. 2001. Protein synthesis costs could account for the tissue-specific effects of sub-lethal copper on protein synthesis in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, 53, 265–277. DOI: 10.1016/S0166-445X(01)00171-0

TAYLOR, L., MCGEER, J. 2000. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchusmykiss*) in hard and soft water: evaluation of chronic indicators. *Environmental Toxicology and Chemistry,* 19 (9), 2298–2308. DOI:/10.1002/etc.5620190920/full

WOOD C, M., FARRELL, A. P., BRAUNER, C. J. 2011. Fish Physiology: Homeostasis and Toxicology of Essential Metals. Fish Physiology, 31 (1), Academic Press, 520 p.

ZANETTE, J. 2013. Genes and Proteins Related with Biotransformation in Tropical Fishes. In: ALMEIDA, E. A., RIBEIRO, C. A. O. Pollution and Fish Health in Tropical Ecosystems. CRC Press. chapter 2, 15-34.

ZIMMER, A. M., BARCAROLLI, I. F., WOOD, C. M., BIANCHINI, A. 2012. Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. *Aquatic Toxicology*, 122-123, 172–80. DOI:10.1016/j.aquatox.2012.06.010