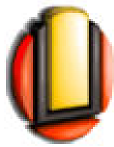

UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG

INSTITUTO DE CIÊNCIAS BIOLÓGICAS - ICB

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS – FISIOLOGIA ANIMAL COMPARADA

ALTERAÇÕES NO METABOLISMO MITOCONDRIAL E EIXO SOMATOTRÓPICO NO TELEÓSTEO *Poecilia vivipara* APÓS EXPOSIÇÃO CRÔNICA AO COBRE

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Fisiológicas – Fisiologia Animal Comparada da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de MESTRE.

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Rio Grande

Fevereiro, 2016

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AGRADECIMENTOS

Durante a realização desta dissertação, contei com a ajuda de várias pessoas e instituições. Desta forma, agradeço:

Primeiramente à minha família, pelo apoio incondicional que sempre recebi. Mesmo com a distância que nos separa, não sei o que seria de mim sem vocês.

Ao meu orientador Adalto Bianchini, pela confiança que sempre depositou em mim. Aprendi muito contigo durante estes dois anos de convivência, tanto em termos científicos como pessoais. Espero que ainda possamos trabalhar juntos por muitos anos.

Ao Iuri Salim Abou Anni, pelas amostras e dados concedidos, assim como pela agradável convivência que tivemos neste período.

À Sandra Isabel Moreno Abril, pela paciência em me ensinar as técnicas de biologia molecular usadas neste trabalho.

À Roberta Daniele Klein, pela ajuda nas técnicas de dosagem hormonal por ELISA, assim como pelas risadas durante o processo.

A todo o Grupo de Pesquisa em Toxicologia Aquática pela convivência agradabilíssima e pelas discussões sempre produtivas durante nossas reuniões.

A todos os funcionários do programa da pós em fisiologia animal comparada, desde secretários, faxineiros, até os técnicos e professores.

Às instituições: ICB (FURG) e IB (UFPel), pelo espaço concedido.

Às agências de fomento: FAPERGS, CAPES, CNPq (INCT-TA) e IDRC pelo auxílio financeiro que foi concedido direta ou indiretamente.

Por fim, a todos os meus amigos, sejam de Rio Grande ou de Pelotas, que me fazem companhia nos mates, nos almoços, nos jantares, nos bares e nas festas.

A todos, meu muito obrigado.

Sapere aude!

Habe Mut dich deines eigenen Verstandes zu bedienen!

"Ouse saber!

Tenha a coragem de usar o seu próprio conhecimento!"

Immanuel Kant em: "Resposta à Pergunta: Que é esclarecimento?"

RESUMO

O cobre (Cu) é um metal essencial à vida, porém é toxico quando em altas concentrações. Seus efeitos incluem redução de crescimento e alteração no metabolismo energético. Portanto, o objetivo do presente estudo foi avaliar os efeitos da exposição crônica ao Cu sobre o metabolismo mitocondrial e a regulação do eixo somatotrópico em diferentes tecidos do teleósteo *Poecilia vivipara* através da avaliação da expressão dos genes que codificam o GH, GHR1, GHR2, IGF1, IGF2, COX I, COXII, COXIII e ATP5A1, bem como através da análise da concentração de GH e GHR. Peixes recém-nascidos (<24 h) foram mantidos sob condição controle (sem adição de Cu na água) ou expostos ao metal (5 e 9 µg/L) por 345 dias em água salgada (salinidade 24; temperatura 28°C). Após exposição, foram coletadas amostras de cérebro, músculo esquelético e fígado. No músculo, houve uma redução na expressão gênica da COX III e do GHR2 após exposição a 5 µg/L de Cu, bem como da COX II e III, do IGF 1 e IGF2 e do GHR2 após exposição a 9 µg/L de Cu. No fígado, houve um aumento na expressão gênica da ATP5A1 após exposição a 9 µg/L de Cu. Portanto, a exposição crônica ao Cu causa uma redução na expressão de genes relacionados à cadeia transportadora de elétrons no músculo, o que pode reduzir a produção de ATP. Além disso, causa uma dessensibilização ao GH associada à diminuição na expressão gênica do GHR2, levando a uma diminuição na expressão gênica do IGF1 e IGF2. Esta resposta pode estar relacionada a uma alteração na razão entre as isoformas do GHR2 e não a uma diminuição na concentração total de GHR2. No fígado, a exposição crônica ao Cu induz uma resposta compensatória no metabolismo energético, a qual pode estar relacionada ao papel deste órgão no processo de detoxificação de metais. Portanto, a diminuição no crescimento de peixes induzida pela exposição crônica ao Cu está relacionada à redução na capacidade de produção de ATP e desregulação do eixo somatotrópico no músculo esquelético, impedindo que este tecido produza adequadamente fatores tróficos importantes para a manutenção do anabolismo. Além disso, a exposição crônica ao Cu pode causar um aumento no consumo de ATP hepático, o que contribuiria indiretamente para a redução do crescimento.

Palavras-chave: cadeia transportadora de elétrons, cobre, crescimento, desregulação endócrina, exposição crônica, fosforilação oxidativa, mitocôndria.

INTRODUÇÃO GERAL

Metais

Os metais são constituintes naturais da crosta terrestre e são redistribuídos para os ecossistemas da terra por processos geoquímicos de erosão e lixiviação (Santore et al., 2000). Os metais foram e continuam sendo essenciais para o desenvolvimento e perpetuação da tecnologia humana por apresentarem atributos como boa condutividade elétrica e de calor, alto ponto de fusão e de ebulição e uma elevada dureza (Nogueira et al., 2005). Desta forma, o processo de desenvolvimento da civilização humana caminha lado a lado a um processo antrópico de extração de metais da natureza (Mazzuco, 2008).

Por outro lado, os metais são elementos importantes para o funcionamento dos diversos níveis da organização biológica, e alguns deles possuem papel essencial para as numerosas formas de vida encontradas no nosso planeta (Rezende e Lacerda, 1986; Chapman et al., 1996). Neste contexto, as atividades humanas de mineração alteram drasticamente os ciclos geoquímicos dos metais, fazendo com que eles sejam encontrados em ambientes aos quais eles não faziam parte ou então que sejam encontrados em concentrações muito mais elevadas do que as naturalmente ocorrentes (Mazzuco, 2008), gerando assim uma contaminação ambiental.

Os metais liberados no ambiente possuem destinos diversos, podendo interagir com animais ou plantas e causar efeitos biológicos. Quando estes elementos se encontram em elevadas concentrações, tais efeitos podem ser tóxicos, colocando os metais como possíveis poluentes. A toxicidade dos metais varia de acordo com características intrínsecas a cada elemento, assim como sua disponibilidade biológica, concentração e forma química (Rezende e Lacerda, 1986; Chapman et al., 1996).

A importância do cobre e seus mecanismos de toxicidade

O cobre é um dos metais liberados no ambiente a partir de diversas atividades humanas. Ele é um elemento de transição que pode ser encontrado

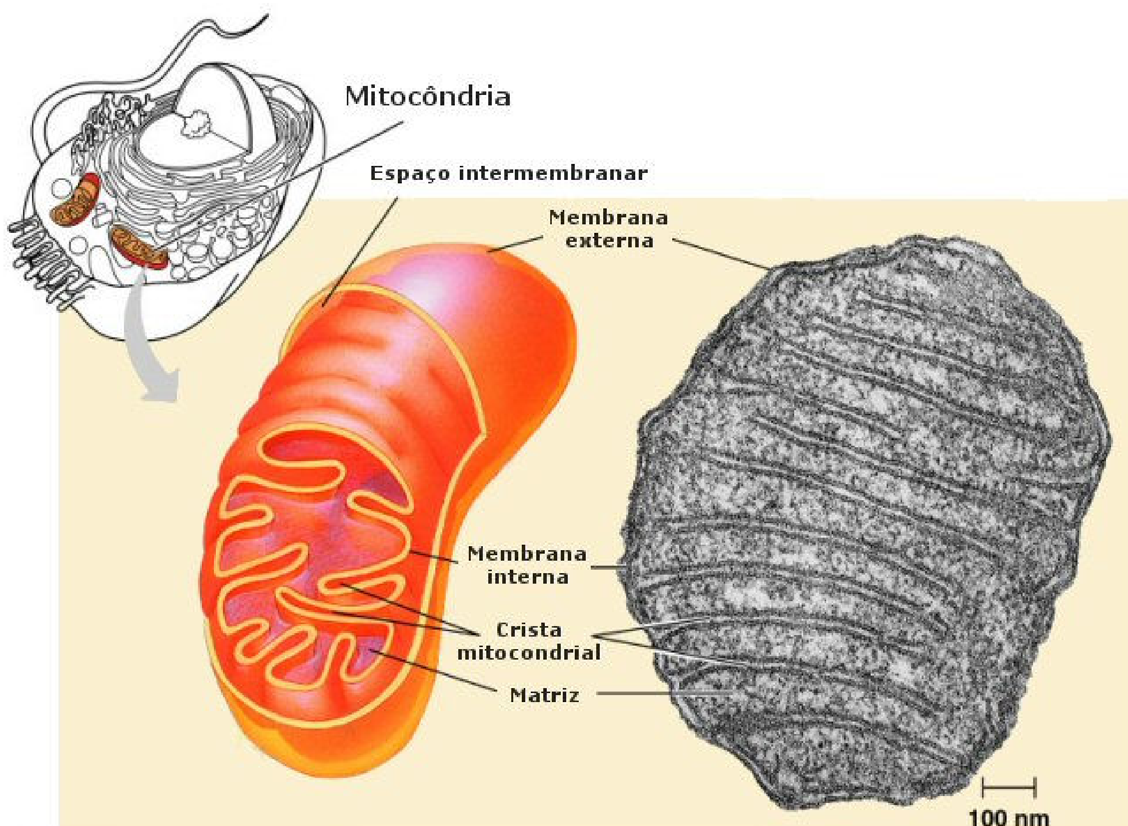
no ambiente em quatro formas químicas distintas: o cobre elementar (Cu^{0}), o íon cuproso (Cu^{1+}), o íon cúprico (Cu^{2+}) e o íon trivalente (Cu^{3+}), sendo a forma cúprica a mais reativa. O Cu^{2+} reage preferencialmente com ligantes inorgânicos via oxigênio, como por exemplo, H_2O_2, OH^-, CO_3^- e SO_4^{2-}, bem como a compostos orgânicos, através de grupos fenólicos e carboxílicos (Barceloux, 1999). Sendo assim, o cobre pode ser absorvido por plantas e animais e cumprir papéis bioquímicos e fisiológicos. No caso dos animais, a ingestão também é uma forma relevante na aquisição de cobre.

De fato, o cobre é um metal essencial para a vida, possuindo diversas funções importantes, tais como estruturação de proteínas reguladoras da homeostase celular (Knight et al., 1994), regulação de processos de resposta ao estresse oxidativo (Bopp et al., 2008; Leary et al., 2009), sinalização de hormônios esteroides (Dang et al., 2000) e respiração mitocondrial (Belyaeva et al., 2011). Apesar de seus papéis fisiológicos, quando o cobre se encontra em concentrações elevadas no ambiente, ele pode se acumular em diversos tecidos como fígado, rim, brânquias e intestino e causar efeitos tóxicos, comprometendo o funcionamento celular. O mecanismo de toxicidade mais conhecido do cobre envolve alterações na capacidade de regulação iônica e osmótica de animais aquáticos, visto que este metal pode inibir a transcrição e atividade da Na^+/K^+-ATPase e a atividade da anidrase carbônica (Zimmer et al., 2012). Outro mecanismo de toxicidade bem estabelecido para o cobre é o estresse oxidativo, considerando que este metal aumenta a produção de espécies reativas de oxigênio (ROS) e diminui a transcrição e atividade de proteínas que fazem parte do sistema antioxidante das células. Além dos efeitos já citados, o cobre pode alterar o consumo de oxigênio, equilíbrio ácido-base, excreção de amônia (Grosell et al., 2004), permeabilidade de membrana, sinalização de hormônios esteroides (Dang et al., 2000) e a proliferação celular (Monteiro et al., 2009), bem como causar danos celulares, gerar processos apoptóticos e alterar o metabolismo energético.

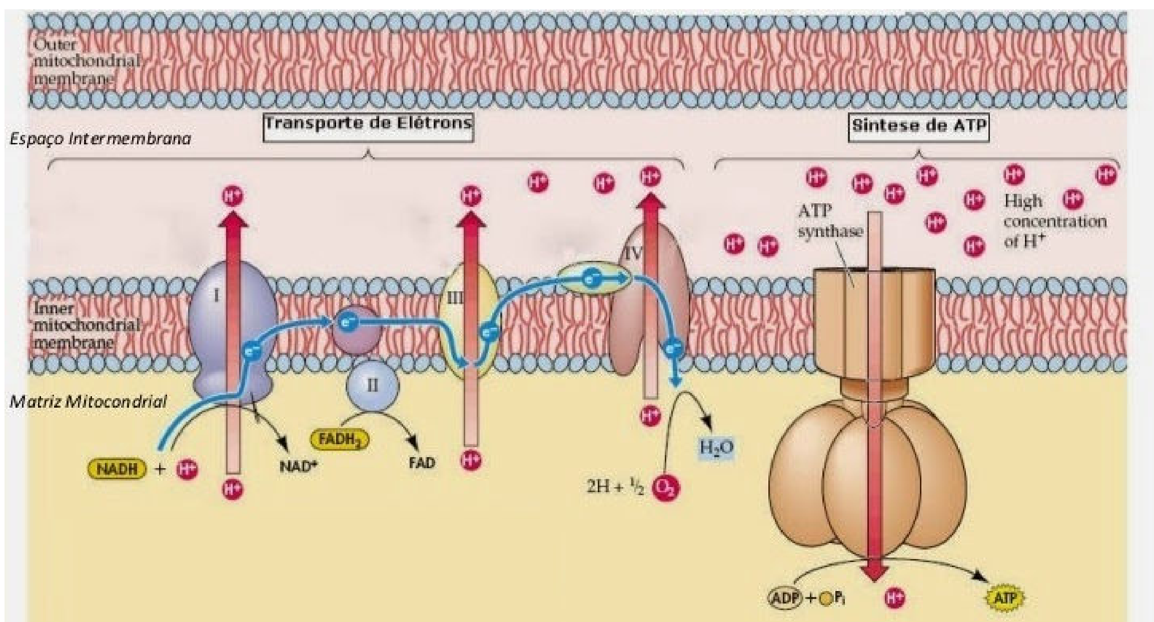
A maioria dos trabalhos toxicológicos envolvendo o cobre foi realizada considerando curtos períodos de exposição este metal. Desta forma, os efeitos tóxicos do cobre podem ser considerados bem conhecidos em termos de exposição aguda. Este cenário é bem diferente quando consideramos os potenciais efeitos crônicos do cobre após exposição em longo prazo. Os

103 estudos que são considerados crônicos apresentam períodos de exposição que
104 variam entre 60 e 100 dias, e mostram que, diferentemente das ações agudas,
105 efeitos crônicos do cobre envolvem grandes ajustes bioquímicos e fisiológicos,
106 tais como alterações imunológicas, redução do crescimento e mudança nos
107 padrões de liberação de catecolaminas e glicocorticóides (Handy, 2003).
108
109 **O cobre e o metabolismo energético**
110
111 Existem evidências que o cobre pode alterar o metabolismo energético a
112 partir de ações em várias vias energéticas e, consequentemente, alterar a
113 produção de ATP. Por exemplo, este metal pode alterar a atividade de enzimas
114 da via glicolítica, alterando assim o padrão de oxidação da glicose e de
115 produção do piruvato. O cobre pode ainda alterar a atividade da lactato
116 desidrogenase, enzima responsável pela transformação do piruvato em lactato
117 em meio anaeróbico. Ainda, o cobre pode modificar a atividade de enzimas do
118 Ciclo de Krebs, alterando assim a produção das coenzimas reduzidas NADH e
119 $FADH_2$. Por fim, este metal pode alterar o funcionamento da mitocôndria,
120 organela responsável pela maior parte da produção celular de ATP. É
121 interessante ressaltar que a grande parte das evidências que caracterizam as
122 referidas ações do cobre é proveniente de experimentos de exposição aguda
123 ao metal. Desta forma, os efeitos da exposição em longo prazo ao cobre sobre
124 o metabolismo energético são pouco conhecidos (Lauer et al., 2012).
125 Até onde é sabido, o único estudo demonstrando os efeitos da
126 exposição extremamente longa a concentrações ambientalmente relevantes do
127 cobre sobre o metabolismo energético em animais aquáticos foi realizado pelo
128 nosso grupo de pesquisa. Neste trabalho, foi demonstrado que a exposição ao
129 cobre por 345 dias não causou alteração significativa na atividade da piruvato
130 quinase e lactato desidrogenase no fígado, brânquias e músculo esquelético do
131 peixe teleósteo *Poecilia vivipara*. Da mesma forma, a atividade da citrato
132 sintase no músculo e nas brânquias também não foi alterada. Porém, a
133 exposição crônica a 9 µg/L de cobre levou a um aumento na atividade desta
134 enzima no fígado de *P. vivipara* (Anni, 2015). Este resultado indica que a
135 exposição ao metal levou a um aumento na via aeróbica de produção de
136 energia no fígado. Assim, um dos objetivos da presente dissertação foi avaliar

137 os componentes desta via relacionados com o metabolismo mitocondrial,
138 sendo eles a cadeia transportadora de elétrons e a fosforilação oxidativa.
139
140 **Metabolismo mitocondrial: cadeia transportadora de elétrons e**
141 **fosforilação oxidativa**
142
143 A mitocôndria é a organela responsável por manter a produção aeróbica
144 de ATP das células e este processo está intimamente relacionado a sua
145 morfologia. A mitocôndria é limitada por uma membrana externa que oferece
146 proteção e controle sobre o tráfego de substâncias. Já o interior desta organela
147 é subdivido por uma segunda membrana, chamada de membrana interna, que
148 se encontra disposta em forma de cristas, formando as chamadas cristas
149 mitocondriais. A membrana interna da mitocôndria delimita dois espaços
150 internos, chamados de matriz mitocondrial e espaço intermembranas (Fig. 1).
151

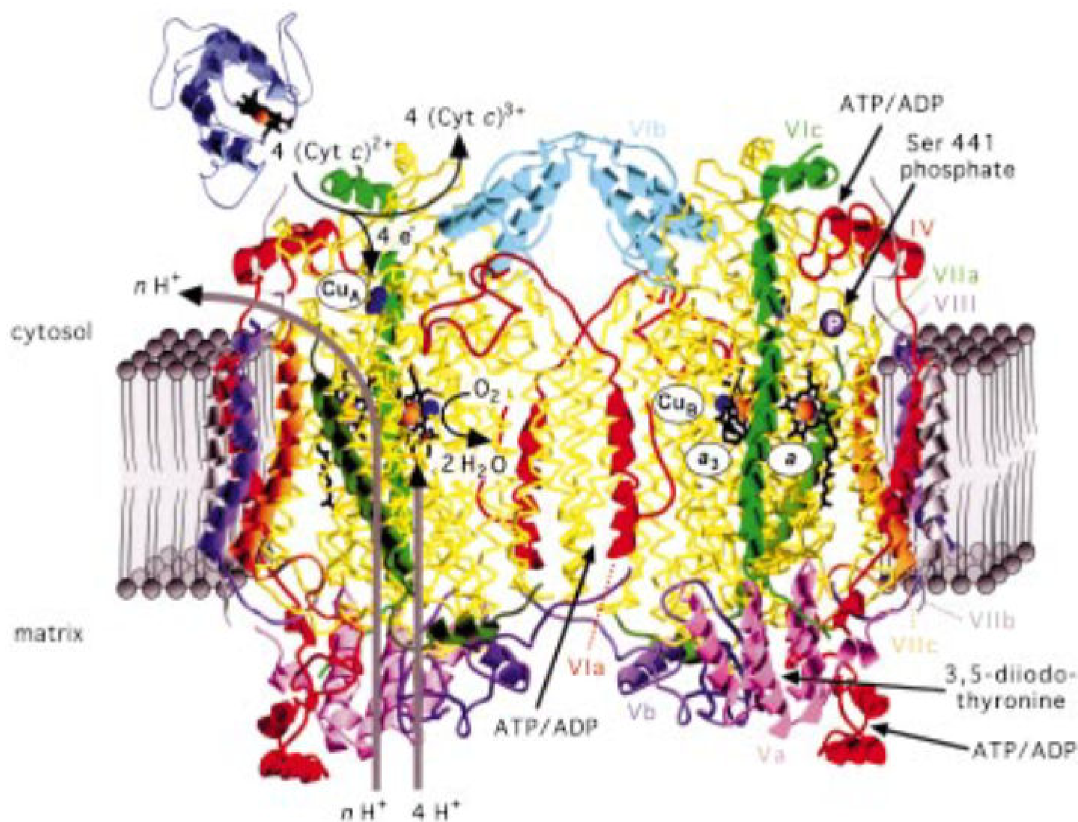


152
153 Figura 1 – Esquema mostrando as estruturas morfológicas que constituem uma
154 mitocôndria. Fonte: http://www.cientic.com/tema_celula_img4.html

155 Além disto, esta membrana abriga uma série de complexos proteicos
156 que formam a cadeia transportadora de elétrons. Os complexos I e II
157 pertencentes a esta cadeia, são responsáveis por oxidar as coenzimas
158 reduzidas NADH e $FADH_2$, produzidas no Ciclo de Krebs. Os elétrons liberados
159 no processo são transferidos para seu aceptor final, o oxigênio, pelos
160 complexos III e IV. A energia potencial liberada pelo transporte destes elétrons
161 através da cadeia transportadora faz com que os complexos I, III e IV
162 bombeiem prótons da matriz mitocondrial para o espaço intermembranas,
163 gerando uma energia potencial armazenada na forma de um gradiente
164 eletroquímico (Navarro e Boveris, 2007). Este gradiente é dissipado por um
165 quinto complexo proteico (Complexo V; ATP sintase) que permite a passagem
166 destes prótons de volta a matriz mitocondrial. Este processo transforma a
167 energia potencial eletroquímica em energia cinética, possibilitando um giro em
168 uma das porções que constitui a ATP sintase. Esta energia cinética é usada
169 para produzir o ATP a partir de ADP e fosfato orgânico, num processo
170 chamado fosforilação oxidativa (Devenish et al., 2000). A cadeia transportadora
171 de elétrons e a fosforilação oxidativa estão esquematizados na figura 2.
172
173 

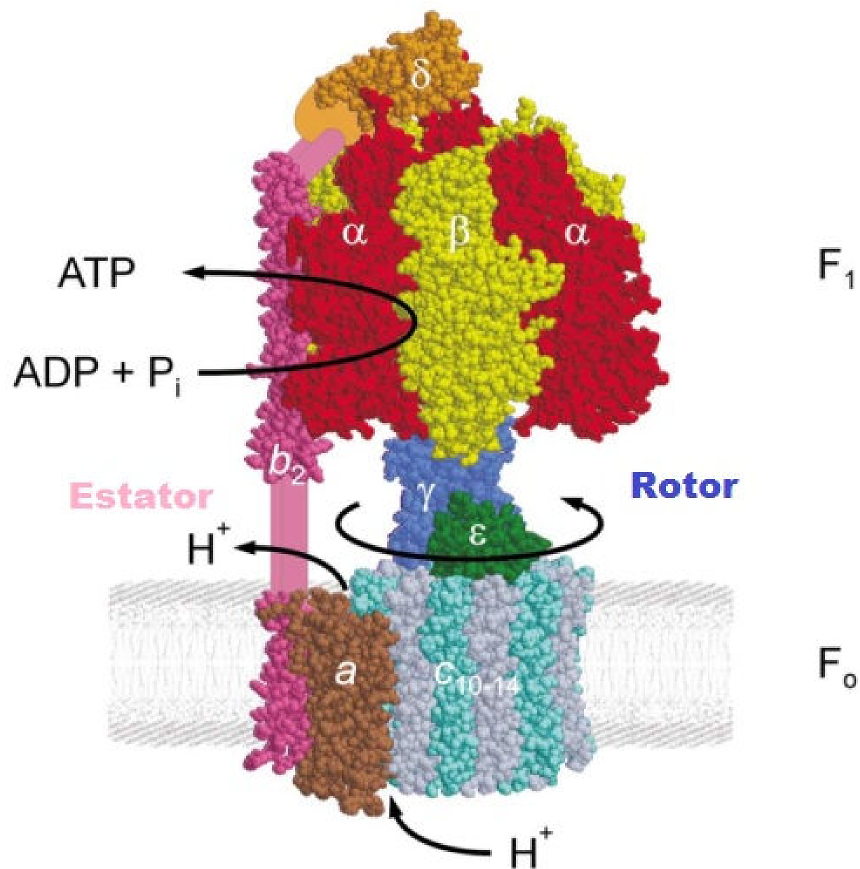
174 Figura 2 – Esquema demonstrando os complexos proteicos que constituem a cadeia
175 transportadora de elétrons e a fosforilação oxidativa, assim como suas respectivas funções.
176 ATP synthase = ATP sintase; High concentration of H^+ = alta concentração de H^+; Outer
177 mitochondrial membrane = membrana mitocondrial externa; Inner mitochondrial membrane =
178 membrana mitocondrial interna. Fonte: http://matiassinantropicos.blogspot.com.br/

179 Para avaliar os efeitos do cobre sobre o metabolismo mitocondrial, foi
180 analisada a expressão de alguns genes relacionados à cadeia transportadora
181 de elétrons e da fosforilação oxidativa, sendo estes associados à formação do
182 complexo IV e da ATP sintase, respectivamente.
183 O complexo IV ou citocromo c oxidase, é um dos complexos proteicos
184 responsáveis pelo transporte de elétrons até o oxigênio e pelo bombeamento
185 de prótons da matriz mitocondrial para o espaço intermembranas. Este
186 complexo faz parte de um seleto grupo de proteínas que são sintetizadas a
187 partir de subunidades pertencentes a dois genomas distintos. Três destas
188 subunidades são transcritas pelo genoma mitocondrial (COX I, COX II e COX
189 III) e dez outras subunidades são formadas pelo genoma celular (Fig. 3). As
190 três subunidades mitocondriais possuem grupos prostéticos essenciais para
191 sua atividade e que são formados pela interação com o íon cobre (Ludwig et
192 al., 2001).
193



194
195 Figura 3 – Representação esquemática da citocromo c oxidase e suas subunidades em
196 coração bovino. Fonte: Ludwig et al. (2001).

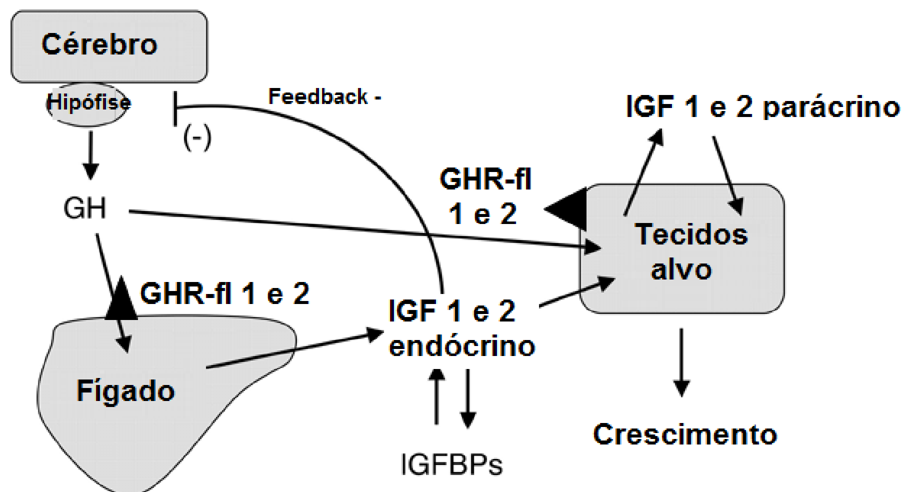
197 A ATP sintase é um complexo proteico formado por pelo menos 17
198 subunidades e é responsável pela produção aeróbica de ATP pela mitocôndria.
199 Esta proteína pode ser dividida em duas grandes porções, uma que se projeta
200 da membrana interna da mitocôndria, chamada de porção F_1, e uma que fica
201 integrada a esta membrana, chamada de porção $F_{0.}$ A porção F_1 da ATP
202 sintase funciona como um motor que apresenta um movimento rotacional
203 gerado pela hidrólise de ATP. Este movimento é gerado por um rotor que se
204 encontra atrelado a um estator formado pelas subunidades *a* (ATP5A1) e *b*
205 (ATP5B) (Devenish et al., 2000; Arechaga e Jones, 2001) (Fig. 4).
206



207
208 Figura 4 – Esquema representando a ATP sintase de *Escherichia coli* e suas subunidades.
209 Fonte: Weber e Senior (2003).
210
211 **O cobre e o crescimento em peixes**
212
213 Outra ação tóxica do cobre muito bem caracterizada é a inibição do
214 crescimento em peixes. Marr et al. (1996) demonstraram que a exposição

215 crônica (60 dias) a uma baixa concentração de cobre dissolvido na água (4,6
216 µg/L) diminuiu o crescimento da truta *Oncorhynchus mykiss*. Além disso, Kim et
217 al. (2004) demonstraram que a exposição crônica (60 dias) ao cobre via dieta
218 (50 mg/kg) diminuiu o crescimento do peixe *Sebastes schlegeli*. Por sua vez,
219 James et al. (2008) mostraram que a exposição crônica (100 dias) a uma alta
220 concentração de cobre dissolvido na água (100 µg/L) reduziu o crescimento
221 dos peixes *Carassius auratus* e *Xiphophorus helleri*.
222 É interessante notar que a maioria dos estudos que avaliam os efeitos
223 do cobre sobre o crescimento em peixes foi realizada com tempos de duração
224 que vão de 60 até 100 dias. Até onde se sabe, existem apenas dois estudos
225 que avaliaram os efeitos do cobre por períodos mais prolongados, sendo estes
226 realizados nos peixes teleósteos *Salvelinus fontinalis* (McKim et al., 1971) e *P.
227 vivipara* (Anni, 2015). Ambos os estudos utilizaram um tempo de exposição ao
228 cobre de aproximadamente 11 meses, sendo estes os únicos trabalhos que
229 avaliaram o efeito do cobre durante mais de um estágio de vida dos animais.
230 McKim et al. (1971) avaliou 5 concentrações de cobre (3,4; 5,7; 9,5; 17,4 e 32,5
231 µg/L) durante a fase de alevinos-juvenis e demonstraram que a exposição a
232 17,4 µg/L de cobre gerou efeitos drásticos na sobrevivência e crescimento dos
233 peixes. Por sua vez, Anni (2015) foi o primeiro a avaliar o efeito da exposição
234 crônica ao cobre desde o nascimento até a fase adulta dos peixes. Neste
235 estudo, as concentrações testadas foram 5 e 9 µg/L, sendo que ambas
236 causaram uma diminuição significativa no peso final dos indivíduos avaliados.
237 O crescimento é um processo fisiológico regulado pelo eixo
238 somatotrópico e a interação hipotálamo-hipófise é responsável pelo controle da
239 produção e liberação do hormônio do crescimento (GH) na corrente sanguínea.
240 O hipotálamo é o primeiro centro cerebral a receber estímulos intrínsecos e
241 extrínsecos, sendo responsável pela integração destes estímulos. A partir
242 desta integração, ele sincroniza o crescimento em vertebrados com o ambiente
243 no qual o animal se encontra, assim como com suas características internas.
244 Para regular o crescimento, o hipotálamo envia sinais para a hipófise, o
245 segundo centro cerebral a regular o crescimento. No que se refere ao
246 crescimento, o hipotálamo regula a atividade hipofisária a partir da secreção do
247 hormônio liberador do hormônio do crescimento (GHRH). Já a hipófise, é o
248 centro cerebral responsável pela produção e liberação do GH, que ao atingir a

249 corrente sanguínea, se liga a seu receptor específico (GHR), o qual pode estar
250 presente em diversos órgãos (Butler et al., 2001).
251 Já foram propostos dois clados para o GHR: receptores do hormônio do
252 crescimento tipo 1 (GHR1) e tipo 2 (GHR2). Além disto, cada um destes
253 receptores apresentam uma forma completa (GHR1-fl e GHR2-fl) e uma forma
254 truncada (GHR1-t e GHR2-t). As isoformas trucadas do GHR são receptores de
255 membrana produzidos por "splicing" alternativo do mRNA de GHR-fl, mas não
256 possuem a porção intracelular desta proteína. Desta forma, quando o GH se
257 liga ao GHR-t, não desencadeia as respostas celulares características do GHR-
258 fl, sendo então um mecanismo molecular de regulação do eixo somatotrópico,
259 podendo levar os tecidos a apresentarem uma dessensibilização ao GH
260 (Fuentes et al., 2012). A ligação direta do GH no GHR-fl presente na
261 membrana plasmática de células alvo estimula a produção dos fatores de
262 crescimento semelhantes à insulina (IGF1 e IGF2) pela ativação da via
263 JAK2/STAT5 (Argetsinger et al. 1993) (Fig. 5).
264



265
266 Figura 5 – Esquema sintetizando a regulação do eixo somatotrópico
267
268 É interessante observar que a produção de IGFs pode se dar de duas
269 formas distintas. Caso o GH se ligue a células do fígado, irá estimular este
270 tecido a produzir e liberar IGFs na corrente sanguínea, onde exercerão suas
271 funções de forma endócrina. Caso o GH se ligue a outros tecidos periféricos,
272 como o músculo ou brânquias, irá estimular a produção local de IGFs, que irão
273 exercer suas funções de forma parácrina/autócrina (Reinecke et al., 2005).

274 Independente da forma de ação, os IGFs possuem uma função anabólica,
275 estimulando a expressão de genes envolvidos no processo de miogênese,
276 assim como pela diminuição na expressão de genes envolvidos na degradação
277 de proteínas e atrofia muscular. Já nas brânquias, estes tecidos cumprem
278 funções osmorregulatórias (Fuentes et al., 2012).

279 Outro ponto interessante de regulação do eixo somatotrópico é pelo
280 estado de associação dos IGFs a suas proteínas de transporte. Os IGFs não
281 são transportados em uma forma livre na corrente sanguínea, mas sim
282 associados a proteínas de transporte chamadas proteínas de ligação ao IGF
283 (IGFBP) (Fig.5). Quatro isoformas de IGFBP já foram caracterizadas para
284 peixes, sendo que cada uma delas possui distintas forças de ligação aos IGFs.
285 Desta forma, a quantidade de IGFs que se encontra disponível para ligação à
286 órgãos alvo depende da proporção entre as isoformas de IGFBP presentes na
287 circulação. Por fim, os IGFs possuem a capacidade de inibir a produção de GH
288 através de uma alça de retroalimentação longa que atua a nível hipotalâmico
289 (Reinecke et al., 2005) (Fig. 5).

290

291 **O modelo animal: *Poecilia vivipara***

292

293 O teleósteo *P. vivipara* (Fig. 6), popularmente conhecido como "barrigudinho", é
294 uma espécie da família Poeciliidae que se distribui ao longo de toda a costa
295 brasileira (Santos et al., 2011). Assim como todos os poecilídeos, esta espécie
296 possui uma estratégia reprodutiva do tipo vivípara, onde as fêmeas carregam
297 seus embriões em uma estrutura semelhante a placenta de mamíferos até a
298 eclosão. Já os machos, possuem uma estrutura que possibilita a fecundação
299 externa, chamada de gonopódio (Meredith et al., 2011). Além disso, *P. vivipara*
300 apresenta diversas características que a torna uma espécie interessante para
301 ser usada como um modelo em experimentação animal. Por exemplo, *P.*
302 *vivipara* apresenta uma ampla tolerância a variações das condições
303 ambientais, bem como facilidade de manutenção e reprodução completa em
304 cativeiro (Paulo et al., 2012). De fato, esta espécie vem sendo utilizada como
305 organismo modelo em diferentes estudos com contaminantes químicos
306 ambientais, incluindo o cobre, no âmbito do Instituto Nacional de Ciência e
307 Tecnologia de Toxicologia Aquática (INCT-TA) (www.inct-ta.furg.br).

308

309
310 Figura 6 – *Casal de Poecilia vivipara*. Fêmea à esquerda e macho à direita. Fonte:
311 http://www.viviparos.com/Galeria/Felipe2.htm
312
313 **OBJETIVOS**
314
315 **Objetivo geral:**
316
317 • Avaliar os efeitos da exposição crônica ao cobre sobre alguns dos
318 mecanismos fisiológicos relacionados ao crescimento em *Poecilia*
319 *vivipara*: regulação endócrina e metabolismo energético.
320
321 **Objetivos específicos:**
322
323 • Caracterizar parcialmente os genes que codificam o GH, GHR 1, GHR 2;
324 IGF 1 e IGF 2 em *P. vivipara*;
325 • Avaliar o efeito da exposição crônica ao cobre sobre a expressão de
326 genes envolvidos na regulação endócrina do crescimento: GH (cérebro),
327 GHR 1 e GHR 2 (cérebro, fígado e músculo) e IGF 1 e IGF 2 (fígado e
328 músculo) de *P. vivipara*;
329 • Avaliar o efeito da exposição crônica ao cobre sobre a expressão de
330 genes que codificam proteínas envolvidas cadeia transportadora de
331 elétrons e fosforilação oxidativa: subunidades 1, 2 e 3 da citocromo c

332 oxidase e subunidade alfa da ATP sintase (fígado e músculo) de *P.*
333 *vivipara*;

334 • Determinar o efeito da exposição crônica ao cobre sobre a concentração
335 do GH (cérebro) e GHR (fígado e músculo) de *P. vivipara*.

336 **CAPITULO I - Artigo 1**

337

338 **Chronic effects of copper on the expression of genes encoding for**
339 **proteins related to mitochondrial function in the viviparous guppy *Poecilia***
340 ***vivipara***

341

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348 Artigo a ser submetido à revista: Aquatic Toxicology

Chronic effects of copper on the expression of genes encoding for proteins related to mitochondrial function in the viviparous guppy *Poecilia vivipara*

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ABSTRACT

Chronic effects of copper (Cu) on the transcriptional regulation of genes related to the mitochondrial function were evaluated in the viviparous guppy *Poecilia vivipara* acclimated to salt water. Target genes included three mitochondrial-encoded subunits of the cytochrome c oxidase (COX I, II and III), which are involved in the mitochondrial respiratory chain, as well as one subunit of the ATP synthase (ATP5A1), a protein complex involved in the oxidative phosphorylation. Newborn (<24-day old) guppies were exposed to environmentally relevant concentrations of dissolved Cu (nominally 0, 5 and 9 µg/L) in salt water (salinity 24 ppt and 20°C) for 345 days. Following exposure, mRNA levels of the target genes were assessed by RT-qPCR in skeletal muscle and liver. Skeletal muscle of guppies exposed to 5 µg/L Cu had reduced mRNA levels of COX III. Also, guppies exposed to 9 µg/L showed reduced mRNA levels of COX II and III. These findings indicate that Cu exposure may have affected the mitochondrial respiratory chain. On the other hand, increased mRNA levels of ATP5A1 were observed in the liver of guppies exposed to 9 µg/L Cu, thus suggesting a higher activity of oxidative phosphorylation. Therefore, findings reported in the present study indicate that chronic exposure to dissolved Cu induces tissue-specific responses in key aspects of the mitochondrial metabolism. Our results suggest that chronic Cu exposure is leading to a reduced ATP production in the skeletal muscle through a lowered expression of gene encoding for proteins involved the mitochondrial respiratory chain. This effect is paralleled by an increased ATP consumption in the liver, indicated by an increased expression of the gene encoding for ATP5A1, likely associated with the role of this organ in metal detoxification. The combination of these effects can explain a previously observed reduction in growth of *P. vivipara* exposed to the same experimental conditions.

Keywords: chronic exposure, cytochrome c oxidase, energy metabolism, heavy metal, mitochondrial metabolism, oxidative phosphorylation.

INTRODUCTION

Copper (Cu) is an essential metal for life at trace levels (Pena et al., 1999). For example, it is a structural component of some proteins, the cuproproteins (Failla et al., 2001). Also, Cu is a cofactor of some enzymes involved in the antioxidant system (Vutukuru et al., 2006; Craig et al., 2007; Almroth et al., 2008; Bopp et al., 2008; Leary et al., 2009) and the mitochondrial respiratory chain (Kim et al., 2012). Besides its physiological roles, Cu can be characterized as a chemical pollutant, which can be released in the environment as a waste from industrial and harbor activities, as well as together with the domestic sewage (Heath, 1995; Langston and Bebianno, 1998). When present at excessive concentrations in the environment, Cu can be accumulated in animal tissues and induce hazardous effects (Grosell, 2012).

Many studies demonstrate that Cu can induce homeostatic and metabolic disturbances such as alterations in oxygen consumption (De Boeck et al., 1995; Manyin and Rowe, 2009), ammonia excretion (Grosell et al., 2003; Blanchard and Grosell, 2006), ionic and osmotic regulation (Lauren and McDonald, 1987; Grosell and Wood, 2002), and acid-base regulation (Boitel and Truchot, 1990; Bielmyer et al., 2005; Blanchard and Grosell, 2006). The majority of these effects can be directly or indirectly related to disturbances in the energy metabolism. In fact, it has been shown that Cu can affect the energy status of aquatic animals, disturbing many steps of the cellular respiration process. Indeed, toxic effects of Cu on glycolysis has already been shown in the mussel *Lamellidens marginalis* (Satyaparameshwar et al., 2006), the crab *Carcinus maenas* (Hansen et al., 1992) and the fish *Prochilodus lineatus* (Carvalho and Fernandes, 2008). Likewise, Cu-induced alterations in the Krebs cycle activity have been shown in the tilapia *Tilapia mossambica* (Balavenkatasubbaiah et al., 1984) and the yellow perch *Perca flavescens* (Couture and Kumar, 2003). Also, exposure to Cu can alter mitochondrial metabolism in fish (Garceau et al., 2010) and reduce ATP production in crabs (Lauer et al., 2012).

The majority of studies concerning the toxic effects of Cu are related to short-term exposures. Indeed, information gathered after exposure to this metal for more than 4 weeks are extremely rare (Handy, 2003). As far as we know,

447 the longest studies evaluating chronic effects of Cu were reported by McKim et
448 al. (1971) and our research group (Anni 2015). Both studies used fish as animal
449 models and lasted for approximately 11 months. However, only Anni (2015)
450 evaluated the long-term effects of Cu on the energy metabolism. This author
451 showed that chronic exposure to Cu did not alter the pyruvate kinase and
452 lactate dehydrogenase activity in several tissues (gills, liver and muscle) of the
453 viviparous guppy *Poecilia vivipara*, thus suggesting that metal exposure have
454 not affected glycolysis. However, the citrate synthase (CS) activity was higher in
455 liver of guppies exposed to 9 µg/L Cu, indicating that long-term exposure to Cu
456 can alter the Krebs cycle activity. Unfortunately, Anni (2015) did not evaluate
457 other components of the aerobic production of energy, but one can hypothesize
458 that long-term exposure to Cu may also alter the mitochondrial capacity to
459 produce energy.

460 Mitochondria are responsible for maintaining the aerobic production of
461 ATP. This process is fueled by the chemical energy stored in many substrates
462 such as carbohydrates, which are oxidized in the Krebs cycle to produce
463 electron carriers such as NADH and $FADH_2$. These reduced coenzymes are
464 oxidized by protein complexes embedded in the inner mitochondrial membrane
465 (Navarro and Boveris, 2007), the so-called mitochondrial respiratory chain. In
466 turn, the electrons released are transferred through some of these protein
467 complexes until to reach their final acceptor, the oxygen (Pereira et al., 2001).
468 The potential energy produced by this electron transfer is coupled to the
469 pumping of protons out of the mitochondrial matrix to its intermembrane space,
470 thus generating a potential energy stored as a chemical and electrical gradient
471 (Mitchell and Moyle, 1965).

472 The cytochrome c oxidase (COX; Complex IV) is one of the protein
473 complexes responsible for transferring electrons to oxygen and for pumping
474 protons to the mitochondrial intermembrane space (Pereira et al., 2001). It is
475 formed by 13 subunits and the largest three of them (COX I, COX II and COX
476 III) are encoded by mitochondrial genome (Kadenbach et al., 2000). The
477 subunits COX I and COX II possess copper prosthetic groups that are essential
478 for the correct function of the COX complex (Khalimonchuk and Rodel, 2005).
479 The other 10 COX subunits are encoded by the cell nucleus (Kadenbach et al.,
480 1983). The potential energy resulting from the chemical and electrical gradient

481 formed in the mitochondrial cristae is finally dissipated as protons flow through
482 the F_oF_1 ATP synthase (ATP synthase; complex V) back to the mitochondrial
483 matrix, driving the synthesis of ATP by the phosphorylation of ADP, in a process
484 called oxidative phosphorylation (Boyer, 1997; Nakamoto et al., 1998). ATP
485 synthase is a protein complex composed by at least 17 subunits that forms a
486 membrane-protruding portion (F_1) and a membrane-integrated portion (Fo). The
487 F_1 portion of ATP synthase is a motor driven by the hydrolysis of ATP (Boyer,
488 1997). The movement within this motor is generated by a rotor that rotates in a
489 stator constituted by the subunits α (ATP5A1) and β (ATP5B) (Devenish et al.,
490 2000; Arechaga and Jones, 2001).
491 Considering the background above, the present study aimed to assess
492 the expression of some genes related to the mitochondrial respiratory chain
493 (COX I, II, and III) and the oxidative phosphorylation (ATP5A1) in the viviparous
494 guppy *P. vivipara* long-term (345 days) exposed to environmentally relevant
495 concentrations (5 and 9 µg/L) of Cu. This fish species is euryhaline, being
496 widely distributed along the South America Atlantic coast (Froese and Pauly,
497 2011). Also, it has been recently indicated as a promising experimental model in
498 the evaluation of several aquatic contaminants (INCT-TA, 2013), including Cu
499 (Zimmer et al., 2012; Machado et al., 2013; Silva et al., 2014).
500

501 **MATERIAL AND METHODS**
502

503 *Fish rearing and experimental design*
504

505 Mating pairs of *P. vivipara* were collected at the "Arroio do Gelo" stream
506 (Cassino Beach, Rio Grande, southern Brazil), transferred to the laboratory and
507 kept at fixed room temperature (25°C) and photoperiod (12 h light: 12 h dark
508 cycle). They were acclimated in glass tanks containing continuously aerated salt
509 water (24 ppt) for 15 days. Fish were fed twice a day with a commercial diet
510 (Alcon Basic; 45% crude protein, 5% lipids, 2% calcium, 0.7% phosphorus and
511 10% humidity) until apparent satiation.
512 After acclimation, fish couples were isolated in 20-L plastic tanks under
513 the same conditions employed in the acclimation period. Pregnant females were
514 isolated and newborn guppies (<24 h after birth; wet body mass: 6.3 ± 0. 1 mg;

515 standard body length: 7.16 ± 0.13 mm) were collected and used in the
516 experiment. All experimental procedures were previously approved by the
517 Ethics Committee for Animal Use of the University (CEUA/FURG; permit #
518 P014/2012).

519 Newborn guppies were kept under control conditions (no Cu addition in
520 the water) or exposed to Cu (nominally: 5 and 9 µg/L Cu). Copper
521 concentrations tested were selected considering the current Brazilian water
522 quality criteria for Cu in sea water (5 µg/L) and fresh water (9 µg/L) (CONAMA,
523 2005). Exposure media containing Cu were prepared by dilution of a Cu
524 standard solution (1 mg/L Cu as $CuCl_2$; Vetec Química Fina, São Paulo, Brazil).
525 Experimental media were completely renewed every week. Each experimental
526 treatment was performed in duplicate. Fish were kept in 10-L aquaria containing
527 salt water (24 ppt; pH 7.66 ± 0.21) continuously aerated (oxygen saturation
528 >90%). Fish density was always below 1 g/L. Room temperature (28°C) and
529 photoperiod (12 h light: 12 h dark cycle) were fixed. Fish were daily fed with the
530 same commercial diet used in the acclimation and reproduction periods. Fish
531 were kept at the experimental conditions for 345 days. After this period, all fish
532 were sexually mature. They were then killed by sectioning the spinal cord and
533 had the skeletal muscle and liver dissected, immersed in RNAlater (Ambion),
534 held at 4°C for 24 h, and then stored in ultrafreezer (-80°C) until analysis.

535

536 *Expression of COX (I, II and III) and ATP5A1 partial transcripts*

537

538 For each sample (skeletal muscle and liver), total RNA was extracted
539 (Qiazol reagent, Qiagen) and the cDNA was synthetized using the High
540 Capacity cDNA Reverse Transcription kit (Applied Biosystems) and a mix of
541 anchored oligo (dT) primer (Applied Biosystems) with random hexamer primer
542 (Applied Biosystems). Forward and reverse primers to amplify the partial
543 mRNAs of the genes encoding for COX (I, II and III) and ATP5A1 were
544 designed based on sequences of base pairs previously characterized for *P.*
545 *vivipara* (Table 1).

546 The relative expression of the target genes were assessed using the
547 GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and the gene-specific
548 primers in a real time PCR machine (qPCR; 7300 Real-Time PCR System;

549 Applied Biosystems). Reactions were performed in duplicate following the
550 manufacturer instructions and using the following protocol: 50°C for 2 min, 95°C
551 for 2 min, 45 cycles at 95°C for 15 s, and 60°C for 30 s. Melting curve analysis
552 was also performed at the end of each PCR run to ensure amplification of a
553 single product. The relative values for the total target gene expression in tissues
554 samples were analyzed by the $2^{-\Delta\Delta ct}$ method (Schmittgen and Livak, 2008),
555 using the average expression of EF1α and β-Actin as housekeeping genes
556 (Silva et al., 2014).
557

558 *Data presentation and statistical analysis*

559

560 Data are shown as mean ± standard error. Differences among
561 experimental groups were assessed using analysis of variance (ANOVA)
562 followed by the Tukey's test. ANOVA assumptions (normal distribution of data
563 and homogeneity of variances) were previously checked using the Kolmogorov-
564 Smirnov and Levene's test, respectively. Also, independency of data was
565 checked using the Durbin-Watson test. In cases where the experimental data
566 failed to meet the ANOVA assumptions, data were mathematically (square-root)
567 transformed and the ANOVA assumptions re-evaluated. In all cases, the
568 significance level adopted was 95% ($p<0.05$). Statistical analyses were
569 performed using the software Statistica 10 (StatSoft, USA).
570

571 **RESULTS**

572

573 The mRNA expression of genes encoding for COX I, COX II, COX III and
574 ATP5A1 were assessed in the skeletal muscle and liver of the viviparous guppy *P.*
575 *vivipara* kept under control condition or chronically (345 days) exposed to
576 environmentally relevant concentrations of Cu (nominally: 5 and 9 μg/L).
577 There was no significant effect of chronic Cu exposure on the expression of
578 gene encoding for COX I in the skeletal muscle and liver of the guppy *P. vivipara* (Fig.
579 1). However, mRNA expression of the gene encoding for COX II was significantly
580 reduced in the skeletal muscle of guppies exposed to 9 μg/L Cu for 345 days (Fig. 2).
581 Likewise, mRNA expression of the gene encoding for COX III was significantly reduced
582 in the skeletal muscle of guppies exposed to 5 and 9 μg/L Cu (Fig. 3). On the other

583 hand, the mRNA expression of the gene encoding for ATP5A1 was significantly
584 increased in the liver of guppies exposed to 9 µg/L Cu for 345 days (Fig. 4).
585
586 **DISCUSSION**
587
588 Previous studies from our laboratory have demonstrated that long-term
589 (345 days) exposure to Cu under the same experimental conditions (salinity,
590 temperature, pH, photoperiod, exposure time and Cu concentrations) employed
591 in the present study increased the whole-body Cu concentration in the
592 viviparous guppy *P. vivipara*. This finding was explained by Cu accumulation in
593 gills, liver and gut of guppies, which was paralleled by an increased expression
594 of the Cu-transporting ATPase (ATP7B). Also, it is worth noting that a reduction
595 in the fish body mass was observed after the chronic Cu exposure (Anni, 2015).
596 The chronic Cu effect on fish growth described above could be
597 associated with at least two possibilities: alterations in the somatotropic axis
598 and/or changes in energy metabolism. In a companion paper, we have
599 addressed the possible role of Cu as an endocrine disruptor evaluating the
600 effects of chronic Cu exposure on key parameters of the somatotropic axis in *P.*
601 *vivipara* (Zebral et al., 2016). However, Anni (2015) also demonstrated that the
602 long-term (345 days) exposure to Cu did not alter the hepatic and muscular
603 glycolysis, but increased the citrate synthase (CS) activity. This finding would
604 suggest that chronic Cu exposure could be inducing alterations in Krebs cycle.
605 Therefore, we have hypothesized that chronic Cu effects could be related to
606 alterations involving other aspects of the energy production under aerobic
607 conditions.
608 Considering the background above, in the present study we have
609 evaluated the chronic (345 days) effects of exposure to Cu at environmentally
610 relevant concentrations (5 and 9 µg/L) on the expression of key mitochondrial
611 genes related to the respiratory chain and the oxidative phosphorylation in
612 tissues (liver and skeletal muscle) of *P. vivipara* acclimated to salt water (24
613 ppt). The mRNA expression of COX (subunits I, II and III) and ATP5A1 was
614 evaluated in the skeletal muscle and liver of *P. vivipara* after exposure to
615 dissolved Cu. As far as we know, this is the first study reporting such long-term
616 effects of Cu on some aspects of energy metabolism in fish.

617 Our results show that chronic exposure to 5 μg/L Cu reduced the mRNA
618 expression of COX II in the skeletal muscle (Fig. 2). In addition, long-term
619 exposure to 5 and 9 μg/L Cu also reduced the mRNA expression of COX III in
620 this tissue (Fig. 3). It is important to note that COX is the latter protein complex
621 of the mitochondrial respiratory chain. It is formed by 13 subunits, with the
622 subunits I, II and III being originated from the mitochondrial genome
623 (Kadenbach et al., 2000). This protein complex is responsible for transferring
624 electrons to its final acceptor, the oxygen. The COX complex is also responsible
625 for pumping protons to the mitochondrial intermembrane space generating a
626 potential energy stocked as electrochemical gradient in the mitochondrial
627 cristae (Khalimonchuk and Rodel, 2005). Considering the argumentation that
628 COX activity corresponds to the expression of its subunits (Kim et al., 1995;
629 Hardewig et al., 1999), our findings indicate that the activity of this protein
630 complex was also reduced after long-term Cu exposure in the guppy *P.*
631 *vivipara*.

632 As argued by Arnold (2012), COX has a pivotal role in the control of
633 cellular metabolism, being regulated by oxidative phosphorylation, proton
634 pumping efficiency, ATP and reactive oxygen species (ROS) production.
635 Interestingly, it has already shown that Cu exposure, specially under acute
636 conditions, increases the tissue levels of ROS (Halliwell, 1984; Harris and Gitlin,
637 1996; Gaetke and Chow, 2003) and reduces cellular antioxidant system in fish
638 (Dorval and Hontela, 2003; Craig et al., 2007; Almroth et al., 2008; Bopp et al.,
639 2008; Eyckmans et al., 2011), including the guppy *P. vivipara* (Machado et al.,
640 2013). These effects resulted in cell damages related to oxidative stress (Costa
641 et al., 2002; Main et al., 2010; Machado et al., 2013). Thus, the reduced mRNA
642 expression of COX II and COX III in the muscle of *P. vivipara* may be related to
643 oxidative stress generated by chronic Cu exposure.

644 Additionally, some studies have shown that Cu exposure diminishes the
645 mitochondrial ATP formation (Viant et al., 2002; Lauer et al., 2012). Altogether
646 these findings combined with those reported in the present study suggest that
647 chronic Cu exposure reduces the ability of muscle mitochondria to generate
648 properly the electrochemical gradient used to synthesize ATP. This could
649 partially explain the negative effects of Cu that are directly or indirectly related
650 to disturbances in energy status. In addition, disturbances in the capacity of

651 muscle mitochondria to produce energy could be related to the Cu-induced
652 reduction in growth of fish (McKim et al., 1971; Marr et al., 1996; Al-Ogaily et
653 al., 2003; Kim et al., 2004; Liu et al., 2010), including the viviparous guppy *P.*
654 *vivipara* (Anni, 2015). Indeed, somatic growth relies on the proper proliferation
655 of skeletal muscle cells, a process that depends greatly on the energy released
656 by ATP hydrolysis (Glass, 2003; 2005; Velloso, 2008).

657 Differently from the skeletal muscle, chronic exposure to Cu did not alter
658 the mRNA expression of COX I, II and III in the liver. This finding indicates a
659 tissue-specific effect of chronic Cu exposure on the expression of the gene
660 encoding for COX in *P. vivipara*, as already described in the freshwater-
661 acclimated killifish *Fundulus heteroclitus* (Ransberry et al., 2016). The
662 difference observed between Cu effects in skeletal muscle and liver of *P.*
663 *vivipara* may be explained by the high capacity of liver in protecting itself
664 against the toxic effects of heavy metals (Mason and Jenkins, 1995) and ROS
665 (Cazenave et al., 2006). In fact, liver is the main organ involved in Cu
666 detoxification (Kuo et al., 2006; Uren Webster et al., 2013; Silva et al., 2014).

667 As observed for COX, we noticed a similar tissue-specific effect of Cu
668 exposure on the expression of mRNA encoding for ATP5A1. Indeed, it was only
669 altered in the liver of guppies exposed to 9 µg/L Cu. Interestingly a previous
670 study from our laboratory has demonstrated that long-term (345 days) exposure
671 to 9 µg/L Cu induced an increase in CS activity in the liver of *P. vivipara* (Anni,
672 2015). These findings suggest that the liver of guppies chronically exposed to
673 Cu show increased aerobic production of energy. This is likely a compensatory
674 adjustment to deal with the energy-demanding process related to Cu
675 detoxification. Indeed, depletion of ATP causes up-regulation of mitochondrial
676 enzymes, such as ATP synthase (Grover et al., 2008). Therefore, the
677 differential effect of Cu on the transcriptional regulation of ATP5A1 in skeletal
678 muscle and liver of *P. vivipara* may be explained considering the pivotal role of
679 the liver in the clearance of heavy metals.

680

681 **CONCLUSIONS**

682

683 Findings reported in the present study are evidences that chronic
684 exposure (345 days) to dissolved Cu at environmentally relevant concentrations

(5 and 9 µg/L) may induce disturbances in energy production associated with a suppression in the mitochondrial respiratory chain in the skeletal muscle of the viviparous guppy *P. vivipara* acclimated to salt water (24 ppt salinity). Based on findings previously reported in the literature, we hypothesize that the observed Cu effects may be linked to oxidative stress. In addition, our results indicate that a compensatory adjustment in energy production would be occurring in the liver of the viviparous guppy *P. vivipara* in order to cope with the energy-demanding process involved in Cu detoxification. Finally, our findings support the idea that the reduction observed in growth of the viviparous guppy *P. vivipara* after chronic exposure to dissolved Cu is due, at least in part, to a decreased mitochondrial production of energy in the skeletal muscle, as well as an increased energy expenditure in the liver.

ACKNOWLEDGMENTS

The authors thank Sandra Isabel Moreno Abril and Roberta Daniele Klein (Universidade Federal do Rio Grande - FURG, Rio Grande, RS, Brazil) for laboratory assistance. Financial support was provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil; grant #573949/2008-5 - Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES, Brasília, DF, Brazil; grant # 230/2010 - Ciências do Mar), and the International Development Research Center (IDCR, Ottawa, Canada; Project #104519-003). YDZ and ISAA were MSc and PhD fellows from the Brazilian CAPES, respectively. AB is a research fellow from the Brazilian CNPq (grant #304430/2009-9) and is supported by the International Canada Research Chair Program from IDRC.

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925 **Table 1**. Primers used for real-time PCR (qPCR) analysis of the mRNA
926 expression of genes encoding for cytochrome c oxidase subunit I (COX I),
927 cytochrome c oxidase subunit II (COX II), cytochrome c oxidase subunit III
928 (COX III) and ATP synthase subunit alpha (ATP5A1) in the viviparous guppy
929 *Poecilia vivipara* kept under control condition (no copper addition in the water)
930 or exposed to dissolved Cu (nominally: 5 and 9 µg/L) for 345 days.
931

Primer		Sequence (5'-3')
COX I	Forward	TTCCTTGGACTTGCAGGCAT
COX I	Reverse	AGGTGGACTGAGAGGACCTC
COX II	Forward	GCCGTAGAATACTCCAGGCC
COX II	Reverse	ACTCTTGAGCAGTCCCATGC
COX III	Forward	GGCCAACGAAAACAAGCCAT
COX III	Reverse	GAGCCGTAGACTCCATCTGC
ATP5A1	Forward	GGTATCGCCAGAGTGTACGG
ATP5A1	Reverse	TTTCCCAGAGCATCCACCAC

932

933 **CAPTION TO FIGURES**

934

935 **Figure 1**. Relative expression of the cytochrome c oxidase subunit I (COX I) in
936 the liver and skeletal muscle of the viviparous guppy *Poecilia vivipara* kept
937 under control condition (no copper addition in the water) or exposed to
938 dissolved copper (nominally: 5 and 9 µg/L) for 345 days. β-actin and EF-1 were
939 used as housekeeping genes (Ct average). Data are mean ± standard error (n =
940 8-10). Different letters indicate significant different mean values among the
941 experimental groups ($p < 0.05$, ANOVA, Tukey's test).

942

943 **Figure 2**. Relative expression of the cytochrome c oxidase subunit II (COX II) in
944 the liver and skeletal muscle of the viviparous guppy *Poecilia vivipara* kept
945 under control condition (no copper addition in the water) or exposed to
946 dissolved copper (nominally: 5 and 9 µg/L) for 345 days. β-actin and EF-1 were
947 used as housekeeping genes (Ct average). Data are mean ± standard error (n =
948 9-10). Different letters indicate significant different mean values among
949 experimental groups ($p < 0.05$, ANOVA, Tukey's test).

950

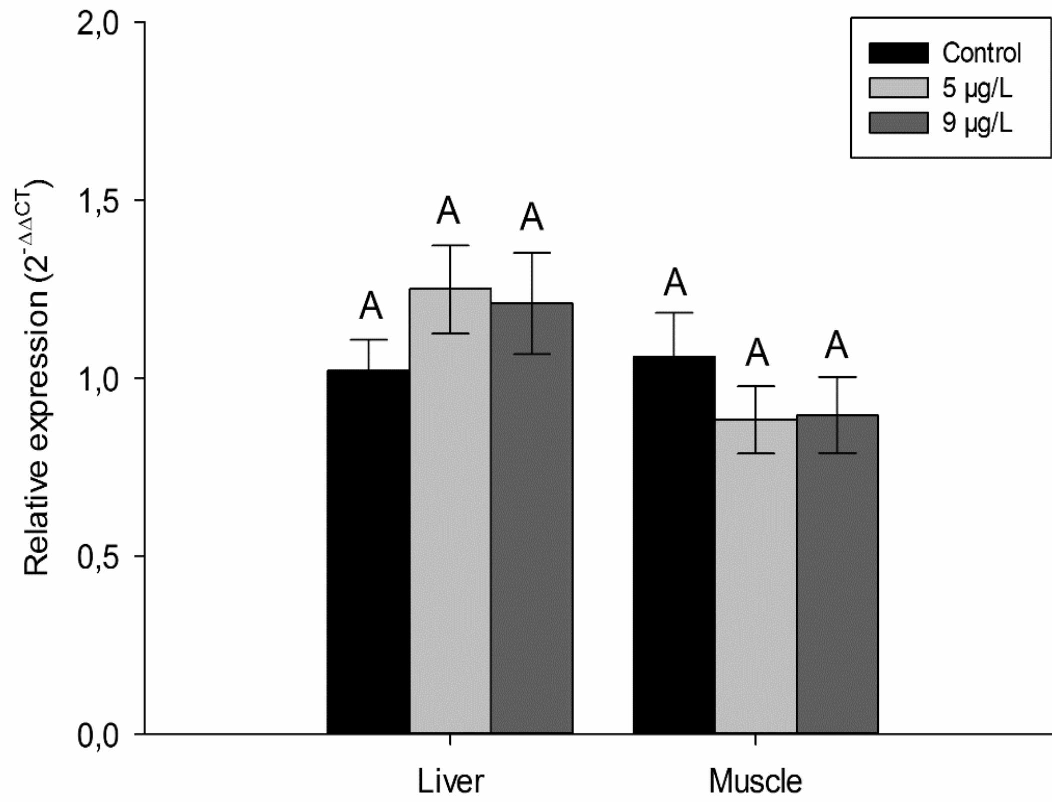
951 **Figure 3**. Relative expression of the cytochrome c oxidase subunit III (COX III)
952 in the liver and skeletal muscle of the viviparous guppy *Poecilia vivipara* kept
953 under control condition (no copper addition in the water) or exposed to
954 dissolved copper (nominally: 5 and 9 µg/L) for 345 days. β-actin and EF-1 were
955 used as housekeeping genes (Ct average). Data are mean ± standard error (n =
956 9-10). Different letters indicate significant different mean values among
957 experimental groups ($p < 0.05$, ANOVA, Tukey's test).

958

959 **Figure 4**. Relative expression of the ATP synthase subunit alpha (ATP5A1) in
960 the liver and skeletal muscle of the viviparous guppy *Poecilia vivipara* kept
961 under control condition (no copper addition in the water) or exposed to
962 dissolved copper (nominally: 5 and 9 µg/L) for 345 days. β-actin and EF-1 were
963 used as housekeeping genes (Ct average). Data are mean ± standard error (n =
964 6-9). Different letters indicate significant different mean values among
965 experimental groups ($p < 0.05$, ANOVA, Tukey's test).

966 Figure 1

967



968

969 Figure 2

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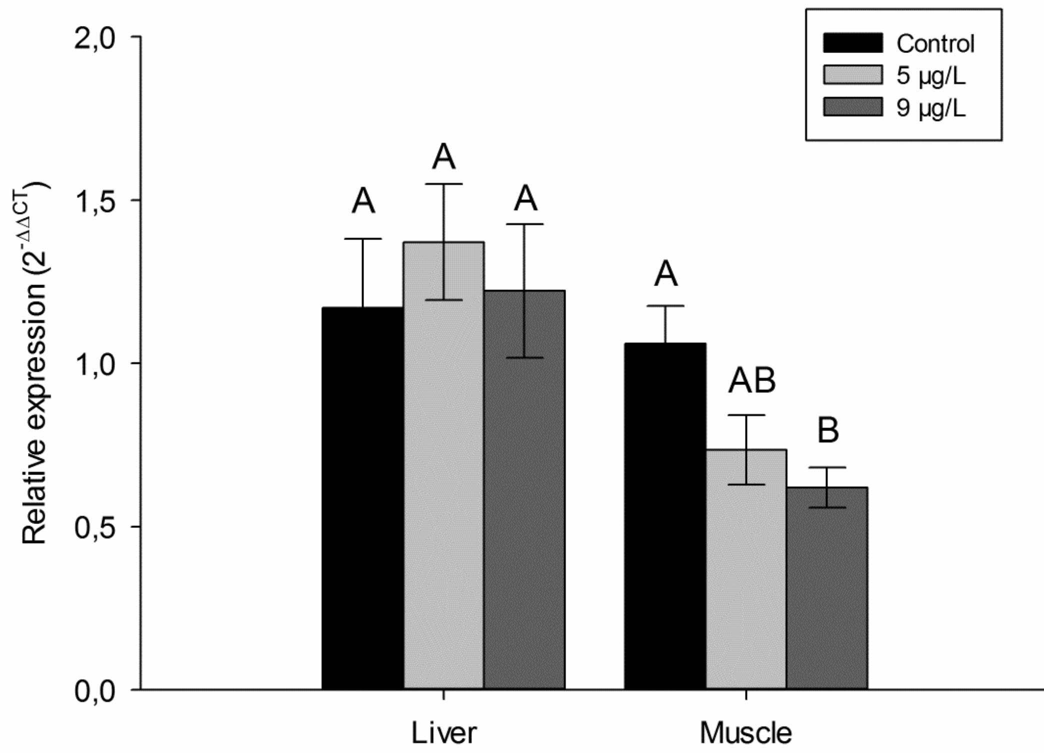

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972 Figure 3

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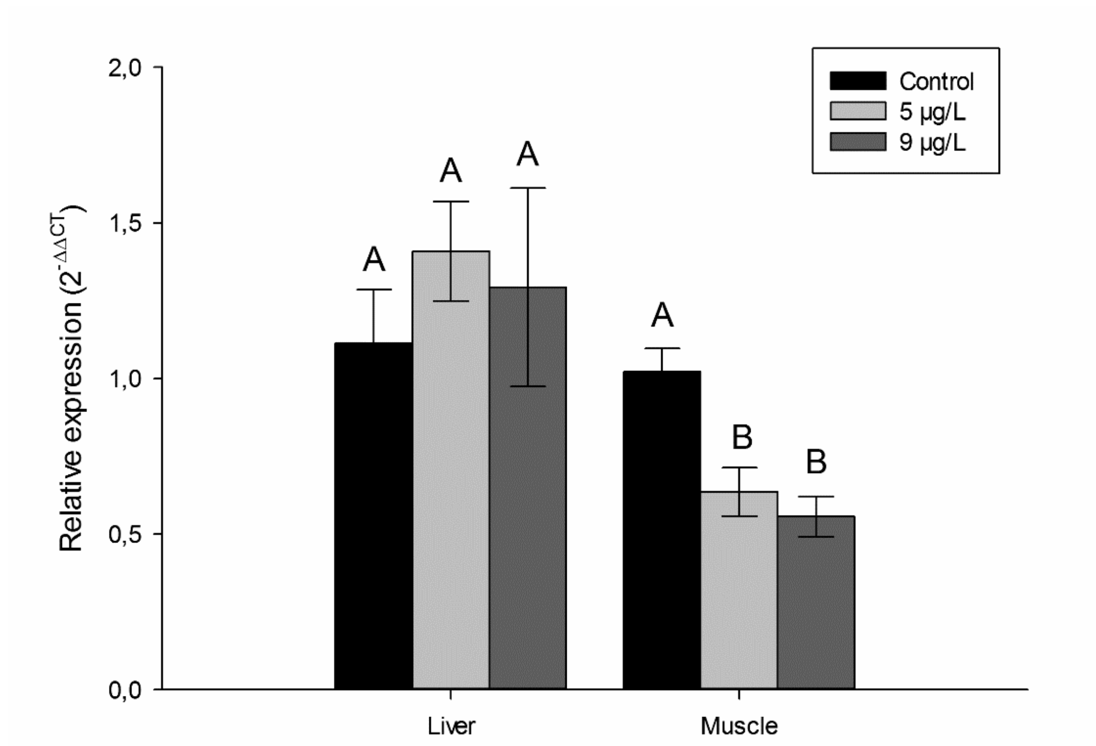

974

975 Figure 4

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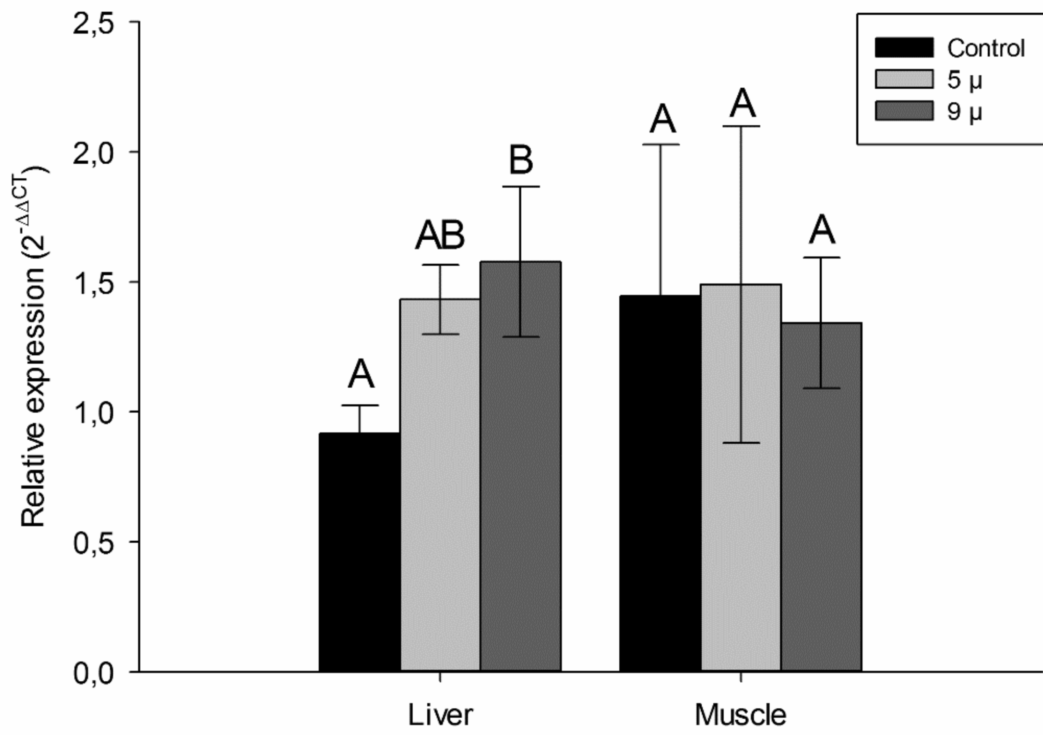

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CAPITULO II – Artigo 2

Copper as endocrine disruptor: chronic effects on the expression of genes and hormones associated with growth of the viviparous guppy *Poecila vivipara*

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Artigo a ser submetido à revista: Aquatic Toxicology

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993 hormones associated with growth of the viviparous guppy *Poecila vivipara*

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ABSTRACT

Chronic effects of dissolved copper (Cu) on the regulation of the somatotropic axis were evaluated in the viviparous guppy *Poecilia vivipara*. Newborn guppies were kept under control conditions or exposed to environmentally relevant concentrations of Cu (nominally: 5 and 9 µg/L) in salt water (salinity 24 ppt) for 345 days. After exposure, transcriptional regulation of the gene encoding for the growth hormone (GH) was evaluated in brain, while the expressions of genes encoding for the growth hormone receptor 1 (GHR1) and 2 (GHR2) were analyzed in brain, skeletal muscle and liver. In turn, the expression of genes encoding for the insulin-like growth factor 1 (IGF1) and 2 (IGF2) was evaluated in skeletal muscle and liver. In addition, we assessed the GH concentration in brain, as well as the GHR concentration in skeletal muscle and liver. mRNA levels of the target genes were assessed by RT-qPCR. Tissue concentrations of target proteins were assessed using ELISA kits. Chronic Cu exposure affected the transcriptional regulation of target genes only in skeletal muscle. A reduced level of mRNA expressing GHR2 was observed in guppies exposed to 5 µg/L Cu, while reduced levels of mRNA expressing GHR2, IGF 1 and IGF 2 were observed in guppies exposed to 9 µg/L Cu. However, GHR concentration was not affected. These findings indicate that the reduced growth previously reported in *P. vivipara* chronically exposed to Cu is related to an insensitivity of the skeletal muscle to GH, which is associated with a reduction in the expression of GHR2. This effect is leading to impairment in the expression of IGF1 and IGF2, which are important trophic factors in the maintenance of the muscular anabolic state. In addition, we hypothesize that this insensitivity is due to alterations in the proportion of the different isoforms of GHR2, and not by a reduction in the production of total GHR. Also, we conclude that paracrine/autocrine actions of muscular IGF1 and IGF2 are more relevant in Cu-induced growth inhibition than endocrine actions mediated by liver IGF1 and IGF2. Finally, fish growth impairment induced by chronic Cu exposure is associated with a disruption of the somatotropic axis regulation. Therefore, Cu can be considered as an endocrine disruptor in the guppy *P. vivipara*.

Keywords: chronic exposure, copper, endocrine disruptor, growth, hormone somatotropic axis.

1059 ## INTRODUCTION

1060

1061 Copper (Cu) ions are essential for the correct function of cells by acting
1062 as co-factor of many enzymes (Knight et al., 1994). They are also involved in a
1063 great number of important cellular processes such as respiratory chain (Mercer
1064 and Llanos, 2003), hormone signaling (Dang et al., 2000) and antioxidant
1065 defense (Bopp et al., 2008; Leary et al., 2009). Besides their important
1066 physiological roles, Cu ions can be toxic when present at elevated
1067 concentrations (White and Rainbow, 1982). In aquatic animals, acute Cu effects
1068 are well known and usually involve damages in target tissues through
1069 disturbances in ionic and osmotic regulation (Craig et al., 2010), oxidative stress
1070 (Chen et al., 2011; Machado et al., 2013), enzyme inhibition (Liu et al., 2010)
1071 and loss of the selective permeability of plasma membrane (García et al.,
1072 2007). Conversely, information regarding chronic effects of Cu is scarce. In
1073 addition, most experiments are performed for less than 4 weeks (Handy, 2003).
1074 In general, chronic Cu effects are associated with biochemical and physiological
1075 adjustments such as immunological alterations (Dethloff and Bailey, 1998), and
1076 high levels of circulating corticosteroids (Pelgrom et al., 1995; Flik et al., 2002).
1077 In turn, tissue Cu accumulation involves an up-regulation of the expression of
1078 genes encoding for intracellular Cu-transporting proteins (Silva et al., 2014;
1079 Anni, 2015).

1080 Also, fish growth inhibition is a well-known effect of chronic exposure to
1081 Cu. It has been characterized in experiments with duration time ranging from 60
1082 to 100 days. Marr et al. (1996) demonstrated that 60 days of exposure to 4.6
1083 µg/L Cu reduced the growth rate of the rainbow trout *Oncorhynchus mykiss*.
1084 Similarly, Kim et al. (2004) reported a reduced growth of the fish *Sebastes*
1085 *schlegeli* after Cu administration via diet (50 mg/kg) for 60 days. In addition,
1086 James et al. (2008) showed that the growth rate of the fishes *Carassius auratus*
1087 and *Xiphophorus helleri* was reduced after exposure to approximately 100 µg/L
1088 Cu for 100 days. The longest experiments on the chronic Cu effects in fish were
1089 conducted for approximately 11 months, using the fishes *Salvelinus fontinalis*
1090 (McKim et al., 1971) and *Poecilia vivipara* (Anni 2015). McKim et al. (1971)
1091 assessed the toxic effect of five concentrations of Cu (32.5; 17.4; 9.5; 5.7 and
1092 3.4 µg/L) during the larval-juvenile period. They demonstrated that exposure to

1093 17.4 µg/L Cu caused drastic effects on fish survival and growth. In turn, Anni
1094 (2015) was the first author to assess the long-term effects of Cu exposure
1095 throughout the entire life cycle of a fish species, from newborn to adult. This
1096 author tested two environmentally relevant Cu concentrations (5 and 9 µg/L)
1097 and both caused a reduced fish growth (body mass).

1098 In fish and other vertebrates, growth is regulated by the growth hormone
1099 (GH)/insulin-like growth factor (IGF) system, the so-called somatotropic axis.
1100 Indeed, GH has a pivotal role in the regulation of this axis. It is produced by the
1101 pituitary neurons and is the main hormone regulating somatic growth. After
1102 production, GH is released into the blood stream and reaches several
1103 peripheral tissues (Butler et al., 2001). This hormone exerts its actions through
1104 specific binding to growth hormone receptors (GHR), which are present in target
1105 tissues (Lee et al., 2001). Two clades of GHR have been proposed for fish, the
1106 growth hormone receptor type 1 (GHR1) and the growth hormone receptor type
1107 2 (GHR2) (Jiao et al., 2006).

1108 The GHR is a class-1 cytokine membrane receptor expressed in all
1109 organs. It is assumed that GHR exerts it actions through activation of the
1110 JAK2/STAT5 pathway, triggering the production of IGF1 and IGF2 in target
1111 tissues (Smit et al., 1996). These hormones are known to stimulate cell
1112 proliferation and differentiation, leading to a diversity of anabolic processes,
1113 such as muscle growth (Fuentes et al., 2013) and osmoregulatory-dependent
1114 alterations in gills morphology (McCormick, 2001). The liver is the main tissue to
1115 mediate GH-dependent adjustments. Hepatic IGF1 and IGF2 are released in
1116 the blood stream and exert their actions in many peripheral tissues through an
1117 endocrine action (Björnsson et al., 2004). Alternatively, GH can also mediate
1118 liver-independent production of local IGF1 and IGF2 in peripheral tissues, such
1119 as skeletal muscle, kidney, intestine and gills (Butler and Le Roith, 2001;
1120 Herrington and Carter-Su, 2001; Leroith et al., 2001). In this case, IGF1 and
1121 IGF2 exert their anabolic actions in an autocrine/paracrine way (Reinecke,
1122 2010). The somatotropic axis can be modulated by a series of environmental
1123 cues, such as nutritional status, temperature, photoperiod, salinity and pollution.
1124 Considering the last case, estrogens and heavy metals are the most relevant
1125 examples (Deane and Woo, 2009).

1126 In light of the above, the present study aimed to assess the physiological
1127 mechanisms responsible for the endocrine regulation of growth in the viviparous
1128 guppy *Poecilia vivipara* exposed throughout its life cycle (345 days) to two
1129 environmentally relevant concentrations of dissolved Cu (5 e 9 µg/L) in salt
1130 water (salinity 24 ppt). This euryhaline species is widely distributed along the
1131 Atlantic coast in Brazil (Froese and Pauly, 2011). Indeed, the Brazilian network
1132 on aquatic toxicology has indicated *P. vivipara* as an experimental model for
1133 ecotoxicological studies (INCT-TA, 2013).
1134 To achieve our goal, tissue concentrations of GH and GHR were
1135 assessed by the ELISA method in brain (GH and GHR), muscle (GHR) and liver
1136 (GHR) samples. In addition, analyses of relative expression of genes encoding
1137 for GH, GHR1, GHR2, IGF1 and IGF2 were also performed. Partial sequences
1138 for the mRNA encoding for these proteins are described for the first time. The
1139 target genes were assessed in terms of transcriptional regulation in key tissues
1140 for growth control: brain (GH, GHR1 and GHR2), skeletal muscle (GHR1,
1141 GHR2, IGF1 and IGF2) and liver (GHR1, GHR2, IGF1 and IGF2).
1142
1143 **MATERIAL AND METHODS**
1144
1145 *Fish rearing*
1146
1147 Mating pairs of *P. vivipara* were collected in the "Arroio do Gelo" stream
1148 (Rio Grande, RS, southern Brazil), transferred to the laboratory, and maintained
1149 in salt water (salinity 24 ppt) continuously aerated for 15 days. Room
1150 temperature (25°C) and photoperiod (12 h light: 12 h dark cycle) were fixed.
1151 Fish were fed twice a day with a commercial diet (Alcon Basic; 45% crude
1152 protein, 5% lipids, 2% calcium, 0.7% phosphorus and 10% humidity) until
1153 apparent satiation. After the acclimation period, couples of guppies were
1154 separated and maintained in 20-L plastic tanks under the same conditions used
1155 for acclimation. Pregnant females were transferred to breeding boxes. Newborn
1156 guppies (<24 h after birth) were separated and distributed among the
1157 experimental groups. All experimental procedures were previously approved by
1158 the Ethics Committee for Animal Use of the University of Rio Grande
1159 (CEUA/FURG; permit # P014/2012).

1160

1161 *Experimental design*

1162

1163 Newborn guppies (wet body mass: 6.3 ± 0. 1 mg; standard body length:
1164 7.16 ± 0.13 mm) were randomly distributed in three experimental groups. One
1165 group of fish was kept under control conditions (no Cu addition in the water),
1166 while the other two groups of fish were exposed to dissolved Cu (nominally: 5
1167 and 9 μg/L) for 345 days. Each experimental group was performed in duplicate.
1168 Exposure media were prepared by dilution of a Cu standard solution prepared
1169 with $CuCl_2$ (Vetec Química Fina, São Paulo, Brazil). The Cu concentrations
1170 tested were selected considering the current Brazilian water quality criteria for
1171 Cu in sea water (5 μg/L) and fresh water (9 μg/L) (CONAMA, 2005).

1172 Fish were kept in 10-L aquaria filled with salt water (salinity 24 ppt; pH
1173 7.66 ± 0.21) continuously aerated (oxygen saturation >90%), under controlled
1174 temperature (28°C) and photoperiod (12 h light: 12 h dark cycle). Experimental
1175 media were completely renewed every week. Fish stocking density was always
1176 <1 g/L. Fish were fed daily until apparent satiation with the same commercial
1177 diet used during the acclimation period. When all fish were sexually mature (345
1178 days after the beginning of the experiment), the experiment was ended and fish
1179 were killed by spinal cord sectioning. Brain, skeletal muscle and liver were
1180 dissected. Tissues designated for ELISA quantifications were immediately
1181 stored in ultrafreezer (-80°C). In turn, tissues designated for mRNA
1182 quantifications were immersed in RNAlater (Ambion), held at 4°C for 24 h, and
1183 then stored in ultrafreezer (-80°C) until analysis.

1184

1185 *Quantification of tissue concentration of GH and GHR*

1186

1187 Tissue GH concentration was assessed in brain homogenates, while
1188 GHR concentration was analyzed in brain, muscle and liver homogenates.
1189 Quantifications were assessed using the immunoassay technique with
1190 commercial ELISA kits (GH: MBS701414 and GHR: MBS055120; MyBiosource,
1191 San Diego, California, USA). Tissue homogenization and protein quantification
1192 proceeded as described by the manufacturer.

1193 The GH and GHR concentrations were normalized by the total protein
1194 concentration in the tissue homogenates. Total protein content was determined
1195 using the Bradford reagent (Bio-Rad, USA) as described by the manufacturer.
1196
1197 *Identification of GH, GHR1, GHR2, IGF-1 and IGF-2 partial transcripts*
1198
1199 All mRNA sequences used in the present study were found in the NCBI
1200 nucleotide collection (nr/nt) (http://www.ncbi.nlm.nih.gov/).The GH sequences
1201 previously characterized for *Kryptolebias marmoratus* (JN383973.1) and
1202 *Odontesthes bonariensis* (AY187284.2), and previously predicted for *Poecilia*
1203 *formosa* (XM_007564985.1) and *Poecilia reticulata* (XM_008416502.1) were
1204 employed to search for conserved regions. Sequences were evaluated with
1205 ClustalW multiple alignment using the Bioedit Sequence Alignment Editor
1206 software (7.2.5) (Hall, 1999). Thus, forward and reverse PCR primers were
1207 designed to amplify a GH conserved fragment expected to amplify the *P.*
1208 *vivipara* transcript. The same strategy was used to design the forward and
1209 reverse PCR primers for the other target genes.
1210 For each target gene, a different combination of related species was
1211 used in the clustalW multiple alignment. GHR1 conserved region was identified
1212 by alignment of the previously characterized sequence for *K. marmoratus*
1213 (JN383975.1) and predicted sequence for *P. reticulata* (XM_008418329.1), *P.*
1214 *formosa* (XM_007557592.1) and *Xiphophorus maculatus* (XM_005794909.1).
1215 For GHR2, conserved region was found by alignment of the previously
1216 characterized sequence for *K. marmoratus* (JN383976.1) and predicted
1217 sequence for *X. maculatus* (XM_005812440.1) and *P. formosa*
1218 (XM_007542180.1). Conserved region in IGF-1 sequence was obtained by
1219 aligning the complete *Epinephelus coioides* (AY513719.1) and *Paralichthys*
1220 *lethostigma* (DQ221741.1) IGF-1 mRNA, and predicted sequence for *P.*
1221 *reticulata* (XM_008400899.1) and *P. formosa* (XM_007555315.1). For IGF-2,
1222 conserved region was found by alignment of previously described sequence for
1223 *Poecilia butleri* (DQ337477.1), *Cnesterodon decemmaculatus* (DQ337475.1),
1224 *Limia melanogaster* (DQ337478.1) and *Paralichthys olivaceus* (AF091454.1).
1225 All primers designed are shown in Table 1.
1226

1227 *Expression of GH, GHR1, GHR2, IGF1 and IGF2 partial transcripts*

1228

1229 Effects of chronic Cu exposure on the mRNA expression for target genes
1230 were determined in brain, muscle and liver samples. Total RNA was extracted
1231 with Qiazol reagent (Qiagen) as described by the manufacturer. cDNA was
1232 synthesized with the High Capacity cDNA Reverse Transcription kit (Applied
1233 Biosystems) and a mix of anchored oligo (dT) primer (Applied Biosystems) with
1234 random hexamer primer (Applied Biosystems).

1235 Real time PCR (qPCR; 7300 Real-Time PCR System; Applied
1236 Biosystems) was used to quantify the relative expression of target genes using
1237 the GoTaq qPCR Master Mix (Promega, Madison, WI). The gene-specific
1238 primers used were designed using conserved regions for fish species related to
1239 *P. vivipara*, as mentioned above. Expressions of all genes were analyzed in
1240 duplicate using the following protocol: 50°C for 2 min, 95°C for 2 min, 45 cycles
1241 at 95°C for 15 s, and 60°C for 30 s. To ensure amplification of a single product,
1242 melting curve analysis was performed at the end of each PCR run. The relative
1243 values for the total target gene expression in tissues samples were analyzed by
1244 the $2^{-\Delta\Delta ct}$ method (Schmittgen and Livak, 2008), using the average expression
1245 of β-Actin and EF1α as housekeeping genes (Silva et al., 2014).

1246

1247 *Data presentation and statistical analysis*

1248

1249 Data are expressed as mean ± standard error. Differences among the
1250 experimental groups were assessed using analysis of variance (ANOVA)
1251 followed by the Tukey's test. Parametric assumptions of the ANOVA model
1252 were checked. Normal distribution of residuals was evaluated by the
1253 Kolmogorov-Smirnov test, while the homogeneity of variances was assessed by
1254 the Levene's test. Durbin-Watson test was also used to evaluate the
1255 independency of observations. When data fail to meet the ANOVA
1256 assumptions, square-root transformation was applied and the ANOVA
1257 assumptions were tested again. Pearson's correlation analysis was also
1258 performed. In all cases, the level of significance adopted was 95% ($\alpha = 0.05$).
1259 All analyses were performed using the SigmaPlot 12.0 software (Systat, USA).

1260

1261 **RESULTS**

1262

1263 In all tissues analyzed, chronic Cu exposure did not alter the
1264 concentration of GH (Fig. 1) and GHR (Fig. 2).
1265 All designed gene transcripts could be partially identified and sequences
1266 showed high query cover and identity when compared to sequences described
1267 for other fish species. The GH partial mRNA of *P. vivipara* had 153 nucleotides
1268 and 98% similarity compared to that described for the GH partial sequence of *P.*
1269 *latipinna* (AF134609.1), with 77% of query coverage. The partial GHR1 mRNA
1270 of *P. vivipara* comprised 71 nucleotides, demonstrating 97% similarity with that
1271 described for the predicted GHR1 sequence of *P. latipinna* (M_015024111.1),
1272 with 97% of query coverage. The partial GHR2 mRNA of *P. vivipara* had 96
1273 nucleotides and showed 78% when compared to the GHR2 complete sequence
1274 described for *K. marmoratus* (JN383976.1), with 77% of query coverage. The
1275 partial IGF1 mRNA of *P. vivipara* comprised 126 nucleotides and showed 98%
1276 similarity with the complete IGF1 sequence described for *Cichlasoma dimerus*
1277 (KM005102.1), with 96% of query coverage. Finally, the partial IGF2 mRNA of
1278 *P. vivipara* comprised 155 nucleotides, demonstrating 100% similarity with the
1279 complete IGF2 sequence described for *P. butleri* (DQ337477.1), with 100% of
1280 query coverage.
1281 Chronic exposure to Cu did not affect the GH mRNA expression in the
1282 brain (Fig. 3). Similarly, Cu exposure did not alter the GHR1 mRNA expression
1283 in any tissue analyzed (Fig. 4). Also, it did not change the GHR2 mRNA
1284 expression in the brain and liver of guppies (Fig. 5). Conversely, chronic
1285 exposure to 5 and 9 µg/L Cu significantly reduced the GHR2 mRNA expression
1286 in the skeletal muscle of guppies (Fig. 5). Also, chronic exposure to 9 µg/L Cu
1287 significantly reduced the IGF1 mRNA expression (Fig. 6) and the IGF2 mRNA
1288 expression (Fig. 7). In the skeletal muscle, IGF1 and IGF2 mRNA expressions
1289 were significantly correlated with the GHR1 mRNA expression ($R = 0.6$, $p<0.001$
1290 and $R= 0.68$, $p<0.001$, respectively). Also, IGF1 and IGF2 mRNA expressions
1291 were correlated with the GHR2 mRNA expression ($R = 0.69$, $p<0.001$ and 0.73,
1292 $p<0.001$, respectively).

1293

1294 **DISCUSSION**

1295

1296 Studies evaluating the chronic effect of heavy metals on the endocrine
1297 system are scarce. In the case of Cu, the majority of the long-term experiments
1298 performed up to now had a duration period ranging from 60 to 100 days.
1299 Indeed, information on the chronic effects of Cu for more than 100 days is quite
1300 rare (Handy, 2003). In the present study, we evaluated the effect of the chronic
1301 exposure to dissolved Cu throughout the entire life of the viviparous guppy *P.*
1302 *vivipara*. In fact, newborn guppies (<24 h after birth) were exposed to Cu until
1303 all individuals have reached the sexual differentiation, i.e., 345 days after the
1304 beginning of the experiment. The Cu effects on metabolism and growth of *P.*
1305 *vivipara* under the same experimental conditions have been previously reported
1306 (Anni, 2015; Zebral et al., 2016).

1307 Anni (2015) reported that guppies exposed to 9 µg/L Cu for 345 days
1308 showed higher whole-body Cu concentrations. This accumulation of Cu in the
1309 fish body was explained by an accumulation of Cu in gills, intestine and liver of
1310 guppies. This author also demonstrated that tissue Cu accumulation was
1311 related to an increased expression of mRNA encoding for the Cu-transporting
1312 protein ATP7B in the intestine and liver of *P. vivipara*. In addition, Anni (2015)
1313 reported that chronic exposure to 5 and 9 µg/L reduced fish body mass. It is
1314 worth noting that growth inhibition induced by Cu is also well described after
1315 short-term exposure to Cu in freshwater (Al-Ogaily et al., 2003) and saltwater
1316 fish (Liu et al., 2010), as well as after long-term exposure in freshwater fish
1317 (McKim et al., 1971). Therefore, in the present study we evaluated the possible
1318 effects of the chronic Cu exposure on the somatotropic axis of the guppy *P.*
1319 *vivipara* in order to elucidate the physiological mechanisms involved in inhibition
1320 of fish growth induced by chronic exposure to Cu. Analyses performed involved
1321 the concentrations of GH and GHR and the mRNA expression of GH, GHR1,
1322 GHR2, IGF1 and IGF2. As far as we know, this is the first study to evaluate the
1323 alterations in the somatotropic axis induced by chronic Cu exposure and its
1324 possible relationship with fish growth inhibition.

1325 Unfortunately, the guppy *P. vivipara* do not provide sufficient blood
1326 volume to quantify the circulating concentrations of GH. However, data on the
1327 mRNA and tissue concentration of this hormone in the brain clearly indicate that
1328 GH levels were not altered after chronic Cu exposure. The great majority of

studies concerning growth inhibition and alterations in the somatotropic axis are related to food deprivation experiments and will be used for comparison with our results.

Differently from the observed in the present study, growth inhibition induced by starvation provokes a rise in the level of circulating GH. After food deprivation, the channel catfish *Ictalurus punctatus* showed elevated GH mRNA expression (Peterson et al., 2009). Similarly, starvation caused higher levels of circulating GH in the fine flounder *Paralichthys adspersus* (Fuentes et al., 2012), rainbow trout *O. mykis* (Gabillard et al., 2006; Norbeck et al., 2007) and tilapia *Oreochromis mossambicus* (Fox et al., 2006). One explanation to the starvation-dependent rise in plasma GH is the reduced levels of hepatic GHR (Gray et al., 1992) and the consequent reduction in plasma IGF1 concentration (Banos et al., 1999; Pierce et al., 2001; Beckman et al., 2004). As a consequence of this process, the negative regulation of IGF1 in the production of GH is also reduced (Pierce et al., 2005; Norbeck et al., 2007; Kling et., 2012). It is interesting to note that the hepatic expressions of IGF1 and IGF2 mRNA were not altered by chronic exposure of *P. vivipara* to Cu.

It is worth noting that IGF1 and IGF2 are proteins released into the circulatory system right after their production, and are not retained in the liver (Duan, 1997; Duan, 1998). Thus, hepatic expression of IGF1 and IGF2 mRNA are directly comparable to its circulating levels (Beckman, 2011). Therefore, we can infer that circulating levels of IGF1 and IGF2 were also unaffected by chronic Cu exposure in the present study. The liver concentration of GHR and the hepatic expression of GHR1 and GHR2 mRNA were also unaffected by chronic exposure to Cu. These findings are in complete agreement with the observed levels of plasma GH. Therefore, the endocrine actions of the hepatic-derived IGF1 and IGF2, just as plasmatic concentrations of GH, are unlikely involved in the Cu-induced reduction in growth of *P. vivipara* reported by Anni (2015).

Despite the relevance of the liver in endocrine regulation, other peripheral tissues, such as the skeletal muscle, are also implicated in the regulation of the somatotropic axis (Fuentes et al., 2013). Interestingly, it was in this tissue that the majority of the chronic Cu-induced effects were observed when considering the GH-IGF system. In this case, reduced mRNA expression

1363 of GHR2, IGF1 and IGF2 was observed after chronic exposure of *P. vivipara* to
1364 Cu. The skeletal muscle can express different proportions of the complete
1365 isoforms (GHR1-fl and GHR2-fl) and truncated isoforms (GHR1-t and GHR2-t)
1366 of GHR (Fuentes et al., 2013). The truncated isoforms of GHR are membrane
1367 receptors produced by alternative splicing of the GHR-fl lacking its intracellular
1368 portion. Therefore, the direct binding of GH to GHR-t does not trigger any
1369 intracellular response (Dastot et al., 1996). On the other hand, GHR-fl activation
1370 by GH triggers a series of intracellular responses mediated by the JAK2/STAT5
1371 cascade signal (Argetsinger et al., 1993; Zhu et al., 2001). The phosphorylation
1372 of STAT5 promotes the expression of IGF1 and IGF2, which are hormones
1373 implicated in the stimulation of cell proliferation and differentiation (Davey et al.,
1374 2001).

1375 Within the muscle cell, IGF1 and IGF2 sustain an anabolic state through
1376 stimulation of myogenic processes, as well as inhibition of protein degradation
1377 and muscle atrophy in an autocrine/paracrine way (Fuentes et al., 2013).
1378 Indeed, there is a very interesting example on how the skeletal muscle can
1379 modulate the somatotropic axis and therefore alter growth control. It has been
1380 demonstrated that the naturally slow-growing fish *Paralichthys adspersus*
1381 expresses proportionally more GHR-t than GHR-fl in its skeletal muscle cells.
1382 Therefore, this tissue has a natural resistance to GH, leading to low expression
1383 of muscular IGF1. Paradoxically, circulating levels of IGF1 in this fish are very
1384 similar to those described for other fish species (Fuentes et al., 2012). In a
1385 similar manner, the Cu-induced reduction in muscular mRNA expression of
1386 IGF1 and IGF2 seen in the present study can be a result of a Cu-induced
1387 insensitivity of skeletal muscle cells to GH, which was characterized by the
1388 observed reduction in GHR2 mRNA expression. The exciting example
1389 described by Fuentes et al. (2012) can help connecting the Cu-induced
1390 muscular insensitivity to GH seen in the present study and the fish growth
1391 inhibition induced by Cu exposure reported by Anni (2015) and other several
1392 authors (McKim, et al., 1971; Al-Ogaily, et al., 2003; Luo, et al., 2010; Nekoubin
1393 et al., 2012).

1394 Although we were able to assess the Cu-induced alterations in the GHR2
1395 mRNA expression n the skeletal muscle, the muscle concentration of GHR was
1396 not affected by the chronic Cu exposure. In this case, at least three hypotheses

can be considered to explain this discrepancy. Firstly, the antibody used in the ELISA kit could be designed to bound to a specific area present only in the GHR1, thus excluding the GHR2 from the assessment. In this case, protein concentration and gene expression would be in conformation, considering that GHR1 mRNA expression in the skeletal muscle was not altered by Cu exposure. Secondly, the region targeted by the ELISA antibody could be common to GHR1 and GHR2. Therefore, cross-assessment of GHR1 could prevent the proper evaluation of Cu-induced alterations in GHR2 concentration. Thirdly, the antibody used in the ELISA kit could be targeting an extracellular region of the GHR. In this case, we would be assessing the total amount of GHR, including the GHR-t and GHR-fl. Thus, if the Cu-induced alterations in the concentration of GHR involve adjustments in the balance between the full-length and the truncated form of the GHR, we would not be able to assess this alteration using the ELISA kit.

Considering the third hypothesis as the most plausible, it is tempting to suppose that we are assessing the expression of the full-length isoform of the GHR2. If that is true, the reduced expression of GHR2 mRNA in the skeletal muscle could indicate that chronic exposure to Cu stimulates the alternative splicing of the GHR2-fl, leading to a greater production of the GHR2-t. If this hypothesis is true, we could not assess this alteration in terms of protein concentration because the ELISA kit would not distinguish the two isoforms, thus explaining why the muscular concentration of GHR remained unaltered after chronic exposure to Cu. Unfortunately, the ELISA kit manufacturer does not provide information regarding which isoform of GHR and which part of the protein is targeted by its antibody. Considering this fact, as well as our data on gene expression, the high correlations seen between the mRNA expression of GHR2 and the mRNA expressions of IGFs thus further support the idea that we are actually assessing the full-length isoform of the receptor and its intracellular region. Certainly, the complete cloning of the GHR2-fl and the GHR2-t sequences, as well as the assessment of the GHR2-t expression would help to elucidate this question. Finally, it is worth noting that the information given by Fuentes et al. (2012) also supports our current hypothesis.

The proposed relationship between the Cu-induced growth inhibition and the reduced mRNA expression of muscular IGF1 and IGF2 does not explain the

growth reduction reported by Anni (2015) following chronic exposure to 5 µg/L Cu. The only gene to have its mRNA expression altered after exposure to this Cu concentration was the muscular GHR2. Therefore, the Cu-induced reduction in *P. vivipara* growth should be, at least in part, be linked to growth-related actions of GHR2 that are independent of IGF1 and IGF2. In fact, nuclear translocation of GHR is implicated in cellular proliferation induced by GH through activation of the MEK/ERK signaling cascade in a way that is independent of the JAK2/STAT5 phosphorylation (Liang et al., 1999; Figueiredo et al., 2016). Less nuclear translocation of GHR2 in muscle cells may explain part of the Cu-induced growth delay reported by Anni (2015). Additionally, it is important to note that findings reported in the present study resulted from a very long-term exposure (345 days) to Cu. Since we do not provide data related to intermediate sampling times, a possible influence of Cu on the somatotropic axis after a shorter time exposure cannot be ruled out.

We demonstrated in the present study that the Cu-induced growth inhibition in *P. vivipara* is related to a reduced muscular expression of GHR2 mRNA. Since Anni (2015) reported that this tissue did not accumulated Cu, it is likely thus possible that Cu is acting indirectly on the muscle. As argued by Handy (2003), chronic Cu effects are related to large physiological and biochemical adjustments, such as elevated levels of circulating corticosteroids. Similar endocrine adjustments dependent on Cu could explain the reduced expression of GHR2. In fact, it has been shown that thyroid hormones and insulin are implicated in GHR expression modulation and that Cu can modulate the Akt/FoxO signaling in an insulin-like manner (Hamann et al., 2014). Additionally, insulin signaling and Cu homeostasis seem to be linked (Yang et al., 2014). Thus, interactions of thyroid hormones, insulin and insulin-dependent processes with chronic Cu exposure could be implicated in the modulation of the muscular GHR2 expression observed in the present study.

CONCLUSIONS

Findings reported in the present study show that the viviparous guppy *P. vivipara* exposed throughout its entire life cycle to environmentally relevant concentrations of dissolved Cu in salt water displays alterations in the

1465 somatotropic axis characterized by reduced expression of GHR2 in the skeletal
1466 muscle, paralleled by reduced expression of local IGF1 and IGF2. Therefore,
1467 we can conclude that the well established inhibitory effect of Cu exposure on
1468 fish growth involves skeletal muscle insensitivity to GH. This effect leads to
1469 impairment in the local expression of IGF1 and IGF2, which are key trophic
1470 factors in the maintenance of myogenic processes. Also, our findings show that
1471 the paracrine/autocrine actions of muscular IGF1 and IGF2 are more relevant in
1472 the chronic Cu-induced inhibition of fish growth than endocrine actions
1473 mediated by liver IGF1 and IGF2. Finally, as far as we know, the present paper
1474 is the first to relate the well established chronic effect of Cu on fish growth to a
1475 disruption in the somatotropic axis. Therefore, our findings support the idea of
1476 considering Cu as an endocrine disruptor in the viviparous guppy *P. vivipara*, at
1477 least under chronic conditions.
1478

1479 **ACKNOWLEDGMENTS**

1480

1481 Financial support was provided by the Conselho Nacional de
1482 Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil; grant
1483 #573949/2008-5 - Instituto Nacional de Ciência e Tecnologia de Toxicologia
1484 Aquática), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior
1485 (CAPES, Brasília, DF, Brazil; grant # 230/2010 - Ciências do Mar), and the
1486 International Development Research Center (IDCR, Ottawa, Canada; Project
1487 #104519-003). YDZ was a MSc fellow from the Brazilian CAPES. ISAA, SIMA
1488 and RDK were PhD fellows from the Brazilian CAPES. AB is a research fellow
1489 from the Brazilian CNPq (grant #304430/2009-9) and is supported by the
1490 International Canada Research Chair Program from IDRC.

1491

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1682 **Table 1**. Primers used for real-time PCR (qPCR) analysis of the mRNA
1683 expression of genes encoding for growth hormone (GH), growth hormone
1684 receptor 1 (GHR1), growth hormone receptor 2 (GHR2), insulin-like growth
1685 factor 1 (IGF1) and insulin-like growth factor 2 (IGF2) in the viviparous guppy
1686 *Poecilia vivipara* kept under control condition (no copper addition in the water)
1687 or exposed to dissolved Cu (nominally: 5 and 9 µg/L) for 345 days.
1688

Primer		Sequence (5'-3')
GH	Forward	AGAACCTACGAACTGCTGGC
GH	Reverse	TTGCCACGGTCAGGTAAGTC
GHR1	Forward	AACACCTCAGGTTCAGAGCG
GHR1	Reverse	CAAACAGCTGAAACTGGGGC
GHR2	Forward	TGCCTTCAAACCAACGTAAACT
GHR2	Reverse	TCGCTGAAGTCTCCAAAGTCC
IGF1	Forward	ATCTCCTGTAGCCACACCCT
IGF1	Reverse	CAGACAAACTGCAGCGTGTC
IGF2	Forward	CAGTAGGCCAAACAGCAGGA
IGF2	Reverse	GCATAGAGGAGGACGACACG

1689

1690 **CAPTION TO FIGURES**

1691

1692 **Figure 1**. Growth hormone (GH) concentration in brain of guppies *Poecilia*
1693 *vivipara* kept under control condition (no copper addition in the water) or
1694 exposed to dissolved copper (nominally: 5 and 9 μg/L) for 345 days. Data are
1695 mean ± standard error (n = 6). Different letters indicate significant different
1696 mean values among experimental groups ($p<0.05$, ANOVA, Tukey's test).

1697

1698 **Figure 2**. Growth hormone receptor (GHR) concentration in liver, muscle and
1699 brain of guppies *Poecilia vivipara* kept under control condition (no copper
1700 addition in the water) or exposed to dissolved copper (nominally: 5 and 9 μg/L)
1701 for 345 days. Data are mean ± standard error (n = 4-11). Different letters
1702 indicate significant different mean values among experimental groups ($p<0.05$,
1703 ANOVA, Tukey's test).

1704

1705 **Figure 3**. Relative expression of growth hormone (GH) in brain of guppies
1706 *Poecilia vivipara* kept under control condition (no copper addition in the water)
1707 or exposed to dissolved copper (nominally 5 and 9 μg/L) for 345 days. β-actin
1708 and EF-1 were used as housekeeping genes (Ct average). Data are mean ±
1709 standard error (n = 9-10). Different letters indicate significant different mean
1710 values among experimental groups ($p<0.05$, ANOVA, Tukey's test).

1711

1712 **Figure 4**. Relative expression of the growth hormone receptor 1 (GHR1) in liver,
1713 muscle and brain of guppies *Poecilia vivipara* kept under control condition or
1714 exposed to dissolved copper (nominally: 5 and 9 μg/L) for 345 days. β-actin and
1715 EF-1 were used as housekeeping genes (Ct average). Data are mean ±
1716 standard error (n = 9-10). Different letters indicate significant different mean
1717 values among experimental groups ($p<0.05$, ANOVA, Tukey's test).

1718

1719 **Figure 5**. Relative expression of the growth hormone receptor 2 (GHR2) in liver,
1720 muscle and brain of guppies *Poecilia vivipara* kept under control condition (no
1721 copper addition in the water) or exposed to dissolved copper (nominally: 5 and
1722 9 μg/L) for 345 days. β-actin and EF-1 were used as housekeeping genes (Ct
1723 average). Data are mean ± standard error (n = 9-10). Different letters indicate

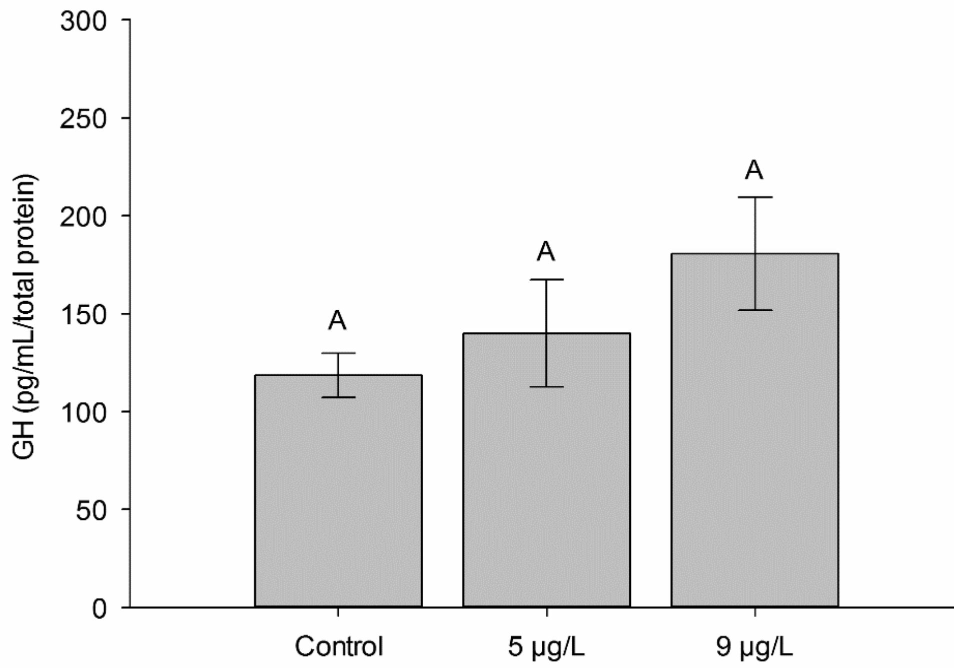
1724 significant different mean values among experimental groups ($p<0.05$, ANOVA,
1725 Tukey's test).
1726

1727 **Figure 6**. Relative expression of the insulin-like growth factor 1 (IGF1) in
1728 muscle and liver of guppies *Poecilia vivipara* kept under control condition (no
1729 copper addition in the water) or exposed to dissolved copper (nominally: 5 and
1730 9 μg/L) for 345 days. β-actin and EF-1 were used as housekeeping genes (Ct
1731 average). Data are mean ± standard error (n = 9-10). Different letters indicate
1732 significant different mean values among experimental groups ($p<0.05$, ANOVA,
1733 Tukey's test).
1734

1735 **Figure 7**. Relative expression of the insulin-like growth factor 2 (IGF2) in
1736 muscle and liver of guppies *Poecilia vivipara* kept under control condition (no
1737 copper addition in the water) or exposed to dissolved copper (nominally: 5 and
1738 9 μg/L) for 345 days. β-actin and EF-1 were used as housekeeping genes (Ct
1739 average). Data are mean ± standard error (n = 8-10). Different letters indicate
1740 significant different mean values among experimental groups ($p<0.05$, ANOVA,
1741 Tukey's test).
1742

1743 Figure 1

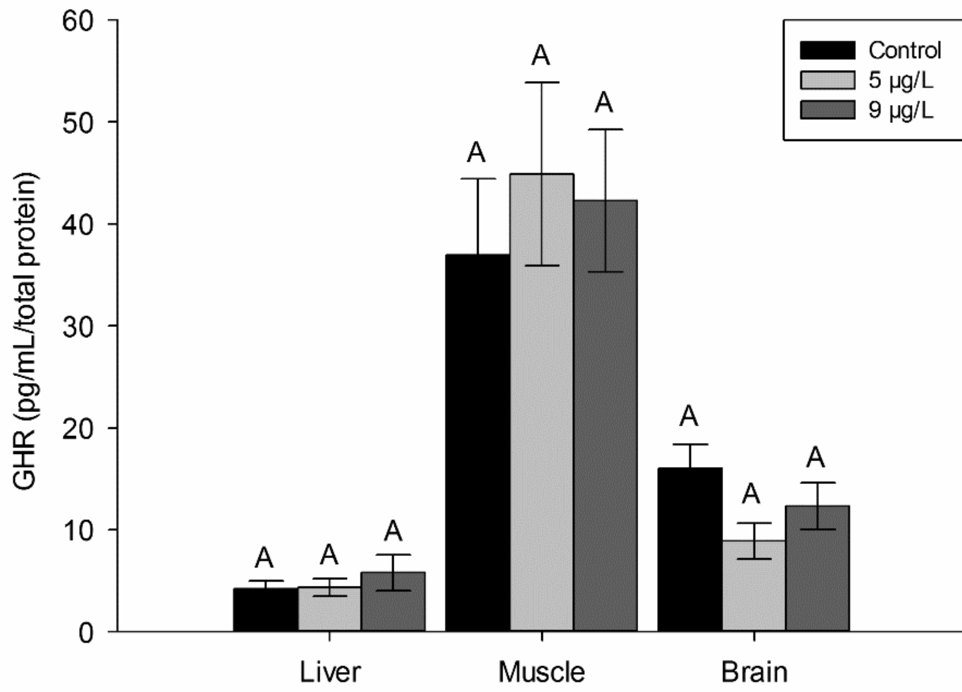
1744



1745

1746 Figure 2

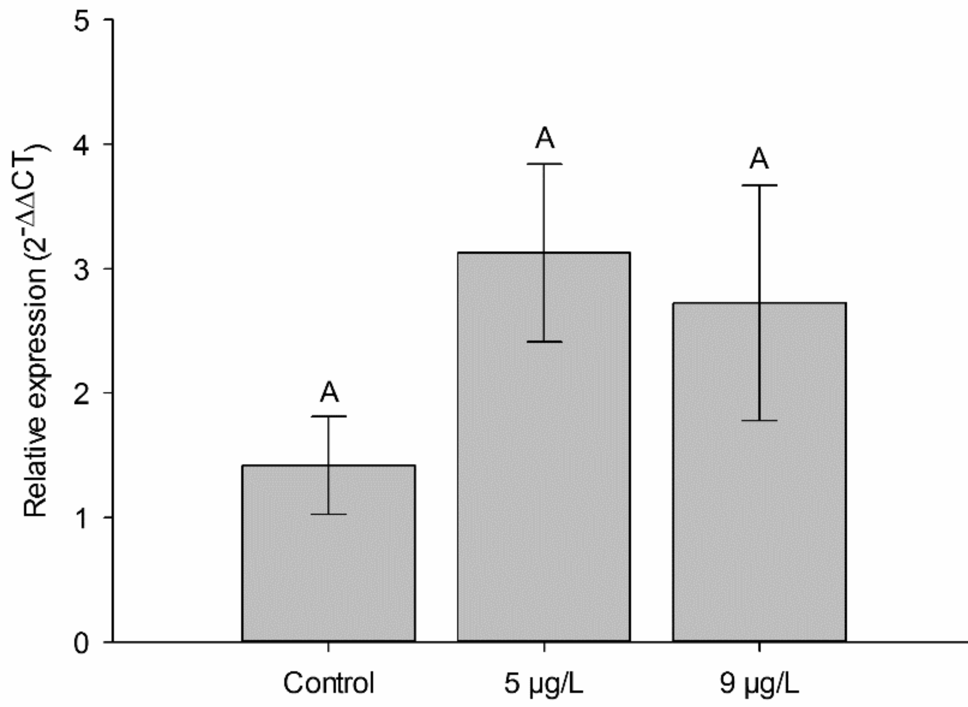
1747



1748

1749 Figure 3

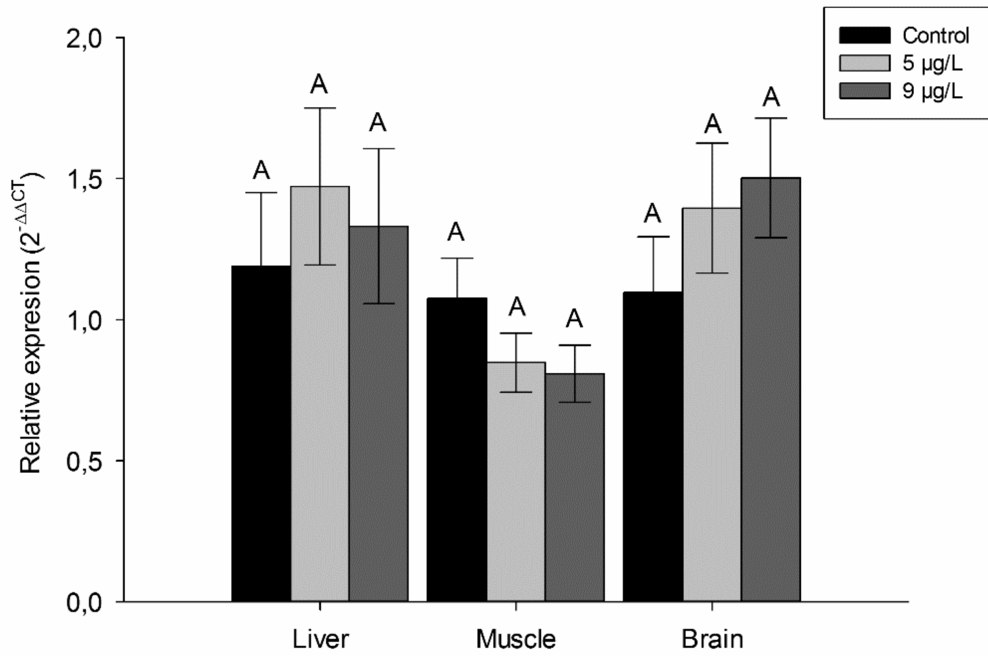
1750



1751

1752 Figure 4

1753



1754

1755 Figure 5

1756

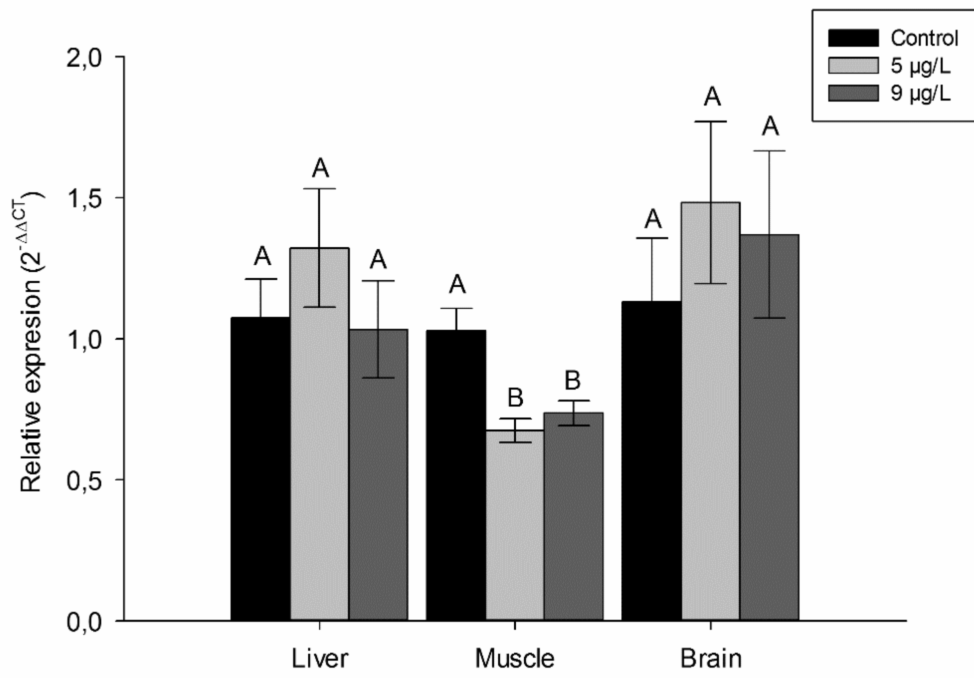


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Figure 6

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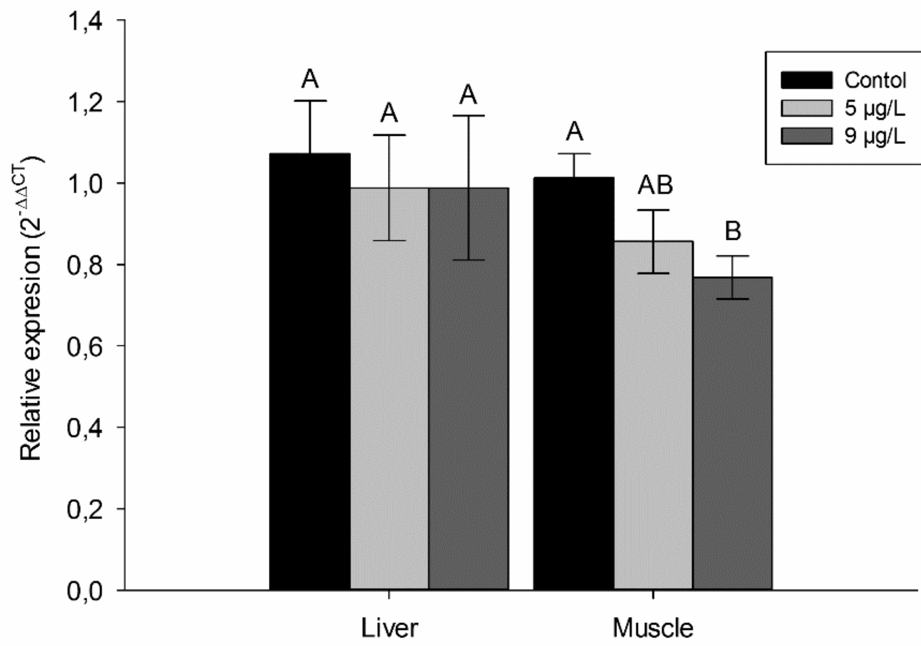

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1761 Figure 7

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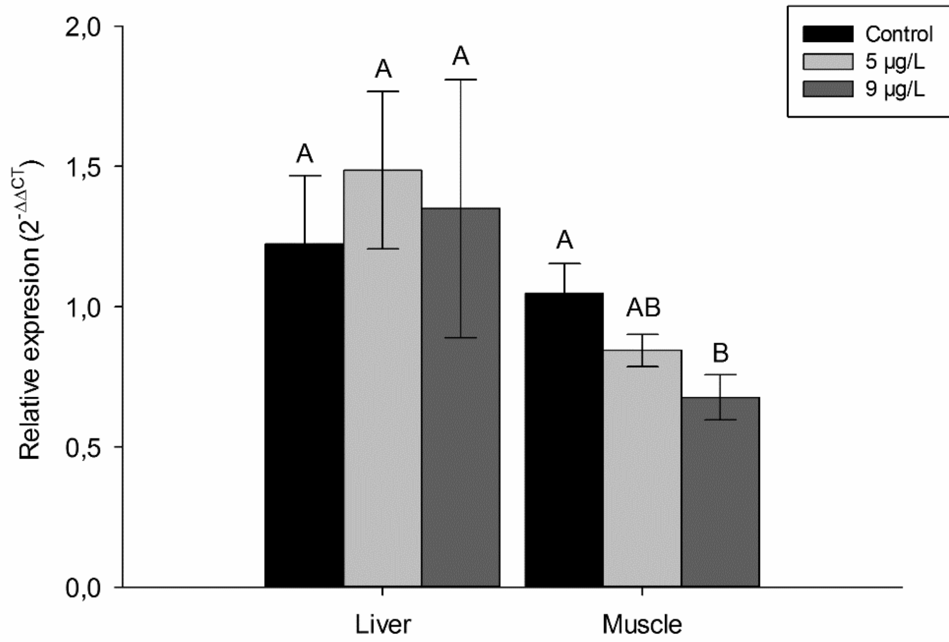

1763

CONCLUSÕES GERAIS

Os estudos no âmbito do nosso grupo de pesquisa acerca dos mecanismos de toxicidade envolvendo a exposição crônica ao cobre se iniciaram com o trabalho de Anni (2015), onde foram demonstradas algumas das vias moleculares responsáveis pela acumulação do cobre frente à exposição crônica a este metal. Foi demonstrado ainda, que tal exposição está relacionada a prejuízos no crescimento e alteração na via aeróbica de produção energética.

Considerando o exposto acima, os objetivos da presente dissertação foram estabelecidos com o intuito de aprofundar estes conhecimentos, principalmente no que se refere às ações do cobre sobre o crescimento. Sendo assim, foram adotadas duas linhas de análise. Na primeira delas, foram analisados os possíveis mecanismos endócrinos relacionados ao prejuízo do crescimento que estavam associados à exposição crônica ao cobre. De fato, diversos estudos, além daquele de Anni (2015), já haviam demonstrado um efeito negativo da exposição ao cobre sobre o crescimento em peixes, mas surpreendentemente, nenhum havia averiguado as ações do cobre sobre os mecanismos reguladores do crescimento. Na segunda linha de investigação, foram avaliados os efeitos da exposição crônica ao cobre sobre o metabolismo mitocondrial, a fim de averiguar possíveis prejuízos da exposição ao metal sobre a capacidade de produção energética desta organela, considerando que Anni (2015) já havia demonstrado alguns efeitos do cobre sobre enzimas do Ciclo de Krebs. Portanto, no presente estudo foi testada a hipótese de que as ações do cobre relacionadas ao prejuízo no crescimento de peixes estariam associadas a alterações sobre mecanismos de regulação endócrina e produção aeróbica de energia. Para avaliar as ações sobre os mecanismos de regulação endócrina, foram analisados alguns componentes do eixo somatotrópico em termos de expressão gênica (GH, GHR1, GHR2, IGF1, IGF2) e proteica (GH e GHR).

Com base nos resultados obtidos, foi observado que a exposição crônica ao cobre não alterou a expressão gênica e nem proteica do GH no cérebro. Da mesma forma, também não foram observadas alterações na expressão dos genes GHR1, GHR2, IGF1 e IGF2 no fígado, nem da concentração de GHR

1798 neste tecido e no músculo esquelético. Por outro lado, no músculo esquelético
1799 foi observada uma redução na expressão gênica de GHR2 no após exposição
1800 a 5 e 9 µg/L de cobre, bem como uma diminuição na expressão gênica de IGF1
1801 e IGF2 após exposição a 9 µg/L deste metal. A partir destes resultados,
1802 conclui-se que a redução observada no crescimento de *P. vivipara* após
1803 exposição crônica ao cobre está relacionada a um processo de
1804 dessensibilização ao GH no músculo esquelético, a qual ocorre devido a uma
1805 diminuição na expressão gênica do GHR2, associada a uma diminuição na
1806 expressão de IGF1 e IGF2, hormônios estes que são produzidos de forma
1807 dependente da sinalização do GH e possuem a função de manter os músculos
1808 em um estado anabólico. Com base ainda nos resultados obtidos, é sugerido
1809 que esta resposta pode estar relacionada a uma alteração na razão entre as
1810 isoformas truncada e completa do GHR2 no músculo esquelético, e não a uma
1811 diminuição na concentração total de GHR2. Além disso, conclui-se que a
1812 diminuição do crescimento de *P. vivipara* após exposição crônica ao cobre está
1813 mais relacionada a alterações na ação parácrina/autócrina dos IGF1 e IGF2 do
1814 que suas ações endócrinas. Com isso, sugere-se que o cobre seja considerado
1815 um desregulador endócrino.
1816 Para avaliar as ações do cobre sobre o metabolismo mitocondrial, foram
1817 selecionados alguns genes alvo que expressam alguns componentes da cadeia
1818 transportadora de elétrons (COX I, COX II e COX III) e da fosforilação oxidativa
1819 (ATP5A1) para serem avaliados em termos de sua regulação transcripcional no
1820 músculo esquelético e fígado de *P. vivipara*. Foi demonstrado que a exposição
1821 crônica ao cobre não alterou a expressão hepática de nenhum dos genes
1822 codificantes das subunidades da COX. Da mesma forma, a expressão
1823 muscular do gene codificante da ATP5A1 não foi alterada. Por outro lado, a
1824 expressão gênica de COX II e COX III foi diminuída no músculo esquelético e a
1825 expressão do gene codificante da ATP5A1 foi aumentada no fígado.
1826 Interessantemente, a exposição ao cobre gerou ações tecido-específicas sobre
1827 o metabolismo energético. Neste caso, o músculo esquelético apresentou
1828 diminuição na expressão de genes relacionados à cadeia transportadora de
1829 elétrons, indicando um prejuízo sobre a capacidade deste órgão em produzir
1830 ATP, o que pode ter sido limitante para a proliferação de células musculares,
1831 levando assim a um menor crescimento. Já o fígado, apresentou um aumento

na expressão do gene de uma das proteínas relacionadas à fosforilação oxidativa. Considerando a importância deste órgão para a detoxificação de metais, o efeito observado no fígado após exposição crônica ao cobre pode estar relacionado a um aumento na produção de ATP para compensar os gastos energéticos com o processo de detoxificação e excreção hepática do excesso de cobre.

Por fim, conclui-se que as hipóteses formuladas no presente estudo foram corroboradas pelos resultados obtidos e que a redução do crescimento observada em *P. vivipara* após exposição crônica ao cobre está relacionada tanto a alterações em nível de regulação endócrina quanto de metabolismo energético. Interessantemente, foi demonstrado que a maioria destas alterações ocorre no músculo esquelético, evidenciando assim que este tecido está no centro do processo de inibição do crescimento associado à exposição crônica ao cobre. Certamente, um melhor entendimento dos mecanismos de regulação do crescimento a partir da alteração na produção das isoformas de GHR-fl e GHR-t no músculo, e suas possíveis relações com o status energético deste tecido, poderão contribuir muito para a melhor compreensão dos processos que inibem o crescimento em peixes.

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