Efeitos do cobre sobre a atividade de enzimas-chave do metabolismo energético do órgão respiratório de animais aquáticos

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SUMÁRIO

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2. RESUMO GERAL

Animais que habitam ambientes aquáticos costeiros devem apresentar mecanismos para a manutenção de sua homeostase para tolerar as alterações recorrentes que ocorrem nestes ambientes. Entre essas alterações estão variações na salinidade e, algumas vezes, a presença de contaminantes, como o metal cobre. A manutenção da homeostase requer energia, fazendo com que os animais aumentem a produção de ATP através da estimulação das vias metabólicas responsáveis pela sua geração, como a via glicolítica e o ciclo de Krebs. Dessa maneira, é de extrema importância conhecer como a salinidade e o cobre interferem no metabolismo energético de animais aquáticos costeiros, em especial em espécies representantes de diferentes grupos abundantes nestes ambientes e que possuem características fisiológicas especificas, como os crustáceos, os peixes e os equinodermos. Em vista disso, o objetivo desta tese foi avaliar os efeitos do cobre no metabolismo energético no caranguejo *Neohelice granulata*, no peixe *Fundulus heteroclitus* e no pepino-do-mar *Trachythyone crassipeda*. Foram selecionadas enzimas consideradas marcapasso da via glicolítica (hexoquinase, fosfofrutoquinase e piruvato quinase) e do ciclo de Krebs (citrato sintase ou isocitrato desidrogenase). Além disso, a enzima lactato desidrogenase também foi avaliada já que em situações de hipóxia, ela passa a ser uma importante via de reciclagem de NAD^+. O órgão escolhido para analise foram as brânquias (caranguejo e peixe) e árvores respiratórias (pepino-do-mar) por serem o órgão que está em maior contato com a água, sendo mais susceptíveis à acumulação de cobre e estarem associadas com a iono e osmorregulação em peixes e crustáceos e com a excreção de amônia em crustáceos e holotúrias.

O caranguejo *N. granulata* foi exposto (96 h) ao cobre nas salinidades 2 (1 mg Cu/L) e 30 (5 mg Cu/L) e, além das enzimas citadas anteriormente, também foram avaliados o potencial de membrana mitocondrial e atividade da citocromo c oxidase de mitocôndrias branquiais isoladas. Em geral, os resultados mostraram que o cobre afeta os parâmetros analisados, sendo mais tóxico em brânquias anteriores de caranguejos aclimatados à salinidade 2.

No peixe *F. heteroclitus*, foi avaliado o efeito da exposição aguda ao cobre (30 μg/L) por 96 h em peixes aclimatados a diferentes salinidades (0; 3,5; 11; e 35). Nesta espécie, um efeito mais toxico do cobre foi observado nos animais aclimatados à água doce. Isto sugere que, além de um efeito direto do cobre sobre a enzima Na^+,K^+-ATPase como observado em outros estudos, o metal causa um efeito indireto, já que uma menor quantidade de ATP está disponível para o funcionamento da bom de sódio-potássio. Os peixes de água salobra (salinidades 3,5 e 11) também podem estar com suas reservas energéticas depletidas, pois o cobre diminui a atividade da isocitrato desidrogenase. Em relação aos peixes de água salgada o mecanismo de toxicidade do cobre é outro que não um efeito sobre a iono/osmorregulação ou o metabolismo energético.

O pepino-do-mar *T. crassipeda* foi exposto (96 h) a diferentes concentrações de cobre (0, 5, 9, e 20 μg Cu/L) na salinidade 33. A exposição a todas as concentrações de cobre testadas acarretou em um aumento da atividade da piruvato desidrogenase sugerindo uma situação similar à estivação, onde ocorre uma mudança do substrato energético preferencial em pepinos-do-mar. Além disso, a diminuição da atividade da lactato desidrogenase provocada pela exposição a todas as concentrações de cobre testadas é extremamente danosa a animais de baixo metabolismo, que tendem a obter energia preferencialmente por via anaeróbica.

Com base nos resultados apresentados nesta tese, pode-se concluir que a exposição aguda a concentrações subletais do metal afeta o metabolismo energético, em especial a via glicolítica, nos animais estuarinos (caranguejo *N. granulata* e peixe *F. heteroclitus*) e marinhos (pepino-do-mar *T. crassipeda*) estudados, porém a extensão e o tipo de alteração variam conforme a espécie estudada e não é possível identificar um padrão único de resposta dos diferentes grupos animais (equinodermos, crustáceos e peixes) ao metal como é observado em animais de água doce.

Palavras-chave: cobre, metabolismo energético, salinidade, equinodermo, crustáceo, peixe

3. INTRODUÇÃO

O ambiente aquático costeiro está sujeito à interferência de diversos fatores externos, como circulação de ventos, aporte de água continental, correntes marítimas, marés, além da ação antrópica. Isto faz com que este ambiente seja susceptível a mudanças, seja através de variações em sua salinidade, temperatura, oxigênio dissolvido, ou até mesmo a presença de contaminantes (Bianchi, 2006). Desta forma, os organismos que habitam este ambiente também precisam estar preparados para enfrentar estas alterações de maneira que possam manter sua homeostasia, através de adaptações fisiológicas e bioquímicas (Vitale *et al*., 1999).

Por exemplo, para enfrentar alterações na salinidade, os organismos aquáticos possuem mecanismos de regulação iônica e osmótica que visam a manutenção do volume celular, e que variam de acordo com a espécie e a salinidade do meio. Animais osmorreguladores são aqueles capazes de regular a composição osmótica de seus fluídos corporais através de mecanismos ativos ou passivos que mantêm estáveis a composição iônica e o fluxo de água entre os meios interno e externo, enquanto que nos animais osmoconformadores as flutuações no ambiente provocam variações similares na concentração dos fluídos corporais destes animais (Schmidt-Nielsen, 2002; Marshall & Grosell, 2005). O ponto em que não há gradiente osmótico entre o meio externo e o meio interno é chamado de ponto isosmótico.

Quando os animais aquáticos encontram-se em salinidades abaixo do seu ponto isosmótico, eles estão hiperosmóticos em relação ao meio externo, e tendem a perder íons por difusão e ganhar água por osmose, de forma que devem absorver íons e perder água. Quando em salinidades acima de seu ponto isosmótico, estes animais encontram-se hiposmóticos em relação ao meio externo, e tendem a ganhar íons por

difusão e perder água por osmose, passando então a excretar ativamente íons e ingerir água (Marshall & Grosell, 2005).

Diferentes grupos animais habitam os ambientes aquáticos costeiros, incluindo os crustáceos decápodes, que hiper ou hiporregulam dependendo da salinidade do ambiente em que se encontram, sendo que algumas espécies sao osmoconformadoras (Péqueux, 1995); os peixes, que osmorregulam em salinidades diferentes daquela equivalente ao seu ponto isosmótico (Marshall & Grosell, 2005); e os equinodermos, que são animais exclusivamente marinhos e tendem a ser osmoconformadores ainda que façam regulação da concentração de alguns de seus íons corporais, e podem tolerar pequenas variações na salinidade, especialmente se habitam a zona intermareal (Foglietta & Herrera, 1996; Vidolin *et al*., 2007).

A presença de contaminantes, como dito anteriormente, também provoca mudanças nos ambientes aquáticos e causa grande preocupação nas áreas costeiras, pois pouca informação está disponível sobre os efeitos que estes contaminantes exercem sobre a biota destes locais (Ferrer *et al*., 2006). Os ambientes costeiros são especialmente susceptíveis à contaminação por sofrerem pressão de diversas atividades como de pesca, turismo, aquicultura, urbanização, portuárias, exploração petrolífera, construção naval e de diversos complexos industriais (Vitousek *et al*., 1997).

Um dos contaminantes mais estudados em ambientes de água doce é o cobre. Apesar de ser essencial, este metal é tóxico em elevadas concentrações (Harris, 2000) e seu lançamento nos corpos aquáticos deve ser regulamentado. Muito da informação e do conhecimento da toxicidade do cobre está disponível apenas para animais dulcícolas. É sabido que diversos parâmetros químicos da água, como matéria orgânica dissolvida, pH, dureza e composição iônica podem fornecer proteção contra

os efeitos tóxicos do cobre (Pagenkopf, 1983; Erickson *et al*., 1996). A toxicidade aguda do cobre em invertebrados e peixes geralmente é maior em água doce, devido às menores concentrações dos possíveis ligantes (Cl^-, SO_4^{2-}, matéria orgânica natural, $S_2O_3^{2-}$, sulfetos, Br^-, e $B(OH)_4^-$) ou competidores (Na^+, Mg^{2+}, Ca^{2+}, K^+, e Sr^{2+}) do cobre neste meio, quando comparadas com aquelas presentes em águas salobras e marinhas (Santore *et al*., 2001), sendo que alguns estudos jah comprovaram que a salinidade influencia na toxicidade aguda do metal (Grosell *et al*., 2007; Pinho *et al*. 2007; Pinho & Bianchini, 2010).

Para regulamentar a emissão de metais, incluindo o cobre, em ambientes aquáticos foi desenvolvido o Modelo do Ligante Biótico (Biotic Ligand Model - BLM). O BLM é um modelo matemático utilizado para predizer a toxicidade de metais em organismos de água doce, e leva em consideração a especiação e a complexação do metal dissolvido em solução e a competição entre o íon livre do metal e outros cátions pelos sítios de ligação no ligante biótico (Paquin *et al.*, 2002a, 2002b). O ligante biótico é o sítio de ação de ligantes químicos, onde o metal pode se ligar (Di Toro *et al.*, 2001) e exercer sua ação tóxica.

A brânquia foi escolhida como o ligante biótico para a modelagem da toxicidade do cobre pelo BLM, pois inúmeros estudos mostraram uma alta acumulação do metal neste órgão, a qual foi fortemente relacionada, em peixes e crustáceos de água doce, com uma inibição da atividade da Na^+,K^+-ATPase, enzima responsável pela regulação iônica e osmótica (Grosell *et al.,* 2007). Esta inibição enzimática causa uma diminuição no transporte ativo de Na^+ e Cl^-, que resulta em distúrbio na regulação iônica e osmótica, podendo levar o organismo inclusive à morte (Grosell *et al*., 2002). Além disso, a brânquia dos animais aquáticos é o órgão

que permanece mais tempo em contato com a água, estando mais susceptível a um efeito de metais.

Os mecanismos para a manutenção de homeostasia, tanto para adaptações às variações de salinidade da água quanto para defesas contra a toxicidade do cobre, requerem uma maior produção de energia, em função de aumento no metabolismo corporal (Tseng & Hwang, 2008; Martins & Bianchini, 2009). Morgan e Iwama (1991) sugerem cinco padrões de alterações de consumo de oxigênio em animais aquáticos em resposta a variações de salinidade:

1) nenhuma alteração na taxa metabólica;
2) taxa metabólica é mínima em salinidade isotônica e aumenta em salinidades mais baixas ou mais elevadas;
3) taxa metabólica aumenta linearmente coma salinidade;
4) alta taxa metabólica em água doce, diminuendo em meio isotônico (para animais que não toleram água salgada);
5) taxa metabólica mais elevada em água salgada, mas diminuída em outras salinidades.

O aumento da demanda energética devido a osmorregulação se deve a um aumento da Na^+, K^+-ATPase branquial, e em alguns peixes, parece ser suprida por aumento no uso de glicose exógena e, possivelmente, lactato (Sangiao-Alvarellos, *et al*., 2003). Entretanto, o custo energético da osmorregulação é dependente da história natural, grau de eurialinidade, e estágio ontogenético dos animais (Altinok & Grizzle, 2003). Por exemplo, para o peixe eurialino *Fundulus heteroclitus*, cerca de 6 a 10% da energia corporal total é direcionada para a osmorregulação em água salgada, sendo que em água doce este percentual é um pouco menor (Kidder III *et al*., 2003).

O metabolismo energético dos animais é composto de diversas vias bioquímicas, tanto catabólicas quanto anabólicas. A primeira via catabólica a ser descrita foi a glicólise, que é um processo anaeróbico onde ocorre a conversão de uma molécula de glicose em duas moléculas de piruvato, com a produção de duas moléculas de ATP e de NADH. Isso ocorre através de 10 reações sequenciais catalisadas por enzimas, em que se destacam três enzimas responsáveis pelo controle da via: a hexoquinase, a fosfofrutoquinase e a piruvato quinase (Berg *et al*., 2002). Estas enzimas são consideradas enzimas marcapasso, pois controlam a velocidade da via, catalisam reações irreversíveis, e são altamente reguladas.

A hexoquinase é a primeira enzima da via e catalisa a fosforilação da glicose em glicose-6-phosphato. Este produto pode seguir na via glicolítica ou ter outros destinos, como a via das pentoses-fosfato. A fosfofrutoquinase é a terceira enzima da via, catalisando a reação de fosforilação da frutose-6-fosfato em frutose-1.6-bifosfato, e, por ser uma enzima marcapasso, está sujeita a uma complexa modulação alostérica, que inclui sua inibição quando os níveis de ATP e citrato forem elevados, ou sua ativação por altos níveis de AMP, ADP e frutose-2,6-bifosfato. A piruvato quinase catalisa a última reação da via, onde ocorre a transferência de um grupo fosforil do fosfoenolpiruvato para o ADP formando piruvato e ATP (Nelson & Cox, 2008).

O piruvato produzido na glicólise pode ter diversos destinos dentro do metabolismo. Em termos de produção de energia, o destino mais comum é a sua conversão em acetil CoA pelo complexo enzimático piruvato desidrogenase para ser utilizado pelo ciclo de Krebs. Entretanto, em situações anaeróbicas, o piruvato é reduzido a lactato pela lactato desidrogenase, promovendo a regeneração de NAD^+ e permitindo a continuação da via glicolítica. Outros processos também utilizam o

piruvato, como a síntese do aminoácido alanina e de ácidos graxos (Campbell & Farrell, 2006).

O ciclo de Krebs ou ciclo do acido tricarboxílico é uma via catabólica central em que o citrato formado a partir de oxaloacetato e acetil CoA é oxidado produzindo CO_2, sendo que a energia desta oxidação é armazenada na forma das coenzimas reduzidas NADH e $FADH_2$. Isto ocorre por meio de oito etapas catalisadas por enzimas, onde os intermediários podem provir de outros pontos do metabolismo ou serem desviados para a síntese de outras moléculas (Nelson & Cox, 2008). A regulação do ciclo de Krebs ocorre nas etapas catalisadas pelas suas enzimas marcapasso: citrato sintase, isocitrato desidrogenase e α-cetoglutarato desidrogenase.

A citrato sintase é a enzima responsável pela condensação do acetil CoA e oxaloacetato para a formação de citrato, e é regulada alostericamente por NADH, succinil-CoA, citrato e ATP, que a inibem, e por ADP, que a estimula. A oxidação do isocitrato a α-cetoglutarato e CO_2 é catalisada pela isocitrato desidrogenase, que é inibida por NADH e ATP, e estimulada por ADP. A α-cetoglutarato desidrogenase catalisa a oxidação do α-cetoglutarato a succinil-CoA e CO_2, sendo regulada pelos seus inibidores alostéricos succinil-CoA, ATP e NADH. Após uma volta completa do ciclo de Krebs, uma molécula de piruvato terá produzido três moléculas de NADH e uma molécula de $FADH_2$ (Berg *et al*., 2002).

As coenzimas reduzidas, NADH e $FADH_2$, produzidas pela glicólise e pelo ciclo de Krebs fornecem prótons e elétrons para os complexos da cadeia de transporte de elétrons mitocondrial. Conforme os elétrons das coenzimas reduzidas são transportados pelos complexos, ocorre o bombeamento de prótons para o espaço intermembrana da mitocôndria, causando um gradiente protônico. Este gradiente gera um potencial eletroquímico entre as membranas mitocondriais, e é indispensável para

a produção de ATP pelo processo de fosforilação oxidativa. Desta forma, a oxidação completa de uma molécula de glicose em dióxido de carbono e água gera 32 moléculas de ATP, e envolve a formação de piruvato pela glicólise, a conversão de piruvato à acetil CoA pela piruvato desidrogenase, a oxidação de acetil CoA pelo ciclo de Krebs, a utilização de NADH e $FADH_2$ produzidos pela glicólise e pelo ciclo de Krebs na cadeia de transporte de elétrons, e a fosforilação oxidativa, sendo dependente de oxigênio (Campbell & Farrell, 2006). A figura 1 ilustra a relação entre a glicólise, o ciclo de Krebs, a cadeia de transporte de elétrons, o potencial de membrana mitocondrial e a oxidação fosforilativa.

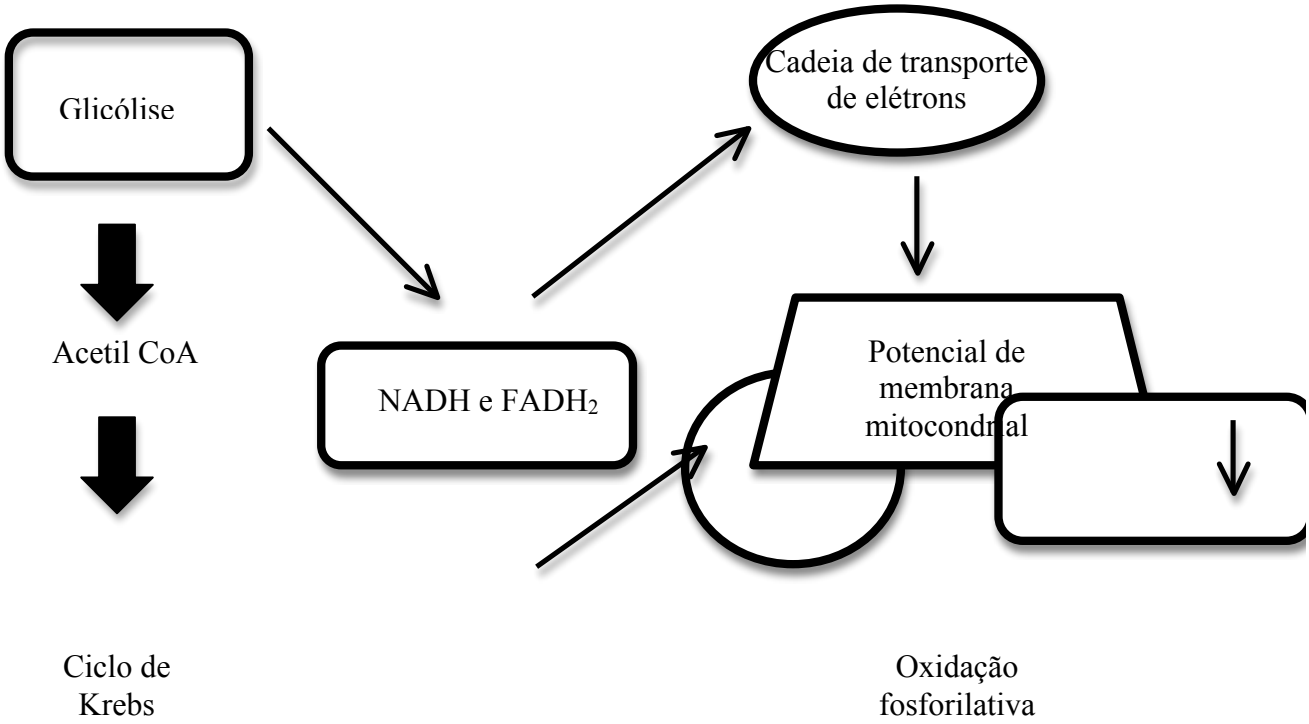

Fig. 1. Relação entre a glicólise, o ciclo de Krebs, a cadeia de transporte de elétrons, o potencial de membrana mitocondrial e a oxidação fosforilativa.

Poucos são os estudos que avaliam e comparam o efeito do cobre sobre o metabolismo energético de organismos aquáticos, em especial nos animais estuarinos, costeiros e marinhos. Além disso, a maioria da informação disponível utiliza como modelo biológico peixes de água doce, não contemplando grupos de invertebrados e nem situações com variações de salinidade. Assim sendo, esta tese contempla dados referentes ao efeito da exposição ao cobre principalmente nas enzimas–chave do metabolismo energético do órgão respiratório de espécies de três grupos animais distintos: o crustáceo *Neohelice granulata*, o peixe *F. heteroclitus* e o equinodermata *Trachythyone crassipeda*.

O caranguejo *N. granulata* (anteriormente *Chasmagnathus granulata* ou *C. granulatus*) é um crustáceo decápoda braquiúro pertencente à família Varunidae, que habita marismas ao longo do sul da costa atlântica da America do Sul, desde o Rio de Janeiro, Brasil, até a Patagônia, Argentina (Boschi, 1964). Este animal tem sido utilizado amplamente em estudos, por ser eurialino e de extrema importância ecológica nas marismas. Tolera salinidades desde a agua doce até a salinidade 40, hiper-osmorregulando em baixas salinidades (água doce e salobra) e hiper-osmorregulando em altas salinidades (salinidade 40) (Novo *et al*., 2005; Bianchini et al., 2008).

O peixe *F. heteroclitus* é um pequeno teleósteo da família Fundulidae, conhecido popularmente como *killifish* ou *mummichog*, que habita marismas e estuários da costa atlântica da America do Norte, distribuindo-se da Nova Escócia, no Canadá, até a Flórida, nos Estados Unidos (Schulte, 2007). Este animal é um dos modelos biológicos mais estudados, devido principalmente a sua alta eurialinidade, tolerando inclusive uma transferência abrupta da água doce para água do mar e vice-versa (Griffith, 1974). Além de ser extremamente eurialino, também é euritérmico e

tolerante a hipóxia e contaminantes (Whitehead *et al*., 2011). O pepino-do-mar *T. crassipeda* é um equinodermo pertencente à família Cucumariidae da classe Holothuroidea, é estenoalino, demersal e ocorre em zonas tropicais da costa brasileira, porém pouco se sabe sobre a sua biologia (Cherbonnier, 1961).

Considerando o acima exposto, a hipótese central desta tese é que a exposição ao cobre causa uma diminuição na atividade de enzimas chave do metabolismo energético das brânquias de *N. granulata* e de *F. heteroclitus* e das árvores respiratórias de *T. crassipeda*, implicando em uma menor quantidade de energia produzida para lidar com as alterações fisiológicas provocadas pelo metal, além de um maior efeito do metal nas espécies eurialinas ocorrer em baixa salinidade. Por fim, espera-se que os resultados apresentados nesta tese ajudem a elucidar melhor os efeitos fisiológicos do cobre em animais estuarinos e marinhos, fornecendo dados relativos a espécies fisiologicamente diferentes.

4. OBJETIVO GERAL

Em face do anteriormente exposto, o objetivo principal desta tese foi avaliar os efeitos da exposição aguda ao cobre dissolvido na água sobre o metabolismo energético de três espécies aquáticas: o caranguejo decápode *Neohelice granulata*, o peixe *Fundulus heteroclitus*, e o pepino-do-mar *Trachythyone crassipeda*.

5. OBJETIVOS ESPECÍFICOS

Para atender ao objetivo geral desta tese, os seguintes objetivos específicos foram estabelecidos:

a) avaliar o efeito da exposição aguda ao cobre dissolvido na água sobre a atividade das enzimas da via glicolítica (hexoquinase, fosfofrutoquinase, piruvato quinase e lactato desidrogenase) e do ciclo de Krebs (citrato sintase) nas brânquias do caranguejo *Neohelice granulata* aclimatado a diferentes salinidades da água;

b) determinar o efeito da exposição ao cobre dissolvido na água sobre a atividade da citocromo c oxidase e o potencial de membrana de mitocôndrias isoladas das brânquias do caranguejo *N. granulata* aclimatado a diferentes salinidades da água;

c) avaliar o efeito da exposição aguda ao cobre dissolvido na água sobre a atividade das enzimas da via glicolítica (hexoquinase, fosfofrutoquinase, piruvato quinase e lactato desidrogenase) e do ciclo de Krebs (isocitrato desidrogenase) nas brânquias do peixe *Fundulus heteroclitus* aclimatado a diferentes salinidades da água;

d) analisar o efeito da exposição aguda a diferentes concentrações de cobre dissolvido na água sobre a atividade das enzimas da via glicolítica (hexoquinase, fosfofrutoquinase, piruvato quinase e lactato desidrogenase) e do ciclo de Krebs (citrato sintase) nas árvores respiratórias do pepino-do-mar *T. crassipeda* aclimatado à água do mar.

6. ARTIGO

Copper effects on key metabolic enzymes and mitochondrial membrane potential in gills of the estuarine crab *Neohelice granulata* at different salinities

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Abstract

The estuarine crab *Neohelice granulata* was exposed (96 h) to a sublethal copper concentration under two different physiological conditions (hyperosmoregulation: water salinity 2 ppt, 1 mg Cu/L; isosmotic: salinity 30 ppt, 5 mg Cu/L). After exposure, gills (anterior and posterior) were dissected and activities of enzymes involved in glycolysis (hexokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase), Krebs cycle (citrate synthase), and mitochondrial electron transport chain (cytochrome c oxidase) were analyzed. Membrane potential of mitochondria isolated from anterior and posterior gill cells was also evaluated. In anterior gills of crabs acclimated to 2 ppt, copper exposure inhibited hexokinase, phosphofructokinase, pyruvate kinase, and citrate synthase activity, increased lactate dehydrogenase activity, and reduced the mitochondria membrane potential. In posterior gills, copper inhibited hexokinase and pyruvate kinase activity, and increased citrate synthase activity. In anterior gills of crabs acclimated to 30 ppt, copper exposure inhibited phosphofructokinase and citrate synthase activity, and increased hexokinase activity. In posterior gills, copper inhibited phosphofructokinase and pyruvate kinase activity, and increased hexokinase and lactate dehydrogenase activity. Copper did not affect cytochrome c oxidase activity in either anterior or posterior gills of crabs acclimated to salinities 2 and 30 ppt. These findings indicate that exposure to a sublethal copper concentration affects the activity of enzymes involved in glycolysis and Krebs cycle, especially in anterior (respiratory) gills of hyperosmoregulating crabs. Changes observed indicate a switch from aerobic to anaerobic metabolism, characterizing a situation of functional hypoxia. In this case, reduced mitochondrial membrane potential would suggest a decrease in ATP

production. Although gills of isosmotic crabs were also affected by copper exposure, changes observed suggest no impact in the overall tissue ATP production. Also, findings suggest that copper exposure would stimulate the pentose phosphate pathway to support the antioxidant system requirements. Therefore, despite *N. granulata* is very tolerant to copper, acute exposure to this metal can disrupt the energy balance by affecting biochemical systems involved in carbohydrate metabolism.

Keywords: copper, gills, glycolysis, Krebs cycle, mitochondrial membrane potential, salinity

1. Introduction

Aquatic organisms are subject to a series of environmental challenges such as salinity, temperature, and dissolved oxygen changes, as well as exposure to chemical contaminants. Coping with these challenges requires a series of physiological and biochemical adaptations to maintain the metabolic homeostasis. Regarding contaminants, copper is one of the most studied metals, especially because it is essential to all living organisms, playing a key functional role in hemocyanin, the respiratory pigment in crustaceans. However, it is toxic to aquatic animals when at excessive concentrations in the water (White and Rainbow, 1982). Copper toxicity is usually associated with the amount of metal bound to a biotic ligand like the fish gill membrane (Paquin et al., 2000, Paquin et al., 2002, Arnold et al., 2005).

Metal bioavailability depends on the water chemistry. In fact, the amount of copper complexing [Cl^-, SO_4^{2-}, natural organic matter, $S_2O_3^{2-}$, sulfides, Br^-, and $B(OH)_4^-$] and competing (Na^+, Mg^{2+}, Ca^{2+}, K^+, e Sr^{2+}) agents augments at increasing salinities, offering a certain degree of protection against copper toxicity (Wright, 1995). However, some evidences indicate that animal physiology is even more important than water chemistry to explain changes in copper toxicity across different water salinities. This would suggest that copper might display differential mechanisms of toxicity depending on the water salinity and animal that are being tested (Grosell et al., 2007).

Among the different physiological and biochemical disturbances induced by copper, it was shown that exposure to this metal negatively affect glycolysis (Hansen et al., 1992; Satyaparameshwar, 2006; Carvalho and Fernandes, 2008), Krebs cycle (Balavenkatasubbaiah et al., 1984; Couture and Kumar, 2003), ionic and osmotic

regulation (Staag and Shuttleworth, 1982; Wilson and Taylor, 1993; Grosell et al., 2004a,b; Pinho et al., 2007), acid-base balance (Boitel and Truchot, 1990; Wilson and Taylor, 1993; Skaags and Henry, 2002; Bielmeyer et al., 2005; Blanchard and Grosell, 2006), ammonia excretion (Larsen et al., 1997; Grosell et al., 2003; Blanchard and Grosell, 2006), oxygen consumption (Katticaran and Salih, 1992; Santos et al., 2000; Manyin and Rowe, 2009), and growth (Santos et al., 2000; Manyin and Rowe, 2009). Most of these effects can be directly or indirectly associated with an insufficient production of energy to maintain cell metabolism and homeostasis. At extreme conditions, this situation can even lead to death.

Most of the information on physiological and biochemical effects of copper on aquatic animals is derived from studies on freshwater fish and crustaceans. Therefore studies in brackish and saltwater animals are needed for a better understanding of the toxic effects of copper across a wide range of water salinities. *Neohelice granulata* is a euryhaline crab inhabiting salt marshes from estuaries and lagoons along the Atlantic coast of South America. It tolerates water salinities ranging from 0 to 40 ppt, with an isosmotic point close to 30 ppt, hyperosmoregulating at lower salinities and hyposmoregulating at higher salinities. Biochemical and physiological mechanisms associated with the species ability to iono- and osmoregulate at different salinities was recently reviewed (Bianchini et al., 2008). Also, tolerance to copper was previously evaluated and shown to be very high and dependent on both acclimation salinity and presence of food in the water. In the absence of food, copper concentration inducing 50% mortality after 96 h of exposure (96-h LC_{50}) ranged from 0.028 to 91.2 mg Cu/L for water salinities ranging from 0.1% seawater to 30 ppt. In the presence of food, it ranged from 0.422 to 120 mg Cu/L, respectively. The higher concentration of copper

not inducing mortality was reported as being 1 and 5 mg Cu/L in crabs acclimated to brackish and seawater, respectively (Bianchini et al., 2003).

Considering this background, the effects of sublethal concentrations of copper on the activity of enzymes associated with glycolysis (hexokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase), Krebs cycle (citrate synthase), and electron transport chain (cytochrome c oxidase) were evaluated in gills (anterior and posterior) of *N. granulata* subjected to two different physiological conditions (hyperosmoregulation: water salinity 2 ppt; isosmotic: water salinity 30 ppt). The influence of copper exposure on the membrane potential of mitochondria isolated from gill cells was also considered in the present study. As a higher amount of energy would be necessary to cope with the extremely low salinity (2 ppt) than with the salinity close to the isosmotic point (30 ppt), we hypothesize that a more significant impact of copper exposure would be seen in gills of hyperosmoregulating crabs than in gills of isosmotic ones.

2. Material and methods

2.1. Crab collection and maintenance

Adult male crabs were collected in salt marshes of the Patos Lagoon estuary (Southern Brazil) and acclimated to laboratory conditions for at least 2 weeks. Crabs were kept in 250-L tanks containing aerated salt water (salinity 2 or 30 ppt), under fixed temperature (20ºC) and photoperiod (12h L: 12h D). Crabs were fed ground beef three times a week.

2.2. Copper exposure

Acclimated crabs (n = 10) were exposed (96 h) to copper in aquaria containing 2 L of water at the desired salinity. Copper (as $CuCl_2$) was added to water from a stock solution (1 g Cu/L). Copper concentrations tested were 1 and 5 mg Cu/L for salinities 2 and 30 ppt, respectively. These concentrations were shown to be the higher non-lethal concentrations of copper to *N. granulata* under the same experimental conditions employed in the present study, at the respective water salinity tested. Furthermore, they are toxicologically equivalent since they have similar calculated concentrations (~$4.5x10^{-6}$ mol/L) of free copper ion (Bianchini et al., 2003), the most toxic dissolved copper species. We are aware that these copper concentrations are not environmentally relevant, but it is important to note their suitability to identify the mechanism(s) of action involved in acute toxicity of waterborne copper in the estuarine crab *N. granulata* acclimated to brackish (salinity 2 ppt) and sea water (salinity 30 ppt).

Experimental media were completely renewed every 24 h and copper was added to the media 24 h prior crab introduction into the test chamber. A control group (no copper added to the water) was also tested in each experimental water salinity. Temperature (20ºC) and photoperiod (12h L: 12h D) were fixed. No food was provided to crabs during copper exposure. A first experiment was performed to collect samples for glycolysis and Krebs cycle enzymes assays. A second experiment was performed to collect samples for mitochondrial membrane potential and cytochrome c oxidase activity measurements.

After copper exposure, crabs were cryoanesthetized, and gills (anterior and posterior) were dissected and frozen (-80°C) until enzyme analysis or pooled (2 crabs per pool) and homogenized for mitochondria isolation.

2.3. Glycolysis and Krebs cycle enzymes assays

Glycolysis and Krebs cycle enzymes were assayed according to Lallier and Walsh (1991) with slight modifications. Gills were homogenized in ice-cold buffer (50 mM imidazole; 0.1 mM PMSF; pH 7.8) and centrifuged (10,000 x *g*; 20 min; 4°C). The supernatant was used as enzyme source. Enzymes were assayed spectrophotometrically using a microplate reader (ELx808IU, BioTek Instruments, Inc, Winooski, VT, USA). Glycolysis enzymes (hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase) assays were buffered with 50 mM imidazole (pH 7.4) and the NAD^+/NADH oxidation/reduction was followed at 340 nm. Krebs cycle enzyme (citrate synthase) assay was buffered with 50 mM HEPES (pH 8.1), and DTNB reduction was followed at 405 nm. All assays were performed at 25°C and specific conditions were used for each assay as follows (final concentrations): hexokinase (5 mM $MgCl_2$, 1 mM D-glucose, 0.16 mM NAD^+, 2 U/mL glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 1 mM ATP); phosphofructokinase (10 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 0.12 mM NADH, 1 U/mL aldolase, 3 U/mL triose phosphate isomerase, 1 U/mL α-glycerophosphate dehydrogenase, and 3 mM fructose-6-phosphate); pyruvate kinase (10 mM $MgCl_2$, 30 mM KCl, 2.5 mM ADP, 0.12 mM NADH, 20 U/mL lactate dehydrogenase, and 0.5 mM phosphoenolpyruvate); lactate dehydrogenase (2 mM

pyruvate and 0.12 mM NADH); citrate synthase (0.1 mM acetylcoenzyme A, 0.1 mM DTNB, and 0.5 mM oxaloacetate).

2.4. Mitochondria isolation

Procedures for mitochondria isolation were adapted from Parrino et al. (2000). Gills were homogenized in 6 mL of a buffer solution (530 mM sucrose, 200 μM EGTA, 1mM EDTA, 20 mM HEPES and 0.5% bovine serum albumin; pH 7.5) and centrifuged (1,600 x *g*) for 12 min at 4^{o}C. The supernatant was centrifuged (7,100 x *g*) for 15 min at 4^{o}C. Pellet was suspended with a respiration buffer solution (303 mM sucrose, 90 mM KCl, 25 μM EGTA, 4 mM KH_2PO_4, and 20 mM HEPES; pH 7.5). Isolated mitochondria were kept on ice until cytochrome c oxidase activity and membrane potential measurements, as described below.

2.5. Mitochondrial membrane potential measurement

Mitochondrial membrane potential was assessed using the cationic fluorescence probe JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanineiodaide; Sigma-Aldrich, USA). At low concentrations (less than 300 nM), this probe exists as a green fluorescent monomer (excitation: 485; emission at 530 nm). However, at high concentrations (>1 mM) a very strong red-orange fluorescence occurs (excitation: 485; emission at 530 nm) due to the formation of dye aggregates. Therefore, low membrane potentials will show green fluorescence while high ones will present a red-orange fluorescence, since more

of the dye enters the mitochondria as is accumulated in the matrix, forming the aggregates (Reers et al., 1995).

Measurements were performed in aliquots of isolated mitochondria (10 μL) and JC-1 solution (90 μL) pipetted into wells of a 96-well microplate. The JC-1 solution was prepared from a stock solution (40 μg/L in ethanol) by 200-fold dilution in a buffer solution containing 110 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 20 mM HEPES, 10 mM sodium succinate, and 10 mM ATP. After incubation (20ºC) for 1 h in the dark, fluorescence generated in the reaction mixture was read (excitation: 485 nm; emission: 590 nm).

2.6. Cytochrome c oxidase assay

After the mitochondrial membrane potential measurement, isolated mitochondria were ruptured by three rounds of freeze/thaw, and cytochrome c oxidase activity was assayed according to Kirby et al. 2007), following the oxidation of reduced cytochrome c at 550 nm. The reaction medium contained (final concentration): 20 mM KH_2PO_4 (pH 7.0), 0.45 mM n-dodecyl-β-D-maltoside, and 15 μM reduced cytochrome c. After addition of an aliquot of isolated mitochondria, absorbance was read for 10 s.

2.7. Data analysis

Data were expressed as mean ± SEM. Differences between gills (anterior and posterior) and crab groups (control and copper-exposed) acclimated to the same water salinity (2 or 30 ppt) were assessed by two-way analysis of variance (ANOVA)

followed by the Tukey's test. Difference in enzyme activity between crabs acclimated to salinities 2 and 30 ppt was assessed for both anterior and posterior gills of control crabs using the Student *t*-test. Data normality and homogeneity of variances were previously verified. In all cases, the significance level adopted was 95% ($\alpha = 0.05$).

3. Results

As expected, no crab mortality was observed in both control and copper-exposed crabs over the 96-h period of test in salinity 2 and 30 ppt.

In control crabs, no significant difference was observed in hexokinase activity between anterior and posterior gills of crabs acclimated to salinity 2 ppt. However, anterior gills showed a lower enzyme activity than posterior ones in crabs acclimated to salinity 30 ppt. Hexokinase activity was higher in both anterior and posterior gills of crabs acclimated to salinity 2 ppt than in those acclimated to salinity 30 ppt. In copper-exposed crabs, a reduced hexokinase activity was observed in both anterior and posterior gills of crabs acclimated to salinity 2 ppt. However, copper exposure increased the enzyme activity in both anterior and posterior gills of crabs acclimated to salinity 30 ppt (Fig. 1).

Phosphofructokinase activity was higher in anterior than in posterior gills of control crabs acclimated to salinity 2 or 30 ppt. In both anterior and posterior gills, no significant effect of water salinity on enzyme activity was observed. In copper-exposed crabs, a reduced phosphofructokinase activity was observed in anterior gills of crabs acclimated to salinity 2 ppt and in both anterior and posterior gills of those acclimated to salinity 30 ppt (Fig. 2).

Pyruvate kinase activity was similar in anterior and posterior gills of control crabs acclimated to salinity 2 ppt. However, it was lower in anterior than in posterior gills of crabs acclimated to salinity 30 ppt. Anterior gills of crabs acclimated to salinity 2 ppt showed higher pyruvate kinase activity than those of crabs acclimated to salinity 30 ppt. However, no effect of the acclimation salinity was observed in posterior gills. In copper-exposed crabs, a reduced pyruvate kinase activity was observed in both anterior and posterior gills of crabs acclimated to salinity 2 ppt. In those acclimated to salinity 30 ppt, copper exposure reduced the enzyme activity only in posterior gills (Fig. 3).

No significant difference in lactate dehydrogenase activity was observed between anterior and posterior gills of control crabs acclimated to salinity 2 or 30 ppt. Also, no effect of the acclimation salinity was observed on enzyme activity in anterior gills. However, a higher lactate dehydrogenase activity was observed in posterior gills of control crabs acclimated to salinity 2 ppt than in those acclimated to salinity 30 ppt. Copper exposure increased the enzyme activity in anterior and posterior gills of crabs acclimated to salinity 2 and 30 ppt, respectively (Fig. 4).

Citrate synthase activity was lower in anterior than in posterior gills of control crabs acclimated to either salinity 2 or 30 ppt. Anterior and posterior gills of control crabs acclimated to salinity 2 ppt showed a higher enzyme activity than those of control crabs acclimated to salinity 30 ppt. Copper exposure inhibited citrate synthase activity in anterior gills of crabs acclimated to 2 or salinity 30 ppt. However, an increased enzyme activity was observed in posterior gills of crabs acclimated to salinity 2 ppt after exposure to copper (Fig. 5).

No significant difference in cytochrome c oxidase activity was observed between anterior and posterior gills of control crabs acclimated to either salinity 2 or

30 ppt. Anterior gills of control crabs acclimated to salinity 2 ppt showed higher enzyme activity than those of control crabs acclimated to 30 ppt. However, no significant effect of acclimation salinity was observed in posterior gills. Copper exposure did not affect cytochrome c oxidase activity in both anterior and posterior gills of crabs acclimated either to salinity 2 or 30 ppt (Fig. 6).

Mitochondria isolated from anterior gills of control crabs acclimated to salinity 2 ppt showed higher membrane potential than the posterior ones, while no difference was observed between gills of control crabs acclimated to salinity 30 ppt. Copper exposure reduced the membrane potential of mitochondria isolated from anterior gills of crabs acclimated to 2 ppt, while no effect was observed in posterior gills. Also, no change in membrane potential of mitochondria isolated from anterior or posterior gills of crabs acclimated to 30 ppt was observed after copper exposure (Fig. 7).

4. Discussion

In the present study, significant differences in enzyme activities were observed between gill types (anterior and posterior gills) of the estuarine crab *N. granulata* acclimated to 2 ppt (hyperosmoregulating crabs) or 30 ppt (isosmotic crabs). Like in other decapod crustaceans, anterior and posterior gills from *N. granulata* have distinct morphology and physiology, with anterior gills being specialized in gas exchange and ammonia excretion, and posterior gills being responsible for salt transporting processes (Genovese et al., 2000; Luquet et al., 2000; for review: Bianchini et al., 2008). Therefore, differences in enzyme activities observed in the present study

within pair of gills (anterior and posterior) would be likely related to their respective functional role.

In addition to differences associated with the gill type, as expected changes in the activity of several enzymes were also observed according to the crab acclimation salinity. In fact, acclimation to different salinities was also shown to induce several structural, biochemical and physiological changes in *N. granulata* (Genovese et al., 2000; Luquet et al., 2000; for review: Bianchini et al., 2008). In a broad view, gill enzymes from control *N. granulata* acclimated to salinity 2 ppt exhibited higher activity than those acclimated to salinity 30 ppt. In fact, one expects that crabs require more energy at salinity 2 ppt, where they are hyperegulating, than at salinity 30 ppt, where they are at their isosmotic point, especially at the gills level. Nery and Santos (1993) evaluated the carbohydrate metabolism of *N. granulata* during acclimation to different water salinities and noticed that the substrate for energy production required for osmoregulation varies seasonally and is tissue-dependent. In summer, crabs submitted to hypo-osmotic stress (fresh water) may use free amino acids and glycerol to sustain the adequate tissue glucose levels, especially through gluconeogenesis, in cases where animals are not fed. In turn, crabs subjected to hyper-osmotic stress (salinity 40 ppt) likely use only glycerol for gluconeogenesis. Crabs used in the present study were also collected in the summer, were kept without food for 48 h before introduction in the test chamber, and were not fed during the 96-h period of experiment. Therefore, glycogen levels in their tissues were likely depleted, and fuel necessary for glucose-dependent processes was likely obtained through gluconeogenesis.

Glycolysis is the first stage of carbohydrate metabolism, an anaerobic process producing two molecules of ATP from one molecule of glucose through 10 enzymatic

reactions. Glucose is first phosphorylated to glucose-6-phosphate by hexokinase, one of the enzymes involved in glycolysis regulation. Together with phosphofructokinase and pyruvate kinase, it also coordinates the whole metabolic pathway functioning (Nelson and Cox, 2009). The complete aerobic oxidation of glucose in carbon dioxide and water involves the Krebs cycle and the oxidative phosphorylation, and yields 32 molecules of ATP in the end. The final product of glycolysis is pyruvate that under aerobic situation is converted to acetyl coenzyme A, a compound that enters the Krebs cycle, but under anaerobic condition it is converted to lactate by lactate dehydrogenase. Pyruvate also has other destinations in the metabolism, such as gluconeogenesis and amino acid synthesis. In turn, Krebs cycle is responsible for the production of reduced coenzymes NADH and $FADH_2$ employed by the electron transport chain to generate the mitochondrial membrane potential necessary for ATP production through the oxidative phosphorylation (Campbell and Farrell, 2006).

Considering this background, any copper-induced disturbance in the activity of one or more enzymes involved in the biochemical processes described above could disrupt the energy balance at the gills level, with important impacts on energy-dependent processes such as ionic/osmotic and acid-base regulations in aquatic animals. In the present study, the effect of sublethal concentrations of copper on the activity of enzymes involved in glycolysis, Krebs cycle and electron transport chain was evaluated in anterior and posterior gills of the estuarine crab *N. granulata* acclimated to different salinities (2 and 30 ppt). The effect of copper exposure on membrane potential of mitochondria isolated from anterior and posterior gills was also considered. Results reported in the present study indicate that copper exposure affected the activity of glycolytic and Krebs cycle enzymes in gills of the estuarine

crab *N. granulata*. However, the extent of the observed effects varied according to the crab acclimation salinity.

In anterior gills of hyperosmoregulating crabs, hexokinase (Fig. 1), phosphofructokinase (Fig. 2), pyruvate kinase (Fig. 3), and citrate synthase (Fig. 5) activities were reduced after copper exposure. However, lactate dehydrogenase activity was markedly increased (Fig. 4). These findings indicate that exposure to waterborne copper led crabs to a situation of physiological hypoxia, inducing a change from aerobic to anaerobic metabolism. This statement is based on the fact that lactate dehydrogenase activity is considered as an index of anaerobic potential of a tissue while citrate synthase activity reflects its aerobic capacity (Somero and Childress, 1980).

In turn, posterior gills of hyperosmoregulating crabs exposed to copper showed reduced hexokinase (Fig. 1) and pyruvate kinase (Fig. 3) activities, while phosphofructokinase (Fig. 2) and lactate dehydrogenase (Fig. 4) activities remained unchanged. In this case, citrate synthase activity (Fig. 5) was increased. Taken altogether, these findings suggest that acetyl coenzyme A needed to keep the Krebs cycle functioning is derived from another source than the pyruvate originated from glycolysis, possibly from lipid degradation. Luvizotto-Santos et al. (2003) showed that gill lipid content was decreased during hypo-osmotic stress, indicating that lipids might be serving as energy substrates for acetyl coenzyme A formation in the estuarine crab *N. granulata*. Glycerol from lipid degradation and free amino acids might also be used for glucose synthesis through gluconeogenesis, as proposed by Nery and Santos (1993).

The decreased hexokinase activity observed in both anterior and posterior gills of hyperosmoregulating crabs exposed to copper (Fig. 1) can be explained

considering a hypoglycemia condition, as proposed by Medesani et al. (2001). These authors also exposed *N. granulata* to copper in brackish water (salinity 12 ppt), but at a lower copper concentration (0.1 mg/L) for a longer period of time (2 weeks). In fact, they observed a reduced hemolymph glucose level in copper-exposed crabs, as well as insensitivity to the crustacean hyperglycemic hormone (CHH). This hormone is responsible for the maintenance of the hemolymph glucose level in crustaceans and is generally elevated in animals under stressing conditions (Lorenzon, 2005).

Phosphofructokinase activity was inhibited in anterior gills of hyperosmoregulating crabs exposed to copper, but not in posterior gills of these animals (Fig. 2). Additionally, phosphofructokinase activity (Fig. 2) was lower than hexokinase activity in anterior gills of copper-exposed crabs, as well as in posterior gills of control crabs (Fig. 1), indicating the predominance of the pentose phosphate pathway over glycolysis in both situations (Chang and Connor, 1983). Pentose phosphate shunt is responsible for the production of NADPH, an essential cofactor for glutathione reoxidation, thus counteracting a possible oxidative stress condition caused by copper exposure (Carvalho and Fernandes, 2008). A difference in phosphofructokinase activity was also observed between anterior and posterior gills of control crabs (Fig. 2), with the latter showing a lower enzyme activity, indicating a shift in the glucose-6-phosphate fate. In turn, the pyruvate kinase inhibition observed in both gill types (Fig. 3) could be due to a copper-induced displacement of the Mg^{2+} necessary for the proper function of kinases, leading to a phosphoryl transfer inhibition. This effect could affect not only the hexokinase activity, but also the phosphofructokinase activity.

In isosmotic crabs, posterior gills showed higher hexokinase activity than anterior ones (Fig. 1). This finding associated with the phosphofructokinase activity

data (Fig. 2) suggests a differential fate of glucose-6-phosphate in anterior and posterior gills. In the first, glycolysis seems to be the main pathway, while in the latter the main pathway seems to be the pentose phosphate shunt. In this context, it is important to note that phosphofructokinase activity (Fig. 2) was lower than hexokinase activity (Fig. 1) in both anterior and posterior gills of copper-exposed crabs, as it was seen in anterior gills of hyperosmoregulating crabs. These findings indicate that glucose-6-phosphate phosphorylated by hexokinase could be feeding the pentose phosphate shunt. In this case, an effect on NADH production would be then expected in anterior gills of copper-exposed crabs since Krebs cycle would be compromised, as indicated by the observed reduction in citrate synthase activity (Fig. 5). Although posterior gills of copper-exposed crabs showed a reduced pyruvate kinase activity (Fig. 3) and an increased lactate dehydrogenase activity (Fig. 4), no change was observed in citrate synthase activity (Fig. 5). These findings indicate that energy in posterior gills is still mainly obtained from the aerobic metabolism even after copper exposure, with acetyl coenzyme A being provided by another source than glycolysis. Finally, the observed increase in hexokinase activity in both anterior and posterior gills of isosmotic crabs exposed to copper suggests a hyperglycemia condition. Since crabs were fasting during the 96-h period of copper exposure, it is possible that glucose needs were provided through gluconeogenesis (Nery and Santos, 1993).

Regarding mitochondrial parameters, a clear absence of copper effect on cytochrome c oxidase activity was observed in all experimental conditions, i.e. anterior and posterior gills of crabs acclimated to 2 and 30 ppt. This lack of response was also observed by Hansen et al. (1992) in the crab *Carcinus maenas* exposed to copper. Since this enzyme depends on copper to function properly, one could consider

that it would be insensitive to copper. Despite the lack of copper effect on cytochrome c oxidase activity, membrane potential of mitochondria isolated from anterior gills of hyperosmoregulating crabs exposed to copper was reduced. However, no copper effect was observed in isosmotic crabs. Mitochondrial membrane potential is crucial for ATP production by mitochondria. It is sustained by the transport of H^+ by four enzymatic complexes that together form the electron transport chain, since electrons move from one complex to the other. Cytochrome c oxidase is also named complex IV and is responsible for the transport of electrons from cytochrome c to oxygen, which is reduced to water (Campbell & Farrell, 2006). Since no copper effect on cytochrome c oxidase was observed, the copper-induced decrease in membrane potential observed in mitochondria isolated from anterior gills of hyperosmoregulating crabs could be thus explained considering an impairment of one of the other enzymatic complexes or even a membrane uncoupling. If enzymatic complexes are affected, less H^+ is pumped to the mitochondrial intermembrane space reducing the H^+ motive force and consequently the membrane potential. If uncoupling occurs, H^+ leaks from the intermembrane space also reducing the H^+ motive force and the membrane potential, although oxygen is still reduced. In fact, mild uncoupling occurs naturally to diminish reactive oxygen species formation (Abele et al., 2007). It is important to note that both possibilities have the same consequence: a reduction in mitochondrial ATP production.

5. Conclusions

Findings reported in the present study show that copper affects glycolysis and Krebs cycle enzymes, being more toxic to anterior gills of hyperosmoregulating crabs.

In this case, copper exposure would be leading to a shift from aerobic to anaerobic metabolism, characterizing a situation of functional hypoxia. Also, data suggest that copper exposure is likely inhibiting the mitochondrial ATP production in anterior gills of crabs acclimated to low salinity. Although the activity of some gill enzymes of isosmotic crabs was also affected by copper exposure, data suggest that no significant impact on the gill ATP production occurred. Furthermore, they suggest a possible induced stimulation of the pentose phosphate pathway to support the requirements of the antioxidant system in gills of isosmotic crabs exposed to copper. Finally, data reported in the present study demonstrate that even though *N. granulata* is very resistant to copper, acute exposure to excessive concentrations of this metal in the water can cause disruption in gill biochemical systems and a consequent imbalance of energy.

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Legends to figures

Figure 1. Hexokinase activity in anterior and posterior gills of control and copper-exposed crabs at salinities 2 and 30 ppt. Different small and capital letters indicate differences between treatments at salinity 2 and 30 ppt, respectively. * denotes difference between anterior or posterior gills of control crabs acclimated to 2 and 30 ppt. Data are expressed as mean ± SEM (n = 10).

Figure 2. Phosphofructokinase activity in anterior and posterior gills of control and copper-exposed crabs at salinities 2 and 30 ppt. Different small and capital letters indicate differences between treatments at salinity 2 and 30 ppt, respectively. No significant difference was observed between anterior or posterior gills of control crabs acclimated to 2 and 30 ppt. Data are expressed as mean ± SEM (n = 10).

Figure 3. Pyruvate kinase activity in anterior and posterior gills of control and copper-exposed crabs at salinities 2 and 30 ppt. Different small and capital letters indicate differences between treatments at salinity 2 and 30 ppt, respectively. * denotes difference between anterior or posterior gills of control crabs acclimated to 2 and 30 ppt. Data are expressed as mean ± SEM (n = 10).

Figure 4. Lactate dehydrogenase activity in anterior and posterior gills of control and copper-exposed crabs at salinities 2 and 30 ppt. Different small and capital letters indicate differences between treatments at salinity 2 and 30 ppt, respectively. * denotes difference between anterior or posterior gills of control crabs acclimated to 2 and 30 ppt. Data are expressed as mean ± SEM (n = 10).

Figure 5. Citrate synthase activity in anterior and posterior gills of control and copper-exposed crabs at salinities 2 and 30 ppt. Different small and capital letters indicate differences between treatments at salinity 2 and 30 ppt, respectively. * denotes difference between anterior or posterior gills of control crabs acclimated to 2 and 30 ppt. Data are expressed as mean ± SEM (n = 10).

Figure 6. Cytochrome c oxidase activity in anterior and posterior gills of control and copper-exposed crabs at salinities 2 and 30 ppt. Different small and capital letters indicate differences between treatments at salinity 2 and 30 ppt, respectively. * denotes difference between anterior or posterior gills of control crabs acclimated to 2 and 30 ppt. Data are expressed as mean ± SEM (n = 10).

Figure 7. Mitochondrial membrane potential of anterior and posterior gills of control and copper-exposed crabs at salinities 2 and 30 ppt. Different small and capital letters indicate differences between treatments at salinity 2 and 30 ppt, respectively. Data are expressed as mean ± SEM (n = 10).

Figure 1

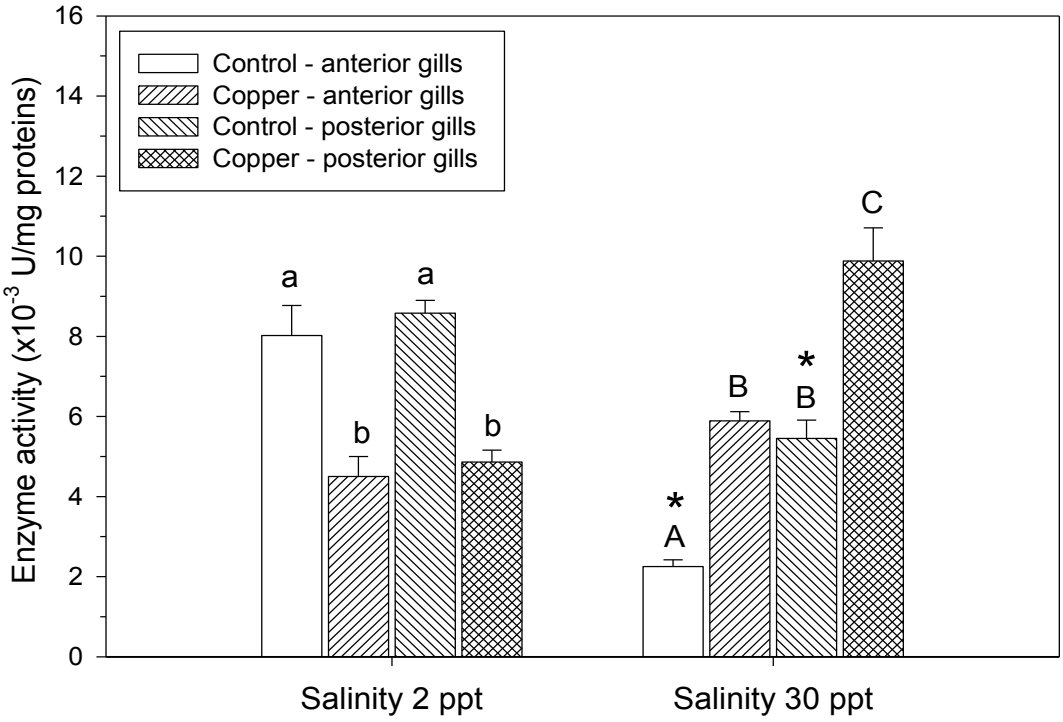

Control - anterior gills
Copper - anterior gills
Control - posterior gills
Copper - posterior gills
Enzyme activity (x10-3 U/mg proteins)
0
2
4
6
8
10
12
14
16
a
b
a
b
*
A
B
*
B
C
Salinity 2 ppt
Salinity 30 ppt

Figure 2

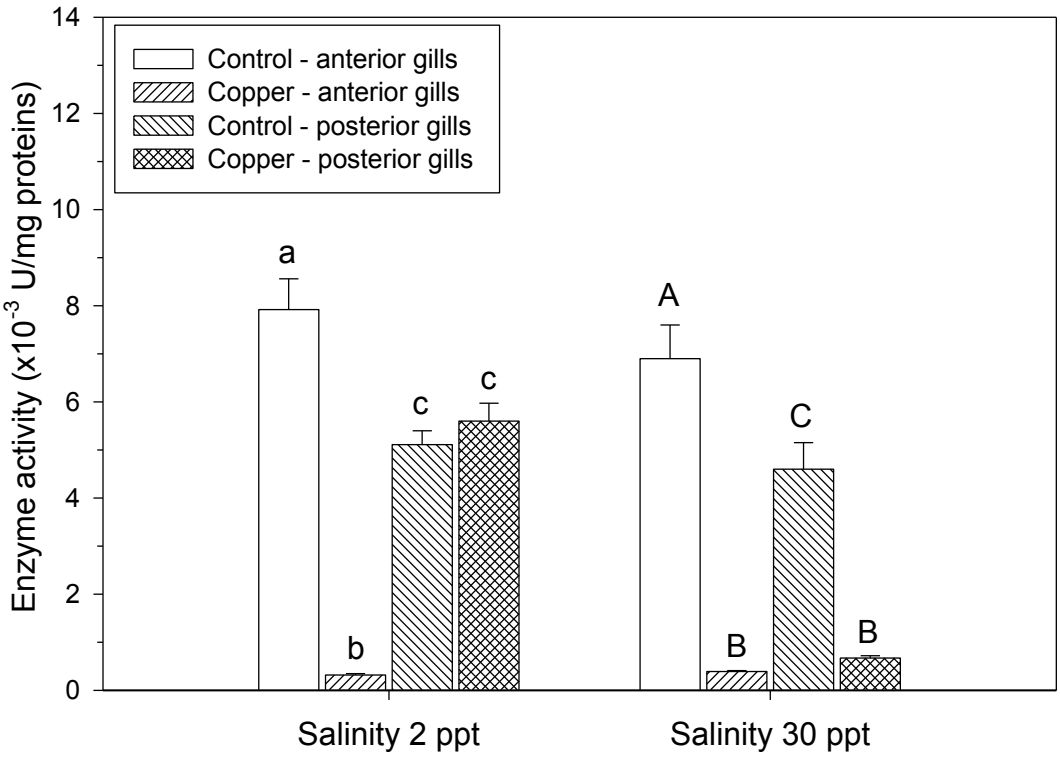

Control - anterior gills
Copper - anterior gills
Control - posterior gills
Copper - posterior gills
Enzyme activity (x10-3 U/mg proteins)
14
12
10
8
6
4
2
0
a
b
c
c
A
B
C
B
Salinity 2 ppt
Salinity 30 ppt

Figure 3

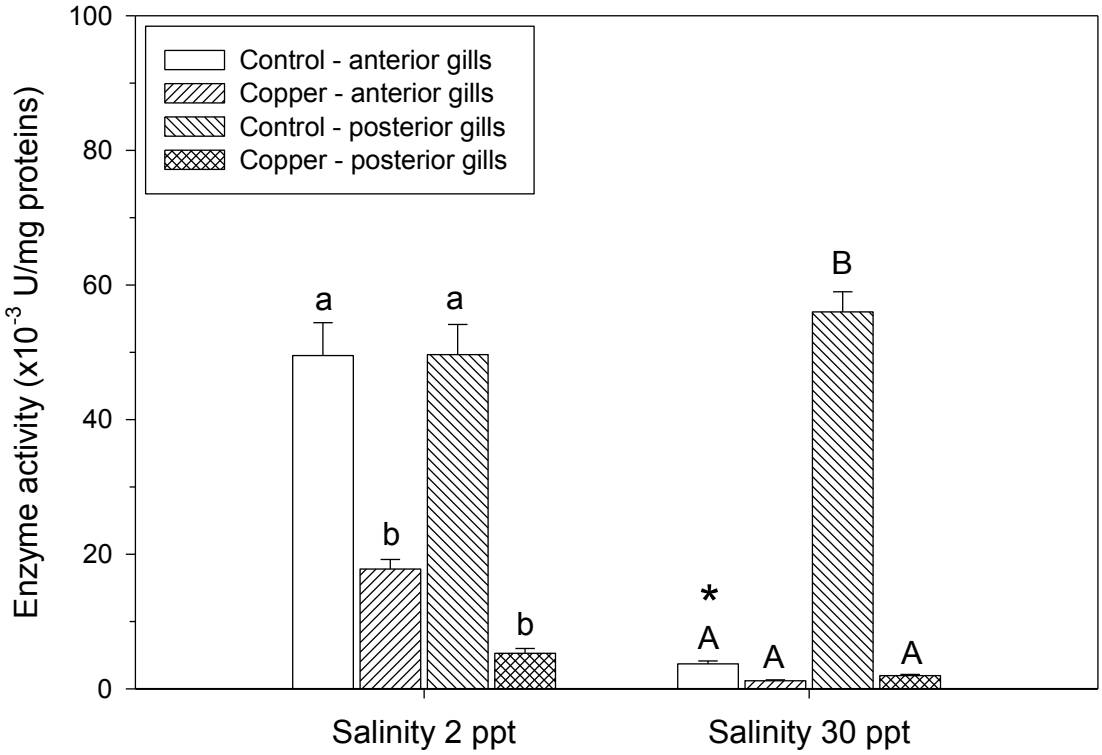

Control - anterior gills
Copper - anterior gills
Control - posterior gills
Copper - posterior gills
Enzyme activity (x10-3 U/mg proteins)
100
80
60
40
20
0
a
b
a
b
*
A
A
B
A
Salinity 2 ppt
Salinity 30 ppt

Figure 4

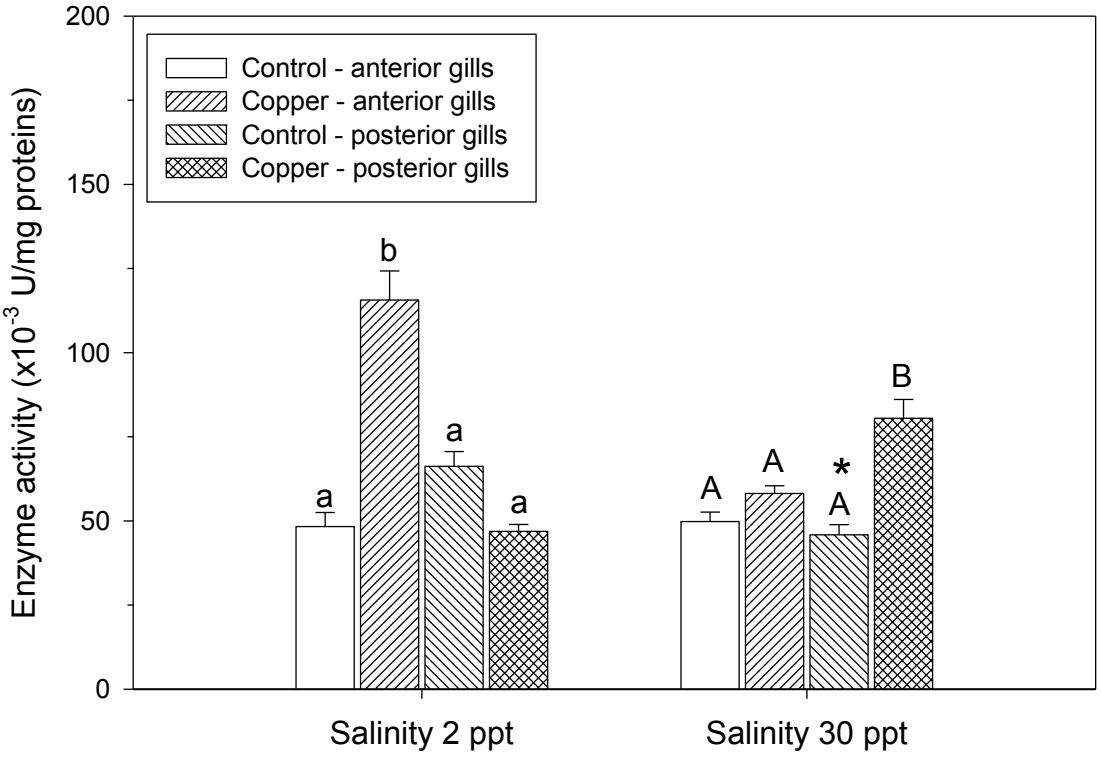

Control - anterior gills
Copper - anterior gills
Control - posterior gills
Copper - posterior gills
Enzyme activity (x10-3 U/mg proteins)
200
150
100
50
0
a
b
a
a
A
A
A
*
B
Salinity 2 ppt
Salinity 30 ppt

Figure 5

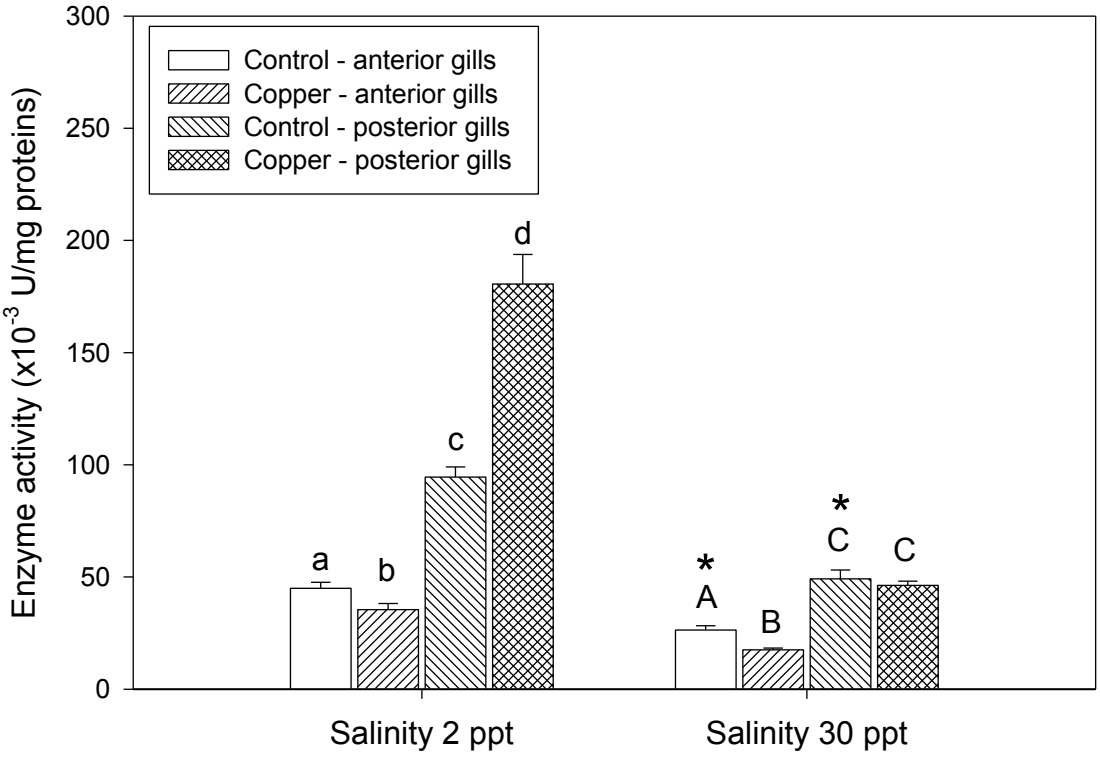

Control - anterior gills
Copper - anterior gills
Control - posterior gills
Copper - posterior gills
Enzyme activity (x10-3 U/mg proteins)
300
250
200
150
100
50
0
a
b
c
d
*
A
B
*
C
C
Salinity 2 ppt
Salinity 30 ppt

Figure 6

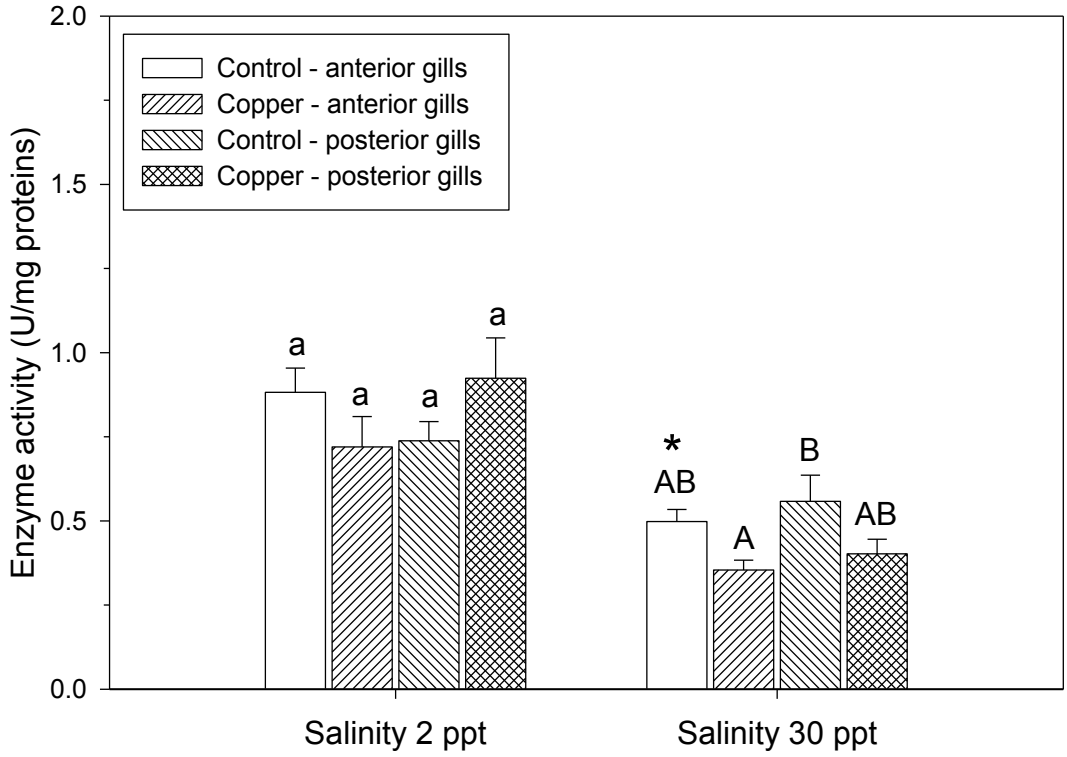
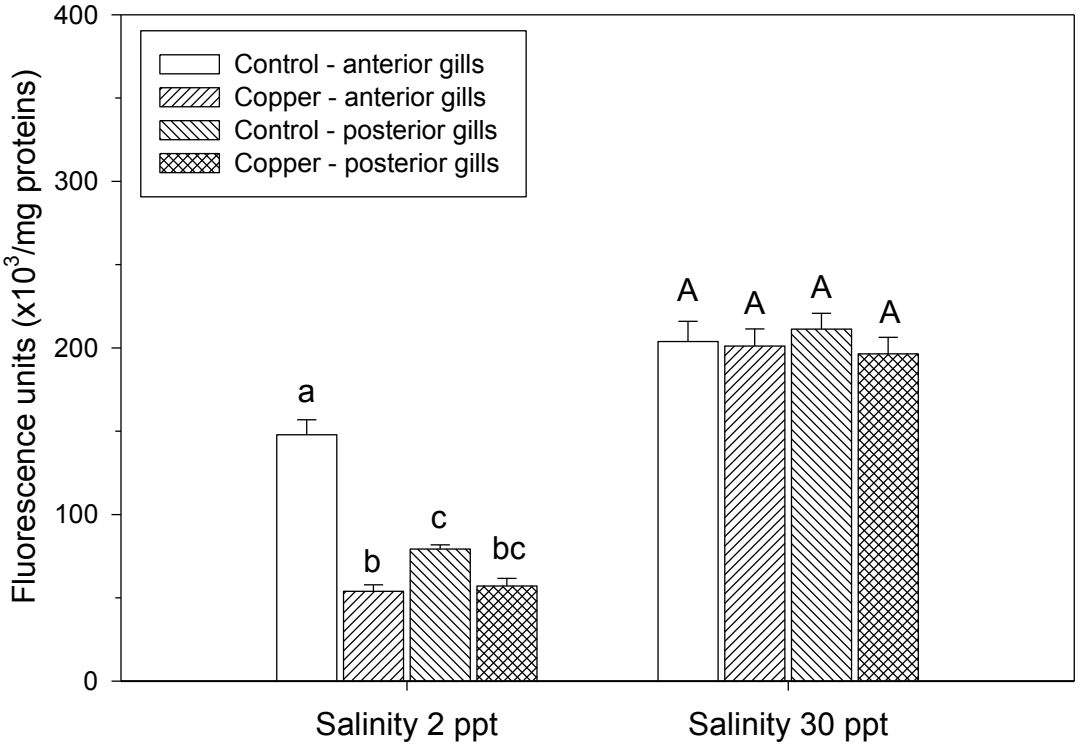

Control - anterior gills
Copper - anterior gills
Control - posterior gills
Copper - posterior gills
Enzyme activity (U/mg proteins)
2.0
1.5
1.0
0.5
0.0
a
a
a
a
*
AB
A
B
AB
Salinity 2 ppt
Salinity 30 ppt

Figure 7


Control - anterior gills
Copper - anterior gills
Control - posterior gills
Copper - posterior gills
Fluorescence units (x10^3/mg proteins)
400
300
200
100
0
a
b
c
bc
A
A
A
A
Salinity 2 ppt
Salinity 30 ppt

7. ARTIGO

Copper toxicity across salinities in the euryhaline fish *Fundulus heteroclitus*: A metabolic approach

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Abstract

Copper induces ionic and osmotic disturbances in both fresh- and seawater fish. However, this mode of copper action is not seen in the euryhaline killifish *Fundulus heteroclitus* exposed to copper in salt water, indicating that another mode of action, such as energy production impairment, could be involved. Therefore, we evaluated the effects of exposure (96 h) to copper (30 μg Cu/L) on the activity of enzymes involved in glycolysis and Krebs cycle in gills of *F. heteroclitus* across a wide range of salinities (0, 3.5, 11, and 35 ppt). In all experimental salinities, hexokinase activity was not affect by copper exposure. In turn, phosphofructokinase activity was inhibited in fresh water and increased in the other experimental salinities. Pyruvate kinase activity was reduced in fresh water and increased in salinity 3.5 ppt. Lactate dehydrogenase activity was decreased in fresh water and augmented in salinities 3.5 and 35 ppt. NADP-dependent isocitrate dehydrogenase activity was dwindled in fresh water and salinities 3.5 and 11 ppt. Taken altogether, these findings indicate that enzymes involved in energy production in *F. heteroclitus* are more sensitive to copper in freshwater fish, reducing its ability for energy production through glycolysis and Krebs cycle. They also suggest that the well-known ionic and osmotic disturbance observed in freshwater fish acutely exposed to copper could not be only associated with a direct metal effect on the activity of gill ion-transporting proteins, but also with an indirect effect on the activity of energy-demanding gill ion-transporting proteins. In turn, fish acclimated to salt water are able to keep the ability for energy production after copper exposure. Therefore, a different mechanism other than ionic/osmotic regulation and energy disturbances would be involved in copper toxicity in *F. heteroclitus* acclimated to salt water.

Key words: *Fundulus heteroclitus*, Copper, Salinity, Energy metabolism

1. Introduction

Euryhaline fish can cope with changes in environmental salinity by altering their iono- and osmoregulatory physiological strategy. In fresh water, fish are hyperosmotic in relation to the environmental osmotic concentration. Therefore, they are subjected to diffusive ions losses and water gain. To counteract theses processes, freshwater fish eliminates the excess of water by excreting copious volume of diluted urine and compensate the ions losses by actively absorbing salts, especially Na^+ and Cl^- across the gill epithelium (Marshall, 2003). In seawater fish, diffusive fluxes of water and ions are occurring in the opposite direction, since fish are hyposmotic in relation to the environmental osmotic concentration. To maintain the ionic and osmotic balance, they drink sea water to compensate the diffusive water loss through permeable epithelia, especially the gills (Wood and Marshall, 1994). However, they have to desalinize the ingested sea water by actively absorbing salts, especially Na^+ and Cl^-, across the gastro-intestinal tract. This process helps out the diffusive water absorption taking place at the intestinal epithelium. Salts absorbed at the digestive tract from the ingested sea water and those diffusively gained through the water permeable surfaces, especially the gills, are actively secreted at the gill epithelium. When fish plasma is isosmotic to the environmental medium, which corresponds to approximately 11 ppt salinity for teleost fish, Na^+ and Cl^- fluxes are in equilibrium. Therefore, only passive ion movements are taking place and no energy is expended for the active ions uptake or secretion across the gill epithelium (Loretz, 1995; Larsen et al., 2002; Marshall and Grosell, 2005). It is important to note that gill cells have a whole biochemical machinery to absorb or secrete ions, which is composed by ion-transporting proteins. The activity of these proteins is mainly coordinated by Na^+/K^+-

ATPase, an ATP-demanding enzyme. Therefore, adaptation to fresh or sea water requires energy and changes in the energy production rate are expected in a wide range of environmental salinities.

Fish are also subjected to other environmental stressors than water salinity. Water contamination by chemicals, such as trace metals, can also induce changes in fish energy demand. Copper is one of the most studied metals and its toxicity is well documented, especially in freshwater fish. Biochemical and physiological responses of freshwater fish to copper exposure include increased concentration of plasma ammonia, inhibition of gill Na^+/K^+-ATPase activity, disruption of acid-base equilibrium, decreased content of whole-body glycogen, alterations in the antioxidant system, and metabolic depression (Lauren and McDonald, 1985, 1987a,b; De Boeck et al., 1995; Karan et al., 1998; De Boeck et al., 2001; Grosell et al., 2002; Vutukuru et al., 2005; De Boeck et al., 2006; Liu et al., 2006; De Boeck et al., 2010). In contrast with freshwater fish, much less studies were conducted to identify the copper effects in seawater fish. However, they show also disruptions in ionic and osmotic regulation, acid-base balance, and ammonia excretion (Staag and Shuttleworth, 1982; Wilson and Taylor, 1993; Larsen et al., 1997; Grosell et al., 2004a,b; Blanchard and Grosell, 2006).

In both freshwater and seawater fish, gills are considered as the main organ involved in copper uptake and consequently a major target for its toxicity (Paquin et al., 2002). As previously stated, fish needs energy for detoxification, repair processes, and maintenance of the whole-body homeostasis in order to cope with the stress induced by waterborne copper exposure. In this context, there are many metabolic pathways that provide energy to the cells, including glycolysis and Krebs cycle. Glycolysis metabolizes glucose, and is composed by a series of enzymatic reactions

leading to the production of ATP, NADH, and specially pyruvate. Hexokinase, phosphofructokinase, pyruvate kinase, as well as lactate dehydrogenase under anaerobic conditions, are key enzymes involved in glycolysis and their activities can regulate the glucose flow through this biochemical pathway. In turn, Krebs cycle is the final common pathway for the oxidation of amino acids, fatty acids, and carbohydrates. It is regulated by the activity of three enzymes that are part of the cycle; isocitrate dehydrogenase is one of them. The Krebs cycle does not consume or generate ATP, but is responsible for NADH and $FADH_2$ production, being thus indirectly dependent on oxygen. In turn, reduced co-enzymes are responsible for the transport of electrons to the electron transport chain that maintains the proton gradient necessary for ATP production through the mitochondrial oxidative phosphorylation (Campbell and Farrell, 2006). As mentioned above, copper can affect fish physiology by disrupting several functions. However, little is known about a copper-induced disruption in energy metabolism by affecting the activity of enzymes involved in glycolysis and Krebs cycle, especially in the gills (Tóth et al., 1996; Beaumont et al., 2000; Carvalho and Fernandes, 2008).

Considering the background above, the aim of the present study was to evaluate copper effects on the activity of key enzymes involved in energy metabolism (hexokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase, and NADP-dependent isocitrate dehydrogenase) across a wide range of water salinities, from fresh to sea water, in the common killifish *Fundulus heteroclitus*. It is important to note that a large bulk of information is already available for this fish species, including biochemical and physiological aspects of osmoregulation and responses to environmental stressors other than salinity (Wood and Marshall, 1994; Marshall, 2003; Scott et al., 2004a,b; Blanchard and Grosell, 2005; Scott et al., 2005; Scott and

Schulte, 2005; Blanchard and Grosell, 2006; Schulte, 2007; Scott et al., 2008). We hypothesize that fish acclimated to the isosmotic point would need less energy to osmoregulate, exhibiting lower enzyme activities in the gills in comparison to fish acclimated to the other salinities. Regarding animals exposed to copper, we expect a more significant impact in freshwater acclimated fish, since copper is more toxic in this condition.

2. Material and methods

2.1. Animal collection and maintenance

Adult killifish *F. heteroclitus* (2-5 g wet body mass) were collected near Shediac, Bay of Fundy, NB, Canada (46° 20'N, 64° 40'W) and maintained in static charcoal-filtered fiberglass tanks containing salt water at salinity 10 ppt for several weeks. Room temperature (18-20°C) and photoperiod (12h L: 12h D) were fixed. Killifish were fed once daily with commercial flakes, but food supply was withheld 48 h before the beginning of the experiment. Prior copper exposure, killifish were acclimated to different salinities (0: fresh water, 3.5 ppt: brackish water, 11 ppt: brackish water, and 35 ppt: full strength sea water) for 2 weeks. The water used for fish acclimation and copper exposure was obtained by addition of artificial sea salt (Instant Ocean sea salt, Aquarium Systems) to dechlorinated Hamilton City tap water (Ontario, Canada) to reach the desired salinities.

2.2 Copper exposure

Killifish were kept (96 h) in 8-L tanks at a density of 6 fish/tank containing water at the desired salinity (0, 3.5, 11, and 35 ppt). Copper (30 μg Cu/L nominal) was added to the water 24 h prior fish introduction into the exposure chamber to allow the complete copper equilibration with the experimental medium. The LC50Total and dissolved copper concentrations in the exposure media were analyzed in non-filtered and filtered (0.45 μm-mesh filter) samples by graphite furnace atomic absorption spectroscopy (Spectra AA model 220Z, Varian, Palo Alto, CA), respectively. The respective controls (no copper addition into the water) were also run at the experimental salinities. Measured copper concentrations in the different experimental treatments are shown in Table 1. Every day, the experimental media were partially (80%) renewed using new solution prepared as described above. Killifish were not fed during the exposure period. After 96 h of exposure, killifish were euthanized with a lethal dose of tricaine methanesulfonate anaesthetic (0.8 g/L MS-222, Syndel Laboratories, Vancouver, BC, Canada) neutralized with NaOH. Gills were then quickly dissected, frozen in liquid nitrogen, and stored at -80°C until analysis.

2.3. Enzyme activity analysis

The activity of the glycolysis enzymes were assayed according to Lallier and Walsh (1991). Isocitrate dehydrogenase activity was determined according to Alp et al. (1976). Briefly, gills were homogenized in ice-cold buffer solution (50 mM imidazole, pH 7.8) containing 0.1 mM PMSF. Homogenates were centrifuged at 10,000 x *g* for 20 min at 4^{o}C. Supernatants obtained were used as enzyme source.

Enzymes activities were determined by spectrophotometry using a microplate reader (Molecular Devices, Menlo Park, CA, USA) at 25°C. NAD^+/NADH (NADP/NADPH) oxidation/reduction was followed by measuring the absorbance (340 nm) of the reaction mixture over time. Enzyme activities were normalized considering the protein content in the homogenates (Bradford Reagent, Sigma, St. Louis, MO, USA). Assays were buffered with a 50 mM imidazole (pH 7.4) solution containing the following reagents: hexokinase: 5 mM $MgCl_2$, 1 mM D-glucose, 0.16 mM NAD^+, 2 U/mL glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 1 mM ATP; phosphofructokinase: 10 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 0.12 mM NADH, 1 U/mL aldolase, 3 U/mL triose phosphate isomerase, 1 U/mL α-glycerophosphate dehydrogenase, and 3 mM fructose-6-phosphate; pyruvate kinase: 10 mM $MgCl_2$, 30 mM KCl, 2.5 mM ADP, 0.12 mM NADH, 20 U/mL lactate dehydrogenase, and 0.5 mM phosphoenolpyruvate; lactate dehydrogenase: 2 mM pyruvate, and 0.12 mM NADH; and NADP-dependent isocitrate dehydrogenase: 1 mM $MnCl_2$, 8 mM $MgCl_2$, 0.5 mM NADP, and 5 mM D-L-isocitrate.

2.4. Statistical analysis

Data were expressed as mean ± S.E.M (n = 5). Analysis of variance (ANOVA) followed by the Tukey test was performed to identify significant differences among the enzyme activity in control killifish acclimated to the different experimental salinities. In turn, Student *t* test was used to identify differences between control and copper-exposed killifish at each experimental salinity. ANOVA assumptions (data normality and homogeneity of variances) were previously verified. Comparisons showing $p<0.05$ were considered significantly different.

3. Results

Activity of all enzymes analyzed was significantly affected by the acclimation salinity. Under control conditions (no copper addition into the water), gills of fish acclimated to freshwater showed the lowest hexokinase activity. Gills of killifish acclimated to salinity 11 ppt also exhibited lower hexokinase activity than those acclimated to salinity 3.5 or 35 ppt. In all experimental salinities, gill hexokinase activity was not affected by copper exposure (Fig. 1).

Phosphofructokinase activity was higher in gills of control fish acclimated to fresh water than in gills of the control fish acclimated to any of the other experimental salinities. Copper exposure reduced the phosphofructokinase activity in gills of fish acclimated to fresh water while an increased activity was observed in gills of those acclimated to any of the other experimental salinities (Fig. 2).

In control fish, gills of fish acclimated to fresh water and full strength seawater (salinity 35 ppt) showed higher pyruvate kinase activity than the gills of those acclimated to brackish water (salinities 3.5 and 11 ppt). Pyruvate kinase activity decreased after copper exposure in gills of freshwater fish while it increased in gills of fish acclimated to salinity 3.5 ppt (Fig. 3).

Lactate dehydrogenase activity was higher in gills of control fish acclimated to freshwater than in the gills of those acclimated to any of the other experimental salinities. In the other hand, the lower enzyme activity was observed in gills of fish acclimated to full strength sea water (salinity 35 ppt). Copper exposure inhibited the lactate dehydrogenase activity in gills of fish acclimated to fresh water. However, an increased enzyme activity was observed in gills of fish acclimated to brackish water at salinity 3.5 ppt or to full strength sea water (Fig. 4).

Gills of control fish acclimated to fresh water and brackish water at salinity 11 ppt showed lower NADP-dependent isocitrate dehydrogenase activity than gills of those acclimated to brackish water at salinity 3.5 ppt or to full strength sea water (salinity 35 ppt). NADP-dependent isocitrate dehydrogenase activity was reduced in gills of fish acclimated to fresh water or to brackish waters (salinities 3.5 and 11 ppt) after copper exposure (Fig. 5).

4. Discussion

Fish acclimation to fresh or salt water requires an increase in the activity of energy-demanding and passive mechanisms to maintain plasma Na^+ and Cl^- homeostasis (Evans et al., 2005). Response to environmental salinity changes is then achieved by synthesis and activation of enzymes and ion-transporting proteins, which are highly energy consuming (Tseng and Hwang, 2008). As expected, results reported in the present study showed a clear difference in the activity of metabolic enzymes in gills of control killifish acclimated to the different experimental salinities. In fact, gills of fish acclimated to fresh water showed a lower capability of exogenous glucose utilization. This statement is based on the lower hexokinase activity observed in these fish when compared to the enzyme activity found in gills of fish acclimated to brackish or sea water. However, freshwater fish exhibited the highest glycolytic capacity, as indicated by their higher phosphofructokinase and pyruvate kinase activities.

Although gills of brackish and seawater fish showed a higher capability for glucose utilization than gills of freshwater fish, gill phosphofructokinase activity was at least 3-fold higher than gill hexokinase activity. This finding points to the

predominance of glycolysis over the pentose phosphate pathway in gills of *F. heteroclitus*. This is supported by the high pyruvate kinase activity found in gills of this fish. Furthermore, lactate dehydrogenase activity was higher than NADP-dependent isocitrate dehydrogenase activity indicating the prevalence of the anaerobic metabolism over the aerobic metabolism in gills of *F. heteroclitus*, especially in specimens acclimated to fresh water.

A preference for glycolysis over Krebs cycle is explained by the fact that gills rely mainly on glucose and lactate as fuel (Mommsen, 1984; Perry and Walsh, 1989). Tseng et al. (2007) identified a novel group of cells in gills of the tilapia *Oreochromis mossambicus* which are called glycogen-rich cells due to their high capacity of glycogen storage. These cells are located beside ionocytes, providing them with pyruvate or lactate via monocarboxylase transporters (Hwang and Lee, 2007; Tseng and Hwang, 2008). Therefore, energy required by gills for ionic and osmotic regulation during salinity changes could be supplied by glycogen-rich cells or plasma glucose. Since the killifish *F. heteroclitus* was fastening during copper exposure, plasma glucose was likely provided by liver glycogenolysis.

In *Sparus aurata* transferred from brackish water (salinity 12 ppt) to sea water (salinity 35 ppt), plasma glucose level and gill hexokinase activity were increased (Sangiao-Alvarellos et al., 2003). The rainbow trout *Oncorhyncus myskiss* also showed higher plasma glucose levels and gill hexokinase, phosphofructokinase and pyruvate kinase activity with increasing salinity from fresh water to salt water (Soengas et al., 1995). In the present study, this pattern of response was not observed in the killifish *F. heteroclitus* as a function of increasing water salinities. However, it is important to note that even though *O. mykiss* is a euryhaline fish, it does not tolerate rapid variations in salinity like *F. heteroclitus*. In fact, an up-regulation of

genes expressing proteins involved in glycolysis and lipid metabolism, as well as creatine kinase was observed during osmotic acclimation in *F. heteroclitus* (Whitehead et al., 2011).

Regarding copper effects, our data clearly show that physiological alterations induced by the acclimation salinity also affected the enzymes response to metal exposure. This statement is based on the fact that gills of fish acclimated to fresh water showed a completely different pattern of response than gills of those acclimated to brackish or sea water. Although salt water is as challenging as fresh water when osmoregulation is considered, it seems that exposure to copper is more deleterious to *F. heteroclitus* in fresh water. Based on the results reported in the present study, freshwater killifish exposed to copper showed a reduced capability of energy production either by glycolysis or Krebs cycle. This finding suggests that a situation of functional hypoxia could be occurring in the freshwater acclimated killifish after exposure to copper. In the other hand, activities of enzymes were increased in gills of 3.5 ppt and seawater fish after copper exposure, indicating a higher demand of energy under this experimental condition. Since copper exposure also inhibited the NADP-dependent isocitrate dehydrogenase activity in gills of brackish water fish (salinities 3.5 and 11 ppt), glycolysis activity seems enough to maintain the adequate levels of ATP.

Most of studies focused on copper effects on energy metabolism were performed in liver and muscle of freshwater fish. No change in phosphofructokinase activity was found in white or red muscle of *Salmo trutta* exposed to 5 μg Cu/L (Beaumont et al., 2000). However, phosphofructokinase and pyruvate kinase activities were shown to be reduced in liver of *Prochilodus lineatus* after copper exposure (Carvalho and Fernandes, 2008). Also, lactate dehydrogenase activity was reported to

be increased in gills, liver and heart of *Cyprinus carpio* exposed to copper (Tóth et al., 1996). However, a reduced lactate dehydrogenase activity was found in liver of *S. aurata* exposed to copper (Antognelli et al., 2003). Therefore, in addition to the fact that gills were the organs analyzed in the present study, this is the first one performed in a euryhaline fish across a wide range of salinities.

Results reported in the present study show that the only enzyme that had its activity affected by copper exposure in all experimental salinities was phosphofructokinase. A number of allosteric modulators can change the activity of this enzyme. For example, ammonia is shown to activate phosphofructokinase activity (Sungden and Newsholme, 1975). In this context, it is important to note that an increased level of plasma ammonia was observed in *F. heteroclitus* exposed to copper in sea water, but not in those exposed to the metal in fresh water (Blanchard and Grosell, 2006). This fact could explain why killifish acclimated to brackish and sea water showed increased enzymes activities.

Copper is generally considered as an iono- and osmoregulatory toxicant in fish (Grosell et al., 2002, 2004a,b). However, sometimes no ionoregulatory disturbances are observed in seawater fish after exposure to waterborne copper (Grosell et al., 2003). It was reported that *F. heteroclitus* exposed to copper accumulated more metal in the gills at low salinities (0, 5, and 11 ppt) than at high salinities (20, and 28 ppt). Furthermore, a decreased Na^+ uptake associated with gill Na^+/K^+-ATPase inhibition was only detected in freshwater fish (Blanchard and Grosell, 2005, 2006). Considering that adenosine triphosphatases like Na^+/K^+-ATPase rely on ATP to perform ion transport across the cell membrane, a copper-induced disturbance in ATP production through glycolysis and Krebs cycle, as observed in the present study, would certainly impair the activity of ATPases and consequently fish ability to

regulate plasma ionic and osmotic concentration. Therefore, our findings can help to better understand the copper effects on fish physiology across a wide range of salinities, as well as to support the idea that physiology is pivotal for interactions between salinity and acute copper toxicity (Grosell et al., 2007). This statement is based on the fact that the activity of key enzymes involved in glycolysis and Krebs cycle were inhibited in gills of fish exposed to copper in fresh water. This condition is probably reducing the amount of ATP available and consequently the gill Na^+/K^+-ATPase activity. In the other hand, gills of killifish acclimated to brackish and sea water showed to be able to increase the activities of metabolic enzymes after copper exposure, being thus able to provide more energy to support the likely raised demand of energy under this experimental condition.

5. Conclusions

Exposure to an environmentally relevant concentration of copper (14-18 μg Cu/L) affects the activity of enzymes in energy metabolism in gills of the euryhaline killifish *F. heteroclitus*. However, the copper effect is dependent on the acclimation salinity. Gills of killifish acclimated to fresh water are more sensitive to copper exposure, showing reduced activity of lactate dehydrogenase and Krebs cycle enzymes. Therefore, these fish are consequently less able to keep the level of energy production needed to cope with the increased ionic and osmotic disturbances induced by waterborne copper exposure. In brackish (salinity 3.5 ppt) acclimated killifish, energy production through the Krebs cycle is also compromised, but lactate dehydrogenase activity is increased, which could help these fish to maintain adequate levels of energy production.

Taken altogether, findings reported in the present study provide more evidence that copper is more toxic to euryhaline fish acclimated to freshwater than to those acclimated to brackish or full strength sea water. Furthermore, they point out that the mechanism of acute copper toxicity in salt water killifish is not associated with disturbances in energy production through glycolysis and Krebs cycle. Therefore, other mechanism than disturbances in ionic/osmotic regulation and gill energy production disturbances would be involved in the acute copper toxicity in the killifish *F. heteroclitus* acclimated to salt water.

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Table 1. Measured copper concentrations in the experimental media used to expose the killifish *Fundulus heteroclitus* to waterborne copper. Since no significant differences between copper concentrations at the beginning and 24 h after exposure or between total and dissolved Cu concentrations were found for each experimental condition and water salinity, data were pooled and only one mean copper concentration was calculated.

Salinity (ppt)	Control (μg Cu/L)	Copper exposure (μg Cu/L)
fresh water (0)	3.30 ± 1.20	18.53 ± 0.86
brackish water (3.5)	4.20 ± 0.23	14.28 ± 1.10
brackish water (11)	4.64 ± 0.63	18.20 ± 1.59
sea water (35)	4.79 ± 0.80	18.42 ± 2.30

Figure captions

Figure 1. Hexokinase activity in gills of the killifish *Fundulus heteroclitus* acclimated to different salinities. Data are mean ± S.E.M. (n = 5). Different letters indicate significant difference among control animals acclimated to different salinities ($p < 0.05$). No significant difference was observed between control and copper-exposed fish for each experimental salinity ($p > 0.05$).

Figure 2. Phosphofructokinase activity in gills of the killifish *Fundulus heteroclitus* acclimated to different salinities. Data are mean ± S.E.M. (n = 5). Different letters indicate significant difference among control animals acclimated to different salinities ($p < 0.05$). * indicates significant difference between control and copper-exposed fish for each experimental salinity ($p < 0.05$).

Figure 3. Pyruvate kinase activity in gills of the killifish *Fundulus heteroclitus* acclimated to different salinities. Data are mean ± S.E.M. (n = 5). Different letters indicate significant difference among control animals acclimated to different salinities ($p < 0.05$). * indicates significant difference between control and copper-exposed fish for each experimental salinity ($p < 0.05$).

Figure 4. Lactate dehydrogenase activity in gills of the killifish *Fundulus heteroclitus* acclimated to different salinities. Data are mean ± S.E.M. (n = 5). Different letters indicate significant difference among control animals acclimated to different salinities ($p < 0.05$). * indicates significant difference between control and copper-exposed fish for each experimental salinity ($p < 0.05$).

Figure 5. NADP-dependent isocitrate dehydrogenase activity in gills of the killifish *Fundulus heteroclitus* acclimated to different salinities. Data are mean ± S.E.M. (n = 5). Different letters indicate significant difference among control animals acclimated to different salinities ($p < 0.05$). * indicates significant difference between control and copper-exposed fish for each experimental salinity ($p < 0.05$).

Figure 1

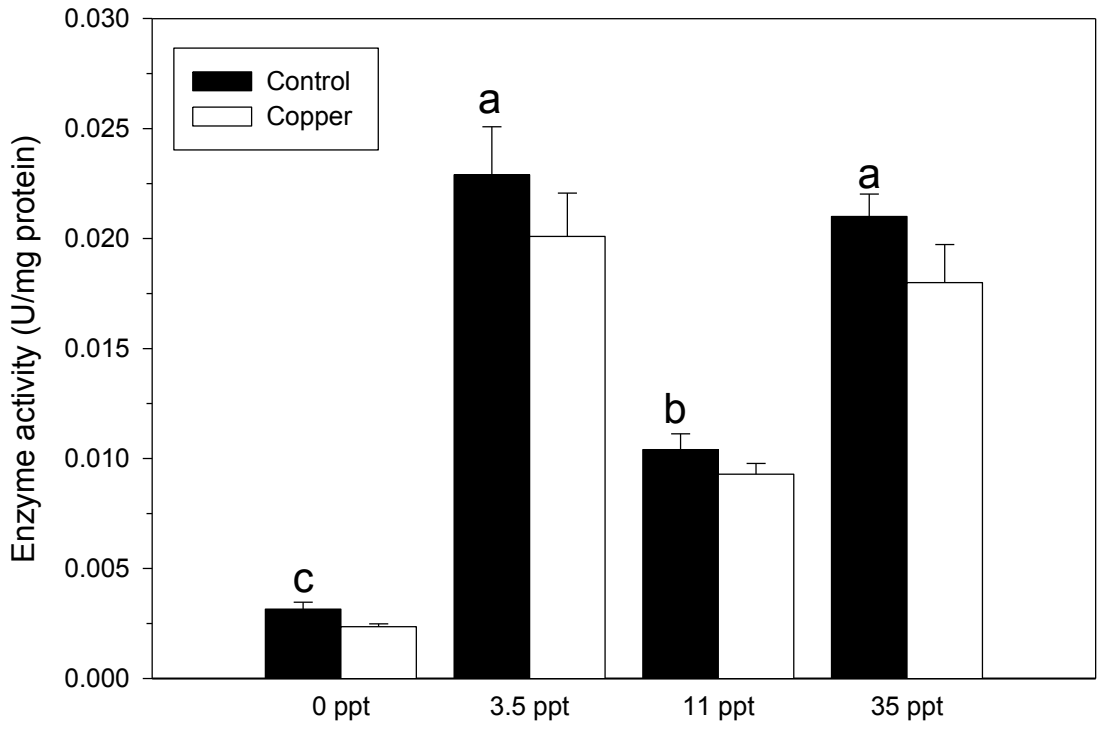
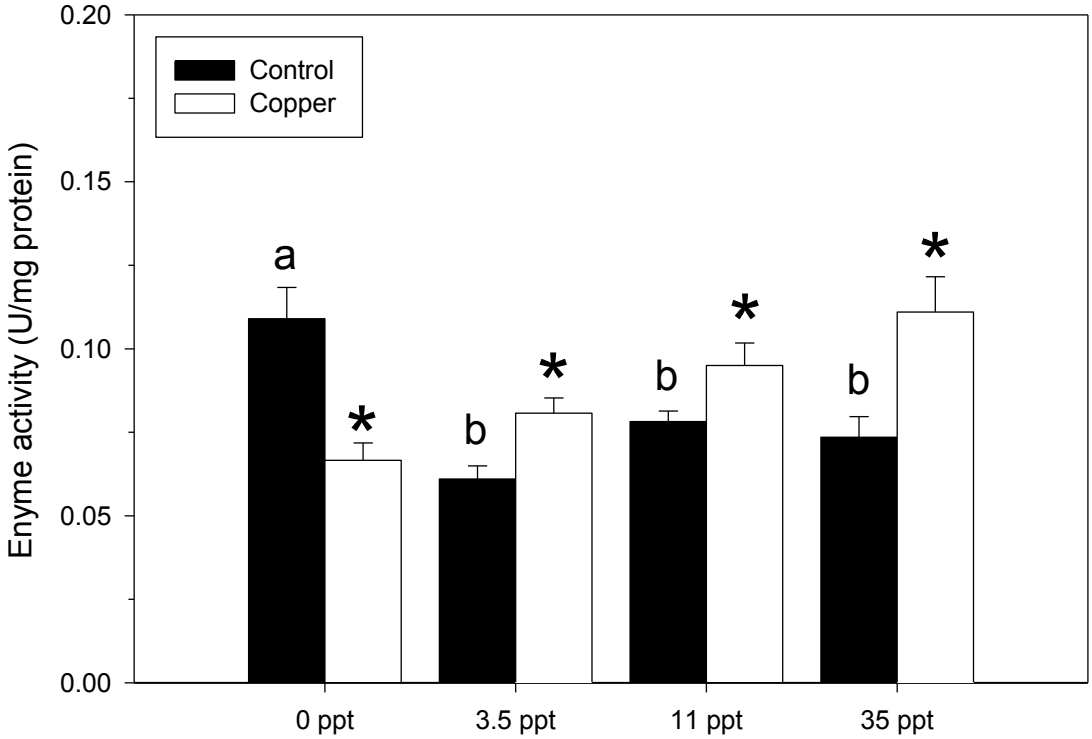

Control
Copper
Enzyme activity (U/mg protein)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
a
a
b
c
0 ppt
3.5 ppt
11 ppt
35 ppt

Figure 2


0.20
0.15
0.10
0.05
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Enyme activity (U/mg protein)
Control
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a
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b
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0 ppt
3.5 ppt
11 ppt
35 ppt

Figure 3

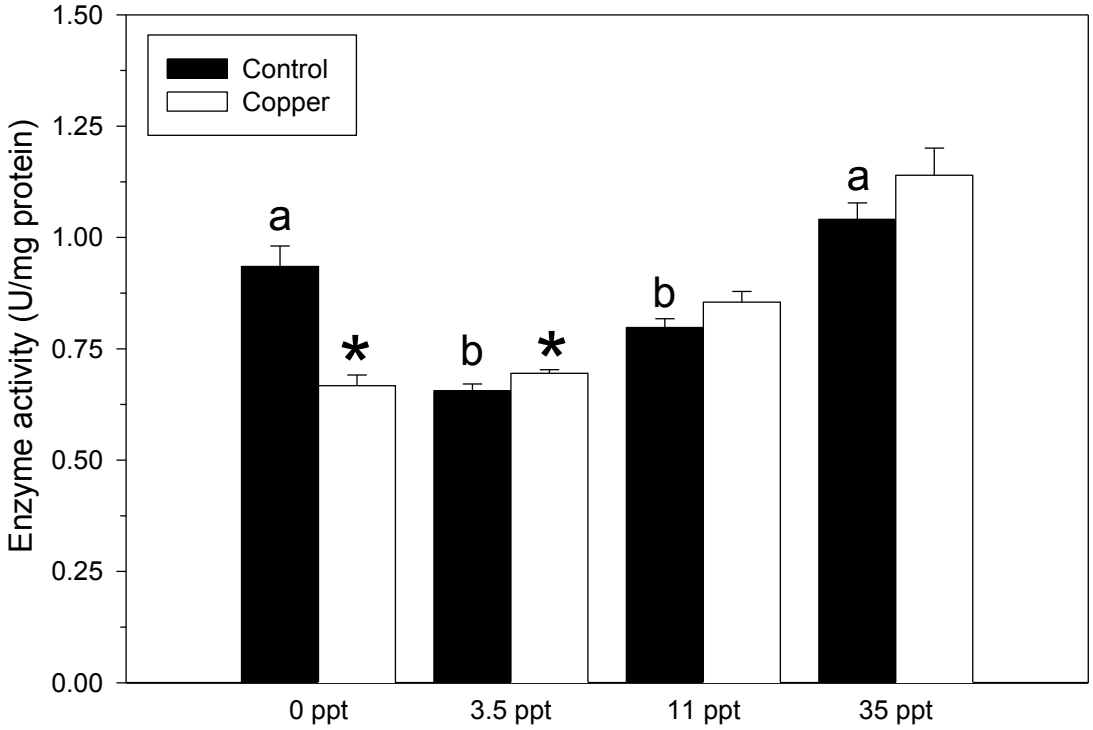

Control
Copper
Enzyme activity (U/mg protein)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
*
b
*
b
a
0 ppt
3.5 ppt
11 ppt
35 ppt

Figure 4

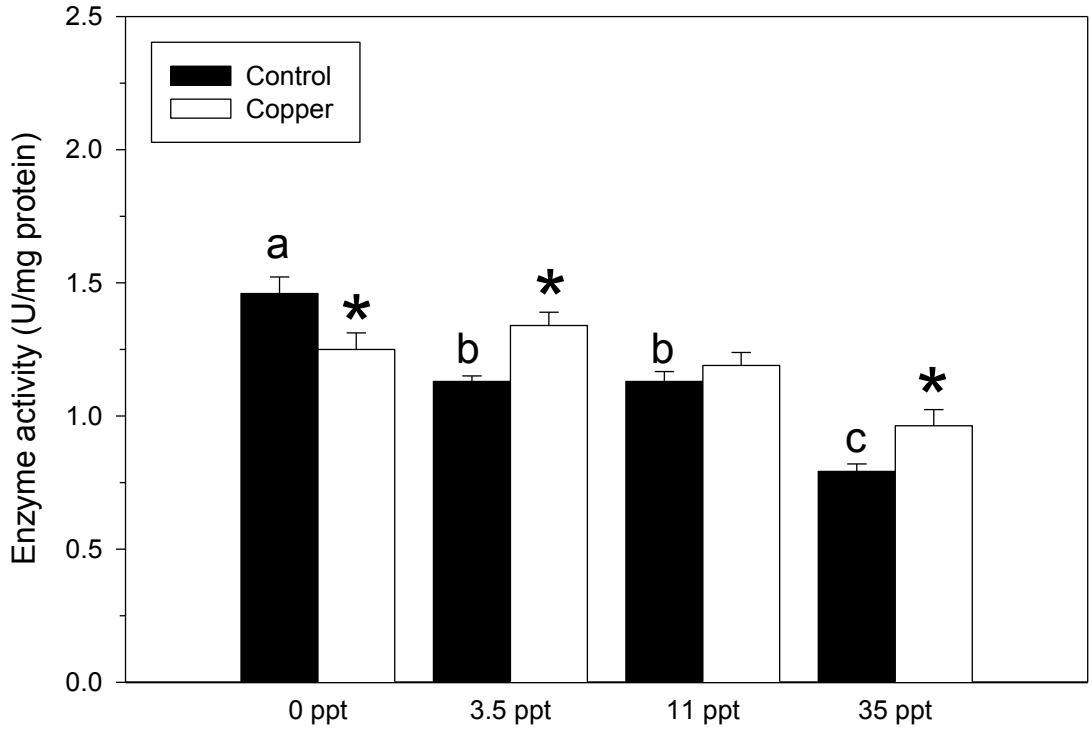
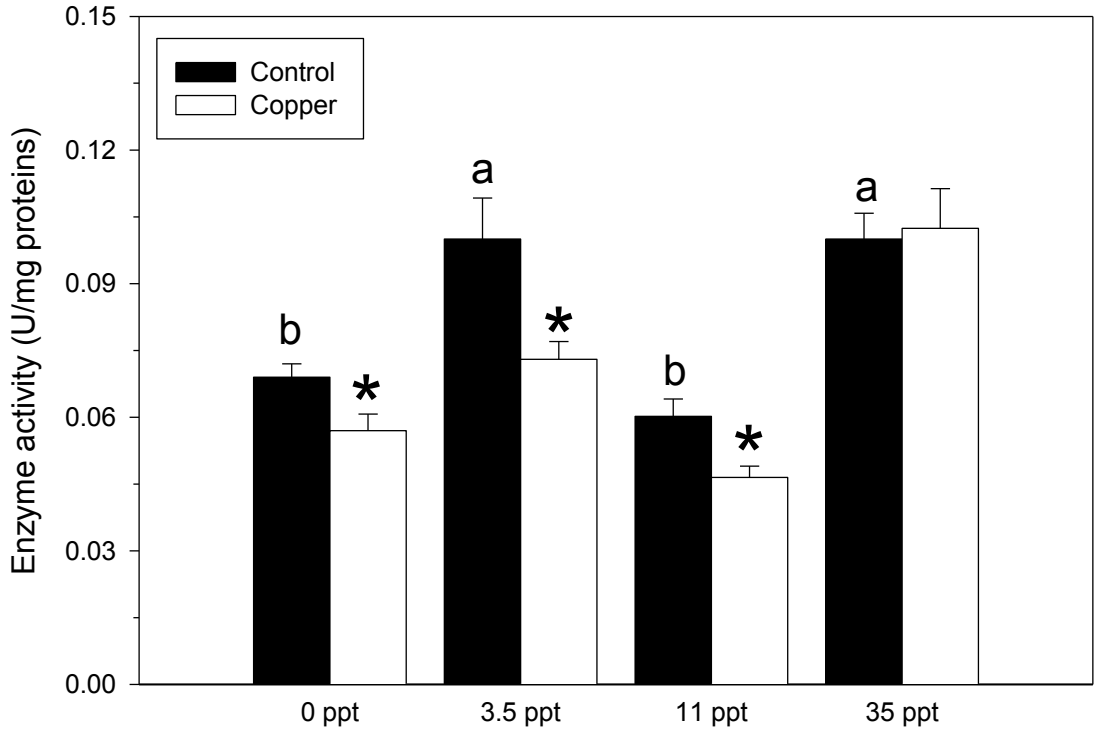

Control
Copper
Enzyme activity (U/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
c
*
*
*
0 ppt
3.5 ppt
11 ppt
35 ppt

Figure 5


Control
Copper
Enzyme activity (U/mg proteins)
0.15
0.12
0.09
0.06
0.03
0.00
b
a
b
a
*
*
*
0 ppt
3.5 ppt
11 ppt
35 ppt

8. ARTIGO

Copper effects on energy metabolism in the sea cucumber *Trachythyone crassipeda*

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Abstract

Sea cucumbers are echinoderms found worldwide and very susceptible to metal contamination due to their association with marine sediments. The aim of this study was to evaluate copper effects on glycolysis and Krebs cycle in respiratory trees of the sea cucumber *Trachythyone crassipeda*. Animals were exposed (96 h) to environmentally relevant concentrations of dissolved copper (5, 9, and 20 μg Cu/L) in sea water (salinity 33 ppt). Pyruvate kinase activity was increased in sea cucumbers exposed to any copper concentration, while hexokinase activity was increased only in sea cucumbers exposed to 9 and 20 μg Cu/L. In turn, lactate dehydrogenase activity was inhibited in all copper concentrations. No change was observed in phosphofructokinase and citrate synthase activity after exposure to copper. Based on these findings, we hypothesize that copper exposure leads the sea cucumber *T. crrassipeda* to an "aestivation-like" condition. Under this condition, glucose would be used through the pentose-phosphate pathway instead of the glycolysis to sustain the antioxidant defense system, with lipids being the major source of energy instead of carbohydrates. The lactate dehydrogenase inhibition induced by copper exposure can be considered very deleterious to animals showing low metabolic rates, such as sea cucumbers, since it can seriously compromise the NAD^+ recycling.

Keywords: copper, energy metabolism, glycolysis, Krebs cycle, sea cucumber

1. Introduction

Echinoderms are exclusively marine invertebrates. In the last decades, they have been widely used as bioindicators of marine polluted areas, since their larval stages are very sensitive to environmental stressors while adults show high capacity for metal accumulation (Kobayashi, 1980; Rygg, 1985; Ringwood, 1992; Xing and Chia, 1997; Laboy-Nieves and Conde, 2001; Storelli et al., 2001). Although most of the studies used sea urchins or sand dollars, especially during the embryo-larval development, other classes of echinoderms also could present good potential for studies on metal toxicity.

Sea cucumbers belong to the class Holothuroidea, and are found worldwide. Furthermore, they carry economic and cultural significance in some parts of the world where these animals are captured in the environment or cultivated for culinary purpose (Conand and Byrne, 1993). Since they produce a series of substances that can be toxic to other animals, they are used for extraction of marine natural products, which present pharmacological properties such as anticancer, anti-inflammatory, antioxidant, and anti-angiogenic (Yamada et al., 2003; Mamelona et al., 2007; Bordbar et al., 2011).

Holothurians inhabit in close contact with the sea bottom and usually feed on detritus or suspended material, thus being susceptible to ingestion of contaminated sediments and metal accumulation (Xing and Chia, 1997; Rojas et al., 1998; Maurer et al., 1999; Warnau et al., 2006; Denton et al., 2009). Although this group of animals can be considered as a good experimental model for metal toxicity in sea water, few studies focused on the effects of metals on their physiology are available in the literature.

Copper effects in marine organisms are not still well known even though its toxicity is documented for echinoderm larvae, mollusks and crustaceans (Boitel and Truchot, 1989; Rivera-Duarte et al., 2005; Nadella et al., 2009; Pinho and Bianchini, 2010). Freshwater and marine fish, such as the rainbow trout *Oncorhyncus mykiss*, the gulf toadfish *Opsanus beta*, and the flounder *Platichthys flesus*, when acutely exposed to dissolved copper showed mainly osmoregulatory disturbances (Stagg and Shuttleworth, 1982; Wilson and Taylor, 1993; Grosell et al, 2004a,b). However, acid-balance and ammonia excretion disturbances were also reported in the killifish *Fundulus heteroclitus* and the cod *Gadus morhua* (Larsen et al., 1997; Blanchard and Grosell, 2006).

Regarding marine invertebrates, acidosis associated with respiratory impairment was shown to be a response to copper exposure in the shore crab *Carcinus maenas* and in adults of the sea urchin *Diadema antillarum* (Boitel and Truchot, 1990; Bielmyer et al., 2005). Also, impairment in carbonic anhydrase activity was reported to occur following exposure of three species of scleractinian corals to copper (Bielmyer et al., 2010). Other biochemical and physiological effects are also reported to be associated with copper exposure. For example, a reduction in antioxidant capacity as observed in the sea anemone *Aiptasia pallida* (Main el at., 2010) while an increased whole-body Na^+ concentration was reported in the copepod *Acartia tonsa* (Pinho et al., 2007).

In freshwater animals, gills are considered the main target organ for copper toxicity since they rapidly accumulate the metal from waterborne exposure, which induces changes in several physiological processes (Grosell et al., 2007). To cope with the negative effects of copper on the organism physiology, mechanisms of detoxification and repair are switched on to prevent extensive damage to

biomolecules. The functioning of these mechanisms requires energy, thus stimulating the metabolic pathways in order to supply the higher metabolism. Direct effects of copper on metabolism cannot be ruled out and would worsen even more the health status of exposed individuals (Lai and Blass, 1984; Tóth et al., 1996; Beaumont et al., 2000; Antognelli et al., 2003; Gul et al., 2004).

In the light of the above, the main objective of the present study was to evaluate the effects of the acute exposure to waterborne copper on the energy metabolism in the sea cucumber *Trachythyone crassipeda*. Endpoints analyzed were the activity of key enzymes of glycolysis (hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase) and Krebs cycle (citrate synthase) in the respiratory trees, the organs responsible for gas exchange and ammonia excretion in sea cucumbers.

2. Material and methods

2.1. Animal collection

Adults of *T. crassipeda* were collected by scuba diving near the "Ilha do Frade", an island of Vitoria city (Espírito Santo state, Southeastern Brazil), kept under laboratory controlled conditions (water salinity 33 ± 2 ppt, temperature: 21± 2°C; photoperiod 12h L: 12h D) and not fed for two days before exposure to copper.

2.2. Copper exposure and tissue collection

Acclimated sea cucumbers were divided into four experimental groups (n = 6 for each group) and kept for 96 h in tanks containing 10 L of natural seawater (salinity 33 ± 2 ppt) contaminated or not with copper. Copper concentrations tested (5, 9, and 20 μg Cu/L) were selected based on the current water quality criteria for Brazilian waters. The lower concentrations corresponded to the quality criterion for marine (5 μg Cu/L) and fresh waters (9 μg Cu/L) while the highest one (20 μg Cu/L) represents a "non-conforming" condition. A control (no copper addition into the sea water) was also run. During the exposure period, laboratory conditions were kept as described for the acclimation period. Sea water used for sea cucumber acclimation and exposure to copper was pumped directly from the ocean, filtered one time through a 50 μm-mesh filter, filtered twice through a 1 μm-mesh filter, and twice sterilized using UV light. Copper was added to the water 24 h prior to animal introduction in the test chamber to allow the complete equilibration of copper with sea water. During the exposure period, experimental media were completely renewed every 24 h. Animals were not fed during the exposure period and no sea cucumber mortality was observed in any treatment. After copper exposure, sea cucumbers were cryoanesthetized and had their respiratory trees dissected. Samples were immediately frozen and transferred to laboratory in dry ice, where they were kept in an ultra-low temperature freezer (-80°C) until the enzymatic analysis, which were performed as described below.

2.3. Enzyme analyses

Activity of glycolysis and Krebs cycle enzymes were assayed according to Lallier and Walsh (1991) with slight modifications. Respiratory trees were homogenized in ice-cold buffer solution (50 mM imidazole; 0.1 mM PMSF; pH 7.8) and centrifuged (10,000 x *g*; 20 min; 4^{o}C). The supernatant obtained was used as enzyme source. Enzyme activities were measured by spectrophotometry using a microplate reader (BioTek, Vermont, USA). Glycolysis enzymes (hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase) assays were buffered with a 50 mM imidazole solution (pH 7.4) and the NAD^{+}/NADH oxidation/reduction was followed by absorbance measurements at 340 nm. Krebs cycle enzyme (citrate synthase) assay was buffered with a 50 mM HEPES solution (pH 8.1), and DTNB reduction was followed by absorbance measurements at 405 nm. All assays were performed at 25^{o}C and the specific conditions used for each assay are detailed as follows (final concentrations): hexokinase (5 mM $MgCl_2$, 1 mM D-glucose, 0.16 mM NAD^{+}, 2 U/mL glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 1 mM ATP); phosphofructokinase (10 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 0.12 mM NADH, 1 U/mL aldolase, 3 U/mL triose phosphate isomerase, 1 U/mL α-glycerophosphate dehydrogenase, and 3 mM fructose-6-phosphate); pyruvate kinase (10 mM $MgCl_2$, 30 mM KCl, 2.5 mM ADP, 0.12 mM NADH, 20 U/mL lactate dehydrogenase, and 0.5 mM phosphoenolpyruvate); lactate dehydrogenase (2 mM pyruvate and 0.12 mM NADH); citrate synthase (0.1 mM acetylcoenzyme A, 0.1 mM DTNB, and 0.5 mM oxaloacetate).

2.4. Statistical analyses

Data were expressed as mean ± S.E.M (n = 6). Analysis of variance (ANOVA) followed by the Tukey test was performed to identify significant ($p < 0.05$) statistical differences among treatments. ANOVA assumptions (data normality and homogeneity of variances) were previously verified.

3. Results

No mortality was observed in either control or copper-exposed sea cucumbers after the 96-h period of copper exposure. Hexokinase activity was higher in animals exposed to 9 and 20 μg Cu/L than in those kept under control conditions or exposed to 5 μg Cu/L (Fig. 1). No significant change in phosphofructokinase activity was observed after exposure to any copper concentration tested (Fig. 2). Pyruvate kinase activity was increased in sea cucumbers exposed to all copper concentrations, with animals exposed to 20 μg Cu/L exhibiting the highest activity (Fig. 3). An inhibition of lactate dehydrogenase activity was observed in sea cucumbers exposed to all copper concentrations tested, with animals exposed to 9 μg Cu/L showing the lowest mean value of enzyme activity (Fig. 4). Like the phosphofructokinase, citrate synthase activity remained unchanged after exposure to any copper concentration tested (Fig. 5).

4. Discussion

Waterborne copper exposure in sea water affected the activity of some glycolytic enzymes in respiratory trees of the sea cucumber *T. crassipeda*. Disruption on glycolysis could lead to energy impairment, especially under hypoxic conditions and in animals that rely on anaerobic metabolism.

Hexokinase is the first enzyme in the glycolysis pathway, being responsible for the phosphorylation of glucose into glucose-6-phosphate. The observed increase in hexokinase activity in the sea cucumber *T. crassipeda* exposed to the higher copper concentrations tested in the present study (9 and 20 μg Cu/L) is not in alignment with the enzyme inhibition reported in posterior gills and midgut gland of the shore crab *C. maenas* and the digestive gland of the mussel *Mytilus galloprovincialis* after copper exposure (Hansen et al., 1992; Canesi et al., 1998). The increased hexokinase activity observed in the present study suggests a raise in the amount of free glucose available in the haemolymph, which could lead to a higher production of glucose-6-phosphate.

Glucose-6-phosphate can have other fates in metabolism than glycolysis depending on the cell energy status and needs. When the ATP/ADP ratio is elevated, glucose-6-phosphate is driven by the pentose-phosphate pathway. This biochemical pathway is responsible for the production of NADPH, a reduced coenzyme used in the antioxidant system and in lipid biosynthesis. The pentose-phosphate pathway is also involved in production of ribose, a five carbon sugar that participates in nucleic acids formation (Campbell and Farrell, 2006). Taking into account that no effect of copper exposure was observed in phosphofructokinase activity, and that hexokinase activity was at least 10-fold higher than phosphofructokinase activity, our results

indicate that glucose-6-phosphate is likely not fueling the glycolysis pathway. It is important to note that phosphofructokinase is highly subjected to regulation by allosteric factors, such as ATP, fructose-1,6-biphosphate, and citrate (Campbell and Farrell, 2006). Therefore, slight variations in the concentration of one of these factors can modify the enzyme activity, enhancing or inhibiting it.

Since it was expected that the response of pyruvate kinase activity to copper exposure would follow that shown by the phosphofructokinase activity, the enhanced pyruvate kinase activity observed after *T. crassipeda* exposure to copper thus indicates that glycolysis is being fueled after the second step of control. In this context, we can point glycerol produced from lipid degradation as being a key substrate to fuel the glycolysis. This molecule can be also used to produce glucose through gluconeogenesis. In this context, it is important to note that sea cucumbers can go under aestivation, a period of inactivity that occurs when adverse survival conditions are present, and characterized by metabolic depression and primary reliance on lipid oxidation to fuel the metabolism (Storey, 2002). In fact, it was reported that the sea cucumber *Apostichopus japonicus* uses protein and lipids as energy sources during aestivation periods, thus activating the antioxidant defense system (Yang et al., 2006; Bao et al., 2010; Fangyu et al., 2011).

Oxidative metabolic changes, such as those observed during aestivation, can increase reactive oxygen species (ROS) production, which in turn can cause damage to lipids, proteins, and DNA (Storey, 2006). Even though the metabolism is depressed, tolerant animals enhance their antioxidant defenses, anticipating the excess of ROS formation once tissues are reoxygenated (Hermes-Lima and Zenteno-Savin, 2002). At this point, it is important to stress that copper also can induce ROS formation through Fenton and Haber-Weiss reactions (Furuno et al., 1996;

Pourahmad and O'Brien, 2000). Based on the findings reported in the present study, we suggest that exposure to copper at environmentally relevant concentrations may lead the sea cucumber *T. crassipeda* into a physiological situation similar to 'aestivation'. This hypothesis relays on the supposition that glucose is being used in the pentose-phosphate pathway and that lipids are the major source of energy. These assumptions are thus based on the combination of responses of the hexokinase, phsphofructokinase, and pyruvate kinase activity to copper exposure.

A higher activity of pyruvate kinase would lead to a higher production of pyruvate. In this case, one expects that more pyruvate would be used by lactate dehydrogenase and the pyruvate dehydrogenase complex to form lactate and acetyl CoA, respectively. Since lactate dehydrogenase activity was inhibited and citrate synthase activity remained unchanged in sea cucumbers exposed to any of the copper concentrations tested, the excess of pyruvate is probably being used for other purposes than energy production, such as the formation of the amino acid arginine. Also, it is important to note that an effect of copper exposure on pyruvate dehydrogenase complex cannot be ruled out. In this context, an enzymatic impairment induced by copper such as the one observed for lactate dehydrogenase could be also occurring with the pyruvate dehydrogenase. Unfortunately, the activity of this enzyme was not evaluated in the present study.

It is known that lactate dehydrogenase recycles NADH to NAD^+, allowing glycolysis to undergo in anaerobic conditions, although the amount of ATP produced is much smaller under this pathway than through the oxidative phosphorylation. Therefore, the observed inhibition of the lactate dehydrogenase activity after exposure to copper can be deleterious to the sea cucumber *T. crassipeda*, especially if it undergoes into a condition of hypoxia. Since citrate is an allosteric activator of

phosphofructokinase, the lack of copper effect on citrate synthase activity would sustain the citrate levels in the tissue. This may explain why the phosphofructokinase activity is not affected in the respiratory trees of *T. crassipeda* exposed to any of the copper concentrations tested in the present study. In fact, no correlation was observed between the activities of these two enzymes in tissues of the shore crab *C. maenas* exposed to dissolved copper (Hansen et al., 1992).

5. Conclusions

Findings described in the present study clearly indicate that exposure to dissolved copper significantly impairs the activity of key enzymes of glycolysis in the respiratory tree of the sea cucumber *T. crassipeda*. It is important to stress that this effect can potentially cause metabolic perturbations in the metabolic fates of both glucose-6-phosphate and pyruvate. For aquatic animals showing low metabolism like sea cucumbers, the copper-induced inhibition of lactate dehydrogenase activity observed in *T. crassipeda* can seriously compromises the whole-animal energy metabolism and homeostasis. We expect that findings reported here can bring more attention to the importance of considering sea cucumbers as an important biological model in future biochemical and physiological studies focused on metal toxicity in sea water.

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Figure captions

Figure 1. Hexokinase activity in respiratory trees of control and copper-exposed sea cucumbers *Trachythyone crassipeda*. Data are expressed as mean ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).

Figure 2. Phosphofructokinase activity in respiratory trees of control and copper-exposed sea cucumbers *Trachythyone crassipeda*. Data are expressed as mean ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).

Figure 3. Pyruvate kinase activity in respiratory trees of control and copper-exposed sea cucumbers *Trachythyone crassipeda*. Data are expressed as mean ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).

Figure 4. Lactate dehydrogenase activity in respiratory trees of control and copper-exposed sea cucumbers *Trachythyone crassipeda*. Data are expressed as mean ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).

Figure 5. Citrate synthase activity in respiratory trees of control and copper-exposed sea cucumbers *Trachythyone crassipeda*. Data are expressed as mean ± SEM (n = 6). Different letters indicate significant differences among treatments ($p < 0.05$).

Figure 1

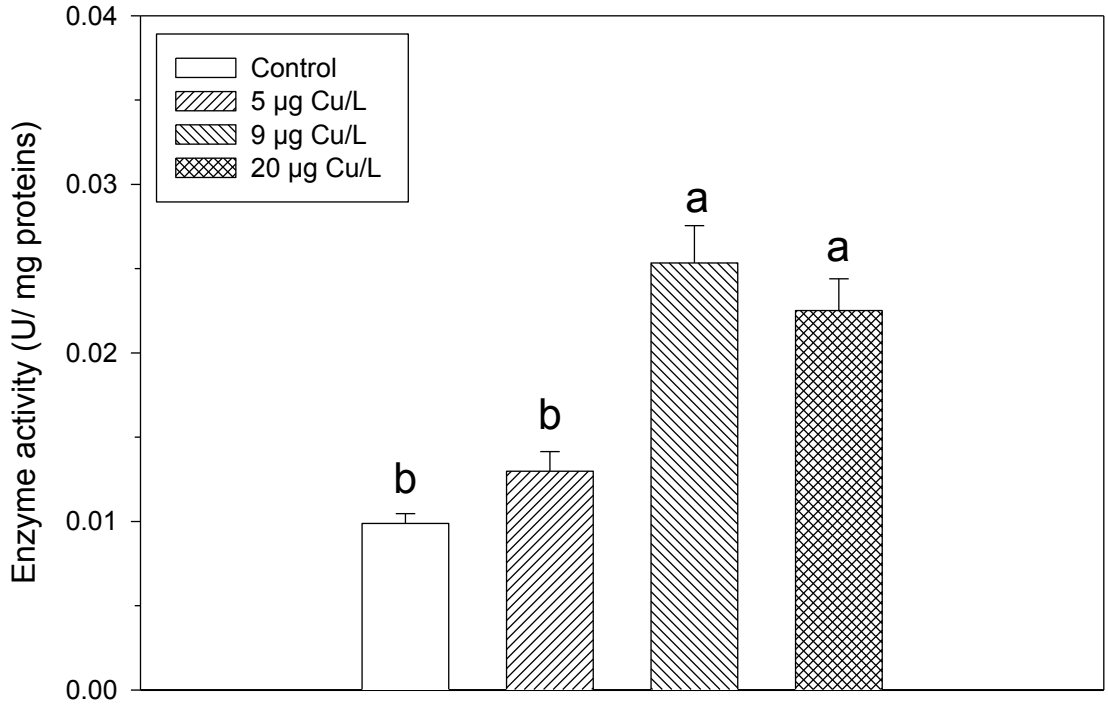
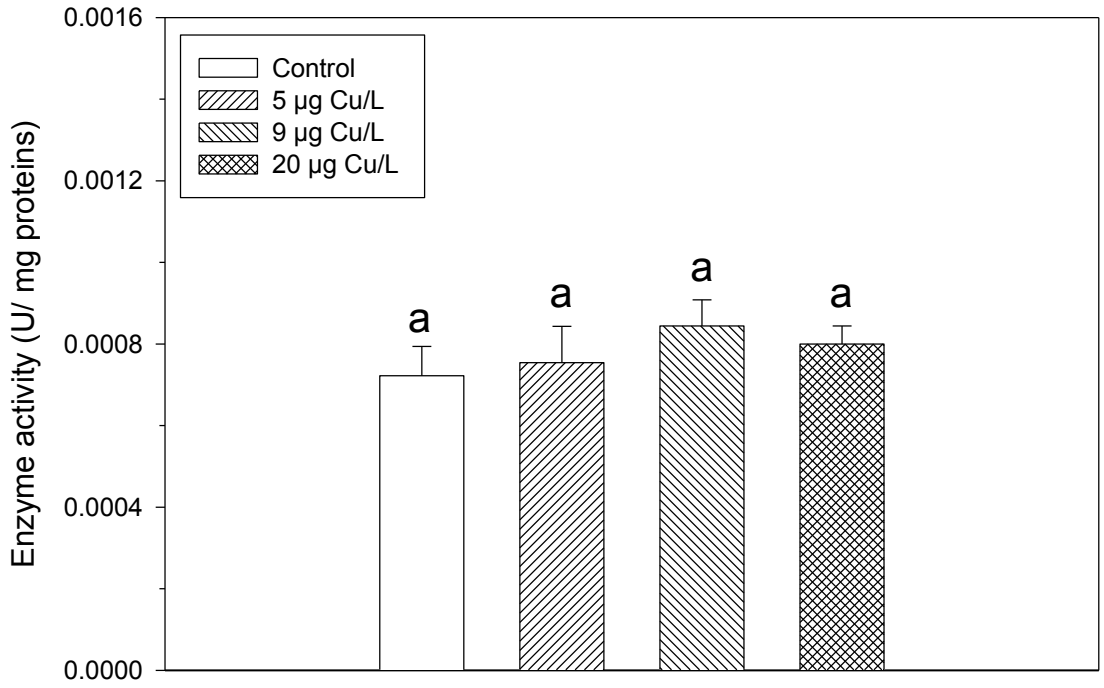

0.04
0.03
0.02
0.01
0.00
Enzyme activity (U/ mg proteins)
Control
5 µg Cu/L
9 µg Cu/L
20 µg Cu/L
b
b
a
a

Figure 2


0.0016
0.0012
0.0008
0.0004
0.0000
Enzyme activity (U/ mg proteins)
Control
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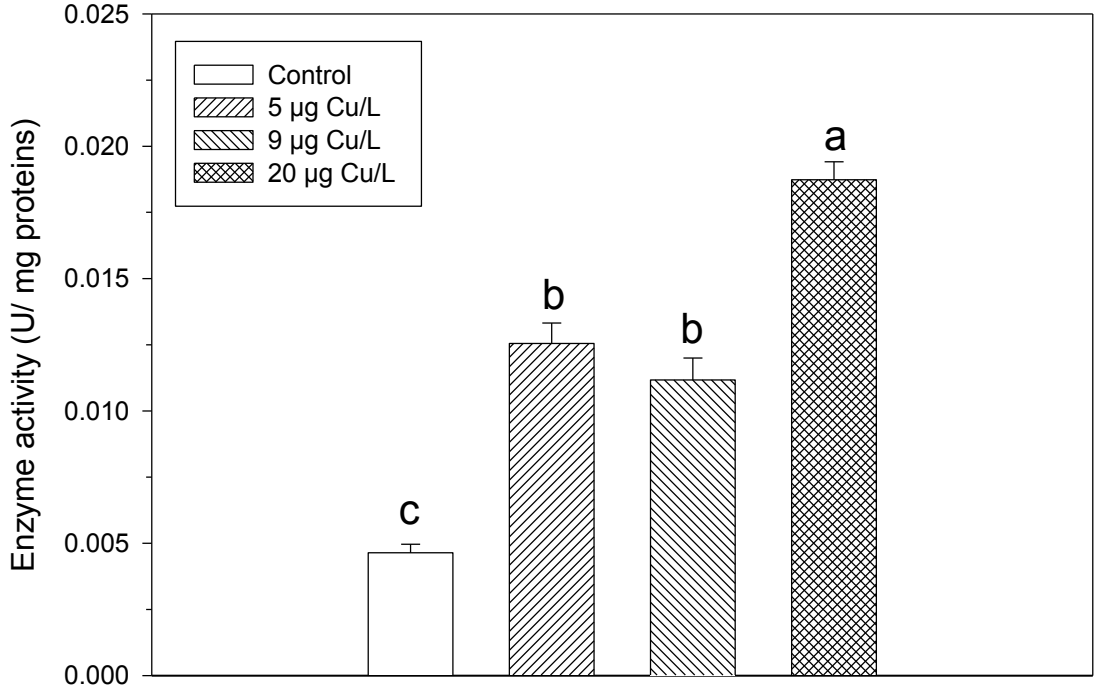
9 µg Cu/L
20 µg Cu/L
a
a
a
a

Figure 3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0.020
0.015
0.010
0.005
0.000
Enzyme activity (U/ mg proteins)
Control
5 µg Cu/L
9 µg Cu/L
20 µg Cu/L
c
b
b
a

Figure 4

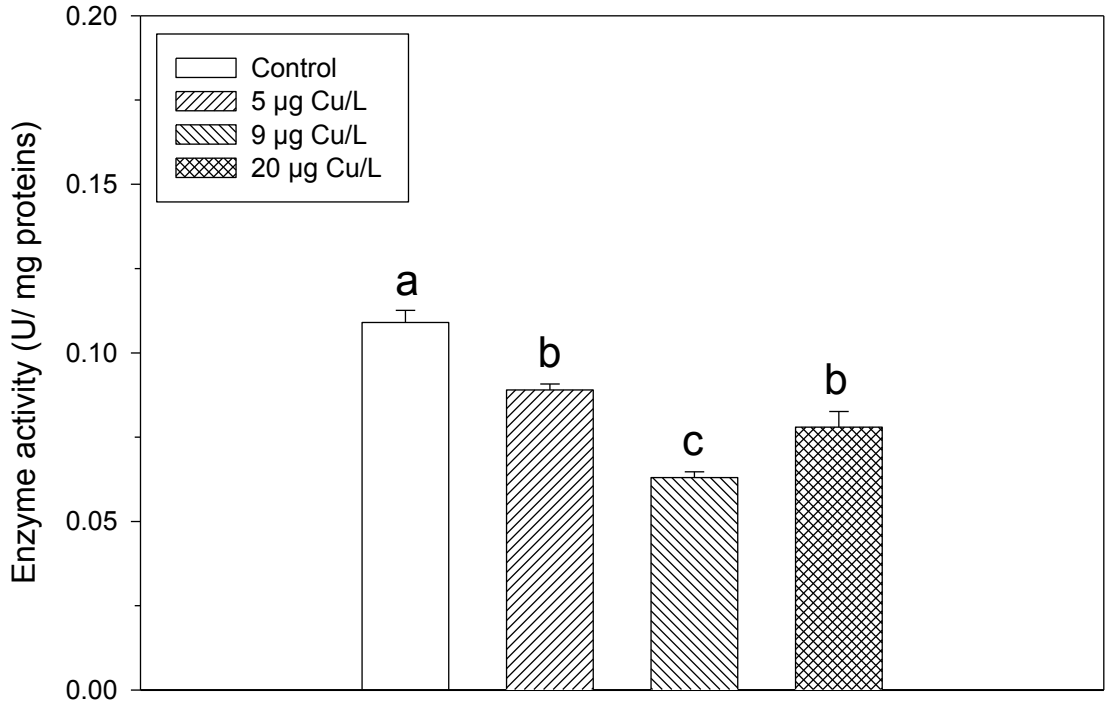
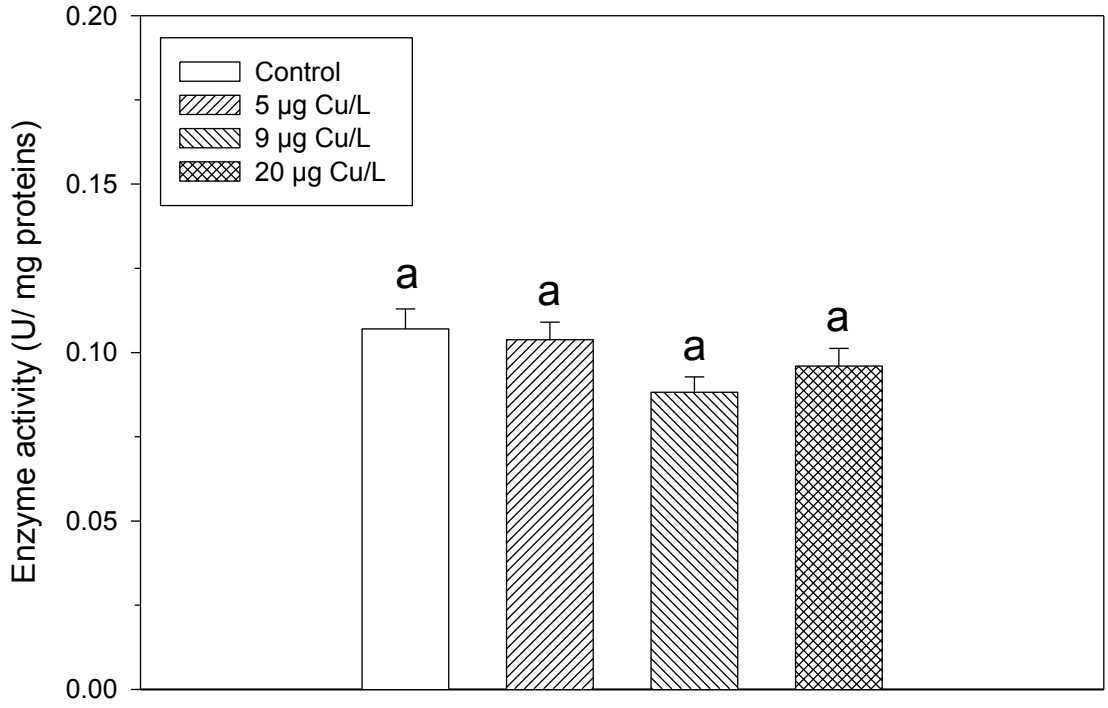

Control
5 µg Cu/L
9 µg Cu/L
20 µg Cu/L
Enzyme activity (U/ mg proteins)
0.20
0.15
0.10
0.05
0.00
a
b
c
b

Figure 5


0.20
0.15
0.10
0.05
0.00
Enzyme activity (U/ mg proteins)
Control
5 µg Cu/L
9 µg Cu/L
20 µg Cu/L
a
a
a
a

9. CONCLUSÕES

Com base nos resultados obtidos nesta tese, conclui-se que:

- as brânquias do peixe *Fundulus heteroclitus* apresentam maior atividade enzimática do que os demais animais em alta salinidade (aproximadamente salinidade 33), o que condiz com sua maior necessidade energética devido ao seu metabolismo, tanto pela osmorregulação quanto pela natação;

- a salinidade de aclimatação *per se* provoca alterações metabólicas tanto no caranguejo *Neohelice granulata* quanto no peixe *F. heteroclitus*;

- a exposição aguda do caranguejo estuarino *N. granulata* a concentrações subletais deste metal afeta a atividade das enzimas envolvidas na glicólise e no ciclo de Krebs, especialmente nas brânquias anteriores, quando os caranguejos estão hiperosmorregulando. Além disso, observa-se uma mudança do metabolismo aeróbico para anaeróbico após a exposição aguda de *N. granulata* ao cobre, caracterizando uma situação de hipoxia funcional nos caranguejos expostos ao metal, sendo que a observada redução no potencial de membrana mitocondrial sugere uma diminuição na produção de ATP. Portanto, apesar de *N. granulata* ser uma espécie altamente tolerante ao cobre, a exposição aguda a concentrações elevadas deste metal pode causar um déficit energético, já que afeta os sistemas bioquímicos envolvidos no metabolismo dos carboidratos;

- no peixe *F. heteroclitus* a exposição aguda a concentrações subletais de cobre é mais danosa aos indivíduos aclimatados à água doce do que aqueles expostos às águas salobras e marinhas. No caso dos peixes aclimatados à água doce, a exposição ao cobre diminui o status energético do animal, devido a uma menor atividade do ciclo de Krebs e da lactato desidrogenase. Por sua vez, *F. heteroclitus* aclimatado e exposto

agudamente ao cobre em águas salobras e marinhas, apesar de sofrer inibição do ciclo de Krebs, é capaz de suprir suas demandas energéticas através do aumento da atividade de algumas de suas enzimas metabólicas, em especial a lactato desidrogenase;

- no pepino-do-mar *Trachythyone crassipeda*, que apresenta um baixo metabolismo, conclui-se que a exposição ao cobre pode ser extremamente danosa, mesmo em baixas concentrações, pois esta inibe a atividade da lactato desidrogenase. Cabe ressaltar que a atividade desta enzima é fundamental para o metabolismo dos animais nas situações de hipoxia, uma vez que esta é responsável pela redução do piruvato formado em condições de anaerobiose, a qual é essencial para a regeneração de NAD^+ e consequente continuidade da atividade da via glicolítica;

- considerando o conjunto dos resultados descritos nesta tese, a exposição aguda a concentrações subletais de cobre afeta o metabolismo energético, em especial a via glicolítica, nas três espécies de animais aquáticos analisadas no presente estudo. Porém, observa-se que a extensão e o tipo de alteração (diminuição ou aumento) variam conforme a espécie estudada e não é possível identificar um padrão único de resposta dos diferentes grupos animais costeiros (equinodermos, crustáceos e peixes) à exposição ao metal, como é observado para peixes e crustáceos de água doce.

Espera-se que, futuramente, os resultados desta tese ajudem a elucidar o mecanismo de toxicidade do cobre dissolvido na água, principalmente em salinidades variadas, em espécies de diferentes grupos animais, e que invertebrados osmoconformadores também sejam utilizados como modelos biológicos em futuros estudos sobre o(s) efeito(s) do cobre e de outros metais.

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