UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
INSTITUTO DE CIÊNCIAS BIOLÓGICAS – ICB
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

TESE DE DOUTORADO

Efeitos combinados do estresse térmico e da exposição ao cobre no metabolismo energético, comportamento trófico e estado oxidativo do coral *Mussismilia harttii*

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Orientador: Dr. Adalto Bianchini

Tese apresentada ao Programa de Pós-graduação em Ciências Fisiológicas da Universidade Federal do Rio Grande - FURG, como requisito parcial a obtenção do título de DOUTORA.

RIO GRANDE
Agosto, 2021

Ficha Catalográfica

F676e Fonseca, Juliana da Silva.
Efeitos combinados do estresse térmico e da exposição ao cobre no metabolismo energético, comportamento trófico e estado oxidativo do coral *Mussismilia harttii* / Juliana da Silva Fonseca. – 2021.
161 f.

Tese (doutorado) – Universidade Federal do Rio Grande – FURG, Programa de Pós-Graduação em Ciências Fisiológicas, Rio Grande/RS, 2021.
Orientador: Dr. Adalto Bianchini.

1. Aquecimento Global 2. Estresse Oxidativo 3. Inibição Enzimática 4. Poluição Química 5. Recifes de Coral I. Bianchini, Adalto II. Título.

CDU 563.6

Catalogação na Fonte: Bibliotecário José Paulo dos Santos CRB 10/2344

SERVIÇO PÚBLICO FEDERAL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO RIO GRANDE
INSTITUTO DE CIÊNCIAS BIOLÓGICAS-ICB

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Aos doze dias do mês de agosto de dois mil e vinte e um às 13:30, via Webconferência no link: https://conferenciaweb.rnp.br/events/defesa-de-tese-de-doutorado-do-ppgcf-academica-juliana-da-silva-fonseca reuniram-se docentes, discentes e comunidade em geral, para a Defesa Pública da ***octagésima oitava*** Tese de Doutorado do Programa de Pós-Graduação em Ciências Fisiológicas, como requisito parcial para obtenção do grau de Doutor, conforme artigo 3º do Regimento Geral da Pós-Graduação *Stricto sensu* da FURG. A tese intitulada **"Efeitos combinados do estresse térmico e da exposição ao cobre no metabolismo energético, comportamento trófico e estado oxidativo do coral *Mussismilia harttii*"**, foi apresentada pela discente **Juliana da Silva Fonseca** seguindo-se da arguição pela Banca Examinadora composta pelo Profº. Dr. Adalto Bianchini (Orientador e Presidente da Banca); Profº. Dr. Flavio Manoel Rodrigues da Silva Júnior (FURG); Profº Dr. Carlos Eduardo da Rosa (FURG); Profº Dr. Robert Tew Boyle (FURG) e Profº Dr. Miguel Mies (USP). Após a defesa e argüição pública, reuniu-se a Banca Examinadora, para avaliação final nos termos do Art. 37 do Regimento e da Norma Complementar da defesa de Dissertação ou Tese do Programa de Pós-Graduação, tendo a candidata sido considerada **APROVADA** por unanimidade. Nada mais havendo a tratar, às 17:30, foi lavrada a presente ata que, após lida e aprovada, vai assinada pelo Coordenador do Programa.

Profº. Dr. Fábio Everton Maciel
Coordenador do Programa de Pós-Graduação

Sumário

Agradecimentos

Agradeço primeiramente à minha mãe pelos ensinamentos, amor, incentivo, esforço e compreensão!

Ao meu orientador, Prof. Dr. Adalto Bianchini, pela confiança depositada em mim e pelos valiosos conhecimentos propiciados durante esses nove anos de vida acadêmica.

À FURG e ao Instituto de Ciências Biológicas por toda a infraestrutura que permitiu a realização deste trabalho, em especial ao Programa de Pós-Graduação em Ciências Fisiológicas e aos seus alunos, professores e funcionários.

Ao projeto Coral Vivo e seus patrocinadores, Programa Petrobras Ambiental e Arraial d'Ajuda Eco Parque pela infraestrutura, logística e financiamento para a realização deste projeto.

Ao programa Ciências do Mar – CAPES, pela concessão da bolsa de doutorado.

Aos membros do grupo de pesquisa, em especial a Laura Marangoni e Joseane Marques pela realização dos experimentos de campo e pelos ensinamentos científicos desde a graduação.

Um agradecimento especial às pessoas que estiveram diretamente envolvidas com o desenvolvimento dessa tese, das quais tive oportunidade de formar grandes parceiras de trabalho, especialmente ao Yuri Zebral, Miguel Mies, Laura Marangoni e Joseane Marques. Sem vocês não teria conseguido!

Aos meus amigos pelas conversas, risadas e paciência nos momentos de consolo.

Aos professores Dr. Carlos Eduardo da Rosa, Dr. Flavio Manuel Rodrigues da Silva Júnior, Dr. Robert Boyle e Dr. Miguel Mies por terem aceitado compor a banca avaliadora e contribuírem com esse trabalho!

Por fim, agradeço às agências de fomento à pesquisa pelo financiamento:
CAPES, CNPq, IDRC e FAPESP.

Resumo geral

Os recifes de corais encontram-se ameaçados por mudanças climáticas globais e impactos locais. Apesar disso, pouco se sabe sobre os efeitos combinados desses estressores em corais. O cobre é amplamente conhecido como perturbador da capacidade energética e oxidativa. Devido a isso, a exposição ao cobre pode reduzir a sensibilidade de corais a eventos de mudanças climáticas. Portanto, é imprescindível a avaliação combinada desses estressores. Assim, o objetivo foi investigar os efeitos combinados do estresse térmico e da exposição ao cobre em enzimas do metabolismo energético (hexoquinase, piruvato quinase, lactato desidrogenase, citrato sintase, isocitrato desidrogenase e cadeia transportadora de elétrons), no comportamento trófico (marcadores de autotrofia e heterotrofia) e no estado oxidativo (peroxidação lipídica e capacidade antioxidante total) do coral *Mussimilia harttii*, endêmico dos recifes do Atlântico Sul. Para isso, os corais foram expostos em um mesocosmo marinho a três temperaturas (25.0, 26.6 e 27.3 ºC) e três concentrações de cobre (2.9, 5.4 e 8.6 µg/L) por até 12 dias. Ainda avaliamos o efeito da exposição ao cobre *in vitro* na atividade de enzimas do metabolismo energético e determinamos o funcionamento do metabolismo do coral em condições de campo. Os resultados mostraram que a combinação dos estressores inibe enzimas do metabolismo energético, além disso, demonstramos na exposição *in vitro* que o cobre pode interagir diretamente com enzimas especificas do metabolismo energético e contribuir para essa inibição. Mostramos também que a combinação dos fatores afeta parâmetros específicos no coral, uma vez enzimas do metabolismo energético se mostraram sensíveis à exposição, enquanto marcadores tróficos e oxidativos não foram alterados. Portanto, é possível que a inibição de enzimas do metabolismo energético possa contribuir para redução na produção de espécies reativas de oxigênio e consequente para a manutenção dos níveis basais dos marcadores tróficos e oxidativos. Os resultados demonstram que *M. harttii* pode ativar mecanismos fisiológicos baseados na diminuição do metabolismo para reduzir o estresse causado pela exposição combinada aos estressores.

Palavras-chave: Aquecimento global, Estresse oxidativo, Inibição enzimática, Poluição química, Recifes de coral.

Abstract

Coral reefs are threatened by global climate change and local impacts. Despite this, little is known about the combined effects of these stressors on coral. Copper is widely known to disrupt energy and oxidative capacity. Because of this, exposure to copper can reduce the sensitivity of corals to climate change events. Therefore, the combined assessment of these stressors is essential. Thus, the aim of this thesis was to evaluate the combined effects of thermal stress and copper exposure on energy metabolism enzymes (hexokinase, pyruvate kinase, lactate dehydrogenase, citrate synthase, isocitrate dehydrogenase and electron transport chain) on trophic behavior (markers of autotrophy and heterotrophy) and in the oxidative status (lipid peroxidation and total antioxidant capacity) of the *Mussimilia harttii* coral, endemic to South Atlantic reefs. For this, corals were exposed in a marine mesocosm to three temperature (25.0, 26.6 and 27.3 ºC) and three copper concentrations (2.9, 5.4 and 8.6 µg/L) for up to 12 days. We further evaluated the effect of *in vitro* copper exposure on the activity of energy metabolism enzymes and determined the functioning of coral metabolism under field conditions. The results showed that the combination of stressors inhibits enzymes of energy metabolism, in addition, we demonstrated in *in vitro* exposure that copper can directly interact with specific enzymes involved in energy metabolism and contribute to this inhibition. We also show that the combination of factors affects specific parameters in coral, since enzymes of energy metabolism were sensitive to combined exposure, while trophic and oxidative markers were not altered. Therefore, it is possible that the inhibition of energy metabolism enzymes may contribute to a reduction in the production of reactive oxygen species and, consequently, to the maintenance of basic levels of trophic and oxidative markers. The results demonstrate that *M. harttii* can activate physiological mechanisms based on decreased energy metabolism to reduce the stress caused by combined exposure to stressors.

Keywords: Chemical pollution, Coral reefs, Enzymatic inhibition, Global warming, Oxidative stress.

1. Introdução geral

Os recifes biogênicos são estruturas rígidas formadas por diferentes organismos marinhos que secretam esqueleto calcário e são resistentes à ação de ondas e correntes marinhas (Leão, 1994). No caso dos recifes de corais, esses são formações construídas principalmente por organismos coralíneos pertencentes à Ordem Scleractinia, dos quais tem maior participação na produção das estruturas rochosas. Os recifes de corais são considerados um tipo de recife biogênico. Embora os corais tenham um papel de destaque na formação das estruturas tridimensionais, é importante ressaltar que é necessária a ação conjunta de vários organismos formando uma complexa teia de associações e sucessões, tornando esses ambientais a maior estrutura produzida por seres vivos do planeta (Figura 1) (Castro e Zilberberg, 2016).

Figura 1: Associação de diversos organismos em recife de coral no Brasil, Recife de Fora, Porto Seguro, Bahia. Imagem retirada de Castro e Zilberberg, 2016.

Os recifes de corais de águas rasas são considerados um dos ecossistemas mais produtivos e biologicamente diversos do mundo. Eles ocupam menos de 1% da superfície do oceano, mas abrigam 25% das espécies marinhas (Spalding et al., 2001; Knowlton et al., 2010; Sheppard et al., 2018). Esse aspecto faz com que os recifes de corais possuam um alto valor econômico e uma elevada diversidade e complexidade biológica (Goldberg, 2013). Mais de 100 países abrigam recifes de corais costeiros, sendo que a maior parte são localidades pobres e dependentes da produtividade desses ecossistemas para a subsistência. Do ponto de vista físico, os recifes de corais protegem as regiões costeiras da ação de ondas e tempestades, incluindo diversas áreas do litoral costeiro. Em relação aos ganhos econômicos, os recifes de corais movimentam a economia através da atividade de turismo e pesca (Moberg e Folk, 1999; Castro e Zilberberg, 2016). Devido a grande diversidade e abundância de espécie, os recifes de corais possuem uma grande beleza cênica, tornando-os pontos turísticos importantes. A alta diversidade biológica também proporciona uma teia alimentar de grande complexidade. Um ambiente rico em alimentos culmina na presença de importantes predadores, incluindo peixes de alto valor comercial utilizados para a alimentação humana. Em consequência de sua grande complexidade biológica, os ambientes coralíneos também fornecem matéria-prima para a fabricação de produtos com alto potencial farmacológico (Castro e Zilberberg, 2016). A competição por espaço nesses ambientes faz com que muitas espécies produzam substâncias químicas com o potencial para serem utilizadas na produção de antibióticos (Dias e Lima, 2016).

Embora os recifes de corais estejam presentes em muitos locais do mundo, as maiores formações coralíneas estão concentradas em regiões rasas tropicais (Castro e Zilberberg, 2016). Os corais formadores de recifes, conhecidos, como corais pétreos ou verdadeiros, são organismos com uma estreita faixa de tolerância ambiental e estão confinados em regiões com águas claras, quentes, de elevada salinidade e profundidade, de aproximadamente 50 metros (Goldberg, 2013). Atualmente, 30% das regiões costeiras tropicais abrigam recifes de corais totalizando aproximadamente 600.000 Km^2 de área coberta (Spalding et al., 2001). Em virtude de sua alta produtividade, os recifes de corais de águas rasas podem atingir milhares de quilômetros de extensão, como a Grande Barreira de Recife de Coral da Austrália, que possui 2.000 km de área construída por organismos coralíneos, podendo ser vista inclusive do espaço (Castro e Zilberberg, 2016).

O sucesso da produtividade e diversidade dos recifes de corais de águas rasas é proporcionado graças à associação simbiótica entre corais e endossimbiontes fotossintetizantes (dinoflagelados do gênero *Symbiodinium,* conhecidos popularmente como zooxantelas). Essa relação é fundamental para o crescimento das estruturas calcárias e possibilita que recifes de corais se desenvolvam em ambientes com baixa oferta de nutriente (Davy et al., 2012). Essa relação mutualística confere benefícios para ambos os organismos. A associação entre cnidários e zooxantelas é importante para a nutrição de muitos corais. Embora eles sejam mixotróficos, essa relação simbiótica permite aos corais contar com uma fonte adicional de nutrição advinda de suas algas fotossintetizantes. De forma geral, o coral hospedeiro fornece gás carbônico (CO_2), nutrientes inorgânicos produzidos pelo seu metabolismo e maior

proteção contra a herbivoria. Em contrapartida, as zooxantelas oferecem os produtos resultantes da fotossíntese, como carbono orgânico e oxigênio (O_2) (Figura 2). Tais produtos fornecidos pelas zooxantelas dão suporte energético às funções fisiológicas vitais do hospedeiro. Uma consequência importante dessa simbiose é que, devido à natureza fotossintética dos simbiontes, corais zooxantelados apresentam maiores taxas de deposição de esqueleto de carbonato de cálcio ($CaCO_3$) (Marangoni, 2016).

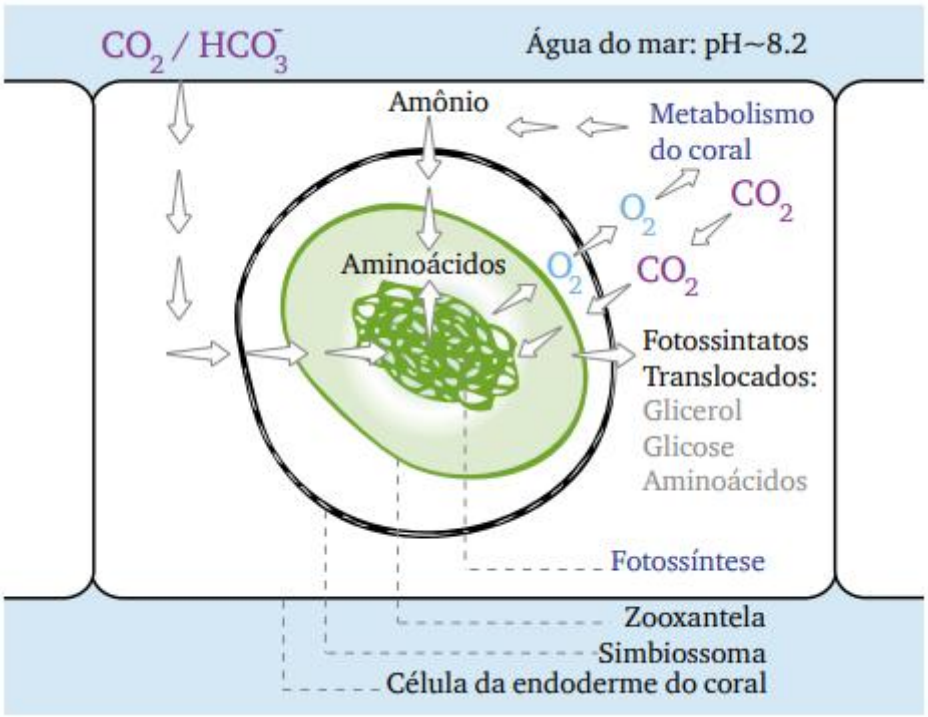

Figura 2: Principais trocas metabólicas da simbiose entre corais e zooxantelas. Imagem retirada de Marangoni, 2016.

A história geológica e biológica de diferentes regiões do planeta interfere na diversidade de espécies de coral nos recifes. Com relação às espécies de corais construtoras de recifes, os recifes do Indo-Pacífico apresentam a maior diversidade de espécie, abrigando cerca de 605 espécies de corais-pétreos zooxantelados (Castro e Zilberberg, 2016). Por sua vez, recifes do Atlântico Sul apresentam uma diversidade mais baixa. Por exemplo, o Caribe apresenta

maior concentração de espécie do Atlântico Sul, com 65 espécies de corais-pétros zooxantelados (Veron, 2000). O Brasil possui os únicos recifes verdadeiros do Atlântico Sul, entretanto com uma baixa diversidade, possuindo apenas 17 espécies de corais-pétros zooxantelados. Embora apresente uma baixa diversidade de espécies coralíneas construtoras, os recifes de corais brasileiros são únicos, possuindo uma fauna distinta, com alto grau de endemismo (49% das espécies) (Castro e Zilberberg, 2016), considerando a presença de corais-pétros zooxantelados (Ordem scleractinia), corais-negros (Ordem Antipatharia), corais-de-fogo ou hidrocorais (famílias Milleporidae e stylasteridae) e octocorais (subclasse Octocorallia). Além disso, eles ocorrem em locais com alto grau de turbidez e concentração de nutrientes, característica única dos recifes brasileiros quando comparado a recifes de outras regiões do mundo (Castro e Pires, 2001; Castro e Zilberberg, 2016; Mies et al., 2020).

Dentre as espécies construtoras de recifes no Brasil, destaca-se *Mussismilia harttii* (Verrill, 1868) (Figura 3), um coral-pétreo zooxantelado que ocorre desde o Rio Grande do Norte até o norte do Espirito Santo. Este desempenha importante papel ecológico na construção dos recifes no Brasil (Castro e Pires, 2001), embora atualmente encontra-se listada como espécie ameaçada de extinção (Leão et al., 2016; IUCN, 2019).

Figura 3: Pólipo do coral *Mussismilia harttii*, popularmente conhecido como coral-vela. Fonte Coral Vivo.

Mudanças climáticas globais (aquecimento e acidificação) e impactos locais (poluição, sobrepesca, turismo desordenado, etc) têm causado sérias preocupações na forma como afeta a biodiversidade dos ecossistemas marinhos. Entre as pressões causadas pelo homem está o aumento da emissão de CO_2 na atmosfera, que tem como consequência o aquecimento global. Dentre os ecossistemas marinhos, os recifes de coral se destacam quanto a sua sensibilidade aos efeitos do aumento da temperatura e da contaminação química, o que ameaça a contribuição dos recifes de coral para as diferentes formas de vida (Doney et al., 2012).

O aquecimento global tornou-se uma realidade preocupante para todos os ecossistemas, em virtude das consequências socioeconômicas e ambientais do aumento da temperatura em escala global. Entretanto, seus impactos ainda devem aumentar nos próximos anos. O Painel Intergovernamental sobre mudanças climáticas (IPCC) prevê um aumento de 0.2 ºC na temperatura

média global por década, sendo esperado um aumento de 2.6 (cenário otimista) a 4.8ºC (cenário pessimista) até o ano de 2100 (IPCC, 2014). O aumento da temperatura já tem causado alterações na biosfera levando a uma significativa mudança na distribuição e abundância das espécies ao redor do globo. Uma lista resumida dessas alterações incluem declínios e mudanças na população de organismos (Reid et al., 1998; Helmuth et al., 2002), expansão de espécies invasoras (Occhipinti-Ambrogi, 2007) e branqueamento de recifes de corais (Hoegh-Guldberg 1999, Suzuki et al. 2007, Hughes et al., 2018).

Os ecossistemas aquáticos são um dos mais vulneráveis aos efeitos do aumento da temperatura, pois a maioria das espécies aquáticas são ectotérmicas. A temperatura tem um papel fundamental na fisiologia dos organismos ectotérmicos, causando alterações nas reações bioquímicas que podem resultar em diferentes disfunções fisiológicas (Hochachka and Somero 2002). Ainda, é importante ressaltar que o aumento da temperatura não é o único estressor fisiológico para organismos aquáticos. Os impactos locais ocupam um lugar de destaque quanto aos seus efeitos na biota, entretanto ainda são poucas informações na literatura sobre os efeitos fisiológicos da combinação do aumento da temperatura e impactos locais em organismos aquáticos (Sokolova and Lannig, 2008), especialmente em corais. Portanto, faz-se necessário entender os efeitos combinados de estressores nesses organismos, uma vez que a poluição química pode reduzir a capacidade de tolerância dos corais aos cenários de mudanças climáticas (Nystrom et al., 2001; Sokolova and Lannig, 2008).

A contaminação química ocorre quando há a entrada de uma determinada substância ou elemento no ambiente ou no organismo em uma

concentração maior do que naturalmente encontrado (Mazzuco, 2008). No caso dos ecossistemas aquáticos, esses estão entre os principais destinos finais de contaminação (Duda, 1993; Muller et al., 2002). Atividades agrícolas, urbanas e industriais são as principais causas do aumento do nível de contaminação em ambientes marinhos (van Dam et al., 2011). Entretanto, a contaminação por metais é uma das mais preocupantes, pois os metais não são degradados, uma vez no meio ambiente permanecem por centenas de anos causando efeitos nocivos aos seres vivos. O cobre (Cu), por exemplo, é um metal comumente encontrado na natureza, sendo o aporte de esgoto o principal fator que eleva as concentrações deste metal no ambiente marinho (Santore et al., 2001).

O Cu pode ser encontrado em diferentes formas na natureza. Na sua forma metálica (Cu^{0}), íons cuprosos (Cu^{+1}), cobre cúbrico (Cu^{+2}) e trivalente (Cu^{+3}). A forma Cu^{+2} está frequentemente no ambiente aquoso, da qual pode interagir com diferentes compostos orgânicos e inorgânicos (Barceloux, 1999). Alterações de parâmetros físico-químicos da água, como a redução da dureza, pH, alcalinidade e da concentração de matéria orgânica pode aumentar a disponibilidade do Cu para a biota (Santore et al., 2001). Esse metal desempenha um papel importante para as diferentes formas de vida. O Cu é um metal essencial e atua como cofator para o funcionamento de várias enzimas, como a catalase, citocromo oxidase, superóxido dismutase, além de ser auxiliar na síntese do grupo heme e na absorção do ferro (Barceloux, 1999). Apesar de ser um metal importante para as funções fisiológicas, o Cu em altas concentrações pode causar efeito tóxico. Assim, com o objetivo de tentar controlar as emissões desse metal no ambiente aquático, a legislação

ambiental brasileira estipula as concentrações máximas de 5 e 9 µg/L de Cu para água salgada e doce, respectivamente (CONAMA 357). Essas concentrações são provavelmente encontradas em áreas costeiras no mundo todo, porém estudos já reportaram em recifes de corais costeiros concentrações ainda mais altas (Jones, 2010). Importante salientar que a legislação também não considera a alteração da disponibilidade e toxicidade deste metal na presença de outros fatores, como altas temperaturas. Isso destaca a necessidade de serem aprofundados estudos sobre os efeitos do Cu associados a outros estressores.

Dentre os mecanismos de toxidade do Cu amplamente conhecidos destacamos aqueles relacionados à inibição da atividade enzimática. Esse metal pode interagir diretamente com grupos sulfidrilas (-SH) presente nas proteínas e alterar a estruturação (Dash, 1989) ou interferir no estado oxidativo dos organismos (Guaratini et al., 2007) e causar a oxidação de proteínas, lipídios e DNA. Em relação ao metabolismo energético, sabe-se que o Cu pode interferir na produção de energia através da alteração da atividade de enzimas-chave envolvidas em diferentes etapas do metabolismo (Vieira et al., 2009; Anni et al., 2019; Zebral et al., 2020) e/ou por sua capacidade em ativar mecanismos de respostas de detoxificação energeticamente custosos (Anni et al., 2019).

A produção de energia nas células envolve a participação de enzimas-chave que realizam reações catabólicas e anabólicas. As reações catabólicas que ocorrem na respiração celular envolve a extração de energia armazenada nas moléculas de glicose, lipídeos e proteínas. Essa energia é utilizada em processos oxidativos que vão resultar na produção de trifosfato de adenosina

(ATP). As etapas da respiração celular envolvendo a quebra da glicose podem ser divididas em glicólise, o ciclo de Krebs, a cadeia transportadora de elétrons e a fosforilação oxidativa. A glicólise é a primeira via catabólica descrita, tal processo ocorre anaerobicamente no citoplasma das células, onde se dá a conversão de uma molécula de glicose em duas moléculas de piruvato, ATP e NADH. Esse processo ocorre através de 10 reações, catalisadas por enzimas diferentes, destacando-se: hexoquinase, fosfofrutoquinase e piruvato quinase. Tais enzimas-chave regulam a velocidade das reações da via glicolítica, catalisam reações irreversíveis e são reguladas por produtos metabólicos (Berg et al., 2002).

A hexoquinase catalisa a primeira reação da via glicolítica transferindo um grupo fosfato para molécula de glicose formando glicose-6-fosfato. Esse produto pode ser mantido na via glicolítica ou ingressar na via das pentoses fosfato. A fosfofrutoquinase transfere um grupo fosfato para frutose-6-fosfato formando frutose-1.6-bifosfato. O último passo da via glicolítica é catalisado pela piruvato quinase, da qual transfere de um grupo fosforil do fosfoenolpiruvato para o ADP, formando como produto o piruvato e o ATP (Navarro e Boveris, 2007; Nelson & Cox, 2008) (Figura 4).

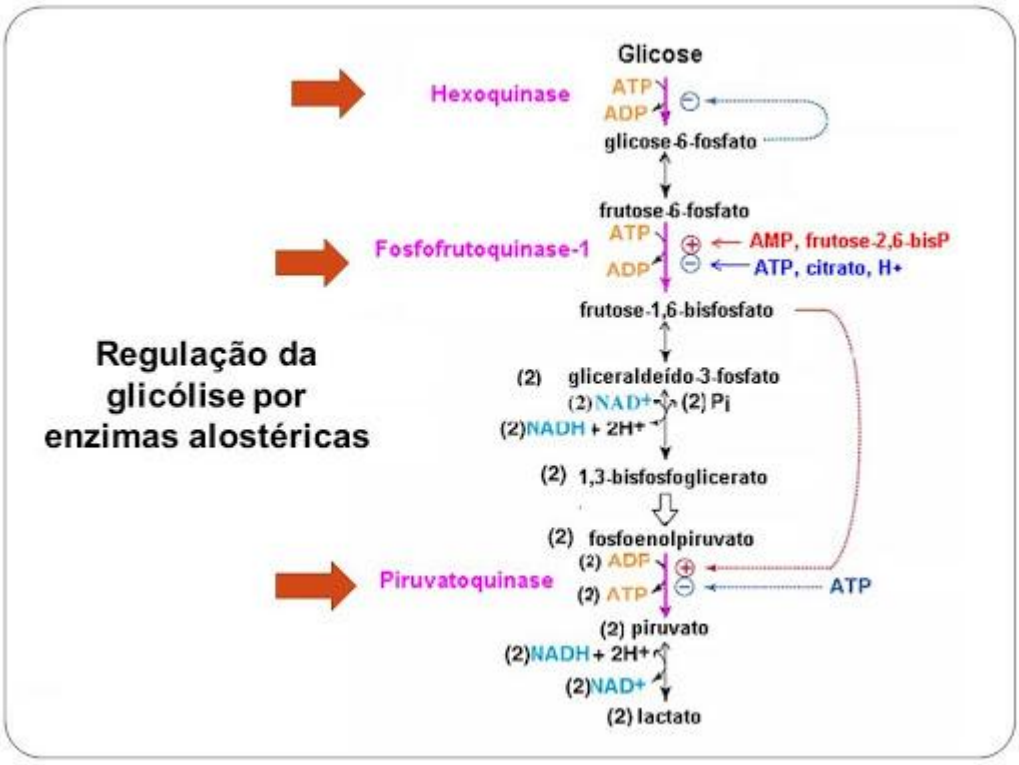

Figura 4: Imagem demonstrando o papel das enzimas-chave da glicólise.

Fonte: http://slideplayer.com.br/slide/1603180/

O piruvato formado na glicólise pode percorrer caminhos distintos no metabolismo. Na ausência de oxigênio, ele é reduzido a lactato pela enzima lactato desidrogenase, da qual utiliza elétrons do NADH e em consequência recicla NAD^+ permitindo a continuação da glicólise (Campbell e Farrell, 2006) (Figura 5).

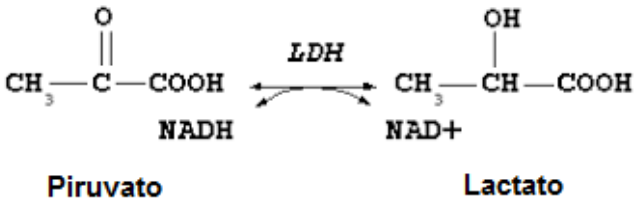

Figura 5: Reação da lactato desidrogenase, enzima-chave do metabolismo anaeróbico. Fonte: https://www.eurofins-biomnis.com/referentiel/liendoc/precis/LDH.pdf

Nos tecidos sob condições aeróbicas, o piruvato entra na mitocôndria e é convertido pela piruvato desidrogenase a Acetil CoA com perda do seu grupo carboxila na forma de CO_2. O Acetil CoA ingressa no chamado ciclo de Krebs e sofre uma condensação com oxaloacetato para a formação de citrato. Essa é a primeira reação do ciclo de Krebs, catalisada pela citrato sintase (Navarro e Boveris, 2007). O ciclo de Krebs é uma via anfibólica do qual o citrato formado pela ação da citrato sintase é oxidado por meio de mais sete reações que produz CO_2 e a energia dessa oxidação é armazenada em coenzimas NADH e $FADH_2$. Além da citrato sintase, a isocitrato desidrogenase também catalisa uma etapa importante no ciclo de Krebs. A oxidação do isocitrato a α-cetoglutarato e CO_2 é catalisada por essa enzima, formando como produto o NADPH, essencial para a reoxidação da glutationa nos sistemas antioxidantes. A oxidação completa do citrato no ciclo de Krebs produz três moléculas de NADH, uma molécula de $FADH_2$ e uma molécula de GTP (Berg et al., 2002) (Figura 6).

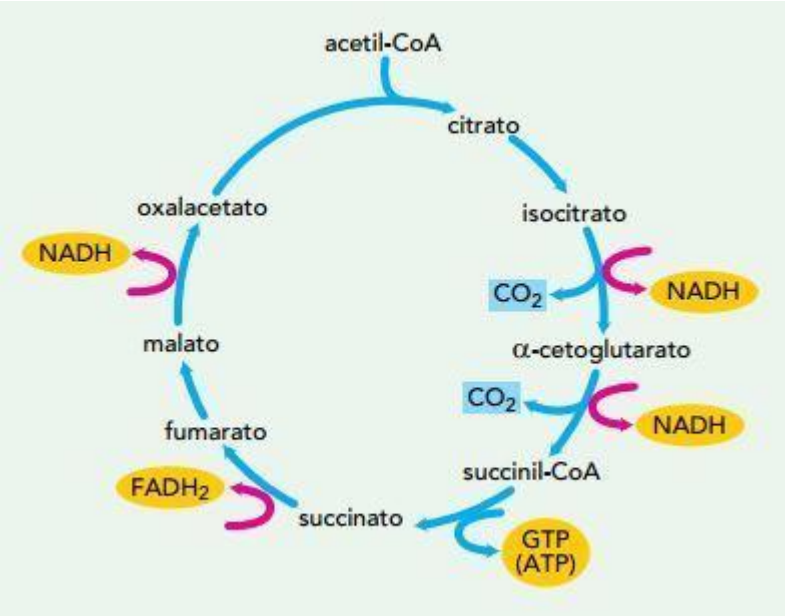

Figura 6: Imagem demonstrando as etapas do ciclo de Krebs. Fonte: http://qnint.sbq.org.br/novo/index.php?hash=molecula.503

As coenzimas reduzidas (NADH e $FADH_2$) formadas na glicólise e ciclo de Krebs fornecem elétrons para os complexos da cadeia de transportadora de elétrons mitocondrial, impulsionando a produção de ATP. A cadeia transportadora de elétrons é composta por quatro complexos proteicos fixos (I, II, III e IV) e dois complexos móveis (ubiquinona e citrocromo c) presentes na membrana interna da mitocôndria. Os complexos fixos I e II recebem os elétrons das coenzimas NADH e $FADH_2$, respectivamente, transferem aos complexos III e IV e consequentemente ao oxigênio reduzindo-o a água. À medida que os elétrons vão sendo transportados pelos complexos, esses bombeam prótons para o espaço intermembranas, causando um gradiente eletroquímico entre as membranas mitocondriais. Tal gradiente se desfaz através da ATP sintase presente na membrana interna da mitocôndria, da qual permite o transporte de prótons para a matriz mitocondrial. Esse processo gera

uma energia cinética indispensável para a produção de ATP via fosforilação oxidativa (Figura 7).

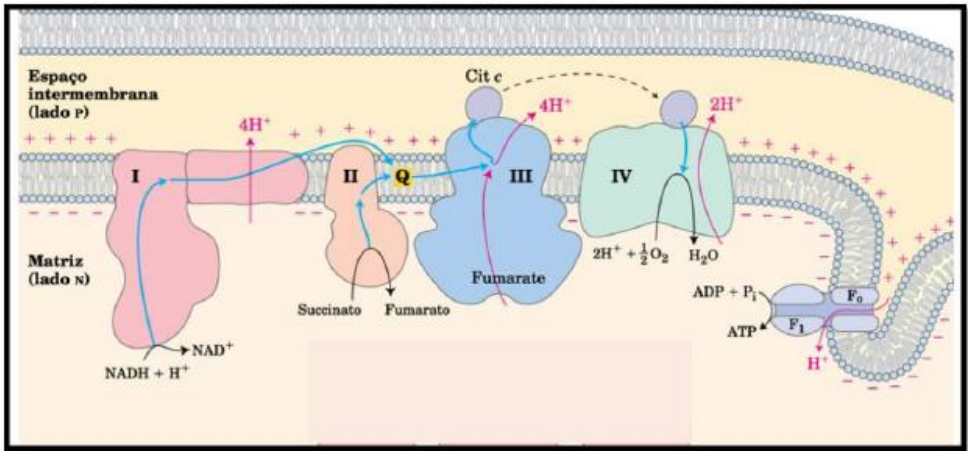

Figura 7: Cadeia transportadora de elétrons. Fonte: Nelson & Cox, 2008.

Outro importante mecanismo de ação do Cu do qual afeta o funcionamento correto da atividade enzimática está relacionado à alteração do estado oxidativo dos organismos. O Cu pode aumentar a geração de espécies reativas de oxigênio (ERO), como o radical hidroxila (OH•), por meio de sua participação em reações de Fenton e Haber-Weiss (reação 1 e 2, respectivamente) (Kalyanaraman et al., 2013). Além disso, o Cu pode inibir a atividade de enzimas antioxidantes, potencializando esses efeitos na geração de estresse oxidativo (Guaratini et al., 2007).

(1) $Fe^{2+}/ Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/ Cu^{+2} + OH^{-} + OH\bullet$ Reação de Fenton

(2) $O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe^{2+}/ Cu^{+}} O_2 + OH^{-} + OH\bullet$ Reação de Haber-Weiss

Reação 1 e 2: Participação do Cu na formação de OH• em reações de Fenton e Haber-Weiss, respectivamente.

As ERO possuem um papel fundamental nos processos fisiológicos e bioquímicos do organismo, para isso há um equilíbrio refinado entre a sua formação e remoção. A produção de tais moléculas ocorre em condições naturais devido ao transporte de elétrons entre os complexos proteicos presentes na membrana interna da mitocôndria e no cloroplasto (no caso de organismos fotossintetizantes). No caso da respiração, o oxigênio (O_2) recebe quatro elétrons reduzindo-se a molécula de água (H_2O). Esse processo é fundamental para a produção de ATP no metabolismo aeróbico (Campbell and Farrell, 2006).

O controle da redução completa do O_2 a H_2O é garantido pela citocromo oxidase, localizada na parte terminal da cadeia transportadora de elétrons. Apesar disso, uma pequena parte pode sofrer uma redução incompleta (10 a 15%) e resultar na formação de produtos intermediários como o radical ânion superóxido (O_2^{-}•) e posteriormente por meio de outras reações produzirem OH• e peróxido de hidrogênio (H_2O_2) (Barbosa et al., 2010). Espécies reativas de nitrogênio (ERN), como óxido nítrico (NO•) e peroxinitrito ($ONOO^-$) também podem ser formadas. Sabe-se que as ERN estão envolvidas na sinalização celular e podem desencadear o branqueamento de cnidários (Peres e Weis, 2006; Weis, 2008; Hawkins e Davy, 2013; Marangoni et al., 2019b). A presença de estressores ambientais pode provocar uma alteração no equilíbrio entre pró-oxidantes e antioxidantes (Guaratini et al., 2007). O estresse oxidativo pode ser definido como um desequilibrio em favor dos pró-oxidantes e/ou em

desfavor dos antioxidantes causando danos no DNA, lipídeos e proteínas (Sies, 1993) ou alteração na sinalização redox (Jones, 2006).

Mais de duas décadas de intensos estudos sobre a fisiologia de corais têm mostrado que o estresse oxidativo desencadeia o processo de branqueamento (Lesser, 1997; Downs et al., 2002; Weis, 2008; Nielsen et al., 2018). Corais podem abrigar milhões de zooxantelas, sendo que a pigmentamentação de seus tecidos é resultado da cor dos pigmentos fotossintetizantes presentes nessas microalgas (Marangoni et al., 2016). O branqueamento caracteriza-se pela redução dessa pigmentação em função da expulsão das algas simbiontes pelo coral hospedeiro ou degradação dos pigmentos fotossintéticos. Dessa forma, é possível visualizar a coloração branca do esqueleto de $CaCO_3$, por essa razão que tal fenômeno é conhecido como branqueamento (Marangoni, 2016).

O estresse oxidativo causa um desequilíbrio na associação coral-simbionte quando há um aumento na produção ERO devido a alterações no transporte de elétrons na cadeia fotossintética dos endossimbiontes (Lesser, 2006; Weis, 2008; Yakovleva et al., 2009). Em condição de estresse, altas quantidades de ERO são difundidas para os tecidos do coral em níveis que excedem a capacidade antioxidante desse organismo, onde danos a biomoléculas podem ocorrer e essa resposta conduzir ao branqueamento (Glynn, 1993; Downs et al., 2002). Assim, o branqueamento seria uma resposta final de defesa do coral, onde a principal fonte de ERO é erradicada, nesse caso as microalgas. Dependendo da intensidade e duração do estresse, o branqueamento pode causar a morte de corais, já que uma importante fonte de nutrição é perdida (Marangoni et al., 2016).

O aumento da temperatura é um dos fatores que mais causa branqueamento de corais. Episódios de branqueamento em massa têm aumentado a frequência e a intensidade desde a década de 80, coincidentemente com picos de eventos climáticos de El Niño (Baker et al., 2008). Dependendo da duração e da intensidade do estresse, o branqueamento pode causar a morte do coral. Porém, os corais também podem se recuperar após um evento de branqueamento e seus tecidos serem recolonizados por zooxantelas. Entretanto, quando isso não acontece pode ocorrer diversas consequências ecológicas associadas à perda do ecossistema recifal. A redução da capacidade fotossintética das zooxantelas afeta o metabolismo do coral, podendo causar a redução da formação das estruturas recifais, do processo reprodutivo e impactar a manutenção vida marinha associada ao recife de coral (Brown, 1997).

Diante de uma situação de estresse, os corais costumam responder alterando seu comportamento trófico predominante autotrófico para heterotrófico (Hughes e Grottoli et al., 2013; Mies et al., 2018; Marangoni et al., 2019b). Os ácidos graxos são considerados importantes marcadores para investigar mudanças no comportamento trófico em corais (Treignier et al., 2008; Mies et al., 2018). Por exemplo, ácido estearidônico (SDA, 18: 4ω3) e o ácido docosapentaenóico (DPA, 22: 5ω3) são fotossintatos translocados dos simbiontes para o hospedeiro (Papina et al., 2003; Mies et al., 2017); enquanto o ácido cis-gondóico (CGA, 20: 1ω9) é amplamente presente no zooplâncton capturado pelos corais (Dalsgaard et al., 2003; Mies et al., 2018). Com base na variação do conteúdo desses ácidos graxos no tecido coral, é possível avaliar mudanças de predominância entre o comportamento autotrófico e heterotrófico

(Mies et al., 2018). Variações nesses marcadores estão relacionadas à resposta ao estresse (Marangoni et al., 2019b).

Embora as algas simbiontes representem as principais fontes de ERO, a produção direta dessas espécies oxidantes também ocorre pelo metabolismo do coral, o que potencializa os danos e acelera o processo de branqueamento. Apesar dos corais hospedeiros e seus endossimbiontes contarem com um sistema de defesa antioxidante, esse pode não ser suficiente para lidar com altas quantidades de ERO, e danos podem ocorrer (Marangoni, 2016).

O sistema de defesa antioxidante tem como objetivo inibir, reduzir e/ou reparar os danos causados pelas ERO e ERN. Esse sistema é dividido em enzimático e não-enzimático. O sistema de defesa antioxidante enzimático é composto pela superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (Grd). A SOD é o primeiro sistema enzimático a atuar, acelerando a dismutação do O_2-• em H_2O_2. Posteriormente, a enzima CAT e GPx impedem o acúmulo de H_2O_2. A CAT catalisa a transformação do H_2O_2 em H_2O e O_2. Tal ação integrada é de grande importância, uma vez que o H_2O_2, por meio das reações de Fenton e Haber-Weiss, resulta na formação do OH• contra o qual não há sistema enzimático de defesa. Para a GPx catalisar a neutralização do H_2O_2 em H_2O, ela necessita de um equilíbrio adequado do ciclo redox da glutationa, uma vez que ela utiliza os elétrons da glutationa reduzida (GSH) e transforma em sua forma oxidada (GSSG). Esse equilíbrio é dado pela Grd, da qual é responsável pela recuperação da GSH, possibilitando a manutenção da integralidade do ciclo redox e, consequentemente, do equilíbrio adequado entre os sistemas de defesa enzimáticos (Barbosa et al., 2010) (Figura 8). Ainda, algumas outras

enzimas antioxidantes são importantes na manutenção do ciclo da GSH. A glutationa S-transferase (GST), por exemplo, catalisa a conjugação da GSH com produtos eletrolíficos formados durante a peroxidação lipídica, facilitando a sua eliminação. Além disso, a glutamato cisteína ligase (GCL) também apresenta um papel importante no ciclo da GSH, catalisando a primeira etapa da formação da GSH a partir da conjugação de cisteína e glutamato (Hermes Lima, 2004).

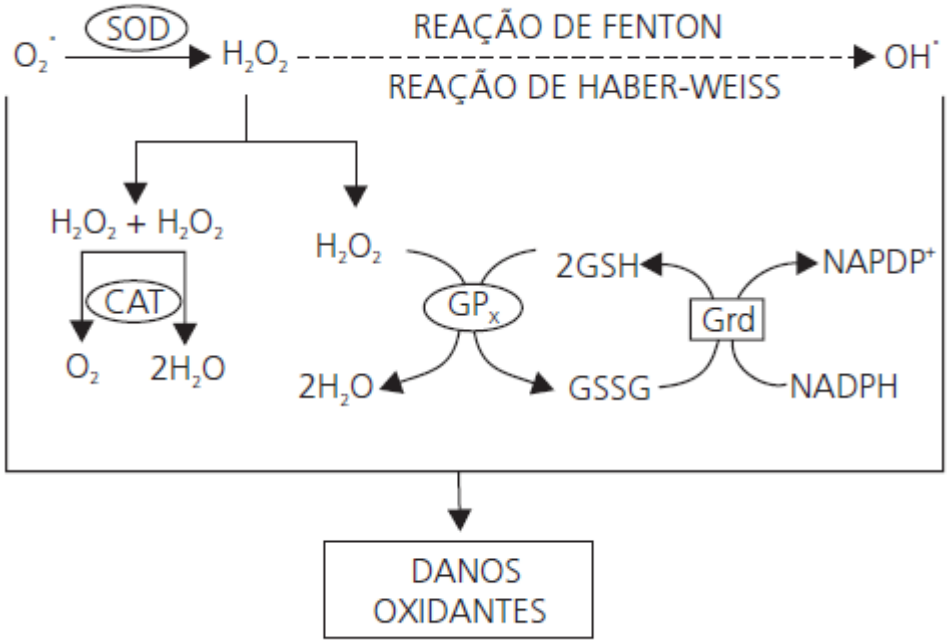

Figura 8: Atuação integrada do sistema de defesa antioxidante enzimático.

Fonte: Barbosa et al. 2010.

Como mencionado anteriormente, o sistema de defesa antioxidante enzimático não é capaz de atuar diretamente na neutralização do OH•. Assim, fica clara a importância do sistema de defesa antioxidante não-enzimático na prevenção e reparação de danos oxidativos (Pamplona e Constantini 2011). Os

antioxidantes não enzimáticos são moléculas de origem endógena ou que podem ser obtidas através da alimentação. Dentre aquelas de origem endógenas podemos destacar a GSH, as tiorredoxinas e o ascorbato. Enquanto aquelas obtidas através da alimentação, temos os carotenoides e tocoferóis (Barbosa et al., 2010; Pamplona e Constantini 2011). Devido à alta complexidade e diversidade de moléculas antioxidantes, a avaliação geral do estado oxidativo dos organismos frente à exposição de estressores torna-se uma condição de alto custo. Pela existência dessa limitação, a determinação da capacidade antioxidante total tem sido uma importante alternativa para avaliar a resposta conjunta dos antioxidantes.

Sabe-se que 95% dos recifes de corais costeiros estão sob ameaça direta à ação combinada de mudanças climáticas globais e impactos locais (Burke et al., 2011; Hughes et al., 2018). Portanto é de extrema necessidade a avaliação dos efeitos combinados desses estressores. Isso é particularmente importante em espécies de corais dos recifes do Atlântico Sul. Os recifes de coral dessa região estão cada vez mais expostos à contaminação por Cu associada a esgotos e rompimentos de barragens (Francini-Filho et al., 2019; Marques et al., 2019). Considerando o exposto, o objetivo dessa tese foi entender os efeitos da exposição do coral endêmico *Mussismilia harttii* a três temperaturas (temperatura média do local de 25.0, e 1.5 e 2.5 ºC acima da média, que são níveis semelhantes aos cenários climáticos futuros emitidos pelo IPCC (IPCC, 2014)) combinadas a três níveis de contaminação por Cu (0, 3 e 5 µg/L acima da concentração média natural) em diferentes parâmetros bioquímicos e fisiológicos.

Na primeira linha de avaliação foi investigado o efeito isolado e combinado desses estressores na atividade de enzimas-chave envolvidas em diferentes etapas do metabolismo energético do coral. Esses resultados compõem o artigo apresentado no capítulo I, publicado no periódico *Chemosphere.* Considerando os resultados obtidos no capítulo I, o segmento objetivou avaliar o metabolismo energético do coral *M. harttii* em condições de campo e o efeito da exposição ao Cu *in vitro* na atividade de enzimas-chave do metabolismo energético. O objetivo do capítulo II foi desvendar um possível mecanismo de ação do Cu na atividade de enzimas do metabolismo energético. Os resultados do capítulo II estão publicados no periódico *Comparative Biochemistry and Physiology, Part C.* O capítulo III está relacionado à avaliação dos efeitos combinados do estresse térmico e da exposição ao Cu em marcadores tróficos e no estado oxidativo do coral hospedeiro e seus simbiontes. A hipótese era que a exposição combinada ao aumento de temperatura e Cu alterasse o estado oxidativo do coral e isso explicaria os efeitos inibitórios na atividade enzimática observados no capítulo I. Os resultados do capítulo III estão publicados no periódico *Environmental Pollution.*

2. Objetivos

2.1. Objetivo geral

O objetivo geral desta tese foi avaliar o efeito isolado e combinado do aumento da temperatura e da exposição ao Cu sobre parâmetros fisiológicos e bioquímicos no coral *Mussismilia harttiii.*

2.2. Objetivos específicos

1) Avaliar o efeito isolado e combinado do aumento da temperatura e da exposição ao Cu na atividade de enzimas pertencentes à glicólise (hexoquinase, piruvato quinase e lactato desidrogenase), ciclo de Krebs (citrato sintase e isocitrato desidrogenase), cadeia transportadora de elétrons (sistema de transporte de elétrons) e via das pentoses-fosfato (glicose-6-fosfato desidrogenase) no coral *Mussismilia harttii.*
2) Analisar o estado metabólico em condições de campo e o efeito da exposição ao Cu *in vitro* na atividade de enzimas-chave envolvidas em diferentes etapas do metabolismo energético (piruvato quinase, lactato desidrogenase, citrato sintase e sistema de transporte de elétrons) no coral *Mussismilia harttii.*
3) Determinar o efeito isolado e combinado do aumento da temperatura e da exposição ao Cu em marcadores tróficos (ácido estearidônico, SDA; ácido docosapentaenóico, DPA; e ácido cis-gondóico, CGA) e no estado oxidativo (peroxidação lipídica e capacidade antioxidante toral) do coral *Mussismilia harttii* e de seus simbiontes.

3. Capítulo I

Este capítulo é representado pelo artigo **"Energy metabolism enzymes inhibition by the combined effects of increasing temperature and copper exposure in the coral *Mussismilia harttii*"** já publicado no periódico *Chemosphere* (DOI: https://doi.org/10.1016/j.chemosphere.2019.124420). O trabalho pode ser encontrado na página seguinte.

Energy metabolism enzymes inhibition by the combined effects of increasing temperature and copper exposure in the coral *Mussismilia harttii*

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Abstract

The combined effects of exposure to increasing temperature and copper (Cu) concentrations were evaluated in the zooxanthellate scleractinian coral *Mussismilia harttii.* Endpoints analyzed included activity of enzymes involved in glycolysis (pyruvate kinase, PK; lactate dehydrogenase, LDH), Krebs cycle (citrate synthase, CS; isocitrate dehydrogenase; IDH), electron transport chain (electron transport system, ETS) and pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PDH). Coral polyps were kept under control conditions (25.0 ± 0.1°C; 2.9 ± 0.7 µg/L Cu) or exposed to combined treatments of increasing temperature (26.6 ± 0.1°C and 27.3 ± 0.1°C) and concentrations of dissolved Cu (5.4 ± 0.9 and 8.6 ± 0.3 µg/L) for 4 and 12 days using a mesocosm system. PK activity was not affected by stressors. LDH, CS, IDH, ETS and G6PDH activities were temporally inhibited by stressors alone. CS, ETS and G6PDH activities remained inhibited by the combination of stressors after 12 days. Furthermore, all combinations between increasing temperature and exposure Cu were synergistic after prolonged exposure. Taken together, stressors applied alone led to temporary inhibitory effects on energy metabolism enzymes of the coral *M. harttii,* however, prolonged exposure reveals strong deleterious effects over the metabolism of corals due to the combination of stressors. The present study is the first one to give insights into the combined effects of increasing temperature and Cu exposure in the energy metabolism enzymes of a scleractinian coral. Findings suggest that moderate Cu contamination in future increasing temperature scenarios can be worrying for aerobic and oxidative metabolism of *M. harttii.*

Keywords: Copper; Climate change; Coral; Electron transport chain; Glycolysis; Krebs cycle.

Highlighs

- Exposure to increasing temperature and Cu inhibits energy metabolism enzymes.
- Stressors applied alone led to temporary effects on enzymatic activity.
- CS, ETS and G6PDH remained inhibited by the combination of stressors after 12 days.
- The interactive effects were all became synergistic after 12 days.
- Combination of stressors affects the aerobic and oxidative metabolism

1. Introduction

Coral reefs are among the most vulnerable marine ecosystems to climate change (Hughes et al., 2017, 2018). According to future projections made by the Intergovernmental Panel on Climate Change (IPCC), mean global temperature will likely experience an increase of 0.2ºC per decade. In the best and worst cases an increase of 2.6ºC and 4.8ºC are predicted until the year of 2100, respectively (IPCC, 2014). These scenarios are extremely worrying for ectothermic organisms, such as corals (Hoegh-Guldberg and Smith, 1989), since the increase in 1–2ºC above the maximum temperature recorded during summer season have resulted in bleaching and death of corals (McWilliams et al., 2005).

In addition to global climate, coral reefs have been threatened by local impacts (Nystrom et al., 2001; van Dam et al., 2011), and concern about the combined effects of global climate change and local impacts in corals has increased considerably in the last decades (Sokolova and Lannig, 2008). In this context, copper (Cu) contamination has been a relevant physiological stressor (van Dam et al., 2011). A variety of anthropogenic sources such as discharges of domestic sewage, agricultural and industrial activities have considerably increased Cu releases into aquatic environments worldwide (Maria and Bebianno, 2011). Although Cu is an essential micronutrient for living organisms (Morgan, 2000), exposure to excessive concentrations of this metal can cause physiological impairment in corals (Bielmyer et al., 2010; Marangoni et al., 2017; Fonseca et al., 2019). Studies on the interactive effects of increasing temperature and Cu exposure are necessary to understand the mechanisms

involved in corals responses to multiple stressors future scenarios (Nystrom et al., 2001; Sokolova and Lannig, 2008).

Temperature has a key role in the metabolism of ectotherms. Increasing temperature may reduce aerobic scope due to a mismatch between oxygen supply and demand (Portner, 2001, 2002). In addition, the exposure to metals, such Cu, may increase energy costs for detoxification, cellular protection and repair (Rainbow, 2002; Kuo et al., 2006; Silva et al., 2014). Therefore, the reduction of aerobic metabolism due to elevated temperatures can reduce organism tolerance to exposure to metals by reducing available energy for detoxification and/or damage repair. These responses create a physiological basis for occurrence of increased deleterious effects in organisms as a consequence of the combined effects of increasing temperature and metals exposure.

Bioenergetics approaches focused in cellular mechanisms have been proposed to evaluate environmental stress (Sokolova et al., 2012). The pyruvate kinase (PK) and lactate dehydrogenase (LDH) are key-enzymes from glycolysis pathway. PK catalyzes the transfer of a phosphate group from phosphoenolpyruvate to ADP, producing pyruvate and ATP. Under anaerobic conditions, pyruvate is converted into lactate by LDH recycling the NADH to NAD+ for glycolysis. On the other hand, in aerobic conditions, pyruvate is oxidized into acetyl-CoA that undergoes into the Krebs cycle for further production of reduced co-enzymes (NADH and $FADH_2$). The citrate synthase (CS) and isocitrate dehydrogenase (IDH) are mitochondrial enzymes performing an essential role in the Krebs cycle.

Reduced co-enzymes (NADH and $FADH_2$) formed in the glycolysis and Krebs cycle are responsible for the production of ATP in the electron transport chain located on the inner membrane of the mitochondria (Nelson and Cox, 2008). The activity of the electron transport system (ETS) was a method used to measure the activity of the complexes of the transport chain. Lastly, glucose-6-phosphate dehydrogenase (G6PDH) is a cytosolic key enzyme of the pentose-phosphate shunt. The main function of the pathway is biosynthesis and NADPH production (Livingstone, 1981). NADPH is an important cofactor for glutathione reoxidation counteracting the oxidative stress condition (Carvalho and Fernandes, 2008). Due to the key role of these enzymes in organism's energy metabolism, they can be used as interesting bioenergetic markers to assess the impacts of multiple stressors (Doucet-Beaupré et al., 2010; Sokolova, 2013). Nonetheless, studies on zooxanthellate corals have not been conducted in this regard prior to date.

Considering the aforementioned, we hypothesized that (i) the exposure of corals to the isolated effects of increasing temperature and Cu may impair their energy metabolism by inhibiting the activity of key enzymes involved in glycolysis (pyruvate kinase, PK; lactate dehydrogenase, LDH), Krebs cycle (citrate synthase, CS; isocitrate dehydrogenase; IDH), electron transport chain (electron transport system, ETS) and pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PDH); and (ii) that the combination of such stressors intensify such deleterious effects. In order to test this hypothesis, *Mussismilia harttii*, a zooxanthellate scleractinian coral considered as a key species in the construction of South Atlantic reefs (Laborel, 1969; Mazzei et al., 2017), was exposed to increasing temperature, different Cu concentrations and

combination of both in a mesocosm system, and endpoints involved in the energy metabolism were evaluated after 4 and 12 days of exposure. The present study is the first one to bring insight into the combined effects of increasing temperature and Cu exposure on the activity of enzymes involved in different stages of energy metabolism in a scleractinian coral.

2. Materials and Methods

2.1 .Coral collection and maintenance

Polyps of six colonies of the scleractinian coral *M. harttii* were collected in July 2012 by scuba diving in the conservation area of the Municipal Natural Park of Recife de Fora (16°24′31″S; 038°58′39″W) in Porto Seguro, Bahia, northeastern Brazil. Mean temperature and dissolved Cu concentration in sea water were 25.2–25.8°C (NOAA, 2012) and 2.9 ± 0.7 μg/L (Fonseca et al., 2017), respectively. Coral polyps were transferred to the mesocosm facility of the Coral Vivo Project (Arraial d'Ajuda, Porto Seguro), individualized, glued on ceramic plates using cyanoacrylate, and acclimated to the experimental conditions for 20 days. Daily and seasonal variations of the environmental parameters were maintained, such as temperature, salinity, pH, light intensity, photoperiod, rainfall, and food sources. Details on the mesocosm experimental system and functioning were previously described by Duarte et al. (2015). The polyps were collected under the permission of the Brazilian Environmental Agency (IBAMA/SISBIO; permit #34256-1).

2.2 . Experimental conditions

Seawater was pumped from a fringing reef at 500 m from the coast (Araçaípe Beach, Arraial d´Ajuda, northeastern, Brazil) into four 5000-L underground primary treatment sumps to receive the desired temperature treatments before being pumped into 36 test aquaria (10-L). Three temperature treatments were tested following scenarios predicted for the next centuries (IPCC 2014): (a) average temperature of local seawater; (b) 1°C above the temperature of local seawater; and (c) 2°C above the temperature of local seawater. Temperature was monitored and controlled by a computerized system. In parallel, seawater was also held into 4 pairs of 1000-L underground secondary treatment sumps to receive the desired Cu treatments. Cu stock solutions were daily prepared from a standard solution of $CuCl_2$ (1 g/L Cu). In the secondary treatment sumps, seawater received 0, 30 and 50 mL of the $CuCl_2$ stock solution to obtain the desired nominal concentrations of 0, 30 and 50 µg/L Cu, respectively. Seawater contaminated with Cu was prepared 24 h prior its use in the experimental system to allow the complete equilibration of Cu with seawater (Lauer et al., 2012). After 24 h of preparation, seawater from the secondary treatment sumps (with and without Cu addition) was mixed in line with seawater coming from the primary treatment sumps (with and without temperature treatment) using peristaltic pumps (flow rate of 0.169 L/min) before reaching the 10-L aquariums. Seawater contaminated with Cu represented 10% of the total seawater flow rate reaching the 10-L aquariums leading to the final nominal concentrations of 0, 3 and 5 µg/L. The concentrations of Cu tested are close or below the limits allowed by the international and Brazilian legislations for the protection of aquatic environments (CONAMA, 2005; EPA, 2005). The

highest concentration tested (5 µg/L) represents the quality criteria for marine environments.

Temperature and Cu treatments were applied alone and combined, for up to 12 days. Treatments were performed in triplicate, with polyps being randomly distributed in aquaria (four polyps per aquarium). After 4 and 12 days of exposure to the experimental treatments, coral polyps (n = 6 per treatment, 2 polyps per aquarium) were collected and frozen in liquid nitrogen for further analyses of biochemical parameters.

2.3. Seawater collection and analysis physicochemical parameters

Water samples were collected every three days over the experimental period from the aquaria to measure Cu concentration. Water samples (non-filtered and filtered with 0.45-µm mesh filters) were acidified with HNO_3 (1% final concentration; SupraPur, Merck, USA) and desalted according Nadella et al. (2009) for total (non-filtered sample) and dissolved (filtered sample) Cu concentrations determination [atomic absorption spectrophotometry with coupled graphite furnace (Perkin-Elmer, USA)]. Data on dissolved organic carbon (DOC) concentration (Total Organic Carbon analyzer, Shimadzu, Japan), pH (pH meter, model HI 9124, Hanna Instruments, USA), pluviometry (local weather station, Veracel Celulose, Brazil) and salinity (optical refractometer, model ITREF 10, Instrutemp, São Paulo, SP, Brazil) were also assessed. Temperature measurements were obtained using loggers (loggers HOBO Water Temp Pro, Onset, USA) installed inside the tanks and at the reef environment to continuously monitor the seawater temperature every 30 min.

2.4. Samples preparation for biochemical analysis

For the determination of enzymatic activity, coral pieces were cut (~200mg), homogenized with 200µL of imidazole buffer (50mM, pH 7.8) containing 0.1mM of PMSF and centrifuged (10,000g, 4^{o}C, for 20min). The supernatant obtained from the coral holobiont (coral tissue and zooxanthellae) homogenate was used as enzymatic source. All evaluations were performed by spectroscopy with a microplate reader (ELx808IU, BioTek Instruments, Inc, Winooski, VT, USA), under 25^{o}C (corals ambient temperature according to NOAA 2012). Enzymatic activity was normalized by the homogenate total protein content (Bradford Reagent, Sigma, St. Louis, MO, USA) and expressed as enzyme units /mg total protein.

2.4.1. Activity of glycolysis enzymes

The activity of the pyruvate kinase (PK) and lactate dehydrogenase (LDH) were performed according to Lallier and Walsh (1991) protocols, with some modifications. Experimental conditions were as follows: PK: 50 mM Imidazole (pH 7.4), 10 mM $MgCl_2$, 30 mM KCl, 0.12 mM NADH, 20U/ml lactate dehydrogenase, 0.5 mM phosphoenolpyruvate and 2.5 mM ADP; LDH: 0.2 M Imidazole (pH 7.4), 0.15 mM NADH and 8 mM sodium pyruvate. PK and LDH activity were determined following decrease of absorbance by the oxidation of NADH at 340 nm.

2.4.2. Activity of Krebs cycle enzymes

Citrate synthase (CS) and isocitrate dehydrogenase (IDH) were performed according to the protocols described by Lallier and Walsh (1991) and

Alp et al. (1976), respectively. Experimental conditions were as follows: CS: 50 mM HEPES (pH 8.1), 0.1 mM acetyl coenzyme A, 0.1 mM DTNB and 0.5 mM oxaloacetic acid. CS activity was recorded following the increase in absorbance due to the reduction of DTNB at 412 nm; IDH: 50 mM Imidazole (pH 7.4), 1 mM $MnCl_2$, 8 mM $MgCl_2$, 0.5 mM NADPH, 5mM DL-Isocitric acid. IDH activity was determined following the reduction of NADP to NADPH at 340nm.

2.4.3. Activity of the electron transport chain

Electron transport system (ETS) was modified from Lannig et al. (2003). Experimental conditions were 0.1 M sodium phosphate (pH 8.5), 0.85 mM NADH, 125µM NADPH, 2 mM iodonitrotetrazolium chloride (INT) and 0.20 % Triton X-100. ETS activity was determined by reduction of INT, which yields an increase in absorbance at 490 nm.

2.4.4. Activity of the phosphate pentoses pathway

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined according to Carvalho and Fernandes (2008). Experimental conditions were: 80 mM glycerin (pH 7.6), 10mM $MgCl_2$, 0.39 mM NADPH and 5mM glucose-6-phosphate. G6PDH activity was determined following the increase in absorbance by the reduction of NADPH at 340 nm.

2.5. Data presentation and statistical analyses

Data were expressed as mean ± standard error. At each time point, the enzymatic activities were compared using two-way ANOVA followed by the Fisher test for multiple comparisons. Data normality and homogeneity were

tested with the Shapiro-Wilk and Cochran C tests, respectively. Data were log-transformed to meet the ANOVA assumptions when necessary. When significant effects on the interaction term were indicated by ANOVA the expected additive inhibition (van Dam et al., 2015; Marques et al., 2017; Marangoni et al., 2019) was calculated to evaluate the interaction. According to Crain et al. (2008) additive effect is assumed when the interaction between the stressors is not significant or when the observed combined effect is the same as predicted; antagonism is assumed when the observed combined effect is smaller than predicted; the synergistic effect was considered when the observed effects is greater than predicted. The confidence level adopted was 95% ($\alpha = 0.05$).

3. Results

3.1. Water physicochemical parameters

Data on physicochemical parameters during the experiment were previously described by Fonseca et al. (2017), since the samples were derived from the same experiment. Total Cu concentrations in the experimental media were 4.6 ± 0.7, 7.7 ± 0.5, and 10.7 ± 1.1 µg/L for the nominal concentrations of 0 (control), 3 and 5 µg/L, respectively. The dissolved Cu concentrations corresponded to 2.9 ± 0.7, 5.4 ± 0.9, 8.6 ± 0.3 µg/L, respectively. Mean values of the temperature treatments were 25.0 ± 0.1°C for control (ambient water temperature), 26.6 ± 0.1°C for 1°C above ambient water temperature and 27.3 ± 0.1°C for 2°C above ambient water temperature.

3.2. Activity of glycolysis enzymes

No effects were observed in PK activity for any of the treatments tested after 4 days (Fig. 1A) or 12 days (Fig. 1B) of exposure. In turn, an inhibitory effect of increasing temperature and exposure to Cu under ambient temperature was observed in LDH activity after 4 days of exposure (Fig. 2A). Fisher´s post hoc comparisons indicated that corals exposed to 8.6µg/L Cu at 25.0ºC (ambient temperature) presented a lower enzyme activity than control corals (25.0ºC and no Cu addition). Corals exposed to increasing temperature (26.6 and 27.3°C) alone also showed a lower LDH activity compared to those exposed to control conditions. A reduced LDH activity was also observed in corals exposed to different combinations of increasing temperature and Cu addition. Corals under 26.6 and 27.3°C, and exposed to concentrations of 5.4 and 8.6 µg/L Cu, showed lower LDH activity than corals maintained at ambient temperature (25.0°C) at same Cu concentrations. Antagonistic interactions were observed on corals exposed to 26.6 and 27.3ºC combined with 5.4 and 8.6 µg/L Cu (Fig 2A). After 12 days, LDH activity recovered to control values in all treatments tested (Fig. 2B).

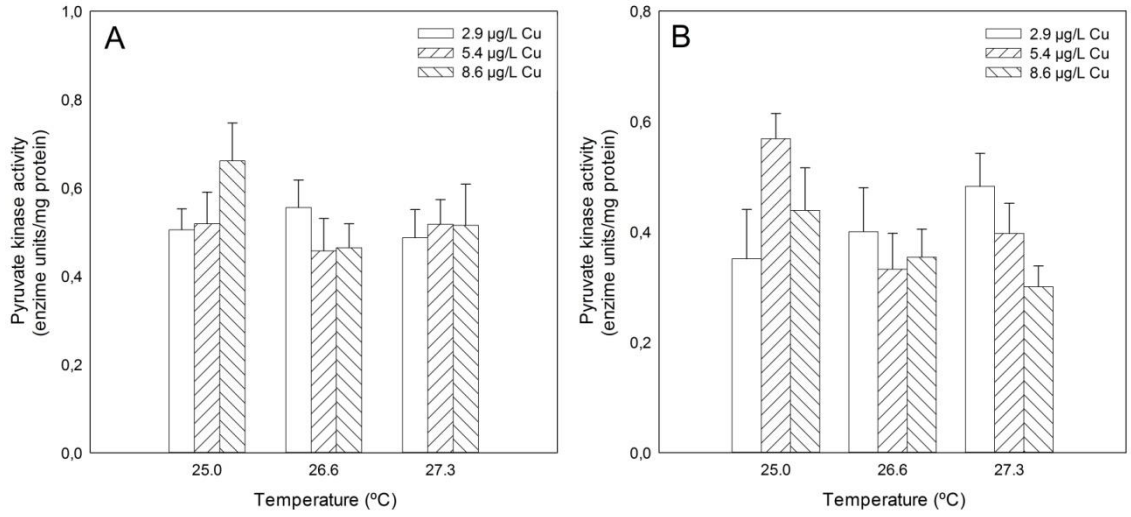

Figure 1: Pyruvate Kinase (PK) activity in the coral *Mussismilia harttii* exposed to increasing temperature, different Cu concentrations and combination of both

for 4 days (A) or 12 days (B). Data are expressed as mean ± standard error. Different lowercase letters indicate significantly different mean values (p≤0.05) among Cu concentrations for the same temperature treatment. Different uppercase letters indicate significantly different mean values (p≤0.05) among temperature treatments for the same Cu concentration.

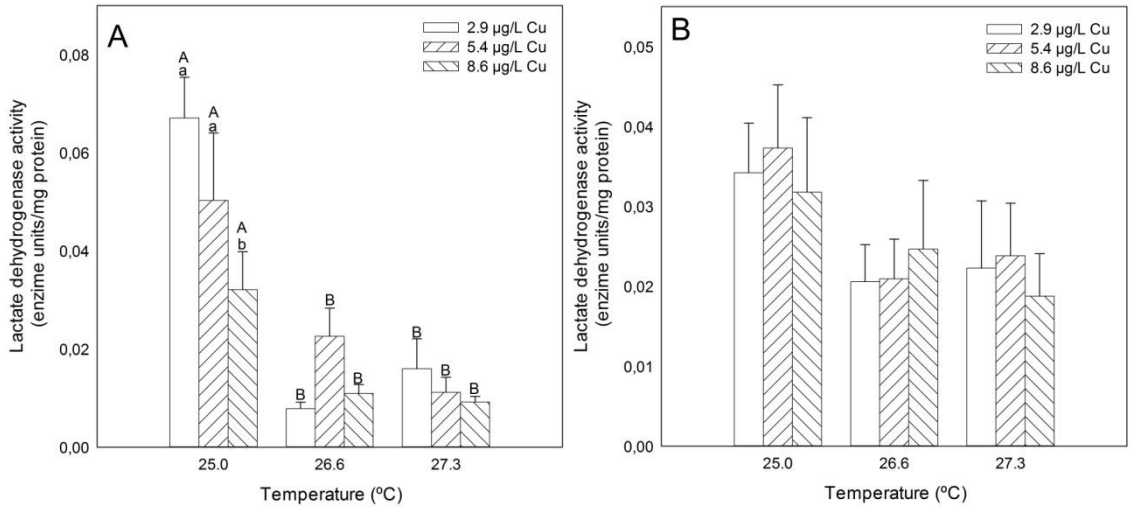

Figure 2: Lactate dehydrogenase (LDH) activity in the coral *Mussismilia harttii* exposed to increasing temperature, different Cu concentrations and combination of both for 4 days (A) or 12 days (B). Data are expressed as mean ± standard error. Different lowercase letters indicate significantly different mean values (p≤0.05) among Cu concentrations for the same temperature treatments. Different uppercase letters indicate significantly different mean values (p≤0.05) among temperature treatments for the same Cu concentration.

3.3. Activity of Krebs cycle enzymes

Significant effects were observed in CS activity after 4 days of exposure to isolated stressors (Fig. 3A). Fisher´s post hoc comparisons indicated a significant reduction in CS activity in corals exposed to increasing Cu concentrations (5.4 and 8.6 µg/L Cu) compared to control corals. Also, corals

exposed to increasing temperature (26.6 and 27.3°C) presented a lower CS activity than those exposed to the control condition. Effects due to the combination of stressors were not observed in CS activity after 4 days of exposure (Fig. 3A). After 12 days, CS activity in the isolated treatments recovered to control values, however, a significant effect was observed by the combination of stressors. Fisher´s post hoc comparisons indicated that corals exposed to 8.6 µg/L Cu at 27.3ºC, presented significantly lower enzyme activity than corals maintained at same Cu concentration at 25.0°C (ambient temperature). Synergistic interactions were observed in corals exposed to 27.3ºC and the higher Cu addition treatment (8.6 µg/L). Also, corals exposed to 8.6 µg/L Cu at 27.6°C, showed a lower CS activity than corals exposed 2.9 µg/L and 5.4 µg/L (Fig. 3B).

Significant effects of increasing temperature and Cu addition alone, and the combination of stressors were observed for IDH activity after 4 days of exposure (Fig. 4A). Fisher´s test indicated that corals exposed to Cu addition treatments (5.4 and 8.6 µg/L Cu) at 25.0°C (ambient temperature) presented significantly lower enzyme activity compared to corals maintained in control condition. Also, corals exposed to increased temperature (27.3°C) alone showed a lower IDH activity than those maintained at 25.0 and 26.6°C. Interestingly, a higher IDH activity was observed in corals exposed to 26.6ºC combined with Cu addition treatments of 5.4 and 8.6 µg/L compared to those exposed to the same Cu concentrations in combination to the other thermal treatments. Antagonistic interactions were observed in corals exposed to 26.6ºC combined with 5.4 and 8.6 µg/L Cu (Fig. 4A). After 12 days of exposure, effects

on IDH activity were no longer observed in any of the treatments tested (Fig. 4B).

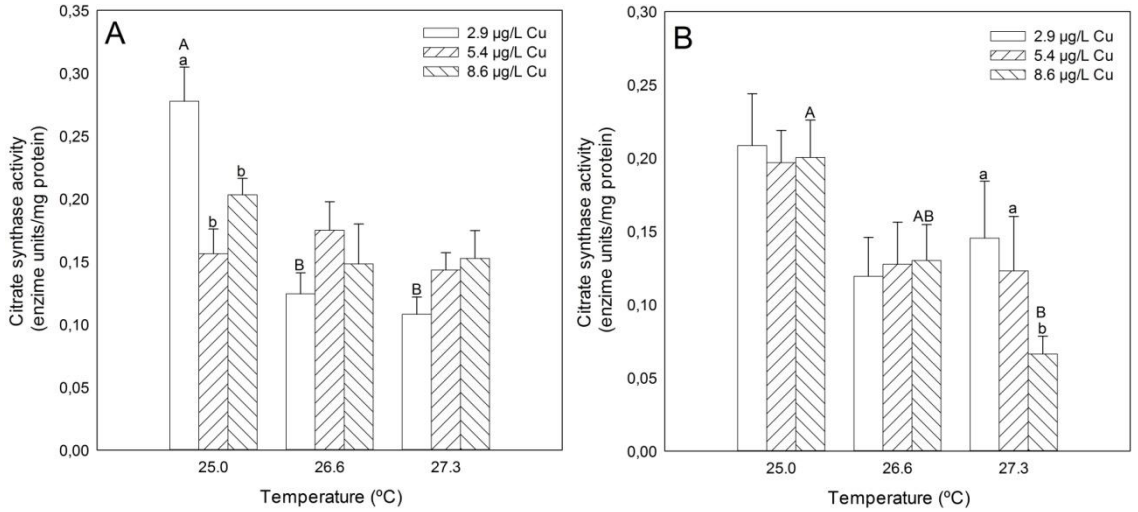

Figure 3: Citrate synthase (CS) activity in the coral *Mussismilia harttii* exposed to increasing temperature, different Cu concentrations and combination of both for 4 days (A) or 12 days (B). Data are expressed as mean ± standard error. Different lowercase letters indicate significantly different mean values (p≤0.05) among Cu concentrations for the same temperature treatments. Different uppercase letters indicate significantly different mean values (p≤0.05) among temperature treatments for the same Cu concentration.

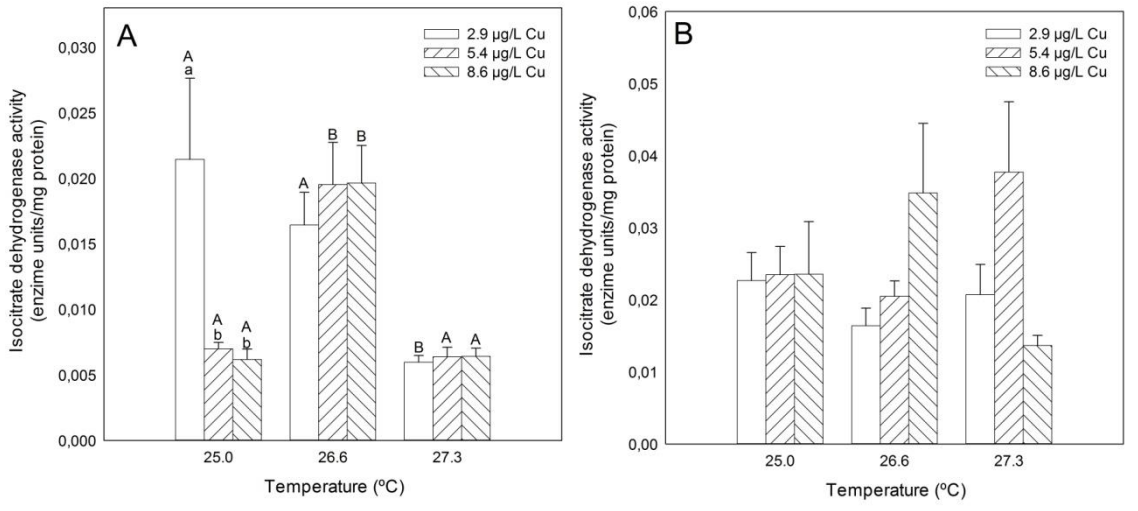

Figure 4: Isocitrate dehydrogenase (IDH) activity in the coral *Mussismilia harttii* exposed to increasing temperature, different Cu concentrations and combination of both for 4 days (A) or 12 days (B). Data are expressed as mean ± standard error. Different lowercase letters indicate significantly different mean values ($p \leq 0.05$) among Cu concentrations for the same temperature treatments. Different uppercase letters indicate significantly different mean values ($p \leq 0.05$) among temperature treatments for the same Cu concentration.

3.4. Activity of electron transport chain

Effects of increasing temperature and Cu addition alone were observed on ETS activity after 4 days (Fig. 5A). Fisher's test indicated an inhibition in ETS activity in corals exposed to 8.6 µg/L Cu. In turn, corals exposed to the increasing temperature (26.6 and 27.3°C) presented lower ETS activity than those exposed to the control condition. Also, corals under 26.6°C and exposed to 8.6 µg/L Cu showed higher ETS activity than those exposed only to increasing temperature (26.6°C) (Fig. 5A). After 12 days of exposure, ETS activity in the isolated treatments recovered to control values, however, a significant effect was observed by the combination of stressors (Fig. 5B). Fisher´s post hoc comparisons indicated that corals exposed to 8.6 µg/L Cu at 27.3ºC, presented significantly lower enzyme activity than corals maintained at same Cu concentration at 26.6ºC. Synergistic interactions were observed in corals exposed to 27.3ºC and the higher Cu addition treatment (8.6 µg/L) (Fig. 5B).

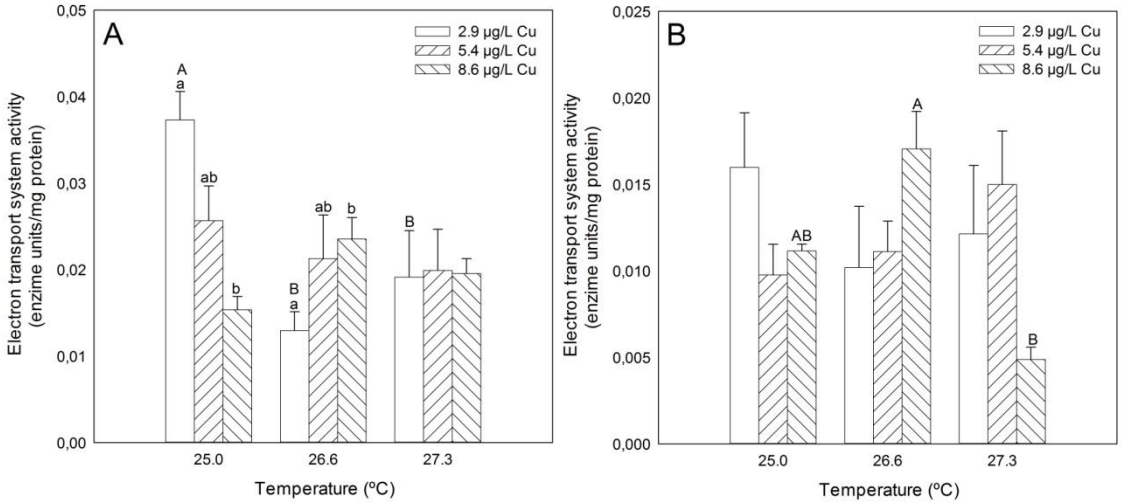

Figure 5: Electron transport system (ETS) activity in the coral *Mussismilia harttii* exposed to increasing temperature, different Cu concentrations and combination of both for 4 days (A) or 12 days (B). Data are expressed as mean ± standard error. Different lowercase letters indicate significantly different mean values (p≤0.05) among Cu concentrations for the same temperature treatments. Different uppercase letters indicate significantly different mean values (p≤0.05) among temperature treatments for the same Cu concentration.

3.5. Activity of the phosphate pentoses pathway

Significant effects of increasing temperature alone, as well as in the combination of stressors were observed for G6PDH activity after 4 days of exposure (Fig. 6A). Corals exposed to increasing temperatures (26.6 and 27.3°C) showed a lower G6PDH activity than those exposed to the control condition. At 26.6°C, corals exposed to 5.4 and 8.6 µg/L Cu showed higher G6PDH activity than those exposed to increasing temperature alone. In addition, at 27.3°C, corals exposed to concentrations of 5.4 and 8.6 µg/L Cu showed lower G6PDH activity than corals maintained at ambient temperature

(25.0°C) and at 26.6ºC under same Cu concentrations. Antagonistic interactions were observed in corals exposed to 27.3ºC combined with 5.4 and 8.6 µg/L Cu (Fig. 6A). After 12 days, enzyme activity recovered back to control values in the increasing temperature treatments alone, however, an effect of Cu addition alone and in the combination of stressors was observed. Higher G6PDH activity was detected in corals exposed to 8.6 µg/L Cu compared to those exposed to 5.4 µg/L Cu at ambient temperature (25.0°C). In addition, corals exposed to 8.6 µg/L Cu at 26.6°C showed an inhibition of G6PDH activity compared to corals maintained at ambient temperature (25.0°C) at the same Cu concentration. Corals exposed to 5.4 and 8.6 µg/L Cu at 27.3ºC presented a significant reduction in G6PDH activity compared to those maintained at ambient temperature (25.0°C) at the same Cu concentrations, and at 26.6ºC combined with 5.4 µg/L Cu. Synergistic interactions were observed in corals exposed to 26.6ºC combined with 8.6 µg/L Cu and to 27.3ºC combined with 5.4 and 8.6 µg/L Cu (Fig. 6B). A summary on the statistical results are presented in supplementary material.

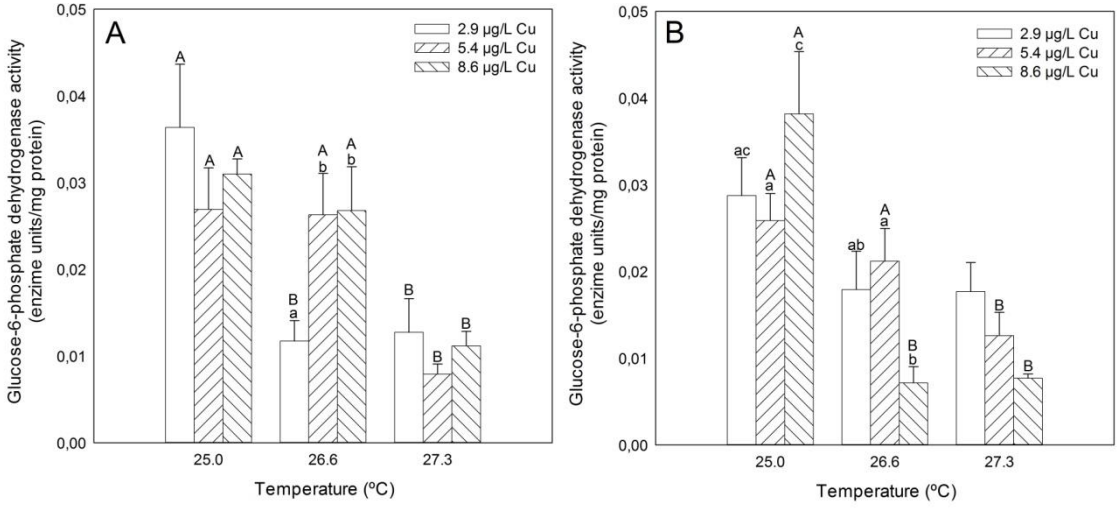

Figure 6: Glucose-6-phosphate dehydrogenase (G6PDH) activity in the coral *Mussismilia harttii* exposed to increasing temperature, different Cu

concentrations and combination of both for 4 days (A) or 12 days (B). Data are expressed as mean ± standard error. Different lowercase letters indicate significantly different mean values (p≤0.05) among Cu concentrations for the same temperature treatments. Different uppercase letters indicate significantly different mean values (p≤0.05) among temperature treatments for the same Cu concentration.

Table S1: Results of ANOVA of the biochemical parameters evaluated in *Mussismilia harttii* exposed to increasing temperature, different Cu concentrations and combination of both for 4 and 12 days. Significant values (p <0.05) are in bold.

	4 days			12 days		
	df	F	*P value*	df	F	*P value*
Pyruvate kinase						
Cu	2	0.3190	0.728597	2	0.9407	0.398806
Temp	2	0.6519	0.526130	2	1.5288	0.229199
Cu*Temp	4	0.7113	0.588730	4	2.0456	0.106306
Lactate dehydrogenase						
Cu	2	3.7554	**0.033014**	2	0.17085	0.843688
Temp	2	28.1287	**0.000000**	2	3.00015	0.063513
Cu*Temp	4	20.153	**0.000001**	4	0.45373	0.768952
Citrate synthase						
Cu	2	7.8830	**0.006516**	2	1.8808	0.135706
Temp	2	11.1192	**0.000151**	2	3.2557	0.050497
Cu*Temp	4	0.5652	0.572817	4	7.2776	**0.002276**
Isocitrate dehydrogenase						
Cu	2	6.42383	**0.016062**	2	0.9822	0.384872
Temp	2	20.5377	**0.000001**	2	0.0270	0.973389
Cu*Temp	4	4.8104	**0.003267**	4	1.8359	0.144673
Electron transport system						
Cu	2	9.2808	**0.003665**	2	0.2185	0.804925
Temp	2	6.54725	**0.011957**	2	0.4492	0.642198
Cu*Temp	4	2.4309	0.065264	4	2.7041	**0.048418**
Glucose-6-phosphate dehydrogenase						
Cu	2	0.5663	0.572207	2	0.5862	0.561666
Temp	2	19.8495	**0.000001**	2	1.55239	0.258733
Cu*Temp	4	2.8325	**0.037298**	4	3.6780	**0.013043**

4. Discussion

4.1. Effects of stressors alone

The glycolytic pathway is considered to be the major energy-generating pathway in invertebrates with low metabolism (Chang and O'Connor, 1983), capable of producing ATP and reduced nicotinamide adenine dinucleotide (NADH) under anaerobic conditions. No significant change in PK activity was observed in corals after 4 and 12 days of exposure to any of the experimental treatments here tested. Inhibition of PK activity in response to metals exposure was reported by many authors (Carvalho and Fernandes, 2008; Lauer et al., 2012; Anni et al., 2019) and these effects are related to a direct competition between metals and bivalent cations essential for the enzyme activity. Lauer et al. (2012) suggested that inhibition of PK activity in Cu-exposed crab *Neohelice granulata* could be due to a Cu-induced displacement of the Mg^{2+} necessary for the proper function of kinases, which in turn may induce variations on the enzyme conformation. Therefore, it is possible to infer that Cu concentrations tested in the present study were probably not sufficient to cause such effect in corals PK activity. In fact, Cu concentrations tested in the present study were a thousand times lower than those tested by the aforementioned studies.

PK activity is related to pyruvate production. In turn, pyruvate can be used by LDH and the pyruvate dehydrogenase complex (PDH) to form lactate and acetyl CoA, respectively. Since LDH and CS activity were inhibited by exposure to increasing temperature and Cu addition, pyruvate may be used for other purposes inside energy production. Murphy and Riachmond (2016) showed that scleractinian corals under hypoxic conditions increase the activity

of alanopine dehydrogenase (ADH) and strombine dehydrogenase (SDH) in order to favor the anaerobic respiration pathway over LDH. ADH and SDH could use pyruvate and recycle NADH to glycolysis (Livingstone, 1991) maintaining energy production through the anaerobic pathway. Also it is important to note that impairment induced by the present stressors tested on the activity of PDH cannot be ruled out.

In aquatic animals showing low metabolism, such as corals, the inhibition of LDH activity can seriously compromise the animal energy metabolism and homeostasis. It has been also observed that organisms under chemical stress can initiate physiological adjustments reducing aerobic metabolism, which can lead to anaerobiosis (Sokolova et al., 2012). However, this mechanism was not here observed for *M. harttii* when exposed to isolated stressors.

CS and IDH were chosen as representative enzyme of the Krebs cycle. Exposure to increasing temperature and Cu addition inhibited the activity of these enzymes. This leads to reduced NADH production for the electron carrier chain. Since CS and IDH are mitochondrial enzymes, its activities are closely related to the activity of the electron transport system (ETS). In the present study we were able to show that Cu exposure mainly affects mitochondrial enzymes (CS, IDH, ETS). Previous investigations have demonstrated that Cu can affect mitochondrial activity in aquatic invertebrates (Silva-Aciares et al., 2011; Lauer et al., 2012; Jorge et al., 2016). Deleterious effects can be possibly related to Cu accumulation in the mitochondrial membranes (Zischka et al., 2011; Lichtmannegger et al., 2016) and increased production of reactive oxygen species (Hosseini et al., 2014; Magriñá et al., 2017), which makes mitochondria

highly vulnerable to Cu (Vogel and Kemper, 1963). Recently, Anni et al. (2019) reported that exposure to Cu can reduce the expression of protein complexes of the electron transport chain in *Poecilia vivipara*. In addition, Jorge et al. (2016) showed in the marine clam *Mesodesma mactroides* presented a reduced activity of the respiratory Complex II after exposed to Cu. Therefore, the results of the present study suggest that exposure to Cu mainly affects corals aerobic metabolism.

G6PDH is considered to be the key enzyme of the pentose phosphate pathway, being responsible for generating ribose units necessary for nucleotide synthesis (Nelson and Cox, 2008). G6PDH activity produces NADPH, which contributes to biotransformation reactions, biosynthesis (lipid synthesis) and antioxidant system (Carvalho and Fernandes, 2008). The exposure to increasing temperature alone inhibited the activity of this enzyme after 4 days of exposure, which can induce metabolic and oxidative damages in coral. However, after 12 days of exposure to stressors alone a reestablishment of enzymatic activity was observed. In this context it is very likely that *M. harttii* may rely on physiological mechanisms to reestablish the energy functions in prolonged stress, such as increased heterotrophy (Levas et al., 2016).

4.2. Combined effects of stressors

The combination of stressors led to an overall inhibitory effect on the enzymatic activity related to corals energy metabolism. Deleterious effects due to the combination of stressors were observed on LDH activity after short exposure. The reduction in this enzyme activity may compromise an important source of energy production for *M. harttii*, considering that glycolysis is

important for the maintenance of energy in organisms with low metabolism. This result evidences the possibility of combined stressors in temporarily reduce energy production through anaerobic metabolism.

In spite of the fact that in most cases the combination of stressors led to inhibition effects on enzymatic activity, the exposure of *M. harttii* to low thermal stress (26.6°C) combined with Cu addition reduced the effects of the metal alone in IDH activity after 4 days of exposure. In this context, an increase in metabolism due to the higher temperature (Hochachka & Somero, 2002) may be related to the higher activity of IDH. Also, the recovery of the IDH activity in the present study may be related to detoxification pathways activation due to higher Cu concentrations. It is important to emphasize that the IDH activity plays an important role in energy and oxidative metabolism, and detoxification pathways (Jo et al., 2001) mainly related to exposure to metals (Smeets et al., 2005).

In parallel, Cu attenuating effect on G6PDH activity was observed for corals in the same thermal stress condition tested (26.6ºC). Metals such as Cu are essential nutrients for the metabolism of organisms, especially invertebrates (White and Rainbow, 1982). In fact, Ferrier-Pagès et al. (2018) showed that Cu is assimilated through heterotrophic feeding and can be important to corals recovery after thermal stress. Thus, we suggest that lower Cu concentrations can have short term beneficial effects over G6PDH activity in corals undergoing small increases in seawater temperature. Interestingly, an increase in ETS activity was also observed in this condition. Therefore, taken together, results indicate an increase in energy demand to repair the possible effects of Cu.

However, at 27.3ºC, this attenuating effect on G6PDH activity is not observed, and exposure to combined stressors inhibits the activity of this enzyme.

Despite of reestablishment of enzymatic activities after prolonged exposure, the inhibition effects of stressors remained on CS, ETS and G6PDH activities. It should be noted that interactions between increasing temperature and Cu exposure were temporarily antagonistic, and after 12 days, all became synergistic. Therefore, the combined effects of stressors seem to be aggravated with time. Inhibition of CS and ETS activity can disrupt the production of ATP via aerobic conditions (Nelson and Cox, 2008), while inhibition of G6PDH activity may reduce the NADPH synthesis for antioxidant enzymes (Carvalho and Fernandes, 2008) leading an oxidative stress.

Taken together, the results from enzymatic activities show that the exposure to stressors (alone and combined) induce a state of energy limitation in *M. harttii.* A possible explanation for this is that increasing temperature and Cu exposure induced oxidative stress (Lesser, 1996; Monserrat et al., 2007), resulting in inhibition of energy metabolism enzymes. Interestingly, an increase in oxidative damage was previously observed in *M. harttii* maintained on the same experimental conditions (Fonseca et al., 2017). Based on these results, we can infer that exposure to these stressors could negatively affect physiological processes such growth, energy storage or reproduction of corals. Interestingly, for both stressors used in this study the potential to affect corals growth has been extensively demonstrated (Bielmyer et al., 2010; Fonseca et al., 2017; Marangoni et al., 2017).

Finally, it is worth noting that the present study tested mean temperatures ranging from 25.0°C to 27.3°C. In addition, dissolved Cu concentrations tested

are close to concentrations allowed by South and North American environmental agencies (CONAMA, 2005 and EPA, 2005, respectively). Considering that maximum temperature values in the site of coral collection was 28.9°C during the summers of 2012 and 2013 (http://www.pmel.noaa.gov), even more severe impacts on coral health should be expected, especially in El Niño episodes. Therefore, findings reported here allowed us to give important insights on the effects induced by increasing temperature in combination with environmentally relevant concentrations of Cu through a new and relevant physiological perspective on corals.

5. Conclusion

The present study is the first one to give insights into the isolated and combined effects of increasing temperature and Cu exposure in the energy metabolism enzymes of a scleractinian coral. Taken together, results show that increasing temperature and Cu concentrations applied alone led to transient inhibitory effects on energy metabolism enzymes of the zooxanthellate scleractinian coral *M. harttii,* suggesting that *M. harttii* may have physiological mechanisms to reestablishing the energy functions under these conditions. However, after prolonged exposure (12 days), significant reduction of CS, ETS and G6PDH activities reveal strong deleterious effects over the aerobic and oxidative metabolism of corals due to the combination of stressors. Thus findings reported in the present study show that moderate Cu contamination in future increasing temperature scenarios can be worrying for aerobic and oxidative metabolism of corals. Furthermore, such bioenergetics approach

employed here may also be used to assess the health status of corals facing others kinds of stressor, such a decreased pH and other sorts of contaminants.

6. Acknowledgments

The present study was supported by the International Development Research Center (IDRC, Ottawa, Canada), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Programa Ciências do Mar, Brasília, DF, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq − Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática, Brasília, DF, Brazil). Thanks to the Coral Vivo Project and its sponsors: Petrobras (Programa Petrobras Ambiental) and Arraial d'Ajuda Eco Park. A. Bianchini is a research fellow of the Brazilian CNPq (Proc. # 307647/2016-1) and supported by the IDRC International Research Chair Program.

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4. Capítulo II

Este capítulo é representado pelo artigo **"Metabolic status of the coral *Mussismilia harttii* in field conditions and the effects of copper exposure *in vitro*"** já publicado no periódico *Comparative Biochemistry and Physiology, Part C* (DOI: https://doi.org/10.1016/j.cbpc.2020.108924). O trabalho pode ser encontrado na página seguinte.

Metabolic status of the coral *Mussismilia harttii* in field conditions and the effects of copper exposure *in vitro*

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Abstract

It is widely known that metals can alter enzyme functioning, however, little is known about the mechanisms of metal toxicity in energy metabolism enzymes of corals. Thus, the present study had two objectives: firstly, we evaluated the activity of eight metabolic enzymes of the coral *Mussismilia harttii* to clarify metabolic functioning under field conditions. After that, we investigated the *in vitro* effect of copper (Cu) exposure in the activity of an enzyme representative of each metabolism stage. We evaluated enzymes involved in glycolysis (hexokinase, HK; phosphofructokinase, PFK; pyruvate kinase, PK and lactate dehydrogenase, LDH), Krebs cycle (citrate synthase, CS and isocitrate dehydrogenase, IDH), electron transport chain (electron transport system activity, ETS) and pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PDH). The *in vitro* tests were performed through contamination of the reaction medium using Cu concentrations of 0, 1.4, 3.7 and 14.2 μg L^{-1}. The results showed that *M. harttii* has elevated activity of HK, PK and CS in field conditions compared to the activity of other energy metabolism enzymes evaluated. Moreover, lower activities of LDH and ETS in exposed samples were observed. In conclusion, in field conditions this species has elevated aerobic metabolism and glucose may be an important energetic fuel. Also, exposure to Cu *in vitro* caused inhibition of LDH and ETS by direct binding.

Keywords: Electron transport system; Glucose; Krebs cycle; Metal; Pentose phosphate pathway.

Highlights

- *M. harttii* in field conditions shows high activity of HK, PK and CS.
- Glucose may be an important energetic fuel to *M. harttii* under field conditions.
- Elevated aerobic metabolism was observed in field conditions.
- *In vitro* exposure to Cu inhibits LDH and ETS activity.
- Cu inhibits LDH and ETS activity by direct binding.

1. Introduction

Essential metals such as zinc, magnesium, copper, iron and calcium are of great importance for biological systems because of their participation in the structure of many proteins (Ekinci and Beydemir, 2010; Alim and Beydemir, 2012). Magnesium (Mg^{2+}), for example, is an important cofactor for energy metabolism enzymes. The deficiency of this metal can cause impairments in metabolic function and may affect many other physiological systems (Hans, 2002). In addition, if Mg^{2+} is removed from the enzymes by substitution with another metal, the enzyme activity may be inhibited (Hansen et al., 1992).

Metals can denature and inhibit enzyme activity by different mechanisms. For example, these elements are known to promote the generation of reactive oxygen species (ROS) through their participation in Fenton reactions (Wardman and Cadeias, 1996) or by depression in the antioxidant defense system (Jomova and Valko, 2011) leading to lipid peroxidation, DNA damage and impairments in enzymatic activity (Stohs and Bagchi, 1995; Ercal et al., 2001; Wang et al., 2004; Masso et al., 2007). Additionally, some metals have the ability to bind to functional groups of enzymes, such as sulfhydryl (-SH), or to replace the metal associated with the enzyme (Viarengo, 1994), leading to loss of function.

Coral reefs are known for being highly productive and for having an immense biological diversity (Costanza et al., 1997). These ecosystems occupy less than 1% of the ocean, however, they serve as shelter for 25% of all marine species (Spalding et al., 2001; Sheppard et al., 2018). Nearshore coral reefs are more likely exposed to pollution (Fabricius, 2005; Leão and Kikuchi, 2005; Smith et al., 2008). Contamination with metals, such as Cu, is a notable local

impact threatening coral reefs in many locations worldwide (Carpenter et al., 2008; van Dam et al., 2011). The pollution of marine environments by Cu occurs by coastal run-off, mining and mainly by the presence of sewage (Jones, 1997). Although it is an essential metal (Belyaeva et al., 2011), exposure to Cu can cause an imbalance of the oxidative status, reduced energy metabolism and induce enzymatic inhibition in corals (Nystrom et al., 2001; Schwarz et al., 2013; Marangoni et al., 2017; Bielmyer-Fraser et al., 2018; Fonseca et al., 2019ab).

Cu can inhibit enzymatic activity when present in elevated concentrations (Viarengo et al., 1996; Santore et al., 2002; Dias and Coelho, 2007; Jin et al., 2015). It has already been demonstrated that this metal can disrupt glycolysis, Krebs cycle and oxidative phosphorylation (Carvalho and Fernandes, 2008; Silva-Aciares et al., 2011; Lauer et al., 2012; Giacomin et al., 2014; Jorge et al., 2016; Fonseca et al., 2019b; Zebral et al., 2020). As it may be expected, ambient stressors, including Cu exposure, can also affect the energy balance of corals (Nystrom et al., 2001; Nakamura et al., 2011; Kaniewska et al., 2012; Vidal-Dupio et al., 2013; Fonseca et al., 2017; Luz et al., 2018). These studies assessed corals energy metabolism by the evaluation of oxygen consumption, photosynthetic capacity, gene expression of metabolic enzymes and ATP levels. It is interesting to note that studies evaluating the energy metabolism of corals from the perspective of the enzymatic activity are scarce (Rivest and Hofmann, 2014; Fonseca et al., 2019b; Pereira et al., 2020), even though such effects are expected to occur prior to changes at the organismal-level, such as coral bleaching, necrosis and death (Moore et al., 2004).

Within this context, understanding about the mechanisms of Cu toxicity are of extreme importance. It has recently been demonstrated *in vivo* that this

metal inhibits the activity of energy metabolism enzymes in *M. harttii* (Fonseca et al., 2019), however, little is known about the mechanism that causes this effect. One alteration in the energy metabolism pathways may disrupt energetic status and compromise physiological maintenance. Consequently, organism's ability to deal with environmental stressors may be diminished (Carvalho and Fernandes, 2008). *Mussimilia harttii* is a zooxanthellate scleractinian coral that has a key role in the construction of South Atlantic reefs, being found along the Atlantic Coast in northwestern Brazil (Laborel, 1969; Mazzei et al., 2017). South Atlantic corals were recently exposed to high concentrations of metals, including Cu, following the Mariana's dam collapse, the worst environmental disaster in Brazilian history (Francini-Filho et al., 2019).

Therefore, the objectives of this study were: 1) describe the energetic metabolism of the scleractinian coral *M. harttii,* endemic to the South Atlantic, under field conditions using the activity of eight enzymes involved in different metabolic pathways: glycolysis [hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH)], Krebs cycle [citrate synthase (CS) and isocitrate dehydrogenase (IDH)], electron transport chain [electron transport system (ETS)] and pentose phosphate pathway [glucose-6-phosphate dehydrogenase (G6PDH)] and 2) assess the *in vitro* effect of Cu exposure in *M. harttii* enzymatic activity.

2. Materials and Methods

2.1. Organisms Collection and analysis of physicochemical parameters

Five polyps from five different *M. harttii* colonies were collected by scuba diving in the in the conservation area "Área de conservação do Parque Natural

Municipal de Recife de Fora" (16°24'31"S; 038°58'39"W; Porto Seguro, Bahia, northeast Brazil) on july 2012. Coral samples were collected with the permission of the Brazillian Environment Agency (IBAMA/SISBIO; permit # 34256-1). At the same time, seawater was collected to evaluate physicochemical parameters. Water samples were acidified with HNO_3 (1% final concentration; SupraPur, Merck, USA) and desalted according Nadella et al. (2009) for dissolved (filtered sample) Cu concentrations determination [atomic absorption spectrophotometry with coupled graphite furnace (PerkinElmer, USA)]. Data on pH (pH meter, model HI 9124, Hanna Instruments, USA), temperature (loggers HOBO Water Temp Pro, Onset, USA), salinity (optical refractometer, model ITREF 10, Instrutemp, São Paulo, SP, Brazil) and dissolved organic carbon (DOC) concentration (Total Organic Carbon analyzer, Shimadzu, Japan) were also assessed. The collected polyps were kept at -80ºC until evaluation of enzyme activity.

2.2. Biochemical analysis

2.2.1. Samples preparation for biochemical analysis

For the determination of enzymatic activity, coral pieces were cut (~200 mg), homogenized with 200 µL of imidazole buffer (50 mM, pH 7.8) containing 0.1 mM of phenylmethylsulfonyl fluoride (PMSF) and centrifuged (10,000 g, 4 ºC, for 20 min). The supernatant homogenate obtained from the coral holobiont (coral tissue and zooxanthellae) was used as the enzymatic source. All evaluations were performed by spectroscopy with a microplate reader (ELx808IU, BioTek Instruments, Inc, Winooski, VT, USA), under 25 ºC (corals ambient temperature according to NOAA 2012). Enzymatic activity was determined based on the protein concentration that provided the highest

enzyme activity. Before each enzymatic analysis, coral tissue homogenates had their protein concentrations standardized in the same range. Therefore, testing different volumes of homogenate in the reaction medium yielded a gradient of protein concentrations. The protein concentration was determined using the Bradford method (Bradford Reagent, Sigma, St. Louis, MO, USA).

2.2.2. Activity of glycolysis enzymes

The activity of the hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were performed according to Lallier and Walsh (1991) protocols, with some modifications. Experimental conditions were as follows: HK: 50 mM imidazole (pH 7.4), 5 mM $MgCl_2$, 1 mM D-glucose, 0.16 mM NAD^+, 2 U mL^{-1} of *Leuconostoc mesenteroides* glucose-6-phosphate desidrogenase and 1 mM ATP; PFK*:* 50 mM Imidazole (pH 7.4), 10 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 0.12 mM NADH, 1 U mL^{-1} aldolase, 3 U mL^{-1} triose-phosphate isomerase, 1 U mL^{-1} de α-glycerophosphate dehydrogenase and 3 mM fructose 6-phosphate; PK: 50 mM Imidazole (pH 7.4), 10 mM $MgCl_2$, 30 mM KCl, 0.12 mM NADH, 20 U mL^{-1} lactate dehydrogenase, 0.5 mM phosphoenolpyruvate and 2.5 mM ADP; LDH: 0.2 M Imidazole (pH 7.4), 0.15 mM NADH and 8 mM sodium pyruvate. The activity of glycolysis enzymes were determined following decrease or increase of absorbance by the oxidation/reduction of NADH at 340 nm.

2.2.3. Activity of Krebs cycle enzymes

Citrate synthase (CS) and isocitrate dehydrogenase (IDH) were performed according to the protocols described by Lallier and Walsh (1991) and

Alp et al (1976), respectively. Experimental conditions were as follows: CS: 50 mM HEPES (pH 8.1), 0.1 mM acetyl coenzyme A, 0.1 mM DTNB and 0.5 mM oxaloacetic acid. CS activity was recorded following the increase in absorbance due to the reduction of DTNB at 412 nm; IDH: 50 mM Imidazole (pH 7.4), 1 mM $MnCl_2$, 8 mM $MgCl_2$, 0.5 mM NADPH, 5 mM DL-Isocitric acid. IDH activity was determined following the reduction of NADP to NADPH at 340 nm.

2.2.4. Activity of the electron transport chain

Determination of activity of the electron transport system (ETS) was modified from Lannig et al (2003). Experimental conditions were 0.1 M sodium phosphate (pH 8.5), 0.85 mM NADH, 125 mM NADPH, 2 mM iodonitrotetrazolium chloride (INT) and 0.20 % Triton X-100. ETS activity was determined by reduction of INT, which yields an increase in absorbance at 490 nm.

2.2.5. Activity of the phosphate pentoses pathway

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined according to Carvalho and Fernandes (2008). Experimental conditions were: 80 mM glycerin (pH 7.6), 10 mM $MgCl_2$, 0.39 mM NADPH and 5 mM glucose-6-phosphate. G6PDH activity was determined following the increase in absorbance by the reduction of NADPH at 340 nm.

2.3. In vitro Cu exposure

An enzyme representative of each stage of energy metabolism was chosen to evaluate the mechanisms of action of Cu in the general functioning of

metabolism. Thus, Cu effects in the activity of PK, LDH, CS and ETS were tested *in vitro*. This experiment was conducted by contamination of the reaction medium with four nominal Cu concentrations: 0, 3, 5 and 20 μg L^{-1} (measured concentrations: 0, 1.4, 3.7 and 14.2 μg L^{-1}, respectively). The first of them (3 μg L^{-1}) is a low and environmentally-realistic concentration. The second (5 μg L^{-1}) is the maximum concentration permitted by Brazilian government for marine environments (CONAMA, 2005). The third (20 μg L^{-1}) is an elevated concentration that is expected to occur in polluted environments (e.g. Jones, 2010). Each fragment from the five different polyps was exposed to four different concentrations of Cu. It is interesting to note that the effect of exposure to such Cu concentrations have already been evaluated in the coral *M. harttii* by our research group and showed to be effective in altering enzymatic activity *in vivo* (Fonseca et al., 2017; Marangoni et al., 2017; Fonseca et al., 2019ab; Marangoni et al., 2019). The reaction medium was contaminated with a stock solution of $CuCl_2$ (5 mg L^{-1}) before the reaction started in order to obtain the desired nominal concentrations. The stock solution was prepared 24 h prior its use in the enzymatic assays to the complete equilibration of Cu with water. The activity of PK, CS, LDH and ETS were evaluated with a kinetic time of 30 min in order to allow more metal interaction in the reaction medium.

2.4. Data presentation and statistical analysis

Data is expressed as mean ± standard error. The *in vitro* effect of Cu exposure was assessed by one-way ANOVA followed by the Fisher test for multiple comparisons. Data normality and homogeneity were tested with the Shapiro-Wilk and Cochran C tests, respectively. When data failed to meet

paramedic assumptions, it was proceeded to log-transformation and data was re-tested.

3. Results

3.1 Water physicochemical parameters of seawater

The levels of dissolved Cu, pH, temperature, salinity and dissolved organic carbon corresponded to 1.4 ± 0.3 μg L^{-1}, 8.29 ± 0.00, 25.2 ± 0.05 ºC, 35.5 ±0.06 ppt, 3.35 ± 0.35 mg L^{-1}, respectively.

3.2. Metabolic evaluation of the coral Mussismilia harttii under field conditions

The results of the protein concentration that provided the highest enzyme activity are shown in table S1, in supplementary material. HK, PK and CS showed elevated activity in comparison to all other enzymes evaluated (Fig. 1).

Table S1 – Activity of hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), citrate synthase (CS), isocitrate dehydrogenase (IDH), electron transport system (ETS) and glucose-6-phosphate dehydrogenase (G6PDH) in the coral *Mussismilia harttii* with different protein concentrations in the reaction medium and kinetic times. Data are expressed as mean ± standard error. Different lowercase letters represent significant differences among protein concentration for the same time ($p<0.05$). Different capital letters indicate differences among kinetic times for the same protein concentration ($p<0.05$). The enzyme activity used in the comparison is marked in bold. The abbreviation “nl” denotes that enzymatic kinetics were not linear.

		Enzymatic activity (mean ± SE)		
		Time (min)		
Enzime	Protein concentrations mg L^{-1}	5	15	30
HK activity (U mg protein^{-1})	0.1	nl	0.34± 0.01	0.56 ± 0.12
	0.3	nl	**0.43 ± 0.08**	0.51 ± 0.11
	0.6	nl	0.39 ± 0.09	0.43 ± 0.11
	0.9	nl	0.28 ± 0.05	0.33 ± 0.08
	1	nl	0.27 ± 0.07	0.30 ± 0.05
PFK activity (U mg protein^{-1})	1	nl	0.0212 ± 0.004	0.014 ± 0.0009
	2	nl	**0.0215 ± 0.001**	0.014 ± 0.002
	3	nl	0.016 ± 0.002	0.010 ± 0.002
	4	nl	0.015 ± 0.001	0.010 ± 0.001
PK activity (U mg protein^{-1})	1	0.44 ± 0.06 **a**^{B}	**0.60 ± 0.09** **a**^{A}	0.65 ± 0.05 **a**^{A}
	2	0.38 ± 0.05 **ab**^{AB}	0.52 ± 0.07 **ab**^{A}	0.35 ± 0.02 **b**^{B}
	3	0.36 ± 0.05 **ab**^{AB}	0.44 ± 0.03 **bc**^{A}	0.22 ± 0.01 **bc**^{B}
	4	0.28 ± 0.04 **b**^{AB}	0.35 ± 0.02 **c**^{A}	0.16 ± 0.01 **c**^{B}
LDH activity (U mg protein^{-1})	1	nl	0.035 ± 0.014	0.0423 ± 0.017
	3	nl	0.034 ± 0.012	0.042 ± 0.025
	5	nl	0.041 ± 0.013	0.045 ± 0.028
	6	nl	**0.044 ± 0.009**	0.050 ± 0.025
CS activity (U mg protein^{-1})	1	**0.26 ± 0.07** **a**	0.27 ± 0.07 **a**	0.24 ± 0.05 **a**
	3	0.24 ± 0.06 **a**	0.22 ± 0.04 **a**	0.15 ± 0.02 **ab**
	5	0.23 ± 0.06 **a**	0.17 ± 0.02 **a**	0.10 ± 0.02 **ab**
	7	0.21 ± 0.05 **a**	0.14 ± 0.02 **a**	0.08 ± 0.01 **c**
IDH activity (U mg protein^{-1})	1	nl	**0.079 ± 0.02**	0.115 ± 0.03
	3	nl	0.066 ± 0.01	0.091 ± 0.02
	5	nl	0.064 ± 0.01	0.08 ± 0.01
	6	nl	0.052 ± 0.01	0.063 ± 0.01
ETS activity (U mg protein^{-1})	23	**0.0154 ± 0.001**^{A}	0.0144 ± 0.0008^{A}	0.0118 ± 0.0005^{B}
	26	0.0151 ± 0.0006^{A}	0.0139 ± 0.0006^{A}	0.0112 ± 0.0004^{B}
	30	0,0152 ± 0.003^{A}	0.0142 ± 0.0008^{A}	0.0111 ± 0.0004^{B}
	36	0.0141 ± 0.001^{A}	0.014 ± 0.001^{A}	0.0104 ± 0.0006^{B}
G6PDH activity (U mg protein^{-1})	7	0.027 ± 0.0006^{B}	0.043 ± 0.006^{AB}	0.055 ± 0.006^{A}
	10	0.022 ± 0.010^{B}	0.048 ± 0.007^{AB}	0.060 ± 0.012^{A}
	12	0.028 ± 0.003^{B}	**0.049 ± 0.005**^{AB}	0.058 ± 0.011^{A}
	15	0.032 ± 0,005^{B}	0.053 ± 0.007^{AB}	0.060 ± 0.014^{A}

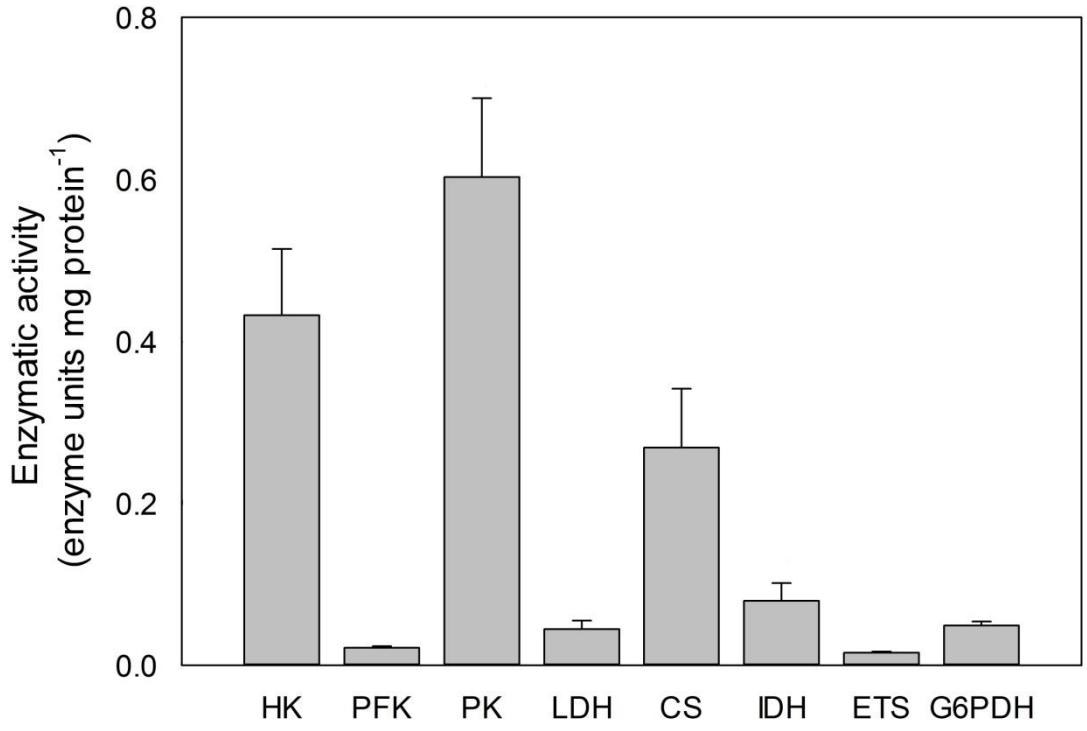

Figure 1: Activity of the hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), citrate synthase (CS), isocitrate dehydrogenase (IDH), electron transport system (ETS) and glucose-6-phosphate dehydrogenase (G6PDH) in the coral *Mussismilia harttii.* Data are expressed as mean ± standard error.

3.3. In vitro effect of Cu exposure

The PK activity was not altered by Cu exposure (Fig. 2). Conversely, the LDH activity was reduced by all Cu concentrations tested (Fig. 3). Despite that, CS activity was unaltered by Cu (Fig. 4). Similarly to LDH, ETS activity was reduced by all Cu concentrations tested (Fig. 5).

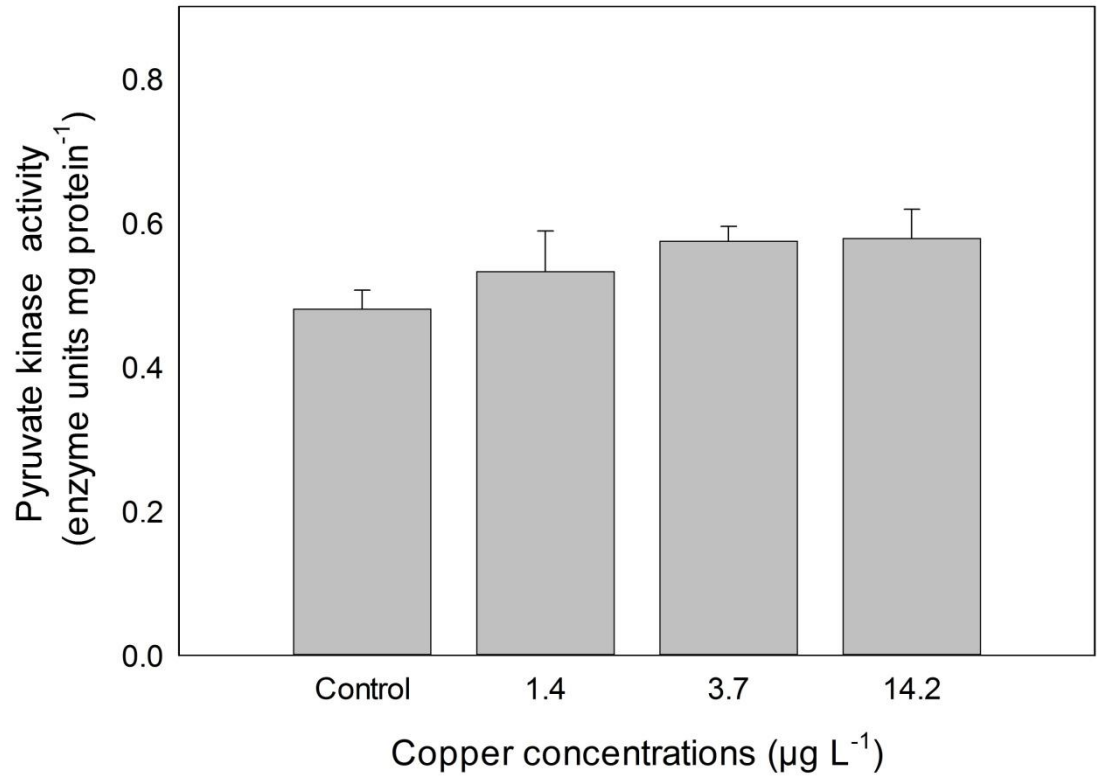

Figure 2: Pyruvate kinase (PK) activity in the coral *Mussismilia harttii* exposed to different concentrations of copper (Cu) *in vitro*. Data are expressed as mean ± standard error.

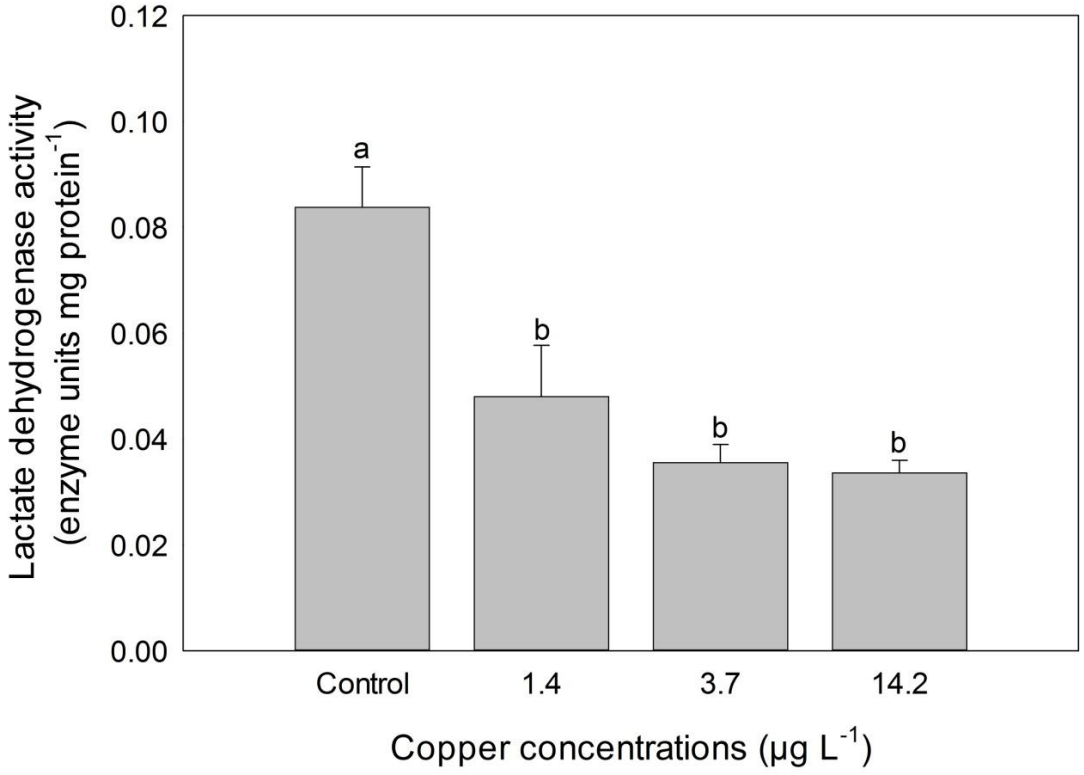

Figure 3: Lactate dehydrogenase (LDH) activity in the coral *Mussismilia harttii* exposed to different concentrations of copper (Cu) *in vitro*. Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$).

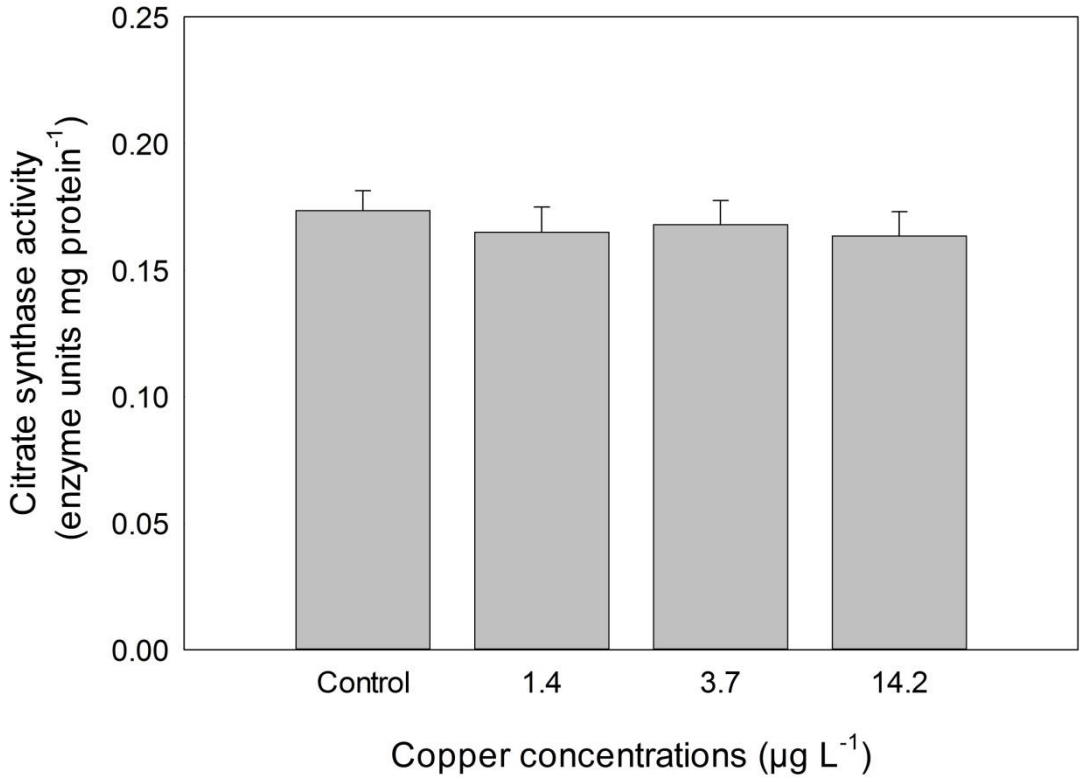

Figure 4: Citrate synthase (CS) activity in the coral *Mussismilia harttii* exposed to different concentrations of copper (Cu) *in vitro*. Data are expressed as mean ± standard error.

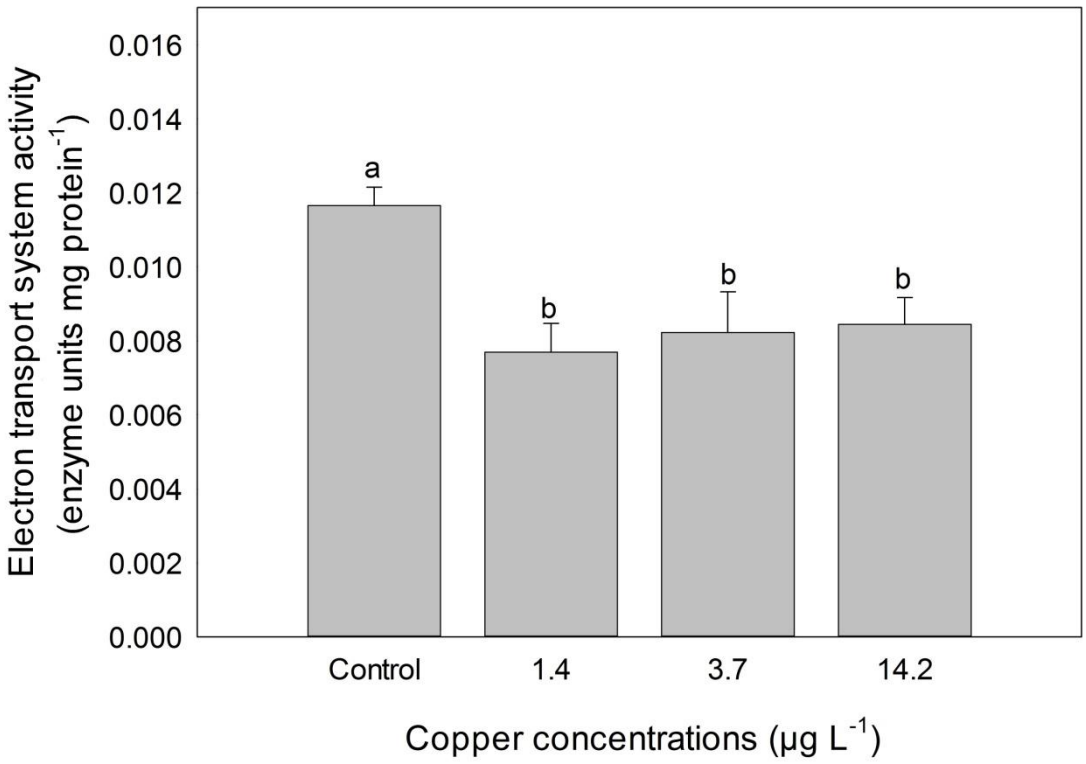

Figure 5 Electron transport system (ETS) activity in the coral *Mussismilia harttii* exposed to different concentrations of copper (Cu) *in vitro*. Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$).

4. Discussion

4.1. Activity of energy metabolism enzymes under field conditions

The present study shows that enzymes related to the glycolysis pathway (HK and PK) had elevated activities. This result reinforces the hypothesis that glucose is the main energetic fuel used by *M. harttii*. This molecule can be obtained by both heterotrophic (predation) (Porter, 1976; Sebens et al., 1996; Houlbrèque and Ferrier-Pagès, 2009) and autotrophic (photosynthesis) processes (Papina et al., 2003; Colombo-Pallotta et al., 2010) and is used to sustain many aspects of corals metabolism. PFK activity, was expected to be

similar to other enzymes related to the glycolysis pathway, nonetheless, this enzyme activity was 20 to 30 times lower than HK and PK activities, respectively. PFK is considered to be a pacemaker enzyme subjected to complex allosteric modulation, such as inhibition by elevated levels of ATP and citrate (Campbell and Farrell, 2006; Lauer et al., 2012). Therefore, the low PFK activity seen in the present study can be a result of allosteric modulation of ATP and/or citrate, taking into account that the activities of PK and CS were higher in comparison to PFK, respectively. Interestingly, Lauer et al (2012) has also observed lower PFK activity in comparison to other enzymes of energetic metabolism.

It is important to note that in the present study, a greater activity of enzymes involved in aerobic metabolism was observed in comparison to LDH, an enzyme related to anaerobic metabolism. Thuesen and Childress (1994) reached similar conclusions in studies with different species of medusae. Nonetheless, Henry and Torres (2013) showed that in the scleractinian coral *F. impensum*, LDH activity is about three times greater than CS activity. It is important to note that this is an azooxanthellate Antarctic coral that lives in low temperatures and in absence of light (Henry and Torres, 2013), therefore, comparisons with this species has to be taken carefully. This result shows that, under field conditions, *M. harttii* has a low anaerobic competence, nonetheless, LDH may represent an important metabolic pathway to produce energy in periods of oxygen deprivation, such as in bleaching and sedimentation processes. It is important to note that scleractinian corals under hypoxic stress could use the activity of alanopine dehydrogenase (ADH) and strombine dehydrogenase (SDH) to reduce pyruvate and recycle NADH to glycolysis

(Murphy and Richmond, 2016). However, the activities of these enzymes were not evaluated in the present study.

Taking into account the results presented above, elevated activity of CS, IDH and ETS was expected. These results show that *M. harttii* may have a low mitochondrial content, considering that this may be a strategy to reduce metabolic cost, aerobic activity and oxidative stress (Brockington, 2001). Alternatively, it is known that analysis of the maximum activity of mitochondrial enzymes requires mitochondrial isolation (Lauer et al., 2012). However, such analysis would need to be performed shortly after sample collection, which makes such a procedure impossible in the present study. Another important issue to be considered is that the evaluation of electron transport chain activity by the ETS method assesses only the complexes I and III (Doucet-Beaupré et al., 2010). The complexes II and IV could provide better information about oxidative phosphorylation because they are involved in $FADH_2$ oxidation and oxygen reduction to water, respectively (Campbell and Farrell, 2006).

4.2. In vitro effects of Cu exposure

Metals are known to exert their toxicity *via* enzyme inhibition, and the mechanisms of enzyme inhibition are mainly involved in the binding of metal to protein. Cu, for example, inhibits enzymatic activity by many mechanisms, such as binding directly to enzymes (Dias and Coelho, 2007) replacing cofactors (Santore et al., 2002) or interacting with -SH (Viarengo et al., 1996), changing protein active conformation (Jin et al., 2015). On the other hand, by indirect mechanisms Cu can also inhibit enzyme activity. This metal is capable of catalyzing the production of harmful radicals/oxidants, such as the hydroxyl

radical (•OH) that interacts with functional groups of proteins, causing enzymatic inhibition (Goldstein et al., 1993; Wardman and Candeias, 1996). In the case of enzymes from the energetic metabolism, Cu can also replace Mg^{2+}, an important enzymatic cofactor, and disrupt enzyme functioning (Lauer et al., 2012). With that in mind, the ability of Cu to inhibit enzyme activity by direct binding was evaluated through *in vitro* exposure using enzymes involved in different stages of energy metabolism.

Metal exposure did not alter PK or CS activity. *In vivo* studies have already demonstrated the ability of Cu to inhibit PK activity (Carvalho and Fernandes, 2008; Lauer et al., 2012; Anni et al., 2019). However, it is noted that the effect is related to the substitution of Mg^{2+} in the protein structure. Interestingly, this mechanism occurs in the presence of high concentrations of Cu. Therefore, the lack of this effect in the present study may be related to the low concentrations of Cu used. Similar to our study, Garceau et al (2010) did not observe an effect of *in vitro* exposure to Cu in CS activity. Since CS is not a metalloenzyme, the mechanisms for inhibiting the activity of this enzyme are still unclear, however, it is believed that it may be caused by an oxidative stress mechanism triggered by exposure to metals (Fonseca et al., 2019b). The Cu concentrations tested *in vitro* are likely not sufficient to trigger an inhibition mechanism caused by the direct binding of Cu to the structure of PK and CS in *M. harttii*.

Conversely, LDH activity was inhibited in all concentrations tested. Other studies have shown that Cu is an important inhibitor of LDH activity *in vivo* and *in vitro* (Dobryszycka and Owczarek, 1981; Elumalai et al., 2002; Pamp et al., 2005; Satyaparameshwar et al., 2006; Koiri et al., 2008; Teodorescu et al.,

2012; Zebral et al., 2020). The mechanism of metal toxicity in LDH activity can be facilitated in the presence of NADH. It is likely that the binding of NADH to the enzyme induces a subtle change in the conformation of the active site, causing a slight opening that exposes amino acid residues to which Cu has an affinity to bind, showing that the formation of ROS has a minor importance for the inactivation of this enzyme (Pamp et al., 2005). LDH is composed of four subunits and contains one cysteine residue per subunit. The binding of metals in –SH groups inhibits the enzyme (Holbrook and Gutfreund, 1973). Cu interacts strongly with these regions and can form fewer stable complexes with some amino acid side chains, i.e., His, Asp, Glu, Ser and thr, that are in high amounts in the structure of LDH. In this way, Cu can change the conformation of the protein and cause a non-competitive inhibition (Vallee and Ulmer, 1972; Pamp et al., 2005). This anaerobic enzyme is responsible for the reduction of pyruvate to lactate and consequent cycling of NADH to NAD^+, that is used in glycolysis pathway (Campbell and Farrell, 2006). The reduction in this enzyme activity may compromise an important source of energy production for *M. harttii*, considering that glycolysis is important when a considerable amount of fast energy is needed. This condition occurs mainly during exposure to chemical stress (Diamantino et al., 2001). Generally, the data presented herein reinforce the evidence that Cu is able to inhibit LDH activity by its interaction at the protein level.

In vitro exposure to Cu reduced *M. harttii* ETS activity. Previous studies have shown that mitochondrial enzymes from aquatic invertebrates are sensitive to Cu (Silva-Aciares et al., 2011; Lauer et al., 2012; Jorge et al., 2016). Such sensitivity may be due to Cu ability to accumulate in mitochondria. The

mitochondria membrane has numerous channel proteins for the entry of essential metals that are part of the structure of complex electron transporters. The presence of high concentrations of metals can cause the displacement of essential metals in the membrane channels. This makes electron transport chain complexes the main targets for metal toxicity (Zischka et al., 2011; Lichtmannegger et al., 2016). Many studies have reinforced this discovery in other invertebrates. For example, *Mesodesma mactroides* exposed to 150 μg L^{-1} of Cu reduced ATP concentration in hemolymph (Giacomin et al., 2014). For the same biological model mentioned above, *in vitro* exposure to Cu reduced the activity of complex II of the electron transport system (Jorge et al., 2016). A similar result has also been obtained by Lauer et al (2012). It was showed in this study that the crab *Neohelice granulata* exposed to Cu (1.000 μg L^{-1}) under different salinities had a reduction in the mitochondrial membrane potential. One may hypothesize that this membrane potential reduction is caused by Cu-dependent inhibition of electron transport system, leading to diminished proton (H^+) pumping to intermembrane space, reducing the electromotive force used by ATP synthase. Interestingly, Silva-Aciares et al (2011) showed that *in vitro* exposure to Cu reduced ATP synthase gene expression in the mollusk *Haliotis rufences*, corroborating the hypothesis presented above. When taken together with the literature, our results clearly indicate that Cu can interact directly with electron transport chain complexes and disrupt *in vivo* ATP production in *M. harttii*.

It is important to mention that Fonseca et al (2019b) has recently reported an inhibition of LDH, CS and ETS activity in *M. harttii* exposed *in vivo* to 5.4 and 8.6 μg L^{-1} of Cu for up to 12 days. The inhibition of enzyme activity

was presumed to be due to a possible oxidative stress condition caused by exposure to the metal. However, the results presented in the present study show that inhibition of LDH and ETS activity can also occur due to the direct interaction of Cu with these enzymes. Despite low ecological relevance, *in vitro* tests are important to identify specific effects and probable mechanisms of action of exposure to chemical stressors. Finally, we would like to stress the fact that this was the first study to describe the energetic metabolism of *M. harttii* under field conditions and revealed possible toxicological mechanisms related to Cu inhibition on the activity of enzymes involved in energy metabolism in this species.

5. Conclusions

In light of the results presented herein, it is concluded that under field conditions, the coral *M. harttii* showed elevated aerobic metabolism and low anaerobic capacity. Also, it is concluded that glucose is a major energetic fuel for this species. For the assessment of *in vitro* effects of Cu exposure, it was demonstrated that LDH and ETS activities were both inhibited by metal exposure. Therefore, it is concluded that Cu may jeopardize energy production by the direct interaction with specific regions of energy metabolism enzymes in the coral *M. harttii*.

6. Acknowledgments

We would like to thank Dr. Joseane A. Marques and Dr. Laura Fernandes de Barros Marangoni for their support in field work. The International Development Research Center (IDRC, Ottawa, Canada), Coordenação de

Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Programa Ciências do Mar, Brasília, DF, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática, Brasília, DF, Brazil). Thanks to the Coral Vivo Project and its sponsors: Petrobras (Programa Petrobras Ambiental) and Arraial d'Ajuda Eco Park. A. Bianchini is a research fellow of the Brazilian CNPq (Proc. # 304430/2009-9) and supported by the IDRC International Research Chair Program.

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5. Capítulo III

Este capítulo é representado pelo artigo **"Isolated and combined effects of thermal stress and copper exposure on the trophic behavior and oxidative status of the reef-building coral *Mussismilia harttii*"** já publicado no periódico *Environmental Pollution* (DOI: https://doi.org/10.1016/j.envpol.2020.115892). O trabalho pode ser encontrado na página seguinte.

Isolated and combined effects of thermal stress and copper exposure on the trophic behavior and oxidative status of the reef-building coral *Mussismilia harttii*

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Abstract

Global warming and local disturbances such as pollution cause several impacts on coral reefs. Among them is the breakdown of the symbiosis between host corals and photosynthetic symbionts, which is often a consequence of oxidative stress. Therefore, we investigated if the combined effects of thermal stress and copper (Cu) exposure change the trophic behavior and oxidative status of the reef-building coral *Mussismilia harttii*. Coral fragments were exposed in a mesocosm system to three temperatures (25.0, 26.6 and 27.3ºC) and three Cu concentrations (2.9, 5.4 and 8.6 µg L^{-1}). Samples were collected after 4 and 12 days of exposure. We then (i) performed fatty acid analysis by gas chromatography-mass spectrometry to quantify changes in stearidonic acid and docosapentaenoic acid (autotrophy markers) and cis-gondoic acid (heterotrophy marker), and (ii) assessed the oxidative status of both host and symbiont through analyses of lipid peroxidation (LPO) and total antioxidant capacity (TAC). Our findings show that trophic behavior was predominantly autotrophic and remained unchanged under individual and combined stressors for both 4- and 12-day experiments; for the latter, however, there was an increase in the heterotrophy marker. Results also show that 4 days was not enough to trigger changes in LPO or TAC for both coral and symbiont. However, the 12-day experiment showed a reduction in symbiont LPO associated with thermal stress alone, and the combination of stressors increased their TAC. For the coral, the isolated effects of increase in Cu and temperature led to an increase in LPO. The effects of combined stressors on trophic behavior and oxidative status were not much different than those from the isolated effects of each stressor. These findings highlight that host and symbionts respond differently to stress and are

relevant as they show the physiological response of individual holobiont compartments to both global and local stressors.

Keywords: Climate change; Heterotrophy; Lipid peroxidation; Pollution; Total antioxidant capacity

Main findings:

Coral and symbiont respond differently to 12 days of exposure to Cu and thermal stress. Stressors do not induce changes in trophic behavior.

1. Introduction

Global warming is an immediate threat to the biodiversity of marine ecosystems (Belkin, 2009; Doney et al., 2012; Frölicher et al., 2018). Coral reefs rank among the most sensitive marine environments because of their physico-chemical stability and short interval between thermal optimum and stress threshold (Glynn, 1993; 1996; Hughes et al., 2018). In recent decades, increase in surface seawater temperature has triggered mass coral bleaching and mortality (Eakin et al., 2010; De'ath et al., 2012; Hughes et al., 2018). The Intergovernmental Panel on Climate Change (IPCC) predictions show that ocean temperature will increase between 2.6 and 4.8 °C by the end of the century and promote further ecological and structural changes on marine environments (IPCC, 2014).

Besides global-scale impacts, coral reefs are also affected by several regional-scale stressors (Carpenter et al., 2008; Knowlton and Jackson, 2008; van Dam et al., 2011). Among them is metal pollution, which is often associated with coastal run-off, mining and sewage (Dubinsky and Stambler, 1996; Jones, 1997). Copper (Cu) is a metal that is an essential micronutrient for cellular functions (Belyaeva et al., 2011), but exposure to high concentrations, often associated with pollution, can cause physiological damage to corals and their symbionts (Bielmyer et al., 2010; Schwarz et al., 2013; Fonseca et al., 2019a).

Both thermal stress and exposure to pollutants are known to disrupt the coral-dinoflagellate symbiosis (Hughes et al., 2003; van Dam et al., 2011). It has been shown that the combination of thermal stress and Cu exposure produce a dramatic reduction in the photosynthetic capacity of the symbiont (Fonseca et al., 2017). The effects of these stressors may be synergistic or not. It has been

shown that they are not in the case of the primary production performed by symbionts associated with *Porites cylindrica* (Nyström et al., 2001). However, they do act synergistically in altering the host coral metabolism (Fonseca et al., 2019b).

Under these stressful conditions, the symbiont photosynthetic machinery produces excessive reactive oxygen species (ROS), causing cellular damage for both host and symbiont (Lesser, 2006; Weis, 2008; Yakovleva et al., 2009). The host responds by expelling symbionts from its tissues, leaving its white skeleton visible under the soft tissue and thus becoming “bleached” (Glynn, 1993; Downs et al., 2005; Howells et al., 2016). Because the symbiotic relationship is obligatory for the host in most cases, bleaching is often accompanied by mortality episodes (Glynn, 1993, 1996; Hoegh-Guldberg, 1999). Therefore, oxidative stress is a critical factor during the bleaching process (Lesser, 1997; Downs et al., 2005; Perez and Weis, 2006; Weis, 2008; Nielsen et al., 2018).

Biochemical parameters associated to oxidative status can be used to evaluate reef ecosystem health (Downs et al., 2005; Prazeres et al., 2012; Marangoni et al., 2019a). Total antioxidant capacity (TAC) and lipid peroxidation (LPO) are relevant tools for predicting bleaching and mortality in coral reef organisms (Prazeres et al. 2012; Marangoni et al. 2019a). LPO indicates the reduction in the integrity of biological membranes (Lesser et al., 2006), while TAC provides an assessment of overall oxyradical scavenging capacity (Huang et al., 2005). The fact that these markers are present in both host and symbiont makes them particularly useful to investigate the stress response at a

biochemical level in different compartments of the holobiont (Gardner et al., 2017).

From an ecophysiological perspective, corals often respond to stress by shifting their predominant trophic behavior from autotrophic to heterotrophic (Hughes and Grottoli et al., 2013; Mies et al., 2018; Marangoni et al. 2019b). Fatty acids are considered robust markers to investigate changes in trophic behavior in corals (Treignier et al., 2008; Mies et al., 2018). That is largely because they are a record of dietary habit and cannot be synthesized *de novo* by most animals (Dalsgaard et al., 2003; Ruess et al., 2005; Budge et al., 2006). For instance, stearidonic acid (SDA, 18:4ω3) and docosapentaenoic acid (DPA, 22:5ω3) are photosynthates translocated from the symbiotic dinoflagellates to the host (Papina et al., 2003; Mies et al., 2017); while cis-gondoic acid (CGA, 20:1ω9) is a fatty acid widely present in crustacean zooplankton captured by corals (Dalsgaard et al., 2003; Mies et al., 2018). Based on the variation in the content of these fatty acids in the coral tissue, it is possible to calculate shifts of predominance between autotrophic and heterotrophic behavior (Mies et al., 2018).

However, the effects of thermal stress and Cu exposure on the trophic behavior of reef-building have not yet been assessed, either individually or combined. In addition, the combined effects of these stressors on the oxidative status has not been assessed at different holobiont compartments. Therefore, we investigated if the isolated and combined effects of thermal stress and Cu exposure promote a shift in trophic behavior and in the oxidative status for the scleractinian coral *Mussismilia harttii,* endemic to the South Atlantic province. Coral reefs in that region have been increasingly exposed to Cu contamination

associated with sewage and dam breaks (Francini-Filho et al., 2019; Marques et al., 2019). Therefore, we evaluated variations in the concentrations of autotrophy- and heterotrophy-specific fatty acids and in the levels of LPO and TAC for both coral and symbiotic algae. In the current scenario, investigations on the combined effects of thermal stress and pollution are warranted in order to predict physiological responses to local stressors in a climate change context (see Nyström et al., 2001; Sokolova and Lannig, 2008; Negri and Hoogenboom, 2011; Banc-Prandi and Fine, 2019).

2. Materials and Methods

2.1 Collection of coral samples

In July 2012, fragments of the coral *Mussismilia hartii* were collected from six different colonies at the Recife de Fora Municipal Park (Porto Seguro, Bahia State, Brazil; (16°24′31″S; 038°58′39″W). Collection was performed with the proper authorization of the Brazilian Environmental Agency (permit #85926584; IBAMA/SISBIO). Collected fragments were fixed on ceramic plates and acclimated in a mesocosm system for 20 days.

2.2 Experimental and mesocosm setup

Twenty-seven experimental 10-L tanks were used to test three temperatures (mean local temperature of 25.0, and 1.5 and 2.5 ºC above the mean, *i. e.* 25.0, 26.5 and 27.5ºC) that are similar to future climate scenarios issued by the IPCC (IPCC, 2014) and three levels of Cu contamination (0, 3 and 5 μg L^{-1} above the mean natural concentration, *i. e.*, 2.9, 6.0 and 8.0 μg L^{-1}) in a fully crossed experimental design. The concentrations of Cu used in the experiment are

below or close the limits allowed by the international and Brazilian legislations for the protection of aquatic environments (CONAMA 2005; EPA 2005). Each of the nine treatments was replicated in three tanks and two *M. harttii* fragments were placed on each tank for sampling after 4 and 12 days of exposure to Cu and thermal stress.

We used a semi-open system in which seawater was collected from the adjacent reef through an underwater pipeline and conditioned in 5000-L underground sumps before being pumped into the 27 experimental flow-through aquariums. Afterwards, before returning to the ocean, water was filtered and sterilized in an additional underground sump where ultraviolet filters and activated carbon cartridges were installed. Due to its direct connection to the reef environment, this system maintained seawater conditions (*e. g.* temperature, turbidity, salinity, pH, zooplankton availability) very similar to those in the natural environment. An automatic temperature control system containing two 15-kW submersible heaters maintained the temperature required for each treatment during both day and night. Simultaneously, seawater captured from the reef environment was held in four pairs of 1000-L underground sumps to receive stock solutions for the application of Cu exposure treatments. The different Cu treatments were prepared daily and 24 hours before its use from a standard solution of $CuCl_2$ (1 g L^{-1}) and added to the underground secondary treatment sumps to obtain the desired concentrations. The seawater that received the Cu treatments was mixed with seawater coming from the primary treatment sumps using peristaltic pumps (flow rate of 0.17 L min^{-1}) before reaching the 10-L tanks. The secondary system seawater (Cu contamination) accounted for 10% of the total seawater flow reaching the 10-L aquariums,

while heated water flow accounted for 90%. A more detailed description of the mesocosm system used in this experiment can be found at Duarte et al. (2015).

2.3. Seawater collection and analyses

Water samples were collected from the tanks after every three days during the experimental period to determine dissolved Cu concentrations and other physicochemical parameters. The concentrations of dissolved Cu were evaluated in filtered (0.45-μm mesh filter) water samples acidified with nitric acid (HNO_3, 1% final concentration; SupraPur, Merck®, Germany). After this, water samples were desalted using the method proposed by Nadella et al. (2009) and the concentrations were determined through Atomic Absorption Spectrophotometry with Graphite Furnace (Perkin-Elmer®, Waltham, USA). Dissolved organic carbon (DOC) concentration was assessed in a Total Organic Carbon analyzer (Shimadzu®, Japan). Data on pH (pH meter HI 9124, Hanna Instruments®, USA) and salinity (optical refractometer ITREF 10, Instrutemp®, São Paulo, Brazil) were evaluated twice a day during the experimental period. Pluviometry was recorded through monitoring by the local weather station, Veracel Celulose, Brazil. Temperature also was monitored (every 30 minutes) using loggers (HOBO Water Temp Pro, Onset, USA) which were placed inside the tanks and also at the reef.

2.4. Fatty acids extraction and gas chromatography

Fatty acid extraction and gas chromatographic analysis were performed in similar fashion to Vidal et al. (1979), with minor modifications. The soft tissue was separated from the skeleton with tweezers and weighed, before being

placed in a glass tube containing 1 mL of n-hexane and dichloromethane (1:1, v:v). Tubes were then agitated for 1 minute before the addition of 10 µg of the internal standard (tricosanoic acid, C23:0). Esterification was performed with 5 mL of 2% of sulfuric acid in methanol. Afterwards, 1 mL of hexane (100%) was added and agitated. Condensers were then attached to the tubes, which were then kept under a 50ºC bath for 30 minutes. Tubes were rested until reaching room temperature and phases separated with a 5-mL saturated solution of NaCl. The bottom phase was discarded and the top phase (approximately 1 mL) was taken for gas chromatographic analysis.

The fatty acids were evaluated in similar fashion to Mies et al. (2017; 2018), with minor modifications. Fatty acids were assessed in a 7890 gas chromatograph couple to mass spectrometer 7010B from Agilent Technologies. Separation was performed in an Agilent J & W ultra-inert column of 30 m in length, 0.25 mm in internal diameter and thickness of 0.25 µm with a 5% phenylmethylsiloxane film. Approximately 1 µL of the sample was introduced in splitless mode and analyzed in mass scan mode. Oven ramp temperature began at 50ºC and was maintained for 2 minutes, before an increase of 9ºC per minute until reaching 300ºC and maintained for 10 minutes. Three different polyunsaturated fatty acids were investigated: SDA, DPA (autotrophy markers) and CGA (heterotrophy marker). Each marker was identified by comparison of their retention times against those in reference material 47033 PUFA No. 1 Marine Source (Sigma-Aldrich, Co®), and their respective mass spectra.

2.5. Sample preparation for oxidative status analyses

Sample preparation was performed according to Hopkinson et al. (2015). The coral samples were cut and homogenized on ice with a buffer for each analysis (1:1 w/v) and with the aid of a sonicator (20 kHz, Sonaer Ultrasonics®, New York, USA). After homogenization, samples were centrifuged (2530 g, 5 min, 4°C), to separate coral (supernatant) and symbiont (pellet) homogenates. Afterwards, 150 µL of buffer was added to the pellet, sonicated (30 kHz) and used for analysis of the symbiotic microalgae. All results were normalized considering the protein concentration of the homogenates.

2.5.1. Lipid peroxidation (LPO)

LPO was determined using the fluorimetric method described in Federici et al. (2007). Coral samples were homogenized in a buffer containing 1.15% potassium chloride (KCl) and 35 µM butylated hydroxytoluene (BHT) diluted in ethanol (95%). This method assesses levels of oxidized lipids by the formation of the tetramethoxypropane chromogen (TMP), which is a product resulting from reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). The assay was performed using 20 µL of homogenate added in a 96-well microtitre plate (in duplicate), containing 10 µL of BHT (1 M) and 160 µL of phosphate buffered saline (1 mM, pH 7.4). Trichloroacetic acid (TCA) (50%) and 1.3% (w/v) thiobarbituric acid (TBA) (dissolved in 0.3% (w/v) NaOH) were then added and the plate incubated at 60ºC for 60 min. The substances react with TBA at a high temperature and produce detectable fluorescence. Quantification (excitation and emission at 520 and 580 nm) was performed using a fluorimeter (Victor 2, Perkin Elmer, 944 Waltham, MA, USA). The results were expressed in µM MDA mg^{-1} of protein.

2.5.2. Total antioxidant capacity (TAC)

TAC was evaluated using the commercial colorimetric kit OxiSelect Total Antioxidant Capacity Assay kit (Cell Biolabs Inc®, San Diego, USA), which detects all classes of low molecular weight antioxidants. The method measures the presence of reduced Cu (Cu^{1+}) by antioxidants present in the sample. The presence of Cu^{1+} is determined by binding to a chromogenic reagent, when coupled it produces a color detected at 490 nm. The absorbance values of the samples are compared to the absorbance of known concentrations of uric acid. The results were expressed in µM of copper reducing equivalents (CRE) mg^{-1} of protein.

2.6. Data and statistical analyses

Effects of thermal stress and Cu exposure in the content of SDA, DPA, CGA, LPO and TAC (the latter two for both coral and symbionts) were assessed by a 2-way factorial analysis of variance (ANOVA). For each experimental time (4-day and 12-day), we performed one ANOVA [fixed factors 'temperature' (three levels, 25.0, 26.6 and 27.3ºC), and 'Cu' (three levels, 2.9, 5.4 and 8.6 µg L^{-1})]. To fulfill ANOVA assumptions, the normal distribution and homogeneity of variances were tested and log-transformed whenever necessary.

To investigate whether the combined effects of thermal stress and Cu exposure induce a shift in trophic behavior in *M. harttii*, we used the Predominant Trophic Mode Index (PTMI, Eq. 1), with minor modifications (see Mies et al. 2018). The PTMI balances differences in autotrophic and

heterotrophic markers, yielding positive values in the case of predominant autotrophy and negative values whenever heterotrophy prevails.

$$(1) \quad \mathrm{PTMI} = [(\frac{\mathrm{autFATM(treatment)}}{\mathrm{autFATM(control)}}) - 1] - [(\frac{\mathrm{hetFATM(treatment)}}{\mathrm{hetFATM(control)}}) - 1]$$

where autFATM stands for µg of (SDA + DPA) g^{-1} of soft tissue, and hetFATM for µg of CGA g^{-1} of coral soft tissue. Control = experimental control (no thermal stress and no Cu exposure), and treatment = manipulative pair at each combination of temperature and Cu. Therefore, seven PTMI calculations were performed for each of the 4-day and 12-day sets of experiments.

3. Results

3.1. Experimental seawater

Throughout the experiment, dissolved Cu concentrations were 2.9 ± 0.7, 5.4 ± 0.9, 8.6 ± 0.3 µg L^{-1}, corresponding to the treatments of 0 (control), 3 and 5 µg L^{-1}, respectively above the mean natural concentration of 2.9 µg L^{-1}. Recorded temperatures were 25.0 ± 0.1 for control, 26.6 ± 0.1 and 27.3 ± 0.1°C, corresponding to the treatments of 1.5 and 2.5°C above ambient seawater temperature. The results of the other physicochemical parameters of experiment are in Table S1 in supplementary material.

Table S1: Results physicochemical parameters of experimental seawater during the exposure of the coral *Mussismilia harttii* to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days.

Physicochemical parameters	4 days	12 days
Dissolved organic carbon (mg L^{-1})	3.35 ± 0.35	1.36 ± 0.46
Salinity (ppt)	35.5 ± 0.06	36.9 ± 0.08
pH	8.29 ± 0.00	8.29 ± 0.00
Pluviometry (mm)	0.23 ± 0.05	0.01 ± 0.00
Temperature (°C)	25.2 ± 0.05	25.5 ± 0.03

3.2. Trophic behavior

Table 1: Results for the 2-way crossed analysis of variance (ANOVA) performed on SDA, DPA (autotrophy markers), CGA (heterotrophy marker), predominant trophic mode index (PTMI), lipid peroxidation (LPO) and total antioxidant capacity (TAC) investigated for *Mussismilia harttii* exposed to thermal stress and Cu contamination for 4 and 12 days. Significant values ($p < 0.05$) are in bold.

		4-days experiment				12-days experiment			
Variable	**Treatment**	**df**	**F**	***p***	**Fisher**	**df**	**F**	***p***	**Fisher**
DAS	*Cu*	2	3.90	**0.02**	*8.6 > 2.9, 5.4*	2	2.47	0.09	
	Temperature	2	0.14	0.86		2	6.93	**<0.01**	*27.3 > 26.6*
	Cu x Temperature	4	0.75	0.56		4	3.31	**0.01**	*26.6 (8.6 < 2.9)*
DPA	*Cu*	2	0.24	0.78		2	0.52	0.59	
	Temperature	2	2.78	0.07		2	3.81	**0.03**	*27.3 > 26.6*
	Cu x Temperature	4	1.81	0.14		4	0.37	0.82	
CGA	*Cu*	2	0.44	0.64		2	2.42	0.10	
	Temperature	2	1.76	0.18		2	4.70	**0.01**	*27.3 > 26.6*
	Cu x Temperature	4	0.42	0.78		4	0.48	0.75	
PTMI	*Cu*	2	2.39	0.10		2	1.01	1.01	
	Temperature	2	1.1	0.34		2	2.76	2.76	
	Cu x Temperature	4	0.59	0.67		4	1.10	1.10	
LPO coral	*Cu*	2	1.93	0.17		2	4.23	**0.03**	*5.4, 8.6 > 2.9*
	Temperature	2	2.63	0.09		2	7.02	**<0.01**	*27.3 > 25.0*
	Cu x Temperature	4	1.48	0.24		4	1.32	0.29	
LPO symbiont	*Cu*	2	1.35	0.28		2	3.29	0.06	
	Temperature	2	3.55	0.05		2	7.81	**<0.01**	*26.6 < 25.0*
	Cu x Temperature	4	0.61	0.65		4	2.01	0.13	
TAC coral	*Cu*	2	2.88	0.08		2	0.49	0.61	
	Temperature	2	0.61	0.55		2	0.10	0.90	
	Cu x Temperature	4	0.36	0.82		4	0.80	0.53	
TAC symbiont	*Cu*	2	0.40	0.67		2	2.64	0.09	
	Temperature	2	3.19	0.06		2	3.48	0.05	
	Cu x Temperature	4	2.53	0.07		4	3.05	**0.04**	*25.0 (5.4, 8.6 > 2.9)*
									26.6 (8.6 > 2.9)
									27.3 (5.4 < 2.9)

After 4 days of exposure, the concentrations of the autotrophy-related markers SDA and DPA in the tissue of *M. harttii* were not affected by temperature (Table 1). Cu concentration, specifically in the treatment of 8.6 µg L^{-1}, induced a significantly higher production of SDA regardless of temperature (Fig.1). The concentration of the heterotrophy marker CGA was not influenced by either temperature or Cu exposure (Fig. 2). After 12 days of exposure, higher temperature was an influencing factor for all three markers (Fig. 1, 2). An

observed trend is that 26.6ºC had a significant effect in decreasing the concentration of all markers, while 27.3ºC significantly increased all of them; but none differed from the control temperature. The interaction between Cu exposure and temperature was only significant for SDA (Fig. 1).

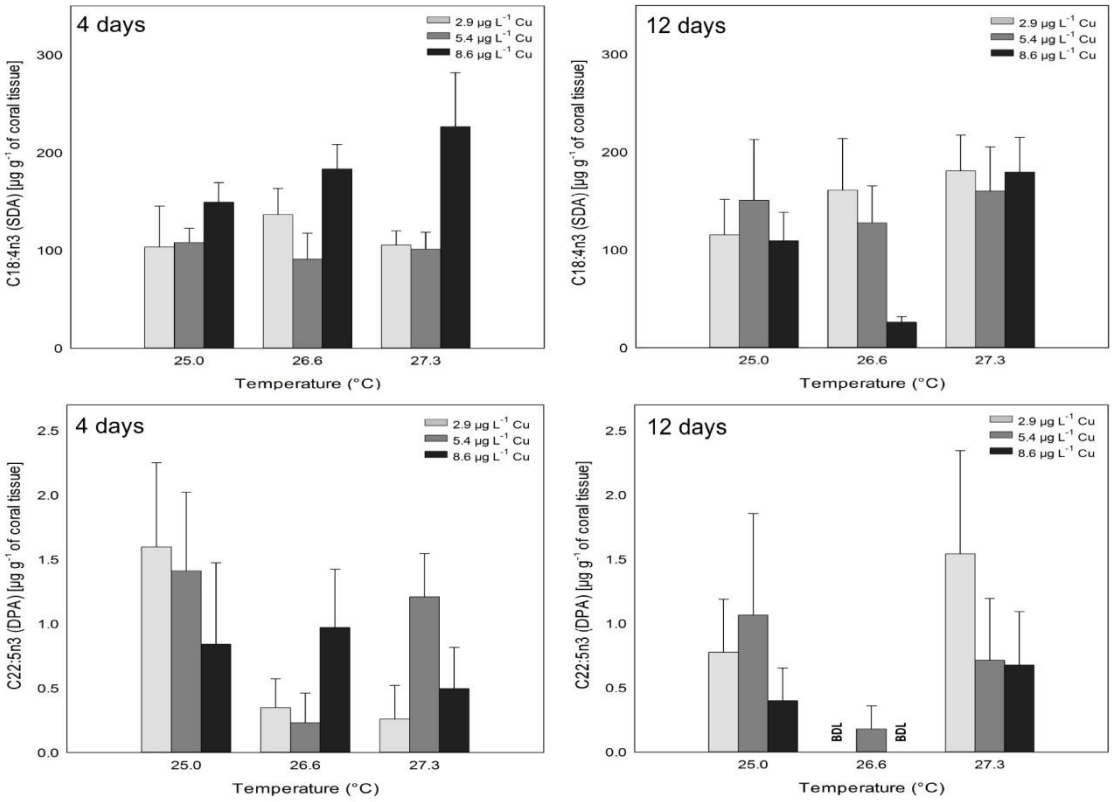

Figure 1: Content of autotrophy fatty acid trophic markers stearidonic acid (SDA) and docosapentaenoic acid (DPA) in the coral *Mussismilia harttii* exposed to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days. BDL: below detection limit.

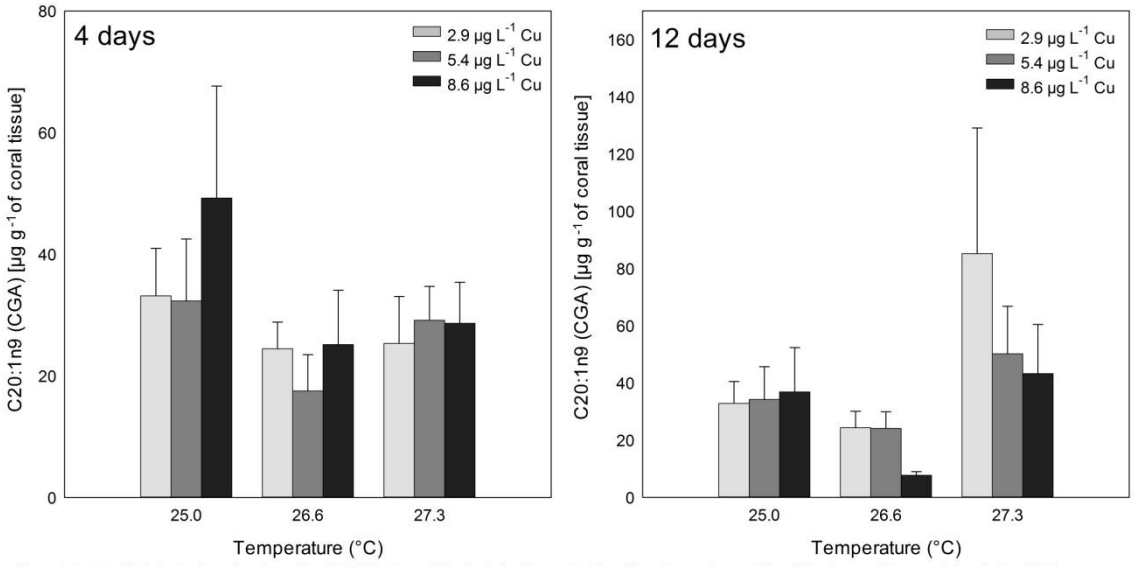

Figure 2: Content of the heterotrophy marker cis-gondoic acid (CGA) in the coral *Mussismilia harttii* exposed to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days.

No significant shifts between trophic modes were detected for either the 4- or the 12-day experiment (Table 1). Neither thermal stress nor exposure to Cu produce impacts on the predominant trophic behavior. The PTMI shows an overall predominance of autotrophy for all treatment combinations. There is an increase in heterotrophy under 27.3ºC for 12 days (Fig. 2), but not enough to promote a shift in the PTMI (Fig. 3).

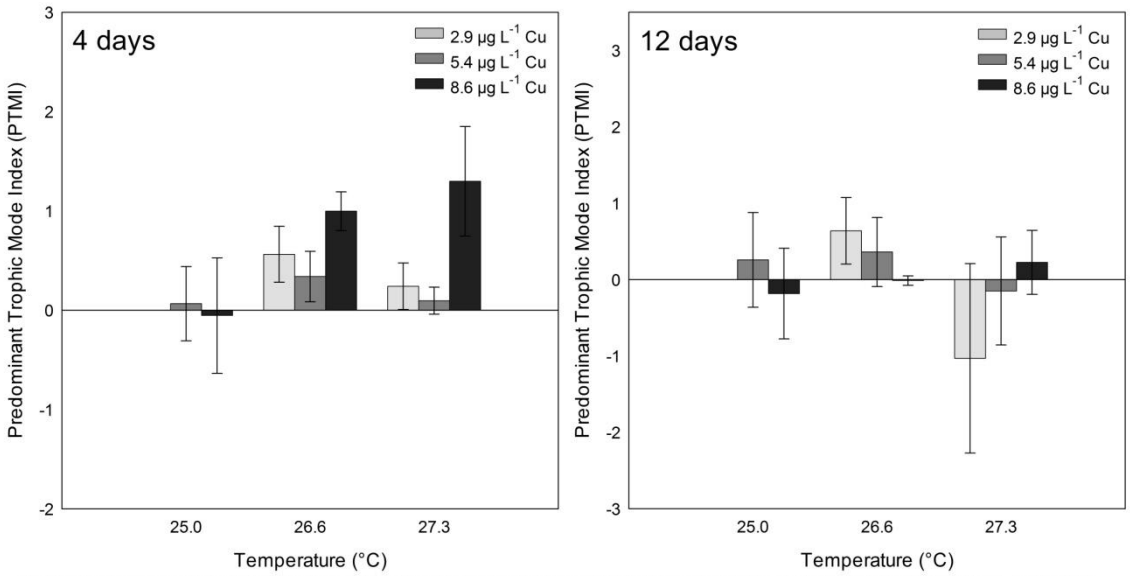

Figure 3: Predominant Trophic Mode Index (PTMI) for the coral *Mussismilia harttii* exposed to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days. Positive values indicate a predominance of autotrophic behavior, while negative values indicate prevalence of heterotrophy.

3.3. Oxidative status

The levels of LPO in the corals were not changed after 4 days (Table 1). However, a significant increase was observed after 12-day exposure to temperature and Cu (Fig. 4, Table 1). Results show that corals exposed at 27.3°C presented higher LPO levels than corals maintained at 25.0°C. LPO levels for corals at 26.6ºC did not differ from those for corals maintained at 25.0 and 27.3ºC. Corals exposed to concentrations of 8.6 and 5.4 µg L^{-1} Cu showed higher LPO levels than corals maintained at 2.9 µg L^{-1} Cu.

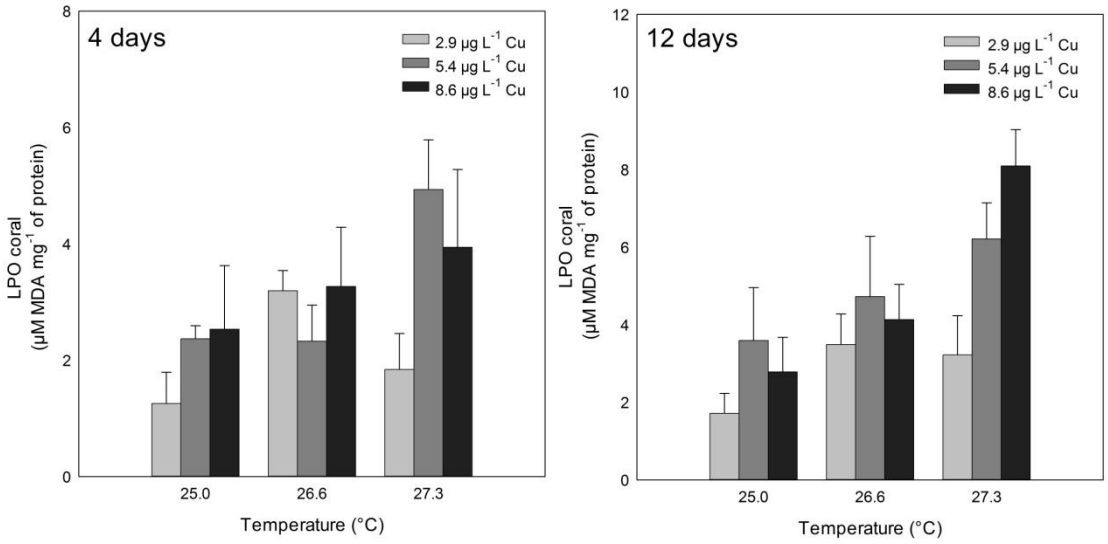

Figure 4: Lipid peroxidation (LPO) for the coral *Mussismilia harttii* exposed to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days.

No effects were observed in the symbiont LPO levels after 4 days of exposure (Table 1). However, effects of temperature were observed in symbionts after 12 days (Table 1, Fig. 5). Exposure to 26.6°C (no Cu addition) reduced LPO levels in symbionts compared to control conditions (25.0°C).

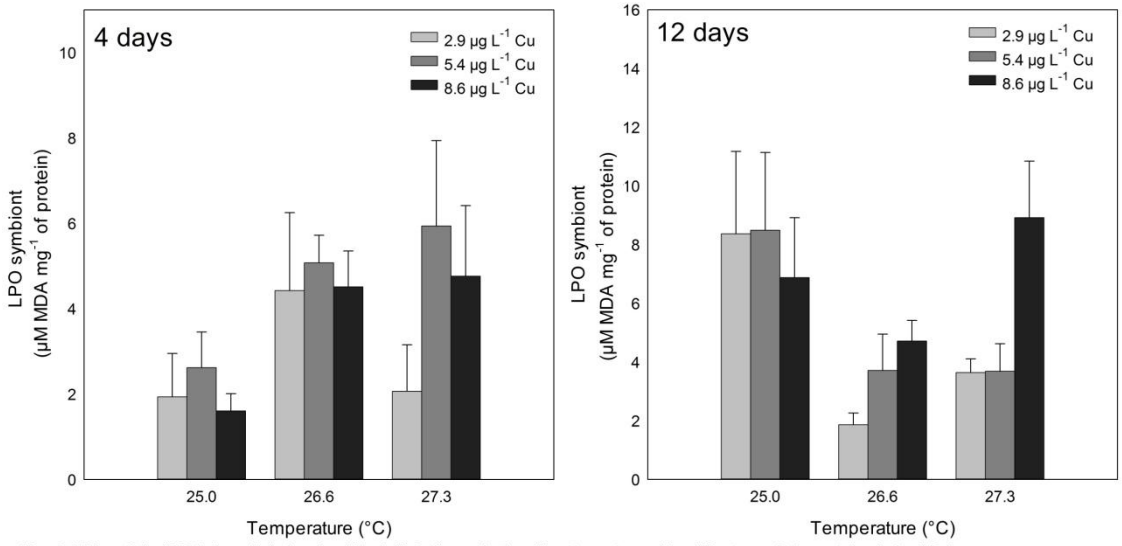

Figure 5: Lipid peroxidation (LPO) for the symbiotic algae of coral *Mussismilia harttii* exposed to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days .

No effects were observed in TAC for corals in exposure to isolated or combined stressors after 4 or 12 days (Table 1, Fig. 6). Similarly, no significant effects were observed in TAC of symbionts after 4 days of exposure. However, after 12 days of exposure significant effects in symbiont TAC were observed for the combination of stressors (Fig. 7). Specifically, results indicate a significant

increase after exposure to Cu alone (5.4 and 8.6 µg L^{-1}) compared to control (2.9 µg L^{-1}). An increase was also observed under 26.6ºC and 8.6 µg L^{-1} compared to 2.9 µg L^{-1} at the same temperature. Exposure to 27.3ºC and 5.4 µg L^{-1} reduced TAC levels in symbionts compared to those maintained at 2.9 µg L^{-1} at the same temperature.

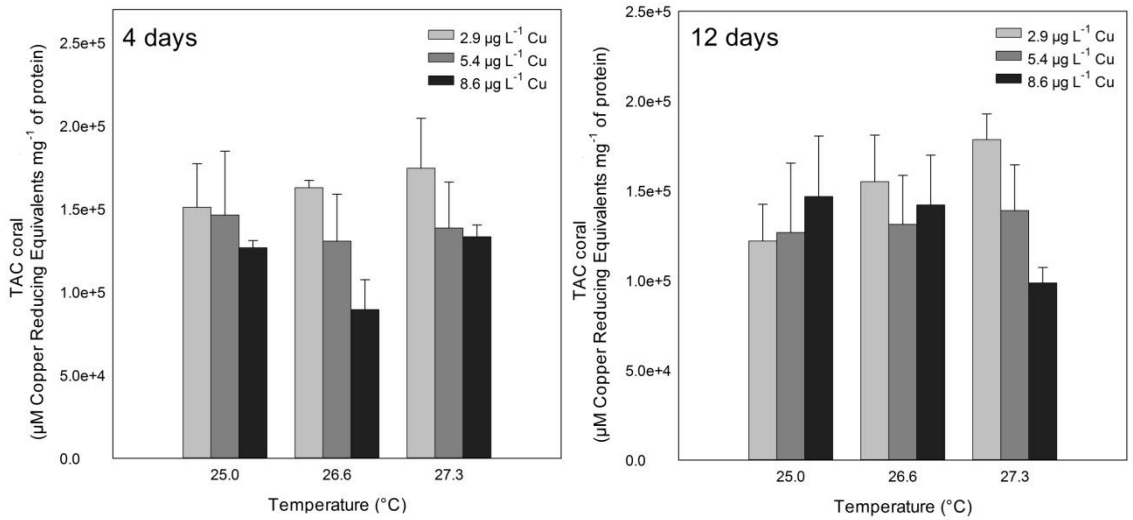

Figure 6: Total antioxidant capacity (TAC) for the coral *Mussismilia harttii* exposed to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days.

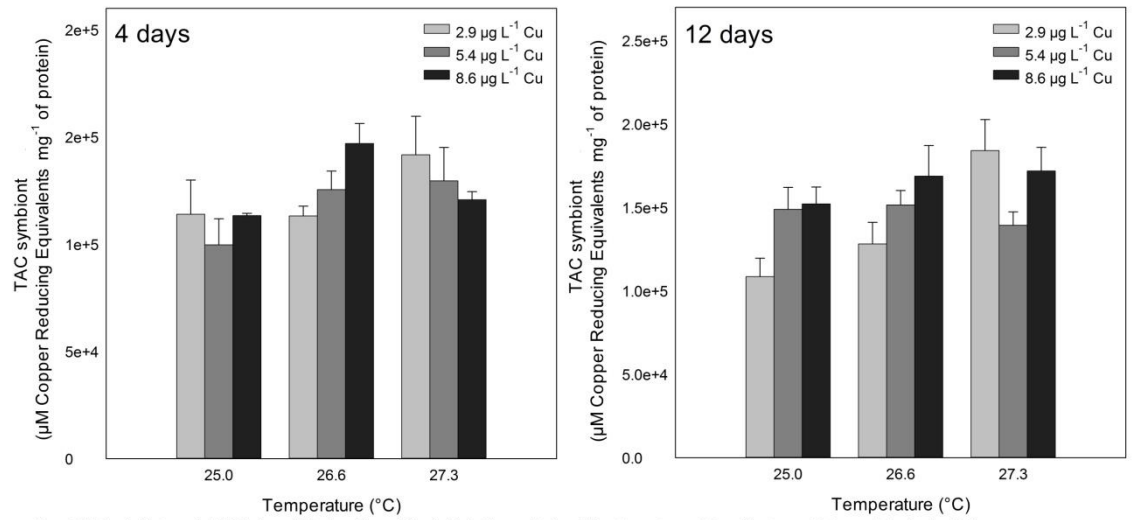

Figure 7: Total antioxidant capacity (TAC) for the symbiotic algae of the coral *Mussismilia harttii* exposed to three different temperatures and three different copper (Cu) concentrations for 4 and 12 days.

4. Discussion

South Atlantic reef-building corals have been described as predominantly autotrophic, with exceptions in cases of climate and anthropogenic disturbances, when heterotrophy becomes predominant (Mies et al., 2018; Marangoni et al., 2019b). Similar trends have also been observed for corals from other locations (Palardy et al., 2005; Roder et al., 2010; Teece et al., 2011). To assess whether the isolated and combined effects of thermal stress and Cu exposure induce a shift in the trophic behavior of *M. harttii*, we evaluated the concentration of fatty trophic markers and calculated the PTMI. Our results show that 4 and 12 days of either isolated or combined exposures to Cu and thermal stress were not sufficient to provoke a shift to heterotrophic predominance. Responses were more intense after 4 days of exposure, in which the concentrations of SDA, a more abundant and definitive marker (see Mies et al., 2018), increased and resulted in a more autotrophic behavior. This was associated with higher Cu contamination and not temperature. It is also possible that this more intense response in the 4-day experiment is associated with an adaptation mechanism, because Cu effects may be more acute than chronic and physiological responses may normalize after that period (Fonseca et al., 2017).

For the 12-day experiment, an effect caused by the combination of thermal stress and Cu maintained an autotrophic predominance although an increase in heterotrophy was observed in this period. It is likely that 12 days is not enough time for 2.5ºC above the mean temperature to trigger a shift in trophic behavior. These shifts have never been detected within the *Mussismilia* genus in an interval shorter than 15 days (Mies et al., 2018; Marangoni et al., 2019b). In

addition, Marangoni et al. (2019b) reported shifts in *M. harttii* trophic behavior only after intense thermal stress (4ºC-weeks; see Kayanne, 2017) over more than 30 days. As for Cu contamination, its effects on trophic mode were more pronounced at the highest concentration tested (8.6 µg L^{-1}) in the 4-day experiment. A previous study showed that *M. harttii* under Cu exposure combined with temperature increase displays unbalanced enzymatic activity in the first 8 days before recovering (Fonseca et al., 2017). Therefore, it is possible that the recovery of enzymatic activity reduced the need for increased autotrophy in the 12-d experiment. In addition, autotrophic behavior was less pronounced for the 12-day experiment; this may have been a response in order to reduce oxidative stress (see Fonseca et al., 2017). It has also been reported that prolonged Cu exposure and thermal stress (12 days) produce severe deleterious effects on enzymes involved in the energy metabolism of *M. harttii* (Fonseca et al., 2019b). It is likely that for longer exposure, *M. harttii* may further increase predation and CGA levels in its tissues (see Marangoni et al., 2019b). An important point to be highlighted is that the increasing temperature was the factor that most influenced the three trophic markers after 12 days. This response is expected considering that this stressor is the one that most affects the coral and zooxanthella association (Lesser 1997; 2006). In addition, exposure to Cu can trigger the increase in detoxification systems, which reduce the effects of combined exposure to thermal stress (Fonseca et al., 2017).

Our findings show that a short-term exposure (4 days) to thermal stress and Cu did not induce changes in the levels of LPO and TAC for both coral and symbiotic algae. This is consistent with results reported for other cnidarians; Main et al. (2010) show that exposure to concentrations of 15 and 50 µg L^{-1} of

Cu induced oxidative stress in the sea anemone *Exaiptasia pallida* only after seven days of exposure. It has also been reported that the combined exposure of thermal stress and Cu did not alter superoxide dismutase activity in the coral *Stylophora pistillata* after an acute exposure of three days (Banc-Prandi and Fine, 2019). This is also in agreement with previous reports that show that few harmful effects associated with calcification, photosynthesis, oxidative stress and energy metabolism occur in the short-term exposure of *M. harttii* to Cu and temperature (Fonseca et al., 2017; 2019b). However, the longer exposure of 12 days increased LPO for *M. harttii* at 27.3ºC and 5.4 and 8.6 µg L^{-1} of Cu. This response was intensified with increases in both isolated Cu and temperature and is a clear indication of oxidative stress in *M. harttii*, likely preceding bleaching. This can happen because (i) higher temperature stimulates metabolic processes and consequently increases ROS production (Lesser, 1997; 2006; Higuchi et al., 2009), and (ii) Cu also induces ROS production through Fenton reactions ($Cu+H_2O_2 \rightarrow Cu^{2+}+OH^{-}+ \bullet OH$; see Wardman and Cadeias, 1996).

It is known that oxidative stress is a major trigger for coral bleaching, a process in which the coral host is challenged by the ROS produced by the symbionts (Lesser et al., 1997; Downs et al., 2002; Lesser et al., 2006). Symbiotic algae are a relevant source of ROS for corals, therefore a faster response of the antioxidant system in the symbionts could be expected. Although our experiments show that TAC remained unchanged for corals, its levels increased significantly after 12 days of exposure to combined stressors for symbionts. This suggests that symbionts respond differently and also that they are indeed likely more tolerant to ROS. Furthermore, prolonged exposure

to isolated temperature reduced the LPO in the symbionts. Such response may be related to (i) activation of mechanisms for the remodeling of membrane lipids which has been suggested as an adaptive response in microalgae in order to reduce damage to biological membranes (Légeret et al., 2016); and (ii) reduction in production of ROS due to an inhibition of photosynthesis. The results also reveal that, for the symbionts, LPO decrease and TAC increase were not always associated. This may have been caused by the action of other more specific antioxidant defenses, such as enzymatic activity associated with superoxide dismutase and catalase, which are not accounted for in the TAC assay (Geracitano et al., 2002; Company et al., 2004; Monferrán et al., 2009).

Prolonged exposure to multiple stressors, either isolated or combined, always exacerbates negative impacts for *M. harttii*. For instance, the prolonged combination of thermal stress and Cu further reduces photosynthetic efficiency, inhibits metabolic enzymatic activity and alters the functioning of calcification enzymes (Fonseca et al., 2017; 2019b). Prolonged exposure to Cu combined with ocean acidification also further reduces photosynthetic rates (Marangoni et al., 2019c). Within that context, our findings show that prolonged exposure also enhances deleterious effects. A 4-day exposure to thermal stress and Cu (isolated or combined effects) was not enough to change the oxidative status, but 12-day exposure did alter for both compartments. However, either a 4- or 12-day exposure to both thermal stress and Cu was not enough to trigger a change in the predominant trophic behavior because of the previously mentioned reasons. Therefore, time of exposure needs to be carefully considered when planning experimentations, especially in the case of tolerant species such as *M. harttii* (see Marangoni et al., 2019b). In fact, further

experiments assessing extremely chronic scenarios (30 days of exposure or more) are welcome.

These results show that, for both the trophic behavior and the oxidative status, the combination of thermal stress and Cu exposure produced little effects in addition to those produced by each stressor individually. It was expected that both heat and Cu stress would trigger the production of ROS, which would significantly alter the oxidative status and impair the photosynthetic efficiency (Lesser, 2006). As a consequence, the coral host would engage in increased heterotrophic feeding in order to compensate for low photosynthate translocation by the symbionts (see Houlbrèque and Ferrier-Pagès, 2009; Mies et al., 2018). However, the holobiont was apparently not stressed to that point. The sole case in which there was an effect by the combination of stressors was in symbiont TAC, suggesting that synergistic effects may not take place in this case. This is similar to reports that the combined effects of thermal stress and pollutant contamination do not produce a synergistic effect on the symbiont primary production (Nyström et al., 2001). However, Fonseca et al. (2019b), reports that there is a synergistic effect on the host coral metabolism. Therefore, the occurrence of synergistic effects may be factor-specific. In addition, these effects may vary according to intensity and our temperature values and Cu concentrations may not have been intense enough to trigger a synergistic effect.

Our findings are relevant in the context of coral reef tolerance to metal pollution. This is particularly important in the case of South Atlantic reefs, which have been recently exposed to tremendous quantities of metals after the break of large mining dams (Francini-Filho et al., 2019). In addition, this work solidifies

M. harttii as an important and tolerant model organism for coral reef investigations. Although currently an endangered species (Leão et al., 2016; IUCN, 2019), *M. harttii* shows tolerance to climate change through heterotrophic plasticity, actively removes solid pollutants from its surface and is particularly tolerant to ocean acidification and metal pollution (Marangoni et al., 2019bc; Santos et al., 2020).

Finally, the results of this study highlight the combined effects of thermal stress and Cu exposure (i) in trophic behavior in a scleractinian coral, and (ii) into the individual responses of the host and symbionts of an important coral specie from South Atlantic reefs.

5. Conclusion

Twelve days of combined exposure to Cu and thermal stress cause different responses in *M. harttii* and its symbionts and the coral oxidative status is significantly altered. However, 12 days is not enough to trigger a shift of predominance from autotrophy to heterotrophy. These results are relevant as they depict the holobiont physiological response to the combination of global and local stressors.

6. Acknowledgments

We thank “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES; Programa Ciências do Mar, Brazil), “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq; Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática, Brazil) and “International Development Research Center” (IDRC; Ottawa, Canada). “Fundação de Amparo à Pesquisa

do Estado de São Paulo" (FAPESP; 2016/18348-1) is acknowledged for the use of the gas chromatograph with triple quadrupole mass spectrometer. We also thank "Coral Vivo Project" and its sponsors Petrobras (Programa Petrobras Ambiental) and "Arraial d'Ajuda Eco Parque". Adalto Bianchini is research fellow of CNPq (Proc. # 307647/2016-1).

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6. Discussão geral

Para avaliar os efeitos combinados do aumento da temperatura e da exposição ao Cu em parâmetros fisiológicos e bioquímicos no *Mussismilia harttii*, nós testamos o efeito de três diferentes temperaturas (temperatura média do local de 25.0, e 1.5 e 2.5 ºC acima da média, que são níveis semelhantes aos cenários climáticos futuros emitidos pelo IPCC (IPCC, 2014)) combinadas a três níveis de contaminação por Cu (0, 3 e 5 µg/L acima da concentração média natural) por até 12 dias de exposição em um mesocosmo marinho. Diante dos efeitos fisiológicos e bioquímicos já conhecidos do Cu em organismos aquáticos a hipótese era de que a exposição combinada ao aumento da temperatura poderia ser mais prejudicial ao coral comparado à exposição isolada desses estressores.

A nossa primeira linha de pesquisa foi à avaliação dos efeitos isolados e combinados do Cu e estresse térmico no metabolismo energético do coral. Nós avaliamos a atividade de sete enzimas envolvidas em diferentes etapas do metabolismo energético: glicólise (hexoquinase, piruvato quinase e lactato desidrogenase), ciclo de Krebs (citrato sintase, isocitrato desidrogenase), cadeia transportadora de elétrons (ETS) e via das pentoses-fosfato (glicose-6-fosfato desidrogenase). De fato, nós mostramos que a exposição prolongada aos estressores combinados inibe sinergicamente a atividade dessas enzimas no coral, comprometendo a produção de energia. A partir desse momento, ficou claro que a exposição combinada aos estressores é muito prejudicial para enzimas do metabolismo energético do coral. A próxima etapa seria o entendimento do mecanismo de ação responsável por esse resultado.

A nossa segunda linha de pesquisa objetivou o entendimento do funcionamento do metabolismo energético em condições de campo e em desvendar um possível mecanismo de ação do Cu na inibição da atividade de enzimas do metabolismo energético do coral. Para isso, nós avaliamos a atividade de oito enzimas envolvidas na glicólise (hexoquinase, fosfofrutoquinase, piruvato quinase e lactato desidrogenase), ciclo de Krebs (citrato sintase, isocitrato desidrogenase), cadeia transportadora de elétrons (ETS) e na via das pentoses-fosfato (glicose-6-fosfato desidrogenase) em corais coletados em campo. Além disso, realizamos os testes de exposição ao Cu *in vitro*. Nesse conjunto de análises, nós demonstramos a capacidade deste metal em inibir a atividade da LDH e ETS pela interação direta com essas enzimas.

Ainda em busca de um possível mecanismo fisiológico e devido ao conhecido papel do aumento da temperatura e da exposição ao Cu no desequilibro do estado redox dos organismos, a nossa terceira linha de estudo focou na avaliação do efeito dos dois fatores (estresse térmico e exposição ao Cu) no comportamento trófico e no estado oxidativo do coral. O comportamento trófico foi investigado através da determinação dos níveis de ácidos graxos específicos da autotrofia (SDA 18: 4ω3; DPA 22: 5ω3) e da heterotrofia (CGA 20: 1ω9) e o estado oxidativo através da análise de LPO e TAC nos corais hospedeiros e algas simbiontes. Curiosamente, nesse caso, os resultados mostraram que a exposição aos estressores combinados não foi suficiente para causar alteração nos parâmetros avaliados. A análise desse conjunto de dados mostra que os resultados deste capítulo têm pouca relação com os resultados

encontrados no capítulo I, considerando a busca do mecanismo inibitório observado nas enzimas metabólicas.

A partir do observado até o momento, está claro que a exposição combinada do Cu ao estresse térmico afeta mais severamente alguns marcadores avaliados do que outros. Neste caso, enzimas do metabolismo energético estão entre os parâmetros mais sensíveis, enquanto alterações no comportamento trófico e no estado oxidativo não são observadas. Outro ponto importante de ser destacado é que o efeito desses estressores na predominância trófica e no estado oxidativo podem variar de acordo com a intensidade e nossos valores de temperatura e concentrações de Cu podem não ter sido intensos o suficiente para desencadear um efeito sinérgico.

Em geral, de acordo com os resultados observados nesta tese ainda não é possível explicar os mecanismos inibitórios do aumento da temperatura e da exposição ao Cu na atividade de enzimas do metabolismo energético relacionando a um desequilíbrio do estado redox do coral. Importante ressaltar que nós mostramos que algumas enzimas especificas do metabolismo energético são inibidas pela interação direta do metal com a proteína, conforme observado do capítulo II. Entretanto, dentro dos parâmetros de estado oxidativo analisados (LPO e TAC) não é possível observar uma alteração do estado redox desencadeada pela exposição combinada aos dois fatores. Um ponto que pode ser considerado é o fato da inibição das enzimas do metabolismo energético causada pela exposição combinada dos estressores ter contribuído para a redução na produção de ERO. Importante destacar que a cadeia transportadora de elétrons é a principal fonte de ERO (Sokova e Lannig, 2008), além disso, um efeito inibitório após a exposição combinada à

temperatura e cobre foi observado na atividade do ETS em *M. harttii* (resultado observado no capítulo I). Essa resposta fisiológica pode ter favorecido a manutenção dos níveis basais dos marcadores tróficos e oxidativos observados no capítulo III. Na conjuntura de resultados apresentados pela tese, essa seria a hipótese mais bem aceita.

Outro aspecto importante de ser considerado é a avaliação de danos em biomoléculas diferentes da atualmente analisada (LPO) e a avaliação da atividade de enzimas especificas envolvidas no sistema de defesa antioxidante. Esses resultados podem contribuir para uma resposta mais robusta do estado oxidativo do coral e auxiliar no entendimento dos efeitos fisiológicos do aumento da temperatura e da exposição ao Cu observados no capítulo I. Todos esses questionamentos serão desvendados em um futuro breve. Um resumo dos resultados e a hipótese apresentada por esta tese pode ser encontrada no esquema apresentado na figura 9.

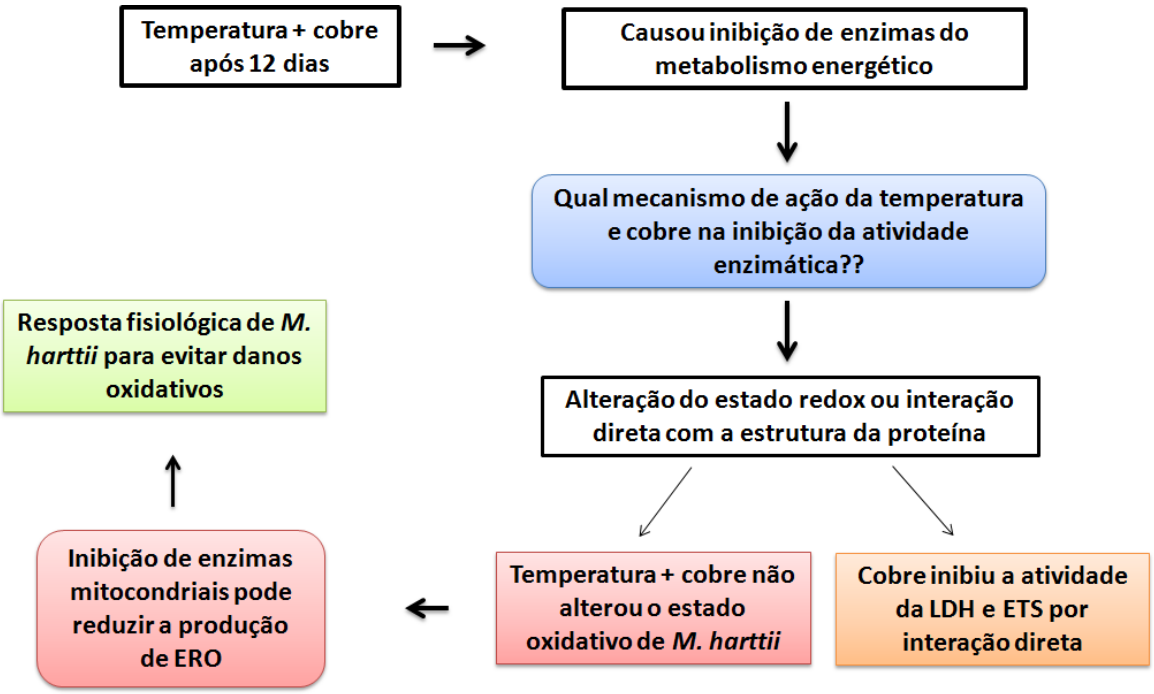

Figura 9: Modelo esquemático sobre os efeitos fisiológicos e bioquímicos da exposição ao aumento de temperatura e cobre (Cu) e mecanismos de recuperação no coral *Mussismilia harttii* após a exposição prolongada (12 dias). A exposição ao aumento da temperatura e Cu inibiu enzimas envolvidas em diferentes etapas do metabolismo energético do coral *M. harttii.* A partir desse momento, os demais capítulos da tese foram focados em entender o mecanismo de ação que conduziu a esta resposta. A exposição ao Cu *in vitro* mostrou a capacidade deste metal em inibir a atividade da LDH e ETS por interação direta. Portanto, a inibição de enzimas do metabolismo energético observada *in vivo* pode estar envolvida com a capacidade do metal em interagir diretamente com a estrutura da proteína. O próximo passo foi entender se a exposição ao aumento de temperatura e Cu causou uma alteração do estado oxidativo de *M. harttii* para então relacionar a inibição de enzimas do metabolismo energético a uma condição de estresse oxidativo. Curiosamente, a exposição combinada aos estressores não alterou os marcadores tróficos e oxidativos analisados. Dentro do conjunto de dados apresentados, a hipótese mais bem aceita é que a inibição de enzimas do metabolismo energético pode estar relacionada a uma resposta fisiológica de *M. harttii* para reduzir danos oxidativos, o que explicaria os níveis basais dos marcadores tróficos e oxidativos observados.

6.1. Informações relevantes para os próximos estudos com *M. harttii*

Recentes estudos têm mostrado que a exposição prolongada a diferentes estressores impacta negativamente a fisiologia de *M. harttii.* Por exemplo, a exposição prolongada do estresse térmico ao cobre reduz a eficiência fotossintética e altera a atividade de enzimas envolvidas no processo de calcificação (Fonseca et al., 2017). Ainda, a exposição prolongada do cobre combinada a acidificação também reduz a eficiência fotossintética (Marangoni et al., 2019a). Porém, dentro dos resultados apresentados na tese, nós mostramos que muitos efeitos deletérios podem ser recuperados após a exposição combinada do cobre ao estresse térmico. Um ponto importante a ser considerado é que o tempo de exposição deve ser cuidadosamente planejado em experimentos, principalmente envolvendo uma espécie tolerante como *M. harttii* (ver em Marangoni et al., 2019b). Demais outros experimentos envolvendo cenários crônicos (30 dias ou mais de exposição) são bem-vindos.

É importante notar também que o experimento de exposição à temperatura e cobre do qual originou os capítulos I e III apresentados por esta tese foi realizado durante a estação do inverno e testou temperaturas variando de 25.0 a 27.3°C. Além disso, as concentrações de cobre testadas estão próximas aos limites estabelecidos pela legislação brasileira (CONAMA, 2005) e já foram detectadas concentrações semelhantes às testadas no Recife de Fora (Marques et al., 2019). Ainda, o valor máximo de temperatura foi de 29.3°C no local de coleta dos corais durante o verão de 2012 e 2013 (http://www.pmel.noaa.gov). Portanto, tais cenários testados de exposição combinada são passíveis de serem encontrados por essa espécie de coral no ambiente e as respostas apresentadas aqui podem ser ainda mais preocupantes em episódios de El Niño.

Apesar disso, as condições experimentadas foram suficientes para mostrarmos mecanismos de defesa em *M. harttii* através de ajustes no metabolismo energético e oxidativo após o estresse causado pela exposição combinada do aumento da temperatura ao cobre. Importante ressaltar aqui que durante o experimento não foram observadas manisfestações visuais de branqueamento nos corais. Essa observação ressalta o poder predito dos marcadores bioquímicos e fisiológicos testados para avaliação do efeito da combinação de estressores ambientais. Os resultados encontrados por esta tese foram importantes para elucidar as bases fisiológicas que justificam aspectos da tolerância de *M. harttii* já observados em outros estudos (Marangoni et al., 2019b; Santos et al., 2020).

7. Conclusão geral

O conjunto de resultados desta tese mostrou que o aumento de temperatura combinado com a exposição ao Cu inibe a atividade de enzimas do metabolismo energético do coral *M. harttii.* Além disso, demonstramos a capacidade do Cu em interagir diretamente com enzimas especificas envolvidas no metabolismo energético e contribuir para esta inibição. Ainda, mostramos que a combinação desses dois fatores (estresse térmico e cobre) afeta mais severamente alguns marcadores do que outros. Neste caso, enzimas do metabolismo energético estão entre os parâmetros mais sensíveis, enquanto alterações no comportamento trófico e no estado oxidativo não são observadas. Dentro dos parâmetros de estresse oxidativo avaliados (LPO e TAC) foi descartado a hipótese de que a combinação do estresse térmico à exposição ao Cu causa desequilíbrio do estado redox e consequentemente inibição da atividade enzimática. Porém a partir do conjunto de dados apresentados é possível que a inibição da atividade de enzimas do metabolismo energético possa contribuir para redução na produção de ERO e consequentemente para a manutenção dos níveis basais dos marcadores tróficos e oxidativos. Os resultados observados nesta tese demonstram que *Mussismilia harttii* pode ativar mecanismos fisiológicos baseados na diminuição do metabolismo energético para reduzir o estresse causado pela exposição combinada aos estressores.

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