Universidade Federal do Rio Grande – FURG
Instituto de Ciências Biológicas – ICB
Programa de Pós-Graduação em Ciências Fisiológicas

Dissertação de Mestrado

EFEITOS INTERATIVOS DO AUMENTO DE TEMPERATURA E DA EXPOSIÇÃO AO COBRE EM PARÂMETROS FISIOLÓGICOS E BIOQUÍMICOS NO CORAL *Mussismilia harttii*

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JULIANA DA SILVA FONSECA

Orientador: Dr. Adalto Bianchini

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Sumário

Resumo geral

O efeito do aumento da temperatura da água e da combinação desse estressor com a exposição ao cobre (Cu) foi avaliado no coral *Mussismilia harttii*. Os parâmetros avaliados incluem aqueles envolvidos no processo de calcificação [anidrase carbônica (AC) e (Ca^{2+}, Mg^{2+})-ATPase], metabolismo energético [capacidade fotossintética máxima (*Fv/Fm*) e concentrações de clorofila a e ATP] e estado oxidativo [capacidade antioxidante contra radicais peroxil (ACAP) e peroxidação lipídica (LPO)]. Pólipos do coral foram coletados, aclimatados em mesocosmo marinho e expostos a três tratamentos térmicos (temperatura ambiente da água do mar e acréscimos de 1ºC e 2ºC nesta temperatura) de forma isolada e combinada a diferentes concentrações de Cu dissolvido [1.9 (sem adição de Cu), 3.8, 5.4 e 8.6 μg L^{-1}] por 4, 8 e 12 dias. A *Fv/Fm* diminuiu ao longo do tempo de exposição ao aumento de temperatura. A interação deste estressor com a exposição ao Cu aumentou este efeito. As atividades da AC e (Ca^{2+}, Mg^{2+})-ATPase aumentaram até 8 dias de exposição, mas não foram afetadas após 12 dias de exposição. A exposição aos estressores causou uma redução da LPO, sugerindo a ativação de mecanismos para remodelamento de lipídeos nas membranas biológicas. Porém, após a exposição prolongada (12 dias) ao aumento de temperatura foi observado aumento da LPO, sugerindo uma menor produção de energia pela redução na transferência de fotossintatos. Por sua vez, a ACAP, a clorofila a e o ATP não foram alterados após a exposição aos estressores. Estes resultados indicam que os estressores avaliados reduzem a fotossíntese e possivelmente a transferência de fotossintatos utilizados como fonte de energia para os corais. Portanto, o aumento de temperatura e a exposição ao Cu podem reduzir a disponibilidade de energia, afetar o crescimento e consequentemente aumentar a susceptibilidade do coral *M. harttii* ao branqueamento.

Palavras-chave: calcificação, cobre, coral, estresse oxidativo, fotossíntese, temperatura.

Introdução geral

Os recifes de coral são ambientes muito produtivos e apresentam grande biodiversidade (Hoegh-Guldberg , 1999). Além disso, são importantes fontes de renda (Carte 1996), possuindo um alto valor econômico, devido à atividade pesqueira dependente desses ecossistemas. De fato, os recifes de coral proporcionam um ambiente rico para peixes e outros organismos marinhos, sendo que 6 milhões de toneladas do pescado capturado anualmente estão associadas a esses ecossistemas (James and Crabbe, 2008).

Os corais são organismos que vivem em ambientes tropicais com temperaturas entre 18 e 30 ºC (van Dam *et al*., 2011). A formação e manutenção dos recifes de coral de águas rasas dependem principalmente dos corais escleractíneos (Classe Anthozoa, Subclasse Hexacorallia, Ordem Scleractinia), conhecidos como corais pétreos ou verdadeiros. Existem cerca de 600 espécies de corais calcificadores (escleractíneos) que contribuem diretamente para formação dos recifes de corais, um habitat característico para peixes, algas e invertebrados (Hoegh-Guldberg, 1999). Os corais escleractíneos realizam o processo de calcificação e possuem simbiose com zooxantelas, dinoflagelados endossimbiontes do gênero *Symbiodinium spp.*, localizadas nas células do tecido oral dos corais (Al-Sofyani & Floos, 2013).

As zooxantelas podem fornecer até 90% do carbono que é direcionado a alimentação dos corais (Papina *et al*. 2003) e da energia para o crescimento (Goreau 1959). Porém, a simbiose entre corais e zooxantelas é vulnerável a variações nas condições ambientais, tais como alterações na temperatura, poluição e intensidade de luz, fatores que podem induzir o fenômeno de branqueamento em corais (Fabricius *et al*., 2005; Hoegh-Guldberg, 1999). Este fenômeno se caracteriza pela expulsão das zooxantelas pelo coral e/ou degradação dos pigmentos fotossintetizantes dessas

microalgas (Downs *et al.*, 2002). Isto afeta os corais, uma vez que sem as zooxantelas, eles são privados dos nutrientes provenientes da fotossíntese (Ferrier-Pagès *et al.*, 1998). Neste contexto, cabe ressaltar que o aumento da temperatura e a poluição química são causas reconhecidas de branqueamento em corais (Nystron *et al*., 2001), sendo que estas são as principais causas propostas para explicar a crescente degradação dos recifes de coral em escala mundial (Anthony *et al*., 2007; Fabricius *et al*., 2005).

No que concerne à fauna de corais esclarectíneos do Brasil, essa se restringe a 18 espécies (Leão *et al*., 2003), o que caracteriza uma baixa diversidade quando comparada àquela dos recifes de outras partes do mundo. Por exemplo, os recifes do Caribe apresentam 918 espécies (Miloslavich *et al*., 2010). Em contrapartida, os recifes brasileiros apresentam um alto grau de endemismo. Dentre as 18 espécies de corais esclarectíneos recorrentes, 6 espécies são exclusivas do Brasil (Leão *et al*., 2003). Dentre as espécies endêmicas, destaca-se *Mussismilia harttii* (Verrill, 1868), um coral zooxantelado que ocorre desde o Rio Grande do Norte até a Bahia. Este desempenha importante papel ecológico na formação dos recifes (Soares, 2011). Porém, segundo o Instituto Chico Mendes da Biodiversidade (ICMBio, 2014), *M. harttii* está listada como sendo uma espécie ameaçada de extinção.

Estudos têm mostrado que *M. harttii* é uma espécie sensível ao aumento da temperatura da água, e que pode sofrer estresse térmico após um longo período de exposição à 28ºC, que é a temperatura média máxima de verão na região de Abrolhos (Soares, 2011). Além disso, a ação de estressores locais, como a contaminação por cobre, pode alterar a atividade de enzimas de calcificação e causar estresse oxidativo em *M. harttii*, aumentando a susceptibilidade desta espécie de coral ao branqueamento (Marangoni *et al*., 2017). Apesar disso, pouco se sabe a respeito das respostas bioquímicas de *M. harttii* ao efeito da exposição a múltiplos estressores, tais como o

aumento de temperatura da água do mar associada às mudanças climáticas e a contaminação da água pelo cobre devida a ações antrópicas.

As mudanças climáticas globais tornou-se uma questão proeminente no final do século passado, sendo que as perspectivas futuras apontam um sério transtorno do potencial impacto dessas mudanças para o bem-estar das gerações futuras (Sokolova *et al.,* 2008). Modelagens recentes preveem um aumento de 0,2ºC na média da temperatura global a cada década, podendo atingir um aumento de 2,6 a 4,8ºC até o ano de 2100, fazendo com que o nível dos oceanos aumente entre 45 e 82 cm (IPCC 2007; IPCC 2014). Este cenário é resultante de um conjunto de atividades humanas, tais como desmatamento, erosão do solo, queima de combustíveis fósseis, entre outras, que colaboram para elevar os níveis atmosféricos dos gases de efeito estufa, principalmente do dióxido de carbono (CO_2) (Freitas *et al*., 2012).

A elevação na temperatura global pode acarretar sérias consequências, tais como aumento na incidência de doenças (Marengo *et al*.,2008; Martens *et al*., 1999), declínio da abundância de zooplâncton (Reid *et al*., 1998), branqueamento em massa de corais (Suzuki *et al*., 2007), mudanças nos habitats marinhos (Beaugrand 2004), introdução de espécies invasivas (Lewis *et al*., 2003), extinção local de populações (Helmuth *et al*., 2002), ameaça ao fornecimento de água (Carere *et al*., 2011), derretimento de geleiras (Marengo *et al*.,2008) e aumento de eventos extremos, como tempestades e furacões (Freitas *et al*., 2012), aumentando assim a hostilidade do ambiente para a vida dos organismos.

Os ecossistemas aquáticos são mais vulneráveis aos efeitos do aumento da temperatura, pois mais de 95% das espécies aquáticas são ectotérmicas (Willmer *et al.*, 2000). O aumento da temperatura tem um papel chave na fisiologia de ectotérmicos, pois pode resultar em alterações na estabilidade de biomoléculas, nas reações

bioquímicas e taxas fisiológicas (Hochachka & Somero 2002). Dentre todos os ecossistemas marinhos, os recifes de coral são os mais vulneráveis a mudanças climáticas (Madin *et al.*, 2012), sendo que o estresse térmico pode causar mudanças na estrutura, dinâmica, produtividade e diversidade de recifes de coral (Calderon-Aguilera *et al*., 2012). Estima-se que entre 50 a 70% dos recifes de coral estão sobre ameaça direta e imediata de mudanças climáticas e poluição química (Wilkinson, 2004).

Em geral, as áreas costeiras e estuarinas são mais impactadas do que as áreas oceânicas, em virtude do desenvolvimento industrial e agrícola (Clark, 2001). Na presença dos impactos antrópicos locais, os organismos têm que lidar com a influência de múltiplos estressores, tais como poluição, hipoxia, sobrepesca, eutrofização, entre outros, além daquela associada às mudanças climáticas. O estresse causado pela contaminação química pode aumentar a susceptibilidade dos organismos aos impactos de mudanças climáticas (van Dam *et al*., 2012). Portanto, o entendimento sobre o efeito interativo de múltiplos estressores, tais como o aumento da temperatura e a poluição por metais, é necessário para compreender os mecanismos envolvidos nas respostas dos organismos a esses estressores e prever os limites de tolerância e sobrevivência dos organismos (Sokolova *et al.*, 2008).

Muitos estudos têm investigado o efeito do aumento da temperatura na toxicidade de metais em ectotérmicos (Heugens *et al*., 2006; Martínez-Jerónimo *et al*., 2006; Yang and Chen, 1996). O aumento da temperatura pode elevar as taxas metabólicas, que por sua vez favorece a captação e acumulação dos metais nos organismos (Brown *et al*., 2004; Heugens *et al*., 2003; Muyssen *et al*., 2010; Wang *et al*., 2014). Além disso, a biodisponibilidade dos metais no ambiente aquático pode ser afetada pelo aumento da temperatura (Rainbow, 2007), uma vez que o aumento da temperatura pode reduzir a solubilidade de compostos que funcionam como

complexantes dos metais na água. Compostos com agrupamentos carboxílicos e fenólicos são exemplos dos quais os metais têm alta afinidade. Desta forma, o aumento da temperatura pode influenciar na especiação de metais no ambiente, aumentando a biodisponibilidade dos metais nas suas formas livres (Bianchini *et al*., 2009). Além disso, o aumento da temperatura altera a permeabilidade das membranas biológicas, o que também pode influenciar no grau de exposição ao metal no ambiente (Kerswell & Jones, 2003, Wood *et al*., 1999).

Os metais são poluentes persistentes no ambiente aquático, estando entre as 20 substâncias mais perigosas do mundo (EPA, 2005). Sabe-se que a incorporação de metais no ambiente ocorre principalmente através de processos naturais, tais como, vento, erosão continental, erupções vulcânicas, entre outros. Entretanto, sabe-se que atividades antrópicas colaboram cada vez mais para elevar os níveis desses contaminantes no ambiente (Bianchini *et al*., 2009). De fato, as atividades industriais, como mineração e fundição, bem como o despejo de dejetos e extração de petróleo são importantes fontes antrópicas de metais no ambiente marinho (Loya & Rinkevich, 1980). Dentre os impactos locais que afetam a saúde do ambiente recifal, destaca-se a contaminação por metais, como o cobre (van Dam *et al*., 2011). A incorporação do cobre é feita pelo uso em materiais condutores (fios e cabos), tubos de encanamento, motores elétricos e interruptores. Além disso, o cobre é utilizado como algicida em reservatórios de abastecimento de água e unidades de aquicultura para o controle de microorganismos (Bianchini *et al*., 2009). O cobre se caracteriza por ser um metal essencial para a manutenção das funções fisiológicas (Morgan, 2000). Porém, em concentrações excessivas pode ser tóxico aos organismos aquáticos (Heath, 1995), podendo inclusive induzir o branqueamento em corais (Bielmyer *et al*., 2010; Jones, 2004, 1997).

Com relação aos efeitos do aumento da temperatura e exposição ao cobre, sabe-se que ambos estressores interferem sobre o metabolismo dos corais (Nystron *et al*., 2001). O aumento da temperatura reduz a capacidade fotossintética (Nystron *et al*., 2001) e aumenta a taxa respiratória dos corais (Coles and Jokiel 1977; Porter, 1999; Rivest & Hofmann 2014), devido ao aumento do metabolismo para a síntese de biomoléculas protetoras ao estresse térmico (proteínas de choque térmico, metalotioneínas, glutationa, etc.), causando o desequilíbrio em outros sistemas fisiológicos, como neste caso, a fotossíntese (Gates & Edmunds 1999). Por sua vez, altas concentrações de cobre reduzem a taxa de respiração (Nystron *et al*., 2001) e inibem o transporte de elétrons para o fotossistema II das zooxantelas (Kuzminov *et al*., 2013; Samson *et al*., 1988).

Dentre as estratégias para se avaliar o efeito biológico de estressores, destaca-se o uso de biomarcadores. Essas ferramentas biotecnológicas podem ser definidas como alterações bioquímicas, celulares e fisiológicas, que fornecem evidências sobre a exposição e/ou efeitos causados por contaminantes (Depledge, 1995) ou variações ambientais. A vantagem da utilização dos biomarcadores consiste no seu potencial preventivo contra os possíveis efeitos biológicos e ecológicos de estressores (Heath, 1995).

A calcificação realizada pelos corais é uma característica fisiológica que se revela como um interessante biomarcador da saúde do ambiente recifal (Marangoni *et al*., 2017; Marques *et al*., 2017). Este processo está intimamente ligado à relação simbiótica entre os corais e as zooxantelas (Freitas *et al*., 2012), uma vez que o branqueamento pode resultar na redução das taxas de calcificação (Bachok *et al*., 2006). A calcificação dos corais resulta na formação da aragonita, um polimorfo de carbonato de cálcio ($CaCO_3$). Estes cristais formam-se a partir da obtenção de cálcio (Ca^{2+}) por

transporte ativo e do carbono inorgânico, principalmente sob a forma de bicarbonato (HCO_3^-), advindos da água do mar ou do metabolismo do coral, e do transporte desses compostos para o fluído de calcificação extracelular (FCE). No FCE, que se localiza entre o tecido orgânico e o esqueleto mineral, forma-se a aragonita (Allemand *et al.*, 2004). Assim, visto que o processo de calcificação tem papel central na fisiologia dos corais, ferramentas para avaliação deste processo podem indicar quão saudável encontra-se o recife ou o quanto este está se desenvolvendo (De'ath *et al.,* 2009; Hoegh-Guldberg, 1999).

A anidrase carbônica (AC) e a (Ca^{2+}, Mg^{2+})-ATPase são enzimas-chave no processo de calcificação realizado pelos corais. A AC catalisa a reação reversível de hidratação do CO_2 em HCO_3^- e prótons (H^+), desempenhando assim um papel fundamental no suprimento de carbono inorgânico dissolvido (CID) para a calcificação dos corais e a fotossíntese realizada pelos simbiontes (Bielmyer *et al.,* 2010). Com relação a Ca^{2+}-ATPase, esta possui um sistema acoplado ao transporte de Ca^{2+} em corais, pois ao mesmo tempo em que direciona Ca^{2+} para o sítio de calcificação, remove $2H^+$ do mesmo. Desta forma, esta enzima estimula a formação de $CaCO_3$ ($Ca^{2+} + CO_2 + H_2O \leftrightarrow CaCO_3 + 2H^+$) (Al-Horani *et al*., 2003) e auxilia na manutenção do estado de saturação da aragonita (Marangoni *et al*., 2016). Por sua vez, sugere-se que a Mg^{2+}-ATPase é a enzima responsável pelo controle temporal e espacial da correta liberação do Mg^{2+} nas superfícies mineralizantes. Portanto, ela desempenha um papel importante na formação de esqueletos de coral (Meibom *et al.*, 2004).

Com relação ao efeito de estressores, sabe-se que a exposição ao cobre pode interferir no funcionamento das enzimas mencionadas acima em corais, incluindo *M. harttii* (Bielmyer *et al.*, 2010; Marangoni *et al*., 2017). Reduções nas atividades da Ca^{2+}-ATPase e AC, devido à exposição ao cobre, podem portanto afetar o processo de

calcificação em *M. harttii*. Neste contexto, cabe ressaltar que o cobre é um metal envolvido na geração de estresse oxidativo via reação de Fenton (Hermes-Lima, 2004; Wardman & Cadeias, 1996). Considerando a fisiologia de corais zooxantelados, é importante salientar que o estresse oxidativo afeta a associação coral-simbionte, na qual o oxigênio produzido na fotossíntese é passado para as células dos corais (Rotchell & Ostrander, 2011), podendo aumentar os danos oxidativo neste último. Assim, sugere-se que o estresse oxidativo esteja relacionado ao processo de branqueamento em corais. De fato, sob determinado grau de estresse, os corais eliminam a fonte dominante de produção de espécies reativas de oxigênio (ERO), expulsando assim as zooxantelas (Downs *et al*., 2002).

O branqueamento é um fenômeno que afeta gravemente os corais (Freitas *et al*., 2012). Na ausência das zooxantelas, os corais perdem acesso aos combustíveis energéticos responsáveis pela produção de ATP, tais como oxigênio, aminoácidos, glicose e alguns peptídeos, que são importantes para o aumento das taxas de calcificação e manutenção de outras funções fisiológicas (Teece *et al*., 2011). Além disso, as zooxantelas provêm ao coral acesso à luz, nutrientes inorgânicos (CO_2) e proteção (Marangoni *et al*., 2016; van Dam *et al*., 2011). Portanto, parâmetros para avaliação da fotossíntese, podem ser indicadores importantes para avaliação da saúde da relação entre o coral e as algas simbiontes. Por sua vez, cabe salientar que fatores ambientais como o aumento de temperatura e a exposição ao cobre podem desencadear o aumento de ERO (Halliwell & Gutteridge, 1999) e resultar na oxidação de lipídios, proteínas e ácidos nucleicos (Monserrat *et al*., 2007). Portanto, biomarcadores de estresse oxidativo são potenciais ferramentas para a avaliação da saúde dos corais, uma vez que danos oxidativos possuem correlação direta com o branqueamento em organismos de ambientes recifais (Prazeres *et al*., 2012).

Como mencionado anteriormente, um cenário de múltiplos estressores é composto pelos impactos gerados pelo homem em escala local, como é o caso da poluição por metais, associados às potenciais mudanças climáticas em escala global. Apesar da crescente preocupação com o impacto biológico destes estressores ambientais, existem poucas informações sobre os efeitos interativos de múltiplos estressores ambientais em corais. Esse fato, aliado a carência de estudos relacionados à fisiologia de espécies coralinas endêmicas no Brasil, faz com que o uso de biomarcadores no coral *M. harttii* para avaliar os efeitos isolados e combinados do aumento da temperatura e contaminação pelo cobre se torne uma importante ferramenta biológica para um maior entendimento das respostas desses organismos aos estressores em questão, bem como para o subsídio na proposição de ações visando à conservação dos recifes de coral no Brasil, que atualmente encontram-se sujeitos tanto aos impactos locais quanto às mudanças climáticas globais.

Objetivo

Objetivo geral

Avaliar o efeito isolado do aumento da temperatura e da combinação deste estressor com a exposição ao cobre sobre parâmetros fisiológicos e bioquímicos no coral escleractínio *Mussismilia harttii*.

Objetivos específicos

- Avaliar o efeito isolado do aumento da temperatura e da combinação deste estressor com diferentes concentrações ambientalmente relevantes de cobre sobre os seguintes parâmetros envolvidos no processo de calcificação: atividades específicas da anidrase carbônica e da (Ca^{2+}, Mg^{2+})-ATPase.

- Determinar o efeito isolado do aumento da temperatura e da combinação deste estressor com diferentes concentrações ambientalmente relevantes de cobre sobre os seguintes parâmetros envolvidos no estresse oxidativo: capacidade antioxidante contra radicais peroxil e peroxidação lipídica.
- Analisar o efeito isolado do aumento da temperatura e da combinação deste estressor com diferentes concentrações ambientalmente relevantes de cobre sobre a capacidade fotossintética máxima das zooxantelas (*Fv/Fm*) e a densidade de endossimbiontes (concentração de clorofila a).
- Avaliar o efeito isolado do aumento da temperatura e da combinação deste estressor com diferentes concentrações ambientalmente relevantes de cobre sobre a concentração de ATP do holobionte.

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Effects of increasing temperature alone and combined with copper exposure on biochemical and physiological parameters in the Brazilian endemic coral *Mussismilia harttii*

Juliana da Silva Fonseca[a], Laura Fernandes de Barros Marangoni[b,c], Joseane Aparecida Marques[b,c], Adalto Bianchini[c,d]

[a]Programa de Pós-Graduação em Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Avenida Itália km 8, Rio Grande, RS, Brazil, 96203-900.

[b]Programa de Pós-Graduação em Oceanografia Biológica, Instituto de Oceanografia, Universidade Federal do Rio Grande, Avenida Itália km 8, Rio Grande, RS, Brazil, 96203-900.

[c]Instituto Coral Vivo, Rua dos Coqueiros, Parque Yaya, Santa Cruz Cabrália, BA, Brazil, 45807-000.

[d]Instituto de Ciências Biológicas, Universidade Federal do Rio Grande. Avenida Itália km 8, Rio Grande, RS, Brazil, 96203-900.

* Corresponding author: Adalto Bianchini
Universidade Federal do Rio Grande – FURG
Instituto de Ciências Biológicas – ICB
Av. Itália km 8, Campus Carreiros
96.203-900 – Rio Grande – RS – Brazil
Phone: +55 53 3293-5193
e-mail: adaltobianchini@furg.br

ABSTRACT

Effects of increasing temperature alone and in combination with exposure to dissolved copper (Cu) were evaluated in the Brazilian endemic coral *Mussismilia harttii* using a marine mesocosm system. Endpoints analyzed included parameters involved in calcification [carbonic anhydrase (CA) and (Ca^{2+}, Mg^{2+})-ATPase activity], metabolism [maximum photosynthetic capacity of zooxanthellae (*Fv/Fm*) and chlorophyll a and ATP concentrations)], and oxidative status [antioxidant capacity against peroxyl radicals (ACAP) and lipid peroxidation (LPO)]. Coral polyps were collected, acclimated and exposed to three different increasing temperature conditions [25.0ºC (control; average temperature of local seawater), 26.6ºC and 27.3ºC] using a marine mesocosm system. They were tested alone and in combination with four environmentally relevant concentrations of dissolved Cu in seawater [1.9 (control; average concentration in local seawater), 3.8, 5.4 and 8.6 μg/L) for 4, 8 and 12 days. *Fv/Fm* reduced over the experimental period with increasing temperature. Combination of increasing temperature with Cu exposure enhanced this effect. CA and (Ca^{2+}, Mg^{2+})-ATPase activities increased up to 8 days of exposure, but were not affected after 12 days of experiment. Short-term exposure to increasing temperature or long-term exposure to the combination of stressors reduced LPO, suggesting a lipid remodeling in biological membranes. Long-term exposure to increasing temperature induced LPO, suggesting a lower energy production in the host coral associated with a likely reduced transference of photosynthates by zooxanthellae. ACAP, as well as concentrations of ATP and chlorophyll a were not significantly affected by the stressors. These findings indicate that increasing temperature combined with exposure to dissolved Cu can increase susceptibility to bleaching and reduce growth in the Brazilian endemic coral *M. harttii*.

Keywords: biomarkers; calcification; copper, coral; oxidative stress; photosynthesis.

1. Introduction

There is growing concern with the rapid increasing in coral reefs degradation worldwide (De'ath et al., 2012). Global climate changes and local impacts are amongst the major threats influencing the integrity these ecosystems (Sokolova et al., 2008). According to the Intergovernmental Panel on Climate Change (IPCC), mean global temperature will experience an increase of 0.2ºC per decade, being predicted an increase of 2.6 to 4,8ºC until the year of 2100 (IPCC, 2014).

Corals are ectothermic organisms (Hoegh-Guldberg and Smith, 1989). Increases of 2-4ºC are known to induce bleaching within days or weeks (Brown, 1997a). Therefore, corals reefs will be one of the most affected ecosystems by climate changes (Madin et al., 2012). Indeed, thermal stress can cause crucial changes in the structure, dynamics, productivity and diversity of coral reefs (Calderon-Aguilera et al., 2012). Also, local stressors such as chemical pollutants, including metals, and increased sedimentation are shown to impact coral physiology (Harland and Brown 1989; Bielmyer et al., 2010; Marangoni et al., 2017). Copper (Cu) is an essential metal for the maintenance of physiological functions (Morgan, 2000). However, excessive concentrations of this metal can induce deleterious effects in aquatic organisms (Heath, 1995), including bleaching (Jones, 1997, 2004; Bielmyer et al., 2010) and metabolic changes in corals (Nystron et al., 2001). Indeed, seawater contamination with metals, including Cu can affect reef environment health (van Dam et al., 2011). Although the effect of only one stressor rarely occurs in nature (Brown, 1997a,b), most studies investigates the effect of isolated stressors in corals (Bielmyer et al., 2010; Kuzminov et al., 2013; Marangoni et al., 2017). In fact, few studies have analyzed the interactive effects of multiple stressors on physiological biomarkers, especially in corals (Brown,

1997a; Rivest and Hofmann, 2014).

Calcification performed by corals is a physiological characteristic which revealed to be an interesting biomarker of reef environment health (Marangoni et al., 2017). The use of biochemical and physiological tools to evaluate the calcification process in corals can indicate how healthy the reef is or how much it is developing (Hoegh-Guldberg, 1999; De'ath *et al.*, 2009). Carbonic anhydrase (CA) and (Ca^{2+}, Mg^{2+})-ATPase are key enzymes in the calcification process. CA catalyzes the reversible reaction involved in the carbon dioxide (CO_2) hydration, thus generating bicarbonate (HCO_3^-) and protons (H^+). Therefore, it plays a key role in the supply of dissolved inorganic carbon (DIC) for coral calcification, as well as the photosynthesis performed by the symbiont algae (Bielmyer et al., 2010). In turn, Ca^{2+}-ATPase transports calcium (Ca^{2+}) into the calcification site and removes $2H^+$, thus increasing the Ca^{2+} and carbonate (CO^{3-}) saturation state (Al-Horani et al., 2003). Regarding Mg^{2+}-ATPase, it has been reported that it controls the Mg^{2+} release into the calcification site, thus playing an important role in the growth of skeletal components in corals (Meibom et al., 2004).

Another important feature of scleractinian corals is its symbiotic relationship with zooxanthellae of the genus *Symbiodinium* spp. These algae are located in the oral tissue cells of corals (Al-Sofyani and Floos, 2013). Zooxanthellae can provide about 90% of the carbon which is directed for coral feeding (Papina *et al*., 2003), thus playing a key role in energy production for coral growth (Goreau, 1959). However, symbiosis between corals and zooxanthellae is vulnerable to environmental stressors, such as changes in temperature and light intensity, as well as pollution. A disruption of this symbiotic relationship can lead to coral bleaching (Hoegh-Guldberg, 1999; Fabricius et al., 2005). This phenomenon is characterized by the expulsion of zooxanthellae by coral

719 and/or the degradation of the photosynthetic pigments of these microalgae (Downs et
720 al., 2002). Bleaching can severely affect corals. In the absence of zooxanthellae, corals
721 are deprived of nutrients provided by photosynthesis (Ferrier-Pagès et al., 1998). As
722 previously mentioned, increasing temperature and chemical pollution are recognized as
723 common causes of bleaching in corals, being the major factors associated with coral reef
724 degradation worldwide (Fabricius et al., 2005; Anthony et al., 2007). Therefore,
725 parameters such as the photosynthetic capacity of zooxanthellae and chlorophyll a
726 concentration may be important tools to evaluate the health and integrity of the
727 symbiotic relationship between corals and zooxanthellae.

728 In light of the above, oxidative stress biomarkers can be considered as potential
729 tools for assessing coral reefs health, since oxidative damage is directly correlated with
730 bleaching in reef organisms (Prazeres et al., 2012). Indeed, increasing temperature and
731 metal exposure can increase the generation of reactive oxygen species (ROS) in aquatic
732 organisms, thus leading to oxidation of biomolecules, such as lipids, proteins and
733 nucleic acids (Halliwell and Gutteridge, 1999; Monserrat et al., 2007). It is reported that
734 oxidative stress affects the coral-symbiont association. In this case, oxygen produced by
735 photosynthesis is transferred to corals cells (Rotchell & Ostrander, 2011), thus
736 increasing the oxidative damage to these cells. Once a threshold level of stress is
737 achieved, corals eliminate the dominant source of ROS, thus expelling the symbiont
738 zooxanthellae (Downs *et al*., 2002). Therefore, corals may present physiological
739 changes preceding the bleaching event, such as reduced antioxidant capacity and
740 increased oxidation of biomolecules (Lesser, 1997).

741 *Mussismilia harttii* is an endemic coral, which is distributed along the Atlantic
742 Coast in northwestern Brazil. It is found from the Rio Grande do Norte to the Espírito
743 Santo coast and has a key role in the construction of South Atlantic reefs (Soares, 2011;

744 Marangoni et al., 2017). Therefore, the evaluation of *M. harttii* responses to global
745 warming itself and in combination with local impacts is crucial to understand how coral
746 reefs in Brazil will be affected by multiple stressors. Also, it is necessary to select
747 potential biomarkers for future use in management programs and conservation strategies
748 in coral reefs. Therefore, the objective of the present study was to evaluate the effect of
749 increasing temperature alone and in combination with environmentally relevant
750 concentrations of dissolved copper using parameters involved in key physiological
751 processes in corals, such as calcification, metabolism and oxidative status in the
752 Brazilian endemic coral *M. harttii*.
753

754 **2. Materials and methods**
755

756 *2.1. Coral collection and maintenance*
757
758 Polyps of six colonies of *M. harttii* were collected by means of scuba diving in
759 the conservation area of the Municipal Natural Park of Recife de Fora (Porto Seguro,
760 Bahia, northwestern Brazil) in July 2012. Coral samples were collected under the
761 permission of the Brazilian Environmental Agency (IBAMA/SISBIO; permit #
762 85926584). Coral polyps were collected, transferred to the experimental facilities of the
763 Coral Vivo Project (Arraial d'Ajuda, Porto Seguro). Polyps of *M. harttii* were
764 individualized, glued on ceramic plates and acclimated to the experimental conditions
765 for 20 days. The experiment was carried out in the marine mesocosm of the Coral Vivo
766 Project (Duarte et al., 2015). It is an open experimental system that continuously
767 exchanges water with the sea, maintaining the same daily and seasonal variations of the
768 environmental parameters, such as temperature, salinity, pH, light intensity,
769 photoperiod, rainfall, and food sources. Seawater is collected from the adjacent reef

770 located approximately 500 m from the coast at the Araçaípe Beach. The mesocosm has
771 forty eight 10-L aquariums, which receive water from tanks and reservoirs, using
772 peristaltic pumps. Details on the mesocosm structure and functioning were previously
773 described by Duarte et al. (2015).

774

775 *2.2. Temperature treatments and combination with copper exposure*
776
777 Coral polyps were exposed to three temperature treatments representing
778 scenarios proposed by the Intergovernmental Panel on Climate Change (IPCC, 2014):
779 (a) average temperature of local seawater; (b) increase of 1^{o}C above the temperature of
780 local seawater; and (c) increase of 2°C above the temperature of local seawater.
781 Seawater was collected at the reef environment and pumped into sixteen 310-L tanks,
782 where the heat treatments were applied using 15,000 w heaters. A computerized system
783 monitored and controlled the desired temperature of each treatment. Flow of heated
784 water represented 90% of the total flow of seawater reaching the 10-L aquariums.
785 Temperature treatments were tested alone and in combination with four different
786 concentrations of dissolved copper (nominal: 0, 1, 3 and 5 μg/L) for 4, 8 and 12 days.
787 Copper stock solutions were prepared daily in eight 1,000-L reservoirs from a standard
788 solution of $CuCl_2$ (1 g/L Cu). These reservoirs contained seawater pumped from the
789 adjacent reef and received 0, 10, 30 and 50 mL of the $CuCl_2$ standard solution to obtain
790 the desired nominal concentrations of 0, 10, 30 and 50 μg/L Cu, respectively. Seawater
791 contaminated with Cu was prepared 24 h prior its use in the experimental system to
792 allow the complete equilibration of Cu with seawater. Seawater contaminated with Cu
793 was then mixed with the heated water before reaching the 10-L aquariums. Therefore,
794 seawater from the tanks (with and without heat treatment) is mixed in line with seawater
795 from the reservoirs (with and without Cu addition) using 16 peristaltic pumps at a flow

of 0.169 L/min. Flow of seawater contaminated with Cu represented 10% of the total flow of seawater reaching the 10-L aquariums. Therefore, final nominal concentrations of Cu in the test aquariums corresponded to 0, 1, 3 and 5 µg/Cu. Treatments were performed in triplicate, where three polyps were arranged randomly in each aquarium. After 4, 8 and 12 days of exposure to the experimental treatments, coral polyps (n = 3 per treatment) were collected for further analyses of biochemical and physiological parameters.

2.3. Seawater collection and analysis

Every three days over the experimental period, seawater samples were collected from the test aquariums to perform measurements of Cu concentrations and some physicochemical parameters. Non-filtered and filtered (0.45-µ mesh filters) samples were stored in 15-mL Falcon-type tubes and acidified with HNO_3 (1% final concentration; SupraPur, Merck, USA). Samples were desalted as described by Nadella et al. (2009) and total (non-filtered sample) and dissolved (filtered sample) Cu concentrations were analyzed by atomic absorption spectrophotometry with coupled graphite furnace (Perkin-Elmer, Waltham, MA, USA). Additionally, data on dissolved organic carbon (DOC) concentration (Total Organic Carbon analyzer, Shimadzu, Japan), pH (pH meter, model HI 9124, Hanna Instruments), pluviometry (local weather station, Veracel Celulose, Brazil), salinity (optical refractometer, model ITREF 10, Instrutemp), and temperature (loggers HOBO Water Temp Pro, Onset, Bourne, MA, USA) were obtained. Temperature measurements were performed using loggers installed inside the tanks and at the reef environment to continuously monitor the seawater temperature every 30 min.

2.4. Sample preparation for biomarkers analyses

Coral polyp samples for biomarkers analyses were prepared as described by Santos et al. (2015). Briefly, they were macerated in liquid nitrogen, separated into aliquots of approximately 200 mg, and homogenized using a sonicator (Sonaer Ultrasonics, Farmingdale, NY, USA). A specific homogenization buffer solution (1:2, w/v) was used for each analysis, as described below. Homogenates were centrifuged (13,000 g; 4 °C) for 10 min. The supernatant was collected and split into aliquots. One aliquot was immediately used for CA and (Ca^{2+}, Mg^{2+})-ATPase activity analysis. Other aliquots were stored in ultrafreezer (-80ºC) for analysis of oxidative stress parameters (ACAP and LPO) and ATP quantification. These aliquots were stored for no longer than one week until analysis. Data were normalized based on the amount of total proteins in the supernatant aliquot. Protein concentration was measured using a commercial reagent kit based on the Bradford method (Bradford Reagent, Sigma-Aldrich, EUA).

2.5. Carbonic anhydrase (CA) activity

CA activity measurement was based on the method described by Henry (1991). Samples were homogenized in a buffer solution containing sucrose (75 mM), tris(hydroxymethyl)aminometano (Tris-Base, 10 mM, pH 8.5), phenylmethanesulfonyl fluoride (PMSF, 1 mM) and dithritiotreitol (DTT, 1 mM). Enzymatic reaction was started by adding 15 µL of sample homogenate in 3 mL of the reaction solution, which had the following composition: Tris-Base (10 mM, pH 8.5), sucrose (75 mM), mannitol (225 mM) and phosphate (10 mM). Subsequently, 390 µL of substrate was added. It was obtained by saturating 250 mL of distilled water with CO_2. As CA is responsible for the

847 CO_2 hydration with consequent release of H^+, enzyme activity was determined based on
848 pH reduction in the reaction mixture. Immediately after substrate addition, pH of the
849 reaction mixture was monitored every 5 s for up to 30 s, using a pH meter (pH 21,
850 Hanna, USA). Additionally, blank measurements were performed by adding 15 µL of
851 the homogenization buffer into the reaction mixture containing reaction solution and
852 substrate. Results were calculated based on the slope of the regression lines after fitting
853 the pH data over time using a linear regression model (dependent variable: pH;
854 independent variable: time). Mean values of the slopes for blank reactions and sample
855 reactions represented the non-catalyzed and catalyzed reaction rates, respectively. Data
856 were normalized based on the amount of total proteins in the sample homogenates,
857 which was determined as described above. Results were expressed in enzyme units/mg
858 protein.

859

860 *2.6. (Ca^{2+}, Mg^{2+})-ATPase activity*
861
862 (Ca^{2+}, Mg^{2+})-ATPase was determined using the colorimetric method described
863 by Vajreswari et al. (1983). Samples were homogenized in a buffer solution containing
864 (hydroxymethyl)aminometano-hydrochloride (Tris-HCl, 100 mM, pH 7.6), sucrose (500
865 mM), DTT (1 mM) and PMSF (1 mM). The reaction medium contained NaCl (189
866 mM), $MgCl_2$ (5 mM), $CaCl_2$ (5 mM), ouabain (1 mM), Tris-HCl (20 mM, pH 7.6) and
867 ATP (3 mM). Sample homogenate (10 µL) was added to 250 µL of the reaction medium
868 and incubated at 30°C for 30 min. The enzymatic reaction was stopped by incubation on
869 ice. The amount of inorganic phosphate released in the reaction medium was determined
870 using the commercial reagent kit "Fosfato" (Doles, Goiânia, GO, Brazil). Sample
871 absorbance was measured at 630 nm using a microplate reader (ELX 808, Biotek,
872 Vermont, USA). Enzyme activity data were normalized based on the amount of protein

in the sample homogenate, which was determined as described above. Results were expressed as mmol Pi/mg protein/min.

2.7. Maximum photosynthetic capacity of photosystem II (Fv/Fm)

The maximum photosynthetic capacity of the symbiont photosystem II *(Fv/Fm)* was measured using a fluorometer with pulse amplitude modulated (Diving-PAM, Walz, Germany). Measurements were performed using a randomly selected polyp per aquarium. Polyps were kept in the dark prior to the experiment, so the photosystem II was turned off. The minimum fluorescence level (*F0*) was obtained by a weak modulated light probe; the maximum fluorescence level (*Fm*) was determined using a saturation pulse of actinic light; and the variable fluorescence level (*Fv*) was calculated as the difference between *Fm* and *F0*. Finally, the maximum photochemical potential of photosystem II was obtained calculating the *Fm/Fv* ratio. *Fm/Fv* values represent exclusively a health picture of zooxanthellae, but can also indicate the physiological state of the holobiont (coral and zooxanthellae).

2.8. Chlorophyll a

Chlorophyll content in coral samples was determined using the methods described by Nusch (1980) and Sartory and Grobbelaar (1984). Chlorophyll was extracted in 400 µL of ethanol (95%) over 24 h. During extraction, samples were kept in the refrigerator and protected from light. After 24 h, samples were centrifuged (200 rpm) at 4° C, for 5 min. Chlorophyll was quantified by measuring the sample absorbance at 665 nm (A_{665}) and 750 nm (A_{750}). Chlorophyll content (CC) was calculated using the following formula: $CC = 12.0 \times (A_{665} - A_{750}) \times Ve/(ww \times d) \times 103$, where "Ve" is the volume of extraction, "ww" is the wet weight of the coral tissue

sample, and "d" is the dilution factor. Results were expressed as ng/mg ww.

2.9. ATP concentration

ATP concentration was based on the luciferase activity. Polyp samples were homogenized in the PMSF (1 mM) and centrifuged (5000g, 4ºC, 5min). Further tests using the same kit, the samples were prepared in perchloric acid (8mM) (Abujamara *et al*., 2014). Due to the high storage time of the samples in the ultrafreezer, PMSF was considered the ideal homogenization buffer for samples with low concentrations of ATP. In this kit, luciferase hydrolyzes the ATP present in the sample, in AMP and this reaction results in the formation of light, at pH 7.8 and 28ºC. Bioluminescence is detected through the wavelength of 535nm. For the assay a kit of ATP was used (Molecular Probes) and the data were normalized by the amount of proteins present in the sample and expressed as µg ATP / mg protein.

2.10. Total antioxidant capacity against peroxyl radicals (ACAP)

Total antioxidant capacity against peroxyl radicals (ACAP) was determined using a fluorimetric method, as described by Amado *et al*. (2009). Samples were homogenized using a buffer solution containing Tris-HCl (100 mM, pH 7.75), EDTA (2 mM) and $MgCl_2$ (5 mM). The reaction solution contained 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 30 mM), KCl (200 mM) and $MgCl_2$ (1 mM). For analysis, the amount of protein in the sample homogenate was adjusted to 0.05 mg/L. Measurements were based on ROS formation. Thermal decomposition of 2,2'-azobis (2-methylpropionamidine) dihydrochloride (ABAP, Sigma-Aldrich, USA) added to the reaction mixture generated ROS (peroxyl radicals). In turn, the substrate 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes, USA) was added

to the reaction solution and cleaved by esterases present in the sample homogenate, thus generating 2',7' dichloroflurescein (H_2DCF), a non-fluorescent compound. H_2DCF was then oxidized by ROS originated from ABAP decomposition, thus generating dichloroflurescein (DCF), a fluorescent compound. Every 5 min and for up to 40 min, fluorescence measurements (excitation: 485 nm; emission: 530 nm) were performed at 37°C using a fluorometer (Victor 2, Perkin Elmer, Waltham, MA, USA). ACAP values were calculated based on the difference between the fluorescence area in the presence and the absence of ABAP. Results were expressed as 1/relative area.

2.11. Lipid peroxidation (LPO)

Lipid peroxidation (LPO) measurement was performed according to the method described by Oakes and Van Der Kraak (2003). This method evaluates the oxidative damage in membrane lipids through the formation of the chromogen tetramethoxypropane (TMP), a product of the reaction between malondialdehyde (MDA), a product of lipid peroxidation, with thiobarbituric acid (TBA). This reaction occurs at high temperature (95ºC) and in the presence of acetic acid. TMP is quantified using (excitation: 520; emission: 580 nm) using the fluorometer (Victor 2, Perkin Elmer, Waltham, MA, USA). Data were normalized based on the amount of total proteins in the sample homogenates, which was measured as described above.

2.12. Data presentation and statistical analyses

Data were expressed as mean ± standard error. Mean data of *Fv/Fm* were compared using two-way analysis of variance (ANOVA) for repeated measures followed by the Fisher test for multiple comparisons. Mean data for other biomarkers at

each experimental time were compared using one-way ANOVA followed by the Fisher test for multiple comparisons. Data normality and homogeneity of variances were previously verified using the Shapiro-Wilk and Cochran C tests, respectively. The confidence level adopted was 95% ($\alpha = 0.05$).

3. Results

3.1. Water physicochemical parameters

Rainfall was higher after 4 days than 8 and 12 days of experiment. DOC concentration was higher after 4 and 8 days than 12 days of experiment. Water salinity increased during the exposure period while pH and temperature did not change significantly throughout the experiment (Table 1). Total Cu concentrations in the experimental medium were 4.6 ± 0.7, 6.3 ± 1.0, 7.7 ± 0.5, and 10.7 ± 1.1 μg/L for the nominal concentrations of 0 (control), 1, 3 and 5 μg/L levels respectively. In turn, dissolved copper concentrations corresponded to 1.9 ± 0.2, 3.8 ± 0.8, 5.4 ± 0.9, e 8.6 ± 0.3 μg/L, respectively. Average values of the temperature treatments were 25.0 ± 0.10ºC for control (ambient water temperature), 26.6 ± 0.08ºC for the increase of 1ºC in ambient water temperature, and 27.3 ± 0.09ºC for the increase of 2ºC in ambient water temperature.

3.2. Enzyme activities

There was a significant increase in CA activity in corals exposed to 26.6 and 27.3ºC for 4 days respect with those maintained at the ambient water temperature (control condition: 25.0ºC). Also, CA activity was increased in coral from all treatments after exposure to combined increasing temperature and Cu exposure for 4 days respect

with those maintained at the control condition (Fig. 1A). After 8 days of exposure, CA activity increased in corals exposed to 27.3ºC compared with those maintained at the control condition. Similarly, there was an increased CA activity in corals exposed to the following treatments which combined increasing temperature and Cu exposure: 26.6ºC/3.8 µg/L Cu, 26.6ºC/5.4 µg /L Cu, 26.6ºC/8.6 µg/L Cu, 27.3ºC/5.4 µg/L Cu, and 27.3ºC/8.6 µg/L Cu (Fig. 1B). However, no significant changes in CA activity were observed after 12 days of exposure to any of the treatments of increasing temperature alone or in combination with Cu exposure (Fig. 1C).

(Ca^{2+}, Mg^{2+})-ATPase activity was not significantly affected by any of the treatments tested for 4 days (Fig. 2A). However, corals exposed to 27.3ºC for 8 days had increased (Ca^{2+}, Mg^{2+})-ATPase respect with those maintained at control condition. Furthermore, (Ca^{2+}, Mg^{2+})-ATPase activity was significantly higher in corals exposed to any of the treatments combining increasing temperature and Cu exposure than in those kept at the control condition for 8 days (Fig. 2B). In turn, (Ca^{2+}, Mg^{2+})-ATPase activity was similar in corals subjected to any of the treatments tested for 12 days and those from the control group (Fig. 2C).

3.3. Metabolic parameters

No significant differences in *Fv/Fm* were observed among temperature treatments at each experimental time. However, coral exposure to increasing temperature reduced *Fv/Fm* over time, leading to a significant decrease after 12 days of exposure to 26.6ºC and 27.3ºC (Fig. 3A). Additionally, *Fv/Fm* was significantly and negatively affected when corals were exposed to 27.3ºC combined with 3.8 µg/L Cu for 4 and 8 days. Also, it was reduced in corals exposed to the following combinations of increasing temperature and Cu exposure for 12 days: 26.6ºC/5.4 µg/L Cu, 27.3ºC/3.8

µg/L Cu, 27.3ºC/5.4 µg/L Cu and 27.3ºC/8.6 µg/L Cu. Furthermore, *Fv/Fm* decreased over time when corals were exposed to combinations of increasing temperature and Cu exposure, being significantly reduced after 12 days of exposure to the following treatments: 26.6ºC/3.8 µg/L Cu, 26.6ºC/5.4 µg/L Cu, 26.6ºC/8.6 µg/L Cu, 27.3ºC/5.4 µg/L Cu, and 27.3ºC/8.6 µg/L Cu (Fig. 3B).

There were no significant changes in chlorophyll level when corals were subjected to increasing temperatures alone or in combination with Cu exposure for up to 12 days (Fig. 4). Similarly, no significant effects on ATP concentration were observed (Fig. 5).

3.4. Oxidative status parameters

No significant effect was observed in ACAP after coral exposure to any of the increasing temperature treatments tested for up to 12 days. Similarly, significant effects were not seen in ACAP after coral exposure to any of the treatments combining increasing temperature and Cu exposure (Fig. 6).

LPO showed a significant reduction in corals exposed to increasing temperatures (26.6ºC and 27.3ºC) when compared to those kept at the control condition. Similarly, a significantly reduced LPO was observed in corals exposed to the following combinations of increasing temperature and Cu exposure for 4 days: 26.6ºC/3.8 µg/L Cu, 26.6ºC/5.4 µg/L Cu, 26.6ºC/8.6 µg/L Cu, and 27.3/3.8 µg/L Cu (Fig. 7A). A reduction in the amount of oxidized lipids was observed in corals exposed to 26.6ºC for 8 days respect with those maintained in the control condition. Similarly, there was a LPO reduction in corals exposed to the following combinations of increasing temperature and Cu exposure for 8 days: 27.3ºC/3.8 µg/L Cu and 27.3/5.4 µg/L Cu (Fig. 7B). On the other hand, LPO was significantly higher in corals exposed to increasing

temperature (26.6 and 27.3ºC) for 12 days than those from the control group. In turn, reduced LPO was observed in corals exposed to the following combinations of increasing temperature and Cu exposure: 26.6ºC/5.4 µg/L Cu and 27.3ºC/3.8 µg/L Cu (Fig. 7C).

4. Discussion

Many studies have evaluated the effect of global warming on coral physiology (Lesser et al., 1990; Schlöder and D'Croz, 2004; Rodolfo-Metalpa et al., 2006; Higuchi et al., 2009; Putnam and Edmunds, 2010; Rivest and Hofmann, 2014). However, only few studies have identified the interactive effect of this stressor with local impacts, and consequently the limits of coral tolerance to these conditions (Nystrom et al., 2001), especially in endemic species from Brazil, such as *Mussismilia harttii.*

Scleractinian corals, including *M. harttii*, are important producers of calcium carbonate ($CaCO_3$) around the world (Falini et al., 2015). Indeed, the calcification process in corals is responsible for 15% of the global $CaCO_3$ production (Bertucci et al., 2013). This process occurs through the formation of an outer skeleton, which is dependent on the enzymatic transformation of DIC from seawater (HCO_3^-) and coral metabolism (CO_2) into $CaCO_3$. In the present study, an increased CA activity was observed after short-term (4 and 8 days) exposure to increasing temperature alone or in combination with environmentally relevant concentrations of dissolved Cu. Interestingly, this response was followed by an enhanced (Ca^{2+}, Mg^{2+})-ATPase activity after 8 days of exposure. However, these responses were transient, with CA and (Ca^{2+}, Mg^{2+})-ATPase activities recovering back to the initial levels after 12 days of exposure. These transient increases in enzyme activities can be explained considering an also

transient increase in metabolism, as demonstrated in *Porites lutea* colonies of the Great Barrier Reef in Australia. In this case, an elevation of 1ºC in seawater temperature induced a significant increase in the calcification rate of *P. lutea* (Bessat and Buigues, 2001), thus indicating an enhanced metabolism at the increased temperature. Indeed, it is well known that exposure to increasing temperature or dissolved metals can alter the metabolism of ectotherms (Cherkasov et al., 2006, Ivanina et al., 2008; Rivest and Hofmann, 2014).

The increased CA activity in *M. harttii* can be explained by the fact that CO_2 production is augmented when metabolism is augmented. In fact, the rate of respiration in corals is enhanced at increasing temperature. This response is directly linked to the effect of temperature on metabolism, as well as the higher energy cost related to repair of the damage associated with the enhanced oxygen consumption and consequent higher ROS formation induced by the thermal stress (Lesser, 1997). As a result of the increased respiration rate, there is an enhanced release of systemic CO_2, which is the substrate for CA to form HCO_3^- and H^+ at the calcification site (Furla et al., 2000). Indeed, about 60 to 80% of the DIC directed to the calcification process in corals is originated from the metabolism (Furla et al., 2000; Bertucci et al., 2013). In turn, the observed latter increase in (Ca^{2+}, Mg^{2+})-ATPase activity can be associated to the enhanced CA activity. In fact, Ca^{2+}-ATPase acts in conjunction with CA at the site of calcification in corals. This enzyme transports Ca^{2+} from the calicoblast cells to the coral calcification sites (Al-Horani et al., 2003) while removes two H^+ formed by the CA reaction (Bertucci et al., 2013). This process avoids the acidification of the calcification site, which is important for maintaining the saturation state of aragonite (Ω_{ar}) and $CaCO_3$ precipitation (Allemand et al., 2011; Al-Horani et al., 2003).Therefore, a possible acidification of coral calcification sites caused by the increased CA activity can explain the latter

increase observed in (Ca^{2+}, Mg^{2+})-ATPase activity in *M. harttii*. This would be a protective response against a potential damage in the calcification process due to exposure to the environmental stressors.

In addition to the role of CA in the calcification process, this enzyme is also important in the CO_2 supply for photosynthesis in the coral symbiont algae (Weis et al., 1989; Bertucci et al., 2013). In corals, zooxanthellae are located in the oral tissue cells, which are difficult to be accessed by the CO_2 used in photosynthesis. As cell membranes are permeable to CO_2, it is converted into HCO_3^- by CA. This would prevent the diffusive CO_2 leakage from the coral cells. Additionally, the CA located near the membrane of the coral symbiont algae performs the conversion of HCO_3^- into CO_2, which is fixed by microalgae for photosynthesis (Allemand et al., 1998; Leggat et al., 2002; Bertucci et al., 2013). Consequently, CA also plays a crucial role in the photosynthetic process. In fact, CA inhibitors exert a negative effect on photosynthesis (Badger, 2003). Therefore, the observed increase in CA activity in *M. harttii* after exposure to the environmental stressors would lead to a higher supply of CO_2 for fixation by the ribulose-1,5-biphosphate carboxylase-oxygenase (RuBisCO), a crucial enzymatic step for the production of carbohydrates in the coral symbiont algae (Bertucci *et al*., 2013).

In light of the above, the observed increase in CA activity in *M. harttii* may be also considered as a transient response of the enzyme in attempt to enhance the maximum capacity of the photosynthetic process in the coral symbiont algae. However, a prolonged exposure of *M. hartii* to the environmental stressors induced damage to the photosynthetic process, which was indicated by the significant reduction in the zooxanthellae *Fv/Fm* after 12 days of experiment. Interestingly, the activity of the calcification enzymes [CA and (Ca^{2+}, Mg^{2+})-ATPase] were also not altered after 12

days of exposure to the environmental stressors. These findings suggest that the prolonged exposure to the stressors may be causing a reduction in energy production in *M. harttii*. In fact, lower calcification rates were observed in the corals *Porites cylindrica* and *Galaxea fascicular* exposed to increased temperature (32ºC) for 45 days. This effect was related to a decrease in energy availability associated with reduced rates of photosynthesis, a process responsible for photosynthates transfer to the coral. Indeed, a reduced ATP level was reported in the coral *Galaxea fascicularis* exposed to 32ºC (Agostini et al., 2013). However, no significant changes in ATP concentration were observed in *M. harttii* after exposure to increasing temperature alone or combined with different Cu concentrations. Differences between studies could be explained considering the maximum temperature tested in our study (27.3^{o}C) and that of 32^{o}C tested by Agostini et al. (2013). Despite the significant effect observed in *Fv/Fm*, it is possible that the maximum temperature tested in the present study was not enough to induce significant changes in ATP concentrations. Another possible explanation is to consider that *M. harttii* used other phosphate compounds than ATP to provide energy when facing the effects induced by the environmental stressors.

In the present study with *M. harttii*, a reduced *Fv/Fm* was observed over the exposure time, with a significant reduction occurring after 12 days of exposure to increasing temperature. Interestingly, the combination of increasing temperature and Cu exposure aggravated this response. This finding indicates that the high productivity of scleractinian corals can be seriously menaced by a scenario of multiple stressors combining increasing temperature (global impact) and Cu exposure (local impact). Indeed, productivity of scleractinian corals is particularly due to their symbiosis with zooxanthellae (Sofyani and Floos, 2013). In corals, the calcification rate is three times higher in the presence of light (Agostini et al., 2013).

It is worth noting that several studies have elucidated the effect of environmental stressors on the dysfunction of the symbiotic relationship between corals and zooxanthellae (Nystron et al., 2001; Kuzminov et al., 2013), including those induced by increasing temperature on the photosynthetic capacity of zooxanthellae (Warner et al., 1996; Al-Horani et al., 2005; Agostini et al., 2013; Krueger et al., 2015; Winter et al., 2016). As described in the present study with *M. harttii*, a reduced photosynthetic capacity of zooxanthellae is generally reported after exposure to increasing temperature. However, differently from our study, only few works have investigated the combined effects of global and local stressors on this relationship (Nystron *et al*., 2001). It is important to note that our findings point out a negative interactive effect of increasing temperature and Cu exposure, which may compromise the coral calcification process that could lead to a significant long-term reduction in coral reefs growth.

Oxidizing species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are normally produced by cellular metabolism, such as respiration (Lesser, 2006, Tang et al., 2014). Additionally, it is important to emphasize that the photosynthetic process also generates ROS (Lesser 2006; Murata et al., 2007; Higuchi et al., 2009). In fact, ROS production has a direct and positive correlation with the amount of oxygen that reaches the photosynthetic system (Lesser, 2006). ROS formed in the photosynthetic chain are diffused into the coral tissue, inducing oxidative damage to biomolecules, including lipids (Marangoni et al., 2016). As previously mentioned, increasing temperature stimulates metabolic processes, thus augmenting oxygen consumption and consequently ROS production in ectotherms (Lushchak, 2011; Fadhlaoui and Couture, 2016). In turn, oxidative stress can be observed when ROS production overwhelms the cell/tissue antioxidant capacity, thus resulting in damage to lipids, proteins and DNA.

In light of the above, a reduced ACAP paralleled by an increased LPO would be expected after *M. harttii* exposure to increasing temperature. Contrarily, a reduced LPO paralleled by a lack of change in ACAP was observed in *M. harttii* after short-term exposure to increasing temperature. This finding could be explained considering the reduced *Fv/Fm*, which likely led to a lower production of ROS by zooxanthellae due to a possible damage in the photosynthetic apparatus induced by increasing temperature. Also, the possible activation of lipid remodeling mechanisms in biological membranes cannot be ruled out. In fact, homeoviscous adaptation of biological membranes has been pointed out as an adaptive response in many organisms (Hazel, 1995), including corals exposed to thermal stress (Papina et al., 2007; Matozzo et al., 2013; Fadhlaoui and Couture, 2016). For example, Papina et al. (2007) reported a reduced proportion of polyunsaturated lipids in the coral *Montipora digitata* and the symbiont algae after exposure to increasing temperature. Additionally, elevated temperatures were shown to increase the amount of saturated lipids in biological membranes to preserve their crystalline state (Dewick, 2009). Acting simultaneously, both mechanisms promote a reduction in the susceptibility of biological membranes to LPO and tissue oxidative damage (Hulbert, 2003). Therefore, we suggest that the reduced LPO observed after short-time exposure of *M. harttii* to increasing temperature is also due to remodeling of lipids in biological membranes. This would represent a defense mechanism in *M. harttii* to avoid the potential oxidative damage to lipids resulting from the increased ROS formation associated with increasing temperature.

Despite the reduced *Fv/Fm* and the possible activation of mechanisms for lipids remodeling in biological membranes, a higher LPO was generally observed in *M. harttii* short-term exposed to increasing temperature combined with Cu exposure than in those exposed to increasing temperature alone. This finding clearly indicates that short-term

exposure to Cu is inducing oxidative stress in *M. harttii*. In fact, Cu exposure is also shown to increase the generation of ROS in zooxanthellae through its involvement in the Fenton reactions ($Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$; Wardman and Cadeias, 1996). Therefore, the $OH^{\cdot}$ formed can be diffused into coral tissue (Rotchell and Ostrander, 2011), inducing oxidative damage to biomolecules, including lipids (Marangoni et al., 2016).

Interestingly, a completely different picture was observed after long-term (12 days) exposure of *M. harttii* to the environmental stressors. Despite the reduced *Fv/Fm*, which would produce a lower amount of ROS, a significant increase in LPO was observed after the long-term exposure to increasing temperature. In this case, it is possible that the defense mechanism involving lipids remodeling was not sustained and did not provide an adequate protection of membranes to oxidative damage. It is important to note that lipid remodeling mechanisms in biological membranes implicate changes in lipid acyl chains composition, as well as changes in cholesterol content. In turn, these changes involve the activity of enzymes such as desaturases and elongases (Hazel and Williams, 1990; Tiku et al., 1996), which are energetically costly. Therefore, a reduced ability to avoid oxidative damage to lipids in *M. harttii* after long-term exposure to increasing temperature can also be explained considering a reduced energy production, as earlier discussed when interpreting the reduced activity of enzymes involved in the calcification process.

Differently from the previous condition (short-term exposure), *M. harttii* long-term exposed to increasing temperature combined with Cu exposure showed a lower amount of oxidized lipids respect with those exposed to increasing temperature alone. It is worth noting that corals pre-exposed to increasing temperature become more tolerant to the toxic effects of Cu through the induction of heat shock proteins (HSPs) and

metallothioneins (Sharp et al., 1994; Black et al., 1995; Nystron et al., 2001). Therefore, long-term exposure to the combination of increasing temperature and dissolved Cu may have reduced coral susceptibility to LPO. As a mechanism to reduce LPO induced by Cu exposure, lipid composition of biological membranes is altered (Rozentsvet et al., 2012; Tang et al., 2014). For example, the amount of the polyunsaturated lipid phosphatidylcholine was altered by the also polyunsaturated lipid plasmanylcholines in the coral *Seriatopora caliendrum* exposed to Cu. In this case, plasmanylcholines were responsible for maintaining membrane properties, such as reducing the uptake of ROS and metals, as well as protecting against LPO. Also, lipid metabolism was altered, leading to an increase in the amount of monounsaturated and saturated lipids in biological membranes (Tang et al., 2014).

Interestingly, changes in LPO were never followed by alterations in ACAP, irrespective the experimental condition tested. It is worth noting that ACAP assay only evaluated the tolerance of *M. harttii* to peroxyl radicals (Amado et al., 2009). Therefore, LPO observed seems to be associated with oxidative damage induced by other oxyradicals than peroxyl radicals. In fact, LPO is mainly associated with the production of OH• (Lesser, 2006), which is the more damaging oxidant species for cell function (Halliwell and Gutteridge 1999). Future measurements of the total oxyradical scavenging capacity (TOSC) would help to explain the response of the antioxidant system of *M. harttii* to the environmental stressors tested in the present study.

It is known that increasing temperatures destabilize the transport of electrons in the respiratory chain and photosynthesis, thus increasing ROS production (Halliwell and Gutteridge, 1999). Additionally, increasing temperature can inhibit the photosystem II repair system through the formation of H_2O_2 (Murata *et al.*, 2007; Winter *et al*., 2016). As observed in the present study, Winter et al. (2016) also reported that exposure to

increasing temperatures (33°C and 35°C) enhanced the photoinhibition in *M. harttii*, with this effect being attributed to a decline in photosystem II repair mechanisms induced by thermal stress.

As observed in the present study with *M. harttii*, exposure to increasing temperature combined with another stressor is reported to cause an even worse damage to the photosystem II, thus enhancing the inhibitory effect on coral photosynthesis. For example, Nystron et al. (2001) reported a reduction of about 56% in the photosynthetic rate in the coral *Porites cylindrica* exposed to increasing temperature (35ºC) combined with Cu (11 µg/L) for 24 h. In fact, metals such as Cu are shown to affect photosynthesis in primary producers (Franco et al., 1999; Baryla et al., 2001; Kuzminov et al. (2013). Cu can inhibit the transport of electrons between PS-II and PS-I and the oxidation sites of photosystem II (Samson et al., 1988; Bielmyer et al., 2010). Furthermore, Cu can reduce the RuBisCo and ATP synthase activities (Kuzminov et al., 2013) and cause negative effects in the energy production by the photosynthetic system. Also, it is known that Cu can replace Mg in chlorophyll a molecules. Such substitution may cause damage to the photosynthetic apparatus and reduce the photosynthetic capacity (Kupper et al., 1996). Therefore, it is suggested that exposure of *M. harttii* to increasing temperature resulted in a reduced capacity of the repairing processes in photosystem II, allowing Cu to cause damage at different stages of the photosynthetic process. In turn, this may have exacerbated the effect of increasing temperature on photosynthesis, especially after a prolonged exposure (12 days) to the environmental stressors.

Most of the oxidative stress responses in corals are associated with bleaching, i.e., the loss of zooxanthellae or photosynthetic pigments from microalgae (Hoegh-Guldberg and Smith, 1989; Glynn et al., 1992; Agostini et al., 2013). Despite, the

chlorophyll a content is a parameter typically used for evaluation of coral bleaching (Hoegh-Guldberg and Smith, 1989), no significant changes in chlorophyll a concentration was observed in *M. harttii* exposed to increasing temperature alone or in combination with Cu. According to Nystron et al. (2001), the photosynthetic rate of zooxanthellae can be reduced by up to 50% as a consequence of exposure to stressors without showing any significant change in chlorophyll a levels. Authors suggested that the loss of zooxanthellae does not cause a significant reduction in photosynthetic pigments, because stress-tolerant symbionts may increase chlorophyll a production to maintain the basal levels of this pigment. Therefore, despite the photosynthetic capacity of the zooxanthellae is reduced following exposure to increasing temperature alone or in combination with Cu, no visual manifestations of bleaching was observed in *M. harttii*. Also, no significant changes in the density of endosymbionts, evaluated through chlorophyll a levels, were noted. These findings indicate that chlorophyll a level may actually not be a reliable biomarker of damage to the photosynthetic capacity or bleaching in corals.

It is worth noting that exposure of *M. harttii* to increasing temperature alone or in combination with different concentrations of Cu was carried out in the winter period in Brazil. In this case, the mean maximum temperature tested was 27.3°C, which represents below average temperature in the summer period in Abrolhos (Soares, 2011), a region near the site of coral collection. It is worth noting that findings from the present study indicate that exposure to increasing temperature alone or in combination with environmentally relevant concentrations of Cu increases the susceptibility of *M. harttii* to bleaching (increased LPO and reduced *Fv/Fm*). Also, it is important to note that the observed reduction in photosynthetic efficiency was enhanced with increasing the exposure time. Therefore, an even worse negative impact on coral health may occur

after chronic exposure of *M. harttii* to environmental stressors in the summer period.

It is important to emphasize that the present study was carried out in a marine mesocosm system, which allows a greater ecological realism (Duarte et al., 2015). In this system, natural variations in environmental conditions (temperature, salinity, photoperiod, pH, etc.) are incorporated, a condition that is difficult to be obtained in experiments performed in conventional laboratories. Currently, many studies have highlighted the importance of using marine mesocosm systems to select potential tools for assessing the health of reef organisms (Santos et al., 2014; Winter et al., 2016; Marangoni et al., 2016, 2017; Marques et al., 2017). Therefore, findings reported in the present study allowed us to elucidate some of the biochemical and physiological effects induced by exposure to increasing temperature alone or in combination with environmentally relevant concentrations of dissolved Cu. Also, they allowed us to identify the main mechanisms involved in the coral *M. harttii* responses to recover the damages caused by the environmental stressors tested (Fig. 8). Scenarios of multiple stressors, as tested in the present study, involving global climate changes combined with local impacts will be even more frequently evaluated in the next decades using coral reef organisms (Nystron et al., 2001). Indeed, these stressors can seriously compromise the resilience of coral species worldwide (Hoegh-Guldberg et al., 2007).

5. Conclusions

Long-term exposure to increasing temperature negatively affected photosynthesis (reduced *Fv/Fm*) and induced oxidative damage (increased LPO) in the Brazilian endemic coral *M. harttii*. The observed reduction in photosynthetic processes can compromise the supply of energy needed for growth and activation of mechanisms

involved in the protection against oxidative damage by reducing the transfer of photosynthates from symbiont algae to corals. Long-term exposure to increasing temperature combined with environmentally relevant concentrations of Cu aggravated the observed effects. Some biochemical and physiological parameters evaluated proved to be potential biomarkers to evaluate coral health in the scope of a scenario of multiple stressors. Indeed, they were altered before the visual manifestation of bleaching could be detected. These parameters include those associated with calcification (CA, [Ca^{2+}, Mg^{2+}-ATPase]), oxidative status (LPO), and metabolism (*Fv/Fm*).

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Figure legends

Figure 1. Carbonic anhydrase (CA) activity in the coral *Mussismilia harttii* exposed to increasing temperature isolated or combined with different concentrations of copper (Cu) for 4 d (A), 8 d (B) or 10 d (C). Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$).

Figure 2. (Ca^{2+}, Mg^{2+})-ATPase activity in the coral *Mussismilia harttii* exposed to increasing temperature isolated or combined with different concentrations of copper (Cu) for 4 d (A), 8 d (B) or 10 d (C). Data are expressed as mean ± standard error. Different letters represent significant difference among treatments ($p<0.05$).

Figure 3. Photochemical efficiency of the photosystem II of symbiotic algae (*Fv/Fm*) in the coral *Mussismilia harttii* exposed to increasing temperature isolated (A) or combined with different concentrations of Cu (B) for 12 d. Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$). The asterisk (*) represents significant difference among the exposure time for the same temperature.

Figure 4. Chlorophyll a of symbiotic algae in the coral *Mussismilia harttii* exposed to increasing temperature isolated or combined with different concentrations of copper (Cu) for) for 4 d (A), 8 d (B) or 10 d (C). Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$).

Figure 5. ATP concentration in the coral *Mussismilia harttii* exposed to increasing temperature isolated or combined with different concentrations of copper (Cu) for) for 4 d (A), 8 d (B) or 10 d (C). Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$).

Figure 6. Antioxidant capacity against peroxyl radicals (ACAP) in the coral *Mussismilia harttii* exposed to increasing temperature isolated or combined with different concentrations of copper (Cu) for 4 d (A), 8 d (B) or 10 d (C). Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$).

Figure 7. Lipid peroxidation (LPO) in the coral *Mussismilia harttii* exposed to increasing temperature isolated or combined with different concentrations of copper (Cu) for 4 d (A), 8 d (B) or 10 d (C). Data are expressed as mean ± standard error. Different letters represent significant differences among treatments ($p<0.05$).

Figure 8. Schematic model depicting the effects of environmental stressors in the coral *Mussismilia harttii*. Exposure to increasing temperature and copper enhance carbonic anhydrase (CA) activity as an attempt to recover the photochemical efficiency of the photosystem II of symbiotic algae (*Fv/Fm*). In turn, reduced *Fv/Fm* induces a decrease in ROS production and consequently in LPO. However, prolonged exposure (12 d) to increasing temperature combined with copper (Cu) addition in seawater causes a greater reduction in *Fv/Fm*. This could reduce the rate of photosynthates transfer to the coral and consequently the energy production. Therefore, less energy would be available to support the activity of enzymes involved in the calcification process. The lack of

1640 increase in the activity of these enzymes after 12 d supports this idea. Also, less energy
1641 would be available to activate the mechanisms involved in the protection against lipid
1642 oxidative damages, thus explaining the increased LPO observed after long-term
1643 exposure to the combination of increasing temperature and Cu exposure.

Figure 1A

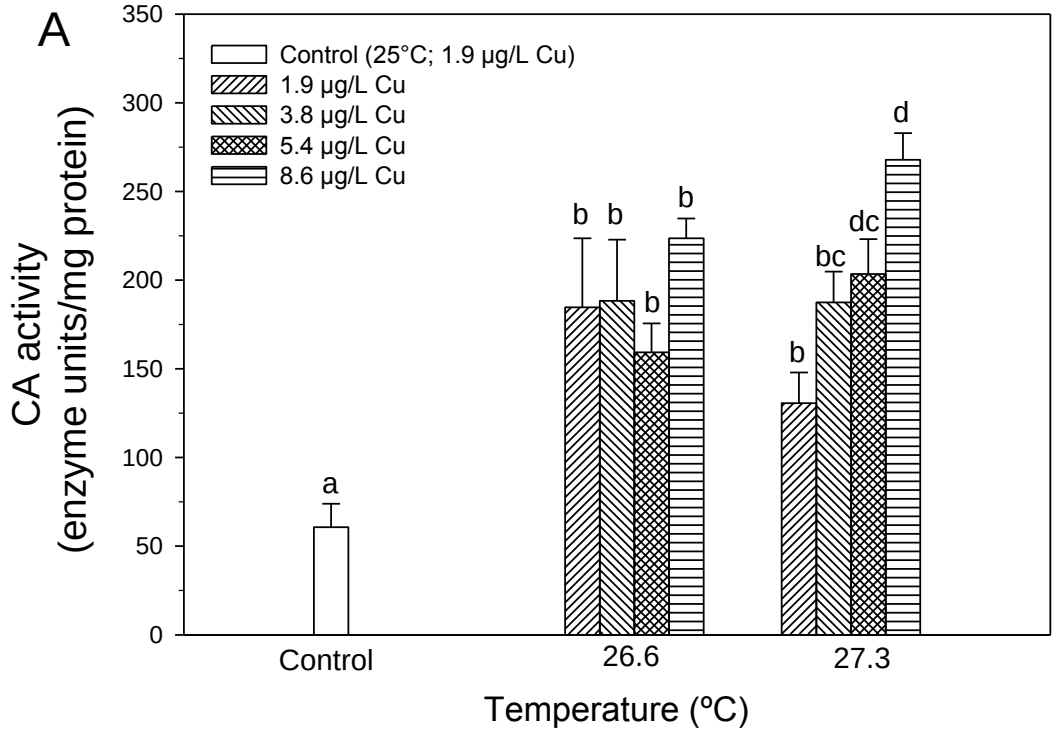

A
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
CA activity
(enzyme units/mg protein)
0
50
100
150
200
250
300
350
a
b b b
b bc dc d
Control
26.6
27.3
Temperature (ºC)

Figure 1B

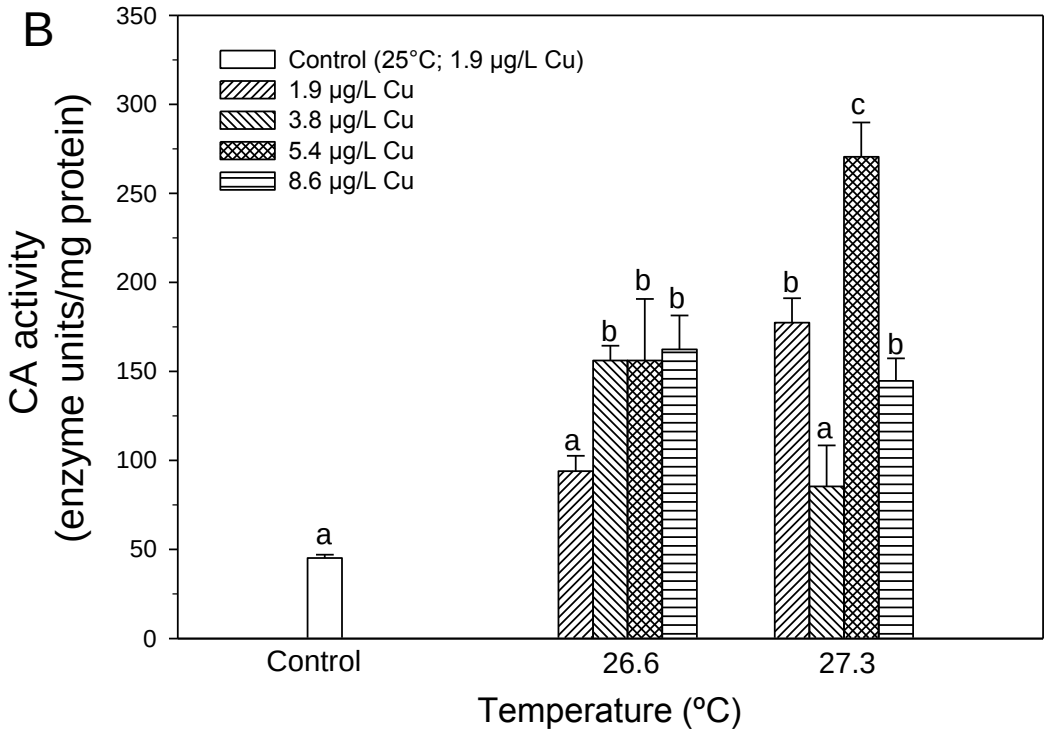

B
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
CA activity
(enzyme units/mg protein)
350
300
250
200
150
100
50
0
a
a
b
b
b
b
a
c
b
Control
26.6
27.3
Temperature (ºC)

Figure 1C

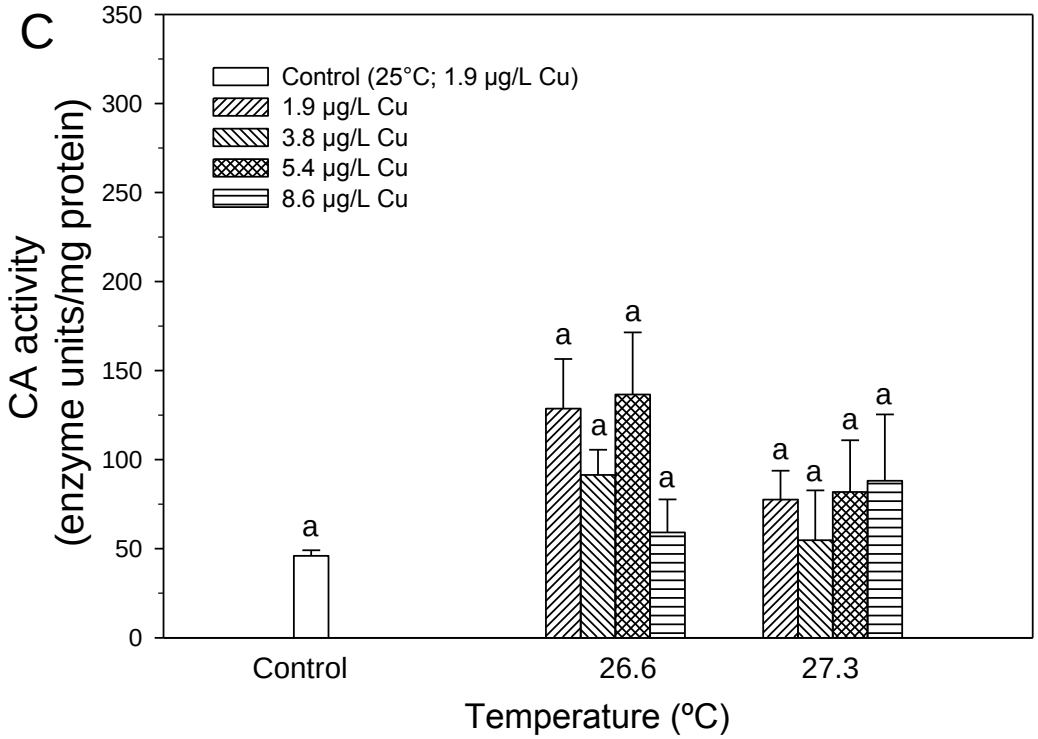

C
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
CA activity
(enzyme units/mg protein)
350
300
250
200
150
100
50
0
a
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 2A

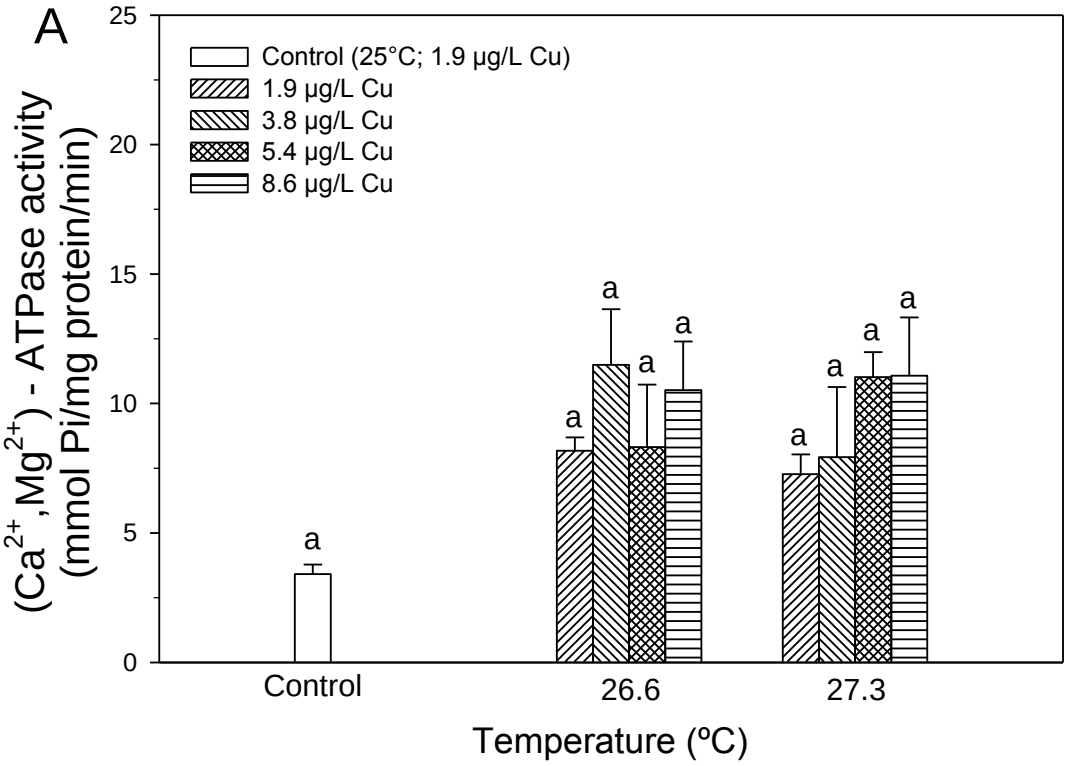

A
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
(Ca2+,Mg2+) - ATPase activity
(mmol Pi/mg protein/min)
0
5
10
15
20
25
a
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 2B

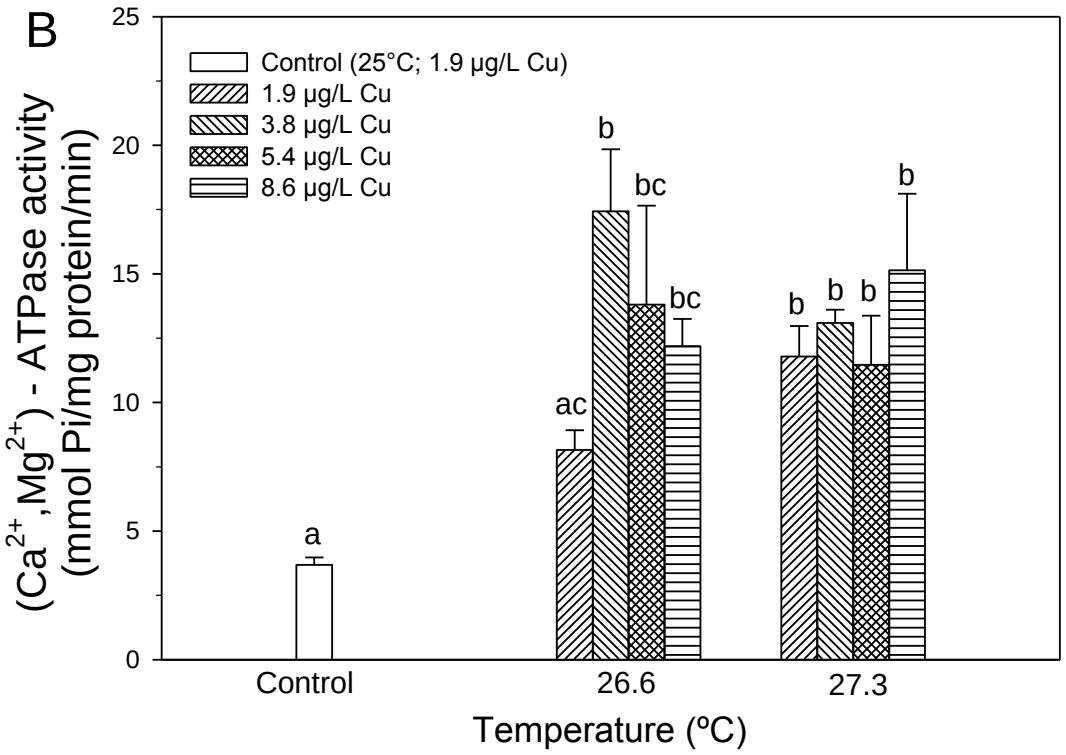

B
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
(Ca2+,Mg2+) - ATPase activity
(mmol Pi/mg protein/min)
0
5
10
15
20
25
a
ac
b
bc
bc
b
b
b
b
Control
26.6
27.3
Temperature (ºC)

Figure 2C

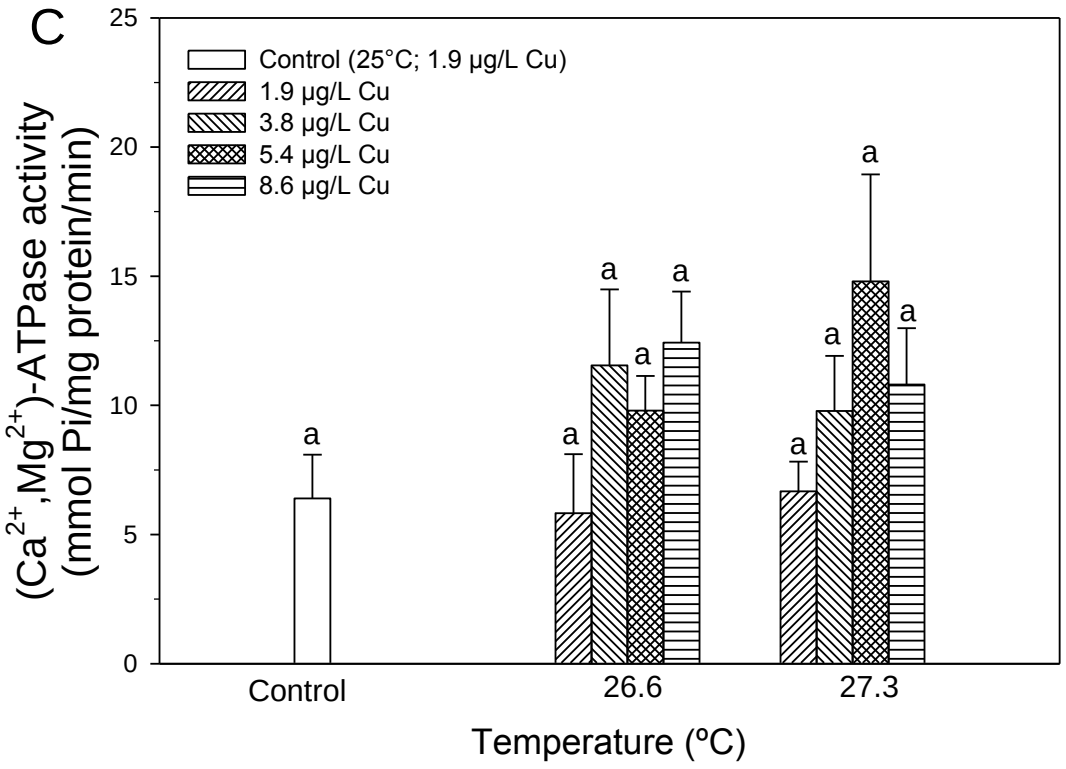

C
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
(Ca2+,Mg2+)-ATPase activity
(mmol Pi/mg protein/min)
0
5
10
15
20
25
a
Control
26.6
27.3
Temperature (ºC)

Figure 3A

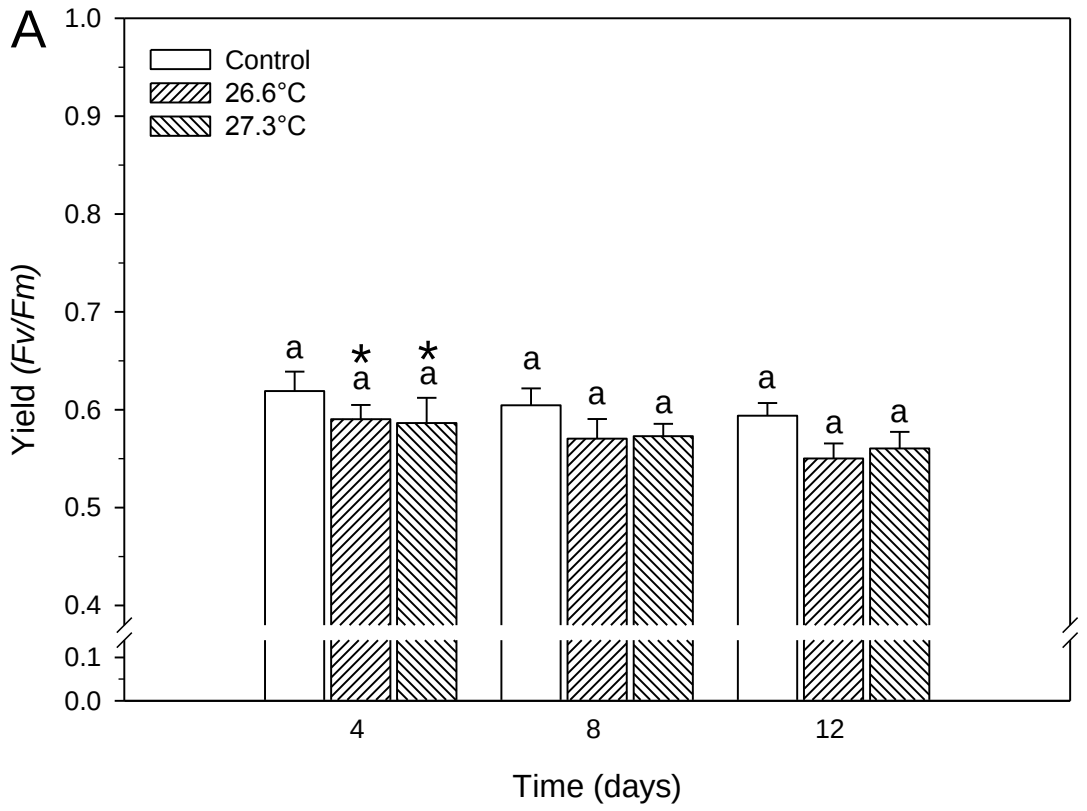

A
Control
26.6°C
27.3°C
Yield (Fv/Fm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.1
0.0
a
*
a
*
a
a
a
a
a
a
a
4
8
12
Time (days)

Figure 3B

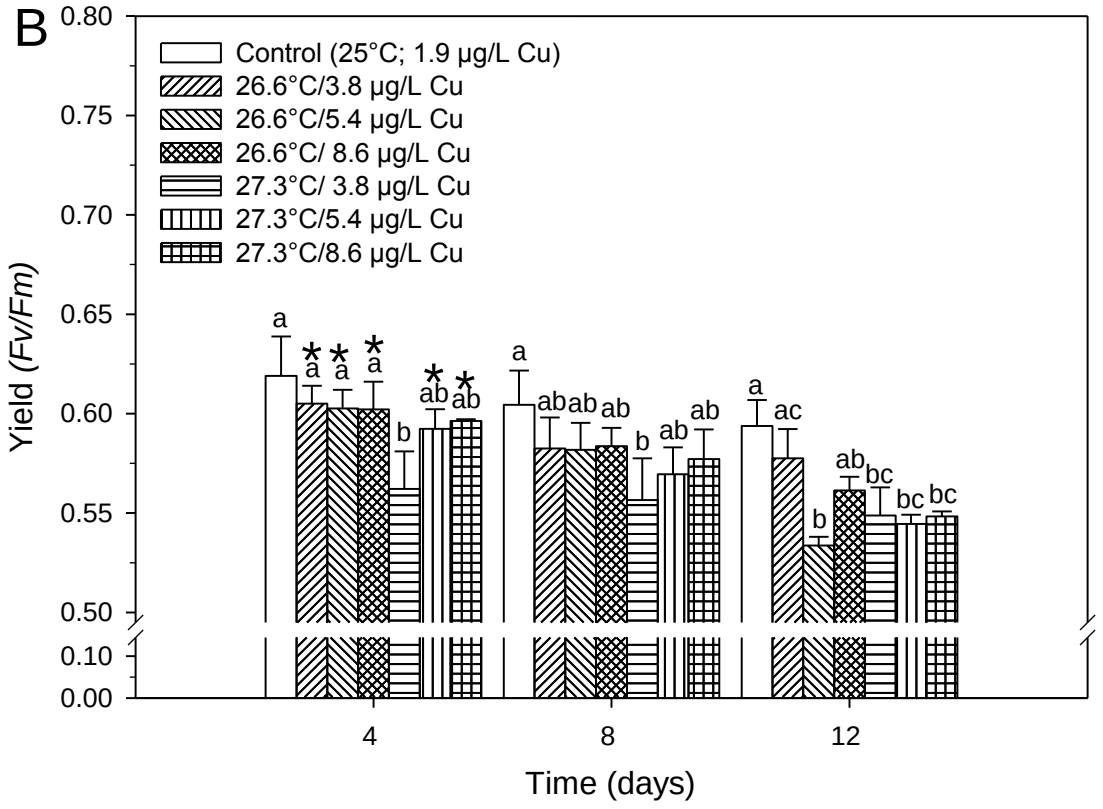

B
Control (25°C; 1.9 µg/L Cu)
26.6°C/3.8 µg/L Cu
26.6°C/5.4 µg/L Cu
26.6°C/ 8.6 µg/L Cu
27.3°C/ 3.8 µg/L Cu
27.3°C/5.4 µg/L Cu
27.3°C/8.6 µg/L Cu
Yield (Fv/Fm)
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.10
0.00
a
a a a
b
ab ab
a
ab ab ab
b
ab
ab
a
ac
b
ab
bc
bc bc
4
8
12
Time (days)

Figure 4A

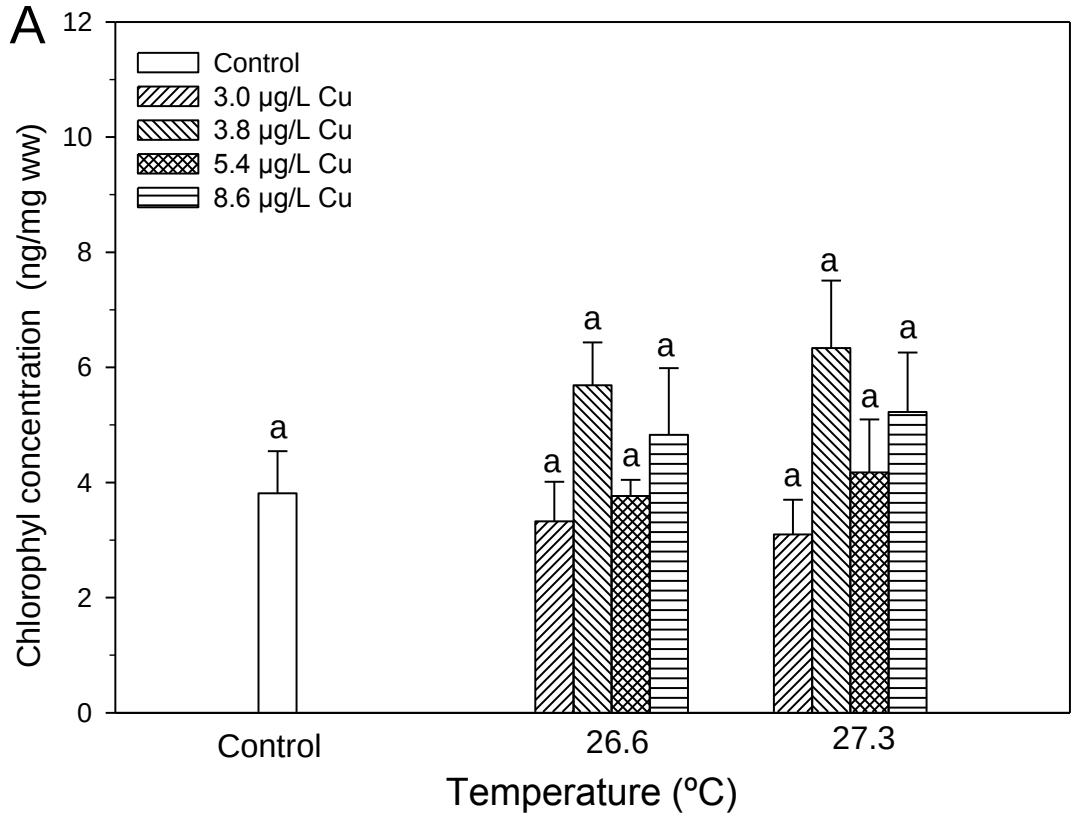

A
Control
3.0 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
Chlorophyl concentration (ng/mg ww)
0
2
4
6
8
10
12
a
Control
26.6
27.3
Temperature (ºC)

Figure 4B

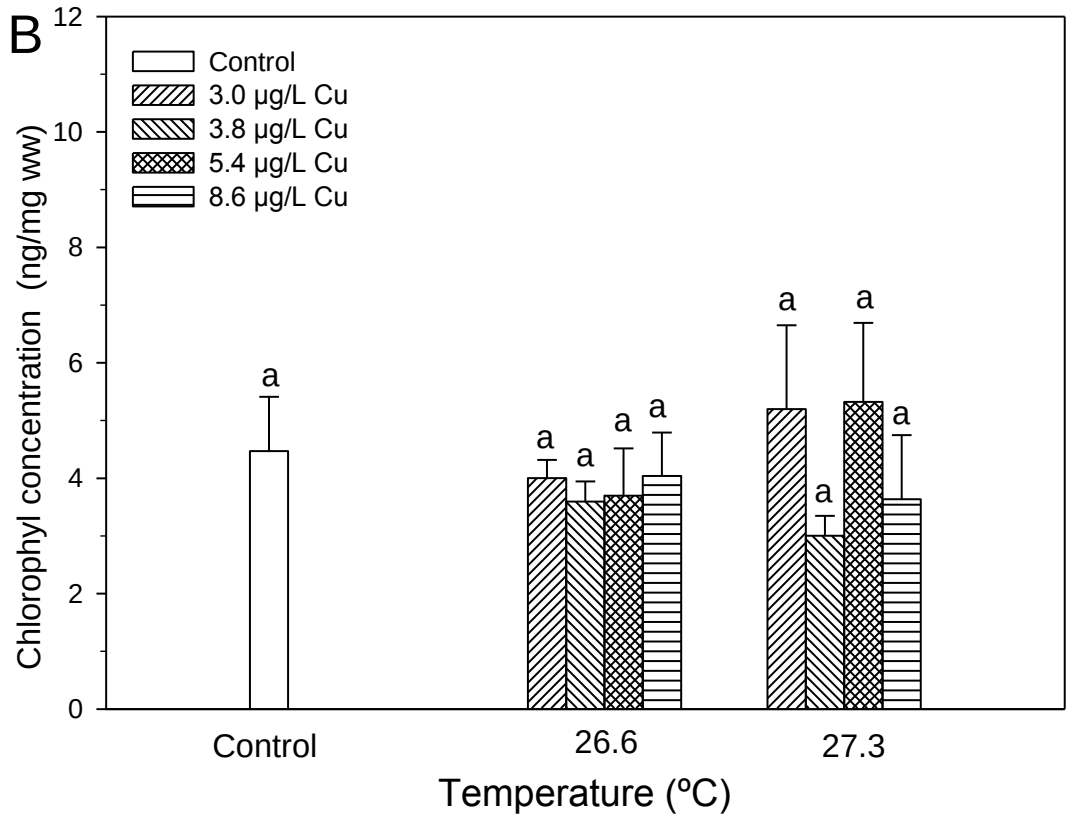

B
Control
3.0 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
Chlorophyl concentration (ng/mg ww)
0
2
4
6
8
10
12
a
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 4C

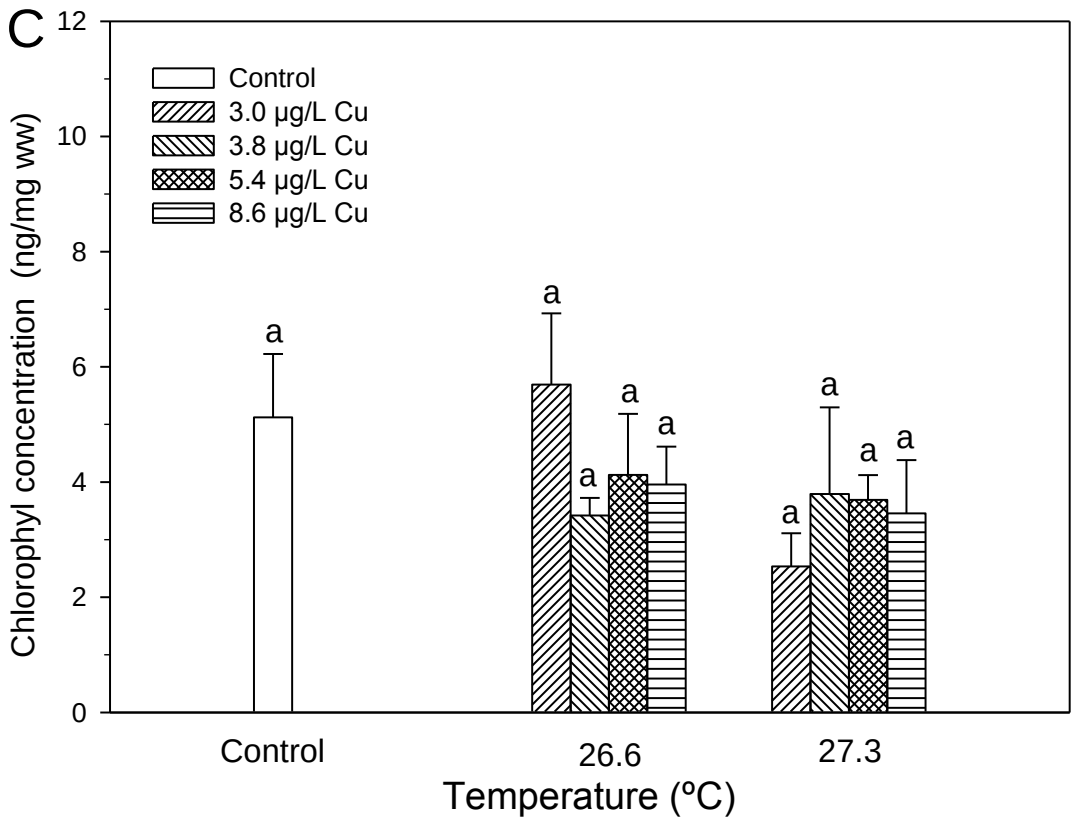

C
Control
3.0 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
Chlorophyl concentration (ng/mg ww)
0
2
4
6
8
10
12
a
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 5A

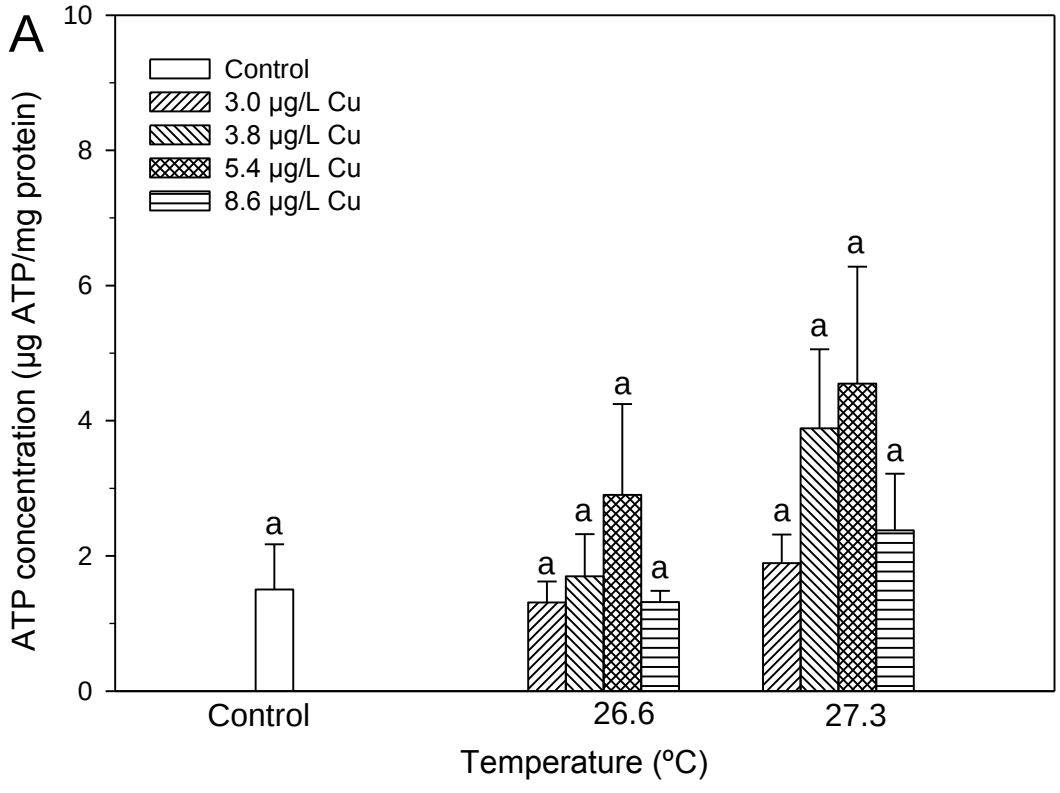

A
Control
3.0 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
ATP concentration (µg ATP/mg protein)
0
2
4
6
8
10
a
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 5B

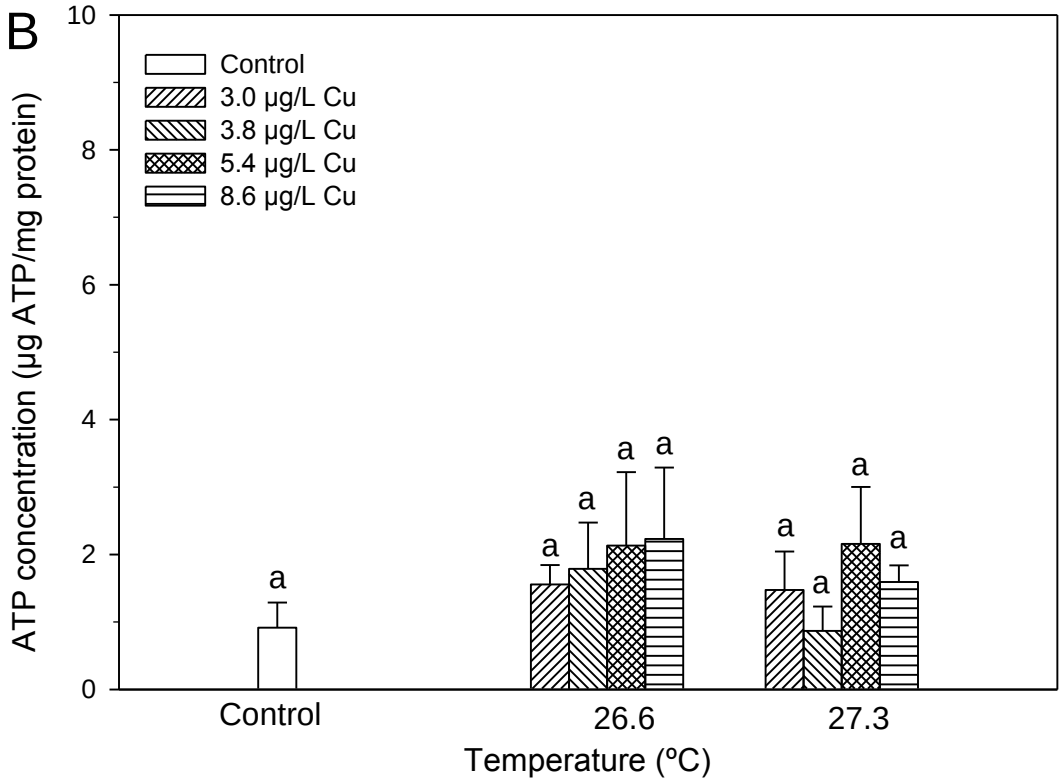

B
Control
3.0 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
ATP concentration (µg ATP/mg protein)
0
2
4
6
8
10
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 5C

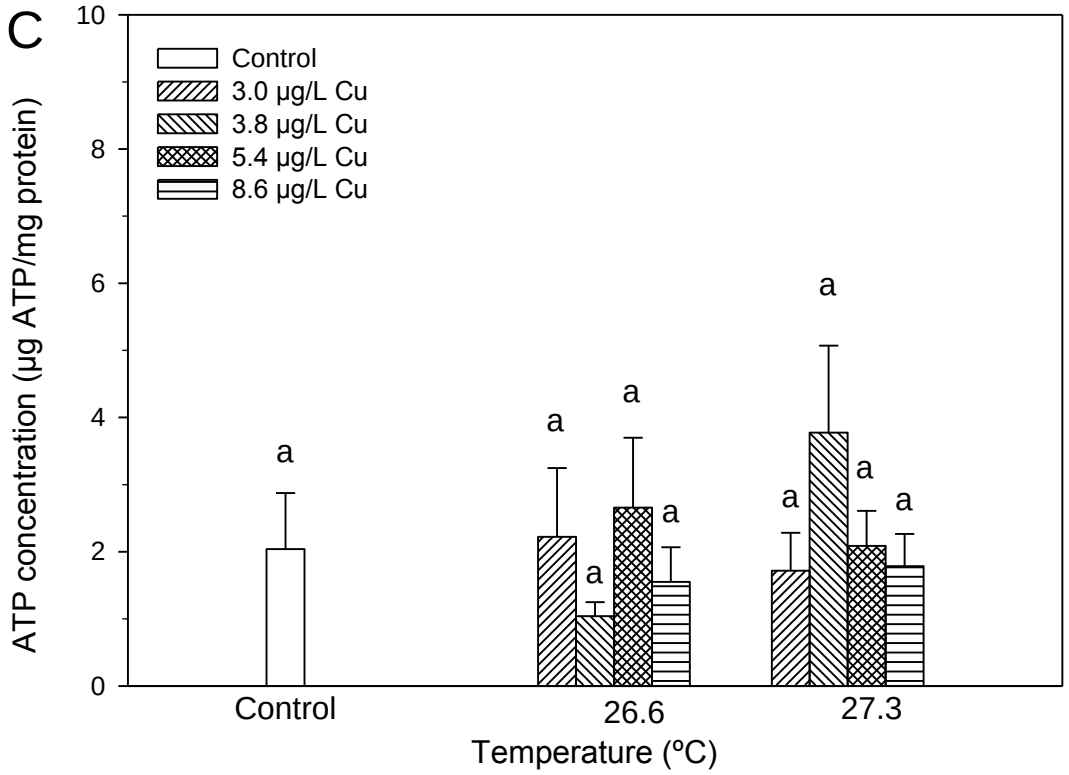

C
Control
3.0 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
ATP concentration (µg ATP/mg protein)
0
2
4
6
8
10
a
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 6A

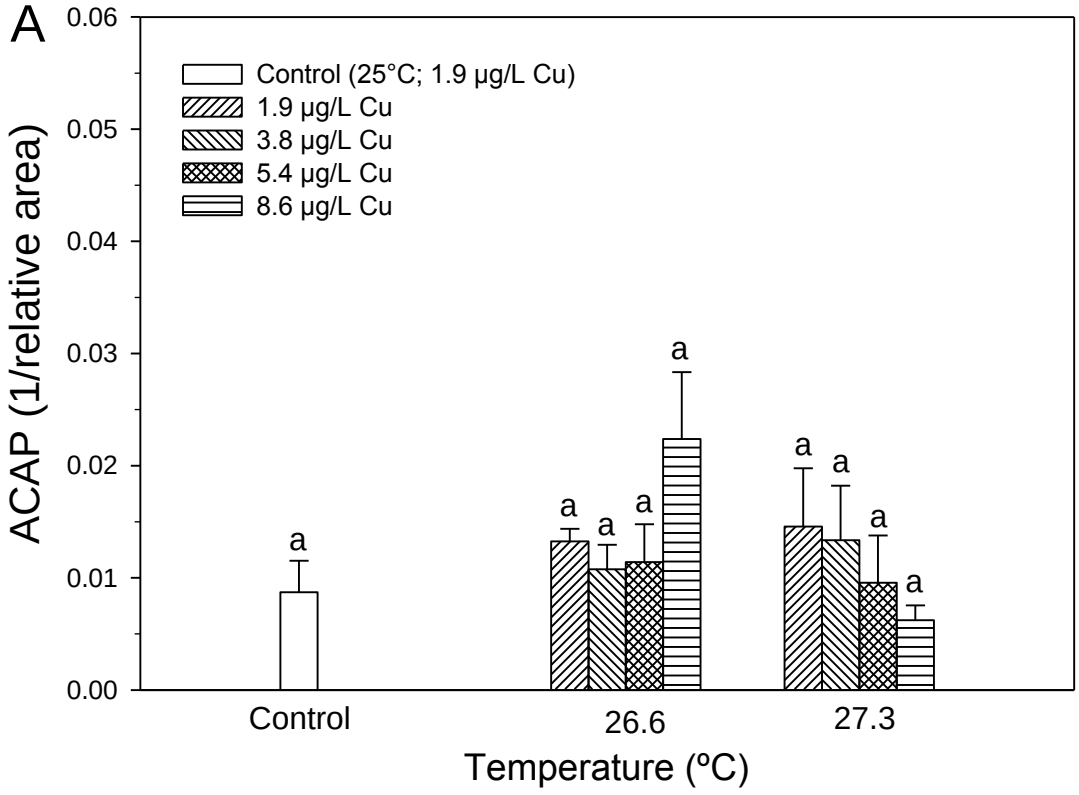

A
ACAP (1/relative area)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
a
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 6B

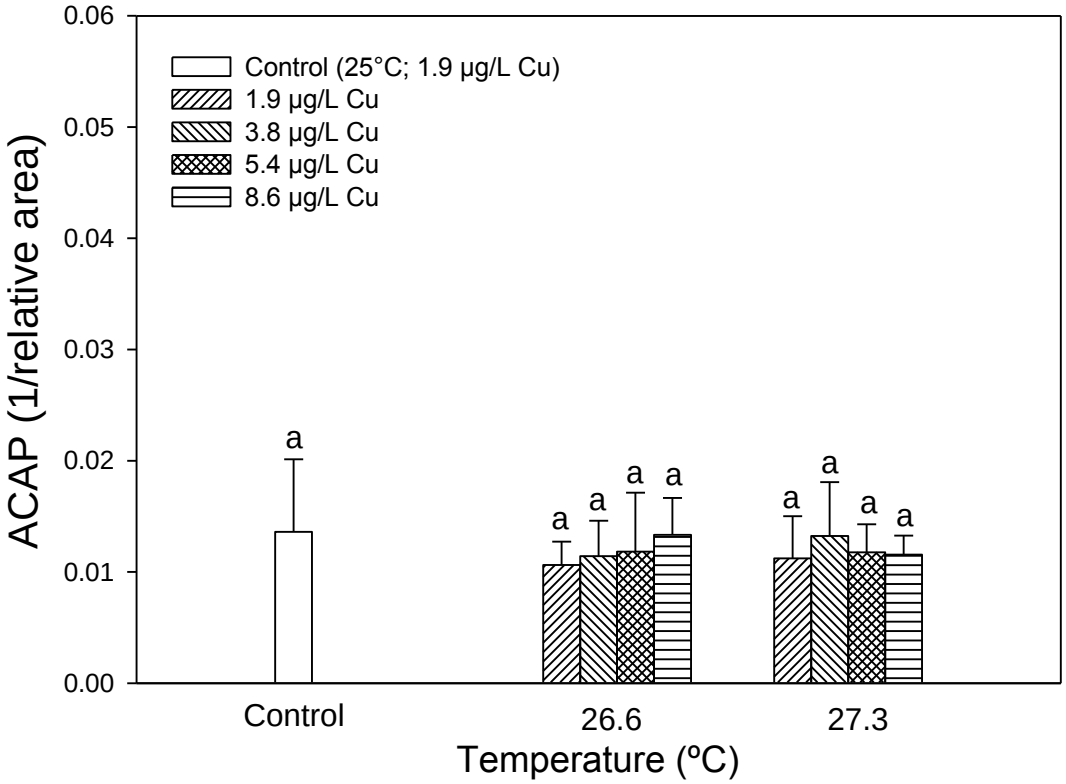

Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
ACAP (1/relative area)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
a
a a a a
a a a a
Control
26.6
27.3
Temperature (ºC)

Figure 6C

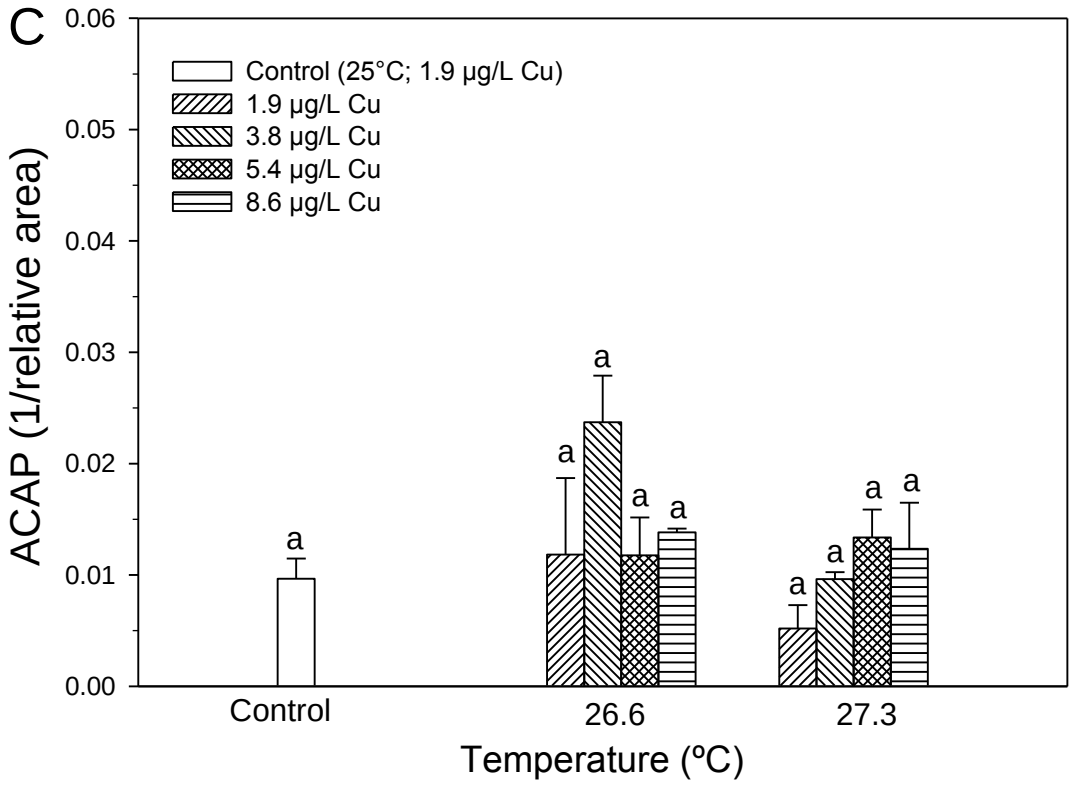

C
ACAP (1/relative area)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
a
a
a
a
a
a
a
a
Control
26.6
27.3
Temperature (ºC)

Figure 7A

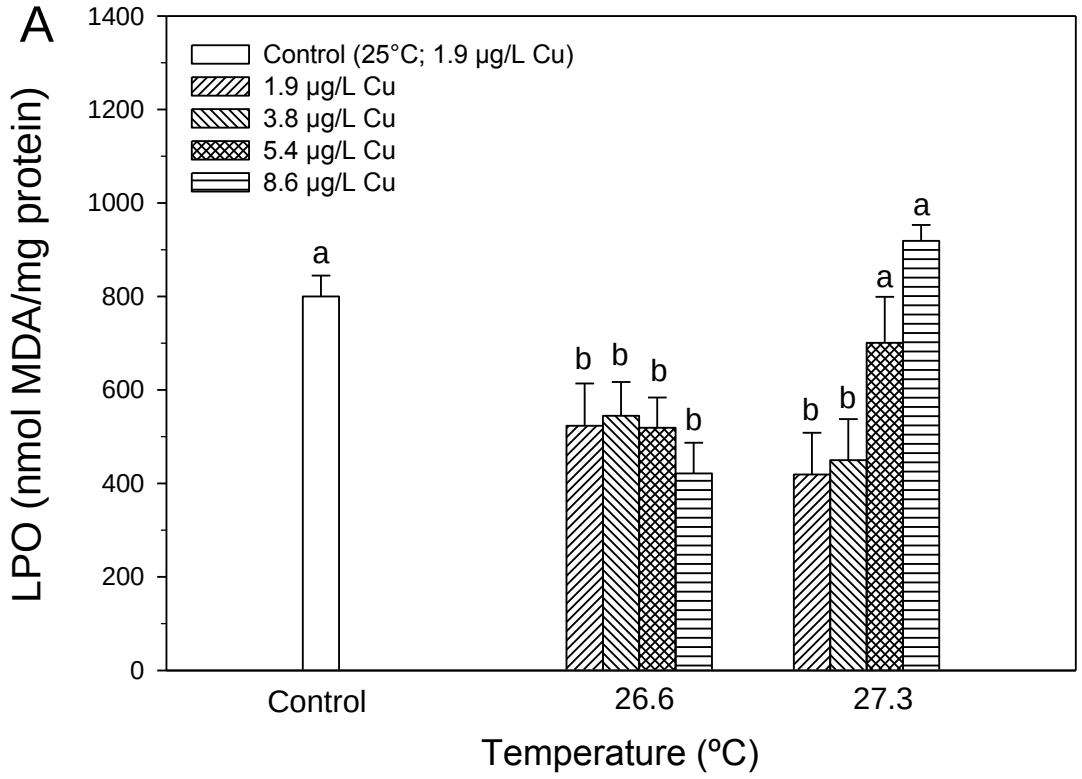

A
LPO (nmol MDA/mg protein)
1400
1200
1000
800
600
400
200
0
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
a
b b b b
b b a a
Control
26.6
27.3
Temperature (ºC)

Figure 7B

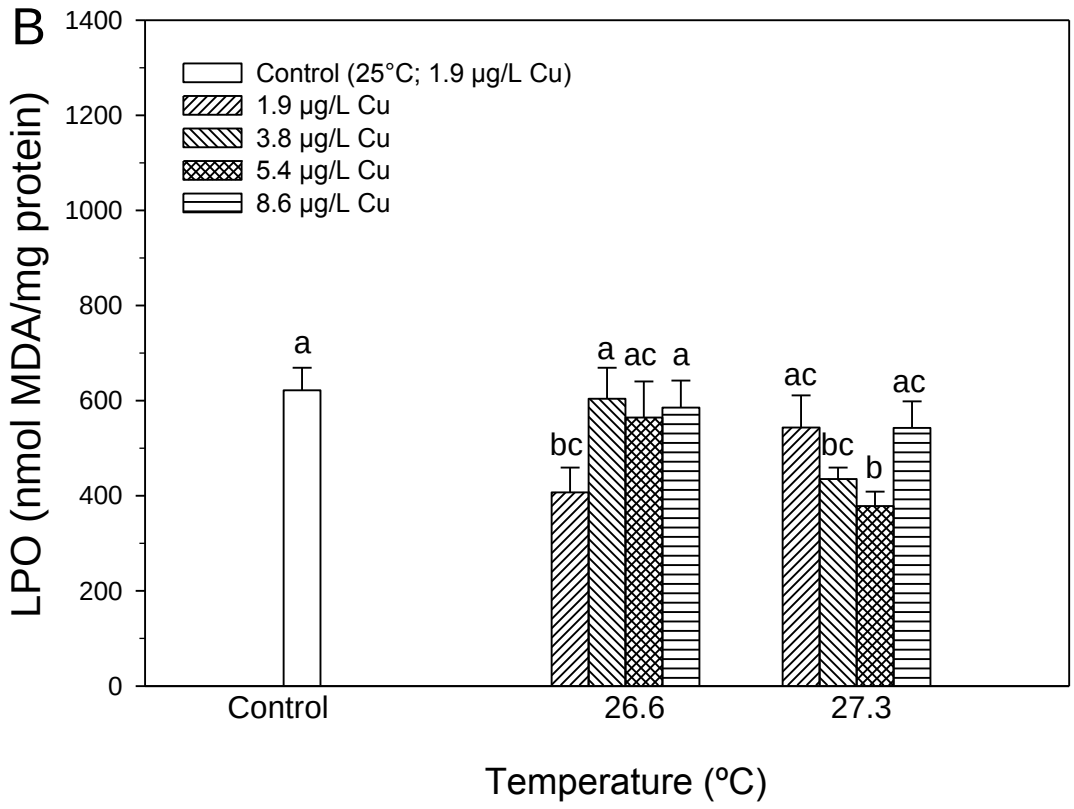
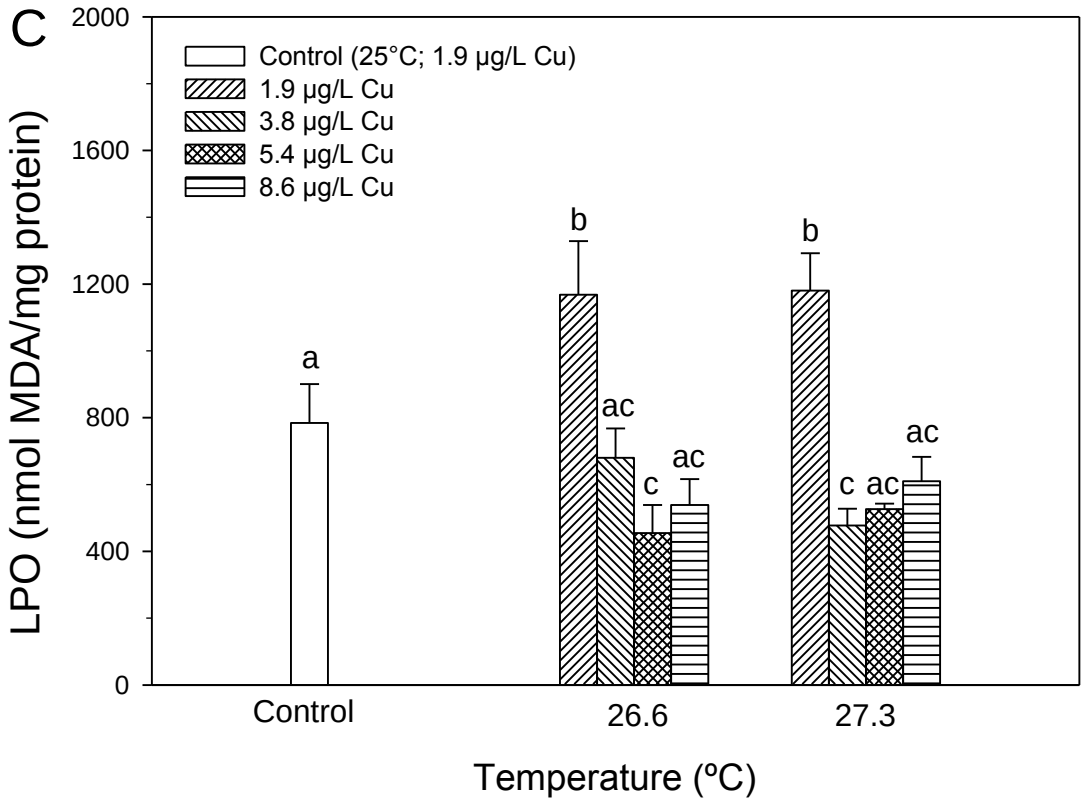
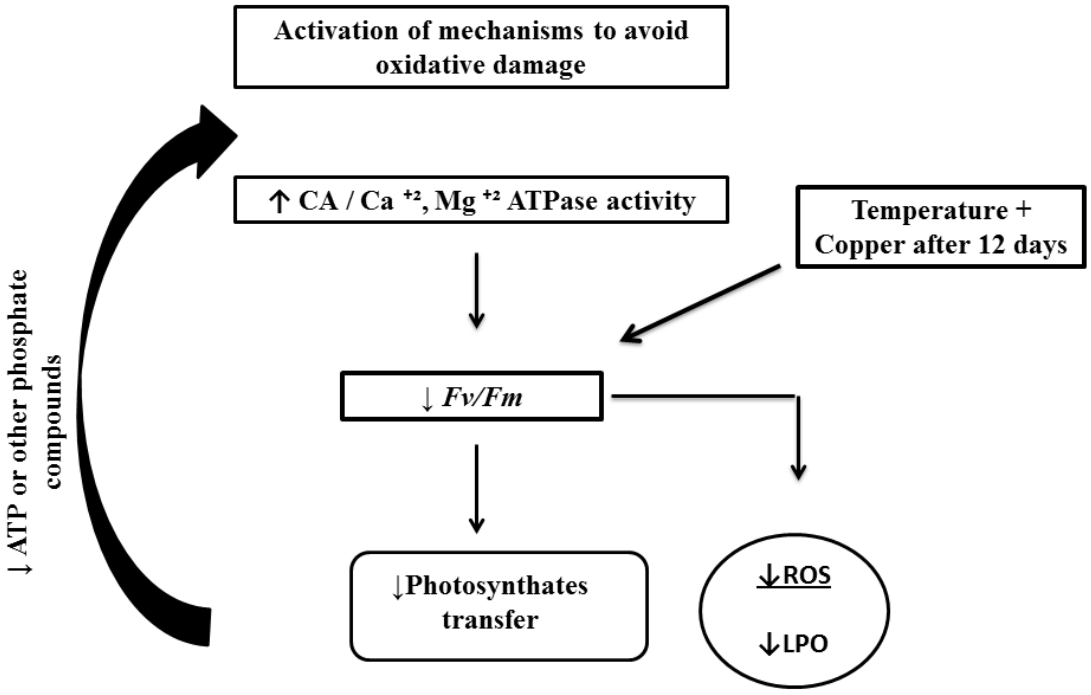

B
LPO (nmol MDA/mg protein)
1400
1200
1000
800
600
400
200
0
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
a
bc
a
ac
a
ac
bc
b
ac
Control
26.6
27.3
Temperature (ºC)

Figure 7C


C
LPO (nmol MDA/mg protein)
2000
1600
1200
800
400
0
Control (25°C; 1.9 µg/L Cu)
1.9 µg/L Cu
3.8 µg/L Cu
5.4 µg/L Cu
8.6 µg/L Cu
a
b
ac
c
ac
b
c
ac
ac
Control
26.6
27.3
Temperature (ºC)

1715 **Figure 8**



1716

Discussão geral:

Considerando-se os resultados do presente estudo com o coral endêmico *Mussismilia harttii*, pode-se concluir que a exposição ao aumento da temperatura de forma isolada e/ou combinada com diferentes concentrações de cobre: (1) aumenta a atividade de enzimas envolvidas no processo de calcificação, sugerindo um aumento do metabolismo causado pela exposição aos estressores ou a ativação de mecanismos para recuperação do processo fotossintético; (2) reduz os danos oxidativos em lipídeos, possivelmente devido à redução do processo fotossintético ou ao remodelamento de lipídeos nas membranas biológicas; (3) diminui temporalmente a transferência de fotossintatos, importantes fontes de energia para o coral, reduzindo assim a energia disponível para as enzimas de calcificação e, consequentemente, para o crescimento, bem como para a ativação de mecanismos de proteção contra danos oxidativos, aumentando assim a susceptibilidade do coral ao branqueamento. Por fim, conclui-se que vários parâmetros bioquímicos e fisiológicos avaliados no presente estudo se mostraram sensíveis à exposição aos estressores (aumento de temperatura e exposição ao cobre dissolvido), indicando que essas ferramentas são potencias biomarcadores para o monitoramento da saúde de corais, uma vez que sofreram alterações, mesmo antes de ter sido observada qualquer manifestação de branqueamento.