UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
INSTITUTO DE OCEANOGRAFIA
PROGRAMA DE PÓS-GRADUAÇÃO EM OCEANOGRAFIA BIOLÓGICA

ECOFISIOLOGIA EM RECIFES DE CORAL: IMPACTOS CLIMÁTICOS E LOCAIS EM CORAIS E HIDROCORAIS DO ATLÂNTICO SUL

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Tese apresentada ao Programa de Pós-graduação em Oceanografia Biológica da Universidade Federal do Rio Grande - FURG como requisito parcial à obtenção do título de DOUTORA

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RIO GRANDE
Setembro 2018

À minha família

'I wish to live in the coral reef,
to swim all day with fishes.
The colors provided are beyond belief,
like an ocean full of riches.
(...)
Such a palette of colors under the sea,
many more wait to be found.
Would you like to live on the reef with me,
You may find the beauty profound.'

[Trecho extraído do poema *Coral Reefs*
da poetiza estadunidense Mel Gibson]

Agradecimentos

Meus agradecimentos à FURG, instituição da qual faço parte desde 2011, quando dei início à minha tão sonhada pós-graduação. Agradeço ao Instituto de Oceanografia e ao Programa de Pós-Graduação em Oceanografia Biológica (PPGOB), por toda a contribuição à minha formação profissional. Igualmente, agradeço o Instituto de Ciências Biológicas, no qual desenvolvi grande parte das minhas atividades acadêmicas e práticas durante meu período de doutoramento.

Agradeço à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelas bolsas concedidas; ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), ao International Development Research Centre (IDRC, Canada) e a International Society for Reef Studies (ISRS), pelo financiamento das minhas atividades de pesquisa.

Meu muito obrigada ao Projeto Coral Vivo e toda a sua equipe, o qual me deu suporte logístico e intelectual com o trabalho de campo e experimentação no sul da Bahia durante todo o período da minha pós-graduação. Foi, e ainda é, um grande aprendizado e estímulo trabalhar em parceria com os professores e fundadores do projeto Dr. Clovis B. Castro e Dr. Débora Pires, e com os integrantes da Rede de Pesquisas Coral Vivo. Meu especial agradecimento aos pesquisadores Dr. Emiliano Nicolas Calderon, Ms. Cristiano Pereira e Dr. Douglas Abrantes, pela grande parceria durante coletas de campo e experimentos “mesocósmicos”.

Agradeço imensamente ao meu pai científico e orientador Dr. Adalto Bianchini, por todo o aprendizado, exemplo profissional, e pelas inúmeras oportunidades e possibilidades que me ofereceu ao longo desses sete anos de pós-gradução. Também agradeço a todo grupo de pesquisa do Prof. Adalto, o qual me deu suporte em inúmeros momentos decisivos. Obrigada a Camila Dalmolin, Roberta Klein e Juliana Fonseca pela parceria dentro e fora do laboratório. Em especial, agradeço minha grande amiga e companheira de trabalho, Joseane Marques, com a qual dividi muitos desafios e nervosismo, mas também muitos momentos de conquista e alegria! Não poderia ter tido uma melhor companheira e colaboradora durante todo esse período.

Agradeço a Dra. Christine Ferries-Pagès e ao Dr. Renaud Grover, meus orientadores durante o período de doutorado-sanduíche, por todo o conhecimento partilhado e estímulo a minha carreira acadêmica, bem como a todos os pesquisadores e técnicos integrantes do “Ecophysiology team” do CSM (Centre Scientifique de

Monaco), por me receberam tão abertamente e me prestarem apoio em todos os momentos necessários, dentro e fora do trabalho. Agradeço também ao Prof. Dr. Oren Levy, pelas oportunidades de colaboração, e por dividir seu espaço de trabalho e experiências profissionais durante este período. Minha experiência nesse "pequeno grande centro de pesquisa" foi um sonho realizado e contribuiu imensamente para meu amadurecimento profissional.

Meus agradecimentos a todos os amigos e amigas, que de perto ou de longe me deram suporte com todo o carinho e atenção. A minha amiga-irmã, Fernanda Cabrini, minha eterna gratidão por torcer tanto por mim e por se fazer sempre tão presente. Um agradecimento especial também aos meus queridos amigos(as) conquistados ao longo desses anos no extremo sul brasileiro, Barbara, Dédi, Elysa, Michele, Lumi, Amanda, Denis, Victor, Marie, Mario, Marião, Jessica, Micheli, Patrícia, Isabel, com os quais dividi tantos momentos de aprendizagem e diversão.

Agradeço especialmente ao meu sempre tão presente companheiro, Samuel Coelho de Faria, cujo apoio e incentivo nunca faltaram durante todo o meu processo de formação. Serei eternamente grata por todo o aprendizado a seu lado e pelas valiosas trocas de experiências e ideias, tanto no campo profissional como pessoal! Agradeço, com muito amor aos momentos de alegria e distração junto a nossos queridos Inácio, Tobias e Pitica.

Minha eterna gratidão a minha família, especialmente aos meus pais, Gutemberg e Heloisa Helena, e a minha linda irmã Liana, que sempre acreditaram, e continuam a acreditar, nos meus sonhos, objetivos e convicções. Muito obrigada por todo o amor, incentivo, dedicação e compreensão. Apesar de distantes fisicamente, sempre me senti amparada por vocês. Não tenho palavras para agradecer o privilégio de fazer parte dessa família.

Índice

Resumo

Os recifes de coral são os ambientes marinhos de maior biodiversidade, com imensa importância ecológica e econômica. Todavia, encontram-se ameaçados pelas mudanças climáticas globais, como a acidificação e a elevação da temperatura dos oceanos, bem como pelos impactos de origem local, como a poluição química. Tal cenário de múltiplos estressores tem causado danos substanciais à saúde do ambiente recifal em todo o mundo, no entanto, o conhecimento sobre os potenciais efeitos sobre os recifes brasileiros é limitado quando comparado com o disponível para outras regiões recifais do mundo. Neste contexto, em condições simuladas de impactos globais (acidificação marinha) e locais (contaminação por cobre), ou naturais de El Niño, e utilizando-se o coral *Mussimilia harttii* e o hidrocoral *Millepora alcicornis* habitando recifes do Atlântico Sul, buscou-se: (i) avaliar os efeitos da acidificação da água do mar sobre o processo de calcificação; (ii) testar se os efeitos do cobre sobre seletos parâmetros fisiológicos são intensificados em decorrência da diminuição do pH; (iii) testar o potencial de respostas bioquímicas relacionadas à capacidade antioxidante e a danos oxidativos como ferramentas úteis para monitoramento de recifes de coral; (iv) testar se os níveis de espécies reativas de nitrogênio (peroxinitrito) dirigem a ocorrência de branqueamento; e (v) investigar a existência de uma correlação positiva entre o grau de predominância trófica (auto- e heterotrofia) e o branqueamento. Ainda, objetivou-se sintetizar o estado da arte da fisiologia de corais esclarectínios relacionada à simbiose coral-zooxantelas, ao fenômeno de branqueamento e ao processo de calcificação, tendo como foco a divulgação científica para discentes de graduação do Brasil. Os resultados demonstram: (i) que *Mi. alcicornis* apresenta elevada tolerância a diferentes cenários de acidificação marinha; (ii) a predominância de efeitos antagonistas pela interação entre acidificação e exposição ao cobre em *Mu. harttii*; (iii) que variações nos níveis de peroxidação lipídica, bem como a capacidade antioxidante basal das espécies, constituem potenciais biomarcadores para monitoramento ambiental; (iv) o peroxinitrito como possível molécula mediadora do branqueamento em ambas as espécies; e (v) mudanças na prevalência de modo alimentar (auto- e heterotrofia) em *Mi. alcicornis* e *Mu. harttii* durante o branqueamento, com heterotrofia preponderante pós-evento. Estes resultados proverão subsídios para planos de manejo, sobremaneira para os ecossistemas recifais brasileiros ainda carentes de informações fisiológicas básicas, incorporando medidas fisiológicas como indicadores de saúde do ambiente recifal.

Palavras-chave: Biomarcadores, branqueamento, calcificação, estresse oxidativo, mudanças climáticas, recifes de coral.

Abstract

Coral reefs are the most diverse marine environments, with great ecological and economic relevance. However, these ecosystems are threatened by climate change, such as ocean acidification and increasing sea surface temperature, as well as by local pressures, such as chemical contamination. Such scenario of multiple stressors has caused substantial damage to the reef environment worldwide, however, our knowledge on the potential deleterious effects on Brazilian reefs are limited compared to other reef regions. In this context, under simulated conditions of global (seawater acidification) and local (copper contamination) stressors, or natural El Niño conditions, and using the coral *Mussimilia harttii* and the hydrocoral *Millepora alcicornis* inhabiting South Atlantic reefs, the present study aimed to: (i) assess the effects of seawater acidification on the calcification process; (ii) evaluate whether the deleterious effects caused by copper exposure on specific physiological traits would be intensified by seawater acidification; (iii) test the potential of biochemical responses related to antioxidant capacity and oxidative damage as useful tools in reef monitoring programs; (iv) test if reactive nitrogen species (peroxynitrite) drive bleaching occurrence; and (v) investigate the existence of a positive relation between the predominance of feeding mode (autotrophy and heterotrophy) and bleaching. Further, a synthesis of the state-of-the-art of scleractinian coral´s physiology, including the coral-zooxanthellae symbiosis, the bleaching phenomenon and the calcification process, was presented to promote scientific outreach for Brazilian undergrad students. The results show the following evidences: (i) the hydrocoral *Millepora alcicornis* is able to cope with a broad range of seawater acidification scenarios; (ii) the combination of seawater acidification and copper exposure mainly resulted in antagonistic effects on the physiology of *Mussismilia harttii*; (iii) changes in lipid peroxidation values and total antioxidant capacity basal levels in corals and hydrocorals constitute potential biomarkers to be used in reefs monitoring programs; (iv) peroxynitrite may be an important mediator in coral and hydrocoral bleaching process; and (v) shifts in the predominance of auto- and heterophy in *Mi. alcicornis* and *Mu. harttii*, with prevalence of heterotrophy after bleaching occurrence. The present findings may underpin management strategies, especially for Brazilian reef ecosystems still lacking basic physiological information, incorporating biochemical measurements as coral reef health indicators.

Key-words: biomarkers, coral bleaching, calcification, climate change, oxidative stress, coral reefs

Prefácio

A presente tese constitui um dos requisitos do doutoramento pelo Programa de Pós-Graduação em Oceanografia Biológica da Universidade Federal do Rio Grande-FURG. Ela estrutura-se em três capítulos independentes e apresentados como apêndices. Os dois primeiros apêncides encontram-se em formato de artigo científico ou manuscritos, redigidos na língua inglesa. Por sua vez, o terceiro apêndice é apresentado no formato de capítulo de livro, redigido na língua portuguesa.

Previamente aos capítulos, é oferecido ao leitor na língua portuguesa uma introdução geral, assim como a metodologia, a síntese dos resultados encontrados e conclusões relacionadas aos dois primeiros anexos. A versão completa dos resultados, um maior detalhamento da metodologia, assim como a discussão sobre os resultados são apresentados nos respectivos apêndices.

O Apêndice I é composto por dois artigos publicados. O primeiro avalia os efeitos de diferentes níveis de acidificação da água do mar no processo de calcificação de uma espécie de hidrocoral. O segundo explora os efeitos combinados da acidificação da água do mar e exposição a concentrações ambientalmente relevantes de cobre no processo de calcificação e no metabolismo fotossintétco de endossimbiontes em uma espécie de coral esclarectínio.

O Apêndice II compõe-se por dois manuscritos. No primeiro, avaliaram-se respostas bioquímicas relacionadas à condição de estresse oxidativo em corais e hidrocorais durante um evento de branqueamento dirigido pelo fenômeno climático El Niño, objetivando acessar o potencial das mesmas como biomarcadores em programas de monitoramento. Por sua vez, o segundo manuscrito avaliou a produção de espécies reativas de nitrogênio e a plasticidade trófica em corais e hidrocorais durante o supracitado evento de branqueamento.

No Apêndice III, foi realizada uma síntese do estado da arte da fisiologia de corais esclarectínios relacionada à simbiose coral-zooxantelas, ao fenômeno de branqueamento e ao processo de calcificação. Tal capítulo compilou um conjunto de achados ecofisiológicos, e apresentou-os de forma didática e ilustrativa para atingir o público de graduação brasileiro. Ele é parte do livro ‘Conhecendo os Recifes Brasileiros’, o qual tive a também oportunidade de editorar junto de outros parceiros da Rede de Pesquisas Coral Vivo.

1. Introdução

1.1. Os recifes de coral

Os recifes de coral de águas rasas ocupam menos de 0,1% - 0,2% da superfície terrestre (Reaka-Kudla, 1997), no entanto, são considerados ecossistemas inigualáveis no ambiente marinho devido a sua beleza cênica, elevada diversidade e complexidade (Goldberg, 2013). Mais de 100 países abrigam em suas linhas costeiras recifes de coral, e a vida e subsistência de dezenas de milhões de pessoas depende, pelo menos em parte, desses ambientes. Em termos físicos, os recifes de coral protegem as regiões costeiras da ação de ondas e tempestades, enquanto economicamente movimentam a economia de muitas nações tropicais com atividades relacionadas ao turismo e à obtenção de recursos alimentares (Moberg e Folk, 1999; Castro e Zilberberg, 2016). Muitas espécies comercialmente valiosas residem nos ecossistemas recifais: peixes recifais constituem quase um terço da ictiofauna descrita, com estoques 30 a 40 vezes maiores comparados ao das pescarias demersais de regiões temperadas (Golberg, 2013); e muitas espécies de invertebrados e algas são fonte de medicamentos e de compostos bioquímicos utilizados pelo homem como matéria prima na pesquisa farmacológica (Goldberg, 2013).

Sob o prisma geomorfológico, os recifes de coral podem ser definidos como estruturas rochosas construídas por organismos marinhos, incluindo animais e vegetais portadores de esqueleto calcário, resistentes à ação de ondas e correntes marinhas (Leão, 1994). O termo *de coral* é utilizado pelo papel preponderante que o acúmulo dos esqueletos de corais pertencentes à Ordem Scleractinia possuem na construção de recifes biogênicos. Não obstante, é importante ressaltar que a formação dessas estruturas tridimensionais depende da atuação conjunta de uma grande diversidade de organismos que formam uma complexa teia de associações e eventos de sucessão ecológica (Goldberg, 2013; Castro e Zilberberg, 2016).

Os corais construtores de recifes de águas rasas, conhecidos também como corais-pétreos ou verdadeiros, são organismos com estreitas faixas de tolerância ambiental, habitando águas claras e mornas, de salinidade entre 33-36 ‰ S (Goreau et al. 1979; Goldberg, 2013). A incidência luminosa nos recifes coralíneos também é um fator de grande importância, uma vez que a maioria dos corais construtores de recife estabelece uma relação simbiótica com endossimbiontes fotossintetizantes (dinoflagelados do gênero *Symbiodinium*, referidos como zooxantelas) (Goreau et al, 1979; Davy et al. 2012). Desta forma, os recifes de corais restringem-se às regiões

tropicais e subtropicais (até 30° graus de latitude), e à profundidades de ≈50 metros (Goldberg, 2013).

A relação simbiótica entre cnidários e zooxantelas é um componente crucial à formação e manutenção dos recifes de coral, possibilitando que corais prosperem em ambientes oligotróficos. Embora primariamente heterotróficos, os corais podem contar com outra fonte de nutrição advinda de seus endossimbiontes fotossintetizantes, o que caracteriza a politrofia e explica a capacidade de sobrevivência em ambientes fortemente oligotróficos (Davy et al. 2012). De forma geral, os produtos da fotossíntese (oxigênio e carbono fixados fotossinteticamente) fornecidos pelas zooxantelas dão suporte a funções vitais como crescimento e reprodução do coral hospedeiro. Em contrapartida, as zooxantelas – residentes no interior de células da endoderme de corais - beneficiam-se de uma maior proteção contra herbivoria, uma estabilidade topológica na coluna d'água que otimiza o acesso a luz, e à obtenção de compostos metabólicos dos hospedeiro importantes ao seu metabolismo fotossintético (Davy et al. 2012). Ainda, é importante ressaltar que corais zooxantelados apresentam maiores taxas de calcificação na presença de luz. Neste caso, a interação entre os processos de fotossíntese e calcificação explicam as maiores taxas de deposição de carbonato de cálcio, sendo tal incremento na síntese do esqueleto pelos corais associado à presença de zooxantelas (Tambutté et al. 2011).

As espécies que compõem as comunidades recifais nas diferentes regiões do planeta diferem de acordo com as histórias geológica e biológica locais. Com relação às espécies coralíneas, os recifes do Indo-Pacífico apresentam a maior diversidade, abrigando 605 espécies de corais-pétreos zooxantelados, o que corresponde a ≈80% das espécies de corais zooxanteladas (Veron et al. 2009). No oceano Atlântico, o Caribe abriga a maior diversidade α, com ≈60 espécies de corais-pétreos zooxantelados (Veron, 2000). O Brasil, por sua vez, abriga os únicos recifes de coral de águas rasas verdadeiros do Atlântico Sul e apresenta uma diversidade de espécies significativamente menor, com apenas 16 espécies de corais-pétreos zooxantelados. Entretanto, os recifes brasileiros possuem elevada diversidade biológica e apresentam uma fauna coralínea distinta, onde 49% das espécies de corais são endêmicas – considerando os corais-pétreos (Ordem Scleractinia), corais-negros (Ordem Antipatharia), corais-de-fogo ou hidrocorais (Famílias Milleporidae e Stylasteridae) e octocorais (Subclasse Octocorallia) (Castro e Pires, 2001; Castro e Zilberberg, 2016).

Dentre as espécies de corais-pétreos zooxantelados da costa brasileira, destacam-se aquelas do gênero endêmico *Mussismilia*, por possuírem importante papel na construção dos recifes (Castro e Pires, 2001). Os hidrocorais do gênero *Millepora* destacam-se por conferirem aos recifes uma maior complexidade estrutural (Leão et al. 2003), dada a ausência de corais escleractíneos ramosos no Atlântico Sul. Estes, assim como os corais escleractíneos, são organismos calcificadores, estabelecem simbiose com zooxantelas, e apresentam papel ecologicamente relevante em comunidades recifais ao redor do mundo (Lewis, 1989). Nos recifes do Atlântico Sul, *Mi. alcicornis* desempenha papel funcional de corais escleractíneos, como as espécies de *Acropora* no Caribe e no Indo-Pacífico, oferecendo abrigo, substrato e fonte de alimento para diversos organismos recifais (Pereira et al. 2012; Leal et al. 2013, 2015).

1.2. Algumas ameaças aos recifes de coral

As crescentes pressões oriundas de atividades humanas, incluindo as alterações climáticas, têm gerado diversas e profundas consequências nos ecossistemas marinhos (Doney et al. 2012). Dentre os impactos causados pelo ser humano, ressalta-se as crescentes emissões de dióxido de carbono (CO_2) na atmosfera (Doney et al. 2012), tendo como principais consequências diretas o aumento da temperatura superficial e a acidez dos oceanos (Doney et al. 2009, 2012). Neste contexto, os recifes de coral ganham atenção, tanto pela sensibilidade às mudanças climáticas já reportadas, quanto pelo fato de que um quarto das espécies marinhas esta associada a esses ecossistemas (Doney et al. 2012).

Os efeitos causados pelo aumento de CO_2 atmosférico não atuam isoladamente. O uso intensivo de fertilizantes, atividades turísticas desordenadas, desenvolvimento urbano e industrial desenfreado na costa, poluição química, entre outros, causam a degradação de habitats costeiros (van Dam et al. 2011; Goldberg, 2013), sendo por isso considerados pressões adicionais importantes em escala regional. Estima-se que em torno de 50% dos recifes no Brasil encontrem-se ameaçados pela ação combinada dos impactos locais e das mudanças climáticas (Wilkinson, 2008), o que evidencia, imperativamente, a necessidade de considerar integradamente os efeitos combinados e possivelmente sinérgicos desses múltiplos estressores nos ecossistemas marinhos (Doney 2010; Ban et a. 2014).

Branqueamento de corais

De forma geral, corais são organismos sensíveis diante de alterações ambientais, respondendo a estressores relacionados a mudanças na salinidade, sedimentação, temperatura, luminosidade, exposição a contaminantes e a poluentes, entre outros (Brown et al. 1997). Os sinais iniciais de estresse incluem o aumento na produção de muco e a retração tecidual por parte do coral hospedeiro (Goldberg, 2013). No entanto, quando os níveis de estresse são ampliados, a relação simbiótica coral-zooxantela pode entrar em colapso, ocorrendo o "branqueamento de corais", o qual é caracterizado pela expulsão das zooxantelas e/ou pela degradação dos seus pigmentos fotossintetizantes (Downs et al., 2000; Douglas, 2003). Este é um dos mais importantes e complexos processos que se manifestam a partir da fisiologia, bioquímica e genética dos componentes envolvidos nessa simbiose (Goldberg, 2013).

Dependendo da intensidade e duração do estresse, os corais podem se recuperar após o branqueamento, contudo podem morrer se a recolonização pelas algas não ocorrer rapidamente, tipicamente dentro do prazo de 2 a 4 semanas (Baker et al. 2008), dependendo dos requerimentos interespecíficos. No contexto do branqueamento, é importante salientar que mudanças na pigmentação de corais são normais e podem ocorrer independente da presença de estressores. Mudanças cíclicas na pigmentação de corais são referidas como "branqueamento fisiológico", e refletem ajustes na densidade de seus endossimbiontes, concentração de pigmentos fotossintestizantes, assim como diferenças da identidade genética dos endossimbiontes (Fitt et al. 2001; Aprill et al. 2007). Algumas dessas mudanças são dirigidas sazonalmente, com uma maior densidade de endossimbiontes nos períodos mais frios e menores densidades durante o verão (Fitt et al. 2001). No entanto, corais severamente branqueados sofrem redução de 70 a 90% na densidade de microalgas, manifestando a cor do esqueleto de $CaCO_3$ subjacente ao tecido, uma vez que este se torna translúcido na ausência do pigmento de seus endossimbiontes (Douglas, 2003). Ainda, eventos severos de branqueamento são tipicamente acompanhados por perdas significativas de biomassa pelo coral hospedeiro (Glynn, 1996; Fitt et al. 2001).

Apesar da relevância ecológica do fenômeno de branqueamento, os mecanismos de sinalização molecular e celular relacionados a este processo ainda não são totalmente compreendidos. Mais de duas décadas de pesquisa indicam oxidantes, incluindo espécies reativas de oxigênio (ERO) e de nitrogênio (ERN), como pivôs no processo de

branqueamento de corais (Lesser, 1997; Downs et al. 2002; Perez e Weis, 2006; Weis, 2008; Hawkins e Davy, 2013; Hawkins et al. 2013; Ross, 2014; Nielsen et al. 2018). Um dos mecanismos fisiológicos para explicar tal fenômeno propõe que este seja uma resposta final de defesa contra o estresse oxidativo (Downs et al, 2002), o qual é caracterizado por uma produção de ERO em um nível que excede a capacidade antioxidante do organismo (Lesser, 2006).

O estresse oxidativo afeta o equilíbrio da associação entre corais e zooxantelas quando um aumento na produção de ERO ocorre em decorrência da desestabilização da cadeia transportadora de elétrons nas membranas dos tilacóides dos endossimbiontes. O excesso de ERO produzido nas células dos endossimbiontes é difundido para as células do hospedeiro, aumentando os danos oxidativos neste último. Em determinado grau de dano oxidativo, os corais erradicam a fonte dominante de produção de ERO, expulsando assim as zooxantelas (Downs et al. 2002; Rotchell e Ostrander, 2011). Este mecanismo está contemplado na "Teoria oxidativa do branqueamento de corais" (originalmente, "Oxidative Theory of Coral Bleaching") (Downs et al., 2002).

Com relação às ERN, foi demonstrado que o oxido nítrico (ON) apresenta papel relevante em endossimbioses microbianas (Trapido-Rosenthal et al. 2001; Wang e Ruby, 2011), e que esta molécula participa do processo de branqueamento de cnidários (Perez e Weis, 2006; Weis, 2008). Por exemplo, a exposição ao aumento de temperatura induz a produção de altos níveis de ON na anêmona *Aiptasia pallida*, levando a dissociação da simbiose estabelecida pela mesma com suas zooxantelas endossimbiontes (Perez e Weis, 2006). Por sua vez, a ERN peroxinitrito ($ONOO^-$), que resulta da ligação entre o ON e ERO (especificamente o superóxido, O_2^-), é uma molécula altamente reativa que pode causar danos irreversíveis a biomoléculas, exercendo um papel crucial no processo de apoptose celular (Szabó et al. 2007). A exposição de anêmonas em simbiose com zooxantelas ao $ONOO^-$ ocasionou branqueamento (Hawkins e Davy, 2013), por isso essa molécula é sugerida como parte do processo de sinalização celular deste processo (Perez e Weis, 2006; Weis, 2008). No entanto, o papel do $ONOO^-$ no processo de branqueamento em cnidários ainda permanece obscuro (Hawkins e Davy, 2013).

Episódios de branqueamento podem provocar alterações na estrutura das comunidades recifais, uma vez que a capacidade fotossintética, crescimento e capacidade reprodutiva dos corais são afetados (Brown, 1997). A maioria dos corais

construtores de recifes apresenta baixa tolerância em relação ao aumento da temperatura superficial das águas, sendo que o aumento da temperatura da água em 1-2°C acima das médias máximas observadas durante meses de verão é um dos fatores mais bem conhecidos por ocasionar o branqueamento de corais (Goldberg, 2013). É importante salientar que o número de episódios de branqueamento aumentou drasticamente desde a década de 1980 (Baker et al., 2008), com previsões de eventos cada vez mais frequentes.

Um dos impactos gerados pelas mudanças climáticas é o aumento da temperatura superficial da água do mar, o qual tem sido intensificado por eventos climáticos, como o El Niño, a fase quente do fenômeno El Niño – Oscilação Sul (Claar et al. 2018). De fato, períodos de El Niño têm sido fortemente associados à ocorrência em larga escala de branqueamento de corais (Hughes et al. 2018). Por exemplo, o super El Ninõ de 1983 não apenas ocasionou o branqueamento em larga escala de corais em Galápagos e em outras áreas da região tropical do Pacífico oriental, como também causou a mortalidade desses organismos em ≈ 95%. Neste cenário, as taxas de bioerosão nesses recifes por organismos, como os ouriços-do-mar, causou o aumento no consumo das estruturas de carbonato de cálcio em relação à produção das mesmas, levando as estruturas recifais ao colapso (Glynn 1984; Glynn e Colgan, 1992).

Recentemente, o terceiro evento em larga escala de branqueamento de corais foi registrado, tendo sido este consequência das anomalias térmicas ocasionadas pelo El Niño 2015-2016. Neste evento, níveis sem precedentes relacionados ao estresse térmico e a ocorrência de branqueamento de corais foram registrados considerando o período entre 1871 a 2017 (Claar et al. 2018; Lough et al. 2018, Hughes et al. 2018).

Acidificação dos oceanos e seus potenciais efeitos sobre organismos calcificadores

Os oceanos representam o maior sumidouro de CO_2 emitido por atividades humanas, absorvendo aproximadamente um quarto do total das emissões desse gás (Canadell et al. 2007). O pH dos oceanos é uma função de sua alcalinidade, a qual é governada pelos níveis de carbonato, bicarbontao, entre outros componentes (Kleypas et al. 2006). Quando o CO_2 atmosférico é absorvido pela superfície dos oceanos, mudanças no sistema tampão dióxido de carbono/ ácido carbônico/ bicarbonato e no pH da água do mar ocorrem subsequentemente (Gattuso e Hansson, 2011). O problema ambiental denominado “acidificação dos oceanos” decorre do excesso de CO_2 absorvido

pelos oceanos devido as crescentes emissões desse gás na atmosfera (Doney et al. 2009). Especificamente, o CO_2 absorvido reage com uma molécula de água, formando ácido carbônico (H_2CO_3); grande parte desse ácido se dissocia, liberando prótons (H^+), bicarbonato (HCO_3^-) e carbonato (CO_3^{2-}), como demonstrado na equação abaixo:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow H^+ + CO_3^{2-}$$

Quanto maior a quantidade de CO_2 absorvido, maior é a concentração de H^+, o que acarreta a diminuição do pH das águas (Doney et al. 2009). Este sistema tampão resiste a alterações de pH por meio da reação dos íons de CO_3^{2-} já existentes com H^+ livres para formar HCO_3^- (Portner et al. 2014). No entanto, os íons CO_3^{2-} são essenciais aos organismos calcificadores para a produção de seus esqueletos de carbonato de cálcio ($CaCO_3$) (Tambutté et al. 2011). Em níveis típicos de pH da água do mar (pH = 8,0 ± 0,3), íons CO_3^{2-} se encontram em nível suficiente para que a precipitação de estruturas calcárias por organismos calcificadores ocorra facilmente. No entanto, com a diminuição do pH da água do mar, a concentração de CO_3^{2-} é reduzida, assim como o estado saturação (**Ω**) do carbonato (Goldberg, 2013), o qual é determinado pelo produto das concentrações de cálcio (Ca^{+2}) e carbonato (CO_3^{2-}), divididos pelo produto de solubilidade (K_{sp}) do $CaCO_3$ (que é uma função de temperatura, salinidade e pressão), como representado na equação a seguir:

$$\Omega\text{-carbonato} = [Ca^{+2}] \times [CO_3^{2-}] / K_{sp}CaCO_3$$

Valores iguais a 1 indicam 100% de saturação, enquanto valores menores ou maiores que 1 indicam subsaturação e supersaturação, respectivamente (Goldberg, 2013). As águas superficiais das regiões tropicais dos oceanos apresentam diferentes níveis de supersaturação de acordo com o tipo de mineral a ser formado. Organismos que secretam suas estruturas de $CaCO_3$ na forma de calcita com alto teor de magnésio são considerados os mais suscetíveis aos efeitos da acidificação doa oceanos, seguidos pelos organismos que precipitam aragonita e calcita pura (Kleypas et al. 2006; Doney et al. 2009).

No que se refere aos corais escleractíneos e hidrocorais, estes precipitam seus esqueletos na forma mineral aragonita, e modelos sugerem que um aumento de 15% nas concentrações de CO_2 em relação aos níveis atuais ocasionará limitações severas em relação à disponibilidade de carbonato para esses organismos em regiões tropicais (Cao e Caldeira, 2008). Ainda, o aumento em 20% de CO_2 em relação aos níveis atuais (480 ppm) é visto como crítico, onde os valores de **Ω** do carbonato combinados ao aumento

da temperatura em ≈2°C causariam um declínio rápido dos recifes de coral devido a diminuição das taxas de calcificação, aumento da bioerosão, competição entre corais e macroalagas, e branqueamento de corais (Hoegh-Gulberg et al. 2007; Diaz-Pullido et al. 2011).

1.3. Utilização de biomarcadores no monitoramento de recifes de coral

Marcadores biológicos ou biomarcadores podem ser definidos como alterações biológicas nos fluídos corporais, células ou tecidos, que podem ser detectadas desde o nível molecular até o sistêmico, e que são indicativas da exposição a estressores ambientais (McCarthy e Shugart, 1990; Bradley, 2012). Com base no entendimento dos processos em nível celular, o uso de biomarcadores bioquímicos permite prever efeitos biológicos ligados à degradação ambiental (Depledge et al., 1995; Downs et al. 2005). Portanto, suas respostas podem ser usadas preventivamente, auxiliando no desenvolvimento de estratégias de manejo e conservação (Bradley, 2012; Downs et al. 2005).

Regulamentações ambientais podem beneficiar-se da utilização de biomarcadores, podendo o gerenciamento de recursos ser mais eficaz se realizado de forma interdisciplinar e integrativa (Bradley, 2012). Neste contexto, a identificação de potenciais biomarcadores para a avaliação da saúde de corais é considerada uma ferramenta poderosa para fornecer informações sobre a condição de saúde dos recifes de coral (Downs et al. 2005). Segue que a utilização de biomarcadores celulares ecologicamente relevantes, aplicados conjuntamente aos métodos tradicionais de monitoramento de recifes de coral (*e.g.* levantamento visual de branqueamento de corais, avaliação da qualidade da água e monitoramento da temperatura superficial das águas) pode melhorar significativamente o gerenciamento de recursos dos mesmos.

A simbiose entre coral e zooxantelas e o processo de calcificação são características que podem constituir biomarcadores da saúde do ambiente recifal (Marques et al. 2016). A atividade de enzimas associadas ao processo de calcificação [Anidrase carbônica (AC) e Ca-ATPase] em corais e outros organismos calcificadores tem sido descrita como um biomarcador sensível à exposição a metais e variações ambientais (Bielmyer et al., 2010; Prazeres et al. 2012, Marangoni et al. 2017; Marques et al. 2017). Especificamente, a AC é uma metaloenzima que catalisa a reação de hidratação reversível do CO_2 em bicarbonato (Moya et al., 2008; Bertucci et al., 2013),

sendo essencial aos corais na aquisição de carbono inorgânico utilizado pelas zooxantelas na fotossíntese e pelo hospedeiro na calcificação (Bertucci et al., 2013). Já a Ca-ATPase participa do transporte de cálcio (Ca^{2+}) para o sítio de calcificação de corais, ao mesmo tempo em que remove prótons deste sítio, direcionando a reação de calcificação ($Ca^{2+} + CO_2 + H_2O \leftrightarrow CaCO_3 + 2H^+$) no sentido da formação de $CaCO_3$ (Al-Horani et al., 2003). Ainda, relaciona-se à manutenção do pH alcalino (>8,2) no sítio de calcificação, o que garante a eficiência do processo (Tambutté et al. 2011).

Parâmetros relacionados ao metabolismo fotossintético, como o potencial fotoquímico e a concentração de pigmentos fotossintetizantes, são frequentemente utilizados na avaliação da saúde e integridade da relação simbiótica entre corais e zooxantelas (e.g. Jones et al. 1999; Fitt et al. 2001; Silverstein et al. 2014; Ezzat et al. 2016). Por sua vez, a condição de estresse oxidativo desempenha papel fundamental no colapso da relação simbiótica entre cnidários e seus endossimbiontes (Downs et al. 2002; Lesser, 2006). Desta forma, o sistema de defesa antioxidante e os danos oxidativos têm sido propostos como indicadores de saúde do ambiente recifal (Downs et al. 2005, 2011, 2012; Prazeres et al. 2012). Ilustrativamente, a peroxidação lipídica e a capacidade de combate às ERO constituem bons instrumentos preditivos para avaliar a susceptibilidade ao branqueamento/mortalidade de organismos recifais que estabelecem simbiose com microalgas (Downs et al. 2005; Huang et al. 2005; Prazeres et al. 2011, 2012; Fonseca et al. al., 2017; Marangoni et al., 2017).

2. Hipóteses

Nesse contexto, em condições simuladas de impactos globais (acidificação marinha) e locais (contaminação por cobre), ou naturais de El Niño, e utilizando-se do coral *Mussimilia harttii* e/ou do hidrocoral *Millepora alcicornis* do Atlântico Sul, testaram-se: (i) a acidificação da água do mar ocasiona efeitos deletérios sobre o processo de calcificação de corais e hidrocorais do Atlântico Sul; (ii) os efeitos deletérios do cobre sobre a fisiologia de corais são intensificados em decorrência da diminuição do pH da água do mar; (iii) respostas bioquímicas relacionadas a capacidade antioxidante e a danos oxidativos constituem-se em ferramentas úteis no monitoramento de recifes coral do Atlântico Sul frente a anomalias térmicas; (iv) os níveis de espécies reativas de nitrogênio (peroxinitrito) dirigem a ocorrência de branqueamento; e (v) a correlação positiva entre a predominância trófica (heterotrofia) e o evento de branqueamento em corais e hidrocorais do Atlântico Sul.

3. Objetivos

Geral:

Avaliar os efeitos de estressores ambientais relacionados a impactos antrópicos e fenômenos climáticos sobre a fisiologia de seletas espécies de corais e hidrocorais representativas dos recifes do Atlântico Sul.

Específicos:

Apêndice I:

- Avaliar o efeito da acidificação da água do mar no processo de calcificação do hidrocoral *Millepora alcicornis;*

- Avaliar o efeito da acidificação da água do mar, bem como da exposição a concentrações ambientalmente relevantes de cobre, de forma isolada e combinada, na resposta de biomarcadores relacionados ao processo de calcificação e metabolismo fotossintético de endossimbiontes no coral *Mussismilia hartti*.

Apêndice II:

- Avaliar respostas bioquímicas relacionadas ao estresse oxidativo em *Mussismilia harttii* e *Millepora alcicornis* durante um evento de branqueamento influenciado pelo fenômeno climático El Niño, *in situ,* com intuito de acessar o potencial informativo dos biomarcadores em programas de monitoramento;

- Avaliar a produção de peroxinitrito e a variação na predominância trófica em *Mussismilia harttii* e *Millepora alcicornis* durante um evento de branqueamento, *in situ,* influenciado pelo fenômeno climático El Niño.

Apêndice III:

- Síntese do estado da arte da fisiologia de corais esclarectineos relacionada à simbiose coral-zooxantelas, ao fenômeno de branqueamento e ao processo de calcificação, tendo como foco a divulgação científica para discentes de graduação do Brasil.

4. Metodologia

4.1. Apêndice I

O sistema experimental

Os experimentos descritos no Anexo I dessa tese (artigo 1 e manuscrito 2) foram realizados no mesocosmo marinho do Projeto Coral Vivo (Arraial d'Ajuda, BA), o qual configura um sistema experimental aberto que troca água permanentemente com o mar, mantendo no meio experimental as variações diárias e sazonais de parâmetros ambientais como temperatura, pH, salinidade, concentração de nutrientes, fotoperíodo, entre outros (Duarte et al. 2015).

Resumidamente, esse sistema experimental capta a água do mar a 500 m da costa em uma franja recifal (Praia de Araçaípe, Arraial d'Ajuda, BA) e a conduz para cisternas subterrâneas, onde tratamentos de aumento da temperatura e redução de pH da água do mar podem ser aplicados, de forma isolada ou combinada. Um Sistema Arduíno de código fonte aberto (Creative Commons) auxilia na aplicação e manutenção dos tratamentos, que podem ser fixos ou contemplar variações temporais, em geral senoidais, das características da água captada. Dezesseis tanques de 130 L no formato "raceway", organizados em quatro réplicas para cada tratamento, recebem água das cisternas, com taxa de renovação de 4 vezes por hora (Duarte et al. 2015).

Em paralelo ao sistema descrito acima, uma estrutura com 48 aquários é destinada a ensaios ecotoxicológicos. Nesta área experimental, soluções estoque de contaminantes são diluídas na água bombeada das cisternas submetida aos tratamentos de temperatura e pH desejados. Após esta diluição, a água é bombeada para os aquários de teste, por meio de bombas peristálticas. A água dos aquários de teste (10 L) é renovada 3 vezes por hora, e o descarte da água de rejeito é feita por filtros de carvão ativado. Os aquários permitem testar 4 tratamentos por variável (temperatura ou pH) e 4 concentrações de contaminante, totalizando 16 combinações de tratamentos em triplicata (Duarte et al. 2015).

Neste estudo, foram conduzidos dois experimentos no Mesocosmo Marinho. No primeiro, o hidrocoral *Millepora alcicornis* (n=4 por tratamento) foi exposto a diferentes níveis de acidificação marinha por até 30 dias. No segundo experimento, realizou-se a exposição do coral *Mussismilia harttii* (n=3 por tratamento) a diferentes níveis de acidificação da água do mar e diferentes concentrações de cobre dissolvido no meio, isolada- e combinadamente, por até 35 dias. Os parâmetros físico-químicos da

água, a saber temperatura, pH, salinidade e incidência luminosa, foram monitorados diariamente.

Acidificação da água do mar

Reatores de gás carbônico (CO_2), dispostos em três das quatro cisternas (5.000 L) acidificaram a água bombeada do recife. A medida do pH da água do mar e o seu ajuste conforme os tratamentos experimentais desejados foi realizado continuamente com ao auxílio de pHmetros acoplados ao sistema controlador computadorizado. Os tratamentos adotados no experimento incluíram o cenário atual (valores de temperatura e pH encontrados no ambiente) e aqueles previstos pelo Painel Intergovernamental de Mudanças Climáticas (IPCC, 2014). Foram consideradas reduções de 0,1; 0,3 e 0,6 unidade de pH abaixo do valor registrado na água do mar no local do estudo, os quais representam os cenários mais otimista (RCP2.6) e pessimista (RCP8.5).

Exposição ao cobre

As soluções estoque de cobre foram preparadas diariamente em reservatórios (1.000 L), a partir de uma solução padrão de $CuCl_2$ (1 g/L Cu). Os reservatórios receberam água do mar bombeada do recife adjacente e as soluções foram preparadas 24 h antes do seu uso para permitir o equilíbrio e especiação do metal na água do mar. Diferentes volumes (10, 30 e 50 ml) da solução padrão de cobre foram adicionados nos reservatórios. Assim, após diluição com água bombeada das cisternas, esperava-se obter as concentrações nominais de 1, 3 e 5 µg/L Cu acima daquela encontrada naturalmente no local de captação de água.

Análise de cobre na água

Para cada tratamento, amostras de água dos aquários de teste foram coletadas a cada 4 dias, para análise e monitoramento da concentração de cobre dissolvido. Também foram coletadas amostras de água do Recife de Fora no momento da coleta dos corais, bem como da região próxima à captação de água do Mesocosmo Marinho. Amostras filtradas (filtro de 0,45 µm de malha) foram coletadas em tubos tipo Falcon (15 mL), acidificadas (HNO_3 1%) e utilizadas para determinação da concentração de cobre dissolvido. As concentrações de cobre foram determinadas por espectrofotometria

de absorção atômica com forno de grafite acoplado (Perkin-Elmer, EUA) em amostras previamente dessalinizadas (Nadella et al., 2008).

Medidas fisiológicas

O potencial fotoquímico máximo do fotossistema II (PSII) foi avaliado por medidas de fluorescência da clorofila *a* utilizando-se um fluorímetro de pulso de amplitude modulada - Diving-PAM (Walz, Alemanha). O nível de fluorescência mínima (F_0) foi obtido por uma sonda fraca de luz modulada, enquanto o nível máximo de fluorescência (F_m) foi detectado após um pulso de saturação de luz actínica. A fluorescência variável (F_v) foi calculada como F_m- F_0, e o potencial fotoquímico máximo do PSII foi obtido por meio da razão de F_v/F_m. Os valores de fluorescência (Yield) foram considerados como medidas do estado de saúde dos dinoflagelados endossimbiontes (Jones et al. 1999) .

A taxa de calcificação dos hidrocorais foi avaliada pelo método da anomalia de alcalinidade descrito por Chisholm e Gattuso (1991), que se baseia na proporção da redução da alcalinidade total da água em 2 moles, a cada 1 mol de $CaCO_3$ precipitado. Os resultados foram expressos em µmol $CaCO_3$. cm^{-2}. h^{-1}.

Análises bioquímicas

A quantificação de clorofila *a* foi realizada conforme o método descrito por Schmidt et al. (2011), com algumas modificações. Aproximadamente 1 cm^2 de coral foi sonicado (70 KHz, Sonaer/EUA) em água Milli-Q até o total desprendimento tecidual do esqueleto. O homogeneizado resultante foi utilizado no processo de extração de clororofila em etanol (95%) a 4°C, por 24 h, e protegidas da luz. Após a extração, as amostras foram centrifugadas (4°C, 5200 rpm, 5 min) e o sobrenadante utilizado para quantificação de clorofila *a*. Os resultados foram expressos em ng de clorofila *a*/g proteína.

A preparação das amostras para análise das enzimas associadas ao processo de calcificação foi realizada conforme descrito por Downs (2005), com algumas modificações. As amostras foram maceradas em nitrogênio liquido e alíquotas de 150-200 mg foram homogeneizadas (1:2; massa/volume) em tampão específico para cada ensaio com auxilio de um sonicador. Em seguida, as amostras homogeneizadas foram

centrifugadas (4°C, 13000 *g*, 10 min,) e a fase intermediária imediatamente coletada para as análises.

A atividade da Ca-ATPase foi determinada de acordo com Chan et al. (1986) para *Mu. harttii*, enquanto que para *Mi. alcicornis* seguiu-se o protocolo descrito por Vajreswari et al. (1983). Ambas as metodologias baseiam-se na quantificação colorimétrica de fosfato inorgânico liberado em meio de reação. Os resultados foram expressos em mM fosfato inorgânico/mg proteína/min. Já a atividade da anidrase carbônica (AC) foi determinada conforme o método descrito por Henry (1991), o qual baseia-se na medida da redução de pH pela catalise da hidratação do CO_2, com correspondente liberação de H^+. Os resultados foram expressos em unidades de AC/mg proteína. Em todos os casos, considerou-se o conteúdo de proteínas totais nos homogeneizados para a normalização dos dados utilizando-se um kit comercial de reagentes baseado no método de Bradford (Bradford Reagent, Sigma-Aldrich, EUA).

Análises estatísticas

No artigo publicado do Anexo I, a avaliação do efeito dos diferentes tratamentos de diminuição do pH da água do mar sobre as taxas de calcificação foi realizado por meio de uma Análise de Variância (ANOVA) de medidas repetidas, considerando os dados transformados pelo arcoseno da raiz quadrada. Por sua vez, os dados de atividade enzimática foram avaliados por uma ANOVA de duas vias (tempo de exposição e tratamento de pH). Ambos os casos foram seguidos pelo Teste de Comparações Múltiplas de Fisher, quando necessário. Os pressupostos de homocedasticidade e normalidade dos dados foram checados previamente, utilizando-se os testes Levene e Shapiro-Wilk, respectivamente. O nível de significância adotado foi de 95% ($\alpha = 0,05$).

No manuscrito submetido do Anexo I, os efeitos da redução do pH e da exposição ao cobre nos valores *F*v/*F*m, clorofila *a* e na atividades enzimáticas foram avaliados utilizando-se ANOVA de duas vias (tempos 15 e 35 dias de exposição), seguido pelo teste a posteriori de Tukey. Quando necessário, os dados foram logaritimizados para atender os pressupostos de homocedasticidade e normalidade, os quais foram também checados utilizando-se os testes de Levene e Shapiro-Wilk, respectivamente. Na ocasião de um efeito de interação detectado, a inibição aditiva esperada (*expected additive inhibition*) (Bliss, 1939; Schmidt et al., 2014; van Dam et al., 2015; Marques et al., 2017) foi calculada para a avaliação do tipo de interação:

quando o efeito observado foi igual ao efeito previsto; antagonista quando o efeito combinado observado foi menor que o esperado; e sinérgico quando o efeito observado foi maior que o previsto (Crain et al., 2008).

4.2. Apêndice II

Desenho amostral

Colônias das espécies *Mussismilia harttii* e *Millepora alcicornis* foram visualmente monitoradas por meio de mergulho autônomo em 3 diferentes pontos do Parque Municipal Natural Marinho do Recife de Fora (Porto Seguro, Bahia) (Figura 1, Manuscrito 1 – Anexo II). O monitoramento foi realizado quinzenalmente, durante o período de 15 de dezembro de 2015 a 15 de junho de 2016, totalizando 12 tempos de amostragem. Para isso, transectos de 20 m foram fixados aleatoriamente a aproximadamente 2 m de profundidade em cada um dos pontos, totalizando 36 transectos realizados. Para a detecção de branqueamento, as colônias foram fotografadas ao lado de "PVC Coral Health Chart" (CoralWatch, University of Queensland, Australia, Siebeck et al. 2006) e avaliadas conforme o protocolo "Coral Watch" seguindo algumas modificações (www.reefquest.org).

Paralelamente, foram coligidas amostras biológicas (N=4 por espécie) em cada um dos transectos realizados, para posteriores análises laboratoriais. A coleta das amostras bilógicas foi feita manualmente, com auxílio de um alicate inoxidável, por meio de mergulho autônomo. Os fragmentos das colônias coletados foram cuidadosamente transportados em água do mar coletada no local e protegidas do sol, para a Base de Pesquisas do Projeto Coral Vivo, onde foram imediatamente congeladas em nitrogênio líquido.

Os pontos selecionados para a realização do monitoramento baseou-se em previsões feitas pela Agência Nacional Oceânica e Atmosférica (sigla em inglês NOAA) relacionadas a ocorrência de branqueamento de corais na costa brasileira no período de verão 2015-2016 (https://coralreefwatch.noaa.gov/satellite/analyses_guidance/global_coral_bleaching_2014-17_status.php).

Monitoramento de estresse térmico

Loggers de temperature (HOBO, modelo UA-002-64), programados para registro de medição a cada 15 minutos, foram fixados em cada um dos pontos amostrados para registrar a temperatura durante o período de monitoramento. A estimava da ocorrência de estresse térmico para corais e hidrocorais foi realizada de acordo com o método desenvolvido pelo programa de monitoramento de recifes de coral da NOAA [NOAA Coral Reef Watch (CRW) program].

A condição de estresse térmico para corais e hidrocorais foi determinada de acordo com valores calculados como "Degree Heating Weeks -DHW" expressos em graus celsius (°C). Este índice é calculado a partir do acúmulo de valores de temperaturas anômalas que excedem temperaturas máximas mensais para determinada região durante um período de 12 semanas (Liu et al. 2003, 2006, 2013). Por exemplo, valores de DHW de 2°C correspondem a duas semanas com 1°C acima da media dentro do período de 12 semanas, ou uma semana com 2°C acima da média (Kayenna, 2017). Considera-se que valores de DHW acima de 4°C induzem o branqueamento de corais, e que valores acima de 8°C resultam na mortalidade dos mesmos (Kayenna, 2017).

Plasticidade Trófica

Ambas as auto- e heterotrofia fornecem lipídios essenciais aos corais (Grottoli et al. 2006), sendo os ácidos graxos frequentemente utilizados em estudos ecológicos como marcadores tróficos (Volkman et al. 1989; Dalsgaard et al. 2003) e como registro de hábitos alimentares (Ruess et al. 2005; Budge et al. 2006). Desta forma, para determinar o modo prevalente de alimentação de corais e hidrocorais utilizou-se o método descrito por Mies et al. (2018). Este se baseia na presença de ácido graxos específicos relacionados a cada modo de alimentação, sendo o ácido cis-gondoico (CGA, 20:1ω9) marcador específico de heterotrofia e os ácidos estearidônico (DAS, 18:4ω3) e docosapentaenoico (DPA, 22:5ω3) marcadores de autotrofia. Realizou-se a pesagem e extração de lipídeos dos fragmentos coloniais (n=12 por espécie e período amostral) seguindo os métodos descritos por Masood et al. (2005) e Mies et al. (2017), para posterior aplicação do Índice de Modo Trófico Predominante (Predominant Trophic Mode Index, PTMI) (Mies et al. 2018). Os resultados obtidos são adimensionais e indicam a prevalência de autotrofia no caso de valores positivos e de heterotrofia para valores negativos.

Análises bioquímicas

Para realizar os as analises de quantificação de peroxidação lipídica e capacidade antioxidante total, ≈0,5 cm^2 de cada espécie foram homogeneizados em tampão específico para cada análise, utilizando-se um sonicador (70 kHz, Sonaer/EUA). O pedaço de esqueleto remanescente foi descartado e o homogeneizado centrifugado a 4°C por 13.000 g durante 10 min. A fase intermediaria do homogeneizado, contendo o tecido do hospedeiro e dos endossimbiontes, foi utilizada para as análises.

A peroxidação lipídica foi determinada utilizando-se o método fluorimétrico descrito por Oakes e Van Der Kraak (2003), o qual baseia-se na medida das substâncias reativas ao ácido tiobarbitúrico (TBARS). Esta metodologia quantifica os danos peroxidativos em lipídios por meio da reação do malondialdeído (MDA), um dos produtos da peroxidação lipídica, com o ácido tiobarbitúrico (TBA). Os resultados foram normalizados considerando o conteúdo de proteínas nas amostras e expressos em nmol MDA/mg proteína.

Por sua vez, a capacidade antioxidante total dos organismos foi medida utilizando-se o kit comercial colorimétrico "OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit" (Cell Biolabs Inc., San Diego, CA, USA), o qual é baseado no método de redução do cobre (II) em cobre (I) por antioxidantes. Os resultados foram expressos em µM de equivalentes redutores de cobre (ERC) / mg de proteína.

A determinação da concentração de peroxinitrito ($ONOO^-$) nos tecidos de corais e hidrocorais foi feita utilizando-se o kit comercial fluorimétrico "Amplitude™ Fluorimetric Peroxynitrite Quantification Kit" (AAT Bioquest®, CA, USA), de acordo com as instruções do fabricante. Fragmentos de ≈0,3 cm foram homogeneizados em tampão fornecido pelo fabricante utilizando-se um sonicador (70 KHz, Sonaer/EUA). O pedaço de esqueleto remanescente foi descartado e o homogeneizado centrifugado a 4°C por 10 000 *g* durante 20 min. A fase intermediaria, contendo os tecidos do hospedeiro e de seus endossimbiontes, foi utilizada para a análise. Os resultados foram expressos em µM de peroxinitrito / mg de proteína.

Assim como descrito anteriormente, considerou-se o conteúdo de proteínas totais nos homogeneizados para a normalização dos dados utilizando-se um kit comercial de reagentes baseado no método de Bradford (Bradford Reagent, Sigma-Aldrich, EUA).

Análises estatísticas

No Manuscrito 1 – Anexo II, as taxas de branqueamento foram expressas em porcentagem, considerando todos os pontos amostrados e agrupados mensalmente. Os dados dos biomarcadores ‘capacidade antioxidante total’ e ‘peroxidação lipídica’ foram agrupados da mesma forma e avaliados utilizando-se analise de variância (ANOVA) de uma via. Quando indicado, a ANOVA foi seguida por Tukey. A homocedasticidade e normalidade dos dados foram checadas previamente utilizando-se os testes de Levene e Shapiro-Wilk, respectivamente. Quando necessário, os dados foram transformados em escala logarítmica para atender aos pressupostos da ANOVA.

Uma análise de componentes principais (PCA) foi aplicada para se reduzir a dimensionalidade dos dados, integrar todas as variáveis e detectar um padrão de correlação entre essas, considerando-se os parâmetros de estresse térmico (DHW e temperaturas máximas) e biológicos (capacidade antioxidante total, peroxidação lipídica e branqueamento). Utilizou-se a função prcomp no pacote *vegan* (Oksanen et al., 2013) no ambiente R (R Core Team 2017), e o pacote *factoextra* (Kassambara e Mundt, 2016) para visualizar graficamente os dados por meio da função fviz_pca_biplot.

No Manuscrito 2 – Anexo II, os padrões de branqueamento e produção de peroxinitrito foram ajustados ao modelo “Distance Weighted Least Squares Model” utilizandos-se o software Statistica 7.0. Uma análise de componentes principais (PCA) foi também realizada utilizando-se os parâmetros branqueamento, plasticidade trófica, temperaturas máximas, DHW e peroxinitrito no ambiente R (R Core Team 2017). O pacote *factoextra* (Kassambara e Mundt, 2016) também foi utilizado para visualizar graficamente os dados por meio da função fviz_pca_biplot.

Devido o aparente padrão oposto entre branqueamento e peroxinitrito evidenciado pela PCA, aplicou-se uma Análise de Correlação Cruzada para Séries Temporais para testar a existência de um atraso na possível associação entre tais parâmetros. Uma vez que os dados em séries temporais podem estar autocorrelacionados, os graus de liberdade são superestimados em análises de correlação tradicionais, o que infla a probabilidade de se cometer o erro Tipo I (Fuller, 2009; Pyper e Peterman, 1998). Portanto, optou-se na presente análise por corrigir os graus de liberdade baseado na soma dos produtos cruzados das autocorrelações das variáveis supracitadas sobre diferentes atrasos (Chelton, 1984; Pyper e Peterman 1998), ao invés de se remover a autocorrelação dos dados. Nos Resultados, o termo Padj

refere-se ao P-valor calculado com o número correto de graus de liberdade, adotando-se $\alpha = 0,05$.

5. Síntese dos resultados

5.1 Anexo I

Efeitos da acidificação marinha no processo de calcificação do hidrocoral Millepora alcicornis

Utilizando-se um mesocosmo marinho do Projeto Coral Vivo, o hidrocoral *Mi. alcicornis* foi exposto a diferentes cenários de acidificação marinha [pH 8,1 (controle), 7,8, 7,5 e 7,2]. Após 16 e 30 dias de exposição, foram realizadas medidas fisiológicas (taxas de calcificação) e bioquímicas [atividade da Ca-ATPase e anidrase carbônica (AC)] relacionadas ao processo de calcificação desses organismos.

Hidrocorais expostos a pH 7,5 por 16 dias, e a pH 7,2 por 16 e 30 dias, apresentaram diminuição nas taxas de calcificação em relação aos organismos mantidos em condição controle (Figura 1, Artigo – Anexo I). A atividade da Ca-ATPase não foi alterada em hidrocorais expostos ao pH 7,8; no entanto observou-se um aumento significativo da atividade dessa enzima ao longo dos 30 dias de exposição nos hidrocorais mantidos em pH 7,5 e 7,2. Ainda, a atividade da Ca-ATPase demonstrou-se significativamente maior em hidrocorais expostos ao pH 7,5 e 7,2 após 30 dias, em relação aos mantidos na condição controle (pH 8,1) e em pH 7,8 (Figura 2, Artigo - Anexo I).

Com relação à AC, a atividade também não foi alterada em pH 7,8, não obstante observou-se alterações na atividade da mesma ao longo do tempo em hidrocorais expostos ao pH 7,5 e 7,2. Especificamente, a atividade da AC demonstrou-se menor em pH 7,5 após 16 dias, retornando a valores controle após 30 dias. Por sua vez, a atividade dessa enzima em hidrocorais expostos a pH 7,2 também foi reduzida após 16 dias, porém aumentou após 30 dias (Figura 3, Artigo – Anexo I).

Os resultados encontrados fornecem as primeiras evidências bioquímicas relacionadas ao processo de calcificação em uma espécie de hidrocoral frente a cenários de acidificação marinha. De forma geral, o processo de calcificação em *Mi. alcicornis* não foi afetado em uma ampla faixa de redução de pH da água do mar. Ainda, os resultados indicam que a Ca-ATPase desempenha um papel chave na manutenção das taxas de calcificação de *Mi. alcicornis* frente a cenários de acidificação da água do mar. Por fim, frente a um cenário severo de acidificação marinha (pH 7,2), *Mi. alcicornis* apresentou sua taxa de calcificação diminuída.

Efeitos combinados da acidificação marinha e da exposição ao cobre no coral escleractínio Mussismilia harttii

No mesocosmo marinho do Projeto Coral Vivo, o coral *Mu. harttii* foi exposto por até 35 dias a diferentes cenários de acidificação da água do mar [pH 8,1 (controle), 7,8, 7,5 e 7,2] e a diferentes concentrações ambientalmente relevantes de cobre (Cu) dissolvido [concentrações medidas: 1,0 (concentração natural da água), 1,6, 2,3 e 3,2 µg/L] de forma isolada e em combinação, totalizando 16 tratamentos. Após 15 e 35 dias de exposição, foi realizada a avaliação de respostas bioquímicas relacionadas ao metabolismo fotossintético dos dinoflagelados endossimbiontes [quantidade de clorofila *a* (Clor *a*) e capacidade fotoquímica máxima (*F*v*F*m)] e ao processo de calcificação (atividade da Ca-ATPase e AC) em *Mu. harttii.*

De forma geral, a combinação desses estressores não intensificou os efeitos deletérios no metabolismo fotossintético de endossimbiontes (Figuras 1AB e 2AB, Manuscrito – Anexo I). Com relação a Ca-ATPase, esta também não indicou aumento de efeitos deletérios em decorrência da interação de estressores. Interessantemente, observou-se um efeito positivo sobre a atividade dessa enzima com a adição de Cu na água do mar (1,6 µg/L) em todos os tratamentos de acidificação marinha testados (Figura 3AB, Manuscrito – Anexo I). Por sua vez, potenciais efeitos deletérios foram observados em relação ao equilíbrio ácido-base em *Mu. harttii* devido interações aditivas e sinérgicas entre estressores (Tabela 3, Manuscrito - Anexo I) que resultaram na redução da atividade da AC (Figura 4AB, Manuscrito – Anexo I).

Em suma, os resultados evidenciam que 76% das interações entre a acidificação da água do mar e o aumento na concentração de Cu dissolvido na água do mar são antagonistas, e apenas 24% destas causaram efeitos aditivos ou sinérgicos possivelmente deletérios ao estado fisiológico de *Mu. harttii*.

5.2 Anexo II

Biomarcadores de estresse oxidativo como potenciais ferramentas no monitoramento de recifes de coral: um estudo de caso em um recife do Atlântico Sul sob influência do El Niño – Oscilação Sul (2015-2016)

Durante o período de monitoramento realizado de Dezembro de 2015 a Junho de 2016, foram identificados períodos de estresse térmico para corais e hidrocorais (indicados como DHW com valores iguais ou superiores a 4°C) para os meses de Abril

a Maio (Figura 2, Manuscrito 1 – Anexo II). Em concordância, *Mi. alcicornis* apresentou maiores taxas de branqueamento (até 73%) de Março a Maio (Figura 3, Manuscrito 1 – Anexo II), enquanto *Mu. harttii* apresentou taxas de branqueamento de até 34% entre Abril e Maio (Figura 4, Manuscrito 1 – Anexo II).

A capacidade antioxidante total apresentou variações significativas para as duas espécies durante o período de monitoramento (Tabela 1, Manuscrito 1 – Anexo II). Um aumento significativo foi observado no mês de março para *Mi. alcicornis* (Figura 3C, Manuscrito 1 – Anexo II), enquanto neste mesmo mês um decaimento foi observado para *Mu. harttii* (Figura 4C, Manuscrito 1 – Anexo II). Considerando os valores nos meses em que se observou baixas (até 10%) ou nenhuma taxa de branqueamento, os valores basais de capacidade antioxidante total para *Mi. alcicornis* e *Mu. harttii* foram de 208,2 e 743,9 µM CRE / mg proteína, respectivamente.

Variações nos níveis da peroxidação lipídica foram observadas ao longo do período de 6 meses apenas para *Mi. alcicornis* (Tabela 1, Manuscrito 1 – Anexo II), com aumento nos níveis de Fevereiro a Maio. Em Junho, quando os hidrocorais se encontraram visualmente recuperados, o nível de peroxidação lipídica voltou a seu valor basal (≈7,1 nmol MDA / mg proteina), considerando os valores dos meses em que menos de 10% de branqueamento foi observado (Figura 3B, Manuscrito 1 – Anexo II). Com relação *Mu. harttii*, nenhuma alteração significativa nos níveis de peroxinitrito foi observada durante o período de monitoramento (Tabela 1; Figura 4B; Manuscrito 1 – Anexo II), e os níveis basais encontrados foram de 358,4 nmol MDA / mg proteina.

Com relação a análise multivariada empregada (PCA) para todos os dados acima descritos, confirmou-se a forte associação entre o branqueamento e os biomarcadores relacionados ao estresse oxidativo (peroxidação lipídica e capacidade antioxidante total), e também reforçou a associação entre as respostas biológicas e os parâmetros de estresse térmico (DHW e temperatura máxima) (Figuras 5 e 6, Manuscrito 1 – Anexo II).

De forma geral, os resultados reforçam o potencial de medidas relacionadas ao estresse oxidativo como potenciais biomarcadores prognósticos em organismos recifais estabelecendo simbiose com zooxantelas. Especificamente, a avaliação da peroxidação lipidica em *Mi. alcicornis*, espécie identificada como um potencial bioindicador no presente estudo, sugere esta medida como uma potencial ferramenta complementar em programas de monitoramento em recifes do Atlântico Sul. Por sua vez, os diferentes

níveis basais de capacidade antioxidante total entre ambas as espécies e suas respectivas taxas de branqueamento, sugerem um potencial preditivo desta medida em relação a susceptibilidade de diferentes espécies ao branqueamento.

Produção de peroxinitrito e plasticidade trófica em corais construtores de recifes durante um evento natural de branqueamento sob influência do El Niño – Oscilação Sul (2015-2016)

O agrupamento dos parâmetros [branqueamento, temperatura máxima (MaxTem), *degree heating weeks* (DHW), peroxinitrito, auto- e heterotrofia] em uma análise multivariada (PCA) para *Mu. harttii e Mi. alcicornis*, mostrou que os dois principais componentes (Dim1 e Dim2) explicaram, respectivamente, 58,7% (Dim1 = 40,7%; Dim2 = 18,0%) e 68,8% (Dim1 = 40,1%; Dim2 = 28,7%) da variância total (Figura 5, Manuscrito 2 – Anexo II). Branqueamento, heterotrofia e DHW são positivamente correlacionados com Dim 1 (0,60<R<0,90) para a duas espécies; MaxTemp esta associada apenas para *Mi. alcicornis*, com o componente 1 (R=0,75).

Contudo, o peroxinitrito demonstrou-se associado à autotrofia em *Mu. harttii* no componente 1 (-0,50<R<-0,54), e correlacionado com a heterotrofia em *Mi. alcicornis* no componente 2 (-0,59<R<-0,61). Ao se considerar o branqueamento como um fator discreto (‘antes’, ‘durante’ e ‘depois’ de sua ocorrência), a autotrofia e o peroxinitrito se agrupam antes do evento de branqueamento para *Mu. harttii*. Por sua vez, a autotrofia e o peroxinitrito em *Mi. alcicornis* aparecem antes e durante a ocorrência de branqueamento, respectivamente. A heterotrofia se apresenta durante o processo de branqueamento para as duas espécies (Figure 5, Manuscrito 2 – Anexo II).

O peroxinitrito e o branqueamento apresentaram um padrão oposto, especialmente no caso de *Mu. harttii* (Figure 5, Manuscrito II – Anexo II), embora uma visível associação entre esses dois parametros durante o período monitorado foi detectado em ambas as espécies (Figure 6, Manuscrito 2 – Anexo II). Neste caso, é possível observar um pico de geração de peroxinitrito em *Mi. alcicornis* antes das taxas máximas de branqueamento. Em contrapartida, dois picos de produção de peroxinitrito são observados para *Mu. harttii* precedendo a ocorrência máxima de branqueamento. As correlações, de maneira espécie-/local-específicas, demonstraram um atraso de 15 dias no pico de peroxinitrito em relação aos picos de branqueamento, sugerindo que a geração dessa ERN deflagra o branqueamento 15 dias após da sua produção máxima.

6. Conclusões

(i) *Millepora alcicornis* demonstrou tolerância a diferentes cenários de acidificação marinha, tendo a Ca-ATPase papel chave na manutenção das taxas de calcificação desse hidrocoral; efeitos deletérios sobre a fisiologia dessa espécie foram observados somente no cenário de acidificação mais severo, o que corrobora parcialmente a hipótese 1 dessa tese;

(ii) a predominância de efeitos antagonistas sobre a fisiologia do coral *Mussismilia harttii*, resultantes da interação entre a acidificação da água do mar e exposição ao cobre, sugere que a intensificação dos efeitos deletérios do cobre pela acidificação marinha em corais pode ser menor que o previsto, não corroborando a hipótese 2 dessa tese;

(iii) o aumento da peroxidação lipídica precedente aos episódios de branqueamento, e as diferenças basais na capacidade antioxidante total entre espécies indicam essas respostas bioquímicas como potenciais biomarcadores a serem aplicados no monitoramento de recifes de coral, corroborando a hipótese 3 dessa tese;

(iv) o aumento na produção dos níveis de peroxinitrito antecedendo a ocorrência de branqueamento em corais e hidrocorais sugere que essa molécula constitui um importante mediador do processo de branqueamento, confirmando a hipótese 4 dessa tese. Em contrapartida, a detecção de variações na predominância entre auto- e heterotrofia nas duas espécies monitoradas durante evento de branqueamento, e a observação da relação positiva entre branqueamento e heterotrofia, confirmam a hipótese 5 dessa tese.

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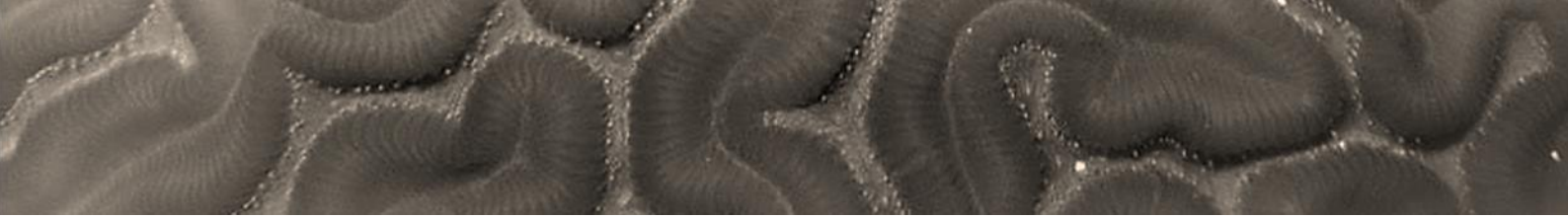

APÊNDICE I

- ***Artigo***

[Publicado no Periódico *Coral Reefs* em Junho de 2017 (36, 1133-1141; DOI 10.1007/s00338-017-1605-6)]

Effects of CO2-driven acidification of seawater on the calcification process in the calcareous hydrozoan *Millepora alcicornis* (Linnaeus, 1758)

- ***Artigo***

[Publicado no Periódico *Aquatic Toxicology* em Novembro de 2018 (206, 123-133; DOI 10.1016/j.aquatox.2018.11.005]

Copper exposure and seawater acidification interaction: Antagonistic effects on biomarkers in the zooxanthellate scleractinian coral *Mussismilia harttii*

Coral Reefs (2017) 36:1133–1141
DOI 10.1007/s00338-017-1605-6

REPORT

Effects of CO_2-driven acidification of seawater on the calcification process in the calcareous hydrozoan *Millepora alcicornis* (Linnaeus, 1758)

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Received: 8 July 2016 / Accepted: 19 June 2017 / Published online: 22 June 2017

Abstract Ocean acidification is expected to intensify due to increasing levels in the partial pressure of atmospheric CO_2 (pCO_2). This could negatively affect major calcifying reef organisms. In this study, the effects of different levels of CO_2-driven acidification of seawater (control: pH 8.1; moderate: pH 7.8; intermediate: pH 7.5; and severe: pH 7.2) on the net calcification rate and activity of enzymes related to the calcification process (Ca-ATPase and carbonic anhydrase) were evaluated in the calcareous hydrozoan *Millepora alcicornis*. The experiment was run for 30 d using a marine mesocosm system. Net calcification ratio was significantly reduced in hydrocorals exposed to intermediate seawater acidification for 16 d and to severe seawater acidification for 16 d or 30 d, compared to animals at control conditions. However, only hydrocorals exposed to severe seawater acidification showed lower net calcification rates than those exposed to control conditions for 30 d. In accordance, the activities of enzymes involved in the calcification process markedly increased in hydrocorals exposed to reduced pH. Ca-ATPase seemed to be more sensitive to seawater acidification than carbonic anhydrase as it increased in hydrocorals exposed to intermediate and severe seawater acidification for 30 d, while carbonic anhydrase activity was only stimulated under severe seawater acidification. Therefore, our findings clearly show that the hydrocoral *M. alcicornis* is able to cope, to some extent, with long-term CO_2-driven acidification of seawater (pH $\geq$ 7.5). In addition, they show that Ca-ATPase plays a key role in the maintenance of calcification rate under scenarios of moderate and intermediate levels of seawater acidification. However, the observed increase in Ca-ATPase and carbonic anhydrase activity was not enough to compensate for the effects of CO_2-driven reduction in seawater pH on the net calcification rate of the hydrocoral *M. alcicornis* under a scenario of severe ocean acidification (pH 7.2).

Keywords Biochemical biomarkers · Calcification · Hydrocoral · Ocean acidification

Communicated by Biology Editor Dr. Anastazia Banaszak

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Introduction

Ocean acidification, defined as a reduction in the pH of the world's oceans, is expected to intensify due to increasing levels in the partial pressure of atmospheric CO_2 (pCO_2) (Fabricius 2008; Stillman and Paganini 2015). According to estimations by the Intergovernmental Panel on Climate Change (IPCC 2014), a decrease of 0.3–0.4 pH units is expected by 2100. If CO_2 emissions continue, ocean acidification will also continue for centuries and will strongly affect marine ecosystems. According to Caldeira and Wickett (2003), atmospheric pCO_2 levels may exceed 1900 ppm in 2300. This would correspond to a reduction in seawater pH of approximately 0.8 units (Caldeira and Wickett 2003).

Oceanic carbonate chemistry is affected by rising pCO_2 levels. Additionally, a variety of taxa are affected by ocean acidification (Kroeker et al. 2013; Stillman and Paganini 2015). Besides the reduction in seawater pH, a rise in pCO_2 levels involves a decrease in the saturation state of calcium carbonate (Ω_{Cc}). Therefore, major marine calcifiers (e.g., corals, hydrocorals, calcifying algae) are expected to face reduced calcification and increased dissolution rates of their skeletons associated with reductions in Ω_{Cc} (Doney et al. 2009). This would imply a serious threat to reef ecosystems. Additionally, there are little data on calcification mechanisms in response to ocean acidification (Doney et al. 2009; Kroeker et al. 2013).

Calcareous hydrozoans of the genus *Millepora* are found in tropical seas worldwide, playing a key ecological role in reef communities (Lewis 1989). Despite their importance and numerous similarities with zooxanthellate scleractinian corals (Lewis 1989), less attention has been paid to the effects of ocean acidification on milleporids compared to scleractinian corals. Additionally, little is known about the biochemical process underling the response of important reef builders, including *Millepora*, to acidified waters.

Ca-ATPase is an enzyme hypothesized to have a primary role in the process of biomineralization. In corals, Ca-ATPase transports Ca^{2+} into the calcification site which removes protons (H^+) from this medium, driving the calcification reaction toward the formation of $CaCO_3$ (Allemand et al. 2011). In turn, carbonic anhydrase (CA) is a metalloenzyme that catalyzes the reversible hydration of CO_2 to bicarbonate (Moya et al. 2008; Bertucci et al. 2013), which is essential for the acquisition of inorganic carbon by the holobiont (i.e., coral and symbiotic algae). Inorganic carbon is used by symbiotic algae in photosynthesis, as well as by the host in the calcification process (Bertucci et al. 2013). Indeed, CA inhibitors have been reported to reduce coral calcification rates (Bertucci et al. 2013). It is thus expected that environmental stressors, such as ocean acidification, may affect the expression of the gene encoding these enzymes and/or the enzymatic activity itself.

The main goal of this study was to determine responses in the calcification process of *Millepora alcicornis* to future scenarios of CO_2-driven acidification of seawater forecasted within the next century and beyond. Specifically, parameters analyzed were net calcification rate and activity of enzymes (Ca-ATPase and CA) involved in the calcification process.

Materials and methods

Sample collection and acclimation

Sixteen branches (approximately 6 cm long) of four *M. alcicornis* colonies (four apical branches of each colony) were collected by scuba diving in the permanent conservation area of the Recife de Fora Natural Municipal Park (Porto Seguro, Bahia, southeastern Brazil; 16°24′20″S, 038°58′58″W). The collected colonies were at least 200 m apart. After collection, branches were transferred to the laboratory of the Coral Vivo Project (Arraial d'Ajuda, Porto Seguro, Brazil), fixed on ceramic plates with cyanoacrylate glue and acclimated in a marine mesocosm system for 20 d.

Experimental setup

The experiment was performed in a large-scale mesocosm designed for reef environment studies built at the Coral Vivo Project facilities between 2011 and 2012. Due to its direct connection and permeability to the reef environment, this system maintained experimental conditions (seawater composition, temperature, pH, turbidity, salinity, plankton density, photoperiod, rainfall, light incidence, among others) similar to those in the natural environment. Treatments were compared to ambient conditions (control) with natural diel fluctuations. A detailed description of the performance of the marine mesocosm system was reported by Duarte et al. (2015).

Different levels of seawater pH were selected based on predictions generated by modeling atmospheric CO_2 emissions (Caldeira and Wickett 2003, 2005; IPCC 2013, 2014). Scenarios simulated three levels of seawater acidification. Therefore, four pH treatments (four replicates per treatment) were tested for up to 30 d. Reductions of 0.3, 0.6 and 0.9 pH units from pH 8.1 ± 0.1 (mean pH $\pm$ SD of ambient seawater at the study site during the experiment) were tested. The experimental setup consisted of 16 independent 310-L tanks continuously supplied with

seawater at a pumping rate adjusted to ~8.3 L min^{-1}. One hydrocoral branch was randomly allocated per tank. Seawater from an adjacent fringing reef (Araçaípe Beach, Arraial d'Ajuda, Porto Seguro, Brazil) was pumped to four 5000-L underground sumps. In each sump, the seawater received one of the four pH treatments. pCO_2 in seawater was manipulated using a CO_2 reactor system immersed in the sumps. CO_2 was bubbled through the natural seawater, thus lowering the pH level. The natural seawater and the CO_2-enriched seawater from each of the sumps supplied four 310-L tanks each. The acidification process was controlled by a computerized system (Reef Angel controller) coupled to pH electrodes (Gehaka 09RBCN) connected to the sumps. Once the treatment pH was attained, the supply of CO_2 was stopped. The mesocosm tanks received natural sunlight and were covered with 70% shade screen to mimic the amount of incident sunlight measured in situ at 2.5 m depth at the hydrocoral collection site (Recife de Fora). The experiment was run in parallel with that performed by Sarmento et al. (2015), who exposed the phytal meiofauna community from the Recife de Fora to ocean acidification. Therefore, hydrocorals from this study and reef phytal meiofauna community analyzed by Sarmento et al. (2015) were kept together in the experimental tanks.

Seawater physicochemical parameters (pH, temperature, salinity, nutrient concentration and light intensity) were monitored throughout the experiment. pH and temperature of the seawater in each tank were monitored every 15 min by the Reef Angel Controller. Temperature data from the pH 7.2 treatment are not presented because the temperature sensor malfunctioned. Salinity (Instrutemp ITREF 10 optical refractometer), nutrients (Hach DR 890 colorimeter with the reagents Nitra Ver X and PhosVer 3 for nitrate and phosphate, respectively) and light intensity (LI-COR, LI 250A Light Meter, LI-193 Underwater Spherical Quantum Sensor) data were measured weekly. As reported by Sarmento et al. (2015), levels of pCO_2 and aragonite saturation state (Ω_{ar}) values were calculated for each treatment based on the total alkalinity (TA) and mean pH data using the software CO2calc version 1.2.9. TA of the seawater supplying the mesocosm system was analyzed following Dickson et al. (2007) using an alkalinity titrator (AS-ALK2, Apollo SciTech Inc., Bogart, GA, USA) and certified reference material (ID: 132, Scripps Institute of Oceanography, La Jolla, CA, USA). The pCO_2 values for each of the treatments were 351.8 (pH 8.1), 939.0 (pH 7.8), 1683.4 (pH 7.5) and 3494.3 µatm (pH 7.2); the Ω_{ar} values corresponded to 3.89, 1.96, 1.23 and 0.64 (Sarmento et al. 2015). Experimental conditions observed in the tanks during the 15- and 30-d exposure periods are summarized in Tables 1 and 2.

Determination of net calcification rates

Net calcification rates were measured ($n = 4$ per treatment) at the beginning of the experiment (day 0, T0) and after 16 and 30 d of exposure using the alkalinity anomaly technique (Chisholm and Gattuso 1991; Yao and Byrne 1998). *Millepora alcicornis* branches were incubated in 200-mL acrylic chambers for 3 h (25 °C, ambient pH 8.1) with filtered seawater (0.45-µm mesh filter). After incubation, seawater was collected, re-filtered (0.20-µm mesh filter) and stored at 4 °C. TA measurements were taken within 24 h using a modified spectrophotometric method (Yao and Byrne 1998). Seawater samples were carefully bubbled with N_2 for at least 4 min. Changes in the colorimetric indicator bromocresol green (Sigma-Aldrich, St. Louis, MO, USA) along with microtitration using 0.1 N HCl solution were measured at 444, 616 and 750 nm using an USB4000-UV–VIS spectrophotometer provided with a PX-2 xenon light source (Ocean Optics, Dunedin, FL, USA). Each seawater sample (30 g) was titrated in a 2.5-cm glass cuvette at a rate of 2.5 HCl mL^{-1} h^{-1} using a 1-mL glass syringe (KD780871) fitted to a syringe pump (KD-100) (KD Scientific, Holliston, MA, USA). The volume of HCl solution required to protonate half of the colorimetric indicator was determined based on the titration time. A certified reference material (ID: 132, Scripps Institute of Oceanography, La Jolla, CA, USA) was used to calibrate the method and to check for quality control of TA measurements. Due to the high morphological complexity of hydrocoral branches, net calcification rates were not related to surface area of branches. They were calculated based on measurements taken at the beginning of the

Table 1 Seawater pH maintained in the experimental tanks at pH 8.1 (control) and after seawater acidification (pH 7.8, 7.5 and 7.2) over 15- and 30-d exposure periods

pH treatment	15-d exposure period				30-d exposure period			
	8.1	7.8	7.5	7.2	8.1	7.8	7.5	7.2
Minimum value	7.71	6.60	6.74	6.39	7.71	6.60	6.22	6.15
Maximum value	8.29	8.41	8.36	8.35	8.34	8.41	8.36	8.35
Median	8.07	7.74	7.47	7.17	8.11	7.77	7.51	7.20
Mean	8.05	7.70	7.49	7.20	8.10	7.74	7.51	7.20
Standard deviation	0.104	0.241	0.184	0.258	0.096	0.185	0.160	0.190
Standard error	0.003	0.006	0.005	0.007	0.002	0.003	0.003	0.004

Table 2 Seawater physicochemical conditions maintained in the experimental tanks at pH 8.1 (control) and after seawater acidification (pH 7.8, 7.5 and 7.2) over 15- and 30-d exposure periods. Values are mean ± standard error

pH treatment	15-d exposure period				30-d exposure period			
	8.1	7.8	7.5	7.2	8.1	7.8	7.5	7.2
Temperature (°C)	24.7 ± 0.2	24.5 ± 0.1	24.6 ± 0.1	–	25.2 ± 0.1	25.0 ± 0.1	25.2 ± 0.1	–
Salinity	35 ± 0.1	35 ± 0.1	35 ± 0.1	35 ± 0.1	35 ± 0.2	35 ± 0.2	35 ± 0.2	35 ± 0.2
Nitrate (mg L^{-1})	0.80 ± 0.00	0.90 ± 0.00	0.90 ± 0.05	0.80 ± 0.00	0.98 ± 0.10	0.98 ± 0.08	0.88 ± 0.03	0.85 ± 0.03
Phosphate (mg L^{-1})	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Light (μmol photons m^{-2} s^{-1})	182 ± 43.7	182 ± 68.5	195 ± 67.9	186 ± 60.5	285 ± 67.6	296 ± 75.4	285 ± 55.0	277 ± 70.9

experiment (T0) and after 16 and 30 d of exposure to the experimental treatments. Data were then expressed as percentage of the initial (T0) value. The T0 calcification rate was determined for each of the incubated branches and used to calculate the percentage increase or decrease in net calcification rates throughout the experiment (16 and 30 d). In each treatment, the same coral branches were used to determine the net calcification rates over the experimental period.

Analysis of Ca-ATPase and carbonic anhydrase activities

For enzyme activity analyses, branches ($n = 4$ per treatment) were collected at the beginning of the experiment (mesocosm-acclimated corals) and after 16 and 30 d of exposure to the experimental treatments. Samples were immediately frozen in liquid nitrogen for further analyses. Sample preparation was performed as previously described (Santos et al. 2015). Briefly, samples were ground in liquid nitrogen, and aliquots (200 mg) were sonicated (Sonaer Ultrasonics, Farmingdale, NY, USA) on ice using the specific homogenization buffer (1:2 w/v) required for analysis of each enzyme, as described below. Homogenized samples were centrifuged (13,000 × g) at 4 °C for 10 min. The intermediary phase was collected and immediately used for enzyme analyses. Data were normalized considering the amount of protein in sample homogenate, which was determined using a commercial reagent kit (Sigma-Aldrich, St. Louis, MO, USA) based on the Coomassie brilliant blue assay (Bradford 1976).

Ca-ATPase activity was measured according to the protocol modified by Vajreswari et al. (1983). Sample homogenates were prepared using a buffer solution containing 500 mM sucrose, 1 mM 1,4-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 100 mM Trizma hydrochloride (pH 7.6). In turn, the working buffer solution contained 189 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 20 mM Trizma hydrochloride (pH 7.6). Samples were incubated at 30 °C for 30 min. Inorganic phosphate (Pi) released by the enzyme activity was measured using a commercial reagent kit (Fosfato, Doles Reagentes, Retiro, Goiás, Brazil) based on the colorimetric method described by Fiske and Subbarow (1925). Measurements were taken at 630 nm using a microplate reader (ELx-800, Biotek, Winooski, VT, USA). Data were expressed as mmol Pi mg $protein^{-1}$ min^{-1}.

CA activity was measured as described by Henry (1991), with some modifications. Briefly, 20 μL of the homogenized sample was added to 2 mL of working buffer solution. The substrate (260 μL of deionized water saturated with CO_2) was added to the reaction mixture, and the pH was measured every 5 s for up to 30 s. Blank measurements were taken simultaneously replacing the sample homogenate with working buffer solution. A linear regression model was used to estimate the slope of the reaction. The regression slope value obtained with the homogenized sample corresponded to the catalyzed reaction ratio. In turn, the regression slope value obtained from blank measurements represented the non-catalyzed reaction ratio. Enzyme activity was calculated subtracting the non-catalyzed reaction ratio from the catalyzed reaction ratio. Data were expressed as enzyme unit (EU) mg $protein^{-1}$.

Data analyses

All results were expressed as mean ± SE. Homogeneity of variances and data normality were checked prior to analysis using Levene's and Shapiro–Wilk's tests, respectively. The influence of pH treatments on net calcification rate was evaluated using two-way (exposure time and pH treatment) analysis of variance (ANOVA) for repeated measures, followed by post hoc Fisher's tests. Because net calcification rates were expressed as percentages, data were arcsine square root transformed before analysis. Enzymatic activities were evaluated using two-way (exposure time and pH treatment) ANOVA, followed by post hoc Fisher's tests. In all cases, the significance level adopted was 95% ($\alpha = 0.05$).

Results

No significant changes in net calcification rate were observed over the experimental period at any pH treatment. However, pH treatment significantly affected the net calcification rate. Hydrocorals exposed to pH 7.5 for 16 d or those exposed to pH 7.2 for 16 and 30 d showed significantly higher reduction in the net calcification rate than those maintained at the control pH (no acidification; pH 8.1) for the respective time of exposure (Fig. 1).

Ca-ATPase activity did not vary significantly over the exposure time in hydrocorals maintained at control condition (no acidification; pH 8.1) or exposed to pH 7.8. However, it was significantly increased over the period of exposure to pH 7.5 and pH 7.2 after 30 d of exposure. Additionally, Ca-ATPase was significantly higher in hydrocorals exposed to pH 7.5 and 7.2 than in those kept at control condition (no acidification; pH 8.1) or exposed to pH 7.8 for 30 d (Fig. 2).

CA activity was not significantly affected over the exposure time in hydrocorals maintained at control condition (no acidification; pH 8.1) or exposed to pH 7.8. However, it was significantly altered over the period of exposure to pH 7.5 and 7.2. CA activity was significantly lower in hydrocorals exposed to pH 7.5 for 16 d and returned to control values after 30 d of exposure. In turn, CA activity was significantly lower in hydrocorals exposed to pH 7.2 for 16 d, but it was significantly higher in hydrocorals exposed to this seawater pH for 30 d (Fig. 3).

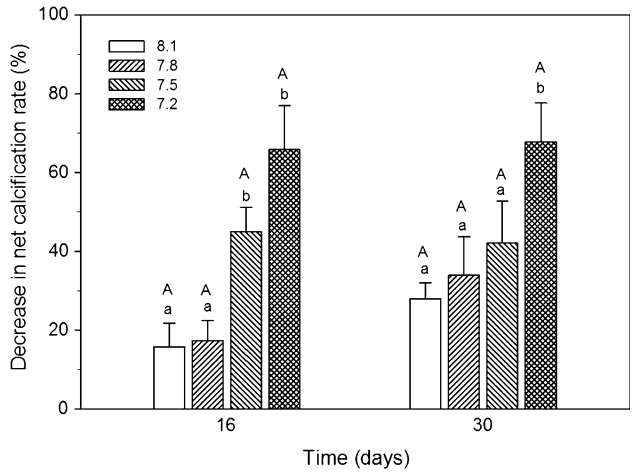

Fig. 1 Mean reduction in net calcification rate compared to initial values in the hydrocoral *Millepora alcicornis* exposed to different levels of seawater acidification. *Error bars* are standard errors ($n = 4$ for each pH treatment). Different *uppercase letters* indicate significantly different mean values ($p < 0.05$) between times of exposure for the same pH treatment. Different *lowercase letters* indicate significantly different mean values ($p < 0.05$) among pH treatments for the same time of exposure

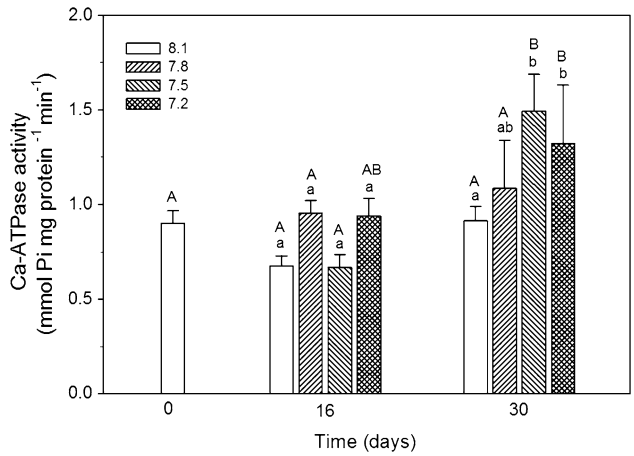

Fig. 2 Mean Ca-ATPase activity in the hydrocoral *Millepora alcicornis* exposed to different levels of seawater acidification. *Error bars* are standard errors ($n = 4$ for each pH treatment). Different *uppercase letters* indicate significantly different mean values ($p < 0.05$) for hydrocorals exposed for 16 and 30 d to the same pH treatment when compared to the pH 8.1 (no acidification treatment) at day 0. Different *lowercase letters* indicate significantly different mean values ($p < 0.05$) among pH treatments for the same time of exposure

Discussion

Previous studies have provided valuable information about the performance of calcifying organisms facing decreased pH/increased pCO_2 concentrations expected to occur within the next century. These studies have been performed in laboratory systems (e.g., Bramanti et al. 2013; Prazeres et al. 2015), in mesocosm systems (e.g., Anthony et al. 2008; Kuffner et al. 2008) and on natural volcanic CO_2 seeps (e.g., Fabricius et al. 2011; Inoue et al. 2013), the last providing great ecological complexity. Only a few studies (e.g., Jokiel et al. 2008; Andersson et al. 2009) have incorporated pH oscillations from the natural environment in their experimental setups; however, oscillations in pH have been shown to affect corals (Dufault et al. 2012; Comeau et al. 2014). In fact, Comeau et al. (2014) suggested that natural diel oscillations in pH could reduce the locally negative effects of ocean acidification on coral calcification. In this context, it is worth noting that the present experiment was run using a mesocosm system that included diel oscillations of environmental parameters (pH, temperature, seawater chemistry, light incidence and nutrients). Therefore, it would be expected that substantial ecological complexity is incorporated into the experimental design.

Our findings indicate a significant negative effect of seawater acidification on the calcification process in the hydrocoral *M. alcicornis*. However, this effect was only observed in scenarios of intermediate (pH 7.5) and severe

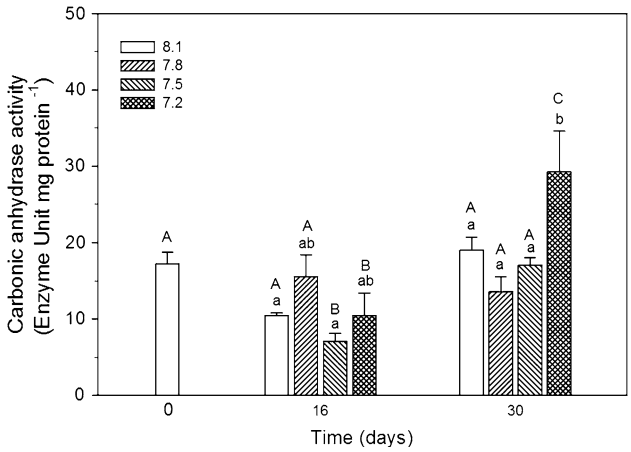

Fig. 3 Mean carbonic anhydrase activity in the hydrocoral *Millepora alcicornis* exposed to different levels of seawater acidification. *Error bars* are standard errors ($n = 4$ for each pH treatment). Different *uppercase letters* indicate significantly different mean values ($p < 0.05$) for hydrocorals exposed for 16 and 30 d to the same pH treatment when compared to the pH 8.1 (no acidification treatment) at day 0. Different *lowercase letters* indicate significantly different mean values ($p < 0.05$) among pH treatments for the same time of exposure

(pH 7.2) CO_2-driven acidification of seawater. Previous studies have also reported reduced growth rates in calcifying organisms (e.g., corals and coralline algae) exposed to lower pCO_2 levels (~700 µatm) than those found to be detrimental (1683 and 3494 µatm) for the hydrocoral *M. alcicornis* in the present study (Langdon et al. 2000; Langdon and Atkinson 2005; Jokiel et al. 2008). As observed for *M. alcicornis*, calcification rate was not affected in the corals *Acropora intermedia* (Anthony et al. 2008) or *A. digitifera* (Takahashi and Kurihara 2013) exposed to intermediate (705 µatm) and high (2142 µatm) pCO_2 levels, respectively. Therefore, for as yet unknown reasons, the hydrocoral *M. alcicornis*, as well as other coral species, has a higher tolerance to CO_2-driven acidification of seawater than other calcifying organisms.

Nutrient enrichment is suggested to ameliorate the effects of increasing acidity in corals (Cohen and Holcomb 2009). For example, *Porites* spp. tolerated high pCO_2 (1000 µatm) when fed with zooplankton (Edmunds 2011) and calcification rates of the coral *Astrangia poculata* were only negatively affected by an increase pCO2 of 780 µatm when kept at low nutrient conditions (Holcomb et al. 2012). It is worth noting that Brazilian reefs are unusual compared to oligotrophic reefs worldwide, presenting comparatively higher turbidity and nutrient levels (Kelmo et al. 2003; Dutra et al. 2006). In fact, almost all southwestern coral reefs on the continental shelf are immersed in turbid waters (Leão and Dominguez 2000), and turbidity has been suggested to affect responses of an endemic Brazilian coral, *Mussismilia harttii*, to temperature changes (Winter et al. 2016). Considering that the study by Winter et al. (2016) was conducted in the same study site as the present study, higher turbidity levels in coastal Brazilian waters could explain, at least in part, the increased tolerance of *M. alcicornis* to high pCO_2 levels (up to 1683 µatm) observed in our study. Additionally, the high variability of responses to elevated pCO_2 levels among calcifying organisms discussed above could be ascribed, to some extent, to species-specific differences and evolutionary constraints among taxa, as well as different experimental setups and methodologies used for measuring calcification rates (Pandolfi et al. 2011; Takahashi and Kurihara 2013; Prazeres et al. 2015; Brown and Edmunds 2016).

The high tolerance of *M. alcicornis* to seawater acidification is characterized by the lack of change in the net calcification rate and enzyme activities (Ca-ATPase and CA) in hydrocorals exposed to pH 7.8. Moreover, hydrocorals exposed for 30 d to pH 7.5 fully recovered from the decrease in net calcification rates observed after 16 d of exposure in the same acidification treatment. Interestingly, this response was paralleled by a significant increase in Ca-ATPase activity. This finding indicates the role of Ca-ATPase as a key enzyme involved in the calcification process in the hydrocoral *M. alcicornis*. Indeed, the 3.2-fold reduction in Ω_{ar} at pH 7.5 ($\Omega_{ar} = 1.23$) compared with the Ω_{ar} in the control (pH 8.1; $\Omega_{ar} = 3.89$) was compensated by a 1.6-fold increase in Ca-ATPase activity. A similar result was recently reported for large benthic foraminifera exposed to seawater acidification (Prazeres et al. 2015). Additionally, the gene encoding for Ca^{2+} plasma membrane ATPase, a protein involved in calcium transport in the coral *Pocillopora damicornis*, was shown to be upregulated at pH 7.8 and 7.4 (Vidal-Dupiol et al. 2013).

Increased Ca-ATPase activity may be related to the maintenance of an elevated Ω_{ar} at the calcification site. It has been reported for scleractinian corals that Ca-ATPase transports Ca^{2+} into the calcification site while removing protons (H^+) from this site (Al-Horani et al. 2003; Cohen et al. 2009). This process facilitates the conversion of HCO_3^-into CO_3^{2-}, thereby increasing the Ω_{ar} necessary for $CaCO_3$ precipitation (Cohen and McConnaughey 2003). It has also been suggested that Ca-ATPase is upregulated in order to maintain calcite saturation state (Ω_{ca}) in foraminifers facing declining pH levels (Prazeres et al. 2015). Therefore, our findings clearly indicate that *M. alcicornis* is able to maintain a steady state in net calcification rate in a wide range of seawater pH (8.1–7.5) by raising the Ca-ATPase activity, which counteracts the reduced Ω_{ar}.

In contrast to findings for Ca-ATPase, CA activity, which is also a key enzyme in the calcification process (Tambutté et al. 2011; Bertucci et al. 2013), was not affected when hydrocorals were exposed to pH 7.5 for

30 d. CA is essential for the acquisition of inorganic carbon in calcifying organisms by converting CO_2 into HCO_3^- (Bertucci et al. 2013). In fact, increased enzyme activity would be needed to raise the rate of CO_2 conversion into HCO_3^- (Vidal-Dupiol et al. 2013). In addition, maintenance of the acid–base balance within tissues would require increased CA activity in acidic conditions (Patel and Bielmyer-Fraser 2015), and in contrast to our findings, genes encoding for proteins involved in the conversion of CO_2 into HCO_3^- (extracellular and cytosolic CAs) were upregulated at pH 7.8 and 7.4 in the coral *P. damicornis* (Vidal-Dupiol et al. 2013).

In the present study, a CA response was only observed in *M. alcicornis* exposed to a scenario of severe acidification (pH 7.2) of seawater. The enzyme activity was 1.5-fold higher in hydrocorals exposed to pH 7.2 than in those kept in control conditions (pH 8.1) for 30 d, which might be related to the need for an increased supply of inorganic carbon substrate in the calcification process to prevent the potential reduction in growth under severe seawater acidification. However, the significant increase in CA and Ca-ATPase activity observed in hydrocorals exposed to this pH did not prevent a significant decrease in net calcification rate at pH 7.2.

Despite the inability of *M. alcicornis* to cope under a scenario of severe acidification (pH 7.2), the hydrocoral was highly tolerant to a wide range of seawater pH (8.1–7.5) that included likely future scenarios of ocean acidification. The high resistance of *M. alcicornis* may also be related to the pronounced oscillations in pH (8.1–7.7) recorded in the control treatment that reflect the pH oscillations of the natural environment. These natural conditions would allow *M. alcicornis* to present physiological and biochemical plasticity to a wide range of seawater pH, including the moderate (pH 7.8) and intermediate (pH 7.5) acidification scenarios tested here. In fact, Comeau et al. (2014) suggest that reef corals may be more resistant to future ocean acidification in locations where diel variations in seawater pH are pronounced, such as the study site in this study. Furthermore, the response of calcifiers will likely be dependent on the habitat and affected by the extent to which pH levels naturally oscillate (Comeau et al. 2014).

Our findings suggest that the tolerance of the hydrocoral *M. alcicornis* to severe acidification in seawater is limited by its ability to express adequate Ca-transporting proteins to face significant reduction in Ω_{ar} under extreme acidification (pH 7.2) of seawater. Indeed, the 6.1-fold reduction in Ω_{ar} at pH 7.2 ($\Omega_{ar} = 0.64$) with respect to Ω_{ar} in control conditions (pH 8.1; $\Omega_{ar} = 3.89$) was not compensated by the only 1.4-fold increase in Ca-ATPase activity observed in hydrocorals exposed to pH 7.2 for 30 d. This is in complete agreement with the fact that undersaturated conditions ($\Omega_{ar} < 1$) result in profound negative influence in skeletal growth (Cohen and Holcomb 2009). The insufficiency in biochemical response to counteract the effects of the most acidic condition (pH 7.2) tested may be related, at least in part, to the effects on the calcification process at the transcriptomic level. This suggestion is based on the downregulation of genes encoding for Ca-ATPase at pH 7.2 (Vidal-Dupiol et al. 2013) and by the fact that *M. alcicornis* did not show physiological plasticity to seawater pH under 7.5.

In summary, our findings give some insights into biochemical aspects related to the calcification process in the hydrocoral *M. alcicornis*, as well as the response of this calcifying organism to different scenarios of seawater acidification. The calcification process in *M. alcicornis* was not affected by a wide range of seawater pH (8.1–7.5) under the experimental conditions tested here. In addition, our findings indicate that Ca-ATPase plays an essential role in the maintenance of a steady state in net calcification rate in the hydrocoral *M. alcicornis*, especially under scenarios of moderate (pH 7.8) and intermediate (pH 7.5) acidification of seawater. Finally, they show that *M. alcicornis* is not able to maintain a steady state in net calcification rate under a scenario of severe acidification (pH 7.2) of seawater.

Acknowledgements The International Development Research Centre (IDRC, Ottawa, Canada; Grant # 104519-003), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Programa Ciências do Mar, Brasília, DF, Brazil; Grant # 84/2010) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática, Brasília, DF, Brazil; Grant # 573949/2008-5) are acknowledged for their financial support. The Coral Vivo Project and its sponsors Petrobras, through the Petrobras Environmental Program, and Arraial d'Ajuda Eco Parque are acknowledged for their support in field research. A. Bianchini (Proc. # 304430/2009-9) and C.B. Castro (Proc. # 303970/2010-3) are research fellows at the Brazilian CNPq. A. Bianchini is supported by the International Canada Research Chair Program (IDRC). L.F.B. Marangoni was a graduate fellow from CAPES.

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Aquatic Toxicology 206 (2019) 123–133

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Copper exposure and seawater acidification interaction: Antagonistic effects on biomarkers in the zooxanthellate scleractinian coral *Mussismilia harttii*

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ARTICLE INFO

Keywords:
Calcification
Ca-ATPase
Carbonic anhydrase
Coral reefs
Metal
Ocean acidification
Photosynthesis

ABSTRACT

Coral reefs are threatened by global and local impacts, such as ocean acidification (OA) and metal contamination. Toxicity of metals, such as copper (Cu), is expected to be enhanced with OA. However, the interaction between these environmental stressors is still poorly evaluated. In the present study, the interactive effects of seawater acidification and increasing Cu concentrations were evaluated in a zooxanthellate scleractinian coral (*Mussismilia harttii*), using biochemical biomarkers involved in the coral calcification process and the photosynthetic metabolism of endosymbionts. Corals were kept under control conditions (no seawater acidification and no Cu addition in seawater) or exposed to combined treatments of reduced seawater pH (8.1, 7.8, 7.5 and 7.2) and environmentally relevant concentrations of dissolved Cu (measured: 1.0, 1.6, 2.3 and 3.2 μg/L) in a mesocosm system. After 15- and 35-days exposure, corals were analyzed for photochemical efficiency (*Fv*/*Fm*), chlorophyll *a* content, Ca-ATPase and carbonic anhydrase (CA) activity. Results showed that 76% of the interactions between reduced seawater pH and increasing Cu concentrations were antagonistic. Only 24% of these interactions were additive or synergistic. In general, the combination of stressors had no significant deleterious effects in the photosynthetic metabolism of endosymbionts or Ca-ATPase activity. In fact, the lowest dissolved Cu concentration tested had a consistent positive effect on Ca-ATPase activity in corals facing any of the reduced seawater pH conditions tested. In turn, potentially deleterious effects on acid-base balance in *M. harttii*, associated with changes in CA activity, were intensified by the combination of stressors. Findings reported here indicate that Cu toxicity in future OA scenarios can be less severe than previously suggested in this coral holobiont.

1. Introduction

Coral reefs are one of the most biologically diverse ecosystems on Earth. They are crucial for the livelihoods of millions of people, generating billions of dollars in tourism-related activities and providing coastal protection (Veron et al., 2009). The tridimensional structure of coral reefs is built by calcifying organisms that are able to secrete carbonate skeletons through the biomineralization process. In this context, special attention has been given to scleractinian corals, which are considered one of the major groups of calcifying organisms. Indeed, they contribute ~15% of the global calcium carbonate production, thus being essential for the construction and maintenance of reefs worldwide (Allemand et al., 2004; Tambutté et al., 2011).

Ocean acidification (OA), defined as a reduction in the pH of world´s oceans, poses a chronic threat to reef ecosystems by (i) reducing carbonate ion concentration, required for the maintenance of carbonate reef structures; and (ii) by reducing the saturation state of calcium carbonate (Ω_{Cc}), which leads to lower calcification and increased dissolution rates of skeletons of major marine calcifiers (Doney et al., 2009; Albright et al., 2018). Since coral skeletons are composed by the aragonite mineral, reductions in aragonite saturation state (Ω_{ar}) are of great concern (Tambutté et al., 2011; Bertucci et al., 2013).

The increasing levels in the atmospheric CO_2 partial pressure (pCO_2) are expected to intensify OA. Declines in surface pH of ocean, in

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https://doi.org/10.1016/j.aquatox.2018.11.005
Received 5 September 2018; Received in revised form 17 October 2018; Accepted 6 November 2018
Available online 10 November 2018
0166-445X/

carbonate ion concentrations, and in Ω_{ar} have been reported (e.g. Bates et al., 2014; Le Quéré et al., 2015). Forecasts indicate a reduction in seawater pH ranging from 0.3 to 0.8 pH units for the next centuries (Caldeira and Wickett, 2003; IPCC, 2014). Furthermore, recent evidences have shown that OA may be actually impairing coral reef growth, and that near-future reductions in the Ω_{ar} may compromise the ecosystem function of coral reefs (Albright et al., 2018).

Besides global pressures, such as OA, coral reefs worldwide are facing multiple local and regional impacts (van Dam et al., 2011; Wolff et al., 2018). In this context, contamination with metals, such as copper (Cu), have been pointed out as a notable local impact threatening coral reefs (van Dam et al., 2011). Main sources of contamination are related to discharges of domestic sewage, industrial effluents and antifouling paints (Turner, 2010). Toxic effects of Cu have been extensively reported in cnidarians (e.g. Nystrom et al., 2001; Grant et al., 2003; Bielmyer et al., 2010; Yost et al., 2010; Schwarz et al., 2013; Marangoni et al., 2017a,b). However, there are few investigations on the combined effects of Cu and global stressors, such as increasing temperature (Fonseca et al., 2017) and OA (Siddiqui and Bielmyer-Fraser, 2015; Bielmyer-Frasier et al., 2018).

Considering that the effect of only one stressor rarely occurs in nature, the evaluation of multiple stressors on calcifying organisms is necessary for a better understanding of the potential effects of global and local pressures on coral reefs. In fact, a currently increased body of research has highlighted the importance of investigating the interactive effects of potential global (e.g. increasing temperature and seawater acidification) and local stressors (e.g. chemical pollution) (Wernberg et al., 2012; Ban et al., 2014; Wolff et al., 2018). In this context, the effect of OA on the speciation of metals in seawater must be considered. Hydroxide (OH^-) and carbonate (CO_3^{2-}) ions form strong complexes with divalent and trivalent metals, and a significant reduction in the concentration of both ions is expected in surface waters due to OA (Millero, 2009; Zeng et al., 2015). As a consequence, changes in the speciation of a number of metals are also expected at a reduced seawater pH. In this case, metals forming strong complexes with OH^- and CO_3^{2-} would be found in the free ionic form, which is known to be toxic to organisms (Millero, 2009; Zeng et al., 2015). Furthermore, estimations made by Millero (2009), as a function of pH and time [based on Caldeira and Wickett (2003) predictions for OA], show that the largest percentage of increase for carbonate-dominated metals is for Cu^{2+}, with a 30% increase. As a consequence, the toxicity of this metal to organisms is expected to be augmented with decreasing pH. To our best knowledge, studies on the interactive effects of these stressors on coral physiology are very scarce in the literature. In fact, only one study has tested the combined effects of OA and Cu pollution on two scleractinian coral species (Bielmyer-Frasier et al., 2018). Therefore, information of OA on Cu toxicity leading to detrimental effects on coral physiology is still very scarce.

Changes in organisms at cellular level due to the presence of toxicants or modified environmental conditions (e.g. reduced pH and increasing temperature) can be used as biomarkers. These tools are known to provide early warning signs of environmental degradation before changes in integrative biological functions or mortality can be observed (Depledge et al., 1995; Downs et al., 2005). In this context, recent investigations have demonstrated that Ca^{2+}-activated ATPase (hereafter referred as Ca-ATPase) and carbonic anhydrase (CA) activities are potential biomarkers to evaluate the physiological status of calcifying organisms, including corals, hydrocorals and foraminifers, exposed to different environmental stressors (e.g. acidifying conditions, increasing temperature, and metal contamination) (Prazeres et al., 2015, 2016; Fonseca et al., 2017; Marangoni et al., 2017a,b; Marques et al., 2017). Furthermore, parameters related to the photosynthetic metabolism, such as photochemical efficiency and content of photosynthetic pigments, have been used to address the health and integrity of the important symbiotic relationship established between corals and dinoflagellates of the genus *Symbiodinium* spp., known as zooxanthellae (e.g. Jones et al., 1999; Fitt et al., 2001; Silverstein et al., 2014; Ezzat et al., 2016).

In light of the above, the present study aimed to evaluate the possible interactive effects of reduced seawater pH and increasing Cu concentrations, using biochemical biomarkers involved in the calcification process in the zooxanthelate scleractinean coral *Mussismilia harttii* and the photosynthetic metabolism of its endosymbiont. The coral *M. harttii* is distributed along the Atlantic Coast (northwestern Brazil) and was chosen as a biological model due to its key role in the construction of South Atlantic reefs (Laborel, 1969; Castro and Pires, 2001). Biomarkers evaluated after acute (15 days) and chronic (35 days) exposures to reduced seawater pH conditions and increasing Cu concentrations in a mesocosm system included holobiont Ca-ATPase and CA activity, as well as chlorophyll *a* content and photochemical capacity of the endosymbiont. Exposure of *M. harttii* to reduced seawater pH followed scenarios predicted for the next centuries (Caldeira and Wickett, 2005; IPCC, 2014). In turn, Cu concentrations tested were close or below the water quality criteria for dissolved Cu in seawater established by environmental regulations in South and North America (USEPA, 2003; CONAMA, 2005).

2. Materials and methods

2.1. Coral collection and acclimation

Four colonies of *M. harttii* were collected by SCUBA divers in 4 different sites in the conservation area of the "Municipal Natural Park of Recife de Fora" (Porto Seguro, Bahia, northwestern Brazil; 16° 25′08.1″S/ 038° 58′54.1″W). The colonies providing samples were at least 500 m apart. Coral fragments were transferred to the experimental facilities of the Coral Vivo Project (Arraial d'Ajuda, Porto Seguro), where polyps were individualized, fixed on ceramic plates with cyanoacrylate glue, and acclimated in a mesocosm system for 30 days. It is important to note that only a limited number of polyps were collected and analyzed because *M. harttii* is classified as threatened by the Brazilian Ministry of Environment (MMA, 2014). Collections were performed under the permission of the Brazilian Environmental Agency (IBAMA SISBIO permit #85926584).

2.2. Experimental setup

Mesocosm systems are considered important experimental tools to generate information on potential stressors affecting biota in wild systems, enabling the incorporation of ecological complexity that is impossible to be achieved in conventional laboratory systems (Luckett et al., 1996; Bellworthy and Fine, 2018). Aiming at an ecologically relevant experimental approach, the present study tested different conditions of reduced seawater pH and increasing dissolved Cu concentrations alone or in combination in the large-scale mesocosm system of the Coral Vivo Project (Arraial d'Ajuda, Porto Seguro). This system was designed for reef environment studies and has been considered to greatly elevate the ability to understand responses to anthropogenic changes (Bellworthy and Fine, 2018). This experimental facility has been described in detail by Duarte et al. (2015).

The experimental setup consisted of polyps (n = 144) randomly distributed in 48 10-L aquaria (3 polyps per aquarium) that were continuously supplied with seawater from a fringing reef (Araçaípe Reef, Arraial d'Ajuda, Porto Seguro). The rate of water renewal was 3-fold compared to the volume of water in the aquaria per hour. The water supplying the aquaria was previously pumped into four underground sumps (5000 L), where CO_2 was injected to reduce the seawater pH at the desired level. Seawater pCO_2 was adjusted using a CO_2 reactor. A computer system (ReefAngel, Freemont, CA, USA) coupled to pH electrodes (Gehaka 09RBCN) connected to the sumps assisted in the control of treatments and data recording. pH and temperature were monitored every 15 min by the computer system, while salinity (Instrutemp ITREF

10 optical refractometer) and light intensity (LI-COR, LI 250 A Light Meter, LI-193 Underwater Spherical Quantum Sensor) were measured once a day in the afternoon. Considering forecasts generated by modeling atmospheric CO_2 emissions reported by Caldeira and Wickett (2005) and the IPCC (2014), three scenarios of seawater acidification were simulated. Reductions of 0.3, 0.6 and 0.9 pH units from the ambient seawater at the study site during the experiment (pH ~ 8.1 NBS) were tested. It is important to note that diel oscillations in pH of seawater from the natural environment were maintained in the experimental system. In fact, these diel variations in seawater pH have been shown to affect corals response to seawater acidification (Dufault et al., 2012; Comeau et al., 2014). Therefore, it is expected that substantial ecological complexity has been incorporated into the experimental design of the present study.

Seawater contamination with Cu was performed using stock solutions of $CuCl_2$ prepared daily in 1000-L reservoirs containing seawater pumped from the fringing reef area. The contaminated media were prepared 24 h before use to allow equilibration between Cu and seawater. Because 10% of the flux going to the experimental aquaria came from these reservoirs (carried in line by auxiliary peristaltic pumps), the different treatments with Cu were prepared by adding 10, 30 and 50 ml of stock solution (1 g/L Cu) into the reservoirs to achieve the desired nominal concentrations of 1, 3 and 5 µg/L above the Cu concentration naturally found in the field. Also, a control (no Cu addition in seawater) was prepared. Considering the treatments of pH and Cu alone and their combinations, a total of sixteen treatments (three replicates per treatment; a total of 48 aquaria) was performed for up to 35 days.

The present experiment was run in parallel with that performed by Marques et al. (2017), who exposed symbiont-bearing foraminifers to the same treatments described here. Therefore, corals from this study and foraminifers analyzed by Marques et al. (2017) were kept together in the aquaria.

2.3. pCO_2 levels and Ω_{ar} in seawater

Levels of pCO_2 and aragonite saturation state (Ω_{ar}) values were calculated for each acidification treatment based on the alkalinity and mean pH data, using the software CO2calc version 1.2.9. Seawater alkalinity supplying the experimental system was analyzed according to Dickson et al. (2007), using an alkalinity titrator (AS-ALK2, Apollo SciTech Inc., Bogart, GA, USA) and a certified reference material (ID: 132, Scripps institute of Oceanography, La Jolla, CA, USA). The methodological procedures and data on pCO_2 levels and Ω_{ar} were previously reported by Sarmento et al (2015), who exposed phytal meiofauna community to ocean acidification. Sarmento et al. (2015) ran a parallel experiment in the same mesocosm system described in the present study using separate experimental tanks. The acidified seawater feeding the experimental aquaria of the present study and the tanks used by Sarmento et al. (2015) came from the same 5000-L underground sumps previously described in this section.

2.4. Dissolved copper concentration in seawater

Every week and for up to 35 days, filtered (0.45-µm mesh filter) seawater samples (10 mL) of each experimental media were collected for analysis of dissolved Cu concentrations. In all cases, water samples were acidified with HNO_3 (1% final concentration; SupraPur, Merck, USA) and desalted according Nadella et al. (2009). Cu concentration was determined by atomic absorption spectrophotometry coupled with a graphite furnace (Perkin-Elmer, Waltham, MA, USA).

2.5. Biomarker analyses

2.5.1. Photochemical capacity of photosystem II (Fv/Fm)

The maximum quantum efficiency of the photosystem II (PSII) photochemistry was obtained based on the *F*v/*F*m ratio, using 8-mm standard glass-fiber optic probe (positioned above the oral disk of polyps) coupled to a portable underwater Pulse Amplitude Modulated (PAM) fluorometer (Diving PAM, Heinz Walz, Effeltrich, Germany). Before measurements, corals were acclimated to dark for 1 h in order to achieve a reliable indication of the PSII activity in dinoflagellates (Jones et al., 1999). Polyps ($n = 3$) were randomly selected in each of the aquaria to perform measurements.

2.6. Chlorophyll a content

Chlorophyll *a* content was determined using a modified method originally described by Schmidt et al. (2011). Briefly, a small piece (~1 cm^2) of coral tissue was sonicated (70 KHz) in MiliQ-water until the whole tissue was detached from the skeleton. The homogenate was used for chlorophyll extraction in ethanol (95%) over 24 h. Extractions were kept at 4 °C and protected from light. After extraction, samples were centrifuged (5200 rpm, 4 °C-, 5 min) and 320 µl of the supernatant was used to quantify chlorophyll *a*. Sample absorbance at 665 nm and 750 nm was measured using a microplate reader (ELX 808, bio Tek Instruments, Winooski, VT, USA). Chlorophyll *a* content (Chl *a*) was calculated following the formula: Chl *a* [ng (mg protein) -1] = 12.0 * (A_{665}-A_{750}) * Ve/ (prot*d) * 103, where 12.0 is the absorption coefficient (per mole) of Chl *a* in 95% ethanol; "A_{665}" is the sample absorbance at 665 nm; "A_{750}" is the sample absorbance at 750 nm; "Ve" is the ethanol volume in the extraction; "prot" is the protein content of coral sample in the well; "d" is the path length of optical cell (cm); and 103 is the correction factor for using the method adapted to microplate reader. The total protein content in the sample homogenate was determined using a commercial reagent kit based on the Bradford assay (Sigma-Aldrich, St. Louis, MO, USA).

2.7. Measurement of Ca-ATPase and carbonic anhydrase (CA) activities

Holobiont sample preparation for enzyme activity analyses was performed following procedures described by Downs et al. (2005), with the modifications reported in Marangoni et al. (2017b). Briefly, samples were ground in liquid nitrogen and than sonicated (70 kHz, Sonaer Ultrasonics, NY, USA) on ice using the specific homogenization buffer (1:2, wv) required for each enzyme assay, as described below. After homogenization, samples were centrifuged (13,000*g*, 10 min, 4 °C) and the intermediary phase was used as enzyme source. The total protein content in sample homogenate was determined using a commercial reagent kit based on the Bradford assay (Sigma-Aldrich, St. Louis, MO, USA).

Measurement of Ca-ATPase activity was performed according to Chan et al. (1986), with some modifications. Sample homogenates were prepared using a buffer solution containing 500 mM sucrose, 1 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF), and 100 mM Tris hydrochloride (Tris-HCl; pH 7.6). Samples were incubated (water bath at 30 °C, for 30 min) in a reaction solution containing 189 mM NaCl, 5 mM $CaCl_2$, 1 mM oubain and 20 mM Tris-HCl (pH 7.6). The reaction was started by adding 3 mM ATP in the reaction solution, and stopped by placing the samples on ice for 10 min. Inorganic phosphate (Pi) released by the enzyme activity in the reaction medium was quantified using a commercial reagent kit "Fosfato" (Doles, Panamá, Goiás, Brazil) based on the method described by Fisk and Subbarow (1925). Absorbance was measured at 630 nm using a microplate reader (ELx-800, Biotek, Winooski, VT, USA). Data was normalized using the total protein content used in the reaction medium and expressed as µmol Pi mg protein^{-1} h^{-1}.

CA activity was measured according to the method described by Henry (1991), with some modifications. The sample homogenate (10 µl) was added to 2 ml of a reaction solution containing 15 mM sucrose, 225 mM mannitol, 10 mM phosphate and 10 mM Tris-Base (pH 8.5). The reaction was started by adding 260 µl of substrate (MiliQ-water saturated with CO_2) to the reaction mixture. The pH was

measured every 5 s for up to 30 s, using a pH meter. Blank measurements were carried out simultaneously by replacing the sample homogenate with 10 µl of the same buffer solution used for sample homogenization. This method is based on the pH reduction caused by the catalytic hydration of CO_2 with consequent release of H^+. Therefore, a linear regression model was used in order to estimate the slope of the reaction. The regression slope obtained with the sample homogenate corresponded to the catalyzed reaction ratio, while the regression slope obtained in the blank measurement was considered the non-catalyzed reaction ratio. Data were normalized considering the total protein content in the reaction mixture and expressed as enzyme units mg protein^{-1}.

2.8. Data presentation and analysis

All data are expressed as mean ± standard error. Combined effects of reduced seawater pH and Cu concentration on *F*v/*F*m values, chlorophyll *a* content and enzyme activities were evaluated using two-way analysis of variance (ANOVA) for data obtained at each exposure time (15- and 35-days exposure). Data were log-transformed to meet ANOVA assumptions when necessary. If indicated, ANOVA were followed by the Tukey's test. When ANOVA indicated a significant effect of the interaction term, the expected additive inhibition (Bliss, 1939; Schmidt et al., 2014; van Dam et al., 2015; Marques et al., 2017) was calculated in order to evaluate the interaction. The combined effect was determined for each combination of reduced seawater pH and Cu concentration. Additivity was assumed when the interaction term was not significant, but both stressors were, or when the observed combined effect was equal to the predicted; antagonism was assumed when the observed combined effect was smaller than the predicted; and synergism was considered when the observed effects were larger than the predicted ones (Crain et al., 2008).

3. Results

3.1. Experimental conditions

Mean light level in the aquaria during the experimental period was 154.4 ± 87.7 µmol photons m-^{2}s^{-1}. In turn, nitrate and phosphate concentrations in the aquaria were 0.89 ± 0.12 and 0.06 ± 0.01 mg/L, respectively. No significant changes in seawater salinity (35.5 ± 1.25) or temperature (26.1 ± 0.8 °C) were observed over the exposure period. Mean seawater pH throughout the experiment in the different pH treatments corresponded to 8.1 (ambient), 7.8, 7.5 and 7.2. Dial oscillations in seawater pH were maintained in the experimental aquaria over the 35-day period of exposure (Table 1). The pCO_2 values for each of the treatments were 351.8 (pH 8.1), 939.0 (pH 7.8), 1683.4 (pH 7.5) and 3494.3 µatm (pH 7.2); the Ω_{ar} values corresponded to 3.89, 1.96, 1.23 and 0.64 (Sarmento et al., 2015). Dissolved Cu concentrations in the experimental media were 1.0 ± 0.13, 1.6 ± 0.12, 2.3 ± 0.04 and 3.2 ± 0.01 µg/L for the nominal concentrations of 0 (ambient), 1, 3 and 5 µg/L, respectively. Data on dissolved Cu concentrations during the experimental period were previously reported by Marques et al. (2017), since both the experiments were run in parallel.

Table 1
Seawater pH in the ambient seawater pH (8.1) and reduced seawater pH treatments (7.8, 7.5 and 7.2) over the 35-days period of exposure.

pH treatment	8.1	7.8	7.5	7.2
Median	8.11	7.77	7.51	7.20
Mean	8.10	7.76	7.51	7.20
Standard deviation	0.096	0.185	0.160	0.190
Standard error	0.002	0.003	0.003	0.004

3.2. Photochemical capacity of photosystem II (Fv/Fm)

No effects of reduced seawater pH, dissolved Cu concentration or the interaction between these two stressors on *F*v/*F*m values were observed after 15 days of exposure ($0.3 \leq F \leq 2.2$, $0.06 \leq p \leq 0.9$; Fig. 1A). After 35 days of exposure, only an effect associated with Cu concentration was observed ($F = 3.0$, $p = 0.04$). Tukey's test indicated a significant difference between *F*v/*F*m values in corals exposed to 1.6 and 3.2 µg/L Cu at pH 8.1 (ambient). Mean *F*v/*F*m values in the lower Cu addition treatment (1.6 µg/L) were slightly higher compared to the other treatments, while mean values in the highest Cu addition treatment (3.2 µg/L) were lower. However, corals from both treatments did not differ from those maintained under control conditions (ambient pH and Cu concentration) (Fig. 1B).

3.3. Chlorophyll a content

Significant effects of reduced seawater pH and Cu addition alone and in combination on coral´s chlorophyll *a* content were observed after 15 days of exposure ($3.1 \leq F \leq 12.1$, $p \leq 0.01$; Fig. 2A). Tukey´s post hoc comparisons indicated a significant increase in chlorophyll *a* content in corals maintained at pH 8.1 (ambient pH) in the presence of 1.6 µg/L Cu when compared to control corals (pH 8.1 and no Cu addition) and corals kept at pH 8.1 in the presence of 2.3 µg/L Cu. Also, corals exposed to pH 7.2 combined with the highest Cu addition treatment (3.2 µg/L) showed increased chlorophyll *a* content when compared to corals exposed to pH 7.2 without Cu addition. Corals exposed to pH 7.5 in the presence of 1.6 µg/L Cu showed increased chlorophyll *a* content when compared to corals exposed to the same seawater pH condition (pH 7.5) combined with 2.3 µg/L Cu. The same pattern was observed in the most severe seawater acidification condition (pH 7.2). In this case, corals exposed to 1.6 µg/L Cu had an increased chlorophyll *a* content when compared to corals exposed to 2.3 µg/L Cu (Fig. 2A). Synergistic interaction was observed between the most acidic condition (pH 7.2) and the higher Cu addition treatment (3.2 µg/L). In turn, antagonism was observed in the other combinations of Cu concentration and pH treatments (Table 2).

After 35 days of exposure, no effect of Cu addition was observed. However, variable effects for seawater acidification and in the interaction term were detected ($2.97 \leq F \leq 3.68$, $p \leq 0.02$; Fig. 2B). Tukey´s test indicated an increased chlorophyll *a* content in corals exposed to the lowest seawater pH condition (pH 7.8) combined with 2.3 µg/L Cu when compared to corals exposed to the same seawater pH condition (pH 7.8) combined with 1.6 and 3.2 µg/L Cu. Also, increased chlorophyll *a* content was observed in coral exposed to the combined treatment of pH 7.8 and 2.3 µg/L Cu when compared to corals exposed to the same Cu concentration, but at different seawater pH conditions (pH 8.1, 7.5 and 7.2). In turn, a significant reduction in chlorophyll *a* content was observed in corals exposed to the combined treatment of pH 7.5 and 1.6 µg/L Cu when compared to corals exposed to the same Cu concentration, but at different seawater pH conditions (pH 8.1, 7.8 and 7.2). Additive interaction was observed between the lower seawater pH condition (pH 7.8) and the Cu concentration of 2.3 µg/L. Antagonism was observed for all the other combined treatments (Table 2).

3.4. Ca-ATPase activity

Effects of Cu addition alone ($8.1 \leq F \leq 30.5$, $p = 0.001$) and in the interaction term ($2.67 \leq F \leq 2.75$, $p = 0.02$) on Ca-ATPase activity were observed at both exposure times. Enzyme activity inhibition was observed after 15 days of exposure to Cu addition treatments (Fig. 3a). Tukey´s post hoc comparisons indicated that corals exposed to 2.3 µg/L Cu at pH 8.1 (ambient pH) showed significantly lower enzyme activity than control corals (pH 8.1 and no Cu addition). Also, reduced Ca-ATPase activity was observed in corals exposed to different

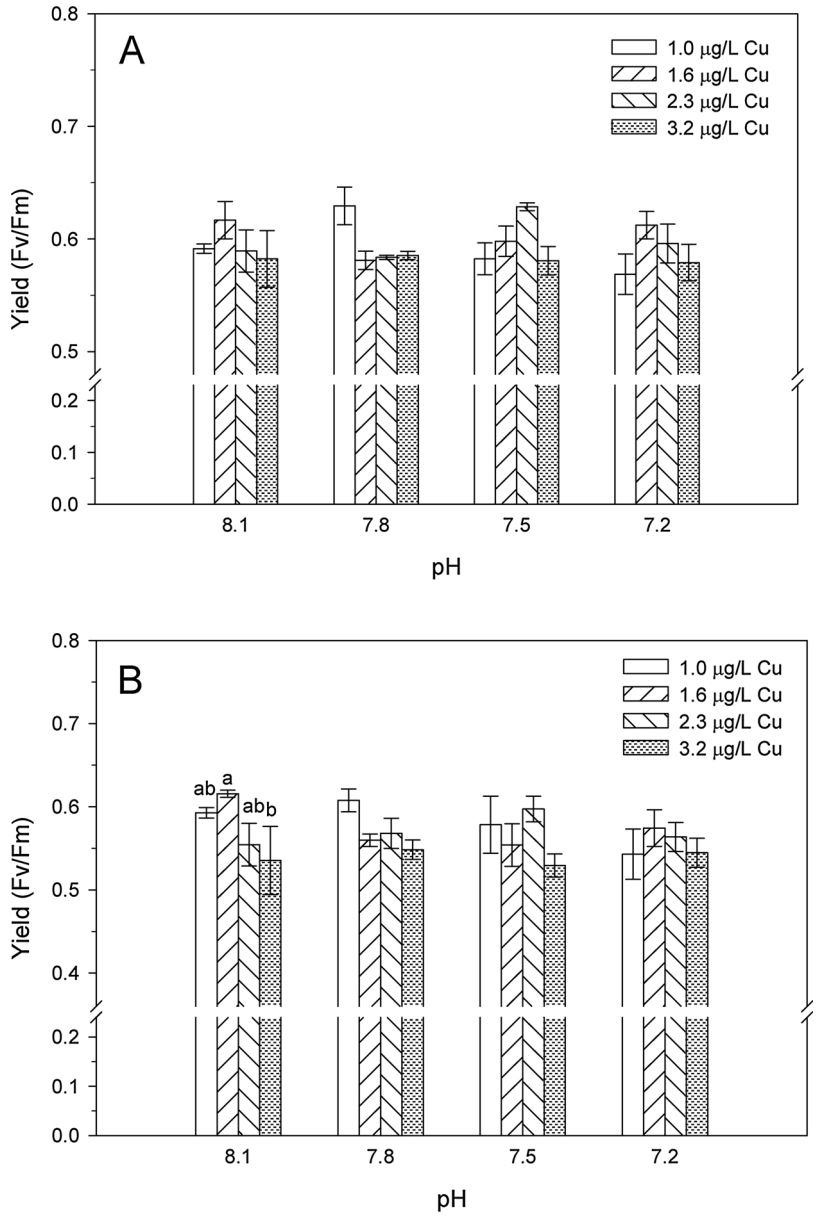

Fig. 1. Photochemical capacity of photosystem II *(Fv/F*m) in the coral *Mussismilia harttii* exposed to different combinations of reduced seawater pH and copper (Cu) concentrations in a mesocosm system for 15 days (A) or 35 days (B). Error bars are standard errors (n = 3 for each treatment). Different lowercase letters indicate significantly different mean values ($p \leq 0.05$) among Cu concentrations for the same pH treatment. Different uppercase letters indicate significantly different mean values ($p \leq 0.05$) among pH treatments for the same Cu concentration.

combinations of reduced seawater pH and Cu addition (Fig. 3A). At pH 7.8, corals exposed to 1.6 and 3.2 µg/L Cu showed lower Ca-ATPase activity than corals maintained at ambient Cu concentration (1.0 µg/L). Also, corals exposed to 1.6 and 2.3 µg/L Cu at pH 7.5 showed lower Ca-ATPase activity than those kept at ambient Cu concentration (1.0 µg/L). Corals exposed to 1.6 µg/L Cu at pH 7.5 also showed lower Ca-ATPase activity than those maintained at pH 8.1 (ambient pH). Synergistic interactions were observed between 1.6 µg/L Cu and pH 7.8; 1.6 µg/L Cu and pH 7.6; and 2.3 µg/L Cu and pH 7.6. Additive interaction was observed between 3.2 µg/L Cu and pH 7.8. In turn, antagonism was observed for all the other combined treatments (Table 2).

After 35 days of exposure, Ca-ATPase activity inhibition was observed after exposure to Cu addition alone (Fig. 3B). Tukey´s test indicated that corals exposed to the two higher concentrations of Cu (2.3 and 3.2 µg/L Cu) at pH 8.1 (ambient pH) showed lower Ca-ATPase activity than corals exposed to the two lower Cu concentrations (1.0 and 1.6 µg/L Cu) at the same seawater pH (8.1). Also, enzyme activity was lower in all reduced seawater pH conditions (pH 7.8, 7.5 and 7.2) than in the ambient pH condition (pH 8.1). In turn, increased enzyme activity was observed in corals exposed to 1.6 µg/L Cu at all reduced seawater pH conditions. For all reduced seawater pH conditions, corals maintained at 1.6 µg/L Cu showed significantly higher enzyme activity than corals maintained at ambient Cu concentration (1.0 µg/L) or exposed to the higher Cu concentrations (2.3 and 3.2 µg/L) (Fig. 3B). Additive interaction was observed between 3.2 µg/L Cu and pH 7.2. In turn, antagonism was observed for all the other combined treatments (Table 2).

3.5. Carbonic anhydrase (CA) activity

Significant effects of reduced seawater pH ($3.5 \leq F \leq 8.0$, $p \leq 0.03$) and Cu addition ($10.1 \leq F \leq 13.8$, $p = 0.001$) alone, and in combination ($2.9 \leq F \leq 4.1$, $p \leq 0.01$) were observed on coral´s CA activity at both exposure times. Enzyme activity inhibition was

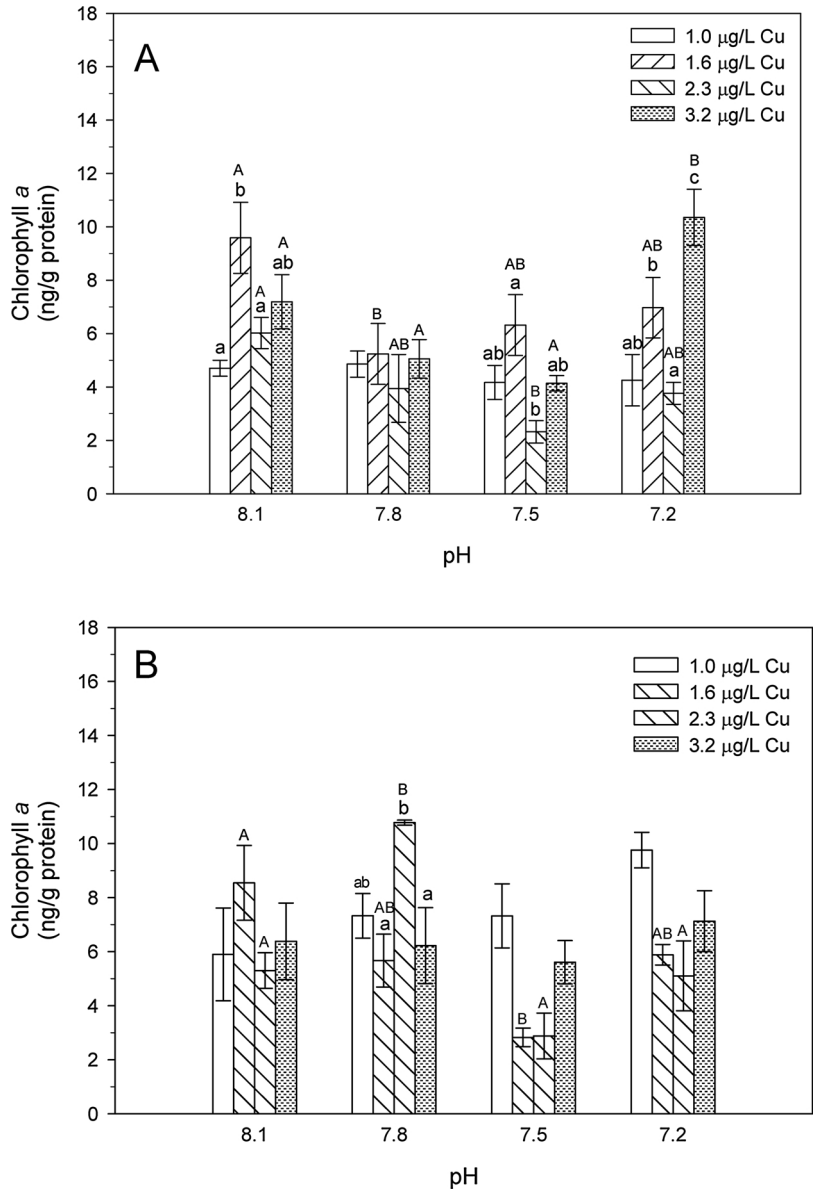

Fig. 2. Chlorophyll *a* content in the coral *Mussismilia harttii* exposed to different combinations of reduced seawater pH and copper (Cu) concentrations in a mesocosm system for 15 days (A) or 35 days (B). Error bars are standard errors (n = 3 for each treatment). Different lowercase letters indicate significantly different mean values ($p \leq 0.05$) among Cu concentrations for the same pH treatment. Different uppercase letters indicate significantly different mean values ($p \leq 0.05$) among pH treatments for the same Cu concentration.

Table 2
Summary of interactive effects based on calculations of predicted additive inhibition and fraction changes compared with the controls. (*) indicates synergistic effect; (+) indicates additive effect; and (–) indicate antagonistic effect.

Combined treatments		Combined effect (15 days)				Combined effect (35 days)			
Cu	pH	Ca-ATPase	CA	Chlr *a*	Fv/Fm	Ca-ATPase	CA	Chlr *a*	Fv/Fm
1.6	7.8	*	–	–	n.a.	–	*	–	n.a.
1.6	7.5	*	–	–	n.a.	–	*	–	n.a.
1.6	7.2	–	–	–	n.a.	–	+	–	n.a.
2.3	7.8	–	+	–	n.a.	–	*	+	n.a.
2.3	7.5	*	–	–	n.a.	–	–	–	n.a.
2.3	7.2	–	–	–	n.a.	–	–	–	n.a.
3.2	7.8	+	*	–	n.a.	–	+	–	n.a.
3.2	7.5	–	–	–	n.a.	–	–	–	n.a.
3.2	7.2	–	–	*	n.a.	–	–	–	n.a.

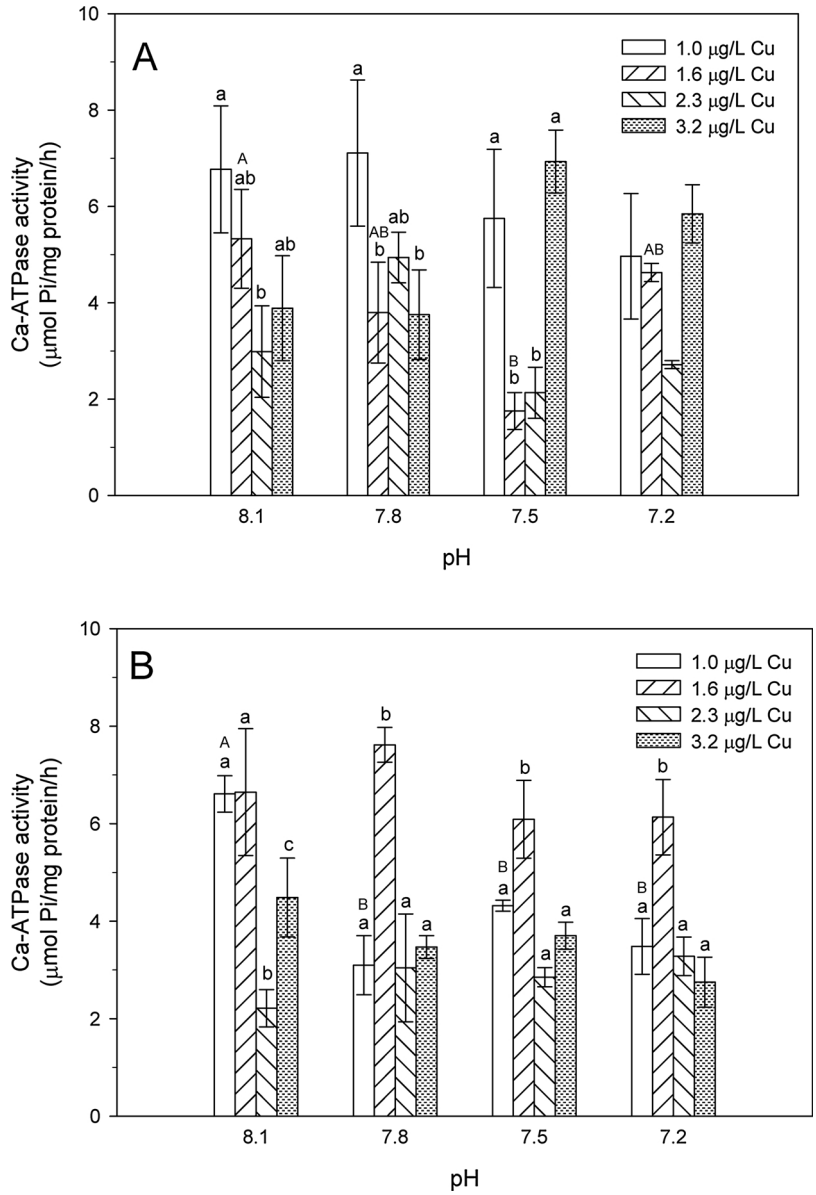

Fig. 3. Ca-ATPase activity in the coral *Mussismilia harttii* exposed to different combinations of reduced seawater pH and Cu concentrations in a mesocosm system for 15 days (A) or 35 days (B). Error bars are standard errors (n = 3 for each treatment). Different lowercase letters indicate significantly different mean values ($p \leq 0.05$) among Cu concentrations for the same pH treatment. Different uppercase letters indicate significantly different mean values ($p \leq 0.05$) among pH treatments for the same Cu concentration.

observed after 15 days of exposure to Cu (Fig. 4A). Tukey´s test indicated that corals exposed to all Cu addition treatments (1.6, 2.3 and 3.2 µg/L Cu) at pH 8.1 (ambient pH) showed significantly lower enzyme activity than control corals (pH 8.1 and no Cu addition). At pH 7.8, corals exposed to the highest Cu concentration (3.2 µg/L) showed lower CA activity than those exposed to any other Cu concentration tested (1.0, 1.6 and 2.3 µg/L) at the same seawater pH (pH 7.8). Also, higher CA activity was observed in corals exposed to pH 7.8 combined with 1.6 µg/L Cu than in those exposed to the same Cu concentration combined to the other pH treatments (pH 7.5 and 7.2) (Fig. 4A). Synergistic interaction was observed between 3.2 µg/L Cu and pH 7.8. Additive interaction was observed between 2.3 µg/L Cu and pH 7.8. In turn, antagonism was observed for all the other combined treatments (Table 2).

As observed after 15 days of exposure, inhibited enzyme activity due to Cu addition alone was observed after 35 days of exposure (Fig. 4B). Corals exposed to the two higher Cu concentrations (2.3 and 3.2 µg/L) at pH 8.1 (ambient pH) showed lower enzyme activity than those exposed to the two lower Cu concentrations (1.0 and 1.6 µg/L). Also, enzyme activity was lower in corals exposed to Cu addition treatments at pH 7.8 than in those maintained at the same reduced seawater pH (pH 7.8) at ambient Cu concentration (1.0 µg/L). Decreased CA activity was also observed due to reduced seawater pH alone (Fig. 4B). Corals exposed to pH 7.5 and 7.2 without Cu addition showed lower enzyme activity than those exposed to pH 8.1 and 7.8 without Cu addition. In turn, corals exposed to the combined treatments of 1.6 µg/L Cu and reduced seawater pH (pH 7.8, 7.5 and 7.2) showed lower enzyme activity than those maintained at pH 8.1 (ambient pH) and 1.6 µg/L Cu. Synergistic interaction was observed between 1.6 µg/L Cu and pH 7.8; 1.6 µg/L Cu and pH 7.6; and 2.3 µg/L Cu and pH 7.8. Additive interaction was observed between 1.6 µg/L Cu and pH 7.2; and 3.2 µg/L Cu and pH 7.8. Antagonism was observed for all the other combined treatments (Table 2).

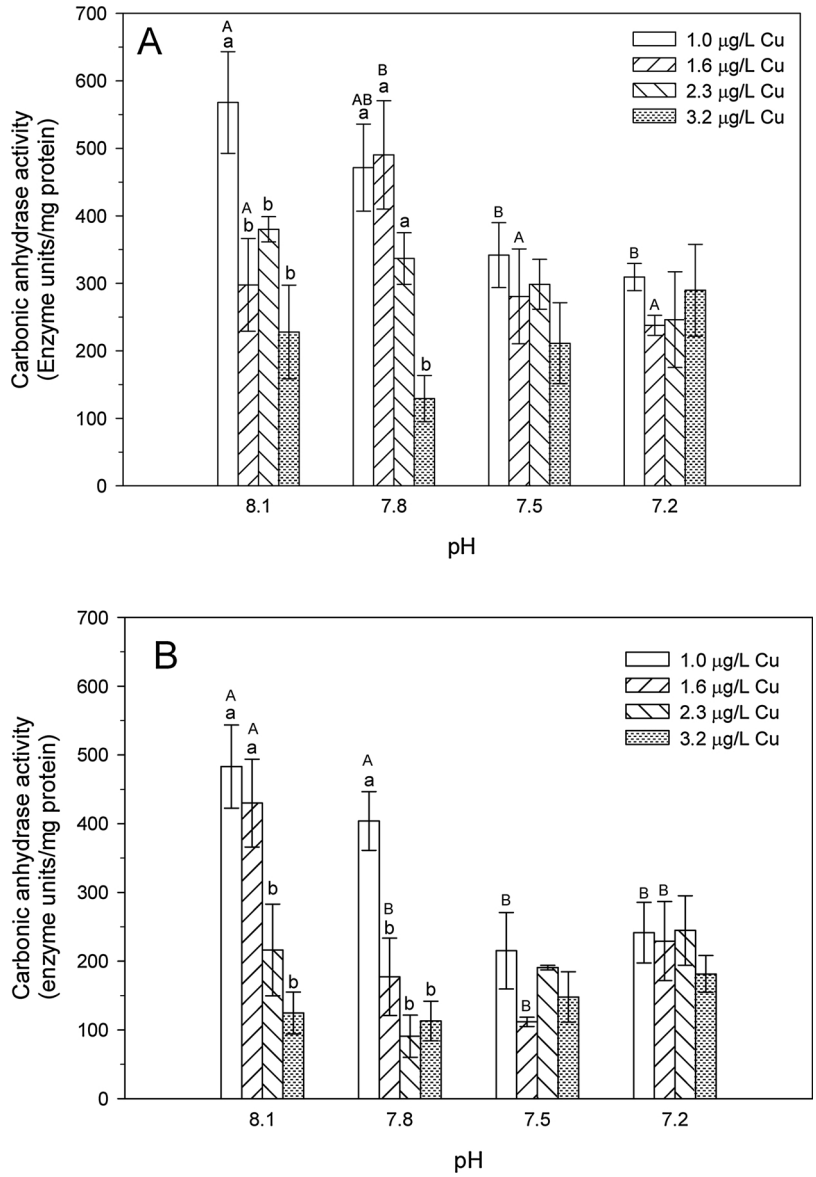

Fig. 4. Carbonic anhydrase activity in the coral *Mussismilia harttii* exposed to different combinations of reduced seawater pH and copper (Cu) concentrations in a mesocosm system for 15 days (A) or 35 days (B). Error bars are standard errors (n = 3 for each treatment). Different lowercase letters indicate significantly different mean values ($p \leq 0.05$) among Cu concentrations for the same pH treatment. Different uppercase letters indicate significantly different mean values ($p \leq 0.05$) among pH treatments for the same Cu concentration.

4. Discussion

Toxicity of metals is expected to be enhanced with ocean acidification. However, there are only few evidences on the occurrence of such interaction (Nikinmaa, 2013; Zeng et al., 2015; Bielmyer-Frasier et al., 2018). In the present study, 76% of the interactions between reduced seawater pH conditions and increasing Cu concentrations evaluated in the coral *M. harttii* were antagonistic, and only 24% were additive or synergistic. Deleterious effects on acid-base balance in *M. harttii* were likely intensified, at some extent, by the combination of stressors. However, the major finding is that the treatment with the lowest dissolved Cu addition tested in the present study had a consistent positive effect on Ca-ATPase activity in corals facing any of the reduced seawater pH conditions. Therefore, our findings suggest that the toxicity of metals in future ocean acidification scenarios can be less severe than previously suggested by forecasts based on metal speciation modeling at lower pH conditions (Millero, 2009; Zeng et al., 2015).

4.1. Effects of stressors alone

No significant change in *F*v/*F*m values was observed in corals acutely (15 days) or chronically (35 days) exposed to any of the experimental treatments tested. However, corals chronically exposed to the lowest (1.6 µg/L) and the highest (3.2 µg/L) Cu concentrations tested showed significant changes in *F*v/*F*m, with a slight enhancement and reduction in the photochemical capacity, respectively. These changes can be explained by the fact that the PSII complex is sensitive to various pollutants, including metals. However, its susceptibility to these stressors is highly variable. Higher concentrations of heavy metals are known to inhibit the PSII photochemical efficiency by replacing the central Mg^{2+} in the chlorophyll molecule (Kupper et al., 1996; Giardi et al., 2001). In turn, low metal concentrations can stimulate the PSII photochemical activity (Giardi et al., 2001). For example, Biscéré et al. (2015) observed increased *F*v/*F*m values in *Stylophora pistillata* exposed to increased cobalt concentrations. However, no alterations were observed in *Acropora muricata*. Bielmyer et al. (2010) reported reduced

Fv/Fm values in *Pocillopora damicornis* exposed to 4 μg/L Cu, while no effects were observed in *Acropora cervicornis* and *Orbicela faveolata* exposed to higher Cu concentrations (up to 10 μg/L) for 35 days. Also, a study performed by Marangoni et al. (2017b) reported no effects on *Fv/Fm* values in *M. harttii* after exposure to Cu concentrations ranging from 3.0 to 6.7 μg/L for 12 days. Altogether, these results highlight that the susceptibility of PSII complex to metals can greatly vary among zooxanthellate coral species, which could be explained by differences in algal symbiont communities (Bielmyer et al., 2010) and the different exposure times tested in the different studies.

A transient positive effect on chlorophyll *a* content was observed after acute exposure to Cu (1.6 μg/L). A recent study performed with two coral species also reported a 2-fold increase in chlorophyll concentration due to seawater enrichment with nickel (Biscéré et al., 2018). Several metals are essential elements to the metabolism of plants and are often used as mineral fertilizers (Kupper et al., 1996). Therefore, chlorophyll synthesis is likely stimulated by seawater with 0.6 μg/L Cu above ambient condition, which was not an excessive enough concentration to produce toxicity.

As observed in the present study, previous reports did not observe alterations in chlorophyll *a* content of corals due to increased pCO_2 levels. Biscéré et al. (2015) did not report any change in the coral *A. muricata* exposed to seawater at pH 7.8. Also, Ventura et al. (2016) did not report changes in chlorophyll *a* content in the symbiotic sea anemone *Anemonia viridis* during long-term *in situ* experiments at natural CO_2 vents, or in a 3-week laboratory experiment with seawater at pH 7.7. These findings are in agreement with the assertion that phototrophs, such as zooxanthellate corals, are able to use bicarbonate (HCO_3^-) effectively and are not considered as being carbon-limited due to the large amount of inorganic carbon present in seawater as HCO_3^- (Raven, 1997; Gattuso et al., 1999). Thus, photosynthesis under CO_2 enrichment would be stimulated only in species lacking carbon-concentrating mechanism (CCM) or when the CCM is not operating (Gattuso et al., 1999).

Ca-ATPase is suggested to drive the calcification reaction towards the formation of $CaCO_3$ by transporting Ca^{2+} into the calcification site, while removing protons (H^+) from this medium (Allemand et al., 2011). Reduced seawater pH and Cu exposure alone had negative effects on Ca-ATPase activity. After chronic exposure, the different seawater pH treatments tested caused a similar level of enzyme activity inhibition (~45%). In turn, Cu at the two highest concentration tested (2.3 and 3.2 μg/L) caused up to 67% of enzyme inhibition. Considering that Ca-ATPase is a key enzyme involved in the coral calcification process by acting as a $Ca^{2+}/2H^+$ exchanger (Allemand et al., 2011), the observed inhibition effects could result in an impairment of the calcification process (Marangoni et al., 2017b). Recent studies on the effects of reduced seawater pH in different calcifying organisms, such as hydrocorals and symbiont-bearing foraminifers, show evidence that Ca-ATPase plays an essential role in the maintenance of calcification rates (Prazeres et al., 2015; Marangoni et al., 2017a; Marques et al., 2017). Hydrocorals and foraminifers exposed to reduced seawater pH conditions showed increased Ca-ATPase activity to counteract the reduced Ω_{Cc} (Prazeres et al., 2015; Marangoni et al., 2017a). In contrast, the zooxanthellate coral *M. harttii* did not show any compensatory response after exposure to reduced seawater pH in the present study. In fact, Ca-ATPase activity was reduced under the experimental conditions tested. Concerning the effects of metals on Ca-ATPase activity in corals, our results are in agreement with previous studies (Marangoni et al., 2017b; Fonseca et al., 2017). In this context, a possible explanation for Cu-induced inhibition of Ca-ATPase activity is the fact that divalent metal ions can compete with Ca^{2+} for binding sites in the organism and modify the functioning of ion transporters (Bianchini and Wood, 2003). Therefore, considering data reported in the literature and the present findings, we suggest that the calcification process in *M. harttii* can be negatively affected by increased Cu concentrations and reduced seawater pH conditions through to two possible mechanisms: (i) lack of substrate (Ca^{2+}) for the precipitation of $CaCO_3$; and (ii) decrease of Ω_{ar} due to the difficulty of maintaining a more alkaline pH at the calcification site.

CA is an essential enzyme for the acquisition of inorganic carbon used in the calcification process by the coral host, as well as the symbiotic algae during photosynthesis (Bertucci et al., 2013). Also, this enzyme is associated with the maintenance of acid-base balance in tissues (Burnett, 1997; Bertucci et al., 2013), and in the removal of carbonic acid produced during the calcification process from the calcification site (Goreau, 1959). As previously reported (Bielmyer et al., 2010), negative effects on CA activity due to Cu exposure alone were also observed. After acute and chronic exposures, all Cu addition treatments tested caused CA inhibition (up to 74%). However, it is important to note that corals exposed to the lower Cu concentration (1.6 μg/L) recovered CA activity to control levels after 35 days of experiment. Our results are in agreement with those reported by Bielmyer et al. (2010), who observed a decreased CA activity in the corals *A. cervicornis* and *O. faveolata* after 35 days of exposure to increased concentrations of Cu (10 and 20 μg/L). This highlights different susceptibilities among coral species to Cu exposure, since CA inhibition in *M. harttii* was observed in the present study at much lower Cu concentrations (2.3 and 3.2 μg/L) after 35 days of experiment. Considering that zooxanthellae are the main sink of essential metals in the symbiotic associations (Ferrier-Pagès et al., 2018), different susceptibility and mechanisms of toxicity observed in coral species exposed to Cu may be related to differences in the algal symbiont communities (Bielmyer et al., 2010).

Reduced seawater pH alone also caused inhibition of CA activity (up to 53%) in the mild (pH 7.5) and most severe (pH 7.2) conditions of seawater reduced pH after acute and chronic exposure. It has been suggested that an increase in CA activity would be necessary under reduced seawater pH conditions to maintain the acid-base balance in tissues (Patel and Bielmyer-Fraser, 2015; Marangoni et al., 2017a), as well to increase the rates of CO_2 conversion into HCO_3^- (Vidal-Dupiol et al., 2013). However, as observed in the present study, an inhibition of CA expression/activity was detected in corals and anemones facing a high pCO_2/reduced pH environment (Moya et al., 2012; Ventura et al., 2016). In this context, decreased CA activity could reflect an acclimation mechanism to reduce CCM energy demands under high pCO_2 conditions (Moya et al., 2012; Ventura et al., 2016). In this case, the ability of calcifying photosynthetic anthozoans to shift the HCO_3-conversion into CO_2 would be energetically favorable, resulting in increased energy availability to other functions. In fact, the apparent absence of negative effects on the photosynthetic metabolism in the present study supports this idea.

4.2. Interactive effects of stressors

The combination of stressors tested in the present study had variable effects on the chlorophyll *a* content after both exposure times. However, most of the combined treatments did not show negative effects on chlorophyll *a* content with respect to the control condition. In fact, 89% of the interactions tested were antagonistic. Furthermore, in contrast to the chronic effects observed when the stressors were tested alone, intensified negative effects on Ca-ATPase activity were observed only after acute exposure to the combination of stressors. After chronic exposure, all interactions had an antagonistic effect on the enzyme activity. Additionally, the lower Cu concentration treatment (1.6 μg/L) seems to be beneficial to corals facing reduced seawater pH conditions. This statement is based on the fact that Ca-ATPase activity was similar to the control levels (pH 8.1 and no Cu addition) at all reduced seawater pH treatments combined with the 1.6 μg/L Cu treatment. Also, it is important to note that this Cu treatment was the only one that did not cause enzyme inhibition when tested alone.

A recent study by Ferrier-Pagès et al. (2018) showed that essential micronutrients to corals, such as Cu, which is assimilated through

heterotrophic feeding, are beneficial to corals undergoing thermal stress and subsequent bleaching. However, unfed coral colonies rely on the uptake of dissolved inorganic compounds by the symbionts, which are the main sink of essential metals in the symbiotic associations (Ferrier-Pagès et al., 2018). In this context, we suggest that the lower dissolved Cu concentration tested (1.6 µg/L Cu) led to positive effects on the symbiotic metabolism. This would result in more energy to be invested in Ca-ATPase activity for a compensatory response of the coral to counteract the deleterious effects of the reduced seawater pH on the calcification process. As previously suggested for corals (Cohen and Holcomb, 2009) and reported for other calcifying organisms (Prazeres et al., 2015; Marques et al., 2017; Marangoni et al., 2017a,b), Ca-ATPase is an important enzyme for the maintenance of the calcification rate. Furthermore, the beneficial effect of the lowest Cu concentration tested (0.6 µg/L Cu above the ambient condition) on the symbiotic metabolism is reinforced by other findings of the present study that indicate positive effects in the photosynthetic metabolism due to this slight increase in Cu concentration (see results for PSII efficiency and chlorophyll *a* content).

Concerning the combined effects of reduced seawater pH and Cu exposure on CA activity, 61% of interactions were antagonistic. However, all interactions between Cu exposure and the milder condition of reduced seawater pH (pH 7.8) were additive or synergetic. Alongside, all interactions between the lower Cu concentration treatment (1.6 µg/L) and the different reduced seawater pH were also additive or synergetic, with significant decreases in CA activity. Considering that overall no significant effects of the exposure to the combination of stressors were observed on the photosynthetic efficiency, we suggest that additive and synergistic effects leading to a reduction in CA activity did not lead to impairment in the photosynthetic carbon metabolism. However, it is worth noting that in the present study CA activity was evaluated in the holobiont. Therefore, possible deleterious effects on *M. harttii* acid-base balance and inorganic carbon supply for the calcification process due to inhibition of CA cannot be ruled out (Bertucci et al., 2013).

Finally, as indicated by the observed predicted inhibition plots, most of the interactions observed in the present study were antagonistic. Despite the fact that the free ion form of Cu is known to increase in more acidic conditions (Millero, 2009; Zeng et al., 2015), with a consequent expected increase in metal toxicity, an increased concentration of protons (H^+) would be also observed at reduced seawater pH (Pascal et al., 2010). In this context, it is important to note that increased concentrations of H^+ can increase the competition with Cu for the binding sites on the organism (Pascal et al., 2010). Thus, this competition could inhibit the toxic effects of Cu on biomolecules, resulting in prevailing antagonistic effects between reduced seawater pH and increased Cu concentrations.

4.3. Conclusions

Information on the toxic interactive effects between reduced seawater pH and metals on scleractinian corals, and other organisms, is scarce. In contrast to metal speciation modeling predictions (e.g. Millero, 2009; Zeng et al., 2015), using a mesocosm, an ecologically relevant experimental approach, and a set of specific biochemical biomarkers, we were able to demonstrate that most interactions (76%) between different levels of reduced seawater pH and increasing Cu concentrations did not increase the Cu toxicity in the zooxanthellate scleractinian coral *M. harttii*. In this context, the predominant antagonistic toxic effects observed seem to be related to an increased competition between H^+ and Cu for binding sites on the organism. Additionally, it is worth noting that increasing Cu concentrations, as low as 0.6 µg/L above the ambient level, could lead to possible beneficial effects on the calcification process in corals facing reduced seawater pH by maintaining the basal level of Ca-ATPase activity. Overall, the present study brings new and relevant evidence on the toxic effects of reduced seawater pH and Cu exposure in corals. Also, it highlights the importance of future investigations on the interaction between multiple stressors for a better understanding of the mechanisms of toxicity associated with these stressors.

Acknowledgments

Dr. Emiliano Calderon, Ms. Cristiano Pereira, Dr. Gustavo Duarte and Coral Vivo staff are acknowledged for their support during experiments. The International Development Research Centre (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) are acknowledged for their financial support. The Coral Vivo Project and its sponsors Petrobras, through the Petrobras Environmental Program, and Arraial dÁjuda Eco Parque are acknowledged for their support in field research. A. Bianchini is a research fellow from the Brazilian CNPq (Proc. # 307647/2016-1). L.F.B Marangoni was a graduate fellow from CAPES.

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APÊNDICE II

- ***Manuscrito I***

[Em preparação, a ser submetido ao Periódico *Ecological Indicators*]

Oxidative stress biomarkers as potential tools in reef degradation monitoring: a study case in a South Atlantic reef under influence of the 2015-2016 El Niño / Southern Oscillation (ENSO)

- ***Manuscrito II***

[Submetido ao Periódico *Coral Reefs*, na Edição Especial *The 2014 - 2017 Global Coral Bleaching Event (CORE-D-18-00300)*]

Peroxynitrite generation and trophic plasticity in reef-building corals during a natural bleaching event influenced by the 2015-2016 El Niño Southern Oscillation (ENSO)

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Biomarcadores de estresse oxidativo como potenciais ferramentas no monitoramento de recifes de coral: um estudo de caso em um recife do Atlântico Sul sob influência do El Niño – Oscilação Sul (2015-2016)

Oxidative stress biomarkers as potential tools in reef degradation monitoring: a study case in a South Atlantic reef under influence of the 2015-2016 El Niño / Southern Oscillation (ENSO)

Oxidative stress biomarkers as potential tools in reef degradation monitoring: a study case in a South Atlantic reef under influence of the 2015-2016 El Niño / Southern Oscillation (ENSO)

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ABSTRACT

The third global-scale coral bleaching event, triggered by the 2015-2016 El Niño, presented unprecedented levels of thermal stress and bleaching occurrence. Identification of potential cellular biomarkers in key reef species can greatly improve coral reef resource manager's ability to make ecological forecasts and develop efficient mitigation strategies. In this context, the present study evaluated ecologically relevant biochemical parameters involved in thermal-stress response in two important reef building species of southwestern Atlantic Reefs - the scleractinian coral *Mussismilia harttii* and the hydrocoral *Millepora alcicornis* - aiming to assess their potential as biomarkers in corals/hydrocorals health assessment. Bleaching frequency, lipid peroxidation (LPO) and total antioxidant capacity (TAC), as well as thermal stress parameters, were monitored during a six-month period in a reef area under influence of the 2015-2016 El Niño event. Results indicate *M. alcicornis* as a promising bioindicator in South Atlantic reefs and reinforce the potential of oxidative stress biomarkers as prognostics tools of reef degradation. LPO is suggested as an informative, cost-effective and logical complement to reef monitoring programs; and TAC basal level is suggested as a potential measurement for predicting corals/hydrocorals susceptibility to bleaching. Findings presented here are expected to contribute with potential biomarker-monitoring techniques to be used as additional tools in traditional reef monitoring programs.

Keywords: Lipid peroxidation, Total antioxidant capacity, bleaching, corals, hydrocorals, thermal stress.

INTRODUCTION

The ecological success of coral reefs is largely attributed to the symbiotic relationship between scleractinian corals and dinoflagellates from the family Symbiodiniaceae, known as zooxanthellae (Hoegh-Guldberg, 1999; LaJeunesse et al. 2018). Despite being the most diverse and productive marine ecosystem on the planet, coral reefs are experiencing extensive degradation worldwide due to climate change (Spalding and Brown, 2015; Hughes et al. 2017). One of the reasons leading to this global degradation is a process, known as bleaching, whereby corals and other invertebrates that establish symbiotic relationships with microalgae, expel their symbionts or experience degradation of algae photosynthetic pigments (Downs et al. 2000). Tropical corals live close to the upper thermal limit of such symbiosis and heat stress is a primary factor in many large-scale bleaching events (Hughes et al. 2018; Lough et al. 2018). In fact, unusually warm sea surface temperature (SST) constitutes the basis of the National Oceanic and Atmospheric Administration (NOAA) Coral Reef Watch (CRW) monitoring program (http://coralreefwatch.noaa.gov/satellite/index.php).

Global climate change impacts (e.g. increasing SST) are being intensified by pulse heat stress events, such as El Niño, the warm phase of the El-Niño Southern Oscillation (ENSO) (Claar et al. 2018). According to climate modelling evidence, extreme El Niño occurrences are expected to increase in response to greenhouse warming, leading to profound ecological and socio-economic consequences (Cai et al. 2014). Indeed, the third global-scale coral bleaching event, triggered by the 2015-2016 ENSO, presented unprecedented levels of thermal stress and bleaching occurrence over the period 1871-2017 (Claar et al. 2018; Lough et al. 2018, Hughes et al. 2018). Specifically, large-scale bleaching has been reported in Southwestern Atlantic reef areas since the 1990s (for a review see Leão et al. 2016). In 1994, severe bleaching was

reported in the Abrolhos bank during summer period (18◦ S, Castro and Pires, 1999); and in 1998, bleaching events were reported in eastern Brazil (12-13◦ S, Leão et al. 2003, 2008). The former reports associated the bleaching events with temperature anomalies. Further, in 2002, 2003 and 2005, mild to moderate bleaching were reported in eastern (12-18° S, Leão et al. 2008) and northeastern Brazil (9° S, Ferreira e Maida, 2006).

Substantial evidences have demonstrated that reactive oxygen species (ROS) are pivotal in the physiology of coral bleaching (Lesser 1997; Downs et al. 2000, 2002; Perez and Weis, 2006; 2008; Ross, 2014; Nielsen et al. 2018). Oxidative stress - when the antioxidant capacity of an organism is overwhelmed by excessive ROS production - leads to several cellular toxicity processes, such as oxidative damage to biomolecules, thus affecting cell functionality (Halliwell and Gutteridge, 2007). Currently, it is widely agreed that the oxidative stress condition plays a key role in the breakdown of the symbiotic relationship between cnidarians and their endosymbionts (Downs et al. 2002; Lesser, 2006). In this case, it is important to note that enzymatic and non-enzymatic antioxidant systems operate in both host and endosymbionts, simultaneously, to modulate stress-induced ROS and, thus, maintain cell homeostasis (Gardner et al. 2017). Thus, variations in the endogenous levels of antioxidants and oxidative damage have been proposed as useful indicators of marine environment health, including coral reefs (Gorbi and Regoli, 2003; Downs et al. 2005, 2011, 2012; Prazeres et al. 2012).

Modified environmental conditions (e.g. higher temperature, reduced salinity or presence of toxicant) can lead to biochemical alterations before injury in some biological function or mortality can be observed. In this context, biological markers (or biomarkers), defined as any biological response in molecular, cellular and physiological levels, can be useful in identifying adverse effects caused by environmental stressors in

marine organisms (McCarthy and Shugart, 1990; Bradley, 2012). Based on an understanding of cellular-level processes, the use of biochemical biomarkers enables to forecast biological effects linked to environmental degradation (Depledge et al., 1995; Downs et al. 2005).

Lipid peroxidation is one of the most prevalent mechanisms of cellular injury and has been extensively reported for marine organisms (Lesser, 2006). In fact, there is growing evidence that biomarkers directly associated with cellular damage, such as lipid peroxidation, are good predictive tools to assess the prospect of organisms bleaching/mortality (Downs et al. 2005; Prazeres et al. 2011, 2012; Fonseca et al. 2017; Marangoni et al. 2017). Also, stress defense mechanisms related to organisms antioxidant apparatus have been suggested as indicators of stress susceptibility (Downs et al., 2002, 2005; Griffin and Bhagooli, 2003; Gorbi and Regoli, 2003; Prazeres et al. 2012 ; Marangoni et al. 2017).

The great majority of studies on oxidative stress evaluate variations of individual antioxidants (e.g. specific enzymes activities) which are useful, for example, to understand the action mode of a stressor. However, antioxidants are part of very complex homeostatic system, acting cooperatively. Thus, quantification levels of a putative protective agent may not reflect the real oxidative status condition of an organism (for a review see Gorbi and Regoli, 2003). In this context, measuring organisms overall oxyradical scavenging capacity (here and after referred as total antioxidant capacity - TAC) provides a more integrated assessment of susceptibility of a tissue/organism to oxidative stress, and methods have been developed for this purpose (see Huang et al. 2005).

Environmental regulations can benefit from biomarkers and resource management can be more effective if coordinated in an interdisciplinary and integrative

approach (Bradley, 2012). In this context, the identification of potential biomarkers for coral health assessment is considered a powerful tool for providing information on the health condition of coral reefs (Downs et al. 2005). Therefore, identification of potential cellular biomarkers in key reef species, to be jointly applied to traditional methods of reef monitoring (e.g. visual bleaching surveys, water quality evaluation and SST monitoring) can greatly improve coral reef resource manager's ability to make ecological forecasts, allowing the development of appropriate and efficient mitigation strategies.

Herein, we evaluated ecologically relevant biochemical parameters involved in thermal-stress response in two important reef building species of southwestern Atlantic Reefs - the scleractinian coral *Mussismilia harttii* and the hydrocoral *Millepora alcicornis* - aiming to assess their potential as biomarkers in corals/hydrocorals health assessment. Bleaching frequency, lipid peroxidation (LPO) and total antioxidant capacity (TAC), as well as thermal stress parameters, were monitored during a six-month period in a reef area under influence of the 2015-2016 El Niño event. Findings presented here are expected to contribute with potential biomarker techniques that may be used as additional tools in monitoring programs that simply document the visual frequency of bleaching, loss of reef organism's abundance and diversity.

MATERIAL AND METHODS

Bleaching survey and biological sampling

Mussismillia harttii and *Millepora alcicornis* colonies were monitored for bleaching occurrence every 15 days, from December 15, 2015, to June 15, 2016 (total of 12 sampling days), by SCUBA divers in three reef sites located at the Recife de Fora Marine Protected Area (Porto Seguro, Bahia, northwestern Brazil) (Figure 1). Reef sites

were as follows: (1) 16°24'25.3" S, 038°58'41.0" W; (2) 16°25'02.6" S, 038°58'55.8" W; and (3) 16°24'39.0" S, 038°59'06.1" W. Twenty-meters long transects were haphazardly laid at approximately 2 meters depth (low tide) in each reef site. Colonies were photographed side by side with a PVC Coral Health Chart (CoralWatch, University of Queensland, Australia) to detect coral bleaching, along the fixed transects. The total number of transects performed was 36. Bleaching frequency was evaluated following the modified coral watch protocol (www.reefquest.org). The reef sites selected in the present study are included in the Brazilian Reef Check monitoring program (www.reefcheck.org, www.recifescosteiros.org.br) and are part of a recent Brazilian Conservation Plan for Endangered Species of Reef Environments (PAN Corais in the Portuguese abbreviation, ICMBio, 2016; Castro et al. 2016). Also, these reef sites were selected according to the National Oceanic and Atmospheric (NOAA) predictions for coral bleaching hotspots in the Brazilian summer of 2015-2016 (https://coralreefwatch.noaa.gov/satellite/analyses_guidance/global_coral_bleaching_2014-17_status.php).

Alongside coral bleaching surveys, fragments (~ 2 cm^2) of *M. harttii* and *M. alcicornis* (most abundant reef builders in these sites; unpublished data, E. N Calderon), coming from different colonies *(n = 4* per species*)*, were sampled for laboratory analyses and frozen in liquid nitrogen.

Thermal stress determination

Temperature data loggers (HOBO Pendant model UA-002-64) were placed in each of the reef sites to record local temperature conditions over all monitoring period. Temperature was recorded every 15 min. Estimations of coral thermal stress in the study area followed methods developed by the National Oceanic and Atmospheric

(NOAA) Coral Reef Watch (CRW) program. The onset of potentially damaging coral bleaching was defined to begin at Degree Heating Weeks (DHW; expressed in units of ℃-weeks) values of 4℃-weeks or greater. DHW are the accumulation of temperature anomalies exceeding the maximum of the monthly mean SST for a given region over a 12-week period (Liu et al. 2003, 2006, 2013). Only anomalies ≥1℃ were used based on the assumption that anomalies < 1℃ SST are insufficient to cause visible stress to corals (Kayenna, 2017). For example, 2℃-weeks correspond to two weeks of constant anomalous SST at 1 ℃ within the 12-week period, or one week at 2 ℃, and so on. DHW values above 4℃-weeks are believed to induce bleaching, in turn, DHW values above 8.0 ℃-weeks are considered to result in widespread bleaching and mortality (Kayenna, 2017). DHW calculated for the study area, considering the three reef sites, can be seen in Figure 2.

Sample preparation for biomarkers analyses

Small pieces (~ 0.5 cm^2) of corals and hydrocorals were homogenized by ultrasound (Frequency 70 kHz, sonicator Sonaer, Farmingdale, NY, USA), using the specific buffer solution for each analysis, as described below. The holobiont homogenized samples were centrifuged (13,000 g) at 4℃ for 10 min and the intermediary phase, containing animal (coral) and zooxanthellae tissues, was collected and immediately used for biomarkers analyses.

Lipid peroxidation

Lipid peroxidation (LPO) was measured using the method based on the thiobarbituric acid reactive substances (TBARS), as described by Oakes and Van Der Kraak (2003). Samples were homogenized in KCl (1,15%) solution containing 35 µM

butylatedhydroxytoluene (BHT). According to the method employed, malondialdehyde (MDA), resulting from lipid peroxidation, reacts with the thiobarbituric acid (TBA) under acidic and high temperature (95°C) conditions, thus generating a fluorescent chromogen (excitation: 515 nm; emission: 553 nm). Measurements were performed using a fluorescence microplate reader (Victor 2, Perkin Elmer, Waltham, MA, USA). Results were normalized by the amount of total protein inside each well and expressed in nmol MDA/mg protein. Protein content in the sample homogenate was determined, using a commercial reagent kit based on the Bradford assay (Sigma-Aldrich, St. Louis, MO, USA).

Total antioxidant capacity

Measurement of total antioxidant capacity (TAC) in coral samples was performed using the "OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit" (Cell Biolabs Inc., San Diego, CA, USA) following manufacture instructions. This assay measures the total antioxidant capacity of biomolecules via single electron transfer (SET) mechanism (Huang et al. 2005), with marginal radical interference. The method is based on the reduction of copper (II) to copper (I) by antioxidants, such as uric acid. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. The net absorbance values of antioxidants were compared with a known uric acid standard curve. Absorbance values are proportional to the sample's total reductive capacity. Absorbance readings were performed using a microplate reader (ELx-800, Biotek, Winooski, VT, USA). Data were normalized considering the total protein content in the sample homogenates and expressed as µM Copper Reducing Equivalents (CRE) g protein^{-1}.

Protein content in the sample homogenate was determined, using a commercial reagent kit based on the Bradford assay (Sigma-Aldrich, St. Louis, MO, USA).

Data presentation and statistical analyses

Results were expressed as mean ± standard error. Bleaching frequency is expressed as percentage, considering all reef sites grouped by month. Biomarkers (TAC and LPO) data were grouped in the same way and evaluated over time using one-way analysis of variance (ANOVA). If indicated, ANOVA were followed by pos hoc Tukey´s test. Homogeneity of variances and data normality were checked prior to the analyses using Levene´s and Shapiro-Wilk´s tests, respectively. Data was log-transformed to meet ANOVA assumptions. In turn, multivariate statistical analyses are required to integrate and interpret multiple biomarker data, and, in this context, the use of Principal Components Analyses (PCA) has been suggested as a promising method (see Allen and Moore, 2004). Thus, aiming to integrate the biological markers response and to examine the association between variables, a PCA analysis was performed considering values of thermal stress (DHW and maximum temperatures) and biological parameters (oxidative stress biomarkers and bleaching frequency). Data were z-transformed prior to PCA. Statistical analyses were performed in R, version 3.3.3 (R Development Core Team, 2015). In all cases, the significance level adopted was 95% ($\alpha = 0.05$).

RESULTS

Thermal stress and bleaching

Mean temperature at the Recife de Fora Marine Protected Area, by grouping data from the three monitored reef sites, was 25.9 ± 1.3°C. Maximum and minimum

temperatures were 34.2 and 20.5°C, respectively. Critical thermal stress for corals, indicated by Degree Heating Weeks (DHW) values of near 4°C-weeks, was observed during April and May (Figure 2). Accordingly, *M. alcicornis* presented the highest bleaching frequency (up to 73%) from March to May (Figure 3a); while *M. harttii* showed highest bleaching occurrence (up to 34%) between April and May (Figure 4a).

Oxidative stress biomarkers

Significant variations in TAC levels were observed over time in both species (Tables 1). Increased TAC levels were observed in March for *M. alcicornis* (Fig. 3c); while in this same month decreased TAC levels were observed for *M. harttii* (Fig. 4c). Based on data of months presenting no or low (less than 10%) bleaching occurrence, TAC basal levels for *M. alcicornis* and *M. harttii* were 208,2 and 743,9 µM CRE / mg protein, respectively.

Significant variations in LPO were observed for *M. alcicornis* over time (Table 1). Increased values of LPO were observed from February to May. In June, when corals visually recovered from bleaching, LPO levels returned to basal levels (~7,1 nmol MDA / mg protein), considering data from months that less than 10% of bleaching was observed for *M. alcicornis* (Fig. 3b). Regarding *M. harttii*, no changes for LPO were observed over sampling time (Table 1; Fig. 4b), and basal levels were 358,4 nmol MDA / mg protein.

Principal component analysis (PCA)

The PCA performed for *M. alcicornis* extracted 5 factors and revealed 67.4% of the total variance. PC1 represented 41.3% of variance, while the second component (PC2) explained 26.1% of variance. A strong positive relationship for LPO, DHW,

maximum temperatures and bleaching was observed, associated with a clustering of the month/time points of April and May. Although weaker, a positive relationship between TAC and bleaching was also observed. As expected, a negative relationship between TAC and LPO was observed. Also, TAC was associated with a clustering of month/time points of March (Figure 5).

The PCA performed for *M. harttii* also extracted 5 factors and revealed 68.6% of the total variance. PC1 explained 46.3% of variance, while PC2 represented only 22.3%. A strong positive relationship for TAC, DHW, maximum temperature and bleaching was observed, associated with a clustering of the month/time points of April and May. As expected, a strong negative relationship between TAC and LPO was also observed, associated with a clustering of month/time points of March. Biplot visual examination reinforces the strong antioxidant capacity response of *M. harttii* in opposition with the occurrence of LPO (Figure 6).

In summary, PCA confirmed the strong association between bleaching and oxidative stress biomarkers (LPO and TAC), as well as reinforced the association between biological responses and thermal stress parameters (DHW and maximum temperatures).

DISCUSSION

The development of biological markers in corals and other coral reef species has been suggested to have a great potential to enhance the ability of resource managers to make ecological forecasts (Downs et al. 2005). In this context, the present study brings new evidence that the quantification of LPO and TAC levels, in corals and hydrocorals, have great potential to be used as auxiliary tools in reef monitoring programs in southwestern Atlantic reefs and other reef locations worldwide.

LPO and TAC as complementary tools in reef monitoring strategies

Unusually warm sea surface temperatures (SST) are used by the National Oceanic and Atmospheric Administration (NOAA) Coral Reef Watch (CRW) monitoring program to generate near-real-time coral bleaching `HotSpots' maps. Further, these HotSpots are summed to estimate DHW, which are used to predict bleaching events (Kayenna, 2017). In the present study, visual bleaching occurred in accordance with the temperature data collected. The estimated DHW for the monitored reef sites indicated that bleaching was expected to occur during the months of April and May. In fact, both species monitored showed significant levels of bleaching during these months, with the highest bleaching occurrence in April and May for *M. alcicornis* (up to 73%) and *M. harttii* (up to 34%), respectively. Also, despite not indicated by DHW, *M. alcicornis* presented bleaching occurrence of ~ 46% during March. These results highlight *M. alcicornis* higher susceptibility to bleaching due to thermal stress.

In parallel to bleaching observations, fluctuations in biomarkers levels tested in the present study were observed before, during, and after bleaching occurrence. LPO levels did not change in *M. harttii* during the sampling period, however, *M. alcicornis* showed significant increased levels from February to May. In turn, TAC levels showed significant changes in both species during the month of March. While *M. alcicornis* presented increased levels of TAC, *M. harttii* showed decreased levels in this month. LPO and TAC results highlight species-specific responses. LPO showed to be more responsive in *M. alcicornis*, and TAC, despite varying in both species, showed a potential predictive capability for *M. harttii*, but not for *M. alcicornis*. These results were reinforced by the statistical multivariate approach (PCA) employed, since a strong positive relation was observed for bleaching and LPO for *M. alcicornis*, while a more positive relation between bleaching and TAC was observed for *M. harttii*.

General indicators can be sensitive and cost-effective tools for the determination of a stress condition, responding in a manner that suggests a potential problem. For example, the determination of oxidative stress (e.g. damage to biomolecules) and-or antioxidant responses in aquatic organisms have been commonly employed as non-specific biomarkers, since many pollutants and environmental factors (e.g. temperature variation) can cause modifications in the balance between pro-oxidants and antioxidants (Monserrat et al. 2007). Reactive oxygen species (ROS) production is a key element in the cellular pathology of bleaching, regardless of stressor (Baker and Cunning, 2016); and oxidative stress biomarkers have been successfully employed to characterize impacted reef areas (e.g. Prazeres et al. 2012; Downs et al. 2012). More specifically, oxidative stress biomarkers related to cellular damage and stress defense have been pointed out as good tools for the prognostic of bleaching/mortality and stress susceptibility in symbiont-bearing reef organisms, such as corals and foraminifers (Downs et al. 2005; Prazeres et al. 2011, 2012). The results presented here concerning damage to lipids (LPO) and TAC reinforce this statement.

Several factors must be considered for designing and executing a biomarker-based monitoring program, such as selecting adequate species to be collected for monitoring. In this context, spatial range and sensitivity to stressors should be considered (Shugart et al. 1992). Based on the literature and in the present findings, we suggest *M. alcicornis* as a potential animal sentinel of southwestern Atlantic reefs, and LPO as a potential biomarker to forecast bleaching occurrence. This affirmation is based on the fact that one and two months before bleaching was observed for *M. alcicornis* and *M. harttii*, respectively, LPO levels in *M. alcicornis* were significantly higher. *M. alcicornis* also showed a higher bleaching frequency than *M. harttii*, which indicates *M. alcicornis* as a better bioindicator due to its higher sensitivity to thermal

stress. Additionally, it is important to note that hydrocorals of the genus *Millepora* play a key ecological role in Brazilian reefs (Leão et al. 2003) and in tropical reef communities worldwide (Lewis, 2006).

Additionally, it is important to point out that the laboratory techniques employed in the present study for biomarker analyses are easily replicable, as well as more time and cost-effective, compared to other biomarkers techniques previously identified as potential tools for reef monitoring strategies (e.g. Downs et al. 2005, 2012; Louis et al. 2017; Parkinson et al. 2018). Furthermore, the little amount of biological sample needed (~ 0.2 cm^2) to perform the assays employed in the present study should make the biomarkers here presented to be considered as a non-destructive approach, since only a tiny fragment of the colonies was used in order to conduct the present study.

Physiological implications

In the present study, an oxidative stress condition was characterized for *M. alcicornis*, before and during bleaching, by the observed increased levels of TAC and lipid damage (LPO). TAC increased levels characterize a compensatory response to increasing thermal stress and lipid damage occurrence; however, TAC increased levels during the month of March were not enough to decrease lipid damage. In turn, after visual bleaching was no longer observed (June), LPO returned back to basal levels, which indicates hydrocorals were recovering from an oxidative stress condition. Also, since no alterations in TAC levels were observed after bleaching occurrence, it is possible to infer that other antioxidant mechanisms, such as specific enzymes, may be acting in order to rebalance the oxidative status of *M. alcicornis*.

In opposition to *M. alcicornis*, TAC levels in *M. harttii* decreased during March and no alterations in LPO levels were observed during the entire sampling period. Also,

bleaching occurrence for *M. harttii* was two-fold lower than observed for *M. alcicornis*. The decreased TAC levels indicate a possible imbalance in the oxidative status of *M. harttii* previous to the occurrence of bleaching. Despite lipid damage did not increase, bleaching occurrence *per se* is a good indicator that increased damage to biomolecules may have occurred. For example, Marangoni et al. (2017) exposed *M. harttii* to increasing concentrations of dissolved copper in seawater, which led this species to an oxidative stress condition evidenced by increasing DNA damage, while no lipid damage was observed. Thus, it is not possible to rule out the occurrence of oxidative damage to other kinds of biomolecules, such as proteins and genetic material.

Excessive sedimentation is one of the main threats to coral reefs, and reef building corals from Brazil coexist with a highly terrigenous environment (Leão & Ginsburg, 1997; Castro et al. 2012; Winter et al. 2016). In fact, almost all reefs located in the Brazilian continental shelf, which includes the reef sites monitored in the present study, are immersed in turbid waters (Leão e Dominguez, 2000). In this context, it has been suggested that *M. harttii* large polyps is a morphological adaptation for a greater resistance to environmental stresses, which gives this species a more efficient mechanism for cleaning themselves of sediment (Leão et al. 2003). From a biochemical point of view, results here presented suggest that *M. harttii* is better adapted to the southwestern Atlantic reefs conditions. According to the TAC levels observed in the present study, it is possible to infer that *M. harttii* naturally operates with maximal capacity against ROS formation. This is evidenced by the decreasing TAC levels in March for this species, which indicate that TAC is not being stimulated as observed for *M. alcicornis*; instead, it is being consumed.

The determination of bleaching susceptibility in reef-building corals is not an easy task due to difficulties of understanding the individual components tolerances in

the holobiont, however, it is agreed that oxidative stress in the host and symbionts play a major hole in the symbiotic partnership dysfunction (Rohwer et al. 2002; Weiss, 2008; et al. 2011). We suggest that holobiont´s TAC determination, such as the ones performed in the present study, can be a useful tool to address susceptibility to bleaching. This is suggested based on the fact that the 3-fold higher basal levels of TAC observed in *M. harttii*, compared to TAC basal levels in *M. alcicornis*, were followed by a 2-fold lower bleaching frequency in the former species. Previous studies suggest that specific adaptations linked to the coral´s antioxidant capacity drive differences in the tolerance of coral species to thermal stress (Gardner et al. 2017) and that measurements of total absorbance capacity of oxyradicals by a tissue or organism provide better understanding of its resistance to ROS (Gorbi and Regoli, 2003). Thus, a possible direct association between TAC and bleaching susceptibility, contemplating a greater number of coral and hydrocoral species, is here suggested as a relevant topic for future investigations.

Conclusions

Results presented here show ecological relevance of parameters measured at biochemical-cellular level that can be important features in reef environmental-monitoring studies, reinforcing the potential of oxidative stress biomarkers as prognostics tools of reef degradation. Specifically, the evaluation of LPO levels in *M. alcicornis*, a promising sentinel organism identified in the present study, is suggested as an informative, cost-effective and logical complement to reef monitoring programs in Atlantic reefs. It may be used in a wide geographical range as the species occurs from Florida to Southeastern Brazil and in Eastern Atlantic islands, such as Ascension, Cape Verde and Canary Islands (Hoeksema et al. 2014). Also, we suggest LPO as a potential

biomarker to be investigated as an auxiliary tool to assess reef environmental health in other reef localities worldwide, as well as further investigations on basal TAC levels for predicting corals/hydrocorals susceptibility to bleaching.

ACKNOWLEDGMENTS

The International Development Research Centre (IDRC, Ottawa, Canada), Coordenação de Aperfeiçooamento 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) are acknowledged for their financial support. The Coral Vivo Project and its sponsors, Petrobras, through the Petrobras Socioenvironmental Program, and Arraial d'Ajuda Eco Parque, are acknowledged for their support in field research. A. Bianchini (Proc. # 304430/2009-9) is a research fellow from CNPq. A. Bianchini is supported by the International Canada Research Chair Program (IDRC). L.F.B. was a graduate fellow from CAPES.

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FIGURES

Figure 1. Brazil coastline showing the state of Bahia, and the Recife de Fora Marine Protected Area (Porto Seguro, Bahia, northwestern Brazil).

Figure 2. Estimations of coral thermal stress defined as Degree Heating Weeks (DHW; expressed in units of °C weeks) for the study area, considering the three reef sites at conservation area of the Municipal Natural Park of Recife de Fora (Porto Seguro, Bahia, northwestern Brazil). The onset of potentially damaging coral bleaching is indicated by the red dashed line, the bleaching risk threshold defined by NOAA CRW (DHW values of 4°C weeks or greater).

Figure 3. (a) Bleaching frequency, (b) Lipid peroxidation (LPO), and (c) Total antioxidant capacity (TAC) in *Millepora alcicornis* from December 2015 to June 2016. Data are expressed as mean ± standard error. Lowercase letters indicate significantly different mean values ($p < 0.05$) in biomarkers levels over the sampling period.

Figure 4. (a) Bleaching frequency, (b) Lipid peroxidation (LPO), and (c) Total antioxidant capacity (TAC) in *Mussismilia harttii* from December 2015 to June 2016. Data are expressed as mean ± standard error. Lowercase letters indicate significantly different mean values ($p < 0.05$) in biomarkers levels over the sampling period.

Figure 5. Principal Component analysis ordination biplot using *Millepora alcicornis* biological markers [Total antioxidant capacity (TAC) and lipid peroxidation (LPO)], bleaching frequency and thermal stress parameters [Degree heating weeks (DHW) and maximum temperatures (TempMax)].

Figure 6. Principal Component analysis ordination biplot using *Mussismilia harttii* biological markers [Total antioxidant capacity (TAC) and lipid peroxidation (LPO)], bleaching frequency and thermal stress parameters [Degree heating weeks (DHW) and maximum temperatures (TempMax)].

Figure 1.

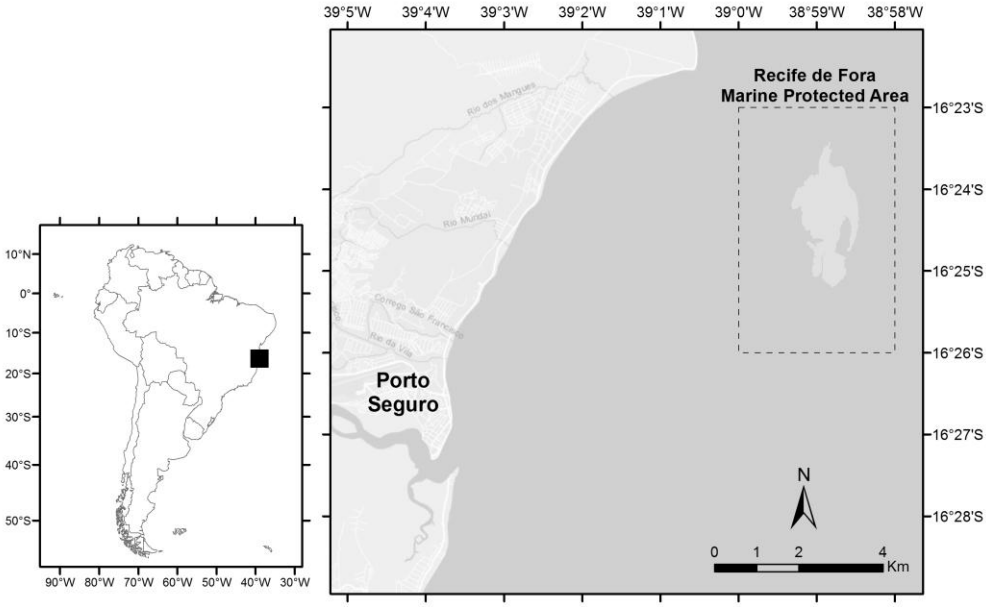


Figure 2.

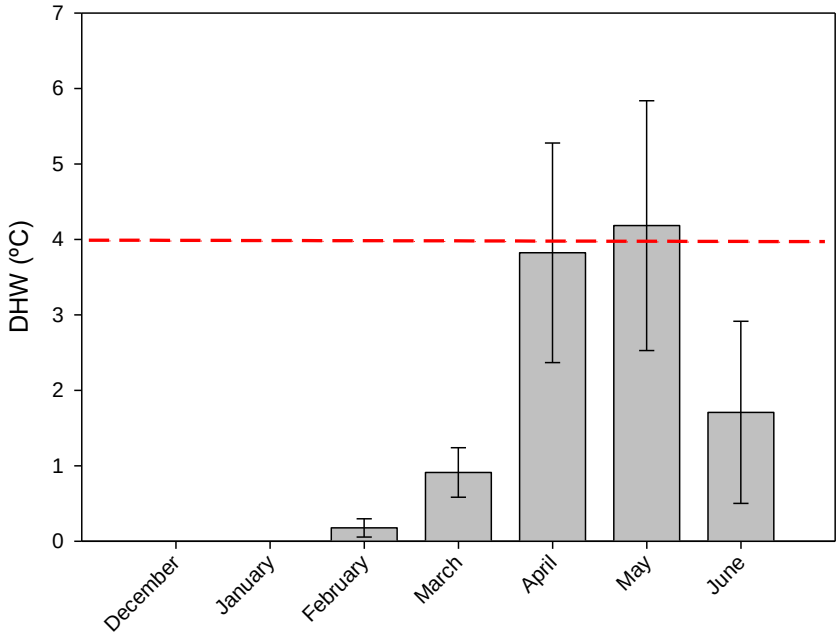


Figure 3.

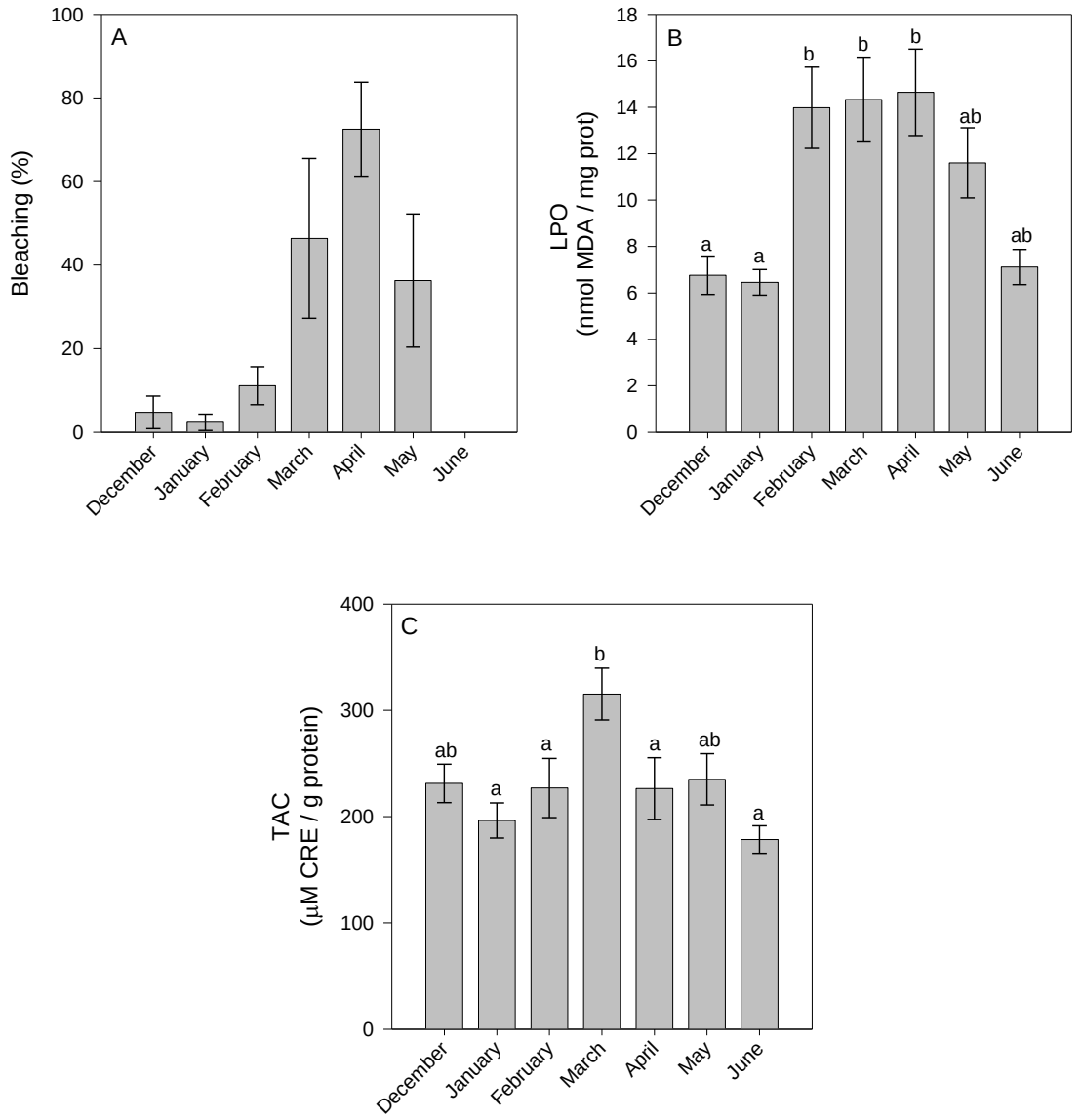

A
Bleaching (%)
100
80
60
40
20
0
December
January
February
March
April
May
June
B
LPO
(nmol MDA / mg prot)
18
16
14
12
10
8
6
4
2
0
a
a
b
b
b
ab
ab
C
TAC
(µM CRE / g protein)
400
300
200
100
0
ab
a
a
b
a
ab
a

Figure 4.

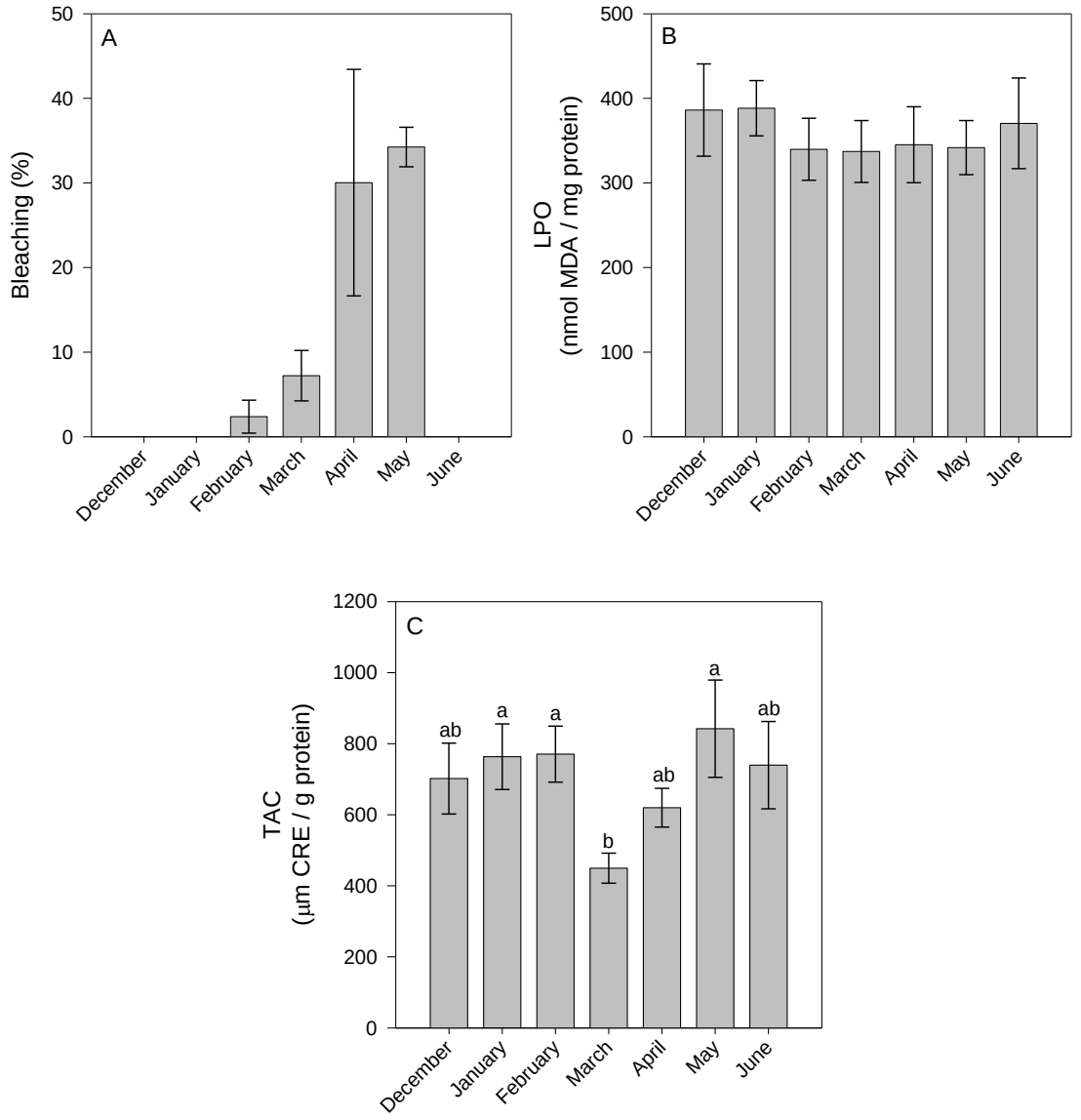

A
Bleaching (%)
0
10
20
30
40
50
December
January
February
March
April
May
June
B
LPO
(nmol MDA / mg protein)
0
100
200
300
400
500
December
January
February
March
April
May
June
C
TAC
(µm CRE / g protein)
0
200
400
600
800
1000
1200
ab
a
a
b
ab
a
ab
December
January
February
March
April
May
June

Figure 5.

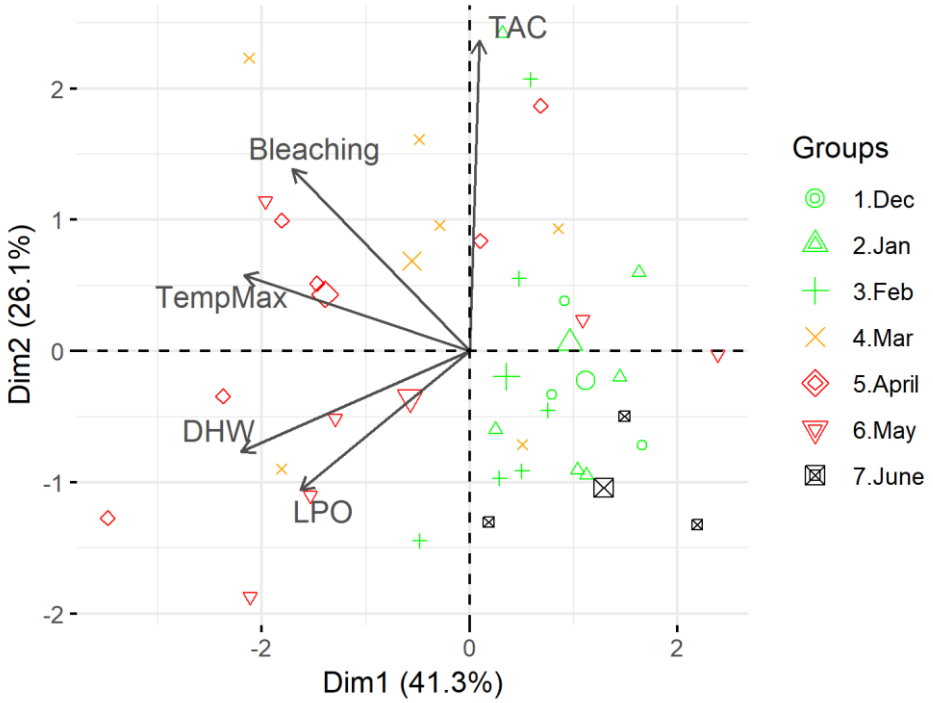
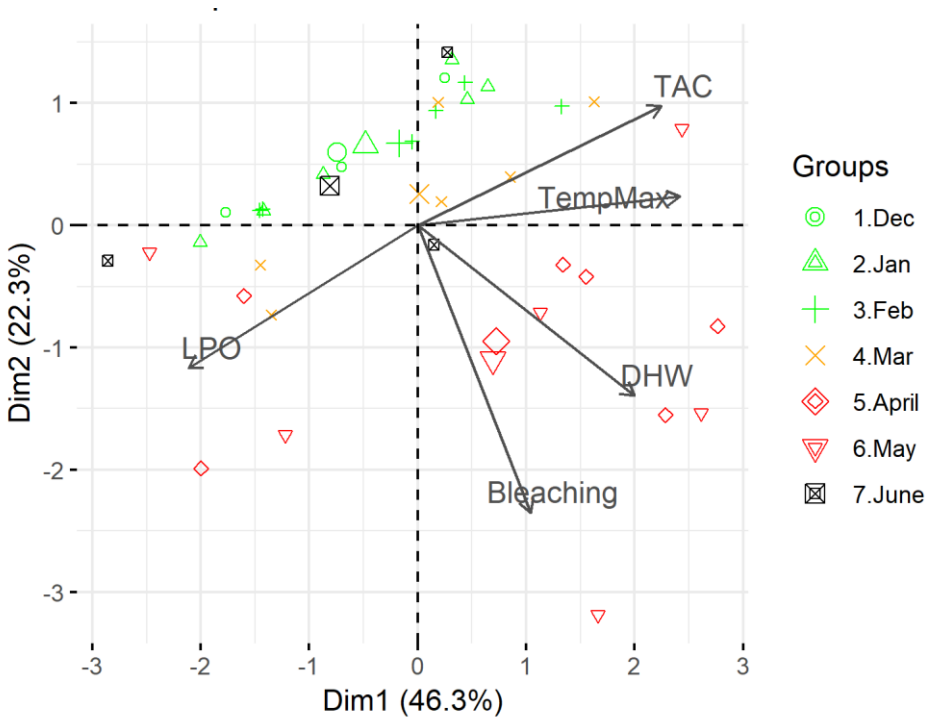


Figure 6.



TABLES

Table 1. Summary of analysis of variance on biochemical biomarkers in *Mussismilia harttii* and *Millepora alcicornis* from December 2015 to June 2016. Significant ($p < 0.05$) results are in bold. TAC: total antioxidant capacity; LPO: lipid peroxidation.

	M. harttii			*M. alcicornis*		
Biomarker	df	F	*p value*	df	F	*p value*
TAC	6	2.51	**0.025**	6	8.85	**0.001**
LPO	6	0.43	0.850	6	5.93	**<0.001**

Manuscrito II

Produção de peroxinitrito e plasticidade trófica em corais construtores de recifes durante um evento natural de branqueamento sob influência do El Niño – Oscilação Sul (2015-2016)

Peroxynitrite generation and trophic plasticity in reef-building corals during a natural bleaching event influenced by the 2015-2016 El Niño Southern Oscillation (ENSO)

Peroxynitrite generation and trophic plasticity in reef-building corals during a natural bleaching event influenced by the 2015-2016 El Niño Southern Oscillation (ENSO)

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Running head: Bleaching in corals and hydrocorals influenced by the 2015-2016 El Niño

Keywords: coral reefs, oxiradicals, oxidative stress, mixotrophy, photosynthesis, heterotrophy.

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ABSTRACT

The present study evaluated a 6-month temporal dynamics of visual bleaching occurrence, peroxynitrite ($ONOO^-$) generation, and feeding plasticity in two important reef building species from South Atlantic reefs, the scleractinian coral *Mussismillia harttii* and the hydrocoral *Millepora alcicornis*, during a natural bleaching/recovery event under the influence of the 2015-2016 El Niño. Our results show that bleaching frequency variability was species-specific, with *Mi. alcicornis* presenting a higher sensitivity to thermal stress. Both species were able to and constantly shifted between feeding modes and a positive relation between heterotrophy and bleaching occurrence was also observed for both species. However, a higher heterotrophic input was displayed by *Mu. harttii*, which suggests this species has more resources for enduring when under stressful events. The present study also brings evidence that $ONOO^-$ may be an important mediator of bleaching in scleractinian corals and hydrocorals experiencing heat stress at field conditions, with consistent observation of higher levels preceding maximum bleaching occurrence in a species-specific time lag. We suggest that the highly cytotoxic molecule $ONOO^-$ may exert a greater influence in the cnidarians bleaching process than previously suggested.

INTRODUCTION

Global climate change impacts on coral reefs are being intensified by pulse heat stress events, such as the El Niño, the warm phase of the El Niño Southern Oscillation (ENSO) (Claar et al. 2018). The 2015-2016 ENSO presented unprecedented levels of thermal stress over the period 1871-2017, which triggered the third global-scale event of mass coral bleaching (Claar et al. 2018; Lough et al. 2018). In fact, corals bleached over 60%, a proportion four times higher than the other two pan-tropical events occurred in 1998 and 2002 (Hughes et al. 2017, 2018).

Coral bleaching episodes are characterized by the disruption of the symbiosis between the coral host and their endosymbiotic *Symbiodinium* dinoflagellates, known as zooxanthellae. This is a complex physiological process resulting from environmental stress that affects the dynamic of internal cellular biology of *Symbiodinium* and their cnidarian hosts (Downs et al., 2000; Lesser, 2006). However, molecular and cellular signaling events underpinning this physiological process are not entirely understood.

Over two decades of research have implicated oxidants, including reactive oxygen and reactive nitrogen species (ROS and RNS, respectively), as pivotal in the physiology of coral bleaching (Lesser 1996, 1997; Downs et al. 2002; Perez and Weis, 2006; Weis, 2008; Hawkins and Davy, 2013; Hawkins et al. 2013; Ross, 2014; Nielsen et al. 2018). RNS include nitric oxide (NO) and peroxynitrite ($ONOO^-$). NO, whose production in animals occurs through the Nitric Oxyde Synthase (NOS), promotes a wide variety of physiological roles in biological systems, including microbial endosymbiosis (Trapido-Rosenthal et al. 2001; Wang and Ruby, 2011) with implications in the bleaching process in cnidarians (Perez and Weis, 2006; Weis, 2008). On the other hand, $ONOO^-$ is a much more reactive RNS and results from the NO´s

binding to ROS (specifically superoxide, O_2^-), causing irreversible damages to biomolecules and playing a critical role in cellular apoptosis (Szabó et al. 2007).

The exposure to elevated temperatures induces the production of high levels of NO in the symbiotic anemone, *Aiptasia pallida*, leading to symbiosis breakdown (Perez and Weis, 2006). The host cells are suggested to be the major source of NO production during heat stress (Perez and Weis 2006), however eukaryotic photosynthesizing organisms also synthesize NO, including different *Symbiodinium* types (A1 and B1, Hawkins and Davy, 2012). $ONOO^-$ has been also shown to induce bleaching (Hawkins and Davy, 2013), and its generation is proposed to be part of the cellular cascade which underpins cnidarian bleaching (Perez and Weis, 2006; Weis, 2008). However, the role of $ONOO^-$ in cnidarian bleaching remains unclear (Hawkins and Davy, 2013), with suggestions that temperature induces bleaching by the action of NO, independently of its conversion to $ONOO^-$ (Hawkins and Davy, 2013). Thus, it is hypothesized that the high reactivity and short half-life of $ONOO^-$ in biological systems (Lesser, 2006) would lead to low *in vivo* $ONOO^-$ levels, apparently insufficient to affect the symbiont loss, challenging the detection and comprehension of its role in biological systems (Szabó et al. 2007).

Reef-building corals are mixotrophic organisms, depending on both photoautotrophy performed by zooxanthellae (Muscatine and Porter, 1977; Muscatine, 1990) and heterotrophic feeding of zooplankton (Porter, 1976; Sebens et al., 1996; Houlbrèque and Ferrier-Pagès, 2009). During bleaching episodes, scleractinian corals may combine alternative sources of fixed carbon, such as energy reserves and increased heterotrophy, in order to recover (Levas et al. 2016). In this context, fatty acids are an important tool for investigating trophic ecology in marine ecosystems (Volkman, 1989; Dalsgaard et al., 2003; Ruess et al., 2005; Budge et al., 2006) and can quantitatively

determine the input of both autotrophic and heterotrophic feeding in corals (Mies et al., 2018). Specifically, stearidonic acid (SDA, 18:4ω3) and docosapentaenoic acid (DPA, 22:5ω3) are fatty acids that have been validated as trophic markers for autotrophic feeding in corals, while cis-gondoic acid (CGA, 20:1ω9) has been validated as a marker for heterotrophic feeding (Mies et al., 2018).

Here we describe the temporal dynamics of $ONOO^-$ generation, visual bleaching occurrence and feeding plasticity in two important reef-building species from South Atlantic reefs, *Mussismillia hartti* and *Millepora alcicornis,* during a natural bleaching/recovery event under influence of the 2015-2016 El Niño. This study is pioneer in reporting $ONOO^-$ generation in corals and hydrocorals during a natural bleaching event at field conditions, since the totality of studies concerning the role of RNS in cnidarians bleaching have been conducted in laboratory conditions (Trapido-Rosenthal et al. 2005; Perez and Weis, 2006; Weis, 2008; Hawkins and Davy, 2012; 2013; Ross, 2014). The trophic plasticity in zooxanthellate calcareous hydrozoans undergoing bleaching is also unknown. Thus, our overarching hypotheses are twofold: firstly, testing for the positive correlation of $ONOO^-$ levels with bleaching occurrence; and second, testing for the (ii) shift of predominance between autotrophy and heterotrophy feeding during bleaching process.

MATERIALS AND METHODS

Coral bleaching survey and biological sampling

Mussismilia harttii and *Millepora alcicornis* colonies were monitored by SCUBA divers for changes in colony color in three reef sites located at the conservation area of the Municipal Natural Park of Recife de Fora (Porto Seguro, Bahia, Northwestern Brazil): (1) Taquaruçu (16° 24' 25.3"S, 38° 58' 41.0"W); (2) Mourão (16°

24' 39.0"S, 38° 59' 06.1"W); and (3) Funil (16° 25'02.6"S, 38° 58' 55.8"W) (Figure. 1). They were selected according to the National Oceanic and Atmospheric (NOAA) predictions for coral bleaching occurrence (https://coralreefwatch.noaa.gov/satellite/analyses_guidance/global_coral_bleaching_2014-17_status.php). Also, they are included in the Brazilian Reef Check monitoring program (www.reefcheck.org, www.recifescosteiros.org.br), and are part of a recent Brazilian Conservation Plan for Endangered Species of Reef Environments (PAN Corais in the Portuguese abbreviation, ICMBio, 2016), and show *Mu. harttii* and *Mi. alcicornis* colonies as most abundant corals in these sites (unpublished data, E. N. Calderon).

Bleaching occurrence was surveyed every 15 days, from December 15th, 2015, to June 15th, 2016. Transects (N=36) of 20 meters with approximately 2 meters depth (low tide) were haphazardly fixed in each reef site, being *Mu. harttii* and *Mi. alcicornis* colonies photographed by side of a PVC Coral Health Chart (CoralWatch, University of Queensland, Australia; Siebeck et al. 2006) along the fixed transects. Bleaching occurrence was evaluated following the modified coral watch protocol (www.reefquest.org). Alongside coral bleaching surveys, pieces (~3 cm) of 4 colonies of each species were sampled and immediately frozen in liquid nitrogen for laboratory analyses. Samples were collected under the permission of the Brazilian Environmental Agency (SISBIO).

Thermal stress determination

Local temperature conditions and estimations of coral thermal stress in each reef site were assessed using temperature data loggers (HOBO Pendant model UA-002-64). Temperature was registered, every 15 min during all monitoring period. Data processing

followed methods developed by NOAA Coral Reef Watch (CRW) program. The onset of potentially damaging coral bleaching was defined to begin at Degree Heating Weeks (DHW; expressed in units of °C weeks) values of 4 °C weeks or greater.

Fatty acids extraction and analysisMillepora alcicornis and *Mussismilia harttii* fragments were weighed and lipid extraction was performed according to Masood et al. (2005) and Mies et al. (2017). Coral tissues were crushed (Conlan et al., 2017) and incubated with 50 µL of tridecanoic acid (C13:0, 1.0 mg mL^{-1}), 1.85 mL of methanol and 100 µL of acetyl chloride for 1 h at 100ºC. Hexane was added, the sample vortexed, and the upper organic phase transferred to a new vial. Evaporation was performed with N_2 until drying, and the content was then resuspended in 100 µL of hexane. Fatty acid methyl esters (FAME) were analyzed on a Trace 1310 gas chromatograph (Thermo Scientific) equipped with a flame ionization detector. They were separated with a DB-FFAP column of 15 m x 0.1 mm ID x 0.1 µm film thickness (J & W Scientific, Agilent Technologies). The program started at 150 ºC for 15 seconds, then increasing 35 ºC per minute to 200 ºC, 8 ºC per minute to 250 ºC and maintained for 4 minutes. 1 µL of the sample were injected and carried by a mixture of hydrogen and nitrogen.

The identification of SDA, DPA and CGA was done by direct comparison of their retention times with the 47033 PUFA Nº1 Marine Source (Sigma-Aldrich) standard mix. For each chromatogram, individual peaks were integrated and normalized by the internal standard. After the extraction, the skeleton fragments were washed with 2% NaClO for 2 min, and dried at 63ºC for 30 h to remove organic tissue. The fragments were then weighed and both soft tissue and skeleton weight were determined.

Predominant Trophic Mode Index To determine which feeding mode prevailed, the data for SDA, DPA and CGA concentration were computed into the equation below, calculating the Predominant Trophic Mode Index (PTMI, Mies et al. 2018):

$$\mathrm{PTMI} = [(\frac{\mathrm{autFATM}(t_2)}{\mathrm{autFATM}(t_1)}) - 1] - [(\frac{\mathrm{hetFATM}(t_2)}{\mathrm{hetFATM}(t_1)}) - 1]$$

where autFATM = [µg of (SDA + DPA) g^{-1} of coral soft tissue], hetFATM = [µg of CGA g^{-1} of coral soft tissue], t_1 = initial sampling period, t_2 = next sampling period and PTMI = Predominant Trophic Mode Index. The result is dimensionless and indicates the prevalence of autotrophy in the case of positive values and the prevalence of heterotrophy for negative values.

Peroxinitrite ($ONOO^-$) quantification

Corals and hydrocorals holobionts were analysed for $ONOO^-$ concentration in biological tissues using the Amplitude™ Fluorimetric Peroxynitrite Quantification Kit (AAT Bioquest®, CA, USA). The reagent DAX-J2™ PON Green 99 specifically reacts with $ONOO^-$, with high selectivity over ROS and other RNS, to generate a green fluorescent product detected at Ex/Em = 490/530nm. Briefly, ~ 0.3 cm of coral/hydrocoral was cut and homogenized in the Assay Buffer solution (provided by the manufacture) using a sonicator (Frequency 70 KHz, Sonaer Ultrasonics, Farmingdale, NY, USA). The remaining piece of skeleton was discarded and homogenates were centrifuged at 10 000 *g* (20 min, 4°C). The intermediary phase was used to perform the assay, according manufactures instructions. Measurements were performed on a solid black 96-well plate using a Multi-Mode Microplate Reader (FilterMax F5, Molecular Devices). Results were normalized considering the total protein content in the sample, which were determined using a commercial reagent kit

based on the Bradford assay (Sigma-Aldrich, ST. Louis, MO, USA). Results were expressed as Peroxynitrite concentration (µM) / mg protein.

Data presentation and statistical analyses

The patterns of bleaching and peroxynitrite generation were adjusted through a Distance Weighted Least Squares Model using the Statistica 7.0 software. A Principal Component Analysis (PCA) was performed to verify the pattern of correlation among traits (bleaching, feeding plasticity, maximum temperature, degree heating weeks and peroxynitrite) after scaling and centralizing the data, using the prcomp function in *vegan* (Oksanen et al., 2013) package in the R environment (R Core Team 2013). The *factoextra* package (Kassambara and Mundt, 2016) was used to visualize graphically the data through the function "fviz_pca_biplot".

Owing the apparently opposite pattern between bleaching and peroxynitrite, we applied cross-correlation analyses using fortnightly log-tranformed data to test peroxynitrite as a driver of bleaching processes. Since data from time series can be autocorrelated, the degrees of freedom are overestimated in correlation analyses, increasing Type I errors and the probability of detecting spurious associations (Fuller, 2009; Pyper and Peterman, 1998). Thus, we corrected the degrees of freedom based on the sum of cross-products of the autocorrelations of tested variables over the examined lags (Chelton, 1984; Pyper and Peterman 1998), instead of removing the autocorrelation from the data. In the 'Results' section, Padj refers to P-value calculated using the corrected degrees of freedom.

RESULTS

Thermal stress conditions and bleaching frequency

Critical thermal stress for corals, indicated by Degree Heating Weeks (DHW) values of near 4°C-weeks, was observed during the first half of April until second half of May at the Taquaruçu reef site (Figure 2). *Millepora alcicornis* showed signs of bleaching during the entire monitoring period, except for the second half of January and first half of June, while 100% bleaching was observed from the second half of March to first half of May (Figure 3A). *Mussismilia harttii* showed bleaching signs during the second half of February (14%) and from April to May, with highest bleaching frequency (67%) during the second half of April and first half of May (Figure 3B).

DHW values of 4 °C-weeks or greater were observed in the first half of April until first half of June at Funil reef site (Figure 2). Bleaching was observed for *Mi. alcicornis* from March to May, with highest bleaching frequency (86%) during the second half of April. In turn, *Mu. harttii* showed bleaching signs during the first half or March (20%) and first half of May (67%) (Figure 3B).

No critical thermal stress indicated by DHW was observed for the reef site Mourão, however, maximum temperatures (up to 34.2°C) were there registered. Bleaching for *Mi. alcicornis* was observed from second half of February to first half of May. The higher bleaching frequency (60%) was also observed during May. *Mussismilia harttii* bleached from March to May, with the higher bleaching frequency (53%) also observed in the first half of May (Figure 3B).

Temperature data over the monitoring period for each of the reef sites are summarized in Table 1.

Predominant Trophic Mode Index

The PTMI data show that both *Mi. alcicornis* and *Mu. harttii* constantly shifted between predominantly autotrophic and heterotrophic, with an overall predominance of heterotrophic behavior (Figure. 4). The responses to thermal stress were different according to species and location. *Millepora alcicornis* displayed a near-equilibrium between feeding modes at Taquaruçu, a single heterotrophic peak at Mourão in March 2016, and two mild peaks at Funil in February and April of 2016. *Mussismilia harttii*, however, displayed several heterotrophic peaks for all locations between January and April of 2016. Both species seemed to have resorted more to heterotrophy at Funil and Mourão. The heterotrophic peaks for *Mi. alcicornis* and *Mu. harttii* did not overlap, showing that their increased heterotrophic activity was asynchronous.

Principal component analysis

Grouping all traits together [bleaching, maximum temperature (MaxTem), degree heating weeks (DHW), peroxynitrite, auto- and heterotrophy] into a multivariate analysis (PCA) for *Mu. harttii* and *Mi. alcicornis*, the two main eigenvectors retain, respectively, 58.7% (Dim1 = 40.7%; Dim2 = 18.0%) and 68.8% (Dim1 = 40.1%; Dim2 = 28.7%) of total variance (Figure. 5). Bleaching, heterotrophy and DHW are positively correlated with Dim1 ($0.60 < R < 0.90$) for both species, while MaxTemp is just associated with *Mi. alcicornis*, also for the eigenvector 1 (R=0.75). However, peroxynitrite is strongly associated with autotrophy in *Mu. harttii* into Dim1 ($-0.50 < R < -0.54$), while it is correlated with heterotrophy in *Mi. alcicornis* into Dim2 ($-0.59 < R < -0.61$). When bleaching is used as a discrete factor ('before', 'during' and 'after'), autotrophy and peroxynitrite are grouped before the bleaching event in *Mu. harttii*. In turn, autotrophy and peroxynitrite appear in *Mi. alcicornis* before and during the

bleaching event, respectively. Heterotrophy is grouped during the bleaching event for both species (Figure 5).

Interestingly, peroxynitrite and bleaching show an opposite pattern, especially for *Mu. harttii* (Figure 5), however both species manifest a visible connection between such traits' peaks over the sampled period of ≈ 6 months (Figure 6). For all reef sites, the same pattern regarding peroxynitrite generation is observed for each species (Figure 6, *left and right panels*). According to Distance Weighted Least Squares plots, *Mi. alcicornis* shows a maximum production of peroxynitrite before the highest bleaching frequency. In turn, two peaks of higher peroxynitrite generation are observed in *Mu. harttii* before the occurrence of the most intense bleaching event. In all cases, peroxynitrite generation decreases after the bleaching occurrence.

Time-series and cross-correlation analyses

Bleaching and peroxynitrite are correlated at no lag (R = 0.808, Padj = 0.003) and at lag -15 days (R = 0.63, Padj = 0.04) for *M. alcicornis* from the locality of Funil (df = 7.3) (Figure YA). Peroxynitrite and bleaching are also correlated in *M. harttii* from the same locality, however at lag -15 days (R = 0.66, Padj = 0.03,) and marginally at -30 days (R = 0.62, Padj = 0.06) (df = 7.6). In Taquaruçu, both traits are not associated for *Mi. alcicornis* (P>0.05), but are correlated at lag -15 days for *Mu. harttii* (R = 0.75, Padj = 0.01, df = 6.8). Any of the species show those traits correlated in Mourão (P > 0.05), where there was no cumulative thermal stress.

DISCUSSION

This study brings new evidence on mechanisms driving the early stages of cnidarians-dinoflagellates symbiosis breakdown during a natural bleaching event

observed in a southwestern Atlantic reef under influence of the 2015-2016 El Niño. Our results suggest that peroxynitrite ($ONOO^-$) may be an important mediator of bleaching in scleractinian corals and hydrocorals experiencing heat stress in field conditions. Also, we give rise to the first evidence on hydrocorals feeding mode plasticity undergoing a bleaching event.

Thermal stress and bleaching frequency

Increasing evidence has shown that DHW provides a reasonable threshold for coral bleaching, being pointed out as an effective mass bleaching index (see Kayanne, 2017; Claar et al. 2018). In the present study, bleaching frequency for both species followed the critical thermal stress for corals (indicated by DHW) in two of the three monitored reef sites, Taquaruçu and Funil. Also, this was reinforced by a strong positive relation between bleaching and DHW for both species. In the reef site Mourão, no critical thermal stress for corals (as DHW) was observed, however, bleaching was observed for both species in a lower frequency. *Millepora alcicornis* bleaching frequency is positively related to maximum temperatures experienced during the monitoring period. Considering that the highest maximum temperatures among reef sites were observed at Mourão (up to 34.2°C), *Mi. alcicornis* bleaching frequency can be explained, at least in part, by maximum temperatures experienced in this specific reef site.

Despite thermal stress being the most documented cause leading to cnidarian-symbiont dysfunction, other environmental stressors can also influence and induce bleaching (Hoegh-Guldberg, 2004). In this context, different features regarding water quality among the studied reef sites could explain bleaching occurrence at Mourão for *Mu. harttii* and *Mi. alcicornis*. This reef site is located in the most landward side of

Recife de Fora conservation reef area, showing higher levels of nitrogen (a land-based nutrient) than the seaward side – Taquaruçu (see Costa et al. 2006). In this context, it is important to note that higher levels of nitrogen in seawater have been linked to reduction of the temperature threshold of coral bleaching (Wiedenmann et al. 2012).

Bleaching frequency variability is also species-specific. In the present study, *Mi. alcicornis* was more sensitive that *Mu. harttii* owing the higher bleaching frequency in all reef sites, reaching the extreme of a 100% during April and May at Taquaruçu site. Information on the thermal tolerance limits of *Millepora* spp. is lacking, however hydrocorals were among the first reef-building species to undergo bleaching during an El Niño event (1982-83) on the Pacific coast of Panama, suggesting them as more sensitive than photosymbiotic scleractinian corals (Glynn and Weerdt 1991), corroborating our findings.

Feeding mode plasticity

Both species were able to and constantly shifted between feeding modes, in agreement with previous reports for other hermatypic coral species (Hoogenboom et al., 2010; Ferrier-Pagès et al., 2011; Mies et al. 2018)., which can be associated with variability in oceanographical and meteorological phenomena (Palardy et al., 2005; Roder et al., 2010; Mies et al., 2018). This can explain why the PTMI variation in *Mu. harttii* and *Mi. alcicornis* was less intense than that for *Mussismilia hispida* at Ubatuba, SP, (southwestern Brazil), which is found in marginal conditions at subtropical latitudes and subject to higher variability in abiotic parameters (Mies et al., 2018). Furthermore, the shifts to heterotrophic behavior in *Mi. alcicornis* and *Mu. harttii* can be partially explained by increases in sea surface temperature (SST) and subsequent bleaching events associated with the ENSO. This affirmation is based on a positive relation

observed between heterotrophy and bleaching occurrence for both species. This is in agreement with previous studies reporting that corals use heterotrophy as a compensation mechanism for the reduced autotrophy resulting from bleaching and oxidative stress (Grottoli et al., 2006; Hughes and Grottoli, 2013; Tremblay et al., 2016).

The higher heterotrophic input displayed by *Mu. harttii* may be explained by the presence of larger tentacles when compared to *Mi. alcicornis*, which is an evolutionary trait specific for predation (Sebens and Koehl, 1984). Therefore, *Mu. harttii* may have more resources for enduring when under stressful events. This statement is reinforced by the fact that *Mu. harttii* presented lower bleaching frequency in all reef sites monitored in the present study, which were nearly 2-fold lower in comparison to the bleaching frequency observed for *Mi. alcicornis.* Thus, it is possible to infer that the higher sensibility observed in hydrocorals to thermal stress compared to some scleractian coral species, observed here and in previous studies (Glynn and Weerdt 1991; Marangoni et al. data not published), may be partially related to the lower heterotrophic input.

Peroxynitrite generation and bleaching

It has been hypothesized that the deleterious effects of high levels of nitric oxide (NO) during bleaching would be related to its conversion to peroxynitrite ($ONOO^-$), which would act as a cytotoxic compound during bleaching. Specifically, $ONOO^-$ through mitochondrial membrane damage would be responsible for the release of potent pro-apoptotic molecules that initiate an apoptotic cascade involved in the bleaching process (Perez and Weis, 2006; Weis 2008). In this context, the present study reinforce this hypothesis by showing that $ONOO^-$ may be an important mediator of bleaching in

scleractinian corals and hydrocorals experiencing heat stress at field conditions, since higher levels of $ONOO^-$ generation preceded maximum bleaching occurrence for both species in all reef sites. Further, a species-specific time lag response was detected regarding $ONOO^-$ generation and bleaching frequency in most cases.

Bleaching usually occurs after a gradual onset of temperature stress that may persist for several weeks. In the present study, bleaching frequency was related mainly to cumulative thermal stress as indicated by DHW, however, this was not observed for the Mourão site. Interestingly, this reef site was the only one where $ONOO^-$ generation and bleaching did not correlate for both species, which suggests that $ONOO^-$ may be a more relevant cytotoxic compound when bleaching is caused by a gradual onset of thermal stress. This is reinforced by previous observations of a sizeable gap between NO synthesis and bleaching under slow heating stress, but not under high temperature shock treatment (Hawkins et al. 2013).

The species-specific time lag response for $ONOO^-$ generation and bleaching occurrence suggests a differential susceptibility to $ONOO^-$ related to the ability of neutralizing and counteracting the deleterious effects of ROS and RNS. In fact, the total antioxidant capacity (TAC) basal levels in *Mi. alcicornis* were observed to be 3-fold lower compared to *Mu. harttii* (Marangoni et al., data not published). Lower TAC levels could explain why *Mi. alcicornis* presents a smaller interval between $ONOO^-$ generation and bleaching, while *Mu. harttii* presents a repeated and longer interval between $ONOO^-$ and bleaching. Also, it is important to highlight that after the bleaching peaks, the concentration of $ONOO^-$ dropped in all cases, suggesting $ONOO^-$ mainly generated by the symbionts, or by the host in response to heat-stressed symbionts.

The *in vivo* production of $ONOO^-$ in the symbiotic sea anemone, *Aiptasia pulchella* occurred under thermal stress; however its generation would be insufficient to

influence symbiont loss since there was not a significant effect of an $ONOO^-$ scavenger on thermal bleaching intensity (Hawkins and Davy, 2013). Despite the fact the present study cannot prove that alternative pathways involving ROS or NO directly are more or less critical than $ONOO^-$ generation in the process of bleaching, we suggest that this highly cytotoxic molecule may exert a greater influence in the cnidarians bleaching process than previously suggested. In this context, it is important to note that most studies into the bleaching mechanisms have used experimentally bleached symbiosis, however the situation regarding $ONOO^-$ generation may be different in reef corals undergoing bleaching in the field (Hawkins and Davy, 2013), as observed in the present study.

In summary, *Mu. Harttii* and *Mi. alcicornis* constantly shifted between feeding modes under the influence of the 2015-2016 El Niño, with a positive relation between heterotrophy and bleaching occurrence. Also, $ONOO^-$ may be an important mediator of bleaching in scleractinian corals and hydrocorals experiencing heat stress at field conditions, with consistent observation of higher levels preceding maximum bleaching occurrence in a species-specific time lag. We suggest that the highly cytotoxic molecule $ONOO^-$ may exert a greater influence in the cnidarians bleaching process than previously suggested.

ACKNOWLEDGMENTS

We would like to thank Prof. Sayuri Miyamoto for providing infrastructure, Dr. Adriano Chaves-Filho for his kind assistance, and Ms. Joseane A. Marques and Dr. Rafael Duarte for helping with R scripts. Dr. Emiliano Calderon, Ms. Cristiano Pereira, Dr. Douglas Abrantes and Coral Vivo´s staff are acknowledged for their support in field work. The International Development Research Centre (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) are acknowledged for their financial support. The Coral Vivo Project and its sponsors Petrobras, through the Petrobras Environmental Program, and Arraial dÁjuda Eco Parque are acknowledged for their support in field research. AB is a research fellow from the Brazilian CNPq (Proc. # 304430 2009-9); SCF is a post-doc fellow from FAPESP (#2017/05310-9), and LFBM was a graduate fellow from CAPES.

AUTHOR CONTRIBUTIONS

LFBM and AB participated on the field work design. AB, MM, AZG, AI and SM contributed with infrastructure/material/technical support. LFBM, CD, MM, AZG, TNSB and AI analyzed the data. SCF contributed with statistical analysis. LFBM, SCF and MM wrote the original draft. All authors read and revised the manuscript.

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FIGURES

Figure 1. Map of Brazil showing the state of Bahia; and Bahia coastline showing the Recife de Fora Marine Protected Area (Porto Seguro, Bahia, northwestern Brazil) and the monitored reef sites: (1) Taquaruçu, (2) Mourão, and (3) Funil.

Figure 2. Estimations of coral thermal stress defined as Degree Heating Weeks (DHW) for the 3 reef sites [(1) Taquaruçu; (2) Mourão; and (3) Funil] at the Recife de Fora Marine Protected Area (Porto Seguro, Bahia, northwestern Brazil), from December, 2015 to June, 2016. Red line indicates 4℃ weeks, the bleaching risk threshold defined by National Oceanic and Atmospheric (NOAA) Coral Reef Watch (CRW) program.

Figure 3. Bleaching frequency for (A) *Millepora alcicornis* and (B) *Mussismilia harttii* from January 2015 to June 2016, at each of the monitored reef sites: Taquaruçu, Funil and Mourão.

Figure 4. *Millepora alcicornis* and *Mussismilia harttii* Predominant Trophic Mode Index (PTMI) from January 2015 to June 2016, at each of the monitored reef sites: (A) Taquaruçu, (B) Funil, and (C) Mourão.

Figure 5. Principal Component Analysis of bleaching, degree heating weeks (DHW), maximum temperature (MaxTemp), autotrophy, heterotrophy and peroxynitrite in *Millepora alcicornis* (A) and *Mussismilia harttii* (B). All traits were grouped between After, Before and During the bleaching event. The two main eigenvectors retain, respectively, 68.8% (Dim1 = 40.1%; Dim2 = 28.7%) and 58.7% (Dim1 = 40.7%; Dim2 = 18.0%) and of total variance.

Figure 6. Pattern of bleaching occurrence (red line) and peroxynitrite concentration (blue line) in *Millepora alcicornis* (*left panel*) and *Mussismilia harttii* (*right panel*) over the period of 2015-2016 El Niño Southern Oscillation from three reef sites at the Recife de Fora Marine Protected Area (Bahia, northwestern Brazil). Bleaching and peroxynitrite are correlated at no lag and at -15 days for *M. alcicornis*, and at lag -15 days for *M. harttii* from Funil. In Taquaruçu, both traits are not associated for *M. alcicornis*, but are correlated at lag -15 days for *M. harttii*. Any of the species show those traits correlated in Mourão.

Figure 1.

Figure 2.

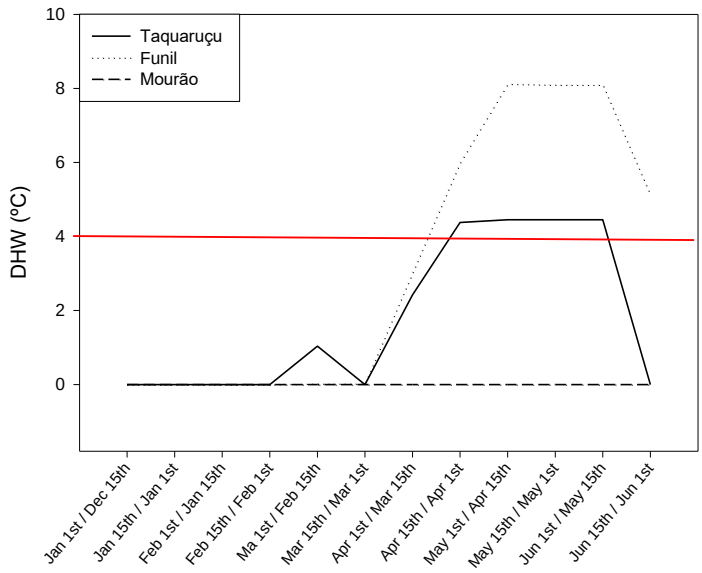
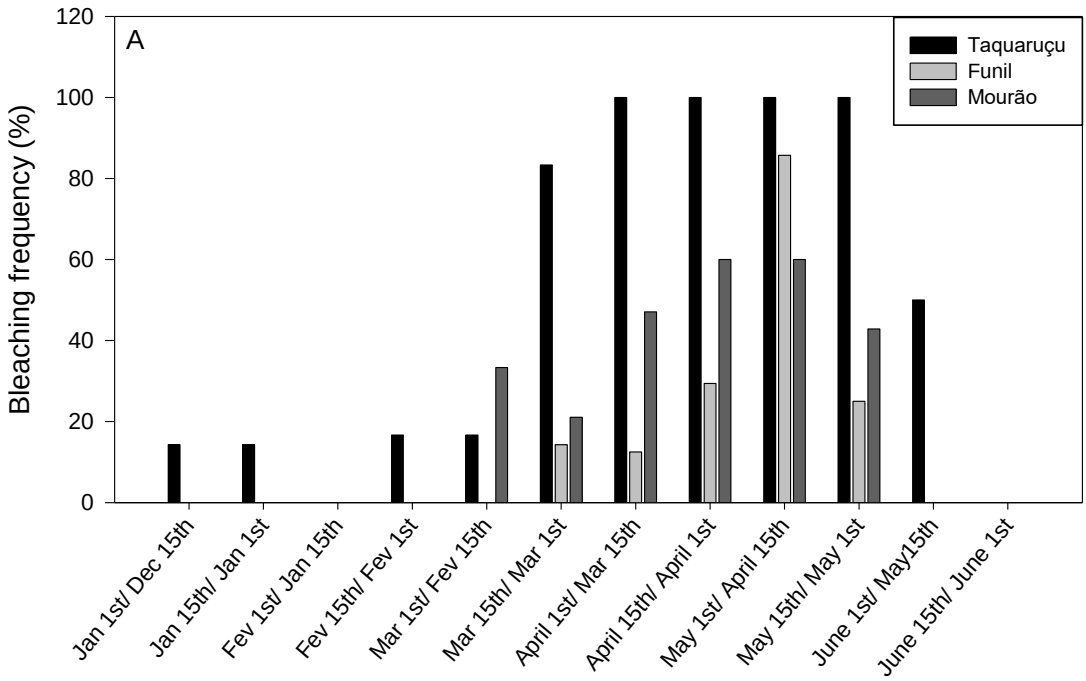

Taquaruçu
Funil
Mourão
DHW (ºC)
10
8
6
4
2
0
Jan 1st / Dec 15th
Jan 15th / Jan 1st
Feb 1st / Jan 15th
Feb 15th / Feb 1st
Ma 1st / Feb 15th
Mar 15th / Mar 1st
Apr 1st / Mar 15th
Apr 15th / Apr 1st
May 1st / Apr 15th
May 15th / May 1st
Jun 1st / May 15th
Jun 15th / Jun 1st

Figure 3.


A
Taquaruçu
Funil
Mourão
Bleaching frequency (%)
120
100
80
60
40
20
0
Jan 1st/ Dec 15th
Jan 15th/ Jan 1st
Fev 1st/ Jan 15th
Fev 15th/ Fev 1st
Mar 1st/ Fev 15th
Mar 15th/ Mar 1st
April 1st/ Mar 15th
April 15th/ April 1st
May 1st/ April 15th
May 15th/ May 1st
June 1st/ May15th
June 15th/ June 1st

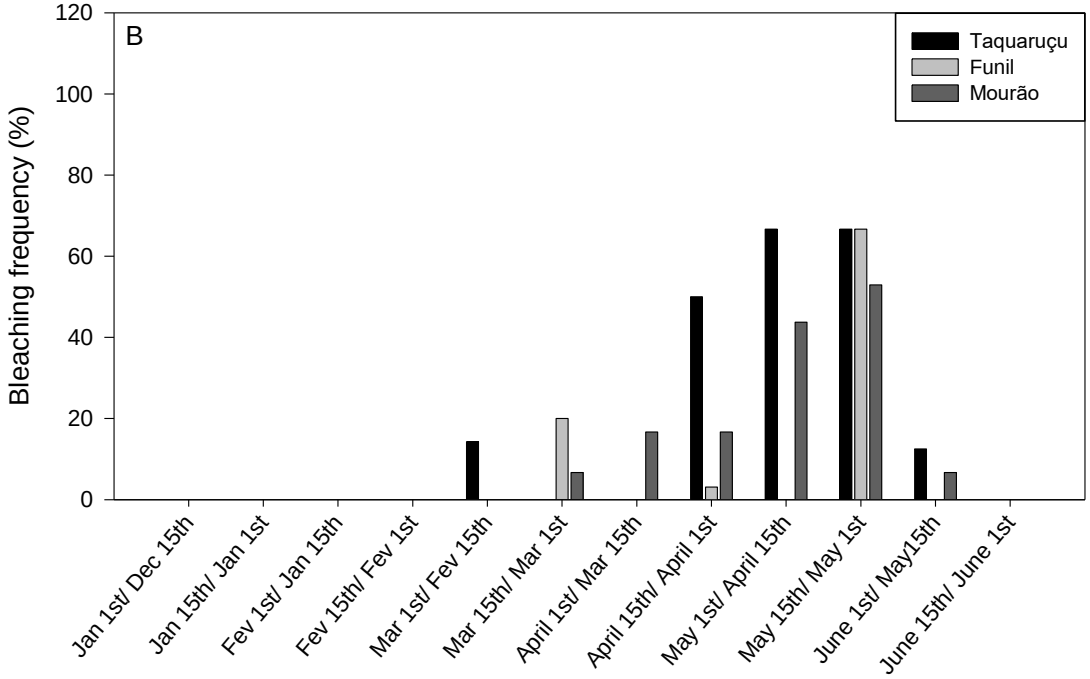
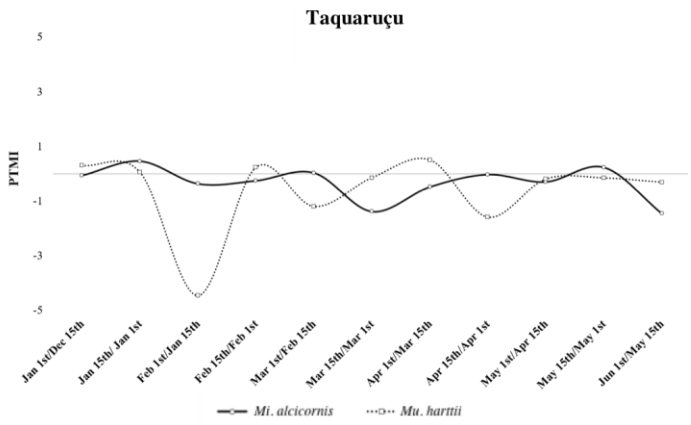
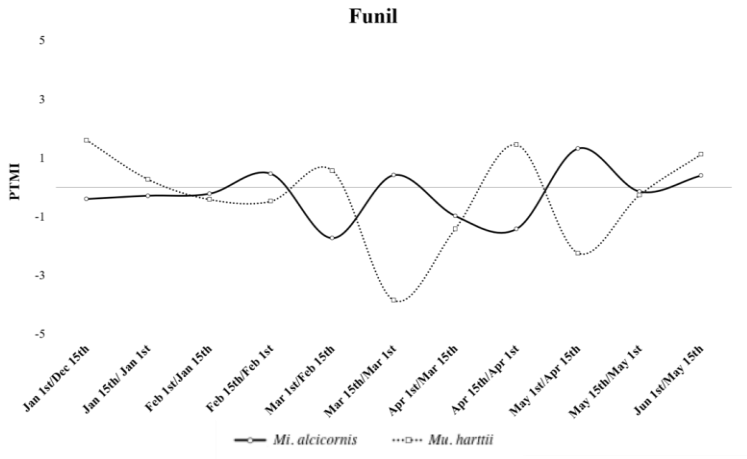

B
Taquaruçu
Funil
Mourão
Bleaching frequency (%)
120
100
80
60
40
20
0
Jan 1st/ Dec 15th
Jan 15th/ Jan 1st
Fev 1st/ Jan 15th
Fev 15th/ Fev 1st
Mar 1st/ Fev 15th
Mar 15th/ Mar 1st
April 1st/ Mar 15th
April 15th/ April 1st
May 1st/ April 15th
May 15th/ May 1st
June 1st/ May15th
June 15th/ June 1st

Figure 4.

A


Taquaruçu
PTMI
5
3
1
-1
-3
-5
Jan 1st/Dec 15th
Jan 15th/ Jan 1st
Feb 1st/Jan 15th
Feb 15th/Feb 1st
Mar 1st/Feb 15th
Mar 15th/Mar 1st
Apr 1st/Mar 15th
Apr 15th/Apr 1st
May 1st/Apr 15th
May 15th/May 1st
Jun 1st/May 15th
Mi. alcicornis
Mu. harttii

B


Funil
PTMI
5
3
1
-1
-3
-5
Jan 1st/Dec 15th
Jan 15th/ Jan 1st
Feb 1st/Jan 15th
Feb 15th/Feb 1st
Mar 1st/Feb 15th
Mar 15th/Mar 1st
Apr 1st/Mar 15th
Apr 15th/Apr 1st
May 1st/Apr 15th
May 15th/May 1st
Jun 1st/May 15th
Mi. alcicornis
Mu. harttii

C

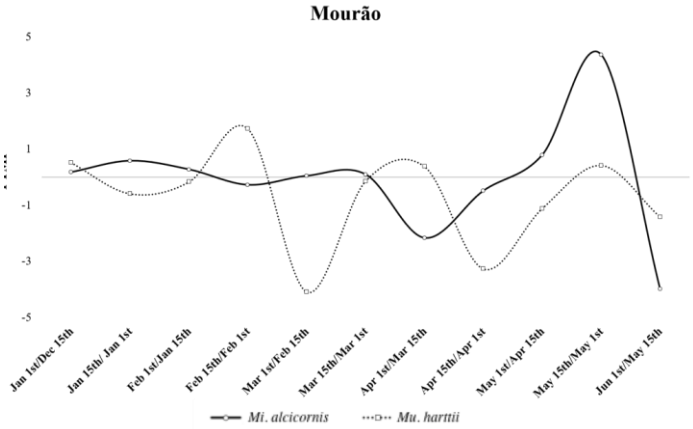
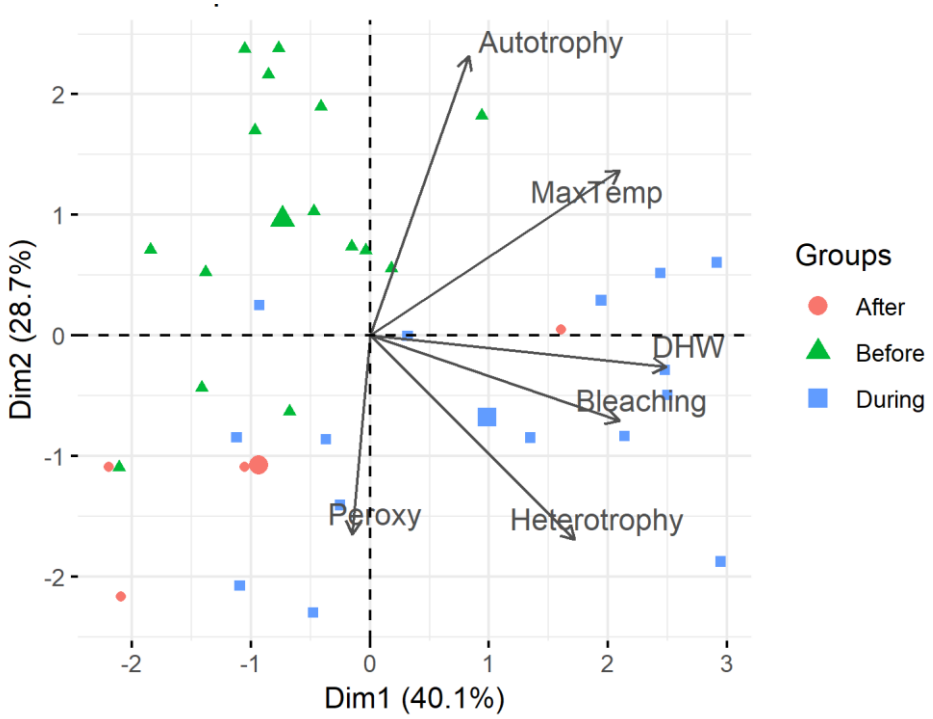

Mourão
5
3
1
-1
-3
-5
Jan 1st/Dec 15th
Jan 15th/ Jan 1st
Feb 1st/Jan 15th
Feb 15th/Feb 1st
Mar 1st/Feb 15th
Mar 15th/Mar 1st
Apr 1st/Mar 15th
Apr 15th/Apr 1st
May 1st/Apr 15th
May 15th/May 1st
Jun 1st/May 15th
Mi. alcicornis
Mu. harttii

Figure 5.

A


Autotrophy
MaxTemp
DHW
Bleaching
Heterotrophy
Peroxy
Dim2 (28.7%)
Dim1 (40.1%)
Groups
After
Before
During

B

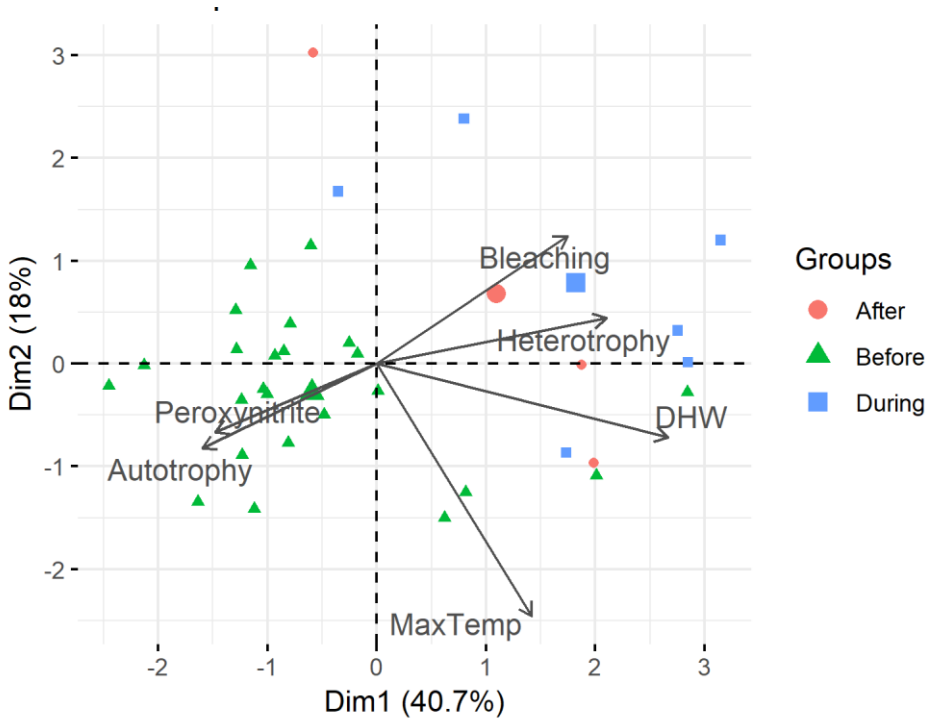
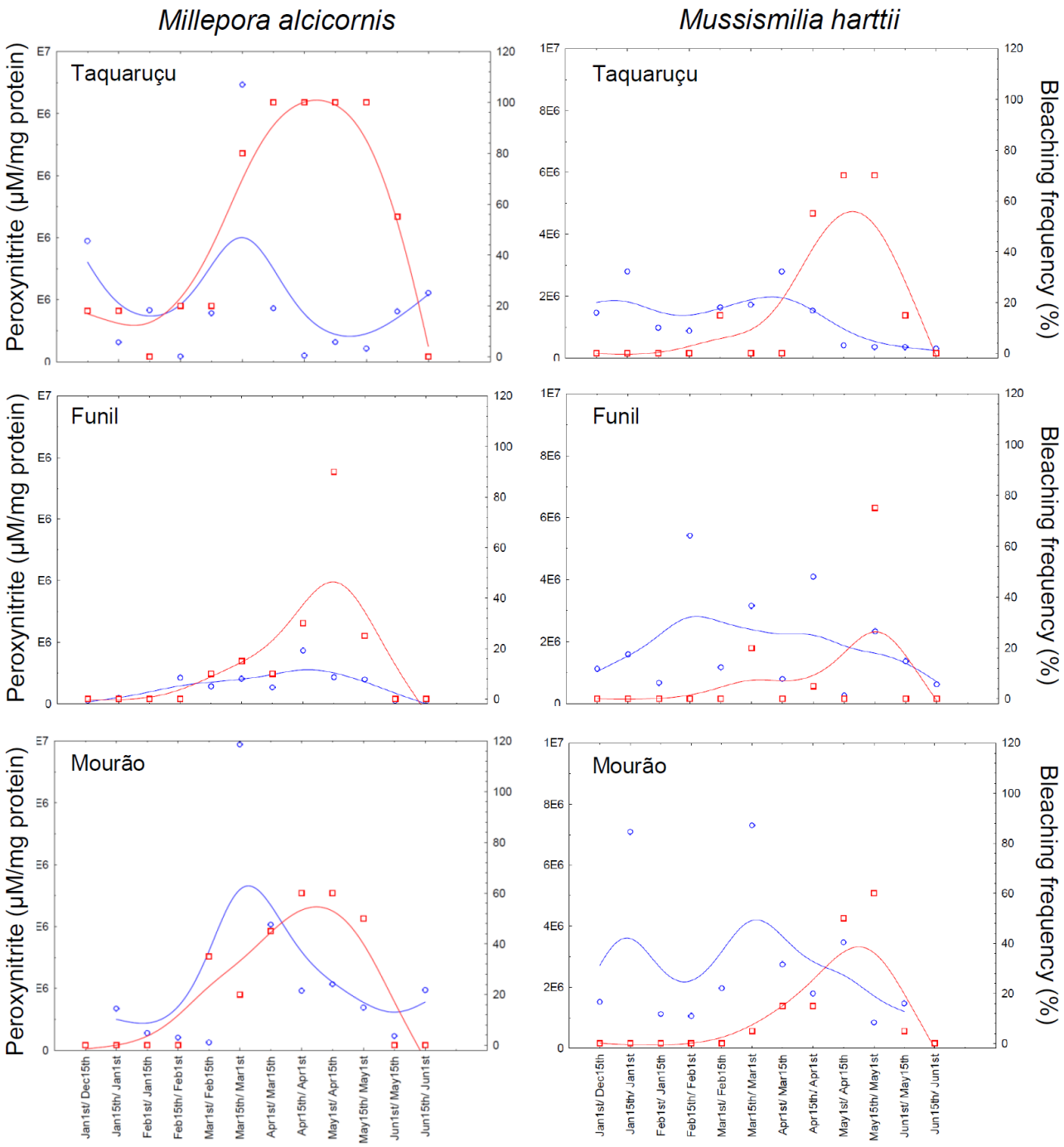

Bleaching
Heterotrophy
DHW
Peroxynitrite
Autotrophy
MaxTemp
Dim2 (18%)
Dim1 (40.7%)
Groups
After
Before
During

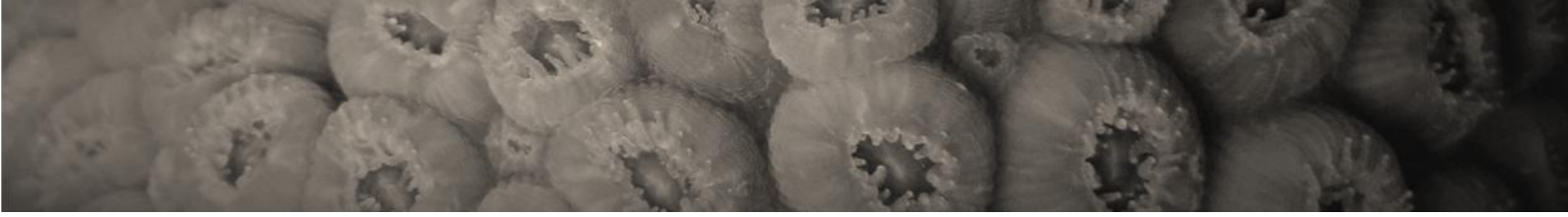
Figure 6.


Millepora alcicornis
Mussismilia harttii
Taquaruçu
Funil
Mourão
Peroxynitrite (µM/mg protein)
Bleaching frequency (%)
Jan1st/ Dec15th
Jan15th/ Jan1st
Feb1st/ Jan15th
Feb15th/ Feb1st
Mar1st/ Feb15th
Mar15th/ Mar1st
Apr1st/ Mar15th
Apr15th/ Apr1st
May1st/ Apr15th
May15th/ May1st
Jun1st/ May15th
Jun15th/ Jun1st

TABLES

Table 1. Seawater temperature (degrees Celsius, ℃) during the 6-month monitoring period in the three reef sites at the Recife de For a conservation area (Porto Seguro, Bahia, northwestern Brazil).

Reef site	**Taquaruçu**	**Funil**	**Mourão**
Minimum temperature	20,53	22,87	22,75
Maximum temperature	27,46	31,13	34,2
Median	24,73	26,52	26,67
Mean	24,73	26,52	26,65
Standard deviation	1,04	0,98	0,94
Standard error	0,19	0,21	0,21



APÊNDICE III

- ***Capítulo de livro***

[Publicado no livro *Conhecendo os Recifes Brasileiros*, 2016, 360 páginas, ISBN 978-85-7427-057-9]

Fisiologia de corais: a simbiose coral-zooxantela, o fenômeno de branqueamento, e o processo de calcificação.

Série Livros Museu Nacional

Conhecendo os Recifes Brasileiros

Rede de Pesquisas Coral Vivo

Projeto Coral Vivo

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Laís Feitosa Machado / Laura Fernandes de Barros Marangoni

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Museu Nacional – Universidade Federal do Rio de Janeiro
Quinta da Boa Vista, São Cristóvão
Rio de Janeiro, RJ, 20940-040

Realização Patrocínio

Ficha Catalográfica

C749 Conhecendo os Recifes Brasileiros: Rede de Pesquisas Coral Vivo / Editores: Carla Zilberberg et al. – Rio de Janeiro: Museu Nacional, UFRJ, 2016.

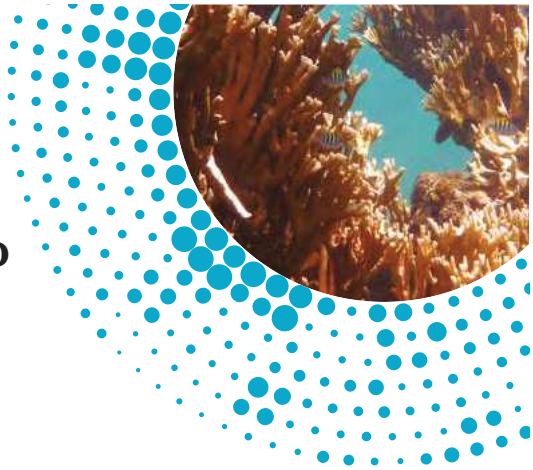
360 p. ; 24 cm. – (Série Livros ; 58)

Inclui bibliografia

ISBN 978-85-7427-057-9

1. Recifes e ilhas de coral – Brasil. 2. Conservação da natureza – Brasil. 3. Divulgação científica – Brasil. I. Zilberberg, Carla. II. Projeto Coral Vivo. III. Museu Nacional (Brasil). IV. Universidade Federal do Rio de Janeiro. V. Série.

CDD 551.42



Fisiologia de corais: a simbiose coral-zooxantela, o fenômeno de branqueamento e o processo de calcificação

Laura Fernandes de Barros Marangoni,
Joseane Aparecida Marques, Adalto Bianchini

Este capítulo aborda processos fisiológicos característicos de corais construtores de recifes de águas rasas. Dessa forma, o termo "coral" será utilizado para designar cnidários da Ordem Scleractinia, conhecidos também como corais pétreos ou verdadeiros. A relação simbiótica entre corais e microalgas (as zooxantelas) será tratada com ênfase nos processos fisiológicos envolvidos nessa associação. Também serão descritos os principais mecanismos relacionados à perturbação dessa simbiose, fenômeno denominado branqueamento de corais. A importância da calcificação realizada por corais na formação de recifes biogênicos e a fisiologia envolvida nesse processo serão descritas, assim como os possíveis impactos da acidificação dos oceanos sobre o mesmo. A fisiologia descrita neste capítulo apresenta grande relevância ecológica e, por isso, tem sido investigada pela comunidade científica e utilizada como descritor da saúde do ambiente recifal. Experimentos de caráter ecofisiológico, como aqueles que vêm sendo realizados pela Rede de Pesquisas Coral Vivo, auxiliam no prognóstico da saúde dos recifes de coral frente a um planeta em transformação. Assim, seguindo a premissa "conhecer para conservar", este capítulo fornece uma fundamentação teórica básica sobre os processos fisiológicos de corais escleractíneos com potencial utilização visando à conservação desses organismos.

Fotografia: Emiliano Calderon - banco de imagens Projeto Coral Vivo.

A relação simbiótica entre corais e zooxantelas

A associação entre cnidários e dinoflagelados do gênero *Symbiodinium* (referidos como zooxantelas) possui especial relevância ecológica em regiões tropicais, onde grande parte dos recifes de coral ocorre. O estabelecimento da associação coral-zooxantela tem importância fundamental na formação desses ecossistemas, pois permite que os corais prosperem em ambientes oligotróficos, onde a oferta de alimento e nutrientes é escassa.

Os corais se assemelham às anêmonas-do-mar, porém, diferentemente delas, são capazes de secretar um exoesqueleto na base de seu corpo, encontrando-se assentados sobre ele. Os corais podem ser classificados como "zooxantelados", quando estabelecem simbiose com zooxantelas, e "azooxantelados", quando não estabelecem simbiose com zooxantelas. Por sua vez, o termo "zooxantela" não possui significado taxonômico, mas é utilizado para referir dinoflagelados endossimbiontes do gênero *Symbiodinium*, um grupo diverso de microalgas[1]. Ao contrário da maioria das formas de vida livre de seu grupo, as zooxantelas residem "enclausuradas" no interior das células da endoderme de corais, em um compartimento intracelular denominado simbiossoma, entendido como a interface entre o coral hospedeiro e suas zooxantelas simbiontes (Figura 1).

Essa simbiose tem importância fundamental na nutrição de muitos corais que, apesar de serem heterotróficos, podem contar com outra fonte de nutrição advinda de seus endossimbiontes fotossintetizantes. Essa politrofia apresentada por muitos corais explica a capacidade de sobreviverem em ambientes oligotróficos. De forma geral, os produtos da fotossíntese fornecidos pelas zooxantelas dão suporte a funções vitais do coral hospedeiro. Ainda, essas microalgas auxiliam na conservação e reciclagem de nutrientes essenciais e contribuem para o aumento das taxas de calcificação do coral. Em troca, as zooxantelas beneficiam-se de uma maior proteção contra a herbivoria, uma posição estável na coluna d'água que otimiza o acesso à luz, e ainda do acesso a compostos provenientes do metabolismo do hospedeiro, essenciais para seu metabolismo fotossintético. Uma consequência importante dessa simbiose é que, devido à natureza fotossintética de seus simbiontes, as espécies de coral zooxantelados ocorrem em regiões rasas, com acesso à luz.

1. Para detalhes, ver Picciani et al. (2016) e Garrido et al. (2016), capítulos 5 e 6 deste volume, respectivamente.

O estabelecimento da simbiose coral-zooxantela envolve mecanismos complexos, como o reconhecimento hospedeiro-simbionte, a incorporação do simbionte pelo hospedeiro, via fagocitose, e o estabelecimento das zooxantelas no compartimento simbiossoma. Além disso, a estabilidade e a persistência dessa simbiose exigem uma coordenação que promova o equilíbrio dinâmico entre o crescimento celular do hospedeiro e da população de endossimbiontes. Apesar da importância dos processos iniciais envolvidos no estabelecimento dessa simbiose, o foco dado no presente capítulo será apenas sobre a natureza energética e a dinâmica bidirecional de produtos metabólicos advindos dessa associação.

Processos fisiológicos associados na simbiose coral-zooxantela

A interface hospedeiro-simbionte: como previamente mencionado, o compartimento intracelular simbiossoma compõe a interface entre o coral hospedeiro e suas zooxantelas endossimbiontes (Figura 1). Sendo assim, para que seja possível a troca de metabólitos entre coral e zooxantela, esses compostos necessitam cruzar essa interface. O simbiossoma é formado por uma série de membranas derivadas do endossimbionte e outra, mais externa, derivada do hospedeiro. Evidências indicam mecanismos de controle ativo nessas membranas, tal como a presença de transportadores de solutos (ATPases) para a translocação de compostos entre o hospedeiro e endossimbiontes. Dessa forma, as “trocas” realizadas nessa simbiose não ocorrem de forma aleatória.

Fotossíntese: a fotossíntese constitui um dos aspectos chave da associação coral-zooxantela, uma vez que por meio desse processo as zooxantelas disponibilizam "combustível" ao hospedeiro para o desempenho de funções básicas essenciais para sua sobrevivência, como manutenção metabólica, crescimento, reprodução e calcificação. Basicamente, o coral utiliza os produtos da fotossíntese (oxigênio e carbono fixado fotossinteticamente) para a geração de energia e síntese de proteínas e lipídeos.

Acredita-se que o glicerol seja a principal forma pela qual o carbono fixado pelas zooxantelas seja translocado para o hospedeiro, sendo que compostos como a glicose e os aminoácidos também já foram identificados (Figura 1). A taxa de translocação de fotossintatos (carbono fixado na fotossíntese) para o coral pode variar conforme as características específicas de cada simbiose. Por exemplo, determinadas espécies de corais podem utilizar mais da heterotrofia para suprir suas necessidades metabólicas do que outras, necessitando, assim, de menores quantidades de fotossintatos. Alguns estudos indicam que até 60% do carbono fixado é fornecido ao coral, enquanto outros indicam fornecimento superior a 90%.

A aquisição de carbono inorgânico (Ci) é vital para a realização da fotossíntese e pode ser advindo (i) da respiração do coral e das zooxantelas na forma de CO_2; (ii) pelo processo de calcificação realizado pelo coral, em que o CO_2 é um subproduto; ou (iii) do ambiente (Figura 1). Neste último caso, o Ci é incorporado pelo coral e então fornecido às zooxantelas, uma vez que elas não possuem contato direto com a água do mar circundante. Em valores típicos de pH da água do mar (≈ 8,2), a maior parte do Ci encontra-se na forma de bicarbonato (HCO_3^-), o que significa que o coral tem que dispor de mecanismos específicos para captar e transportar essa forma de Ci para seus endossimbiontes. Para isso, uma enzima denominada anidrase carbônica cumpre papel fundamental ao realizar a interconversão de HCO_3^- em CO_2, tornando possível o fornecimento de Ci do ambiente para os endossimbiontes fotossintetizantes.

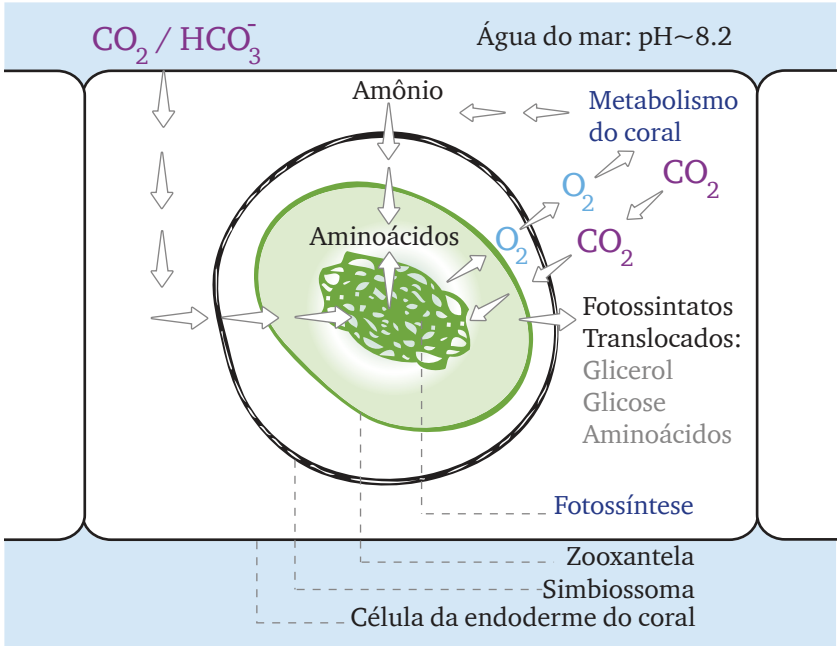


Figura 1. Ilustração das zooxantelas no interior do simbiossoma no tecido de corais e principais trocas metabólicas entre eles.

Reciclagem e conservação de nitrogênio através da interface coral-zooxantelas: dada a grande ocorrência da simbiose entre corais e dinoflagelados em águas tropicais pobres em nutrientes, uma atenção considerável tem sido dada à capacidade desses organismos, quando em associação, de conservar e reciclar o nitrogênio. Hipóteses têm sido propostas para tentar explicar como ocorre a dinâmica de compostos nitrogenados através da interface coral-zooxantelas. A reciclagem considera a hipótese da translocação bidirecional de nutrientes. Nesse caso, o nitrogênio na forma de amônio, resultante de processos catabólicos do hospedeiro (utilização de aminoácidos como substrato na respiração), é assimilado pelos endossimbiontes para que, por fim, seja translocado de volta ao hospedeiro na forma de compostos nutricionalmente importantes, como aminoácidos (Figura 1). Já a hipótese da conservação do nitrogênio considera a utilização preferencial de fotossintatos, no lugar de aminoácidos, como substrato no processo de respiração desempenhado pelo coral. De fato, esse mecanismo está em concordância com uma das formas pela qual se acredita que o coral hospedeiro controle o crescimento da população de seus endossimbiontes,

limitando o acesso deles ao nitrogênio. Nesse caso, a utilização de fotossintatos como substrato na respiração seria vantajosa, por não gerar como produto o composto nitrogenado amônio, como ocorre no caso do catabolismo de aminoácidos.

Controle do hospedeiro sobre os endossimbiontes: cresce o número de estudos que confirmam um determinado grau de controle do coral hospedeiro sobre a fisiologia de suas zooxantelas endossimbiontes. Foi demonstrado, por exemplo, que o coral é capaz de acidificar o interior do simbiossoma como forma de promover a fotossíntese de seus simbiontes, uma vez que a formação de CO_2 a partir da reação de conversão HCO_3^- a CO_2 é favorecida em pH mais ácido[2]. Ainda, evidências indicam que o trânsito de carbono e nutrientes, como nitrogênio e fósforo, na interface entre hospedeiro-simbionte é altamente controlado. Isso permitiria ao coral controlar ativamente o crescimento populacional de simbiontes por meio da limitação de nutrientes.

2. Barrot, K.L.; Venn, A.A.; Perez, S.O.; Tambutté, S.;Tresguerres, M. 2015. *Proceedings of the National Academy of Sciences of the United States of America*, 112(2):607-612.

O fenômeno do branqueamento

Corais saudáveis abrigam milhões de zooxantelas por centímetro quadrado de tecido, sendo a pigmentação de seus tecidos resultante da cor dos pigmentos fotossintetizantes dessas microalgas. Assim, o fenômeno de branqueamento caracteriza-se pelo declínio dessa pigmentação em decorrência da disfunção da simbiose coral-zooxantelas, que resulta na expulsão dos endossimbiontes ou na degradação dos pigmentos fotossintetizantes. Dessa forma, os corais tornam-se visivelmente branqueados quando há uma redução de 70% a 90% na pigmentação de seus tecidos, que se tornam translúcidos. Nessa condição, manifesta-se a coloração branca do esqueleto de carbonato de cálcio subjacente ao tecido, motivo pelo qual o fenômeno é denominado "branqueamento" (Figura 2).

O branqueamento de corais é um dos fenômenos ecológicos associado aos recifes de coral mais estudados da atualidade, caracterizando-se por ser uma resposta ao estresse decorrente de perturbações ambientais. Uma série de fatores, como variações de salinidade, sedimentação excessiva e poluição, entre outros, pode ocasionar esse fenômeno. No entanto, a ocorrência de branqueamento em larga escala na natureza está geralmente associada a temperaturas elevadas combinadas com alta incidência de luz.

Dependendo da duração e da intensidade do estresse, o branqueamento pode causar a mortalidade de corais (Figura 2). Porém, quando a mortalidade não ocorre, os corais que se recuperam após um evento de branqueamento, mediante a recolonização de seus tecidos por zooxantelas, apresentam declínio nas taxas de crescimento e fecundidade, e aumento da susceptibilidade a doenças. As consequências ecológicas do branqueamento não serão aprofundadas neste capítulo, porém, o aumento da mortalidade de corais e o comprometimento funcional desses organismos, devido à ocorrência desse fenômeno, podem resultar em graves consequências para o ecossistema recifal.

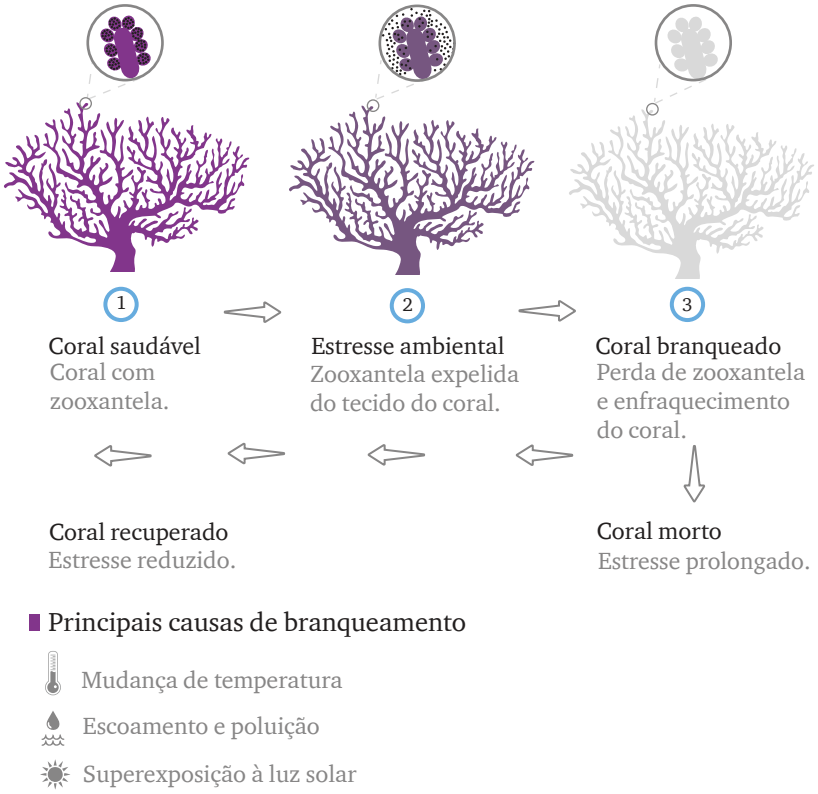


Figura 2. Esquema de branqueamento, recuperação e mortalidade em corais.

Fisiologia do branqueamento

Evidências sugerem que o branqueamento é uma resposta imune do coral hospedeiro frente a endossimbiontes debilitados. Nesse contexto, sabe-se que as espécies ativas de oxigênio (EAO) possuem papel central na perturbação da simbiose coral-zooxantela. A seguir, será feita uma descrição mais detalhada sobre os processos envolvidos nessa perturbação e os mecanismos que levam ao branqueamento.

A condição de estresse oxidativo: organismos aeróbicos utilizam o oxigênio para desempenhar diversos processos metabólicos. Entretanto, como resultado de sua configuração eletrônica, o oxigênio pode sofrer reduções parciais e levar à formação de espécies ativas de oxigênio (EAO). A formação dessas espécies constitui um processo contínuo e fisiológico que cumpre importantes funções biológicas, como a sinalização de processos celulares. No entanto, as EAO podem exercer tanto um papel benéfico, quando em baixas concentrações intracelulares, quanto deletério aos organismos. A produção contínua de EAO durante processos metabólicos levou ao desenvolvimento de mecanismos protetores denominados "capacidade antioxidante", que cumpre o papel de neutralizar os compostos reativos prevenindo os efeitos adversos da EAO. No entanto, perturbações ambientais, como variação de temperatura, radiação excessiva e poluentes, entre outros, podem resultar no aumento da produção de EAO em níveis que excedem a capacidade antioxidante do organismo, levando-o à condição de estresse oxidativo. Tal condição é responsável por causar danos oxidativos às biomoléculas, como lipídios, proteínas e ácidos nucléicos, induzindo a efeitos deletérios e alterando a estrutura e/ou função dessas biomoléculas. Grande parte das EAO é produzida nas cadeias transportadoras de elétrons na mitocôndria, no retículo endoplasmático, em membranas nucleares e no sistema fotossintetizante.

O papel das EAO na perturbação da simbiose coral-zooxantela: os eventos que desencadeiam o fenômeno de branqueamento estão relacionados ao aumento na produção de EAO a níveis que excedem a capacidade antioxidante do coral e de seus simbiontes. Esse evento tem início nos cloroplastos das zooxantelas, local onde ocorre o processo de fotossíntese. Em condições normais, o aparato fotossintético, composto pelos fotossistemas I e II nos tilacóides, opera normalmente, produzindo grande quantidade de oxigênio que se difunde para os tecidos do coral hospedeiro. Nesse processo, as EAO produzidas são neutralizadas por defesas antioxidantes das zooxantelas. No entanto, em condições de estresse, danos ao aparato fotossintético podem ocorrer e gerar grandes quantidades de EAO. Na medida em que a concentração de EAO aumenta, as defesas antioxidantes dos simbiontes são excedidas, com o consequente acúmulo dessas espécies reativas. Em tal condição, além dos danos ao sistema fotossintético aumentarem, as EAO começam a ser difundidas para os tecidos do coral (Figura 3), onde os danos às biomoléculas procedem, conduzindo ao branqueamento. Nesse processo, apesar das zooxantelas serem a principal fonte de EAO, a geração direta de espécies ativas pelo metabolismo do coral, devido a danos às membranas mitocondriais, também contribui para o aumento da concentração de EAO (Figura 3). Apesar dos corais também contarem com um mecanismo de defesa antioxidante, este pode não ser capaz de lidar com altas concentrações de EAO em seus tecidos, o que pode resultar em danos ao DNA, proteínas e lipídios. Dessa forma, o branqueamento seria uma resposta final de defesa do coral contra o estresse oxidativo em que a fonte dominante de produção de EAO, ou seja, as zooxantelas, é erradicada. A capacidade de recuperação do coral após um evento de branqueamento depende da intensidade e da duração do estresse.

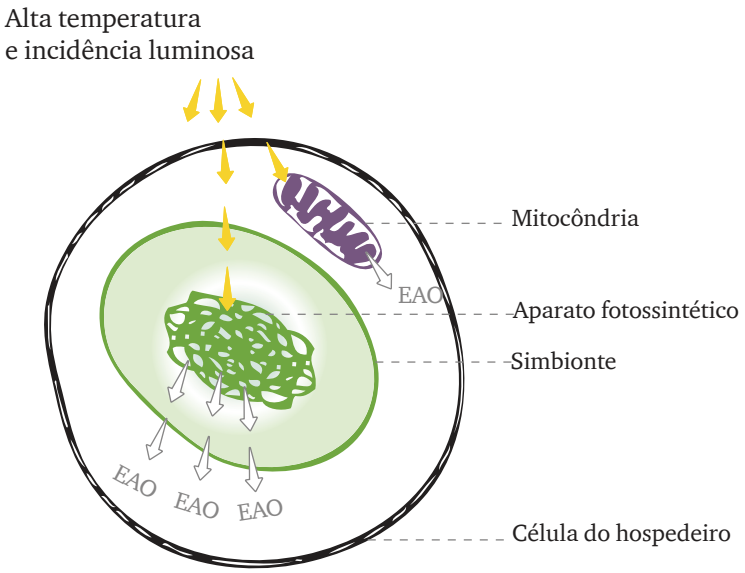


Figura3. Esquema mostrando a difusão de espécies ativas de oxigênio (EAO) das zooxantelas para o coral e os principais locais de produção de EAO no holobionte.

A formação dos recifes de coral e o processo de calcificação

Dentre os principais responsáveis pela formação e manutenção dos recifes de coral de águas rasas, merecem destaque os corais escleractíneos, responsáveis por 15% da produção global de carbonato de cálcio e, por isso, considerados um dos principais grupos de organismos calcificadores. Em sua grande maioria, os corais construtores de recife apresentam forma de vida colonial. As colônias podem chegar a ser compostas por milhares de pólipos (indivíduos geneticamente idênticos), que formam um esqueleto comum de carbonato de cálcio, com grande variedade de formas e tamanhos. Inúmeros esqueletos acrescidos ao longo de muitos milênios formam a estrutura básica dos recifes de coral. O estilo de vida colonial, combinado com processos que aceleram a produção do esqueleto, permite que a taxa de formação do esqueleto desses organismos seja maior que a capacidade de fatores físicos, químicos e biológicos de dissolvê-lo ou degradá-lo. Isso, em grande parte, torna possível a existência dos recifes de coral como são conhecidos hoje.

O processo de calcificação realizado por corais promove a segregação de seus esqueletos de carbonato de cálcio ($CaCO_3$), que são precipitados na forma do mineral aragonita. Para isso, corais suprem o sítio de calcificação com cálcio (Ca^{+2}) e carbono inorgânico (HCO_3^-), obtidos a partir da água ambiente, e eliminam os prótons (H^+) resultantes do processo de mineralização ($Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$). A calcificação é um processo fisiológico ativo que utiliza quantidades significativas de energia, sendo estimado que até 30% da energia metabólica dos corais seja empregada nesse processo. A seguir, serão descritos em maior detalhe os mecanismos fisiológicos que compõem o processo de calcificação. Além disso, será feita uma breve descrição da anatomia corporal dos corais, visando possibilitar uma melhor compreensão do processo de calcificação.

Uma breve descrição da anatomia dos corais: os corais têm como unidade anatômica o pólipo, que consiste de uma estrutura corporal na forma de cilindro, com uma boca central circundada por tentáculos. Por sua vez, os corais coloniais são constituídos por vários pólipos ligados por um tecido denominado cenossarco. Os tecidos que formam os pólipos e o cenossarco constituem duas camadas epiteliais denominadas epiderme e gastroderme, as quais são separadas por uma camada gelatinosa de colágeno chamada mesogleia. O tecido em contato com a água é o tecido oral, enquanto aquele adjacente ao esqueleto é denominado tecido aboral. A epiderme aboral, que está em contato com o esqueleto, é referida como ectoderme calicoblástica, ou calicoderme, e constitui uma camada única de células, denominadas células calicoblásticas (Figura 4).

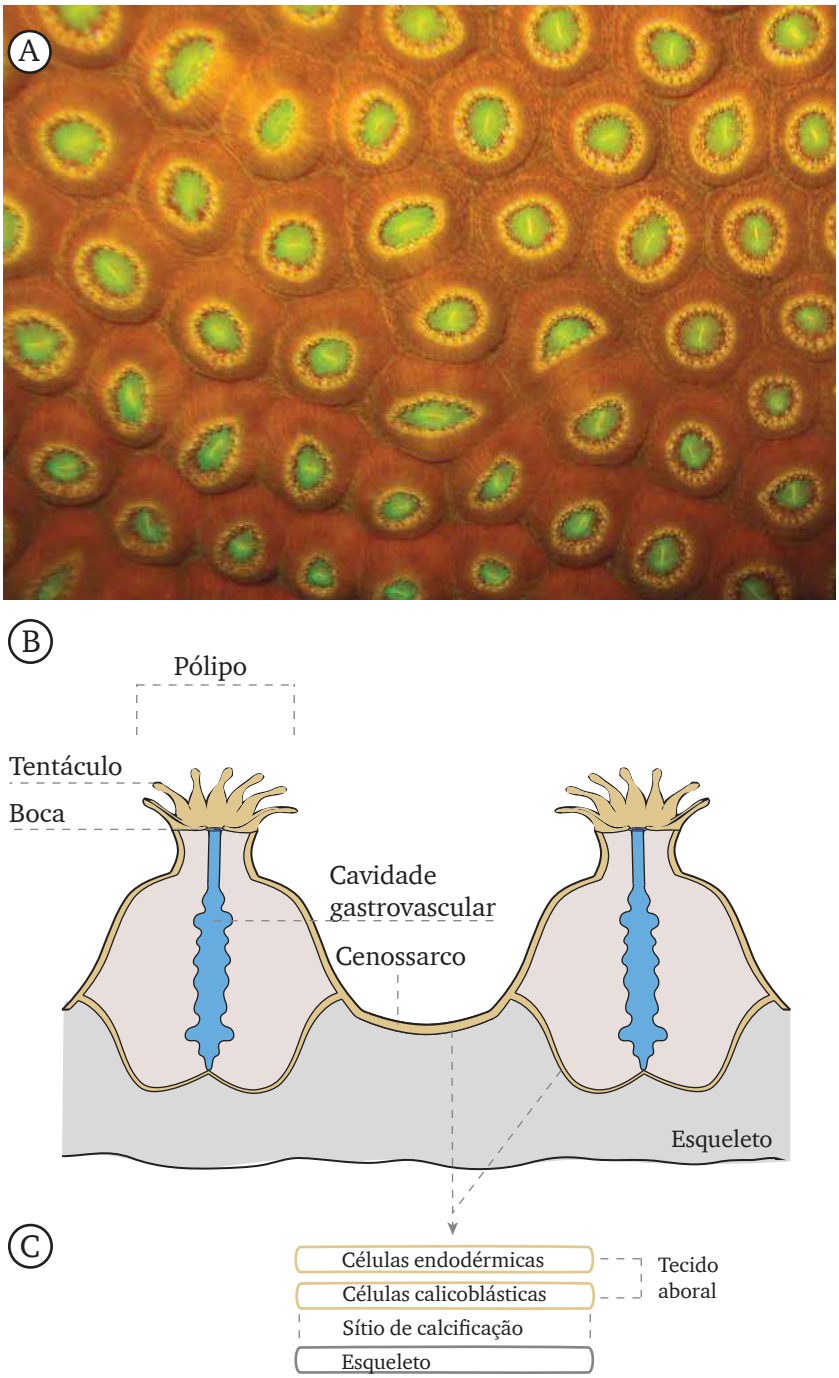


Figura 4. Anatomia básica de corais coloniais. (A) Foto mostrando pólipos conectados pelo cenossarco (foto de *Montastraea cavernosa,* créditos: Laura Marangoni); (B) representação esquemática da foto; (C) visão aproximada do tecido aboral, com destaque para o sítio de calcificação (local de deposição do esqueleto de carbonato de cálcio segregado por corais).

O sítio de calcificação: muitas terminologias têm sido utilizadas para designar a interface entre a calicoderme e o esqueleto. Neste capítulo, ela será tratada como "Meio de Calcificação Extracelular" (MCE). Nesse meio extracelular, que se acredita ser um espaço semifechado, é onde ocorre a deposição do esqueleto de $CaCO_3$ dos corais, sendo também conhecido como "sítio de calcificação" (Figura 4). As células calicoblásticas possuem papel central no que se refere à deposição do esqueleto, uma vez que controlam a composição iônica do MCE e regulam as taxas de calcificação. Para isso, essas células possuem transportadores iônicos, ou seja, proteínas que permitem a passagem de moléculas através da membrana celular. Além disso, secretam uma matriz orgânica que desempenha papel fundamental na nucleação e regulação da morfologia dos cristais de aragonita.

A capacidade dos corais de precipitar $CaCO_3$ na forma de aragonita está relacionada ao que se chama de "estado de saturação da aragonita", representado pelo símbolo Ωar. O Ωar depende da concentração e do equilíbrio dos íons cálcio (Ca^{+2}) e carbonato (CO_3^{2-}) na água do mar, além de outros fatores físico-químicos. Ao ser transportada para o interior do sítio de calcificação, a água do mar circundante tem seu pH e concentração de Ca^{2+}e CO_3^{2-} controlados pelo coral nesse compartimento, que são muito diferentes em relação à água do mar ambiente. A eficiência do processo de calcificação é garantida pela elevação do pH no sítio de calcificação, o que promove um aumento de até 7 vezes do Ωar em relação à água do mar circundante.

Fornecimento de íons e controle do pH: os canais de cálcio e a enzima Ca^{2+}-ATPase presentes na calicoderme contribuem para o abastecimento do sítio de calcificação com Ca^{2+}. Enquanto os canais de cálcio permitem a entrada difusiva Ca^{+2} nas células, a enzima Ca^{2+}-ATPase transporta ativamente um íon de Ca^{2+} ao mesmo tempo em que remove dois prótons (H^+) desse sítio (Figura 5). Dessa forma, a atividade dessa enzima tem papel fundamental não somente no fornecimento de Ca^{2+}, mas também na manutenção de um pH mais alcalino, que garante a eficiência do processo de calcificação. Por outro lado, a enzima anidrase carbônica constitui outro elemento essencial no fornecimento dos íons necessários ao processo de calcificação. Ela promove a reação de hidratação reversível do CO_2, proveniente do metabolismo do coral ou do ambiente, em bicarbonato ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), sendo essencial aos corais na aquisição do carbono inorgânico (HCO_3^-) utilizado no processo de calcificação (Figura 5).

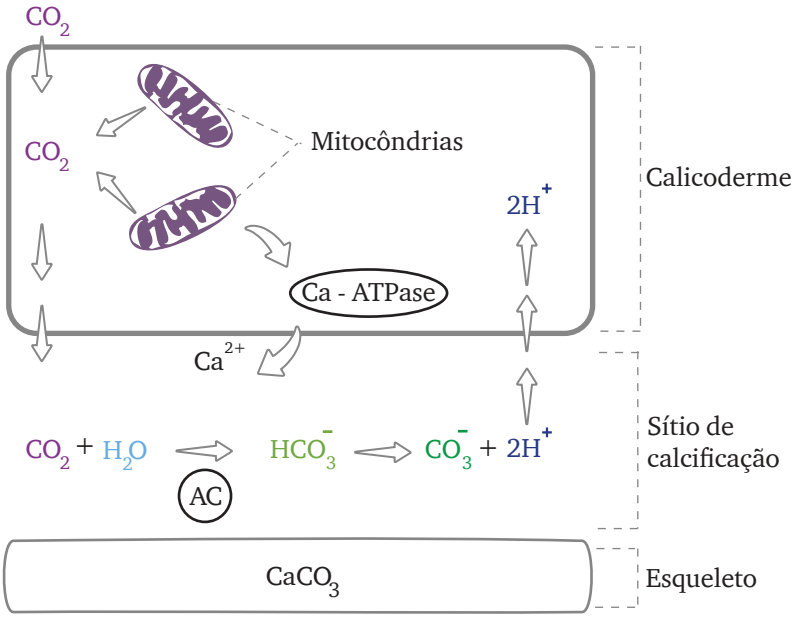


Figura 5. Esquema mostrando o funcionamento das enzimas no interior do sítio de calcificação.

Importância das zooxantelas no processo de calcificação

Corais escleractíneos zooxantelados apresentam taxas de calcificação mais elevadas na presença de luz. Acredita-se que interações entre os processos de fotossíntese e calcificação sejam a razão para maiores taxas de deposição de $CaCO_3$ na presença de luz. As possíveis interações entre esses processos, que seriam benéficas ao processo de deposição do exoesqueleto de corais, são: (i) a assimilação de CO_2 pela fotossíntese levaria a uma modificação do equilíbrio de espécies de carbono inorgânico dissolvido no interior dos tecidos do coral que, de forma indireta, favoreceria o processo de calcificação; nesse caso, a diminuição da pressão parcial de CO_2 no sítio de calcificação favoreceria a manutenção de um pH mais alcalino, otimizando, assim, a precipitação de carbonato de cálcio pelo aumento do Ωar; (ii) a neutralização de prótons (H^+) produzidos na precipitação de $CaCO_3$ por íons hidroxila (OH^-), resultantes do processo de fotossíntese, promoveria a manutenção de um pH mais alcalino, favorável ao processo de calcificação; (iii) a incorporação de compostos inibitórios ao processo de calcificação, como fosfatos, pelas zooxantelas; (iv) a produção de moléculas orgânicas pelas zooxantelas auxiliariam na síntese da matriz orgânica; nesse caso, as zooxantelas forneceriam precursores orgânicos, uma vez que a síntese da matriz orgânica é feita, exclusivamente, pelas células calicoblásticas; e (v) o incremento energético fornecido pelas zooxantelas (oxigênio e fotossintatos) seria fundamental para os processos que requerem energia (transporte de íons e síntese da matriz orgânica); nesse caso, a fosforilação oxidativa de fotossintatos seria o principal processo envolvido na disponibilização de ATP.

A acidificação dos oceanos e o processo de calcificação

O problema ambiental chamado "acidificação dos oceanos" decorre do excesso de CO_2 absorvido pelos oceanos devido às crescentes emissões desse gás na atmosfera. Em linhas gerais, o processo de acidificação acontece quando o excesso de CO_2 sequestrado reage com a água do mar tornando-a mais ácida. No entanto, para entender realmente o que é o processo de acidificação oceânica e seu impacto sobre a fisiologia de organismos calcificadores, é importante entender como o CO_2 se comporta nos oceanos.

O CO_2 absorvido pela superfície do oceano reage com uma molécula de água (H_2O) e forma um ácido fraco, denominado ácido carbônico (H_2CO_3). Grande parte desse ácido se dissocia, liberando prótons (H^+), bicarbonato (HCO_3^-) e carbonato (CO_3^{2-}). Dessa forma, quanto maior a quantidade de CO2 incorporado pelos oceanos, maior é a concentração de prótons (H^+), o que aumenta a acidez das águas (Figura 7). A superfície marinha é ligeiramente alcalina (pH =8,0 ± 0,3), sendo importante ressaltar que o termo acidificação não se refere ao processo pelo qual o oceano se tornará ácido (pH<7), mas sim ao processo de redução do pH.

As espécies de carbono inorgânico mencionadas (CO_3^{2-}e HCO_3^-) fazem parte de um sistema tampão natural importante no controle do pH da água do mar, sendo que o aumento induzido de CO_2 na água modifica o equilíbrio e a abundância relativa dessas espécies. O carbonato (CO_3^{2-}) é essencial aos organismos calcificadores na produção de seus esqueletos e de conchas de carbonato de cálcio ($CaCO_3$). Essas estruturas de $CaCO_3$ são encontradas principalmente nas formas minerais calcita e aragonita, sendo que a capacidade dos organismos de construí-las está relacionada ao estado de saturação da calcita ou da aragonita, respectivamente. O estado de saturação depende, em grande parte, da concentração de CO_3^{2-} na água. Em níveis típicos de pH da água do mar, íons CO_3^{2-} se encontram em nível suficiente para que as estruturas calcárias sejam facilmente secretadas pelos organismos marinhos calcificadores. Porém, pequenas mudanças de pH decorrentes do aumento induzido de CO_2 na água tornam os elementos que compõem os esqueletos calcários mais solúveis e promovem a reação entre H^+ e CO_3^{2-}, com consequente formação de HCO_3^-. Uma vez que a concentração de CO_3^{2-} é reduzida, ocorre também a diminuição do estado de saturação da calcita e da aragonita, com consequente redução nas taxas de calcificação.

Como mencionado, um investimento energético aumenta o pH no sítio de calcificação dos corais e, consequentemente, o estado de saturação da aragonita. Porém, em águas mais ácidas, que apresentam menor estado de saturação, esses organismos necessitam investir ainda mais energia para aumentar o pH no sítio de calcificação e alcançar, assim, um nível de saturação da aragonita suficientemente elevado para que a formação do esqueleto calcário ocorra em taxas mais elevadas que sua dissolução. Em tal contexto, a questão central para a qual ainda não se tem uma resposta definitiva é a seguinte: até que ponto os corais têm a capacidade de compensar energeticamente os efeitos da acidificação dos oceanos sobre o processo de calcificação?

É importante perceber que a dissolução das estruturas de $CaCO_3$ em condições de pH mais ácido está relacionada ao sistema tampão da água do mar, no qual o CO_3^{2-} tem grande importância. A dissolução dessas estruturas acaba por disponibilizar CO_3^{2-} na água do mar, sendo elas responsáveis pela manutenção natural do pH oceânico dentro de uma faixa estreita de variação. A dissolução dessas estruturas também depende da estrutura química em que o $CaCO_3$ é precipitado. A forma mineral aragonita é ≈50% mais solúvel que a calcita pura. Sendo assim, organismos como os corais, que secretam seus esqueletos na forma de aragonita, são mais vulneráveis aos efeitos da acidificação marinha.

Estima-se que a diminuição em torno de 0,1 unidade na média do pH do oceano desde a era pré-industrial já tenha ocasionado uma redução de 20% nas taxas em que os corais formadores de recife produzem seus esqueletos. Além disso, projeções feitas pelo Painel Intergovernamental sobre Mudanças Climáticas (IPCC) sugerem uma diminuição em mais 0,3 unidade de pH até o fim deste século, o que poderia ocasionar um declínio de aproximadamente 50% nas taxas de calcificação de corais. Nesse contexto, o processo de acidificação pode ocasionar o aumento das taxas de erosão da estrutura física dos recifes de coral, o que pode resultar no declínio desse ecossistema, com sérias consequências para todos aqueles que dele dependem.

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GOVERNO FEDERAL
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Agência Brasileira do ISBN
ISBN 978-85-7427-057-9
9 788574 270579