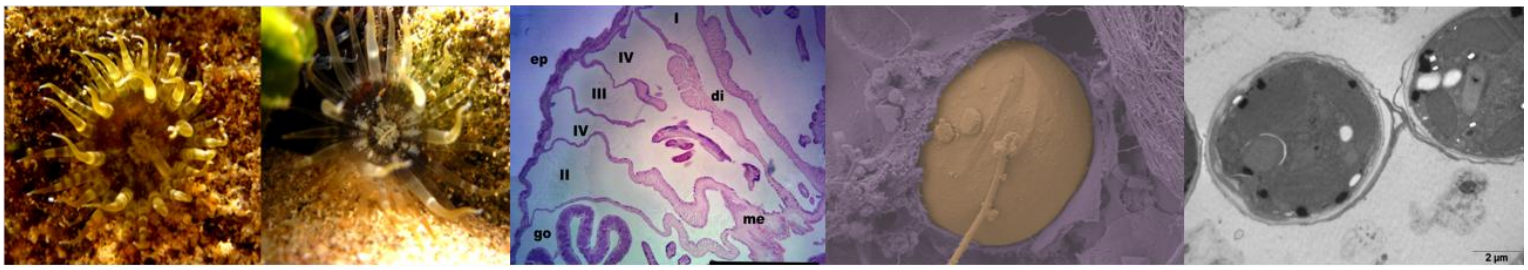

Processos fisiológicos envolvidos na aquisição, perda e recolocação da zooxantela ligados ao branqueamento de cnidários.

Physiological processes associated to acquisition, loss and recovery of zooxanthellae related to cnidarian bleaching.

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Thesis submitted on December 2017 for fulfillment of a Doctorate of Philosophy

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Essa tese é uma versão corrigida, cuja original encontra-se disponível no Instituto de Biociências da USP.

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Dirigido a aquellas personas que no saben nada de fisiología, pero mucho de interacciones

A ti, parte indispensable de esta carrera de fondo. Sin el balón de oxígeno que escondían tus abrazos nunca hubiese llegado hasta aquí. ¿Cómo explicarte a qué he dedicado los últimos cuatro años de mi vida tan lejos de tu sonrisa? Te lo explico lo mejor que sé: con el corazón. El coral vive en amor y compañía de alga, por eso los corales son coloridos. Cuando hace mucho calor, el alga y el coral no se soportan. Se gritan, se dicen cosas feas, y el alga se indigna, coge su color, su fotosíntesis y abandona el abrigo del coral. "Ya no quiero ver películas contigo los domingos. El mar está llenito de peces, ahí te quedas". El coral se pone triste, blanco y muere. Hasta que unos corales optimistas creyeron en segundas oportunidades. Aguantan el calor insoportable blancos tristes y malhumorados. "Si tú no me quieres, otra alga me querrá". Y cuando esto pasa, intentan pescar cualquier alga que pasa por ahí, a ver si seducen una nueva alga que le devuelva el color y la alegría de vivir. "¿Vienes mucho por este lado del Atlántico? ¿De qué mar es ese acento?". Y yo, que soy muy romántica y también creo en las segundas oportunidades, me propongo blanquear a los corales, pero no para que se queden blancos, tristes y malhumorados, sino para ofrecerles nuevas parejas, a ver qué tal se llevan, y volver a llenar de color el mar. Todo por hacer citas a ciegas entre flores de mar y hierba de agua.

Metade de minha alma é feita de maresia

Caminante no hay camino, sino estelas en la mar

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Índice

Prefácio

Esta tese compreende seis capítulos ao todo. O capítulo primeiro contém uma introdução geral sobre a problemática do branqueamento em cnidários, assim como aspectos fisiológicos da recuperação desse processo, apresentando informações relevantes na área, com ênfase nas janelas de conhecimento e justificativa teórica da necessidade de cobrir os vazios de entendimento dentro dessa área.

O segundo capítulo apresenta os resultados sobre "Recuperação do branqueamento e mecanismos de estabelecimento da simbiose na anêmona *Exaiptasia pallida"*. Trata sobre o tempo necessário de recuperação da pigmentação após branqueamento partindo dos endosimbiontes remanescentes no cnidário, avaliando o padrão de repigmentação em diferentes partes do corpo. Apresenta também uma proposta de mecanismo de ruptura da simbiose na qual os endosimbiontes participam ativamente na sua saída e posterior dispersão no meio, abrindo uma nova discussão sobre o agente que dirige tanto o estabelecimento quanto a ruptura da simbiose. Este capítulo está apresentado no formato em que foi submetido ao jornal Microbiome sob o titulo: "Bleaching recovery and assembling/disassembling mechanisms of the *Exaiptasia pallida* (Cnidaria)-*Symbiodinium* spp. (Dinoflagellata) holobiont system". Resultados preliminares desse manuscrito também foram apresentados como pôster no “XIX Simpósio de Biologia Marinha” do CEBIMar, NP-BioMar, e na conferência internacional “Holobiont” no Museu de História Natural de París como apresentação oral com o título “The inner help: bleached *Exaiptasia pallida* (Anthozoa, Actiniaria) farms and allocates remaining endosymbionts ensuring the inheritance of *Symbiodinium* spp. to offspring”, e como pôster com o título “Assembly and disassembly mechanism of an holobiont system” durante a mesma conferência.

O terceiro capítulo, "Flutuação Circadiana da atividade fotossintética e antioxidante da anêmona *Exaiptasia pallida",* trata sobre a avaliação da atividade redox induzida pela fotossíntese da alga hospedada na anêmona, assim como a atividade antioxidante e reparadora do hospedeiro, propondo um possível mecanismo de preparação ao estresse oxidativo para lidar com a atividade fotossintética da zooxantela. O manuscrito apresentado está submetido ao periódico "Journal of Experimental Marine Biology and Ecology", sob o título "Diurnal fluctuation of photosynthesis and antioxidant activity of the *Exaiptasia pallida* (Cnidaria)-*Symbiodinium* spp. (Dinoflagellata) holobiont system".

O quarto capítulo investiga se existe uma alga ideal para cada temperatura dadas as diferentes capacidades fisiológicas de cada clado por separado. O manuscrito apresentado será submetido na revista Coral Reefs sob o título " The holobiont *Exaiptasia pallida-Symbiodinium* sp. is better prepared to overcome warm events than cold temperatures and hosting mixed

endopulation including clades E and F may favor bleaching struggle within the Climate Change Context ".

O quinto capítulo apresenta uma proposta de anêmonas como bioindicadores ambientais como resultado adicional á linha principal desta tese. Considerando que o peróxido de hidrogênio é um derivado de filtros solares e que ao mesmo tempo é um precursor do branqueamento, investigamos se o branqueamento da anêmona *Anemonia sulcata* produzido pela exposição a peróxido de hidrogênio é um bom modelo como bioindicador ambiental através da avaliação da cor e de biomarcadores fisiológicos. Foi desenvolvido durante estágio na Universidade de Cádiz em colaboração com a ecotoxicóloga Dra. Laura Martín Díaz, cujo manuscrito será submetido no jornal Aquatic Toxicology sob o título "Responses of the sea anemone, *Anemonia sulcata*, to increased hydrogen peroxide concentrations in seawater".

Finalmente, o sexto capítulo recolhe as conclusões e considerações gerais do presente doutorado, integrando os resultados obtidos nos capítulos anteriores e indicando as direções futuras para próximas investigações nesta área.

Capítulo I

Introdução geral

Branqueamento de cnidários e aspectos fisiológicos envolvidos na recuperação

Branqueamento de cnidários

O branqueamento é a perda de pigmentos em cnidários simbiônticos, resultado da diminuição marcada ou do desaparecimento das zooxantelas. Este processo pode ser natural e transitório, induzido por mudanças sazonais de luz e temperatura que afetam a fisiologia da alga simbionte e/ou do animal hospedeiro (Fitt et al., 2000). No entanto, pode também ser resultado de estresse ambiental específico, como a acidificação dos oceanos, eventos de temperatura extrema (Cook et al., 1988; Porter et al., 1989; Gates, 1990; Glyn, 1990; Hoegh-Guldberg et al., 2007), ou ainda da ação indireta de microorganismos (p.ex. bactérias - Rosenberg *et al.* 2007). Dentre esses, o estresse térmico tem sido considerado como a principal causa do grave declínio de cnidários simbióticos nos oceanos tropicais verificado em tempos recentes (Hughes et al., 2003; Carpenter et al., 2008). Em nível celular, a perda das zooxantelas pode ser efetivado por diferentes mecanismos, como digestão, exocitose ou necrose, mas estudos recentes indicam a apoptose e a autofagia como uma resposta mais específica ao estresse térmico (Dunn et al., 2007).

A diminuição da população endosimbiôntica (Jones et al., 2000; Grottoli et al., 2004), junto com a redução da eficiência fotossintética dos endosimbiontes remanescentes (Iglesias-Prieto et al., 1992; Lesser, 1996) podem afetar a capacidade fotossintética do holobionte, que é o hospedeiro e demais organismos que vivem em simbiose. Assim, cnidários branqueados perdem sua principal fonte de energia, alterando seu balanço energético (Muscatine, 1967, 1980) o que pode resultar em inanição (Muscatine, 1990; Anthony e Fabricius, 2000) provocando perda de biomassa (Fitt et al., 2000). Consequentemente, o hospedeiro sofre redução das taxas de crescimento, calcificação (no caso de cnidários calcificantes) e reprodução, além de ter incrementada sua vulnerabilidade frente a enfermidades (Buddemeier et al., 2004; Hughes et al., 2007; Rosenberg et al., 2007; Brandt et al., 2009), o que pode levar à morte (Brown, 1997; Hoegh-Guldberg, 1999). Cnidários afetados se tornam competidores menos eficientes em comparação com outros organismos bentônicos (Foster et al., 2008; Green et al., 2008) e, consequentemente, a comunidade associada pode sofrer mudanças dramáticas, induzindo eventos de mortalidade massiva e perda da biodiversidade local (Goreau, 1992; Hale et al., 2011).

Recuperação do branqueamento

Contudo, o processo de branqueamento pode ser revertido, e diversos cnidários têm demonstrado capacidade de recuperação da pigmentação após eventos de branqueamento (Berkelmans et al., 2006; Burt et al., 2008; Jones et al., 2008). Entretanto, previsões sobre fenômenos potencialmente deflagradores de branqueamento apontam para um aumento em frequências e severidade (Hoegh-Guldberg, 1999), o que deve agravar os efeitos dessas perturbações. Assim, impactos crônicos podem ser esperados no caso de cnidários incapazes de recuperar a pigmentação no intervalo de tempo compreendido entre sucessivos eventos de branqueamento, particularmente em áreas de recife (Baker, 2008). Se o tempo necessário para re-pigmentação for maior que o intervalo entre os eventos, a recuperação pode ser comprometida, resultando em maiores taxas de mortalidade (Jones, 2008). Nesse contexto, é relevante estudar o tempo necessário para restauração da pigmentação como uma ferramenta para avaliar os danos de futuros eventos sucessivos deflagradores de branqueamento, assim como os padrões de re-pigmentação em cnidários. Desta forma, o segundo capítulo é dedicado a investigar estes aspectos e entender os padrões de recuperação da pigmentação no plano corporal do holobionte a partir da proliferação de endosimbiontes remanescentes.

Estresse oxidativo associado ao branqueamento

O mecanismo celular do branqueamento em cnidários ainda não é bem conhecido. Diversos estudos apontam que o processo do branqueamento teria sua origem na alga hospedada. Uma vez que um agente externo estressa o holobionte, a alga produziria um excesso de espécies reativas de oxigênio que seriam trasladadas por sua vez ao hospedeiro (Neill et al., 2002; Asada, 2006; Lesser, 2006; Wong et al., 2010). Uma vez ultrapassada a capacidade redutora do hospedeiro, o mesmo poderia sofrer estresse oxidativo que ativaria mecanismos de ruptura da relação, resultando na diminuição da população endosimbiotica e o consequente branqueamento do cnidário. Para entender o estresse oxidativo sofrido pelo hospedeiro, é interessante estudar a capacidade antioxidante de defesa do mesmo. A primeira defesa antioxidante de natureza enzimática seria constituída pela catalase (CAT) e glutationa peroxidase (GPX). A glutationa entraria em um ciclo redox pela glutationa reductase (GR), enquanto a glutationa peroxidase, contribuiria com a redução da glutationa. Quando funcionando em equilíbrio, esta detoxificação é efetiva. Porém quando a produção de espécies reativas é superior ao ritmo de detoxificação, pode ocorrer dano oxidativo sobre lipídios, proteínas e material genético, danificando membranas e organelas celulares. Esse dano oxidativo ao hospedeiro, oriundo da fisiologia da alga hospedada a partir da atividade oxidante "extra", poderia ser o ativador dos mecanismos de branqueamento (Figura 1).

Preparação fisiológica para o estresse oxidativo

De forma geral, existem mecanismos antioxidantes de antecipação ao estresse oxidativo provocado por diversos fatores cíclicos (Malik e Storey, 2009; Weihe et al., 2010; Storey e Storey, 2013; Krivoruchko e Storey, 2015). Estes têm sido descritos para uma variedade de organismos (Fu et al., 2012; Schülke et al., 2012; Klanian, 2013; Zhang et al., 2015), incluindo cnidários aposimbiônticos (Teixeira et al., 2013). Em organismos fotossintéticos, grande parte dos processos fisiológicos e comportamentais estão relacionados com os ciclos físicos diurnos relacionados com a exposição à luz (Kühl et al., 1995; Yacobovitch et al., 2004; Hernando, 2016; Millar, 2016). Esses mecanismos preventivos do estresse são denominados POS, do inglês "Preparation for Oxidative Stress" (Moreira et al., 2016). Porém, POS em animais simbiônticos, não é conhecido. Nesse sentido, o terceiro capitulo da presente tese visa elucidar a sincronização metabólica entre a atividade fotossintética da alga hospedada e a atividade antioxidante da anêmona no contexto do POS.

Branqueamento como estratégia adaptativa

A perda da alga pode fornecer uma oportunidade para o hospedeiro ser reinfectado por novos simbiontes, sendo que diversos autores o consideram como uma fase de adaptação e recuperação às novas condições ambientais (Buddemeier e Fautin, 1993; Baker 2001; Baker et al., 2004; Obura, 2009). Por exemplo, no Panamá e Galápagos o evento do “El Niño” 1982-83 provocou branqueamento e mortalidade significativamente maiores do que o “El Niño” 1997-98, embora o último tenha sido tão forte ou mais que o anterior (Podestá e Glynn, 2001). Na Grande Barreira de Corais os limites de temperatura de branqueamento aumentaram em 2002 com relação a 1998, provavelmente por um processo de aclimatação (Berkelmans, 2009). Neste sentido, Baker (2001) defende que os corais branqueiam para sobreviver à alteração, ou seja, descartam ativamente suas zooxantelas. No entanto, outros consideram que o branqueamento pode ser um mecanismo para minimizar o desequilíbrio na relação hospedeiro-simbionte causado pelo estresse (Obura, 2009).

***Symbiodinium* spp.**

Atualmente, nove clados de simbiontes pertencentes ao gênero *Symbiodinium* são descritos na literatura, sendo nomeados de A até I (Pochon e Gates 2010). Porém, a classificação desse gênero continua em andamento, com a subdivisão de cada clado em "subclados" com base na comparação de sequências nucleotídicas da região do gene ITS2 rDNA (LaJeunesse 2001; Baker, 2003; Coffroth e Santos 2005; Sampayo et al., 2009; Finney et al., 2010; LaJeunesse e Thornhill, 2011). Dos clados/subclados conhecidos, alguns têm exibido capacidades fisiológicas distintas. Por exemplo, nem todas as linhagens de *Symbiodinium*

respondem de maneira igual ao stress térmico (Suggett et al., 2008). Em particular, os clados A e F têm sido descritos como moderadamente tolerantes, enquanto que o B seria mais sensível (McGinty et al., 2012).

O estabelecimento da simbiose

Estima-se que o 85% dos corais adquiram os dinoflagelados por via horizontal nas primeiras fases de seu ciclo de vida, durante a alimentação mediante ingestão ou fagocitose. Uma vez dentro do hospedeiro, os simbiontes residem em vacúolos das células da gastroderme dos pólipos adultos, sendo que a maioria dos corais adultos abriga apenas um clado de simbionte (Baker, 2003). Por exemplo, em Scleractinia é comum encontrar associações com os clados A, B, C ou D, e mais raramente com os clados F ou G (Baker, 2003; LaJeunesse et al., 2010). Porém, a especificidade entre clado hospedado-hospedeiro é variável: enquanto algumas espécies têm sido observadas associadas com um único clado, outras podem abrigar vários tipos de *Symbiodinium* spp. (Pochon et al., 2001). Esta associação pode ser correlacionada com condições ambientais, como incidência de luz ou temperatura (LaJeunesse, 2002; Baker 2003; Finney et al., 2010), e a estrutura das populações simbióticas pode ser trocada em resposta às necessidades fisiológicas provocadas por mudanças ambientais (Baker e Romanski, 2007). Por exemplo, no caso da anêmona *Condylactis gigantea* encontrou-se uma variação da população endosimbiôntica de acordo com a posição em relação à costa e com as variações sazonais de temperatura. Neste organismo, o clado A era o predominante nos ambientes mais variáveis e com picos de temperatura mais pronunciados, enquanto o B predominava em áreas de condições mais estáveis. Além disso, foi verificado que o clado B era mais susceptível ao branqueamento que o A (Venn et al., 2008).

A capacidade de trocar a população endosimbiôntica segundo as condições ambientais tem sido descrita como "symbiont shuffling", ou "mudança de simbionte" (Baker, 2001; Chen et al., 2005). Porém, alguns cnidários branqueados, adquirem do ambiente um clado distinto daquele que estava previamente associado. Essa troca de população endosimbiôntica é denominada "symbiont switching" (Baker, 2003). Num contexto de mudanças climáticas globais, se a origem do dinoflagelado é o ambiente, o cnidário tem a chance de se associar com um clado mais adaptado às condições alteradas, resultando em uma associação que pode ser mais termorresistente e que consegue se aclimatar melhor. Portanto, o branqueamento pode fornecer uma oportunidade para o hospedeiro ser “reinfectado” por clados de *Symbiodinium* mais adequados nessas condições (Buddemeier e Fautin, 1993). Sendo assim, prevê-se que as espécies com tais mecanismos podem apresentar branqueamento como uma estratégia adaptativa e, portanto, uma mortalidade menor após o episódio de stress térmico (Obura, 2009). Assim, no quarto capitulo deste trabalho exploramos a hipóteses de que existe uma associação

mais apropriada para cada temperatura, e investigamos as vantagens fisiológicas de cada associação estabelecida.

As anêmonas como espécies bioindicadoras

O peróxido de hidrogênio é um precursor do branqueamento no nível intracelular (Smith et al., 2005; Sandeman, 2006). Este composto tem elevado potencial oxidativo, é difundido facilmente através das membranas orgânicas e interfere em várias reações celulares (revisado por Lesser, 2006). Além disso, pode originar o radical hidroxila (Haber e Weiss, 1934), um precursor que, por sua vez, desencadeia reações em cascata cujo término pode ser peroxidação lipídica, desnaturação proteica e dano ao DNA (Borg e Schaich, 1984; Lesser, 2006). Além disso, o peróxido de hidrogênio tem sido identificado, dentre outras substâncias oxidativas reativas, como um subproduto de reações fotocatalíticas dos filtros solares quando em contato com a água (Inbaraj et al., 2002; Hanson et al., 2006; Sánchez-Quiles e Tovar-Sánchez, 2014). A exposição a filtros solares tem sido ligada a eventos de branqueamento de cnidários (Danovaro et al., 2008) e o peróxido de hidrogênio da sua decomposição tem sido mostrado como nocivo para o fitoplâncton e outros organismos (Sánchez-Quiles e Tovar-Sánchez, 2014). Porém, os efeitos das concentrações ambientais em anêmonas têm sido pouco estudados (Mitchelmore et al., 2004; Higuchi et al., 2008). Assim, no quinto capitulo da presente tese, foi investigada a possibilidade de usar o branqueamento de anêmonas como um indicador ambiental de estresse oxidativo.

O sistema holobionte: *Exaiptasia pallida*-*Symbiodinium* spp.

Para entender estes mecanismos de recuperação do branqueamento, as interações redox entre hospedeiro-hospedado, a sincronização metabólica do holobionte e as capacidades fisiológicas das diferentes associações, é importante conhecer a relação alga-cnidário. Nesse trabalho usamos o escopo de holobionte, no qual consideramos a associação como uma única unidade funcional. Para isso, foi utilizado o holobionte formado pela anêmona *Exaiptasia pallida* (Anthozoa: Aiptasiidae) e o dinoflagelado *Symbiodinium* spp. Esta anêmona é uma espécie descrita como cosmopolita, com ampla distribuição e de associação simbiôntica generalista (Grajales e Rodríguez, 2016), sendo inclusive considerada como uma peste em aquários. Em adição, devido a facilidade de manipulação e cultivo em laboratório, tem sido usada como modelo em estudos sobre o branqueamento de corais (Thornhill et al., 2013; Baumgarten et al., 2015). Sua transparência na forma aposimbiôntica permite acompanhar com facilidade a repopulação endosimbiôntica durante a recuperação da pigmentação. Adicionalmente, sendo solitária, facilita o desenho experimental em comparação com cnidários coloniais, e por não possuir esqueleto permite isolar melhor as relações metabólicas, sem interferência dos processos calcificantes.

Objetivos gerais do estudo

Propomos um estudo integrado sobre o branqueamento de cnidários e sua recuperação, considerando as interações entre o cnidário hospedeiro e a zooxantela hospedada. O objetivo geral é o estudo do processo fisiológico da perda e realojamento do simbionte relacioando ao fenômeno do branqueamento em *E. pallida* por aumento de temperatura. Os objetivos específicos da proposta são:

a) Estudo da repopulação endosimbiôntica após eventos de branqueamento/estresse termal;

b) Pesquisa das interações redox hospedeiro-hospedado;

c) Avaliação da variação de parâmetros fisiológicos em função da temperatura e do tipo de alga oferecida;

d) Proposta de um modelo de anêmona como bioindicador ambiental.

Para responder aos objetivos foram desenvolvidos diversos protocolos, desde cultivo e manutenção de organismos, até microscopia eletrônica e técnicas micro-espectrofotométricas, para serem utilizadas neste modelo (*E. pallida*), os quais são descritos nos capítulos pertinentes.

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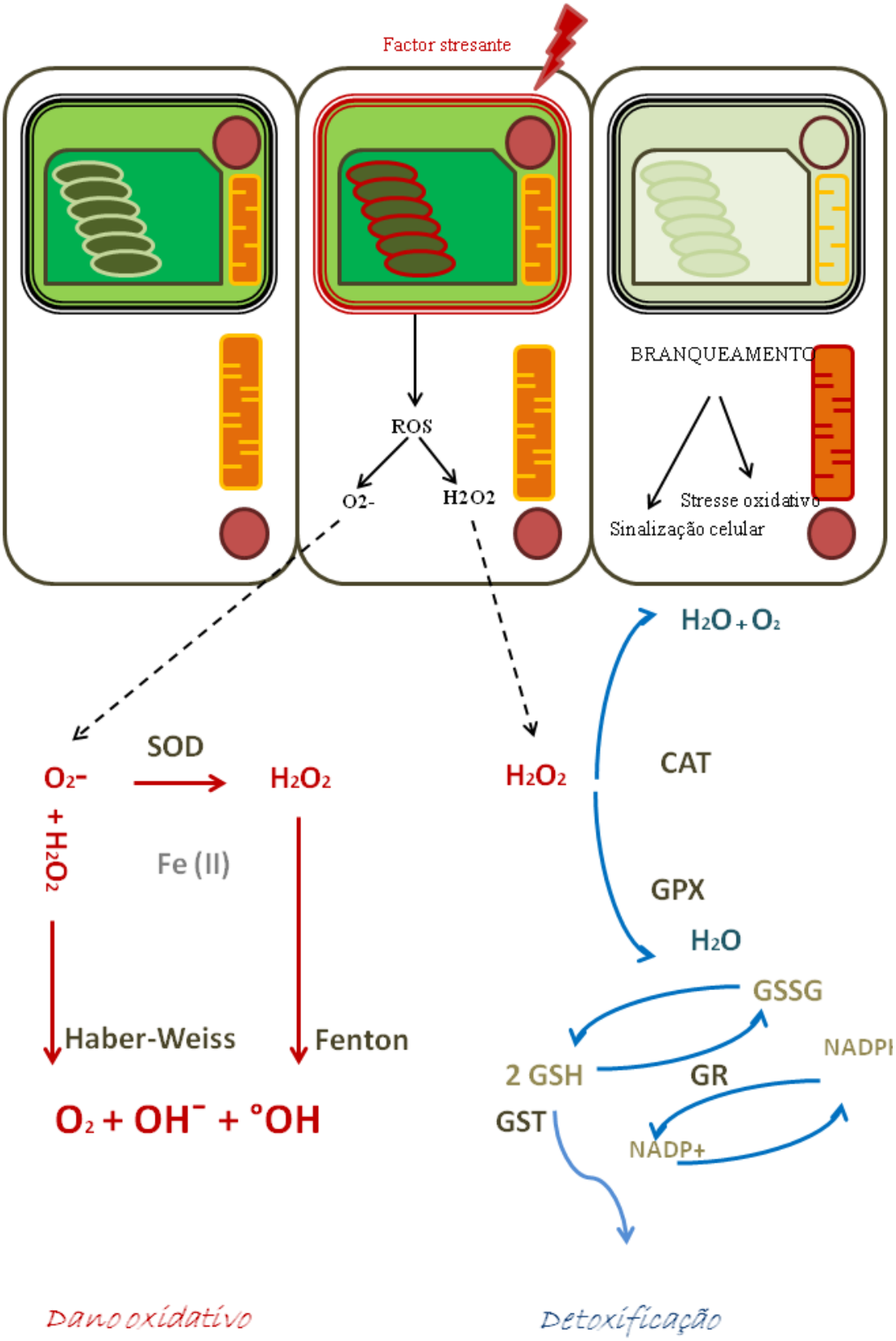
Figura 1. Mecanismo celular de branqueamento em cnidários.



Capítulo II

Recuperação do branqueamento e mecanismos de estabelecimento da simbiose na anêmona *Exaiptasia pallida*

Bleaching recovery and assembling/disassembling mechanisms of the *Exaiptasia pallida* (Cnidaria)-*Symbiodinium* spp. (Dinoflagellata) holobiont system

(Submitted to Microbiome)

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Abstract

Background

Bleaching events are increasing in frequency and strength. It elicits holobiont disassembling in symbiotic cnidarians. However, the association may be reestablished by poorly understood assembling mechanisms. Understanding the recovery time span might allow accurate predictions about ecological consequences of successive bleaching events. In this study, we investigated bleaching recovery of *Exaiptasia pallida*, an actiniarian that lives in symbiosis with the dinoflagellate *Symbiodinium* spp. We measured time of pigmentation of bleached anemones at four functional body parts in holobiont of two sizes using a six levels color scale. We also analyzed histochemical composition and ultrastructure of the symbioma with electron microscopy.

Results

Regardless on anemone's size, we found a sequence of pigmentation recovery after bleaching: tentacles; pedal disc; column-oral disc ($p<0.0001$) that delayed 10 ± 2 days on earlier stages ($p < 0.0001$) (Table 1). Independently on holobiont's size, tentacles pigmented after 8,5 (± 1,9 days), followed by pedal disc (22,5 ± 4,4 days) and pedal laceration. Based on our results,

we propose that one potential holobiont system assembling is sequential proliferation of endogenous symbionts.

We also observed *E. pallida* regurgitating viable endosymbiont clumps embedded on a proteoglycan matrix before concluding pigmentation. Based on ultrastructure observations, we propose such holobiont disassembling mechanism be termed symbiont clumping (flagella interaction; host membrane cell dissipation; endosymbiont membrane interaction; endosymbiont detachment; clumping and clump expulsion).

Conclusions

The time span between successive bleaching events can have a significant impact in the holobiont's recovery. If the intervals are shorter than 40 days, young individuals of the population might not recover maximal pigmentation levels. Less than a month it may alter the structure of the associated community. With less than 10 days intervals, the hosts might not transfer endosymbionts to the offspring, and if it is shorter than a week the endosymbiont clumping, vertical transference and photosynthetic energy supply may be adversely affected.

Who drives the assembling/disassembling mechanisms between the holobiont partners remains unclear. Pigmentation recovery sequence apparently favors light capture and vertical transmission of endosymbiont to host's offspring while the active symbiont clumping disassembling and its expulsion during pigmentation may favor dinoflagellate dispersion.

Key words: bleaching recovery strategy, pigmentation pattern, pigmentation sequence, clumps regurgitation, symbiont clumping, zooxanthellae regulation, vertical transmission, recovery time span, disassembling mechanism, dinoflagellate dispersal strategy.

Background

Bleaching is the pigment loss in symbiotic cnidarians, usually provoked by environmental stress, such as extreme temperature and ocean acidification [1-4]. The decline of endosymbiont populations [5-6] along with the reduction of the photosynthetic efficiency of remaining endosymbionts [7-8] may impair the photosynthetic capacity of the holobiont, ultimately disrupting the energy balance [9-10] and tissue biomass [11]. Thus, bleached cnidarians losses its primary source of energy supply and may starve [12-13]. As a consequence, the host experiences reduced growth rate, calcification (if present), reproduction, and increases its vulnerability to diseases [14-16], ultimately leading to mortality [17-18]. In general, affected cnidarians become weaker competitors in relation to other benthic organisms [19] and the associated community may suffer dramatic changes [20].

Interestingly, some cnidarians have shown the ability to recover from bleaching [21-23]. This process can be natural and transient, driven by seasonal changes in light and temperature on algal and/or animal physiology [24]. Nevertheless, bleaching events are expected to increase in frequency and severity [18] and such disturbances are likely to cause chronic adverse effects in reef areas if affected cnidarians are not able to recover quickly enough [25]. In this context, it seems relevant to diagnose the required time for bleaching recovery. If it is longer than the interval between bleaching events, the recovery may fail, resulting in bleaching-induced mortality [26]. Despite that a thoroughly knowledge of bleaching recovery time span will improve our understanding of the consequences of bleaching events, such studies are scarce.

Herein, we use the holobiont formed by the glass anemone *Exaiptasia pallida* (Anthozoa: Aiptasiidae) and the dinoflagellate *Symbiodinium* spp. to study bleaching recovery time span. This holobiont has been widely used as a model for the study of coral bleaching, as its management and manipulation in laboratory is straightforward [27-28]. Furthermore, its transparency during the aposymbiotic form let us measure the endosymbiotic population growth by the increase of the anemone's pigmentation, of which can be monitored by a color scale as a direct indicator of endosymbiont density and chlorophyll-a content [29]. Traditional methods to characterize the status of cnidarians' pigmentation are based on simple categories ‘‘normal’’, ‘‘partially bleached’’, ‘‘bleached’’ and ‘‘dead’’, disregarding the levels of pigmentation or the distribution along the body parts of the host [30]. In this study, we considered single polyp species to understand if different body parts (tentacle; oral disc; column; pedal disc) recover the endosymbiont population equally after bleaching. Furthermore, we considered two polyp sizes to discuss about potential differences in the time span for recovery and community composition after bleaching events. Finally, based on the evidences found in the pattern of recovery, we discuss about which symbiont drives the bleaching recovery process: microalgae versus anemone.

Materials and Methods

In order to study bleaching recovery, we adapted a methodology [31] to induce bleaching to the anemone *E. pallida*. Individuals of *E. pallida* were subjected to 4 hours cold shock at 4ºC and returned to the aquarium, at 24ºC, 35 ppm salinity, pH 8.2-8.4 and 12:12 photoperiod. They were fed twice a week with *Artemia salina* nauplii. The anemones bleached two weeks after the cold shock and remained white over six months.

For the experiment, eight white and eight dark brown adults of *E. pallida* were individually set in 800 ml aquariums, at 24ºC, 35 ppm salinity, pH 8.2-8.4 and 12:12 photoperiod with white cold light at 1000 lux and gentle aeration. Each batch of eight anemones consisted of four medium (2.5 ± 0.3 cm oral disc) and four small size (0.4 ± 0.2 cm oral disc)

individuals. To avoid environment dinoflagellates contamination, the water was filtered with 0.22 µm micropore and renewed weekly. In each renewal, 15 pedal lacerates were selected and kept in 3 ml finger bowls under the same conditions described above. The experiment lasted 46 days, until all the individuals reached the maximum level of pigmentation at all body parts.

In order to register the pigmentation during heterotrophic deprivation, the anemones' color was evaluated using the CoralWatch Scale provided by the University of Queensland (Australia). This is an inexpensive, rapid and non-invasive method for bleaching assessment which has been validated in other cnidarians [32]. The scale has six levels of tonality, from white (E1) to dark brown (E6), directly correlated with endosymbiont density and chlorophyll-a content [32]. For each anemone, measurements were taken from four main body parts: pedal disc; column; oral disc; and tentacles (Fig. 1a). We considered a body part as pigmented when it maintained at least E5 level during three consecutive observations and completed pigmentation when the body had an even coloration on the four body parts mentioned above.

During the pigmentation process, we observed regurgitation of pellets. To further study its origin, structure and function, we analyzed the histochemical composition and the ultrastructure of both pellets and associated algae from gastrodermis at tentacles and gastrovascular cavity were analyzed using electronic microscopy. For histochemical analysis fixed aggregates were cytospun on coverslips (80 x g, 5 min, 248 FANEN), washed using Milli-Q® water for 20 minutes, and stained on Mallory's trichrome.

Scanning Electron Microscopy

The anemones were anaesthetized in a 1:1 solution of 0.3 M $MgCl_2.6H_20$ and seawater for 1 hour. Cross and longitudinal sections of the column and tentacle of *E. pallida* and the expelled *Symbiodinium* spp. pellets were criticalpoint dried (CPD 030,Balzers), attached on stubs, sputter-coated (SCD 050, Balzers) with gold/palladium, and observed with a scanning electron microscope (Sigma VP FEG, Zeiss).

Transmission Electron Microscopy

The anemones were anaesthetized and fixed as described above. Cross sections of tentacles were cut for better buffer penetration and fixed with Karnovsky's fixative [33] for 24 h at 4°C. All samples were then washed in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C. Following, they were rinsed in 0.1 M NaCl and stained in 1% aqueous uranyl acetate overnight at 4°C. Samples were subsequently dehydrated through immersion in a graded ethanol series at room temperature, and embedded in Spurr's resin [34] (EMbed-812, Electron Microscopy Science). Ultrathin sections were cut using a ultramicrotome (Ultracut UCT, Leica) and a diamond knife, and stained on 200

μm copper grids for 1 minute with lead citrate. Multiple sections from each sample were visualized on EM900 (Carl Zeiss) electron microscope at the Electronic Microscopy Laboratory of the University of São Paulo.

Statistical analysis

Bleaching recovery time span was measured as time of pigmentation of each body part. In order to elucidate if the pigmentation recovery was homogeneous we compared the time of pigmentation at 4 body parts (tentacles; oral disc; column; pedal disc). To assess if it changes through the ontogeny, we compared two anemones sizes: medium (2.5 ± 0.3 cm oral disc) and small size individuals (0.4 ± 0.2 cm oral disc). Differences between body parts and sizes were tested using a two-way ANOVA and determined with multiple comparison tests of Bonferroni using GraphPadPrism statistical package. Significance level was set at $\alpha \leq 0.05$.

Results

Bleached, unfed, but light supplied anemones, are able to survive, reproduce and achieve the maximal pigmentation. However, independently on their size, the recovery was not homogeneous through the polyp with a clear pattern of pigmentation at different body parts after bleaching ($p < 0.0001$) (Table 1). Furthermore, a sequence of endosymbiont distribution was developed equally in all individuals, notwithstanding its ontogenetic stage ($p < 0.0001$) (Table 1). Complete pigmentation was achieved after 31.0 ± 1.2 and 41.5 ± 2.5 days in medium, and smaller individuals respectively (Fig 1b) (Table 2). In all animals, the process started at the middle part of the tentacle, followed by pedal disc, column and, at last, oral disc (Fig. 1c) (Table 3).

After 8.5 ± 1.9 days, the middle part of the tentacles of medium individuals reached maximum pigmentation (E6 in the CoralWatch Scale), while small individuals took 16.5 ± 2.5 days to reach the same pigmentation. In both cases, base of the tentacles remained lighter (E2-E3) and tips white (E1). Pedal disc of all medium sized individuals completed pigmentation after 22.5 ± 4 days, while small individuals took 31.5 ± 3.4 days to reach the same level. The column of medium individuals became pigmented in 25.0 ± 6.2 days, while small anemones took 39.5 ± 2.5 days. Finally the oral disc of medium individuals reached complete pigmentation after 31.0 ± 1.2 days, while smaller individuals got such coloration only after 41.5 ± 2.5 days (Fig 1b).

A prolific pedal laceration was observed after 16 days in medium size (adult individuals) and 26 days in smaller individuals (Fig 1g). Every week, 15 random pedal lacerates from each individual were collected and the rest removed. The vast majority (97%) of the produced lacerates were pigmented as the lacerated pedal disc, and underwent the same pigmentation

pattern as adults. After 10 days, in deprivation of heterotrophic resources and free-living zooxanthellae, lacerates grew and became darker, polarizing the pigmentation to the upper part of the lacerates. When developed into fully formed anemones, they had dark tentacles, white oral disc, white column and white pedal disc (Fig 1h). After 15 days, they had dark pedal disc, but kept the oral disc not pigmented and still not fully pigmented column.

During the experiment, disregarding individual pigmentation level, adult individuals were observed regurgitating dark pellets (E6) around 3 mm in diameter (Fig 2a). The pellets consisted of zooxanthellae aggregates, with both coccoid and flagellated forms. They were ready to free-living, as indicated by their succeeded growth in F/2 Guillard medium over 40 days. These aggregations were embedded in a transparent organic matrix (Fig. 2b) of proteoglycan nature, as evidenced by the affinity to Methyl blue in Mallory's Thrichrome stain (Fig 2c). Its external and internal structure was a dense and cohesive pellet of dinoflagellates (Fig 2d). The clump's surface consisted of an intricate web (Fig 2e-f) and a membrane film (Fig 2g), which seemed to hold together adjacent dinoflagellates. These strands may be the dinoflagellate's own flagella (Fig 2h) and the origin of the membrane may be the symbiosoma or the host membrane (Fig 2i). Within the pellet we found *Symdiodinium* spp. in form of cysts, flagellated (Fig 2 j), and dividing cysts (Fig 2k) with membrane protuberances and invaginations (Fig 2l).

To elucidate the origin of such aggregates and given that the endosymbionts resides in the gastrodermal cells of the host, we investigated the gastrodermis of both gastrodermal cavity (Fig 3a) and tentacles (Fig 3b). In the gastrodermis, we found both hosted endosymbionts (Fig 3c) and free-living zooxanthellaes (Fig 3d). Among hosted endosymbionts, we found both unflagellated and flagellated forms of *Symbiodinium* spp. (Fig 3f), including flagella projections through the host's gastrodermis (Fig 3e). This is the first evidence of hosted flagellated form of zooxanthellae during cnidarians-dinoflagellate symbiosis (Fig 3f). We also found host openings showing hosted endosymbiont (Fig 3g), openings releasing flagellated endosymbionts (Fig 3h) and empty symbiomes cameras (Fig 3i).

Based on our observations, we propose a symbiont clump formation as a disassembling mechanism of the holobiont system, herein named symbiont clumping. Such phenomenon is a five steps mechanism that starts with the interaction of the endosymbiont flagella through the host's gastrodermis (Fig 4a), followed by host's cell membrane disruption between adjacent symbiomes (Fig 4b). Once zooxanthellae share the same symbioma, it may be a zooxanthellae membrane interaction (Fig 4c) that contributes to the membrane film that holds the symbiont clump together. Following steps are the endosymbiont detachment from the symbioma (Fig 4d), aggregate expulsion from the host's gastrodermis to the anemone's gastrovascular cavity (Fig 4e), and then, the expulsion from the gastrovascular cavity to the environment (Fig. 4f).

Discussion

In absence of heterotrophic resources and free-living zooxanthellae in the environment, bleached *E. pallida* farms their reminiscent zooxanthellae and complete maximum pigmentation in 40 days. Within successive bleaching events scenario, other cnidarians that require more time to reestablish their endosymbiont community may become weaker competitors, specially those that in opposition to the anemone *E. pallida* can not perform a full autotrotrophic compensation in deprivation of heterotrophic resources. Metabolism management from heterotrophic to autotrophic (and vice-versa) may enhance resources scarcity tolerance. It increases host's competitiveness against other benthic species and enables the holobiont to occupy further ecological/physiological niches. It could help to explain the current cosmopolitan distribution of this species [35].

During the time span between two consecutive bleaching events three situations of eventual recovery may occur [36]: (1) residual endosymbiont proliferation - our results show that *E. pallida* would need approximately 8 days to recover tentacles pigmentation and that the endosymbiont inheritance would be assured within 16 to 26 days depending on size; (2) another clade of zooxanthellae harbored in the cnidarian leads the endosymbiont proliferation (*shuffling situation*) [37] - it would take the time for an eventual cladal succession involving competition, proliferation and distribution for reestablishment of the "new" symbiotic order; and (3) bleached cnidarian may be re-infected by free-living zooxanthellae (*switching situation*) [37] - it requires that the environment provides free-living zooxanthellae. It would take the time for the random encounter with the bleached cnidarians and to set up the new associations. For future studies, it would be interesting to assess the time needed to accomplish each one of these bleaching recovery strategies.

In *E. pallida*, residual endosymbiont proliferation starts in the tentacles: the most exposed portion to light and also most mobile. This mobility may allow the anemone to modulate the absorption of photosynthetically active radiation by zooxanthellae according to the angle and retraction of the tentacles, as tree leaves do [38], and hence the nutrients supply. Interestingly, the tip of the tentacles remained always white while middle and base of the tentacles were darker, probably for an eventual prey capture and manipulation or sensorial roles [39]. Furthermore, thicker epidermis at the terminal tentacle may harbor a higher density of nematocysts, which may be specialized by its function, size and distribution [40].

The second anemones body part to get repigmented was the pedal disc. One of the asexual reproduction modes in *E. pallida* is pedal laceration [40-43]. As such, allocation of zooxanthellae in this region ensures endosymbiont heritance to their offspring. This vertical transference of zooxanthellae through pedal laceration guarantees an energetic resource to the

progeny in an oligotrophic environment. It provides an advantage when compared to sexual reproduction in a poor nutrient environment without free-living dinoflagellates, because the larva of this species is aposymbiotic and acquires the zooxanthellae from the environment [44-45]. Furthermore, it would be a competitive advantage for space against other benthic organisms that require more time to reproduce, enabling the studied holobiont to occupy further physiological niches. Again, it could help explain the current cosmopolitan distribution of this species [35].

Once the basic energetic and reproductive demands are fulfilled, column and oral disc get darker, increasing the overall photosynthetic surface. We also observed transport of unflagellated endosymbionts by water flow within the anemone's column. It suggests that the column may work as a reservoir of endosymbionts, redirecting algae to other body parts when necessary. Nevertheless, it remains unclear if the mechanism behind the pigmentation recovery would be *in situ* proliferation of the remaining cells, or migration of the endosymbiotic cells from a proliferation center.

The same pigmentation sequence (tentacles; pedal-disc; oral-disc; column) was observed in lacerates, juvenile and adult stages, but 10 days delayed at earlier stages of *E. pallida.* It implies that interval of bleaching events with intervals between 10 and 24 days may permit adult individuals asexual reproduction but would impair small sized individual's reproduction. It may affect the community structure.

Other cnidarians have shown to control the endosymbiont population [46-47], varying from circadian rhythms [11] to several days span [48]. Expelling damaged zooxanthellae has been proposed as an active regulatory mechanism of the host [49-50], and also as a detoxification strategy [51-53]. Nevertheless, loss of healthy zooxanthellae has also been previously reported [54-55]. Interestingly, dividing cells are preferentially expelled from the endosymbiont population [56]. In concordance with previous studies, we found that both dividing cells and flagellated dinoflagellates are expelled and can undergo a complete free-living cycle.

We found endosymbiont clumping, together with the sequence of membranes interaction described above and the structure of the endosymbiont pellet, before the pigmentation process of the host was accomplished. Here we propose a sequence of events of cell-cell communication as a trigger for endosymbiont cell detachment and clumping from the host after host/hosted membrane interactions. Such interactions occur in five main steps, resulting in a disassembly mechanism, ending with endosymbiont clumps that are expelled by the anemone. It starts with i) endosymbiont flagella interaction through the host's gastrodermis, followed by ii) host cell membrane cell disruption, iii) zooxanthellae membrane interaction, iv) endosymbiont

detachment, and v) aggregate expulsion. The clump structure is knitted by its own flagella and reinforced by a proteoglycan matrix. This clumping mucus of proteoglycan nature may contain cellular signals produced by the endosymbiont, possibly interpreted as noxious by the host, promoting the active expulsion of the pellet by the host. It may suggest that the microalgae may trigger the disassembling mechanism as a proactive dispersal strategy driven by the zooxanthellae. Alternatively, this proteoglycan matrix may be produced by the anemone in response to the noxius pellet present in the gastrodermal cavity. Thus, releasing free-living zooxanthellae to the environment could be an endosymbiont farming strategy that spares eventual future symbiont partners of future necessities of their own or of its offspring.

Conclusions

Based on our observations, we propose a mechanism of assembling and disassembling of the holobiont system regarding the proliferation of endogenous symbionts after bleaching. We found a sequence of pigmentation recovery after bleaching notwithstanding its ontogeny but delayed on earlier host stages. Bleached *E. pallida* may prioritize endosymbiont proliferation according to the function of the body part. Firstly, it may promote an endosymbiont proliferation ensuring the photosynthesis supply. Secondly, it ensures the inheritance of *Symbiodinium* spp. to offsprings, providing them the autotrophic resources in an oligotrophic environment as an inner help. Finally, the pigmentation sequence apparently favors light capture and vertical transmission of the endosymbiont to the host's offspring in oligotrophic conditions while the active role in clumping and expulsion of viable dinoflagellates during pigmentation may favor dinoflagellate dispersion. Further studies about the chemical composition of the aggregates and cell to cell communication may reveal who is the trigger of the assembling and dissassembling mechanisms in this holobiont system. Furthermore, studies about the time span needed for other recovery strategies may help to understand future bleaching recovery scenarios.

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Authors' contributions

ICB and MRC conceived and designed the study. ICB conducted the literature research, sampling, data curation, formal analysis, investigation, methodology, project administration and

the original draft. The study was supervised and the manuscript reviewed by MRC and MVK. All authors read and approved the final manuscript.

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Figures and tables

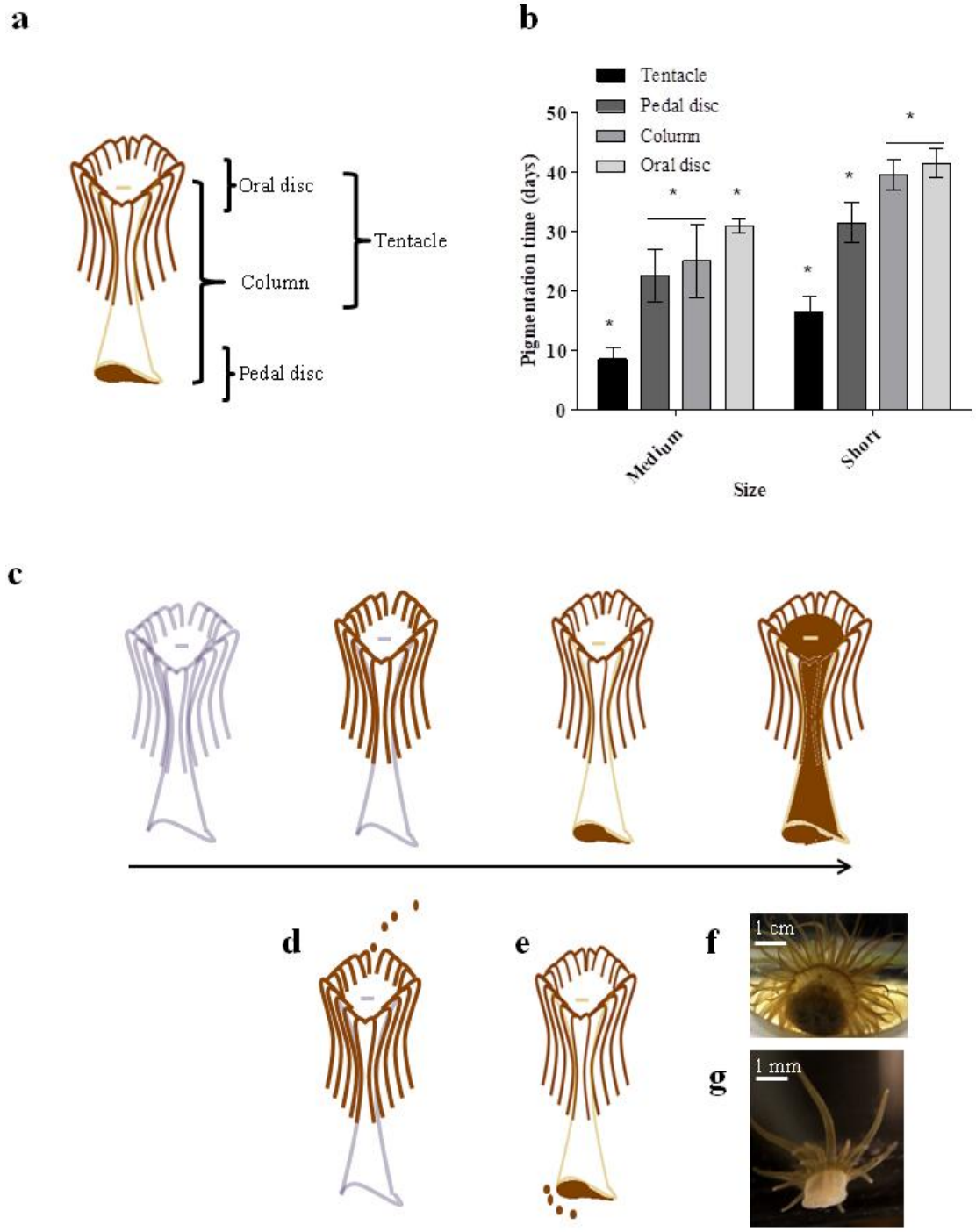

Figure 1. Assembling mechanism of the bleached anemone *E. pallida* regarding proliferation of endogenous dinoflagellate *Symbiodinium* spp.. a) Functional body parts of the anemone considered in this work to assess the bleaching recovery time span: pedal disc; column; oral disc; and tentacles . b) Time of pigmentation of each body part of medium size (oral disc of 2.5 ± 0.3 cm [n=4]) and small individuals (oral disc 0.4 ± 0.2 cm [n=4]). c) Pigmentation sequence observed independently of the size of the individuals. d) Endosymbiont clump regurgitation observed at early anemone pigmentation. e) Pedal laceration of the anemone observed at early pigmentation. f) Pedal laceration of medium size anemone g) Developed lacerate (10 days) with dark tentacles.

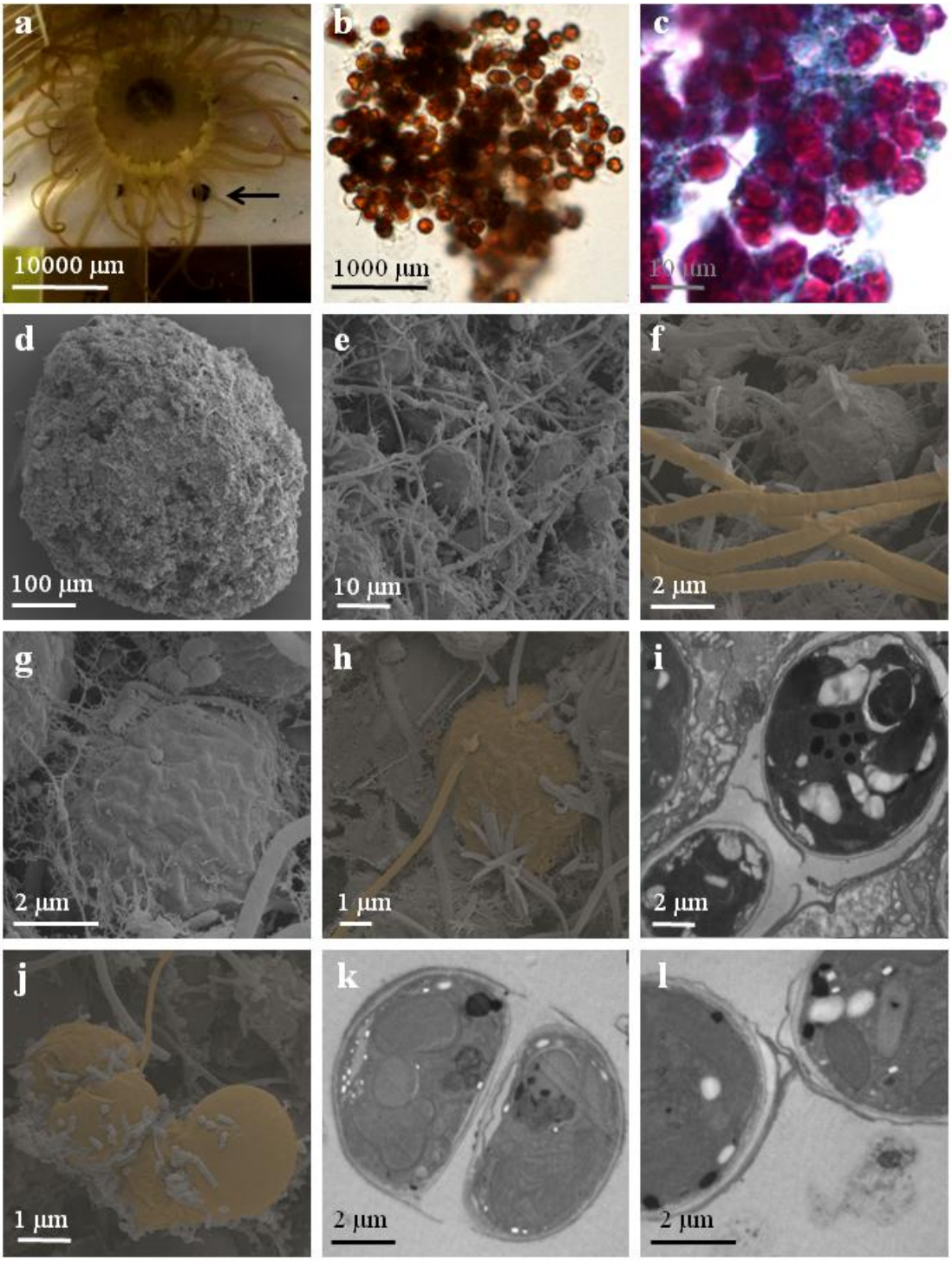

Figure 2. Ultrastructure of the symbiont clump.a) Bleached anemone *E. pallida* and two regurgitated pellets. b) Regurgitated pellet embedded in a transparent matrix. c) Proteoglycan nature of the matrix evidenced by the affinity to the Methyl Blue in the Mallory's Trichrome stain. d) External ultrastructure of the regurgitated pellet. e) Intricate web of strains supporting the surface of the symbiont clump. f) Detail of the strains evidenced on orange. g) Membrane film attaching adjacent cells in the symbiont clump. h) Flagella of the clumped *Symbiodinium* through the membrane film evidenced in orange. i) Transmission electronic microscopy photograph (TEM) of a dividing cell within the symbiont clump.j) Free-living flagellated dinoflagellates (orange) within the symbiont clump digitally colored. k) TEM of host membrane disruption between two adjacent hosted endosymbionts located at the tentacle of the anemone. l) TEM of membrane interaction between adjacent clumped dinoflagellates.

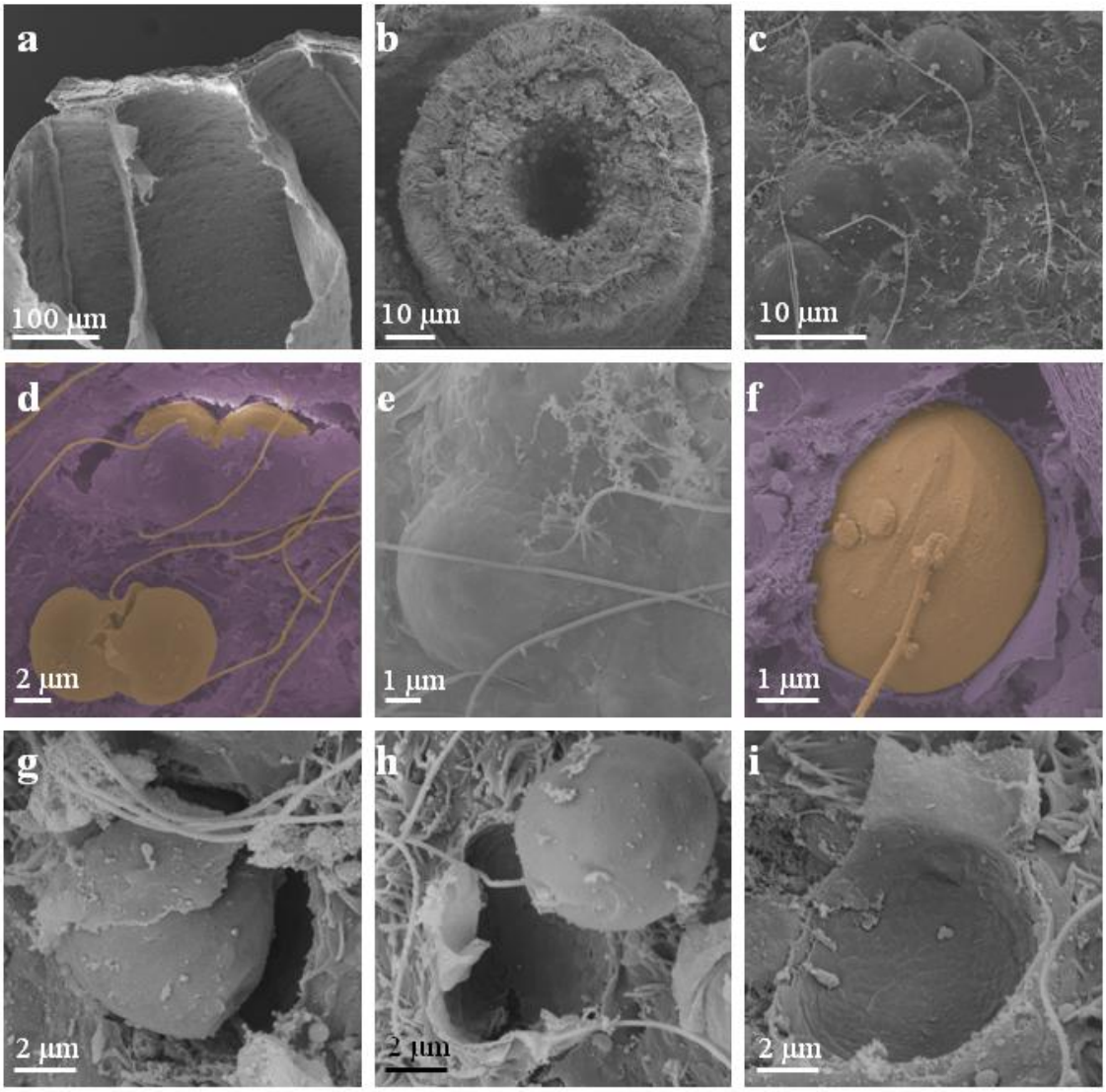

Figure 3. Ultrastructure of the holobiont *E. pallida-Symbiodinium* spp.association.
a) Scanning electron microscopy of longitudinal section of the gastrodermal cavity of the anemone. b) Scanning electron microscopy of cross section of the tentacle of the anemone showing the epidermis, the mesoglea, and the gastrodermis. c) Detail of hosted endosymbionts within the gastrodermis. d) Opening of the symbiosoma showing two hosted endosymbionts and a natant form of the dinoflagellate. e) Flagella projection through the host's gastrodermis. f) Hosted flagellated endosymbiont. g) Opening of the symbiosoma showing the hosted endosymbiont. h) Release of a flagellated endosymbiont. i) Empty symbiosoma within the anemone's gastrodermis. Pictures d and f are digitally colored to mark the host gastrodermis (purple) and the dinoflagellates (yellow).

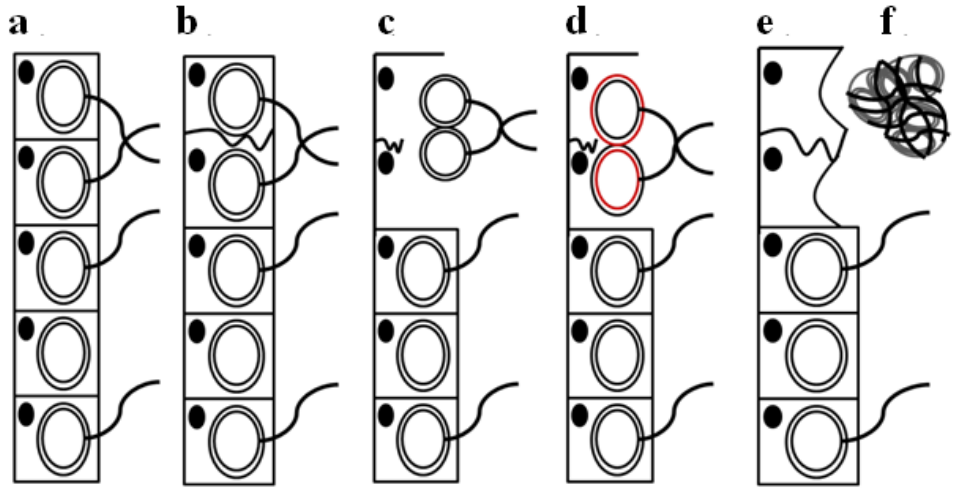

Figure 4. Disassembling mechanism of the holobiont *E. pallida-Symbiodinium* spp..
a) Flagella interaction through the gastrodermis's host. b) Host membrane cell disruption between adjacent symbiomes c) Zooxantella membrane interaction d) Symbiont detachment from the symbiosome f) Symbiont clump expulsion.

Table 1. Summary of the Two-way ANOVA results for pigmentation time accounting for body part, size and the interaction between body part and size.

	Df	F	p
Body part	3	1,390	< 0.0001
Size	2	73,39	< 0.0001
Body part*Size	1	75,06	0,2699
Residual	24		

Table 2. Bonferroni results for pigmentation time accounting for body part and size *P* value < 0.05.

	DP vs C	DP vs DO	DP vs Tnt Med	C vs DO	C vs Tnt Med	DO vs Tnt Med
G	ns	**	***	*	***	***
P	**	***	***	ns	***	***

Table 3. Summary of mean ± SD of time of pigmentation of each body part (tentacle; pedal disc; column; oral disc) medium (M = 2.5 ± 0.3 cm oral disc) and small size individuals (S = 0.4 ± 0.2 cm oral disc) of a bleached anemone *E. pallida* by residual endosymbiont proliferation.

	Tentacle	Pedal disc	Column	Oral disc
M	8.5 ± 1.9	22.5 ± 4.4	25.0 ± 6.2	31.0 ± 1.2
S	16.5 ± 2.5	31.5 ± 3.4	39.5 ± 2.5	41.5 ± 2.5

Capítulo III

Flutuação Circadiana da atividade fotossintética e antioxidante da anêmona *Exaiptasia pallida*

Circadian fluctuation of photosynthesis and antioxidant activity of the *Exaiptasia pallida* (Cnidaria)-*Symbiodinium* spp. (Dinoflagellata) holobiont system

(Submitted to JEMBE)

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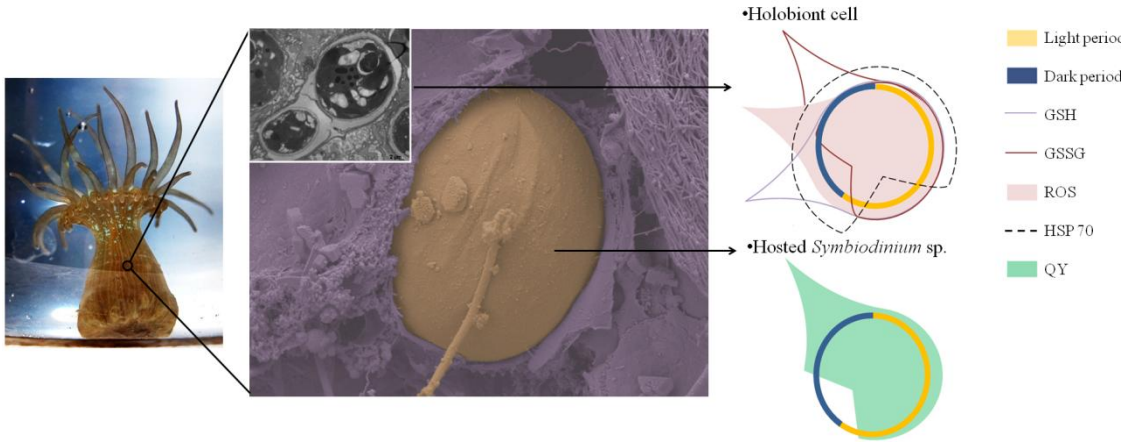
Graphical abstract



Abstract

Photosynthetic organisms undergo circadian fluctuations from nocturnal hypoxia to diurnal hyperoxia. However, little is known about the redox interplay between symbiotic partners related to photosynthesis. Here, we investigated the physiological fluctuations that may prepare the host, *Exaiptasia pallida*, to the oxidant changes associated to photosynthetic activity of its symbiont, the dinoflagellate *Symbiodinium* spp. For that, were measured the i) actual photosynthetic activity and ii) maximum photosynthetic capacity *in hospite* along 10:14 photoperiod (N=84). Within host cells were measured iii) total reactive oxygen species (ROS), iv) lipid peroxidation, and v) total glutathione content. In addition, to elucidate the mechanisms

behind the ROS reduction, we measured vi) total antioxidant capacity, vii) HSP70 expression, viii) oxidized glutathione, and ix) reduced glutathione in the host cells. Our results show that, independently on photoperiod, the endosymbiont performs less than 50% of its maximum photosynthetic capacity ($p = 0.0085$). Furthermore, maximum quantum yield ($p = 0.0009$) and effective quantum yield ($p = 0.0105$) rise previous to the dark period. During light period, both maximal and effective photosynthesis remained constant and dropped at the dark phase. Nevertheless, lipid hydroperoxides, total glutathione levels, and total ferric antioxidant capacity remained constant along the day, independently on photosynthetic activity and photoperiod. In the host cells we detected a drop in HSP70 expression previous to photosynthesis drop, and a complementary fluctuation of glutathione (GSH: $p = 0.002$) and glutathione disulfide (GSSG: $p = 0.0119$) of which the later predominates over the former except at twilight. Our results suggest that the host may constraint the photosynthetic activity *in hospite* to avoid oxidative stress. Furthermore, ROS overproduction does not result in redox imbalance or oxidative damage in the host, and it is not related to changes in the host cells total antioxidant capacity during the circadian period. Nevertheless, GSSG follows the same pattern as the holobiont's Effective Quantum Yield (EQY) and HSP70 expression rises previous to the photosynthetic activity rise, and maximum GSH precedes the maximum ROS. Host's alternates glutathione system related to endosymbiont's photosynthesis. We propose an antioxidant holobiont interplay model as the first evidence of Preparation for Oxidative Stress (POS) mechanism on anemones related to endosymbiont's photosynthesis that relies on HSP70 expression and an alternation of the prooxidant-antioxidant glutathione system.

Highlights

- First report of Preparation for Oxidative Stress on anemones during dark phase related to endosymbiont's photosynthesis
- Host's prevention of oxidative stress may constraint photosynthesis *in hospite*
- Host's HSP70 expression rises previous to the photosynthetic activity rise
- Host's alternates glutathione system related to endosymbiont's photosynthesis

Keywords

Cnidaria, Dinoflagellata, Holobiont physiological interplay, Redox symbiotic synchronization, Symbiotic POS to photosynthesis, Glutathione cycle.

1. Introduction

Environmental physical parameters are not constant at mid- (e.g. days to weeks) and long-terms (e.g. months and seasons) in nature and the oscillations may promote modifications

in the physiological performance of diverse organisms. In photosynthetic organisms, most behavioral and physiological processes are associated with light-dark cycles (Kühl et al., 1995; Yacobovitch et al., 2004; Hernando et al., 2016; Millar, 2016). However, changes in other physical factors, such as anoxia (Krivoruchko and Storey, 2015), dehydration (Malik and Storey, 2009), aestivation (Storey and Storey, 2012), freezing (Storey and Storey, 2013), or air exposure (Weihe et al., 2010) may also stimulate fluctuations on physiological rates. Such factors, when predictable, may provoke an oxidative stress on organisms that, in turn, may trigger preventive antioxidant responses. These mechanisms are also known as "Preparation for Oxidative Stress" (POS; Moreira et al., 2016) and have been described in several Metazoa groups, such as Annelida (Abele et al., 1998), Nematoda (Fu et al., 2012), Echinodermata (Klanian, 2013), Reptilia (Zhang et al., 2015), Mammalia (Schülke et al., 2012), and Cnidaria (Teixeira et al., 2013).

In cnidarians POS has been described only in aposymbiotic individuals. On the other hand, zooxanthellae physiology has been related to seasonal (Warner et al., 2002; Winters et al., 2006; Verde and McCloskey, 2007) and diurnal (Warner et al., 2006; Winters et al., 2003) fluctuations. Oxygen radical production in the sea anemone *Anthopleura elegantissima* has been shown to be a result of photosynthetic activity of its endosymbiotic algae (Dykens et al., 1992). Nevertheless, little is known about the symbiotic relationship between the partners. In this context, a better understanding of the POS in symbiotic cnidarians is a key to discuss the holobiont physiological interplay related to the photosynthetic activity of the microalgae and the host's antioxidant responses.

Herein, studying the eventual metabolic synchronicity associated to the photosynthetic activity of the dinoflagellate *Symbiodinium* spp. with the symbiotic partner *Exaiptasia pallida* (Anthozoa, Aiptasiidae) we hypothesize that photosynthesis *in hospite* may trigger modulation of antioxidant defenses of the host as a POS mechanism. For that we measured the photosynthetic performance of the holobiont along a circadian period, expecting to observe a change on host's redox activity, specifically the variation of host's total reactive oxygen species (ROS). To understand how harmful this oxidative threat is to the host, at each time point, both oxidative status and damage of the host were measured by the analysis of the redox state within the glutathione cycle and lipid peroxidation.

Once the eventual oxidative imbalance of the host produced by the endosymbiont photosynthetic activity was established, we tried to elucidate the mechanisms behind the host's ROS quenching through three different approaches: a) total antioxidant capacity of the host - related to the ferric reducing antioxidant potential; b) protein restoration - preventive production of chaperone proteins such as HSP70 for their role in preventing cellular damage and because

its expression has been previously related to diurnal cycle in other cnidarians (Levy et al., 2011); and c) enzymatic activity - ratio of the oxidized (GSSG) and reduced (GSH) forms of glutathione. Using the acquired data, we argue that the photosynthetic activity of the dinoflagellates produces an oxidant imbalance to the host and put forward a hypothesis on how it deals with this inner threat.

2. Material and methods

Specimens of *E. pallida* were collected at São Paulo Aquarium and maintained in 50 L aquaria with closed-circuit of natural seawater at 36.6 ppm, 24°C ± 0.5°C, and 14h/10h light/dark cycle under natural irradiance. *E. pallida* were fed with *Artemia* sp. metanaupli twice a week and acclimated for one week at experimental conditions prior to the experiment.

For the experiment, we considered three sampling times under dark phase and three sampling times under light phase during the circadian period, starting after 3h of the respective phase condition and at equidistant time points within the 14:10 photoperiod: 3h, 6h and 9h under darkness and 3h, 8h and 13h under light. At each sampling time, photosynthetic performance was measured as maximum quantum yield (MQY) after 10 minutes of dark-acclimation and effective quantum yield (EQY) of the individuals (n = 14; N = 84). Immediately after measuring photosynthesis, triplicates of each sampling time were flash frozen in liquid nitrogen and stored at -80°C for posterior analyses.

Frozen individuals were homogenized with a pestle in a 1.5 mL microcentrifuge tube containing the assay-specific homogenization solution, on ice. For the redox assays (lipid peroxidation, total reactive oxygen species and total antioxidant capacity), the samples (n = 8; N = 48) were homogenized in methanol (1:4 m/v) and then centrifuged for 5 minutes at 14000 rpm. For the HSP70 and glutathione quantifications, samples were homogenized in phosphate buffer (n = 5; N = 30) with 560 µL of Tris-buffered saline solution adjusted at pH 7.4 (50 mM Tris, 150 mM NaCl and 1 mM EDTA). Protein degradation was prevented by using 60 µL of protease inhibitor cocktail (10:1 Sigma-Aldrich). The homogenate was then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant, containing the cytosolic portion of the host cells was used for protein content, HSP70 and glutathione quantifications. All the reagents were dissolved in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2PO_4 and 1.8 mM KH_2PO_4) and adjusted to internal cellular pH (7.4). Total protein content was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher), with bovine serum albumin (BSA) as standard. All microplate data were standardized to total protein content (TP). Absorbance readings were performed in Costar plates (96 flat bottom wells), while fluorescence readings were made in Corning 3603 96 dark wells microplates. The HSP70 assays were carried out in Corning 3590 EIA/RIA microplates. All the assays were measured in microplate readers SpectraMax M4

(Molecular Devices) for absorbance and OPTIMA (BMG Labtech) for fluorescence. All samples were thawed only once, immediately before use, and inoculated in triplicates.

2.1. *Photosynthetic performance*

Photosynthetic performance was measured as maximum quantum yield (MQY, also referred as Fv/Fm) after 10 minutes of dark-acclimation and as effective quantum yield (EQY, also referred as ΔF'/Fm') of the *in vivo* fluorescence of PSII chlorophyll *a* by using the portable fluorometer AquaPen-P AP-P 100 (Photon Systems Instruments). EQY was performed under light-acclimated samples under 1264 ± 1 lux (LX801 Digital Lux Meter).

2.2. *Total reactive oxygen species (ROS)*

Total ROS were measured using the DHR assay (Esposito et al., 2003) adapted from human serum to anemones in the present study. In dark microplates, we inoculated 30 µL of sample and 180 µL of DHR (50 mM in DMSO), using 30 µL of methanol as negative and 30 µL of cumene hydroperoxide 0.175 nM as positive controls. We measured the average rate of Rd production within 60 minutes kinetic reading every 5 minutes (15-40 min) using 485 nm (excitation) and 520 nm (emission) filters.

2.3. *Lipid peroxidation*

Lipid hydroperoxides were measured using the FOX assay (Jiang et al., 1991) adapted from tissue extracts (Hermes-Lima et al., 1995) to anemones in the present study. In transparent microplates, we added 90 µL of $FeSO_4$ 1 mM, 35 µL of H_2SO_4 0.25 M, 35 µL of xylenol orange 1 mM, 160 µL of water, and 20 µL of the supernatant of the samples. The reaction was allowed to proceed during one hour at room temperature. First absorbance reading was determined using a 580 nm filter. Then, 10 µL of cumene hydroperoxide 0.8 mM were added and allowed to react during 15 minutes at room temperature. Second absorbance reading was determined using a similar filter and the results were expressed as cumene (CHP) equivalents *per* total protein (TP) **(**CHP equiv/µg TP).

2.4. *Glutathione assays*

Reduced (GSH) and oxidized (GSSG) forms of glutathione were measured following the Recycling Assay adapted from the enzymatic recycling method for quantitative determination of glutathione and glutathione disulfide levels (Vandputte et al., 1994) adapted to microplate for general tissues (Rahman et al., 2006). The assay was performed on ice, at the lowest irradiance level possible to avoid derivatization. All reagents were diluted in KPE buffer (potassium phosphate buffer 0.1 M with EDTA 5 mM, pH 7.5).

To estimate the reduced glutathione content (GSH), we added 20 µL of each sample (in triplicate) and 120 µL of DTNB:GR to each well (DTNB: 5,5′-Dithiobis(2-nitrobenzoic acid; gr:glutathione reductase) in 96-well transparent microplates, The reaction was allowed to convert GSSG to GSH for 30 seconds, and then 60 µL of β-NADPH were added. The

microplate was immediately read at 412 nm every 30 seconds for 2 min. We calculated the rate of 2-nitro-5-thiobenzoic acid formation as the change in absorbance per minute, and the actual total GSH concentration was determined from the standard curve of GSH from 26.4 to 0.103 nM ml^{-1}. Results were expressed as nM GSH/mg TP.

To estimate the oxidized glutathione content (GSSG), we added 100 µL of sulfosalicylic sample extract in each microcentrifuge tube. Then, 2 µL of 2-vinylpyridine were added, vortex-mixed, and allowed to derivatize GSH for 1h at room temperature. Such procedure was followed by the addition of 6 µL of triethanolamine, which after vortexed was allowed to neutralize for 10 minutes. GSSG assay proceed similarly as that described for the GSH assay. The microplate was read using 412 nm every 30 seconds for 2 minutes. We calculated the rate of 2-nitro-5-thiobenzoic acid formation as the change in absorbance per minute, and the actual total GSSG concentration was determined from the standard curve of GSSG from 26.4 to 0.103 nM ml^{-1}. Results were expressed as nM GSSG/mg TP.

2.5. Ferric reducing antioxidant power

Total antioxidant capacity was determined by the ferric reducing antioxidant power (FRAP) assay from the methanol homogenized samples through the adapted procedure for microplates (Furlan et al., 2015). FRAP solution was prepared immediately before use by mixing 25 ml of acetate buffer (0.3 M pH 3.6), 2.5 ml of 2,4,6-tri-2-pyridyl-*s*-triazine 10 mM (TPTZ, Sigma), and 2.5 ml of ferric chloride 20 mM. In transparent microplates, 265 µL of FRAP solution was mixed with 20 µL of methanol homogenates of the samples and 25 µL of ultrapure water. Methanol and Fe^{2+} were used as negative and positive controls respectively. The reaction was allowed to proceed in dark at 37°C for 30 min, and the TPTZ-reduced complex was determined measuring the endpoint absorbance at 595 nm.

2.6. *Stress protein (HSP70)*

The heat shock protein HSP70 was measured using the enzyme-linked immunosorbent assay (ELISA) adapted from in-house Sandwich-ELISA for human serum (Njemini et al., 2005) to anemones in this study. In Costar EIA/RIA (3390) microplates, we added three replicates of the supernatant containing 40 µg of total proteins of each sample and completed to 50µL with PBS. Microplates were incubated overnight at 4°C and then washed vigorously three times in PBS-Tween 20 0.05%. The microplate surface was blocked to avoid unspecific ligations by adding 200 µL of skimmed milk 5% (Molico), followed by 90 min incubation at 37°C, and another vigorous wash. To tag the protein of interest, 100 µL of the primary antibody (Rabbit IgG, Santa Cruz) diluted in skimmed milk 1% were added to each well and incubated at 37°C for 90 min. The microplate was once again washed vigorously three times in PBS-Tween 20 0.05%, and 100 µL of the secondary antibody (Anti-rabbit IgG, Santa Cruz) diluted in skimmed milk 1% was added to each well and incubated at 37°C for 90 min. The microplate had its final wash (three times in PBS-Tween 20 0.05%), and 100 µL of TMB revelation kit was allowed to

react at room temperature during 10 min in darkness. The reaction was stopped with 50 µL of H_3PO_4 1 M, measured using a 450 nm filter and the results expressed as absorbance units.

2.7. Statistical analyses

The actual photosynthetic activity and maximum photosynthetic capacity was measured as effective quantum yield, and maximum quantum yield. Host's redox activity and state was measured as the integration of the total ROS, LPO, and the GSH+GSSG. Host's antioxidant performance was measured as a) total antioxidant capacity of the host (FRAP), b) stress protein (HSP70), and c) enzymatic activity (GSH and GSSG content). In order to elucidate if the photosynthetic activity and capacity *in hospite* and the antioxidant capacity of the host is constant along the day independently the dark-light condition, we compared three time points at dark (3h, 6h and 9h) and three times at light condition (3h, 8h and 13h). Differences between MQY and EQY at every experimental time were compared by a one-way ANOVA. The rest of the parameter parameters measured at dark and light phases were tested using a two-way ANOVA, considering time as a fixed factor. When differences were determined, a multiple comparison tests of Bonferroni was performed using GraphPad Prism statistical package. ANOVA assumptions were tested with Barlett's test. Significance level was set at $\alpha \leq 0.05$.

3. Results

The MQY of the *E. pallida-Symbiodinium* spp. holobiont was significantly higher than the EQY ($p = 0.0085$). Independently of the circadian phase, the endosymbiont performed less than 50% of its maximum photosynthetic capacity ($p = 0.0085$) (Figure 1a). Furthermore, the maximum photosynthetic capacity *in hospite* did not remain constant throughout the entire day. At 3h of darkness lower MQY was observed when compared with the subsequent sampling times ($p = 0.0009$) and a concomitant reduction in EQY was also registered ($p = 0.0105$). Interestingly, the EQY increases after 6 and 9h of darkness, showing its maximal efficiency at dark conditions previous turning on the photoperiod. After light-on, during the light phase, both MQY and EQY remained constant (Figure 1b).

After 6h of dark phase, we found a significant raise of holobiont ROS production that decreases 3h later and remains constant independently on light or dark conditions ($p = 0.0027$) (Figure 2a). Nevertheless, oxidative damage, measured as lipid peroxidation, and oxidative stress, measured as total glutathione content (Figure 2b), remains constant over time during the circadian period without significant differences. Surprisingly, the total antioxidant capacity of the holobiont also remained constant independently of dark or light phases. Interestingly, there was a decrease of HSP70 production ($p = 0.0013$) after 13h at light phase (Figure 2c), remaining constant without statistical differences over the other light or dark phases.

Our results also indicate a complementary pattern between the oxidized and reduced states of glutathione. With the exception of the measurement at 3h during dark phase (Figure 2d), in which the GSSG oxidized form predominates in relation to GSH. GSH rose after 3h of dark phase ($p = 0.002$) and decreased progressively over dark phase and remained constant during light phase. Conversely, GSSG experienced minimum levels after 3h of dark phase, reaching its maximum after 9h at dark phase and remained at high concentrations during the light period ($p = 0.0119$).

Finally, we present a summary of our results in the Figure 3 as a dial POS model that occurs mainly during the dark phase. It starts with a decrease on HSP70 production at the end of the light phase. It follows with an oxidized status in the host cells characterized by a decrease on GSSG content in the host cells along an increase on the GSH at the beginning of the dark phase. Then, a peak of ROS production in the host cell, right before the peak on photosynthetic performance of the hosted dinoflagellate and together a GSH raise in the host cell. All these parameters come back to baseline levels during the light phase.

4. Discussion

In contrast to other anemones (Kee Alfian et al., 2009; Khoo and Mazlan, 2013), the effective quantum yield of the holobiont *E. pallida-Symbiodinium* is limited to less than 50% of its maximum quantum yield. Such photosynthetic restriction may be host regulated to prevent eventual oxidative stress produced by photosynthetically generated oxygen species. Also, it may be regulated by the symbiont in order to save the metabolic expenditure on producing photosynthesis that would be unnecessary in this condition. In concordance with other studies on cnidarian photophysiology (Sorek and Levy, 2012) our results suggest that photosynthesis *in hospite* does not remain constant along the day. Fluctuations on photosynthetic efficiency show raises before light and dark phases, suggesting a preventive activity for prepare the photosynthetic apparatus and a photoprotective mechanism respectively.

Our results also indicate that the oxygen species present are not constant along the day. Interestingly, the maximum host's ROS precedes both EQY and MQY raise. Nevertheless, it does not produce an oxidative damage at tissue level, as shown by the absence of lipid peroxidation in the host's tissue. Oxidative stress was also not found, since there was no fluctuation on the total glutathione products in the cytosolic fraction of the host. As such, ROS overproduction from endosymbiont's photosynthesis does not generate a redox imbalance to the host in the *E. pallida-Symbiodinium* spp. holobiont, suggesting that there might be a redox quenching mechanism behind. Contrary to our expectations, we haven't found a diurnal fluctuation on total antioxidant capacity in the host. For future studies, it would be interesting to

assess the metabolomic profiles related to photosynthesis pathways to further understanding of the relationship between the partners.

According to our results, the POS mechanism could rely on preventive chaperon protein expression. In concordance to Levy et al. (2011), we have found a diurnal fluctuation of HSP70 levels in the host, which decrease immediately before the photosynthesis decline at the end of the day. This is contrary to the observed for other chaperon proteins, like HSP31, which rises at the end of the day period (Sorek and Levy, 2012) and the gene expression rhythm found for HSP40, HSP70 and HSP90 in *Acropora millepora* (Levy et al., 2011). Additionally, future studies would be interesting to assess the proteomic profiles related to both photosynthesis and respiration metabolism *in hospite*.

Our results suggest that the POS mechanism may rely also on glutathione activity. We have found that there is a pro-oxidant/antioxidant alternation on the glutathione system. Interestingly, the pro-oxidant status follows the same pattern as the EQY. Hence the photosynthesis performed by the endosymbiont induces the oxidized form of glutathione, GSSG. The level of GSSG was lower at the minimal photosynthetic activity and precedes the host's ROS peak. It may involve a POS mechanism preventing the peak of ROS production, which in turn may trigger a detoxification cascade involving other glutathione enzymes, such as glutathione reductase (GR), glutathione S-transferase (GST), and glutathione peroxidase (GPX). This mechanism may be responsible for restoring the basal level of host's ROS. Regarding to the reduced form, GSH, the antioxidant state of the glutathione system predominates over the oxidized form after 3h of dark phase, and decreases progressively over the dark and light phases. For further studies, it would be interesting to understand the role of enzymes with antioxidant activity, such as SOD, which scavenges oxygen production, and CAT, GST, GR, GPX, as well as the metabolic interactions of the products and subproducts, and its membrane trafficking between symbiotic partners. It would be interesting to assess an eventual melatonin role for this glutathione fluctuation, as it has been reported for other organisms (reviewed by Hardeland et al., 2003).

5. Conclusions

Within the three approaches considered in this work, we demonstrate that photosynthetic activity of the endosymbiont produces an innocuous ROS overproduction to the host. We have found that the mechanism behind ROS neutralization may be related to enzymatic activity and protein expression. On one hand, chaperon proteins maintain a basal level of expression that decrease immediately before reduction of photosynthetic activity. On the other hand, glutathione shows daily fluctuation of pro- and anti-oxidant forms related to photosynthesis activity of the hosted algae. Based on our results, we propose an integrated model of diel POS

response in the holobiont system formed by *E. pallida-Symbiodinium* spp. that may be related to the photoperiod. This preventive model is developed mainly during the dark phase and remains in the baseline levels during the light phase. It starts with a decrease on the HSP70 production in the host cells at the end of the light phase, that increases again during the dark phase and remains at baseline levels during the rest of the day. At the beginning of the dark phase, there is an oxidized status of the glutathione system, where the oxidized form predominates over the reduced glutathione. According to our results, it may be a preparation for the ROS overproduction that prevent the raise of photosynthetic performance right before the light phase.

More enzymes may be related to the control of photosynthetic ROS-related production. For future studies, it would be interesting to assess the role of other antioxidant enzymes and chaperon proteins in this symbiotic/metabolic interplay. To enhance the understanding of the symbiotic relation between the partners, we recommend the application of new generation metabolomic, proteomic and transcriptomic approaches to elucidate the integration of the regulatory networks focusing the building up of system biology related to photosynthesis *in hospite* and antioxidant defense of the host.

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Glossary

Abbreviations

CHP: cumene hidroperoxide
DHR: dihydrorhodamine-123
DTNB: 5,5′-Dithiobis(2-nitrobenzoic acid) is also called DTNB or Ellman′s reagent.
EDTA: Ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
EQY: effective quantum yield
FRAP: ferric reducing power assay
GPX: glutathione peroxidase
GR: glutathione reductase
GSH: reduced glutatione
GSSG: oxidazed glutathione
GST: glutathione S-transferase
MQY: maximum quantum yield
POS: preparation for oxidant stress
Rd: rhodamine
ROS: reactive oxygen species
TNB: 2-nitro-5-thiobenzoic acid
TP: total protein
TPTZ: 2,4,6-tri-2-pyridyl-s-triazine
β-NADPH: β-Nicotinamide adenine dinucleotide 2′-phosphate

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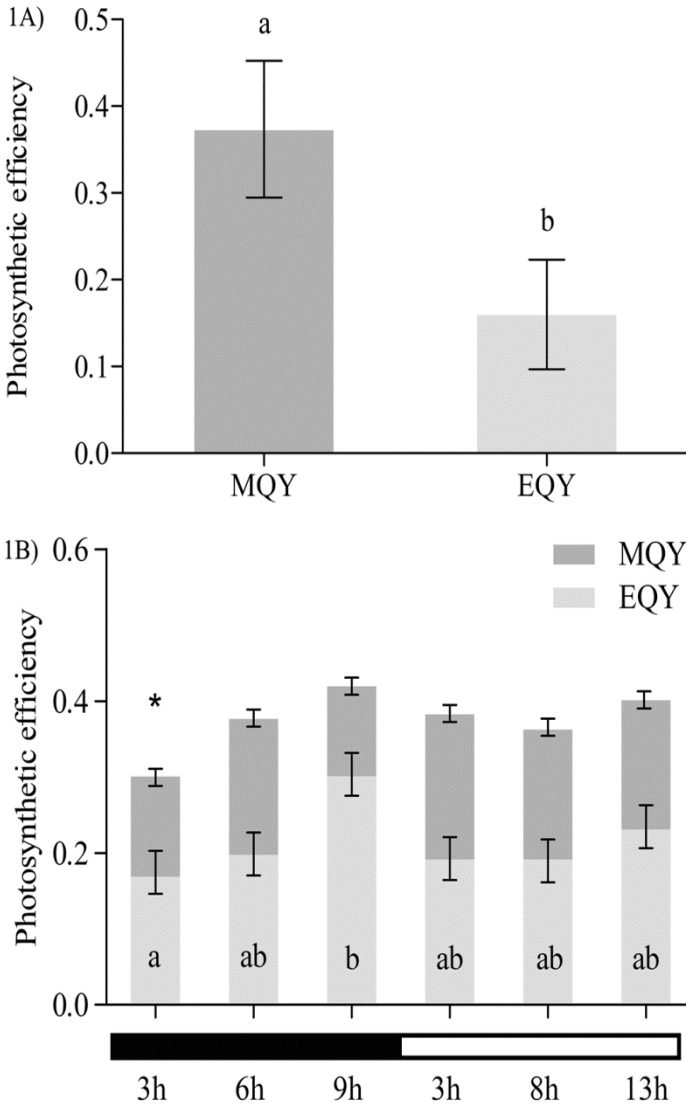

Fig. 1. A) Photosynthetic performance as maximum quantum yield (MQY; like Fv/Fm) and effective quantum yield (EQY; like ΔF'/Fm') (mean ± SD) *in hospite Symbiodinium* spp. within the *E. pallida* holobiont system (n = 84). Asterisk means significant difference between effective and maximum quantum yields ($p < 0.05$); **B)** Photosynthetic performance as maximum quantum yield (MQY; like Fv/Fm; light grey bars) and effective quantum yield (EQY; like ΔF'/Fm'; dark grey bars) (mean ± SD) *in hospite Symbiodinium* spp. within the *E. pallida* holobiont system (n = 84). Black horizontal bar indicates dark phase (hours from light-off) and white horizontal bar indicates the light phase (hours from light-on) during the circadian period. Asterisk means significant difference respecting the other sampling times for MQY ($p < 0.05$). Different letter represent significant differences between sampling times for EQY ($p < 0.05$).

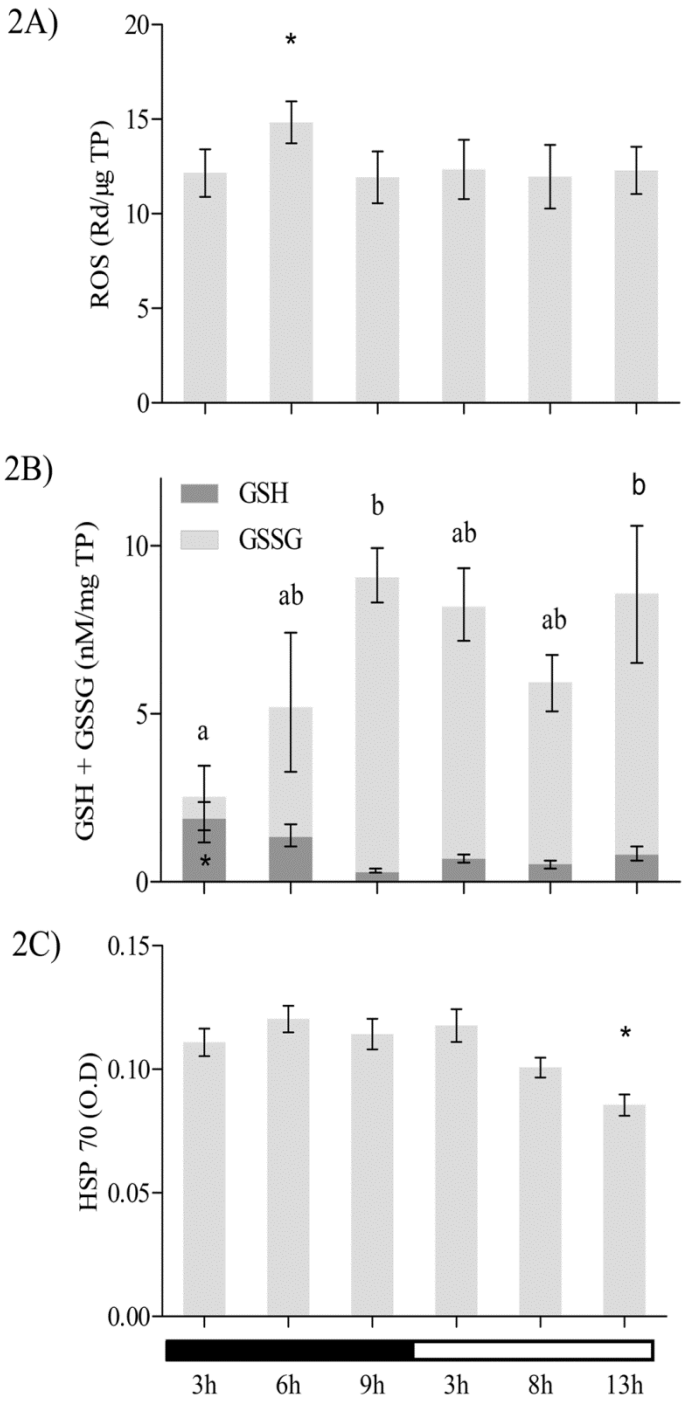

Fig. 2. A) Total reactive oxidant species (ROS) in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as Rd/min µg TP (mean ± SD) (n = 8); **B)** Total glutathione content (GSH + GSSG) in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as nM per mg of total protein (mean ± SD) (n = 5). Level of reduced glutathione (GSH) (dark grey bars) and oxidized glutathione (GSSG) (dark grey bars + light grey bars) in host cells of *E. pallida-Symbiodinium* spp. holobiont system (mean ± SD) (n = 5); **C)** HSP70 in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as optical density at 450 nm (mean ± SD) (n =5). The black horizontal bar indicates dark phase (hours from light-off) and white horizontal bar indicates the light phase (hours from light-on) during the circadian period. Asterisk means significant difference respecting the other sampling times and different letters in **C** represent significant differences between sampling times of GSH ($p < 0.05$).

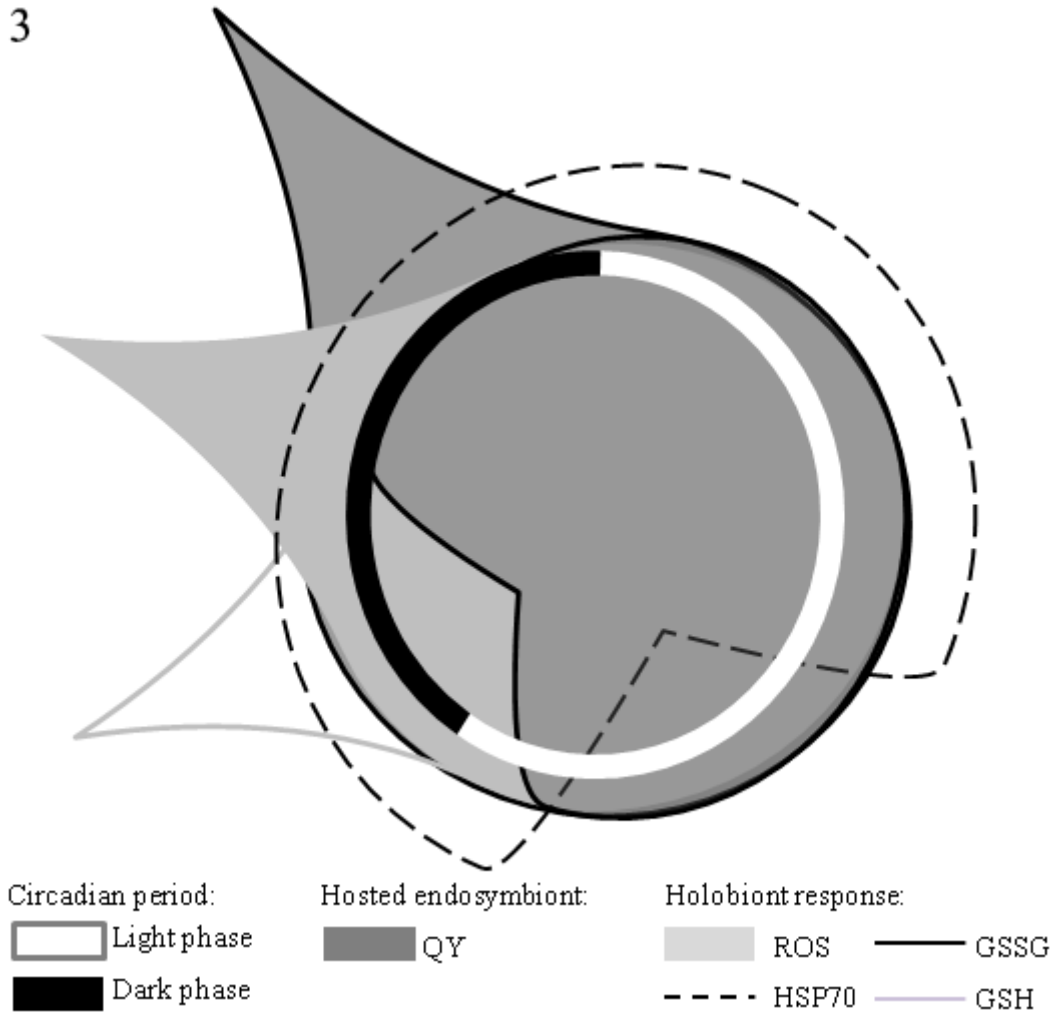

Fig. 3. Summary of the holobiont physiological interplay during a circadian period. Photosynthetic performance (QY) of the *Symbioidinium* spp. *in hospite* within the *E. pallida* holobiont system, and the sequence of redox responses of the host related to stress protein production (HSP70), oxidative status (GSH- GSSG), total reactive oxygen species production (ROS), and reduced glutathione (GSH). Black semicircle indicates dark phase and white semicircle indicates the light phase during the circadian period.

Capítulo IV

The holobiont *Exaiptasia pallida-Symbiodinium* sp. is better prepared to overcome warm events than cold temperatures and hosting mixed endopulation including clades E and F may favor bleaching struggle within the Climate Change Context *(In prep.)*

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Abstract

Thermal bleaching is the main trigger of the declining trend of symbiotic cnidarians in the tropical oceans. Nevertheless, some cnidarians have shown to recover the pigmentation switching the endosymbiont population with new clades from the available environmental *Symbiodinium* sp. pool. In this context, we hypothesize that there is a more suitable *E. pallida-Symbiodinium* sp. combination for every temperature. To test our hypothesis, we considered three treatments of temperature (19 °C; 24 °C; 30 °C) nested into six treatments of offered symbiotic partner (Control; A; B; C; D; E; F) and compared the photosynthetic efficiency of i) clades in culture; ii) *in hospite* versus *in culture* and iii) associations versus control within *E. pallida*. Within the holobiont cells we measured: a) Total reactive oxygen species (ROS); b) Lipid peroxidation (LPO); c) Reduced glutathione (GSH); d) Oxidized glutathione (GSSG); e) Ferric reducing power (FRAP) and f) Stress proteins (HSP70). We found that: i) Cold temperatures triggered ROS- FRAP- LPO system in the host while warm temperatures enhanced FRAP and GSSG with emphasis on clade A. ii) Photosynthesis is inhibited by temperature changes in all clades except D *in culture*, but not *in hospite*; that is enhanced by offering clades D, E and F. Based on our results: i) the holobiont is better prepared to overcome warm events than cold temperatures. Changes on temperature may reduce the environmental *Symbiodinium* sp. pool, leading to a D predominance in unstable environments, F in colder and E-D in warmer scenarios, ii) Symbiosis may protect photosynthesis. Switching into *in hospite* brought photosynthetic advantages for both partners with F at 19 ºC and for E at 30ºC. In both scenarios, mixed endopulation that includes clades E and F may favor bleaching struggle of the holobiont within the context of climate change.

Keywords

Symbiont switching; symbiont shuffling; *Symbiodinium* environmental pool; symbiosis; glutathione reductase; glutathione redox system.

Introduction

Bleaching is a growing concern within the Global Climate Change context. It consists on the loss of pigments in symbiotic cnidarians, usually caused by environmental stress, like extreme temperature events or ocean acidification (Cook et al. 1990; Porter et al. 1989; Gates 1990; Glyn 1990; Hoegh-Guldberg et al. 2007). Thermal bleaching is the main trigger of the declining trend of symbiotic cnidarians in the tropical oceans (Hughes et al. 2003; Carpenter et al. 2008). It begins with a symbiotic population drop (Jones et al. 2000; Grottoli et al. 2004) together with the reduction of photosynthetic efficiency of the residual endosymbionts (Iglesias-Prieto et al. 1992; Lesser 1996). As a consequence, the holobiont suffers from energetic imbalance (Muscatine 1967, 1980) that may result in starvation (Anthony and Fabricius 2000; Muscatine 1990), loss of cnidarian's biomass (Fitt et al. 2000), reduction on growth, reproduction, and calcification (in scleractinian). Thus, the organisms become prone to diseases (Buddemeier et al. 2004; Hughes et al. 2007; Rosenberg et al. 2007; Brandt et al. 2009) and increased mortality rates (Brown 1997; Glynn and D'croz 1990; Hoegh-Guldberg 1999). In such scenario, vulnerable bleached cnidarians may be easily overcompeted by other benthic organisms (Foster et al. 2008; Green et al. 2008) implying drastic changes on the associated community and a massive biodiversity loss (Goreau 1992; Hale et al. 2011).

However, some cnidarians have shown to recover the pigmentation from bleaching (Berkelmans et al. 2006; Burt et al. 2008; Jones et al. 200). It may be natural and transitory, caused by seasonal changes of external factors such as irradiance or temperature that may induce alterations on the physiology of the hosted dinoflagellate and/or the animal host (Fitt et al. 2000). However, these events are expected to increase in frequency and extent (Hoegh-Guldberg 1999) and aggravate its implications from acute and transitory into chronic effects with particular impact on reef areas (Baker 2008; Jones 2008). Although the cellular mechanisms behind cnidarian bleaching and recovery are still poorly understood, recent studies point out the redox activity of the hosted dinoflagellate (Neill et al. 2002; Asada 2006; Lesser 2006; Wong et al. 2010).

Based on evidences of increasing thermal tolerance in coral reefs after successive massive bleaching events in field (Podestá and Glynn 2001; Berkelmans 2009), some authors conceive bleaching as an adaptive/acclimative strategy arguing that the host may be re-infected by different clades and establish a new symbiont order more suitable for the new environmental conditions, increasing the probability to overcome the next changes (Buddemeier e Fautin 1993;

Baker 2001; Baker et al. 2004; Obura 2009). In this context, bleaching could be a mechanism to waive the oxidative imbalance between the cnidarian and the hosted dinoflagellate (Obura 2009) where cnidarians discard actively their dinoflagellates to increase the probabilities to survive the environmental alteration (Baker 2001).

Nine clades of *Symbiodinum* spp. named from A to I have been described (Pochon and Gates 2010) and subdivided according to the ITS2 of the ribosomal DNA (LaJeunesse 2001; Baker 2003; Coffroth and Santos 2005; Sampayo et al. 2009; Finney et al. 2010; LaJeunesse and Thornhill 2011). Some clades show different physiological performance *in culture* according to temperature (Suggett et al. 2008; McGinty et al. 2012; Kruegger et al. 2014), but this remains unclear *in hospite* (e.g. Higuchi 2008). Hosts may harbor one single clade (Baker 2003; LaJeunesse et al. 2010; Thornhill et al. 2013) or several (Pochon et al. 2001; LaJeunesse 2002; Baker 2003) and alter the endocommunity structure according to environmental variations (Pochon et al. 2001; LaJeunesse 2002; Baker 2003; Baker and Romanski 2007; Venn et al. 2008; Finney et al. 2010). This behavior is called "symbiont shuffling" when the clades are already present *in hospite* (Baker 2001; Chen et al. 2005) and "symbiont switching" (Baker 2003) when the new clade comes from the environment.

Within the "symbiont switching" scope, we hypothesized that for every temperature, there is a more suitable association of *Symbiodinium* sp. clade with the anemone *Exaiptasia pallida* (Anthozoa, Aiptasiidae). To test our hypothesis, we considered three treatments of temperature (19°C; 24°C; 30°C) nested into six treatments of dinoflagellate offered (Control; A; B; C; D; E; F). For this purpose, we measured the photosynthetic performance of each clade both *in culture* and *in hospite*, and we assessed within the holobiont cells: a) total reactive oxidant species b) lipoperoxidation c) the redox state of glutathione d) total antioxidant capacity of the host and f) stress proteins (HSP70). Using the acquired data, we discuss future scenarios of *Symbiodinium* sp. pool availability in the environment in the Global Climate Change context and the expected shifts in the endosymbiotic populations based on the eventual benefits of the symbiotic association for both partners.

Material and methods

Specimens of *E. pallida* were collected at the São Paulo Aquarium and maintained in 50 L closed-circuit natural seawater aquaria at 36.6 ppm, 24°C ± 0.5°C, and 14 h/10 h light/dark cycle under natural irradiance and photoperiod. The anemones were fed with *Artemia* sp. metanaupli twice a week. Cultures of *Symbiodinium* sp. (A; B; C; D; E; F) were maintained at 24°C in silica-free sterile f/2-medium (pH 8.1; Guillard and Ryther 1962) and replicated every month. To ensure same conditions in terms of growth phase of every clade, the cultures were synchronized, according to the final volume needed for the experiment and the timing of the

growing curve of each clade. For the experiment, we used a volume of 100 ml for each replica with an initial concentration of 200 cells/ml. Anemones were allowed to one week acclimation at experimental conditions in 100 ml individual aquarium with individual gentle aeration at 24°C. It consisted of a staged acclimation to light conditions with a 4 h delay to ensure that every treatment was applied during the same time span and the same phase within the circadian cycle and ensure time enough for sampling. For the experiment, we considered three treatments of temperature (19°C; 24°C; 30°C ± 0.1°C; monitored by HOBO data-loggers) and six treatments of symbiont partner offer (Control; A; B; C; D; E; F). Anemones in seawater without *Symbiodinium* in the environment were used as control. In addition, three replicates of the *Symbiodinium* sp. cultures were set at each temperature treatment. Due to the difficulties for growing synchronization, the experiment was repeated twice with the cultures ready (Control; D; E; F and Control; A) and the results obtained were concordant (data not shown).

During the experiments, at each sampling time, effective efficiency of photosynthesis of the anemones (n = 9; N = 189) and of the algae cultures (n = 3; N = 54) were measured at each temperature treatment. After 10 minutes in darkness, optimum efficiency of photosynthesis was measured. Immediately after measuring photosynthesis, triplicates were flash frozen in liquid nitrogen and stored at -80°C for posterior analyses. Frozen individuals were homogenized on ice with a pestle against a 1.5 mL microcentrifuge tube containing specific homogenization solutions depending on the assay. For the redox assays (lipid peroxidation, total reactive oxygen species and total antioxidant capacity) samples (n = 3; N = 72) were homogenized in methanol (1:4 w/v) and then centrifuged for 5 minutes at 14000 rpm. For HSP70 and glutathione, the samples (n = 3; N = 72) were homogenized in phosphate buffer (50 mM Tris, 150 mM NaCl and 1 mM EDTA) with 560 µl of Tris-buffered saline solution adjusted at pH 7.4. Protein degradation was prevented by using 60 µl of protease inhibitor cocktail (10:1) (Roche). The homogenate was then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant, containing the citosolic portion of the host cells was used for protein content, HSP70 and glutathione quantifications. All the reagents were diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2PO_4 and 1.8 mM KH_2PO_4) and adjusted to internal cellular pH (7.4). All microplate data were standardized to total protein content (TP). Total protein content was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher) with bovine serum albumin as standard and all assays were carried out in microplate reader Espectra M4 for absorbance and OPTIMA for fluorescence. Absorbance readings were performed in Costar plates (96 wells flat bottom), while fluorescence readings were performed in Corning 3603 96 dark wells microplates. The HSP70 assay were carried out in Corning 3590 EIA/RIA microplates. All samples were thawed only once, immediately before use, and inoculated in triplicates. We

considered that the association between symbiotic partners was stablished when significant differences from the control were found on any of the parameters measured.

Photosynthetic efficiency

Photosynthetic performance was measured as maximum quantum yield (MQY, also referred as Fv/Fm) after 10 minutes of dark-acclimation and as effective quantum yield (EQY, also referred as ΔF'/Fm') of the *in vivo* fluorescence of PSII chlorophyll *a* by using the portable fluorometer AquaPen-P AP-P 100 (Photon Systems Instruments). The EQY was performed under light-acclimated samples under 1264 ± 1 lux (LX801 Digital Lux Meter).

Total reactive oxygen species (ROS)

Total ROS was measured using the dihydrorhodamine 123 assay (DHR) (Esposito et al. 2003) adapted from human serum to anemones in the present work. In dark microplates, we inoculated 30 µL of sample and 180 µL DHR (50 mM in dimethyl sulfoxide). We measured the average rate of Rd production within 60 minutes kinetic reading every 5 minutes (15-40 min), using 485 nm (excitation) and 520 nm (emission).

Lipid peroxidation (LPO)

Lipid hydroperoxides were measured using the FOX assay (Jiang et al. 1991) adapted from tissue extracts (Hermes-Lima et al. 1995) to anemones in the present work. In transparent microplates, we added 90 µL of 1 mM $FeSO_4$, 35 µL of 0.25M H_2SO_4, 35 µL of 1mM xylenol orange, 160 µL of water, and 20 µL of the supernant of the samples. The reaction was allowed to proceed during one hour at room temperature. First absorbance reading was determined using a 580 nm filter. Then 10 µL of 0.8 mM cumene hidroperoxide were added and allowed to react during 15 minutes at room temperature. Second absorbance reading was determined using a similar filter and the results were expressed as CHP equiv/µgTP.

Glutathione assays

GSH and GSSG were measured following the Recycling Assay adapted from the enzymatic recycling method for quantitative determination of glutathione and glutathione disulfide levels (Vandeputte et al. 1994) adapted to microplate for general tissues (Rahman et al. 2006). This methodology is based on the reaction of GSH with DTNB that produces TNB, at a rate proportional to the GSH or GSSG concentration. The assays were performed on ice, at the lowest irradiance level possible to avoid derivatization. All reagents were diluted in KPE buffer (0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5).

Reduced glutathione (GSH)

In 96-well transparent microplates, we added 20 µL of each sample (in triplicate) and 120 µL of DTNB:GR to each well. The reaction was allowed to convert GSSG to GSH for 30 seconds, and then 60 µL of β-NADPH were added. The plate was immediately read at 412 nm every 30 s for 2 min. We calculated the rate of 2-nitro-5-thiobenzoic acid formation as the change in absorbance per minute, and the actual total GSH concentration was determined from the standard curve of GSH from 26.4 nM ml^{-1} to 0.103 nM ml^{-1}. Results are expressed as nM GSH/mg protein.

Oxidized glutathione (GSSG)

We added 100 µl of sulfosalicylic sample extract in each microcentrifuge tube. Then, 2 µL of 2-vinylpyridine were added, vortex-mixed, and allowed to derivatize GSH for 1h at room temperature. Such procedure was followed by the addition of 6 µL of triethanolamine, which after vortexed was allowed to neutralize for 10 minutes. GSSG assay proceed similarly as that described for the GSH assay. The plate was read using a 412 nm every 30 seconds for 2 minutes. We calculated the rate of 2-nitro-5-thiobenzoic acid formation as the change in absorbance per minute, and the actual total GSSG concentration was determined from the standard curve of GSSG from 26.4 nM ml^{-1} to 0.103 nM ml^{-1}. Results were expressed as nM GSSG/mg protein.

Ferric reducing antioxidant power (FRAP)

Total antioxidant capacity was determined by the ferric reducing power of the methanol homogenized samples through the FRAP assay adapted to microplates (Furlan et al. 2015). FRAP solution was prepared immediately before use by mixing 25 ml of acetate buffer (0.3 M pH 3.6), 2.5 ml of 10 mM 2,4,6-tri-2-pyridyl-s-triazine (TPTZ, Sigma), and 2.5 ml of 20 mM ferric chloride. In transparent microplates, 265 µL of FRAP solution was inoculated and mixed with 20 µL of methanol homogenates of the samples and 25 µL of ultrapure water. Methanol and Fe^{2+} were used as negative and positive control respectively. The reaction was allowed to proceed in dark at 37°C for 30 minutes. TPTZ-reduced complex was determined measuring the endpoint absorbance at 595 nm.

Stress proteins (HSP70)

The heat shock protein HSP70 was measured using the enzyme-linked immunosorbent assay (ELISA) adapted from in-house Sandwich-ELISA for human serum (Njemini et al. 2005) to anemones in this work. In Costar EIA/RIA (3390) microplates, we added three replicates of the supernatant containing 40 µg of total proteins of each sample and completed to 50µL with PBS. Microplates were incubated overnight at 4°C and then washed vigorously three times in

PBS-Tween20 0.05%. The plate surface was blocked to avoid unspecific ligations by adding 200 µL of skimmed milk 5% in PBS (Molico), followed by 90 min incubation at 37°C, and another vigorous wash. To tag the protein of interest, 100 µL of the primary antibody (Rabbit IgG) diluted in Molico 1% were added to each well and allowed to incubate at 37°C for 90 min. The microplate was once again washed vigorously three times in PBS-Tween20 0.05%, and 100 µL of the secondary antibody (anti-rabbit IgG) diluted in Molico 1% was added to each well and allowed to incubate at 37°C for 90 min. The microplate had its final wash (three times in PBS-Tween20 0.05%), and 100 µL of TMB revelation kit was added and allowed to react at room temperature during 10 min in darkness. The reaction was stopped with 50 µL of H_3PO_4 1M. Conjugated HSP70 was measured by absorbance using a 450 nm filter. Results were expressed as optical density.

Statistical analyses

In order to elucidate if there is a more suitable symbiotic association (A; B; C; D; E; F vs Control) for each temperature scenario (19°C; 24°C; 30°C) in a short term exposition we compared the physiological performance of i)EQY-MQY in culture (19°C; 24°C; 30°C)ii) *in hospite* (symbiont offer) versus *in culture* (19°C; 24°C; 30°C) and iii) associations versus control (without symbiont offer). For thispurpose we measured: 1.1) EQY, 1.2) MQY (vs. holobiont control and vs. clade culture), 2) ROS, 3) LPO, 4.1) GSH, 4.2) GSSG, 5) FRAP and 6) HSP70. The results were tested using a two-way ANOVA, considering temperature (19°C; 24°C; 30°C) and clade offer (Control; A; B; C; D; E; F) as fixed factors. Differences between the values were determined with multiple comparison against control using Dunnet-post-hoc test with GraphPad Prism statistical package. ANOVA assumptions were tested with Barlett's test. Significance level was set at $\alpha \leq 0.05$.

Results

Photosynthetic efficiency *in culture*

The highest EQY at 24°C was clade E, with 0.41 ± 0.01, followed by A, with half of its efficiency (0.21 ± 0.01), then B, C and F (0.10 ± 0.01) and finally clade D with 0.07 ± 0.01. EQY *in culture* was affected by changes in temperature in all the clades considered ($p < 0.05$). In particular, clades A, B and C were completely inhibited at both temperature treatments, clade E was completely inhibited at 19°C and reduced (24 %) at 30°C, while D resulted enhanced by both treatments of temperature in 40 and 30 % at 19 and 30°C respectively. Conversely, clade F improved by 18% of EQY at 19°C and suffered total inhibition at 30°C.

The highest MQY at 24°C was clade D (0.58 ± 0.01), followed by clade E, 10% less efficient (0.45 ± 0.01), then clade A 10% less efficient (0.35 ± 0.01), followed by clade F, 23 % less efficient (0.12 ± 0.01), and finally clades B and C (0.1 ± 0.01). MQY was affected by temperature in every clade *in culture*, with the only exception of clade D that was enhanced in 7 % at 19°C and remained unchanged at 30°C. Clade A was strongly inhibited, while B and C were completely inhibited by both temperature treatments. Clade E resulted completely inhibited by 19°C and slightly inhibited in 6 % at 30°C. Conversely, MQY of clade F resulted enhanced at 19°C in 20% and completely inhibited at 30°C (Figure1).

Photosynthetic efficiency *in culture* versus *in hospite* (19°C; 24°C; 30°C)

The EQY of the control holobiont remained unchanged by 19°C treatment but suffered a reduction at 30°C. Clade A at 24°C remained invariable *in culture* when compared to *in hospite*, while the holobiont remained unaffected at 30°C and experienced a reduction at 19°C, *in culture* experienced a total inhibition with both 19 and 30°C treatments treatments (Fig 2A). Clade B *in hospite* showed the double of EQY than *in culture* and experienced a rise at 19°C but remained unchanged at 30°C, while *in culture*, EQY of clade B experienced a drop at any temperature change. In clade C, the association also showed greater EQY but suffered little reduction with temperature when compared with a total inhibition *in culture*. In clade D, although at 24°C *in culture* showed lower EQY than *in hospite*, EQY was greater *in culture* when compared to *in hospite*. Clade E showed higher EQY at 24°C *in culture* than *in hospite*, while it rose *in hospite* with the changes in temperature. The EQY of clade F always increased *in hospite*, particularly with changes in temperature.

MQY was not affected by temperature in control anemones. In clade A, MQY was 10 % more efficient *in hospite* than *in culture*. Furthermore, it slightly dropped with treatments of temperature *in hospite* while it was strongly inhibited *in culture*. In clade B, MQY was 30 % more efficient *in hospite* than *in culture* and remained unchanged with treatments of temperature *in hospite* while it was completely inhibited *in culture*. In clade C, MQY was 30 % more efficient *in hospite* than *in culture* and dropped in 10 % with treatments of temperature *in hospite* while it was completely inhibited *in culture*. In clade D, MQY was 18% more efficient *in culture* than *in hospite* and dropped in 10 % with 19°C *in hospite* while it was enhanced *in culture* at this temperature. Interestingly, at 30°C, MQY was enhanced at both *in hospite* and *culture* conditions. In clade E, MQY was slighted enhanced by changes in temperature *in hospite* while completely inhibited at 19°C and 6 % decreased at 30°C *in culture*. In clade F, MQY was 30 % higher *in hospite* than *in culture*. Furthermore, it remained unchanged with treatments of temperature *in hospite* while it enhanced at 19°C *in culture*, but still 10 % less

effective when compared to *in hospite* and it was completely inhibited in culture at 30°C *in culture.*

Associations versus control *in hospite*

1. *Photosynthetic efficiency*

   Overall, both EQY and MQY of *in hospite* were significantly affected by the clade offered, regardless of temperature (Table 1). EQY showed a significant rise when clade F at 19°C was offered and when clade E was offered at 30°C with a rise of 16% and 22% versus control at 24°C respectively (Table 2). MQY showed a significant rise when clade A, C or D were offered at 24°C when, but also at 30°C when clades C, D and F were offered, and at 19°C when clade F was offered. All rised around 15 ± 3 % respecting to the control anemone at 24°C. Finally, we observed the group B-D-F had significant higher photosynthetic efficiency than the then the A-C-E-CONTROL (Figure 2).

2. *Total reactive oxygen species ROS*

   We found significant differences in ROS based on both considered factors together with its interaction (Table 1). Within controls, at 24°C and 30°C ROS was negative while at 19°C was positive. Overall, 19°C showed higher ROS at every offered clade (Table 2) (Figure 3A). Within 19°C, clades A (55 folds) B, (37 folds), E (26 folds) and F (101 folds) had a significant rise on ROS production when compared to control at 24ºC. Clades A and F showed the highest ROS production also within 24ºC and 30ºC treatments.

3. *Ferric reducing power FRAP*

   Overall, we found significant differences in FRAP on all main factors and their interaction (Table 1). At 19°C there were ten folds FRAP and at 30°C almost four folds FRAP respecting to control 24°C. Furthermore, when clade A was offered at 30°C, the host cells got almost five folds more reducing power than control 24°C. Within 24ºC, the offered clades produced an overall decrease of FRAP when compared to control 24ºC.

4. *Lipid peroxidation*

   We found significant effect of temperature on LPO (Table 1). At 19ºC there was a significant rise of LPO independently on the offered clade respecting to 24ºC (Figure 3C). In addition, within 19ºC, we identified a LPO rise with C and D versus control at 19ºC, with approximately double dienes versus control 24°C that was not consistent with the other temperature treatments (Table 2). Furthermore, the minimum LPO was observed in the clade B association at 30°C.

5. *Redox glutathione system*

We found that GSH content in the host cells was affected by temperature and the interaction of temperature and clade offered (Table 1). We observed a GSH overproduction at 30ºC independently on the clade offered. Nevertheless, there was no difference on GSH between the cold treatment versus control (Figure 3D). Although there was not an overall effect on clade offered, at 30°C clade A had three folds GSH content when compared to control 30°C and 125 folds over control 24°C (Table 2). We found that GSSG content in the host cells was affected by temperature and the interaction of temperature and clade offered (Table 1). We observed an inhibition on GSSG production at both temperature treatments of three orders of magnitude at 19ºC and 30ºC (Figure 3 E). Furthermore, clade A at 24ºC produces the double of GSH in the holobiont when compared to other clades (Table 2). The equilibrium *GSH:GSSG* is set at 1:2. At 19°C the glutathione system is at redox equilibrium for every clade with the exception of C (Table 3). At 24°C, GSSG predominates over GSH at every clade, showing an oxidized state at 24ºC in the holobiont cells. At 30°C there is accumulative GSH with emphases on clades A and C, so there is a reduced state of the glutathione system.

6. *Stress proteins HSP70*

Overall, we found significant differences in HSP70 on temperature and offered symbiont partner without interaction between factors (Table 1). The lowee temperature produced less HSP70 respecting to 24ºC and 30ºc. Furthermore, clades B, D and F produced higher HSP70 levels than clades A, C and E. The minimum HSP70 level was at control 19ºC and when clade A was offered at 24ºC (Figure 3F).

Discussion

Here we found that the holobiont *E.pallida-Symbiodinium* was better prepared to overcome warm events than cold temperatures. We propose a mechanism that may prevent oxidative damage during warm but not in cold events with an important role of glutathione reductase. Furthermore, clades E and F were the most suitable associations with the anemone. Switching into *in hospite* brought photosynthetic advantages for both partners with F at 19 ºC and for E at 30ºC. Based on our results, we can predict that free-living clades E and F, may be available in the environment at warm and cold temperatures respectively, while the generalist D can be present at any considered scenario. However, clade D was more efficient in the free-living form and induced lipoperoxidation to the host. In what follows, taking into account the

availability in the environment and the benefits for both partners to establish symbiosis we explore the trade-offs of switching towards cold and warm scenarios.

Cold water events can cause unprecedented bleaching events and mortality (Saxby et al. 2003; Hoegh-Guldberg and Fine, 2004; Lirman et al. 2011). In the holobiont, 19ºC induced LPO in the host probably because of high ROS levels were not reduced despite the increase of FRAP and low HSP70 content. Contrary, at 30ºC a 3-folds increase of FRAP may be related to the GSH increase that counteracted ROS and prevented LPO. Therefore, in concordance with previous studies (Casillas-Barragan et al. in review) the activity of glutathione reductase suggests a main role on oxidative prevention. Contrary to the expected, we have found that the glutathione system of the host is in equilibrium at 19ºC and basal oxidized at 24ºC, but reduced at 30ºC. At 24ºC is oxidized probably because of the inner photosynthesis (Casillas-Barragán et al. *in prep*.), while at 19ºC it remains in equilibrium probably due to a decrease of metabolism induced by low temperature (cf. Hüner et al. 1998). Finally, at 30ºC the reduced glutathione system may be part of the preventive strategy against oxidative damage.

Expected temperatures within global climate change context would inhibit the photosynthesis of five from six of the free-living clades considered here, so could lead to diversity loss of the environmental *Symbiodinium* sp. pool. The most efficient at 24ºC was E, but decayed at 19ºC and tolerated 30ºC while the generalist clade D (Stat and Gates, 2010) was favored by temperature changes and F failed at 30ºC but improved at 19ºC. Currently, we could expect that free-living E predominates in thermally homogeneous areas, while D in heterogeneous and F in cold areas. Nevertheless we cannot confirm their distribution because biogeographical data of free-living *Symbiodinium* sp. are scarce for the lack of accurate methods (Littman et al. 2008) and the complexity of its interactive life cycle (Post et al. 2002; Mouritsen and Richardson, 2003;Casillas-Barragán et al. *in prep*.). Towards colder scenarios, D and F would be favored while in warmer environments only D, and E may survive. In both cases thermos-sensible A, B, C, E could fail or switch into *in hospite* lifestyle.

Based on our results, temperature changes affected all clades *in culture* but not *in hospite*, so symbiosis may protect photosynthesis. The most efficient *in culture* in average subtropical conditions was E, that decreased its photosynthesis *in hospite* and is rarely found in symbiosis in nature. For clade A, it was photosynthetically advantageous to be *in hospite* with temperature. Therefore, A could move from free-living into symbiosis in a changing environment. For B, it was always advantageous to be *in hospite*. In concordance, in *Condylactis gigantea*, clade A predominates in areas with sharp temperature variations, while B, more prone to bleach than A, prevails in thermally homogeneous areas (Venn et al. 2008). For B, C and F was always advantageous to be *in hospite* and, as expected, are common associations in nature (Baker 2003; LaJeunesse et al. 2010; Thornhill et al. 2013, Grajales and Rodríguez, 2016). For D, it was

advantageous to be *in hospite* only at 24ºC but not in other temperatures, where free-living form improved EQY and had the highest MQY.

For *E. pallida* it would be more convenient to associate with E and F because in the presence of E at 30ºC and F at 19ºC the holobiont showed higher EQY. It could be also beneficial for *E. pallida* at 19ºC to associate with E; at 24ºC with A and D and at 30ºC with D and E because improved MQY. On the other hand, It could be also beneficial for *E. pallida* at 30ºC to associate with clade A because it enhanced FRAP in the host. Nevertheless A and F may be potentially oxidant associations because produced more ROS and GSSG. However, these cases did not induce LPO, so maybe the ROS produced worked as mediators within the partners' communication. F, together with B and D, produced HSP70 showing stress remediation. The association *E. pallida-D* happened, evidenced by MQY, HSP70 and LPO increase of the anemone. Even when D associates with diverse cnidarian species in adverse areas (Stat and Gates, 2010) and provides thermal resistance to the host (Rowan, 2004); it is usually as a cryptic endopopulation (Mieog et al. 2007; LaJeunesse et al. 2009; Correa et al. 2009). Although the association may not be beneficial in terms of photosynthesis for the free-living D, it might be in terms of metabolic exchange, physical protection, and/or vector for reproduction (Casillas-Barragan et al. *in prep*).

Contrary to clade D, at 19ºC both anemone and algae E and F resulted benefited while at 30ºC both anemone and clades A, E and F resulted profited. Establishing symbiosis implies a mutually beneficial association where both partners benefits from the relationship. Therefore, clades E and F would be the beneficial switch in both scenarios of global climate change. However, it would be unlikely for a bleached cnidarian switching endosymbiont towards E in a cold scenario, F in a warm and A in any of both scenarios because these clades are thermo-sensible in free-living form and possibly not available in such scenarios. Consequently, it would be a great advantage for the anemone to host clades E and F criptically for symbiont shuffling at challenging temperature shifts and would guarantee survival *in hospite* for the zooxanthellae that eventually could disperse from the host when the external conditions come back to 24ºC. In both scenarios, mixed endopulation including clades E and F may favor bleaching struggle of the holobiont within the climate change context.

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Figures

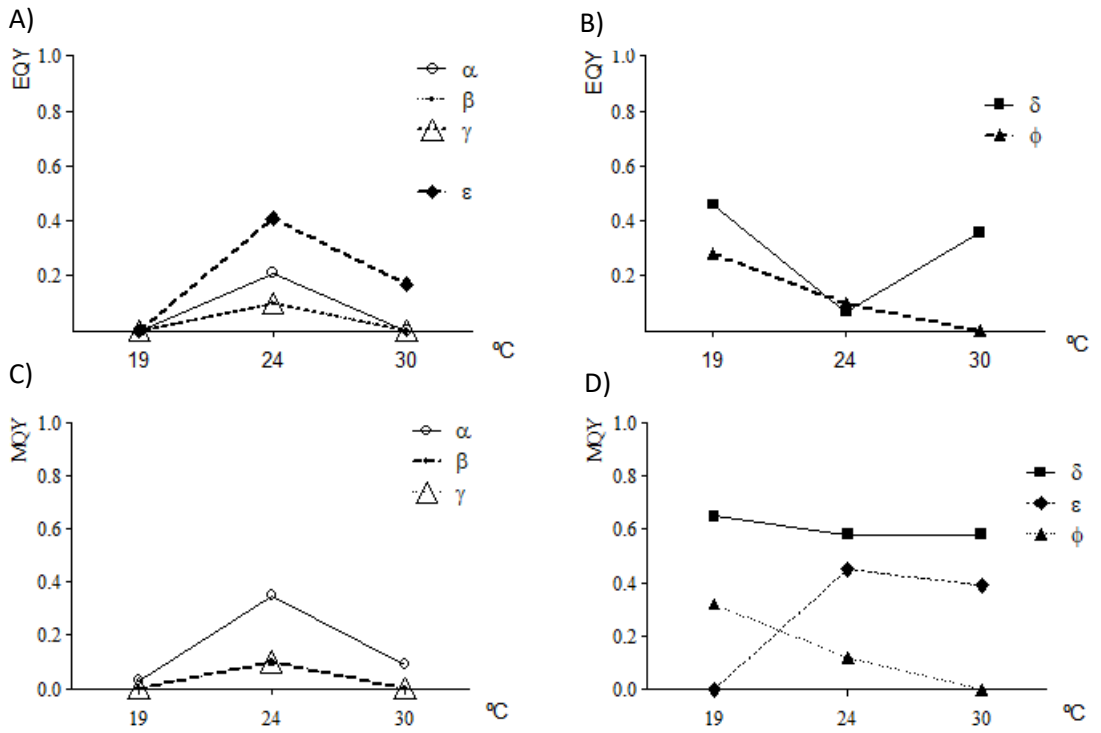

Figure 1. Photosynthetic efficiency *in culture.* Photosynthetic effective quantum yield (EQY; like ΔF'/Fm') and maximum quantum yield (MQY; like Fv/Fm) (mean ± SE) being α ; β; γ; δ; ε; ϕ clades of *Symbiodinium* sp. A; B; C; D; E; F respectively *in culture* (n = 3).

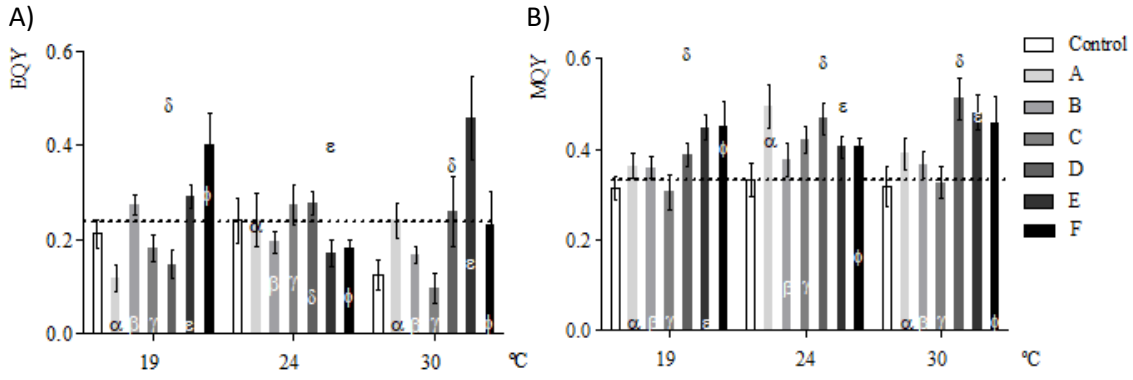

Figure 2. Photosynthetic efficiency *in culture* vs *in hospite.* Photosynthetic effective quantum yield (EQY; like ΔF'/Fm') and maximum quantum yield (MQY; like Fv/Fm) (mean ± SE) being α;β;γ;δ;ε;ϕ clades of *Symbiodinium* sp. A; B; C; D; E; F respectively *in culture* (n = 3) and A; B; C; D; E; F the combination of offered clade and anemone *Exaiptasia pallida* and Control the anemone in filtered sea water (n = 9). Dot points show the photosynthetic efficiency of the anemone control at 24ºC.

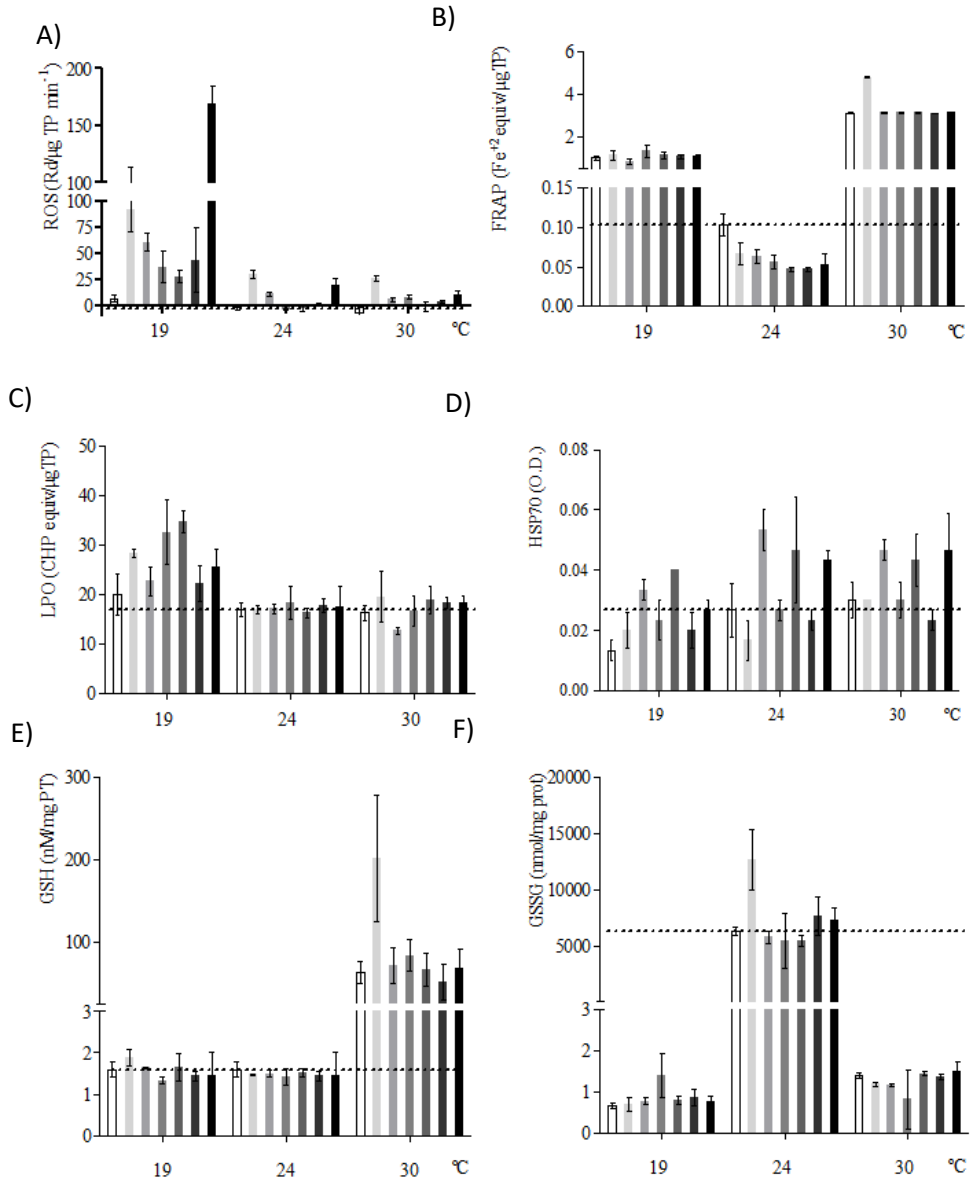

Figure 3. ***In hospite vs control***

The combination of offered clade and anemone *Exaiptasia pallida* in host cells of *E. pallida-Symbiodinium* spp. holobiont system

A) ROS in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as Rd/min µg TP (mean ± SE) (n = 3);

B) FRAP in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as Fe^{+2}equiv/µgTP (mean ± SE) (n = 3);

C) LPO in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as CHP equiv/µgTP (mean ± SE) (n = 3);

D) HSP70 in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as optical density at 450 nm (mean ± SD) (n = 3);

E) GSH in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as (nM/mgTP) (mean ± SE) (n = 3);

F) GSSG in host cells of *E. pallida-Symbiodinium* spp. holobiont system expressed as (nM/mgTP) (mean ± SE) (n = 3);

Dot points show level of each parameter of the anemone control at 24ºC.

Tables

Table 1

Parameters	Source of variation	% of total variation	*df*	*F*	*P*	
EQY	Interaction	24,17	12	4,98	< 0.0001	*
	Clade	7,8	6	3,21	0,005	*
	Temperature	0,04	2	0,0467	0,954	
	Residual		168			
MQY	Interaction	7,86	12	1,48	0,135	
	Clade	15,88	6	5,98	< 0.0001	*
	Temperature	1,95	2	2,20	0,113	
	Residual		168			
ROS	Interaction	23,39	12	7,2	< 0.0001	*
	Clade	27,31	6	16,8	< 0.0001	*
	Temperature	37,94	2	70,1	< 0.0001	*
	Residual		42			
LPO	Interaction	12,40	12	1,184	0,3265	
	Clade	9,16	6	1,748	0,1343	
	Temperature	39,83	2	22,80	< 0.0001	*
	Residual		42			
GSH	Interaction	15,30	12	2,08	0,040	*
	Clade	7,16	6	1,95	0,095	
	Temperature	50,42	2	41,2	< 0.0001	*
	Residual		42			
GSSG	Interaction	8,25	12	2,91	0,005	*
	Clade	4,14	6	2,93	0,018	*
	Temperature	75,17	2	159	< 0.0001	*
	Residual		42			
FRAP	Interaction	3,94	12	15,17	< 0.0001	*
	Clade	2,18	6	16,74	< 0.0001	*
	Residual		42			
HSP70	Interaction	6,70	12	0,5517	0,8672	
	Clade	41,19	6	6,782	< 0.0001	*
	Temperature	9,59	2	4,736	0,0140	*
	Residual		42			

Table 2

	Tª	Control	A	B	C	D	E	F
EQY	19°C	0,21 ± 0,03	0,12 ± 0,03	0,27 ± 0,02	0,18 ± 0,03	0,15 ± 0,03	0,29 ± 0,02	0,40 ± 0,07 *
	24°C	0,24 ± 0,05	0,24 ± 0,06	0,19 ± 0,02	0,27 ± 0,04	0,28 ± 0,03	0,17 ± 0,03	0,18 ± 0,02
	30°C	0,12 ± 0,03	0,24 ± 0,04	0,17 ± 0,02	0,10 ± 0,03	0,26 ± 0,07	0,46 ± 0,09 *	0,23 ± 0,07
MQY	19°C	0,31 ± 0,02	0,36 ± 0,03	0,36 ± 0,02	0,31 ± 0,04	0,39 ± 0,03	0,45 ± 0,03 *	0,45 ± 0,05 *
	24°C	0,33 ± 0,04	0,49 ± 0,05 *	0,38 ± 0,04	0,42 ± 0,03	0,47 ± 0,04 *	0,40 ± 0,02	0,40 ± 0,02
	30°C	0,32 ± 0,04	0,39 ± 0,04	0,36 ± 0,03	0,33 ± 0,04	0,51 ± 0,05 *	0,48 ± 0,04 *	0,46 ± 0,06 *
ROS	19°C	6,50 ± 3,18	91,91 ± 21,28 *	60,79 ± 8,77 *	36,76 ± 15,17	27,47 ± 6,11	43,58 ± 30,63 *	168,56 ± 15,82 *
	24°C	-1,66 ± 2,21	29,23 ± 3,93	10,79 ± 2,25	-4,06 ± 1,14	-2,73 ± 2,92	0,27 ± 1,24	19,84 ± 6,34
	30°C	-7,27 ± 4,63	25,97 ± 2,41	5,27 ± 2,09	7,87 ± 1,36	-1,54 ± 4,47	3,56 ± 1,69	9,68 ± 4,31
LPO	19°C	20,08 ± 4,13	28,28 ± 0,76	22,68 ± 2,84	32,61 ± 6,62 *	34,70 ± 2,11 *	22,09 ± 3,62	25,64 ± 3,52
	24°C	17,01 ± 1,44	16,88 ± 0,76	17,10 ± 0,95	18,34 ± 3,43	16,26 ± 0,94	17,80 ± 1,47	17,43 ± 4,30
	30°C	16,30 ± 1,59	19,52 ± 5,15	12,67 ± 0,67	16,66 ± 2,95	18,98 ± 2,78	18,28 ± 1,17	18,29 ± 1,39
GSH	19°C	1,60 ± 0,19	1,89 ± 0,19	1,64 ± 0,03	1,33 ± 0,08	1,66 ± 0,34	1,45 ± 0,11	1,45 ± 0,56
	24°C	1,60 ± 0,19	1,48 ± 0,01	1,50 ± 0,08	1,41 ± 0,20	1,52 ± 0,09	1,45 ± 0,11	1,45 ± 0,56
	30°C	63,28 ± 13,77	201,63 ± 76,26 *	70,92 ± 21,81	83,76 ± 19,78	66,27 ± 20,12	51,19 ± 21,23	68,00 ± 23,37
GSSG	19°C	0,67 ± 0,07	0,70 ± 0,16	0,78 ± 0,08	1,39 ± 0,53	0,80 ± 0,11	0,88 ± 0,21	0,76 ± 0,15
	24°C	6320,39 ± 339,03	12619,58 ± 2695,38	5783,56 ± 545,35	5475,60 ± 2397,75	5430,82 ± 498,64	7635,74 ± 1693,46	7329,02 ± 1113,01
	30°C	1,39 ± 0,07	1,19 ± 0,05	1,17 ± 0,02	0,82 ± 0,73	1,44 ± 0,04	1,36 ± 0,06	1,49 ± 0,25
FRAP	19°C	1,02 ± 0,10	1,14 ± 0,22	0,84 ± 0,13	1,34 ± 0,29	1,13 ± 0,14	1,07 ± 0,10	1,08 ± 0,10
	24°C	0,10 ± 0,01	0,07 ± 0,01	0,06 ± 0,01	0,06 ± 0,01	0,05 ± 0,00	0,05 ± 0,00	0,05 ± 0,01
	30°C	3,11 ± 0,00	4,81 ± 0,00 *	3,11 ± 0,00	3,11 ± 0,00	3,11 ± 0,00	3,11 ± 0,00	3,11 ± 0,00
HSP70	19°C	0,01 ± 0,00	0,02 ± 0,01	0,03 ± 0,00	0,02 ± 0,01	0,04 ± 0,00 *	0,02 ± 0,01	0,03 ± 0,00
	24°C	0,03 ± 0,01	0,02 ± 0,01	0,05 ± 0,01 *	0,03 ± 0,00	0,05 ± 0,02	0,02 ± 0,00	0,04 ± 0,00
	30°C	0,03 ± 0,01	0,03 ± 0,00	0,05 ± 0,00	0,03 ± 0,01	0,04 ± 0,01	0,02 ± 0,00	0,05 ± 0,01

Table 3

	Tª	Control	A	B	C	D	E	F
GSH:GSSG	19°C	2,39	2,70	2,10	0,96	2,08	1,65	1,91
	24°C	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	30°C	45,53	169,44	60,62	102,15	46,02	37,64	45,64

Capítulo V

Responses of the sea anemone *Anemonia sulcata* to increased hydrogen peroxide concentrations in seawater *(In prep.)*

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Abstract

Cnidarian bleaching is a growing concern in marine environments. It is known that hydrogen peroxide is a precursor of bleaching at cellular level. Although it has also been identified as a subproduct of UV filters, little research has addressed the response of cnidarians against environmental H_2O_2. Furthermore, scarce studies recommend anemones as environmental bioindicators. Here, we hypothesized that *Anemonia sulcata* is a good bioindicator for addressing environmental oxidative stress, and that environmental H_2O_2 produces oxidative damage, alters antioxidant enzyme activity and induces neurotoxicity and genotoxicity. We examined tissue bleaching and physiological effects in *A. sulcata* (n = 5) after exposure of increased H_2O_2 starting from environmental concentrations (nominal values=control, 0.04, 0.4, 4, 40, 400 µM) on long term exposure (0, 7, 14 and 21 days). We considered bleaching as endpoint and measured color and zooxanthellae density. To assess the physiological effects we measured oxidative damage (lipid peroxidation), antioxidant enzyme activity (glutathione peroxidase, reductase, and S-transferase), neurotoxicity (acetyl cholinesterase activity) and genotoxicity (DNA damage) at each sampling time. For this purpose, we adapted several protocols to anemones in this work. We tested our hypothesis though a repeated measures two-way ANOVA test. In this study, bleaching was not induced after 21 days of H_2O_2 exposure. We found significant effects of H_2O_2 exposure in DNA damage ($p = 0.0030$) and GST activity ($p = 0.0146$). We have found no significant effects of H_2O_2 exposure in color, zooxanthellae density, neurotoxicity, GR and LPO. Nevertheless all the biomarkers showed significant differences regarding to time. Based on our results, we don't recommend bleaching of *A. sulcata* as a bioindicator of oxidative stress in the environment. It may be acclimated to oxidative fluctuations associated its intertidal lifestyle or to dial photosynthetic activity of the hosted dinoflagellate. Results of this study suggest tolerance to

long-term oxidative environment and provide insights into the redox metabolic interplay between symbiont partners. Nevertheless, we suggest that *A. sulcata* would be a good model to study plasticity, tolerance, detoxification pathways, and symbiotic relationship between its symbiotic partner and the animal.

Key words

Detoxification model; UV filters; Biomarkers; Biodindicator; Oxidative stress; Glutathione; Symbiosis; Cnidaria; *Symbiodinium* sp.

Introduction

Cnidarian bleaching is a growing concern in marine environments due to worldwide induced mortalities and associated biodiversity loss. Bleaching is the endpoint of various cell-signaling pathways (Douglas, 2003). H_2O_2 has been proposed as a precursor of bleaching (Smith *et al.,* 2005) and algal density control (Sandeman, 2006). H_2O_2 has a high damaging potential because it diffuses easily through the organic membranes, interferes on numerous cellular reactions, and participates of cellular processes such as apoptosis (reviewed by Lesser, 2006). Furthermore, it is a precursor of further oxidative stress because once reduced, it may originate hydroxyl radical $HO^{\cdot}$, a trigger of free radical chain reactions that may end on lipid peroxidation, protein and DNA denaturation (reviewed by Lesser, 2006).

H_2O_2 has also been identified as a subproduct of UV filters in coastal waters (Sánchez-Quiles and Tovar-Sánchez, 2014) via photocatalytic reactions under UV radiation (reviewed by Lesser, 2006). Sunscreen has been shown to cause coral bleaching (Danovaro *et al.,* 2008). It may be discharged into the aquatic environment from bathing areas and industrial inputs (reviewed by Giokas *et al.*,2007). UV filters degradation results in active oxygen species in water (Inbaraj *et al.,* 2002; Hanson *et al.,* 2006). Reported environmental concentrations in nature range from 0.5 to 4 µM in water and from 2 to 16 µM in sediment (Fujii *et al*., 2012) with estimated peaks over 400 times during summer in bathing areas (Sánchez-Quiles and Tovar-Sánchez, 2014). It has been demonstrated to cause toxic effects on phytoplankton (Sánchez-Quiles and Tovar-Sánchez, 2014), but the ecotoxicological effects of these environmental concentrations have been rarely explored.

Little research has addressed the response of cnidarians against environmental H_2O_2 exposure, and always restricted to short term exposure experiments (Mitchelmore *et al.,* 2004; Higuchi *et al.,* 2008). Furthermore, anemones have been used for toxicity tests only related to metal exposure (Duckworth *et al.,* 2017; Siddiqui *et al.,* 2015). Here we propose anemones as a potential indicator of environmental stress and a model to study antioxidant response. In this study, we examined the effects of increased environmental H_2O_2 concentration on tissue

bleaching and physiological effects in the sea anemone *A. sulcata* during long term exposition accounting with environmental concentrations found in nature. The objective of this research was to assess the effect of H_2O_2 exposure at long term exposition on bleaching, oxidative stress, antioxidant enzyme activity, neurotoxicity and genotoxicity in *A. sulcata*. For this purpose, we adapted several protocols to anemones in this work. Finally, based on our results, we discuss the implications of environmental H_2O_2, the adequacy of *A. sulcata* as environmental bioindicator and propose this holobiont as a model to investigate antioxidant pathways for future studies.

Materials and methods

Acclimation

Specimens of sea anemones, *A. sulcata* were provided by Casanova Seafood Company, collected from South Vizcaya Golf in the Northeast Atlantic Ocean. They were maintained individually in a 4 L closed-circuit natural seawater aquaria at 36.6 ± 0.1 ppm, 20 °C ± 0.5 °C, and 12 h/12 h light/dark cycle under LED tube (Cool Daylight Philips) and constant irradiance of 4 ± 0.10 quanta $cm^{-2}s^{-1}$. *A. sulcata* were fed with 2 mL of *Artemia* metanaupli at their oral disc once a week and allowed a one week acclimation at experimental conditions prior to experimentation.

Experimental design

Based on reported environmental concentrations of H_2O_2 (Sánchez-Quiles and Tovar-Sánchez, 2014), we tested 0.04, 0.4, 4, 40 and 400 µM of H_2O_2 during 21 days, in a semi static renovation system. The bioassay was carried out in 4 L aquaria and each treatment was applied in quintuplicate with one organism per aquaria. Physical and chemical parameters were maintained constant during the acclimation and experimental period. Seawater was held constant at 20° C ± 0.25° C. The concentrations of H_2O_2 were freshly prepared by mixing 30 % (w/w) stock solution with natural seawater at the same day of exposure. In order to maintain the exposure concentrations, the aquarium seawater was totally exchanged every other day. Specimens were sampled weekly (0, 7d, 14d, 21d). At each sampling time, endpoints of mortality and color were checked using the CoralWatch Scale provided by the University of Queensland. The scale has six levels of tonality, from white (E1) to dark brown (E6), directly correlated with endosymbiont density and chlorophyll a content. This is an inexpensive, rapid and non-invasive method for bleaching assessment which has been validated for other cnidarians (Siebeck, 2008). Measurements were taken from the middle area of the tentacle and the column. Then, five tentacles were dissected, flash frozen with liquid nitrogen and stored at -80° C for later analysis.

Tissue preparation

Frozen dissected tentacles from each individual were pooled together (n=5) and homogenized during 30 seconds in a tissue disperser (IKA ultra-Turrax T-25) in 1,5 ml of phosphate homogenization buffer (phosphate 50 mM, EDTA, 1 mM and glycerol 10%, pH 7.0 - Krueger *et al.*, 2015). This treatment is able to lysate the host cells without disrupting the symbionts. Subsamples of the homogenate were used for determination of zooxanthellae density using direct counts on a Neubauer haemocytometer. To separate the zooxanthellae the homogenized tissue was centrifuged at 2000 x g during 5 minutes. The pellet containing zooxanthellae cells was then stored for later analysis, while part of the first supernatant fraction (S^2), containing the host cells lysate was used to measure lipoperoxidation, genotoxicity assay and total protein content as described below. The supernatant S^2 was then further centrifuged at 15000 x g during 20 minutes to get the cytosolic fraction S^{15} for the glutathione activity and neurotoxicity assays. All biomarkers were estimated regarding the total protein content (TP), expressed as mg/ml, determined by Bradford Assay (Bradford, 1976). Standards were prepared with bovine serum albumin (BSA) from a 20 mg/ml stock solution diluted in Milliq water at 0, 0.21, 0.42, 0.7, and 1.04 mg/ml. After 5 minutes in dark and RT, endpoint absorbance was recorded at 595 nm. All the assays were performed in 96 flat bottom wells microplates using a spectrophotometer Infinite M200.

Biomarkers

1. Lipid peroxidation (LPO). Oxidative stress was measured using the TBARS assay (Wilbur, 1949) adapted to anemones in the present work. It is based on the detection of breakdown product of the oxidation of carbon double bonds in polyunsaturated lipids of the membrane during oxidative stress: malonaldehyde (MDA). MDA reacts with 2-thiobarbituric acid (TBA), producing tetramethoxypropane (TMP) which forms a chromophore detectable by fluorescence (516/600 nm). Samples were prepared by adding and mixing each of the following reagents in 1.5 ml centrifuge tubes: 150 µl of tissue homogenate, 300 µl of 10% trichloroacetic acid (TCA) in 1 mM $FeSO_4$ and 150 µl of 0.67% thiobarbituric acid (TBA). Standard calibration curve was developed at 0, 0.6, 1.5, 3, 4, 6, 10 µM of 0.001% TMP. Standards and samples were incubated in water bath at 70° C for 10 minutes. In dark 96-well black microplates we added 200 µl of blank (homogenization buffer), standards and samples in duplicate. Endpoint fluorescence was measured using 516 nm excitation and 600 nm emission. Results were expressed as µgTBARS/mg of TP.

2. Neurototoxicity. Acetyl Cholinesterase activity was measured using the method of Ellmann (1961) adapted to anemones in this work. It is based on the detection of the product 2-nitrobenzoato-5-mercaptotiocholine and 5-thio-2-nitrobenzoato produced by the hydrolysation

of tiocholine and DTNB (5 5 dithio-bis-(2-nitrobenzoic) acid). In transparent microplates, we added in duplicate 20 µl of S^{15} supernatant sample and 20 µl of homogenization buffer as blank. To each well, we added 130 µl of DTNB solution (0.75 mM in phosphate buffer 0.1 M, pH 7.5), followed immediately by 50 µl of acetilcholine (0.1 M, pH 7.5). Kinetic absorbance at 405 nm was measured in 50 seconds intervals for 10 minutes at 25° C. The AChE activity was determined as the rate of hydrolysation of acetylcholine to choline and acetate. Results were expressed as nmol thiocholine/min.ml^{-1}.

3. Genotoxicity. Alterations in the genetic material was measured using the alkaline precipitation assay adapted from Gagné and Blaise (1995) to anemones in this work. It is based on the separation of single and double strand breaks from genomic DNA in the presence of trace detergents. Samples were prepared by adding and mixing gently by inversion each of the following reagents in 1.5 ml centrifuge tubes: 50 µl of homogenate sample, 200 µl of SDS 2% (sodium dodecyl sulfate, 10 mM EDTA, 10 mM Tris-base and 40 mM NaOH, pH 12) and 200 µl of 0.12 M KCl. Homogenization buffer instead of samples was used as blanks. The tubes were incubated in water bath at 60° C for 10 minutes and then at 4° C for 30 minutes, followed by centrifugation at 8000 x g during 5 minutes at 4° C. Standard calibration curve was prepared directly on the microplate using salmon sperm DNA (diluted in TEIX: Tris-HCl, EDTA, pH 8) with five points from 0, 0.91, 2.27, 4.55 and 9.09 µg/ml. In black 96-well microplates we added 50 µl of supernatant of the samples and blanks in duplicate and 150 µl of Hoechst fluorescent dye 0.1 µg/ml (63493 Sigma) diluted with a buffer containing 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris-acetate, pH 8.5. Endpoint fluorescence was measured at 360 nm of excitation and 450 nm of emission and the results expressed as DNA equivalents/mg of TP.

4. Antioxidant activity

*4.1. Glutathione peroxidase activity (GPX).*In transparent microplates, we added in duplicate 50 µl of S^{15} supernatant and 50 µl of homogenization buffer as blank. Then, we added 200 µl of daily assay mixture to each well (reduced glutathione, NAPDPH, glutathione reductase). After 2 minutes, we added 50 µl of substrate solution to each well (1.25 mM H_2O_2 in 50mM potassium phosphate buffer with 0.1mM EDTA and 0.15 mM sodium azide, pH 7.0). Kinetic absorbance at 340 nm was measured in 10 seconds intervals during 3 minutes at 30° C. The GPX activity was determined as the rate of loss of NADPH (OD/min)/(6.22*0.8))*0.26); being 6.22 the extinction coefficient for NADPH, 0.8 the path length for 0.26 ml and 0.26 the volume (ml) per well. Results were expressed as µmol NADPH /min per g of TP.

4.2. Glutathione reductase activity (GR). It is based on the consumption of the cofactor NADPH due to the glutathione reductase activity in the sample, which produces the reduction of oxidized glutathione. In transparent microplates, we added in duplicate 20 µl of S^{15} supernatant and 20 µl

of homogenization buffer as blank. Then, we added 200 µl of mixture assay (10 mM oxidized glutathione, and 1 mM NADPH 200 mM sodium phosphate buffer, pH 7.6, incubated at 30° C) to each well. Kinetic absorbance at 340 nm was measured in 2 minutes intervals during 10 minutes at 25° C. The reductase activity was determined as the rate of loss of NADPH (OD/min)/(6.22*0.6))*0.22); being 6.22 the extinction coefficient for NADPH, 0.6 the path length for 0.22 ml and 0.22 the volume (ml) per well. Results were expressed as µmol NADPH/min per g of TP.

4.3. Glutathione S-transferase activity (GST). The activity of GST was measured using the method of Gowland et al. (2002), adapted to anemones in this work. It is based on the conjugation of reduced glutathione with 1-chloro-2-4-dinitrobenzene substrate, which forms a chromophore detectable at 340 nm. In transparent microplates, we added 50 µl of S^{15} supernatant and 50 µl of homogenization buffer in duplicate. Then, we added 200 µl substrate solution (1mM 1-chloro-2-4-dinitrobenzene, 125 mM NaCl and 10 mM HEPES, 1mM glutathione reduced, pH 6.5). The rate of glutathione conjugation was measured at 340 nm at 5 minutes intervals during 30 minutes without blank slope. The results were expressed as optical density/min per g of TP.

Statistical analysis

Statistical differences between H_2O_2 treatments over time were determined with repeated measures Two-way ANOVA test, considering time and concentration as fixed factors, after testing for normality and equal using Kolmogorov–Smirnov and Bartlett's test. Data were log transformed to meet parametrical assumptions when necessary. General differences were followed by a post-hoc multiple comparison Bonferroni test. When the factor time had an effect, we analyzed the data time by time respecting to control of such time with 1 way ANOVA and Dunnet post-hoc analysis respecting to control. The controls of sampling day were compared against t0 with 1 way ANOVA and Dunnet post-hoc analysis, using Prism 5.0 statistical package (GraphPad Software). Significance level was set at $\alpha \leq 0.05$.

3. Results

No mortality or bleaching was registered at any H_2O_2 treatment after 21 days of experiment. Accordingly, color and zooxanthellae density remained unaltered respecting to environmental H_2O_2 concentrations. We haven't found a significant H_2O_2 dose-response related to oxidative stress, neurotoxicity or GPX and GR activity in *A. sulcata*. Nevertheless, we have found significant effects in genotoxicity ($p = 0.0030$) and antioxidant activity related to GST ($p = 0.0146$). Interestingly, all the biomarkers were time-dependant, generally with a significant effect at the 14^{th} day in comparison to days 0, 7 and 21.

Color and cellular density

No significant differences in color at the tentacle or column nor zooxanthellae density were observed due to peroxide exposure. However, after 21 days there was a significant decrease on zooxanthellae density ($p = 0.0426$) when compared to t0 and in day 14 we have found a significant decrease on zooxanthellae density in anemones exposed to 40 and 400 µM of H_2O_2 in comparison to the control of that day ($p = 0.0148$).

3.1.*Oxidative damage-stress. Lipid peroxidation (LPO).*

No significant differences in oxidative damage were observed due to peroxide exposure. Nevertheless, we have found that oxidative damage increases on anemones after 21 days experiment, including controls and with independence on peroxide treatment ($p = 0.0058$) (Fig.1).

3.2. Neurototoxicity. Acetyl Cholinesterase activity

No differences were observed in neuroactivity as a consequence of peroxide exposure, but after 21 days, AChE activity increased in three orders of magnitude respecting to t0 ($P < 0.0001$) (Fig.1).

3.3. Genotoxicity DNA damage.

Anemone's DNA was significantly damaged by H_2O_2 exposure ($p = 0.0037$) and time of exposure ($p < 0.0001$). After seven days, the animals were not affected but after 14 days, those subjected to 40 and 400 µM of H_2O_2 exposure experienced a significant increase on DNA damage respecting to control that persisted after 21 days (Fig. 2).

3.4. Antioxidant enzyme activity

In general terms, glutathione activity increased at day 14 and remains unchanged at days 7 and 21 respecting to t0 (Fig. 3). Both time ($p < 0.0001$) and concentration of H_2O_2 ($p = 0.0146$) had significant effect on GST activity in *A. sulcata* (Figure 4). The GST activity showed a general increasing trend with concentration and time which increased significantly after 21 days at 4 µM H_2O_2 ($p = 0.0232$) and 400 µM H_2O_2 treatments ($p = 0.0082$) respecting to control (Fig. 3).

Discussion

Our results don't show bleaching related to long term exposure to increased environmental H_2O_2 in *A. sulcata*. Other studies neither found bleaching after short-term exposure to environmental H_2O_2 in *Galaxea fascicularis* (Higuchi *et al.*, 2008). Nevertheless,

we observed immediate responses after inoculation, such as tentacle strangulation or a mild bleaching that reversed in less than one week, independently on concentration of exposure (data not shown).

We have found oxidative and DNA damage after 14 days including control individuals. Based on our results, we don't recommend longer periods of time in this condition for this species in toxicity tests. One week is the conservative time span to do this kind of assays to avoid interferences of experimental conditions on the physiological performance of the organism. Furthermore, after 14 days, anemones subjected to 40 and 400 µM of H_2O_2 exposure experienced a significant DNA loss respecting to control that persisted after 21 days. Isolated cnidarian cells of the sea anemone *Anthopleura elegantissima* showed increasing dose-response from 0 to 200 µM genotoxic effects after 1 hour exposure to H_2O_2 , but without loss of cell viability (Mitchelmore *et al.,* 2004). Together, these results suggest that cnidarians may be able to metabolize the genotoxic subproducts generated after short term exposure to H_2O_2 although chronic exposition at high concentrations may overcome this capacity. As a consequence, *A. sulcata* would be a good model for studying the mechanisms behind DNA restoration at short and medium term expositions. Anemones are prime examples of nervous system organized in diffuse nerve nets but neurotoxicity has never been measured on anemones before. Interestingly, neuroactivity increased after 3 weeks of experiment may be as a sensitive response to improve environmental perception in adverse conditions.

In concordance with other studies that didn't found variations on GPx activity induced by oxidative stress produced by increasing temperature (Pey *et al.*, 2017), we haven't found an effect on GPx and GR activity of host cells related to increased long term H_2O_2 exposure. The GST activity increases with time and concentration without oxidative damage resulting from peroxide exposure. As a consequence, GST detoxification shows to be effective in the prevention of oxidative damage and appears to have the main antioxidant role in H_2O_2 reduction because the activity of other glutathione enzymes remained unchanged. Symbiotic cells may cope with oxidative stress related to hyperoxia and thermal stress better than aposymbiotic cells (Richier *et al.,* 2004), suggesting a possible protection of host cells to oxidative stress. Furthermore, GPx diversity and distribution within the cellular compartments of the holobiont has been characterized as inherent to symbiotic lifestyle adaptation of the anemone *Anemonia viridis* (Pey *et al.,* 2017) and recent studies point out that host cells are able to cope with dial oxidative reactive species resulting from photosynthetic activity (Casillas-Barragán et al., *in prep*). To better understand the role of glutathione in the symbiotic lifestyle, it would be interesting to compare the antioxidant responses with the aposymbiotic form of the anemone.

Finally, based on the evidences found in this study, *A. sulcata* shows long term tolerance to an oxidative environment, probably related the intertidal lifestyle or to the adaptation to

symbiosis, on which daily redox cycles induced by photosynthetic *in hospite*, promote preparation for oxidative stress mechanisms in the host. To understand the peroxide detoxification pathways in symbiotic animals, it would be interesting to assess the antioxidant activity of the hosted *Symbiodinium* sp., considering not only glutathione enzymes, but also catalase activity in comparison with the host cells (see Higuchi *et al.*, 2008).

Conclusions

A. sulcata is not an appropriate bioindicator at environmental range concentrations because it shows effects only in abnormally high concentrations not present in nature. We suggest a guideline for toxicity test shorter than 14 days to avoid interferences on the results owing to oxidative stress from similar experimental conditions. Nevertheless, longer term exposures could lead to acclimation processes. In the context of studying stress response pathway, *A. sulcata* is a good model of control to study peroxide tolerance pathways. Also, *A. sulcata* could be an innovative model for studying DNA restoration and neuroactivity.

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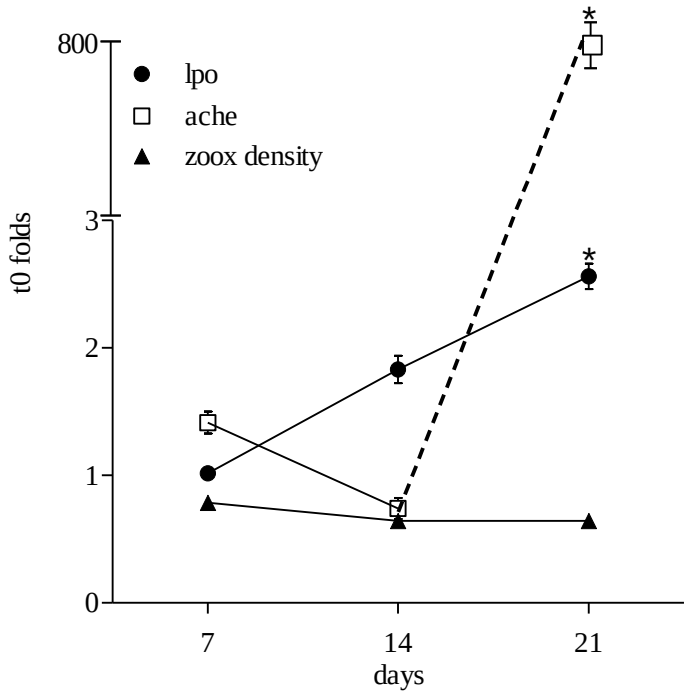
Figures and legends



F**igure 1.** LPO, AChE and endosymbiont density (mean ± SE) respecting to T0 levels in *Anemonia sulcata* after 7, 14 21 days independently on H_2O_2 exposure. Asterisks indicates a significant difference from the control ($p < 0.05$; $n = 30$).

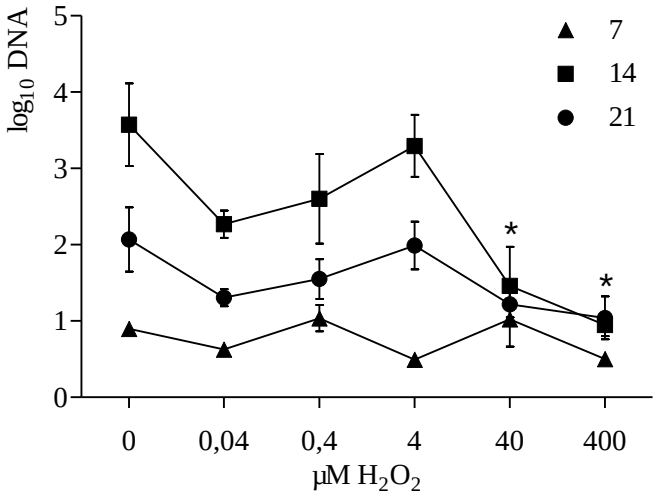


Figure 2. DNA content (mean ± SE) in *Anemonia sulcata* after 7, 14 and 21 days exposure to 0.04; 0.4; 4; 40 and 400 µM of H_2O_2. Asterisks indicates a significant difference from the control ($p < 0.05$; $n = 5$).

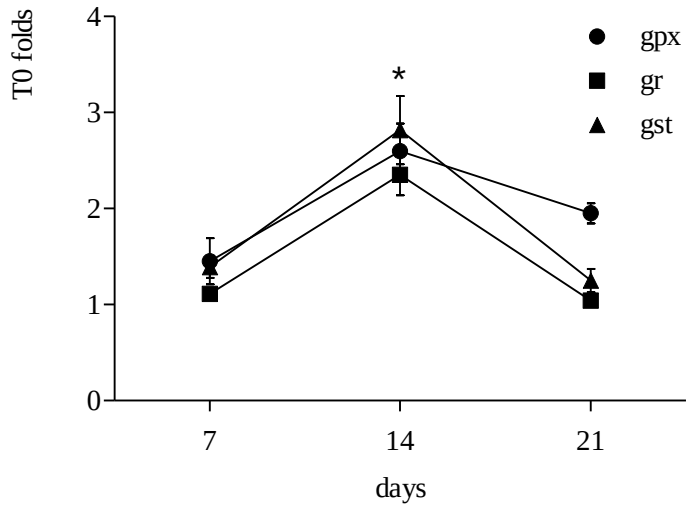


Figure 3. Glutathione enzymes activity (mean ± SEM) in *Anemonia sulcata* after 7, 14 and 21 days independently on H_2O_2 exposure. Asterisks indicates a significant difference from the control ($p < 0.05$; $n = 30$).

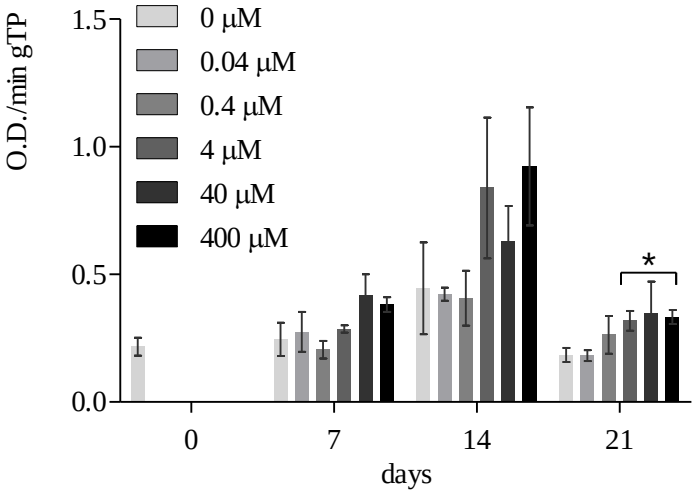


Figure 4. Glutathion *S*-transferase **(**GST) activity (mean ± SE) in *Anemonia sulcata* after 0, 7, 14 21 days exposure to 0; 0.04; 0.4; 4; 40 and 400 µM of H_2O_2. Asterisks indicates a significant difference from the control group ($p < 0.05$; $n = 5$).

Capítulo VI

Conclusões e considerações finais

Branqueamento adaptativo em cnidários

Este estudo coloca evidencias em direção a hipótese de branqueamento adaptativo, que observa o branqueamento como uma fase de aclimatação às novas condições ambientais. Esse processo adaptativo pode acontecer dentro da plasticidade da relação simbiôntica entre os parceiros que conformam o holobionte. Para isso exploramos diferentes vias de recuperação do branqueamento, desde a proliferação dos endosimbiontes remanescentes no holobionte branqueado até a troca do dinoflagelado simbionte disponível no ambiente, assim como o tempo necessário para restauração da pigmentação. Estabelecemos o relacionamento redox entre os parceiros simbiontes com base na fotossíntese. Discutimos sobre as vantagens de diferentes clados de *Symbiodinium* para estabelecer simbiose ou permanecer em vida livre segundo os cenários de temperatura, assim como as vantagens para o cnidário hospedar populações mistas de endosimbiontes e permitir sua predominância segundo as condições ambientais dentro das previsões das mudanças climáticas globais.

Recuperação do branqueamento

As vias de recuperação do branqueamento podem ser variadas e não dependem apenas do animal, mas também da alga hospedeira. Assim, apresentamos uma estratégia de recuperação do branqueamento da anêmona a partir da proliferação dos endosimbiontes remanescentes, que seguem um padrão de pigmentação funcional. Esse padrão prioriza a obtenção de recurso energético pela anêmona e garante o mesmo para a prole através da transmissão vertical do endosimbionte por reprodução assexual (laceração pedal) do hospedeiro. Porém, essa estratégia inclui também a participação do simbionte hospedado, pois observamos a reprodução e dispersão da alga simbionte a partir do organismo hospedeiro. Assim, propomos uma estratégia de dispersão das zooxantelas, na qual utiliza o cnidário é utilizado como vetor de dispersão. Este processo se inicia com a comunicação célula –célula entre os simbiontes, continua com a união de células de vários estágios para formar uma estrutura organizada pelos flagelos das próprias microalgas e suportadas por uma película de natureza proteoglicana, a qual é finalmente, repelida pela anêmona e expulsa ao ambiente.

Preparação para o estresse oxidativo

Apesar dos benefícios metabólicos que a alga fornece para o hospedeiro, a fotossíntese é um processo oxidante. Prévios estudos têm apontado o estresse oxidativo como desencadeante

do branqueamento. Assim, nessa tese aprofundamos o conhecimento acerca da relação basal redox entre os parceiros simbiontes. Descobrimos um mecanismo do hospedeiro de antecipação ao estresse oxidativo no qual o animal se prepara para a atividade fotossintética da alga hospedada. Observamos que a atividade fotossintética da alga, paralela à oxidação da glutationa no hospedeiro, tem seu máximo ao final do período escuro, se antecipando à luz, se mantendo constante durante o período diurno e decaindo no final desse período. Encontramos mecanismos antioxidantes preventivos que começam no final do período diurno e são desenvolvidos durante o período escuro se antecipando à fotossíntese. As proteínas chaperonas mantém um nível basal e exibem seu mínimo no final do período diurno, prévio ao mínimo fotossintético. Este mínimo fotossintético coincide com o máximo de glutationa reduzida, atuando como um possível substrato que se antecipa ao máximo de produção das espécies oxidativas reativas do hospedeiro que, por sua vez, se antecipam ao máximo fotossintético e ao período luminoso.

Estresse oxidativo e temperatura

Encontramos estresse oxidativo produzido em baixas temperaturas independentemente do clado oferecido. Assim, ao contrário do esperado, o holobionte tolera melhor os eventos de temperaturas altas se comparados com os de baixa. Em baixa temperatura, apesar de dobrar a capacidade antioxidante total do holobionte, a mesma foi insuficiente para reduzir os radicais livres. Como consequência, esses radicais induziram dano oxidativo no animal. Já em altas temperaturas, o holobionte quadruplica a capacidade antioxidante assim como o conteúdo em glutationa reduzida da anêmona, conseguindo reduzir os radicais livres e prevenindo o dano oxidativo no hospedeiro. Em particular, o clado A fornece maior capacidade antioxidante em altas temperaturas, e o hospedeiro contém mais glutationa reduzida quando comparado com a associação com os outros clados aqui considerados. Consequentemente, pode ser uma vantagem comparativa para a anêmona se associar com o clado A em episódios oxidantes por temperaturas extremas de altas temperaturas.

Associações *Exaiptasia pallida-Symbiodinium* spp. e temperatura

Outra vantagem comparativa para anêmona se associar com outro clado é oferecer melhoras fotossintéticas. Os clados F e E fornecem mais fotossíntese efetiva em temperaturas frias e quentes, respectivamente. Em adição, estes clados aumentam a capacidade fotossintética máxima do hospedeiro em baixas temperaturas, enquanto os clados A e D em temperaturas normais e os D, E e F em temperaturas altas. Assim, os clados E e F oferecem vantagens fotossintéticas no contexto das mudanças climáticas. Outra vantagem comparativa para o hospedeiro é se associar com clados que fornecem maior quantidade de remedição ao dano proteico, os quais são apresentados pelos clados B, D e F, independente da temperatura. Apesar

de demonstrar que a simbiose pode se estabelecer em períodos de 24 horas por transmissão horizontal, essa interação apenas será possível no caso de a alga estar disponível no ambiente e apta a se associar.

Symbiodinium spp. em vida livre

Porém, os dinoflagelados podem não estar disponíveis na coluna de água, impedindo sua aquisição pelos cnidários. De acordo com as projeções de temperatura ocasionadas pelas mudanças climáticas, encontramos que a diversidade de clados no ambiente pode diminuir em favor de dinoflagelados generalistas. Clados sensíveis a mudanças em temperatura (A, B e C) podem desaparecer em cenários de frio ou de calor por falência fotossintética. Os clados D e F podem ser favorecidos pelo frio, o clado D pelo calor e o E pode tolerar o aquecimento. Assim, o clado generalista D é favorecido por qualquer mudança de temperatura. Por outro lado, a fisiologia desses clados, em vida livre, é diferente daquela quando em associação. Dependendo do cenário, pode ser que não seja vantajoso para a alga estabelecer a simbiose. Por exemplo, o clado D generalista é mais efetivo fotossinteticamente em vida livre que em simbiose, podendo evitar a simbiose em benefício próprio.

Em síntese, a associação de *E. pallida* com os clados A, E e F apresentam benefícios para a anêmona. Porém, o clado A pode estar indisponível no ambiente por ser termosensível. Seria vantajoso para a anêmona a aquisição das algas em ambientes com 24ºC, onde estaria disponível, aumentando a capacidade fotossintética máxima em associação nessas condições, e a capacidade antioxidante do hospedadeiro em caso de eventos de aquecimento. Porém, a alga apenas obtém benefício da associação em caso de mudança de temperatura. No cenário de esfriamento, o clado F seria favorecido em vida livre, ou seja, estaria disponível no ambiente e também seria beneficiado pela associação. O clado E toleraria o aquecimento, ou seja, estaria disponível no ambiente e seria beneficiado pela associação.

As anêmonas como espécies bioindicadoras

Dado que a capacidade fisiológica pode mudar com as algas simbiontes, as anêmonas simbiônticas não são os melhores modelos para estudos ecotoxicológicos. As anêmonas não são boas espécies bioindicadoras do branqueamento por estresse oxidativo devido à sua alta tolerância. Essa alta tolerância pode ser decorrente dos mecanismos preventivos antioxidantes do animal derivados da atividade fotossintética diária da alga hospedada. Também, espécies entre marés como *A. sulcata* acometem períodos de oxidação extrema durante a maré baixa. Por isso, para futuros estudos recomendamos escolher anêmonas aposimbiônticas e bentônicas como espécies bioindicadoras. Contudo, segundo nossos resultados, poderiam ser um

interessante modelo de detoxificação para estudos antioxidantes e de restauração genética e neurológica.

Consideração final

Em conclusão, todas as partes deste estudo apontam a relevância da zooxantela para a recuperação do branqueamento dos cnidários assim como seu papel decisivo no estabelecimento da simbiose. Após confirmar que diferentes clados possuem diferentes capacidades fisiológicas que mudam segundo o estilo de vida livre ou hospedado, e levando em consideração que as mudanças climáticas irão reduzir os clados disponíveis no ambiente, concluímos que o mais apropriado para os dois parceiros seria hospedar populações mistas. Hospedando populações mistas na presença de clados crípticos, a anêmona poderia trocar de população predominante de acordo com as condições ambientais, sendo que a alga seja simultaneamente beneficiada. Desta forma, a alga aumenta o espectro de temperaturas toleráveis e poderia usar o cnidário como vetor de reprodução e dispersão. Dessa maneira, é pertinente o estudo de eventuais sucessões ecológicas das endopopulações simbiontes em diferentes cenários de temperatura. Para isso, seria necessário aprimorar as técnicas genômicas de identificação como também de quantificação dos clados de *Symbiodinium*. Finalmente, em termos de conservação, apresentamos evidências de como as mudanças climáticas podem diminuir a biodiversidade e influenciar negativamente os mecanismos que garantem a persistência dos nossos ecossistemas.

Resumo Geral

O branqueamento por estresse térmico é a principal causa do declínio de cnidários simbióticos nos oceanos tropicais. Contudo, este processo pode ser revertido. Assim, o objetivo deste trabalho é o estudo integrado dos processos fisiológico da perda e recuperação das zooxantelas relacionado ao fenômeno de branqueamento por aumento de temperatura em *E. pallida*, mediante o estudo da resposta do holobionte ao estresse térmico, com ênfase na troca de população simbiótica. Um dos mecanismos de recuperação da pigmentação investigados nesse estudo é a proliferação dos endosimbiontes remanescentes no cnidário, onde foi encontrando um padrão de repigmentação funcional em diferentes partes do pólipo. Neste contexto é proposto um mecanismo de ruptura da simbiose na qual os endosimbiontes participam ativamente de sua expulsão e dispersão no meio, abrindo uma nova discussão sobre o agente diretor da simbiose. Uma vez que o branqueamento tem sido relacionado com estresse oxidativo e a fotossíntese é um processo oxidante, avaliamos a atividade redox induzida pela fotossíntese da alga hospedada, assim como a atividade antioxidante e reparadora do hospedeiro. Descobrimos no hospedeiro um mecanismo preventivo para lidar com a atividade fotossintética da zooxantela abrigada. Por outro lado, a perda da alga a partir do branqueamento pode fornecer uma oportunidade para o hospedeiro ser repopulado por outros simbiontes com diferentes capacidades fisiológicas. Assim, encontramos que existe uma troca de alga ideal para o frio (clado F), e para o calor (clado E). Entretanto, alguns clados podem estar indisponíveis no ambiente por serem termosensíveis em vida livre. Concluímos assim que, dentro dos cenários das mudanças climáticas globais, o mais vantajoso para o animal e para a alga é manter uma endopopulação mista e trocar a população simbiôntica predominante segundo as condições ambientais. Aliás, encontramos que o holobionte estudado está melhor preparado para condições de aquecimento que de esfriamento. Por outro lado, considerando que o peróxido de hidrogênio é um derivado de filtros solares e que ao mesmo tempo é um precursor do branqueamento, investigamos se o branqueamento da anêmona *Anemonia sulcata* produzido pela exposição a peróxido de hidrogênio é um bom modelo como bioindicador ambiental através da avaliação da cor e de biomarcadores fisiológicos. Porém, descartamos *A. sulcata* como espécie bioindicadora por apresentar uma alta tolerância ao estresse oxidativo, provavelmente fornecido pela atividade fotossintética da alga e por ser uma espécie intermareal que suporta grandes intervalos de oxido-redução. Desta forma, os resultados obtidos no presente estudo apontam a relevância do *Symbiodinium* dentro da fisiologia do holobionte, tanto na recuperação do branqueamento quanto no estabelecimento e ruptura da simbiose. Consequentemente, futuros estudos podem aprimorar as técnicas de identificação dos clados

hospedados para entender a eventual sucessão endopopulacional de acordo com as condições ambientais.

Abstract

Thermal bleaching is the main cause of simbiotic cnidarian decay in tropical oceans. However, some cnidarians have shown to recover the pigmentation from such events. The main aim of this study is the holistic approach of the physiological processes associated to zooxanthela loss and recovery after thermal stress in the holobiont *E. pallida*. Firstm we considered pigmentation recovey from the remaining zooxanthelae within the cnidarian host. We found a functional pattern of pigmentation recovery. In this context, we propose a mechanism of symbiosis break in which the endosymbionts participate actively on its release and dispersion to the environment, bringing a new discussion about the role of each symbiotic partner and the driver of bleaching and recovery. Since bleaching has been related to oxidative stress and photosynthesis is an oxidizing process, we evaluated the redox activity of the host as well as the antioxidant and restorative activity, induced by photosynthesis of the hosted algae. We found in the host a preventive mechanism to deal with the photosynthetic activity of the sheltered zooxanthella. On the other hand, loss of endosymbionts from bleaching may provide an opportunity for the host to be repopulated by other symbionts with different physiological capabilities. Thus, we found that there is an ideal clade exchange for the cold (clade F), and for the heat (clade E). However, some clades may not be available in the environment because they are thermosensitive in free-living form. We conclude that, within global climate change scenarios, the most beneficial for the animal and the alga is to maintain a mixed endopopulation and to exchange the predominant symbiotic population according to environmental conditions. In fact, we found that the studied holobiont is better prepared for high than low temperatures. On the other hand, considering that hydrogen peroxide is a derivative of sunscreens and at the same time is a precursor to bleaching, we investigated whether the bleaching of anemone *Anemonia sulcata* produced by exposure to hydrogen peroxide is a good model as an environmental bioindicator through color evaluation and physiological biomarkers. However, we discard *A. sulcata* as a bioindicator species because it presents a high tolerance to oxidative stress, probably provided by algae photosynthetic activity and because it is an intertidal species that supports large oxido-reduction intervals. Thus, the results obtained in the present study point to the relevance of *Symbiodinium* within holobiont physiology, both in the recovery of bleaching and in the establishment and rupture of symbiosis. Consequently, future studies may improve the identification techniques of the host clades to understand the eventual endopopulation succession according to the environmental conditions.

Anexo I

Exaiptasia pallida

Exaiptasia pallida is a widespread species from the tropics and subtropics. Its diagnosis is based on its Cnidom (Grajales and Rodríguez, 2014): spirocysts, basitrichs, microbasic b-mastigophores and p-amastigophores (Fig 1). Its external anatomy is characterized by a wide, regularly shaped pedal disc with an elongated and smooth column, with cinclides in 2–3 longitudinal rows in mid-column. Tentacles long and simple without projections. Six pairs of perfect mesenteries. Acontia well developed. Symbiotic with *Symbiodinium* spp. Asexual reproduction by pedal laceration. (Fig 2).

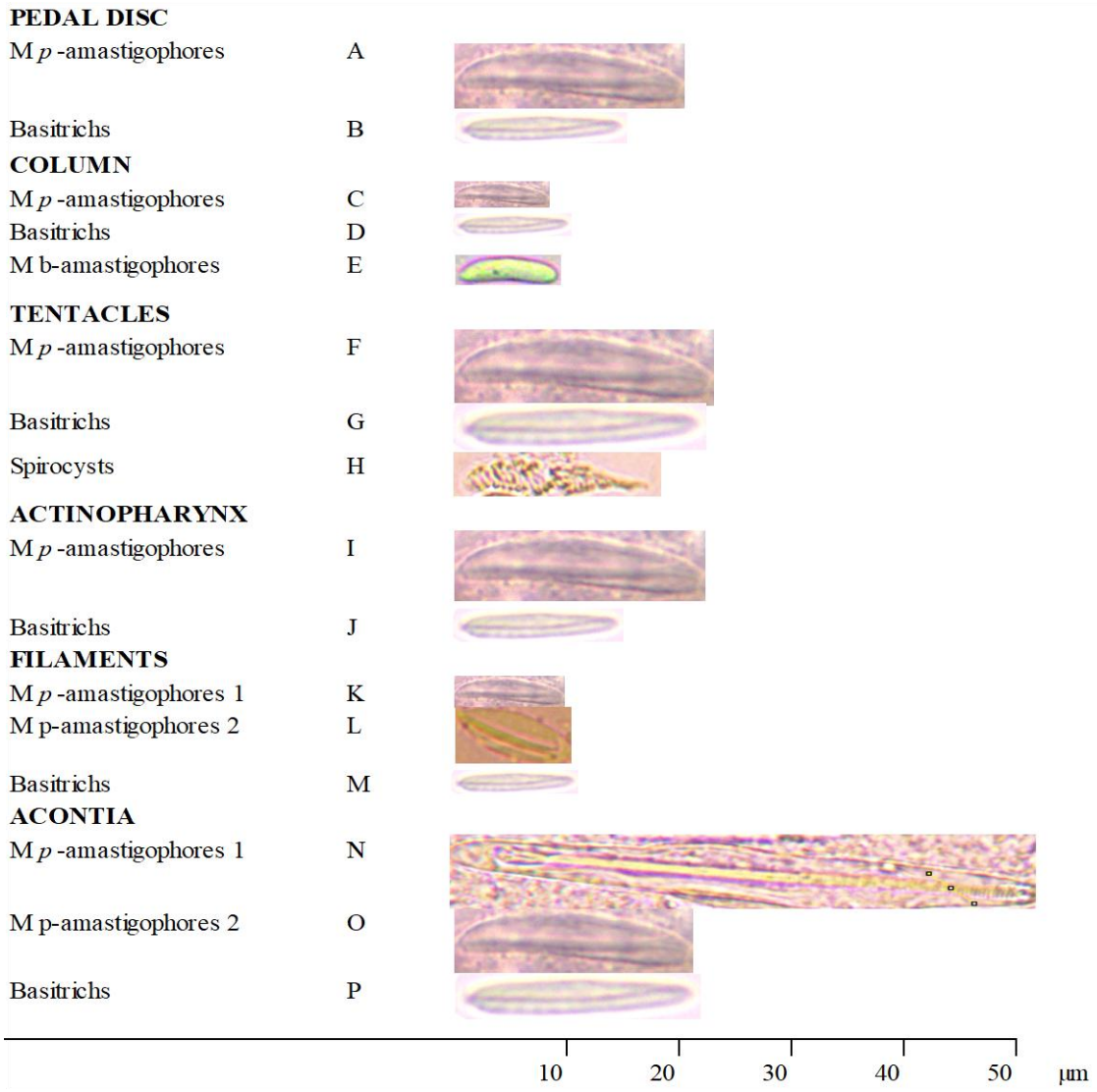


Fig 1. Cnidae plate showing the type, size and distribution of nematocists along the polyp of *Exaiptasia pallida*. (n = 6).

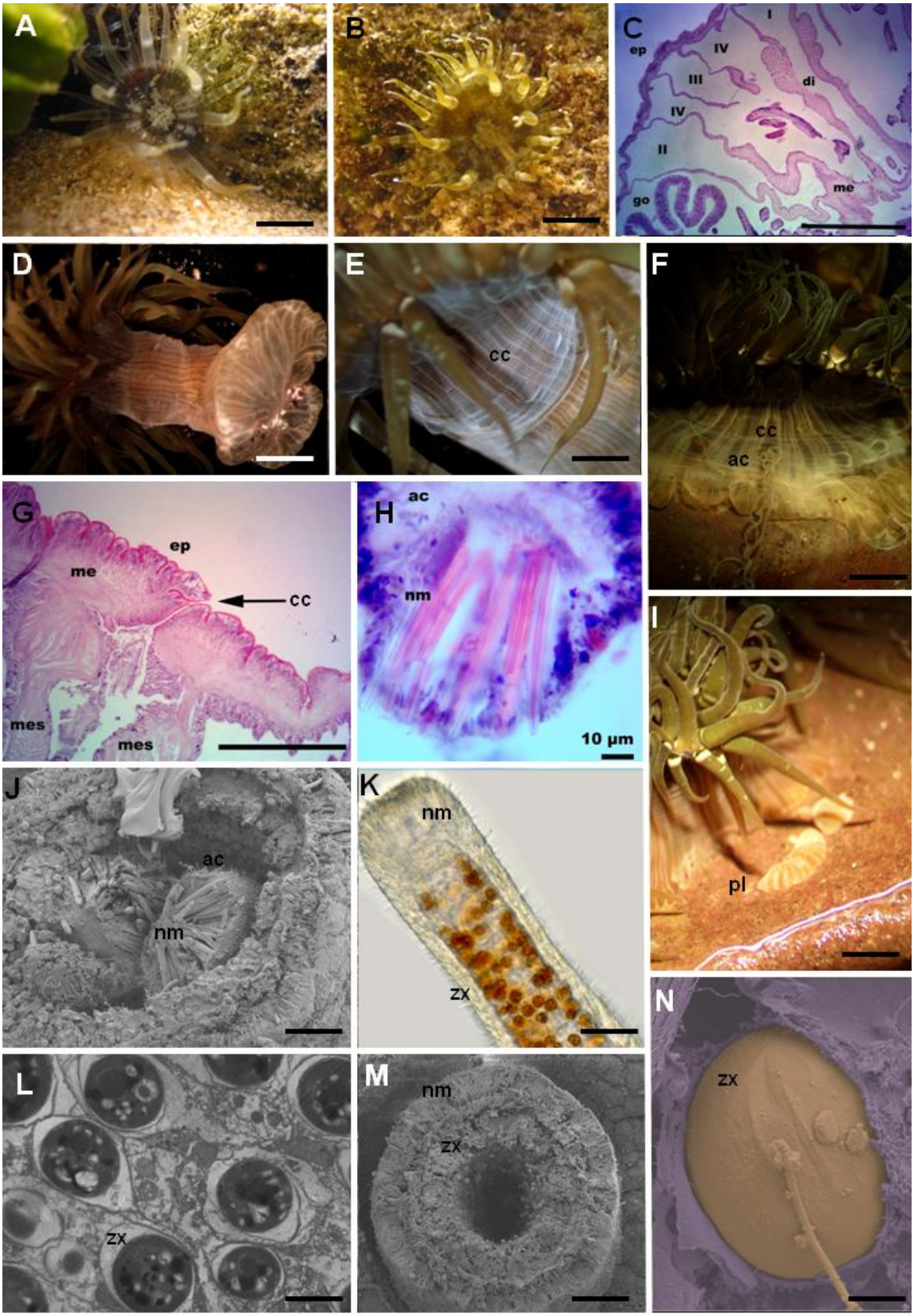


Fig 2. A) Oral view of living bleached *E. pallida* B) Oral view of living brown *E. pallida*. C) Transversal section of *E. pallida* from the polyps column showing the mesenterial cycles. D) Tranversal view showing the column and pedal disc of *E. pallida*. E) Cinclides (cc) in the middle region of the column of *E. pallida*. F) Acontia (ac) fired through the cinclide G) Transversal section of *E. pallida* from the polyps column showing the opening of the cinclide though the mesenterie (me) and epidermis (ep). H) Transversal sectioin of the acontia showing

the nematocists (nm). I) Asexual reproduction of *E. pallida* (pl). J) Transversal sectioin of the acontia showing the nematocists within the tentacle of of *E. pallida* K) the tentacle of of *E. pallida* showing the nematocists in the epidermis and the hosted zooxanthela in the endodermis. L) Hosted Symbiodinum within the tentacle of *E. pallida* M) Transversal section showing the nematocists in the epidermis and the hosted zooxanthela in the endodermis N) Hosted zooxanthela.

Table 1. Size and distribution of cnidae of *Exaiptasia pallida* (mean ± SE) (n = 6; N = 3).

		lenght (µm)	width (µm)
PEDAL DISC			
M p-amastigophores	A	20,96 ± 1,08	3,83 ± 0,10
Basitrichs	B	15,58 ± 3,23	2,29 ± 0,51
COLUMN			
M p-amastigophores	C	6,93 ± 0,84	1,46 ± 0,21
Basitrichs	D	5,97 ± 0,40	1,30 ± 0,14
M b-amastigophores	E	5,38 ± 0,57	1,10 ± 0,64
TENTACLES			
M p-amastigophores	F	25,85 ± 5,13	3,66 ± 0,25
Basitrichs	G	23,47 ± 0,37	3,78 ± 0,46
Spirocysts	H	16,91 ± 2,75	3,31 ± 0,73
ACTINOPHARYNX			
M p-amastigophores	I	25,85 ± 0,47	3,66 ± 0,93
Basitrichs	J	15,98 ± 1,27	2,10 ± 0,18
FILAMENTS			
M p-amastigophores 1	K	8,91 ± 2,05	2,35 ± 0,45
M p-amastigophores 2	L	9,97 ± 4,91	3,98 ± 0,27
Basitrichs	M	5,97 ± 0,40	1,30 ± 0,14
ACONTIA			
M p-amastigophores 1	N	52,55 ± 3,22	5,56 ± 0,34
M p-amastigophores 2	O	21,08 ± 2,51	2,31 ± 0,25
Basitrichs	P	21,28 ± 1,09	2,11 ± 0,47

Anexo II

Symbiodinium sp.

The clades were originally supplied by Mary Alice Coffroth from the Buffalo University to Miguel Mies of the IO with the code: A (Cass KB8 - A1), B (Ap04- B1), C (Mp -AF427469- C1), D (A001 - D1), (E - R1-383), F (Mv - AF360577).