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# Comparative Biochemistry and Physiology, Part A

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## Differential coral bleaching—Contrasting the activity and response of enzymatic antioxidants in symbiotic partners under thermal stress



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### ARTICLE INFO

#### Article history:

Received 22 April 2015

Received in revised form 13 August 2015

Accepted 19 August 2015

Available online 23 August 2015

#### Keywords:

APX

*Acropora millepora*

CAT

Global change

*Montipora digitata*

Oxidative stress

SOD

*Symbiodinium*

Symbiosis

Thermal biology

### ABSTRACT

Mass coral bleaching due to thermal stress represents a major threat to the integrity and functioning of coral reefs. Thermal thresholds vary, however, between corals, partly as a result of the specific type of endosymbiotic dinoflagellate (*Symbiodinium* sp.) they harbour. The production of reactive oxygen species (ROS) in corals under thermal and light stress has been recognised as one mechanism that can lead to cellular damage and the loss of their symbiont population (Oxidative Theory of Coral Bleaching). Here, we compared the response of symbiont and host enzymatic antioxidants in the coral species *Acropora millepora* and *Montipora digitata* at 28 °C and 33 °C. *A. millepora* at 33 °C showed a decrease in photochemical efficiency of photosystem II (PSII) and increase in maximum midday excitation pressure on PSII, with subsequent bleaching (declining photosynthetic pigment and symbiont density). *M. digitata* exhibited no bleaching response and photochemical changes in its symbionts were minor. The symbiont antioxidant enzymes superoxide dismutase, ascorbate peroxidase, and catalase peroxidase showed no significant upregulation to elevated temperatures in either coral, while only catalase was significantly elevated in both coral hosts at 33 °C. Increased host catalase activity in the susceptible coral after 5 days at 33 °C was independent of antioxidant responses in the symbiont and preceded significant declines in PSII photochemical efficiencies. This finding suggests a potential decoupling of host redox mechanisms from symbiont photophysiology and raises questions about the importance of symbiont-derived ROS in initiating coral bleaching.

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### 1. Introduction

Scleractinian corals are important ecosystem engineers building the three-dimensional coral reef framework that supports the immense biodiversity seen in tropical coastal waters. This ecological role is underpinned by the coral's endosymbiosis with dinoflagellates of the genus *Symbiodinium*, which supply carbon-rich photosynthates to the host coral, promote nitrogen recycling and conservation, and enhance

rates of calcification (Muscatine and Porter, 1977). The relationship between the animal and its algal symbionts is therefore a major determinant of individual coral health and ultimately reef function. The disruption of this symbiosis by environmental stress and disease has been termed coral bleaching, since it is phenotypically visible as paling of the coral tissue as a result of a decline in symbiont photosynthetic pigment content and/or cell density (Iglesias-Prieto and Trench, 1994). With the onset of anthropogenic marine pollution and climate change, reef systems worldwide have experienced major perturbations and regime shifts, as well as bleaching events that have led to significant coral mortality (Pandolfi et al., 2003). Today, thermal bleaching triggered by ocean warming is one of the main global challenges facing reef systems.

Thermal stress thresholds and subsequent mortality vary between coral species in the reef community (Loya et al., 2001; Stimson et al., 2002). This differential bleaching susceptibility is, to a certain extent, a result of the specific holobiont composition, i.e. the combination of

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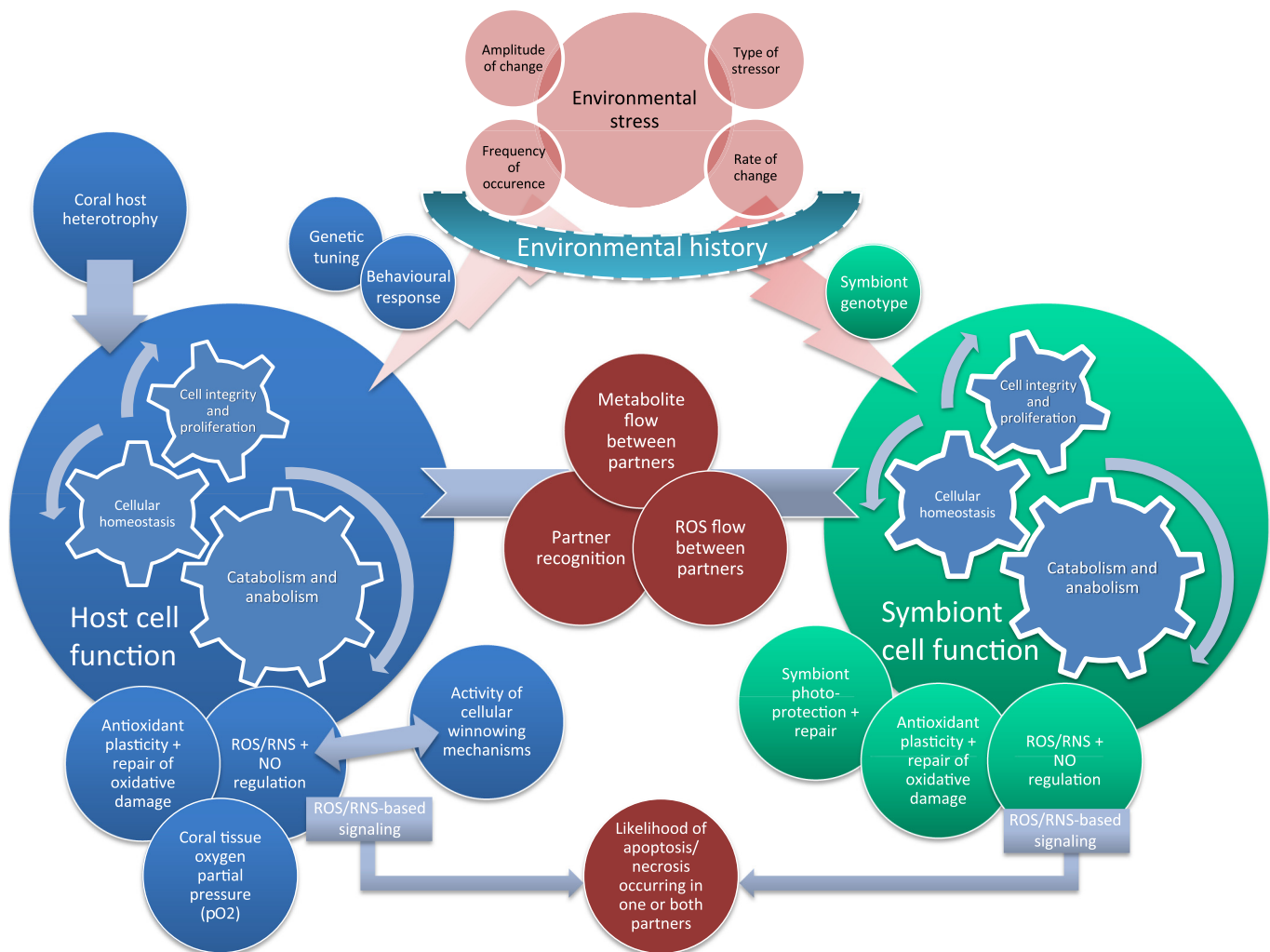
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different host species and one or multiple *Symbiodinium* genotypes (Rowan et al., 1997; Abrego et al., 2008; Sampayo et al., 2008). In addition, physiological acclimation and repeated stress exposure (Iglesias-Prieto and Trench, 1994; Middlebrook et al., 2008; Grottoli et al., 2014), host genetic background (Smith-Keune and van Oppen, 2006; Barshis et al., 2010), symbiont effects on host physiology (Rowan, 2004; Ulstrup et al., 2007; Abrego et al., 2008), and host heterotrophy during episodes of bleaching and recovery (Grottoli et al., 2004; Grottoli et al., 2006), are important factors that define the stability and resilience of the coral symbiosis (Fig. 1). However, the relative importance of different symbionts to the holobiont's resilience is difficult to assess due to complex interactions and trade-offs (Fitt et al., 2001; Abrego et al., 2008; Fitt et al., 2009; Jones and Berkelmans, 2010). Moreover, while some intracellular sites and effects of thermal damage in the host and symbiont have been identified, there is no consensus about the causal chain of events and the supposed 'weak link' in the symbiosis. Indeed, bleaching can be manifested as host-cell detachment and/or release of potentially photosynthetically competent symbionts (Gates et al., 1992; Ralph et al., 2001), as well as breakdown of photosynthetic dark and light reactions (Jones et al., 1998; Warner et al., 1999), and

apoptosis and necrosis in both partners (Dunn et al., 2004; Tchernov et al., 2011; Hawkins et al., 2013; Paxton et al., 2013).

One key process, first suggested by Lesser et al. (1990), is the leakage of reactive oxygen species (ROS) from the symbiont to the host, which then challenges and potentially overwhelms the host's antioxidant capacity under stressful conditions (Downs et al., 2002; Weis, 2008). In addition, high levels of ROS as well as reactive nitrogen species (RNS) in the host gastrodermal cell could stimulate innate immune-like pathways and potentially trigger pro-apoptotic processes in the host (Fig. 1, Perez and Weis, 2006; Dunn et al., 2007; Hawkins et al., 2013). The involvement of ROS in coral bleaching led to the proposal of a unifying mechanistic model of coral bleaching: the 'Oxidative Theory of Coral Bleaching' (OTB). This hypothesis proposes that exposure to temperature and light stress initiates the bleaching cascade primarily by compromising symbiont photosynthesis, leading to the excessive generation of ROS and RNS in the symbiont (reviewed in Weis, 2008). Potential ROS leakage into the host tissue in combination with damage and possible activation of apoptotic pathways in both partners results in the breakdown of the symbiotic relationship (reviewed in Weis, 2008; Lesser, 2011).



**Fig. 1.** The dynamics associated with coral health under stress. Conceptual diagram of key physiological elements that contribute to differential coral bleaching susceptibility under stress. Maintaining cellular functioning under stress is essential to the maintenance of the mutualistic association, because cellular stress directly influences the relationship between coral host and dinoflagellate symbionts with regard to partner recognition, metabolite flow, and ROS flow. Mechanisms related to repair and prevention of oxidative damage play a key role since they determine the threshold for oxidative stress-induced cell death (necrotic or apoptotic) in both partners and potentially also influence host-symbiont recognition. The particular bleaching pathology of the holobiont and the primary impact sites (e.g., host vs. symbiont) are dependent on the specific nature of the stressor and its properties (e.g., heating rate of thermal stress). The particular impact of stress is furthermore modulated by the environmental history of the holobiont as well as specific behavioural responses (e.g. host tissue retraction under high light stress) or genetic factors. This conceptual diagram is not intended to be complete—placing its primary focus on the role of oxidative stress-related mechanisms and their connection to other physiological processes. ROS, reactive oxygen species; RNS reactive nitrogen species; NO nitric oxide.

A large body of literature reports increased antioxidant activity or evidence for oxidative damage as a result of thermal and light stress in both the symbiont and host, supporting the idea that oxidative stress is the trigger of coral bleaching (reviewed by Lesser, 2006; Lesser, 2011). To what extent photoprotective mechanisms in the symbiont and antioxidant capacities in both partners contribute to the overall bleaching susceptibility of the holobiont is therefore an important question that needs to be answered if we are to understand the physiological basis of differential bleaching susceptibilities. In the context of the OTB, differential bleaching susceptibility between holobionts should be related to mechanisms that prevent or combat the excessive generation of ROS under thermal stress (Downs et al., 2002). The goal of this study was to contrast the response of major enzymatic antioxidants (ascorbate peroxidase, catalase, catalase peroxidase, and superoxide dismutase) in both partners under thermal stress in a bleaching susceptible (*Acropora millepora*) and tolerant coral (*Montipora digitata*), and correlate these processes to the phenotypic bleaching response. Both corals are known to harbour different symbiont types (ITS2-types C3 vs. C15) and previous work has established the varying thermal susceptibility for a number of Indo-Pacific corals harbouring these types at the chosen study site (Fisher et al., 2012). Our hypothesis was, following the OTB, that (a) antioxidant defences in *Symbiodinium* will increase in response to elevated temperature and declining photosynthetic performance, followed by (b) an increase in antioxidant defences of the coral host with (c) the occurrence of bleaching in the susceptible coral at high temperatures as a result of (a) and (b). For the tolerant coral *M. digitata* (with *Symbiodinium* type C15), previous work has shown that photoprotective measures might be a key element of thermal tolerance (Fisher et al., 2012), so we hypothesised that no significant antioxidant response would occur in either the symbiont or host, consistent with the OTB that identifies the symbiont as the origin of excessive ROS production under thermal stress.

## 2. Material and methods

### 2.1. Experimental setup and sample processing

Fragments of the scleractinian corals *Acropora millepora* (cream morph) and *Montipora digitata* (green morph) were collected from five different colonies per species ( $N = 5$ ) on the Heron Island reef flat (2–4 m depth) in Feb 2012 (Heron Island Research Station, HIRS, 23°33'S, 151°54'E) under the GBR Marine Park permit G12/34800.1. Each colony provided coral explants that were haphazardly allocated to two 60 L flow-through seawater tanks ( $1.3\text{--}1.5\text{ L min}^{-1}$ ), maintained under shade cloth, exposing the corals to ~25% of the natural irradiance throughout the experiment (i.e., ~400–600  $\mu\text{mol quanta m}^{-2}\text{ s}^{-1}$  on a clear sunny day at noon; LI-COR Quantum light meter LI-189 with cosine sensor, LI-COR Inc., Lincoln, NE, USA). Since all of the prepared explants were thumb-sized (minimising self-shading effects of large branching colonies) and all explants were maintained towards the centre of the tank throughout the experiment, we assume that all explants experienced a homogenous light field. Even though our experiment was conducted in only two tanks, a high tank-volume-to-coral-biomass-ratio with a high water flow minimised any effects of pseudoreplication, while ensuring identical treatment conditions to all explants (*sensu* Brown et al., 2002a).

Corals were acclimated to experimental light conditions for 9 days prior to temperature ramping. Ambient photosynthetically active radiation (PAR) conditions during the experimental period were recorded with an ODYSSEY PAR cosine recorder (DATAFLOW Systems Pty Ltd., Christchurch, New Zealand). Half of the explants were used for analysis of host cellular and apoptotic signalling (Hawkins et al., 2014), whereas the other half were used to assess the activity of enzymatic antioxidants (this study). After sampling on Day 0, temperature in one of the tanks was increased by  $1\text{ }^{\circ}\text{C day}^{-1}$  to a final average temperature of  $33\text{ }^{\circ}\text{C}$  ( $\pm 0.8\text{ }^{\circ}\text{C}$ ), while the minimal temperature of the control tank was set

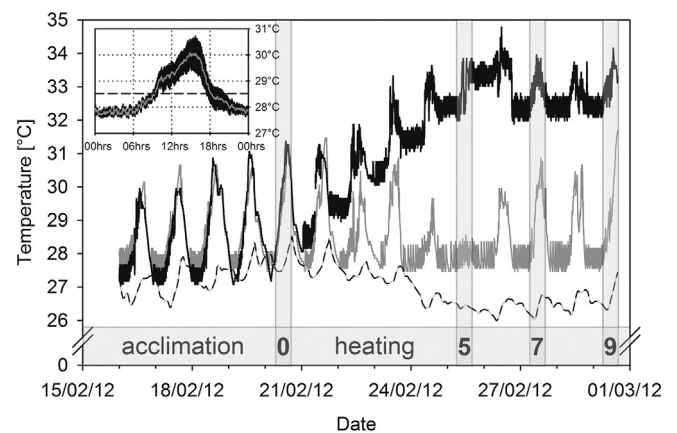
to  $27.5\text{ }^{\circ}\text{C}$ , based on long-term seawater temperature data for the Heron Island reef flat for February (AIMS, 2013). Both tanks expressed a diel temperature cycle that was influenced by tide cycles and solar heating (Fig. 2). Mean light intensities and mean temperature in the control tank were highly correlated during acclimation and the experimental phase (Pearson correlation,  $r = 0.89$ ,  $p < 0.0001$ ,  $N = 14$ ). Average control temperature over the experimental period was  $28.5\text{ }^{\circ}\text{C}$  ( $\pm 0.9\text{ }^{\circ}\text{C}$ ). Sampling occurred immediately after noon on Days 0, 5, 7, and 9, and explants were flash frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  until analysis.

### 2.2. Photobiological variables and sample processing

Effective and maximum quantum yields of algal PSII ( $\Delta F/F_m$ ,  $F_v/F_m$ , respectively), measured using pulse amplitude modulation fluorometry (MI: 9–11, SI: 8, saturation width: 0.8 s, Gain up to 10 for bleached corals, Damp: 2–3; Diving-PAM, Heinz Walz GmbH, Effeltrich, Germany), were monitored on five replicate explants per species and treatment throughout the experiment. Data were recorded daily at noon and 30 min after sunset, with the fibre optic positioned at the same spot on the same explant at each time point. Maximum midday excitation pressure on photosystem II ( $Q_m$ ), hereafter referred to as light pressure, was calculated as  $Q_m = 1 - [(\Delta F/F_m \text{ at noon}) / (F_v/F_m \text{ at dusk on Day 0})]$  (as defined by Fisher et al., 2012).

All samples were processed in a  $4\text{ }^{\circ}\text{C}$  environment, and samples maintained on ice. The whole surface of coral explants was brushed with a water pik using 8 mL lysis buffer (50 mM phosphate, 0.1 mM EDTA, 10% [v/v] glycerol, pH 7.0), and three 500  $\mu\text{L}$  aliquots for chlorophyll *a* (Chl *a*) analysis, symbiont genotyping and cell density determination were taken. Following initial centrifugation ( $2,000 \times g$ , 5 min) to pellet the remaining *Symbiodinium* cells, the supernatant containing the coral tissue was centrifuged again ( $16,000 \times g$ , 5 min) to remove any particulate matter, aliquoted, flash frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$ . The *Symbiodinium* pellets for antioxidant analysis were flash frozen and stored at  $-80\text{ }^{\circ}\text{C}$ . Lysis of *Symbiodinium* pellets after four wash cycles ( $2,000 \times g$ , 5 min) was performed in lysis buffer using 200 mg glass beads (710–1180  $\mu\text{m}$ ; Sigma-Aldrich) in a bead mill (50 Hz, 3 min,  $4\text{ }^{\circ}\text{C}$ , Qiagen tissue lyser, Qiagen N.V., Hilden, Germany). All cell lysates were centrifuged ( $16,000 \times g$ , 5 min), and supernatants aliquoted and frozen at  $-80\text{ }^{\circ}\text{C}$ .

Symbiont cell densities were quantified via haemocytometer counts and normalised to coral explant surface area, which was determined via the wax-dipping method (Stimson and Kinzie, 1991). Symbiont ITS2



**Fig. 2.** Experimental temperature profile. Temperature profile of experimental treatments at  $28\text{ }^{\circ}\text{C}$  (grey) and  $33\text{ }^{\circ}\text{C}$  (black) in a flow-through tank system during acclimation (prior Day 0) and the experimental period (sampling on Days 0, 5, 7, and 9; time-points highlighted by shading). Ambient water temperature at 2.04 m water depth [Heron Island relay pole 3 (AIMS, 2013)] depicted as black-grey dashed line. Average daily temperature variation in the control tank over the whole period is shown as graph insert (mean  $\pm$  95% CI,  $N = 3,937$ , with overall mean of  $28.5\text{ }^{\circ}\text{C}$  shown as dashed line).

identity was determined after previously published methods (Logan et al., 2010). Briefly, ITS2 rDNA regions were by PCR-amplified using the forward primer itsD (5'-GTG AAT TGC AGA ACT CCG TG-3') and reverse primer its2rev2 (5'-CCT CCG CCT ACT TAT ATG CTT-3'). PCR amplicons were visualised via gel electrophoresis to check for correct amplicon size and non-specific amplifications. Successful amplifications were purified using ExoSAP-IT (Global Science) and sequenced in both directions by Macrogen Inc. (Seoul, South Korea). ITS genotypes were identified by comparison against the Geosymbio database (Franklin et al., 2012). Chlorophyll was extracted over 48 h in 1–2 mL N, N-dimethylformamide at 4 °C in the dark. After centrifugation (5 min, 3,900 × g, 4 °C), 3 × 200 µL of the supernatant were spectrophotometrically measured at 646.8 nm, 663.8 nm, and 750 nm in 96-well plates (UVStar, Greiner Bio-One GmbH, Germany). Chl *a* concentrations were determined after optical path length correction (0.555 cm), using the equations of Porra et al. (1989).

### 2.3. Biochemical analysis

Host and *Symbiodinium* samples were analysed for superoxide dismutase (SOD), catalase (CAT; host), catalase peroxidase (KatG; symbiont only), and ascorbate peroxidase (APX) activity, since these enzymes represent the main enzymatic pathways for superoxide and hydrogen peroxide detoxification.

#### 2.3.1. Superoxide dismutase

SOD assays were performed using the riboflavin/nitroblue tetrazolium (RF/NBT) assay in a microtiter plate format (Beauchamp and Fridovich, 1971; Fryer et al., 1998). Lysate or SOD standard (20 µL) was measured in a final reaction mixture of 300 µL potassium phosphate buffer (50 mM, pH 7.8) containing EDTA (0.1 mM), riboflavin (1.3 µM), L-methionine (10 mM), NBT (57 µM), and Triton X-100 (0.025% (v/v)). Absorbance was read at 560 nm both immediately and after 10 min incubation under a homogeneous light field (130 µmol quanta m<sup>-2</sup> s<sup>-1</sup>) at 25 °C using a microtiter plate reader (Biochrom Anthos 2010 Microplate Reader, Biochrom Ltd., Cambridge, UK). Standards and samples were measured using the same reaction mixture and a sigmoidal 5-parameter semi-logarithmic standard curve (24 standard levels) was used to determine SOD activity of samples. One unit of SOD activity inhibited the NBT reduction by 50%.

#### 2.3.2. Ascorbate peroxidase

APX activity was assessed by monitoring the oxidation of ascorbate at 290 nm over 3 min at 25 °C (UV-Vis Spectrophotometer UV-2550, Shimadzu Corp., Japan), using 100 µL lysate in a final reaction mixture of 700 µL potassium phosphate buffer (50 mM, pH 7.0), EDTA (0.1 mM), ascorbate (0.3 mM) and hydrogen peroxide (0.1 mM) (Nakano and Asada, 1981). Activity was determined with  $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ .

#### 2.3.3. Catalase and catalase peroxidase

CAT and KatG activity were determined spectrophotometrically using 100 µL lysate (1:10 diluted for host samples) in a total reaction volume of 700 µL, containing potassium phosphate buffer (50 mM, pH 7.0), EDTA (0.1 mM), and hydrogen peroxide (14 mM). The reaction was monitored for 3 min at 240 nm and 25 °C in quartz cuvettes and activities calculated using an extinction coefficient of 43.6 M<sup>-1</sup> cm<sup>-1</sup> (Beers and Sizer, 1952).

Total soluble protein was determined using the improved Bradford method with BSA as protein standard (Zor and Selinger, 1996). After checking that protein per cell (for *Symbiodinium*) and protein per unit surface area (for host) were not affected by treatments, all enzyme activities were expressed as specific activities (U mg<sup>-1</sup> protein). One enzyme unit is defined as the amount of enzyme that catalyses 1 µmol of superoxide (SOD) or hydrogen peroxide (APX, CAT, KatG) per minute.

### 2.4. Statistical analyses

Baseline physiological variables at the beginning of the experiment (Day 0) were compared using ANOVA, after checking for normality (Shapiro–Wilk test) and equal variance (Levene test).  $F_v/F_m$  and  $Q_m$  results were arcsine square root and Log<sub>10</sub> ( $Q_m + 0.2$ ) transformed, respectively. In case of unequal variances, Welch ANOVA results are reported. Responses of bleaching proxies, photobiology and antioxidants over time were analysed with a repeated measures ANOVA (rmANOVA) with the fixed factors 'species' and 'temperature' and their interaction 'species × temperature'. In case the interaction term was not significant for between- or within-subject effects, it was removed to obtain a minimal adequate model. Results of Pillai's trace test, or where Mauchly's sphericity test was significant, epsilon-adjusted univariate F-test (Greenhouse–Geisser; G-G) are reported. To prevent the loss of the whole replicate data series for the rmANOVA, enzyme activities for missing intermediate replicate points from two colonies (*A. millepora*: 28 °C, Day 5, symbiont data and Day 7, host and symbiont data) were estimated by extrapolating from measurements of the previous and following sampling points. The linear extrapolation located the estimated data points within the distribution of the remaining four replicates. Thus, we conclude that this extrapolation had no effect on the statistical outcome of the analysis. Analysis was conducted with SAS JMP 10.0.0 (SAS Institute Inc., Cary, NC, USA).

## 3. Results

### 3.1. Symbiont identity, photobiological responses, and bleaching

Symbionts in all colonies were identified as *Symbiodinium* ITS2 types C3 (*A. millepora*) and C15 (*M. digitata*), respectively. Both corals exhibited significant differences in their total Chl *a* content at the beginning of the experiment, which was primarily driven by a two-fold higher Chl *a* content per symbiont cell in the *Symbiodinium* C15 population in *M. digitata* rather than by differing symbiont densities (Table 1).

High temperatures elicited significantly different photophysiological responses ( $F_v/F_m$  and  $Q_m$ ) in the two coral species (Table 2, Fig. 3a–d). In *A. millepora* at 33 °C, maximum quantum yield dropped considerably after Day 5, reaching 49% of the control value on Day 8. In the same period,  $Q_m$  increased to 0.75–0.8, whereas in *M. digitata*, declines in  $F_v/F_m$  at 33 °C were minor (94% of the control value on Day 8) with  $Q_m$  reaching a maximum value of 0.2.

*A. millepora*, but not *M. digitata*, visibly bleached at 33 °C. Coral mortality (tissue sloughing and visible algal overgrowth of skeleton) in *A. millepora* was first detected on Day 7 (1 out of 5 replicates) and increased to 60% (3 of 5) on Day 9. Thus, comparative statistics between both corals was only possible until Day 7. The negative effect of high temperature on holobiont Chl *a* content over time was species-specific to *A. millepora*, and declining values after Day 5 were a cumulative effect of the loss of symbiont cells and pigment content per symbiont (Table 2, Fig. 3e, Fig. S1). By Day 7, the four remaining *A. millepora* explants had lost ~70% of their Chl *a* content. In contrast, after 9 days at 33 °C, holobiont Chl *a* content of *M. digitata* was at control levels.

### 3.2. Antioxidant features

Differences in baseline antioxidant enzyme activities between both coral species were detected for symbiont SOD, APX, and KatG, with *Symbiodinium* C3 in *A. millepora* having consistently higher activities than *Symbiodinium* C15 in *M. digitata* at 28 °C (up to 10-fold for APX). Host activities were only significantly different for CAT, where activity of *A. millepora* corresponded to ~72% of the activity in *M. digitata* (Table 1). Comparison between compartments of the coral symbiosis indicated that baseline activity of hydrogen peroxide scavenging via CAT/KatG was consistently higher in the host than the symbionts. Activities were higher by a factor of ~5 in *A. millepora* and ~23 in *M. digitata*.

**Table 1**

Baseline physiological properties of *Acropora millepora* and *Montipora digitata*, harbouring *Symbiodinium* ITS2 types C3 and C15, respectively, at 28 °C on Day 0. Shown are means ± SE (N = 5). Significant differences between both corals at  $p \leq 0.05$  based on ANOVA (\*) or Welch ANOVA (†) are indicated. Symbiont density in  $10^6$  cells/cm<sup>2</sup>, symbiont Chl *a* content in pg cell<sup>-1</sup>, holobiont Chl *a* content in  $\mu\text{g cm}^{-2}$  and enzyme activities in U mg<sup>-1</sup> protein.

	<i>Acropora millepora</i> (C3)	<i>Montipora digitata</i> (C15)	F-statistics
F <sub>v</sub> /F <sub>m</sub>	0.670 ± 0.005	0.646 ± 0.003	F <sub>1,8</sub> = 13.930, p = 0.0058*
Symbiont density	1.76 ± 0.25	1.99 ± 0.28	F <sub>1,8</sub> = 0.353, p = 0.5690
Symbiont Chl <i>a</i> content	1.85 ± 0.11	4.05 ± 0.12	F <sub>1,8</sub> = 174.494, p < 0.0001*
Holobiont Chl <i>a</i> content	3.11 ± 0.38	7.84 ± 0.68	F <sub>1,8</sub> = 37.495, p = 0.0003*
Host SOD	17.26 ± 1.60	15.63 ± 1.60	F <sub>1,8</sub> = 0.517, p = 0.4928
Host CAT	118.15 ± 12.52	164.02 ± 10.60	F <sub>1,8</sub> = 7.821, p = 0.0233*
Symbiont SOD	78.23 ± 6.01	50.41 ± 6.91	F <sub>1,8</sub> = 9.228, p = 0.0161*
Symbiont APX	1.41 ± 0.04	0.13 ± 0.01	F <sub>1,4.935</sub> = 862.112, p < 0.0001†
Symbiont KatG	24.21 ± 0.55	7.32 ± 1.26	F <sub>1,5.448</sub> = 150.251, p < 0.0001†

Baseline SOD activity on the other hand was 3–5 times higher in the symbiont than the host (Fig. 4).

Significant changes over the course of the experiment were observed for hydrogen peroxide scavenging by symbiont KatG and host CAT, independent of species (Table 2, Fig. 4, 5). An influence of temperature, however, was only detected for host CAT and this effect was also independent of species (Table 2). In *A. millepora* at 33 °C, host CAT activity reached ~190 U mg<sup>-1</sup> on Day 5 and remained at this level until the end of the experiment (20–43% above control, Fig. 5c). CAT activity in *M. digitata* at 33 °C was, on average, 23–46% above the control values between Days 5 and 9 (Fig. 5d). Due to the high degree of mortality in *A. millepora* at 33 °C, data for statistical analysis are limited to Days 0–7. Nevertheless, it should be highlighted that the two surviving colonies of *A. millepora* on Day 9 were characterised by a very low Chl *a* content (13% of control values). The symbiont population in these two colonies expressed twice the KatG activity (+105%) and very low APX activities (–80%) relative to the control.

A number of significant correlations in enzyme activity in both symbiotic compartments were found (Table 3). Under ambient conditions, there was a significant positive correlation within the SOD-APX pathway in the symbionts of *A. millepora*, while symbiont KatG activity

was negatively correlated with the activity of this pathway. SOD activities in the symbiont and host compartment were positively correlated in *A. millepora* at 28 °C. The increase in host CAT activity at 33 °C in *A. millepora* was negatively correlated with SOD activity in host and symbiont. In *M. digitata* under ambient conditions, activities of APX and KatG in the symbiont were positively correlated with CAT in the host compartment, but this correlation was not significant at high temperatures. Only symbiont SOD and APX activity were positively correlated at 33 °C.

Although introducing additional uncontrolled variability into the experimental design, the varying mean light levels over the course of our experiment (Fig. 3c, d) allowed an assessment of the light dependence of the antioxidant enzyme activity under ambient conditions. Daily mean light level and antioxidant enzyme activity were correlated only in the symbionts of *A. millepora*, where SOD and APX activity were positively affected by increasing light intensities (Table 3).

#### 4. Discussion

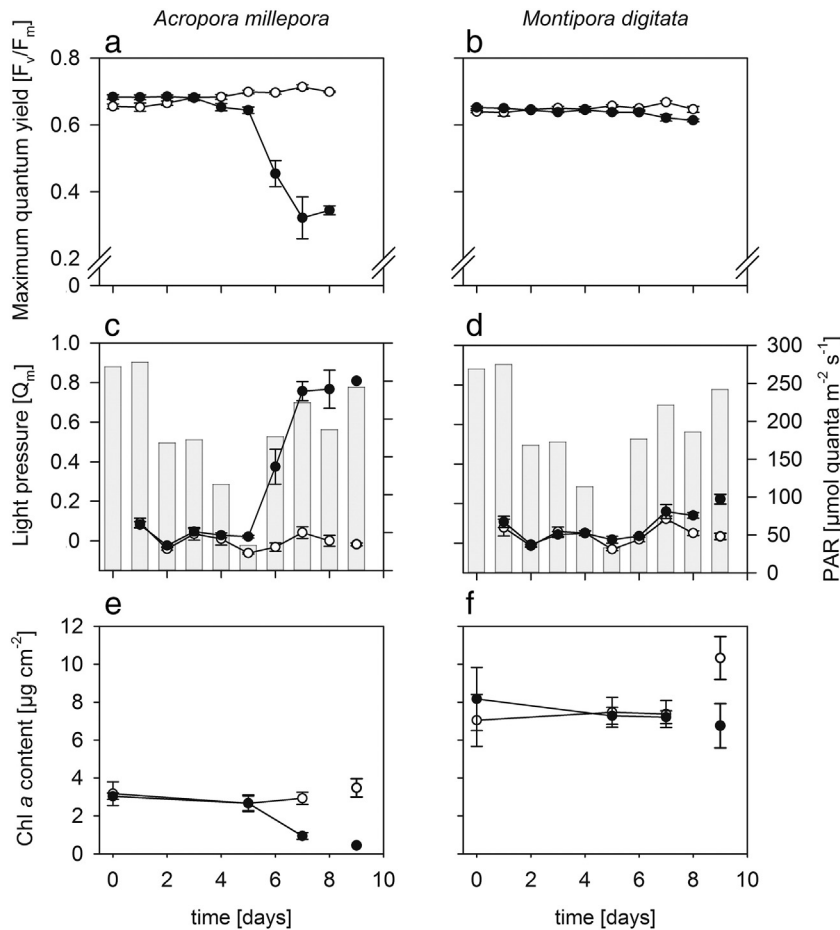
This study contrasted the temperature response of two common Indo-Pacific corals, known to possess different bleaching thresholds,

**Table 2**

Statistical rmANOVA result for bleaching proxies, photobiological and antioxidant variables over available timeframes of 8 days (F<sub>v</sub>/F<sub>m</sub>, Q<sub>m</sub>) and 7 days (all others). Significance at  $p \leq 0.05$  is indicated by an asterisk. N/A indicates a non-significant term in the full factorial model that was removed for the analysis in order to obtain the shown statistical values of the minimal adequate model. Symbiont density in cells cm<sup>-2</sup>, symbiont Chl *a* in pg cell<sup>-1</sup>, holobiont Chl *a* content in  $\mu\text{g cm}^{-2}$ , and enzyme activities are in U mg<sup>-1</sup> protein.

Variable	Between-subject effects				Within-subject effects		
	Species	Temperature	Species × temperature	Time	Time × species	Time × temperature	Time × species × temperature
F <sub>v</sub> /F <sub>m</sub>	F <sub>1,14</sub> = 20.568, p = 0.0005*	F <sub>1,14</sub> = 136.145, p < 0.0001*	F <sub>1,14</sub> = 94.792, p < 0.0001*	F <sub>1,736, 24.308</sub> = 8.368, p = 0.0025*	F <sub>1,736, 24.308</sub> = 1.736, p = 0.1683	F <sub>1,736, 24.308</sub> = 185.076, p < 0.0001*	F <sub>1,736, 24.308</sub> = 129.710, p < 0.0001*
Q <sub>m</sub>	F <sub>1,14</sub> = 6.128, p = 0.0267*	F <sub>1,14</sub> = 41.002, p < 0.0001*	F <sub>1,14</sub> = 20.890, p = 0.0004*	F <sub>7,8</sub> = 28.770, p < 0.0001*	F <sub>7,8</sub> = 0.900, p = 0.5485	F <sub>7,8</sub> = 15.198, p = 0.0005*	F <sub>7,8</sub> = 9.415, p = 0.0026*
Symbiont density	F <sub>1,15</sub> = 4.597, p = 0.0488*	F <sub>1,15</sub> = 0.662, p = 0.4287	F <sub>1,15</sub> = 0.055, p = 0.8185	F <sub>2,14</sub> = 1.483, p = 0.2605	F <sub>2,14</sub> = 1.996, p = 0.1727	F <sub>2,14</sub> = 0.806, p = 0.4663	F <sub>2,14</sub> = 4.4054, p = 0.0328*
Symbiont Chl <i>a</i> content	F <sub>1,15</sub> = 72.899, p < 0.0001*	F <sub>1,15</sub> = 0.107, p = 0.7486	F <sub>1,15</sub> = 6.689, p = 0.0207*	F <sub>2,14</sub> = 4.736, p = 0.0269*	F <sub>2,14</sub> = 2.752, p = 0.0982	F <sub>2,14</sub> = 7.142, p = 0.0073*	F <sub>2,14</sub> = 3.078, p = 0.0780
Holobiont Chl <i>a</i> content	F <sub>1,15</sub> = 39.842, p < 0.0001*	F <sub>1,15</sub> = 2.650, p = 0.1244	F <sub>1,15</sub> = 5.441, p = 0.0340*	F <sub>1,439, 21.589</sub> = 0.1158, p = 0.8250	F <sub>1,439, 21.589</sub> = 0.732, p = 0.4496	F <sub>1,439, 21.589</sub> = 8.132, p = 0.0048*	F <sub>1,439, 21.589</sub> = 5.137, p = 0.0231*
Host SOD	F <sub>1,16</sub> = 0.001, p = 0.9818	F <sub>1,16</sub> = 0.521, p = 0.4810	N/A	F <sub>2,15</sub> = 0.654, p = 0.5340	F <sub>2,15</sub> = 0.795, p = 0.4698	F <sub>2,15</sub> = 0.707, p = 0.5090	N/A
Host CAT	F <sub>1,16</sub> = 44.655, p < 0.0001*	F <sub>1,16</sub> = 10.852, p = 0.0046*	N/A	F <sub>2,15</sub> = 9.277, p = 0.0024*	F <sub>2,15</sub> = 2.732, p = 0.0973	F <sub>2,15</sub> = 4.312, p = 0.0332*	N/A
Symbiont SOD	F <sub>1,16</sub> = 1.752, p = 0.2042	F <sub>1,16</sub> = 7.260, p = 0.0160*	N/A	F <sub>1,403, 22.452</sub> = 0.150, p = 0.7855	F <sub>1,403, 22.452</sub> = 5.549, p = 0.0186*	F <sub>1,403, 22.452</sub> = 0.470, p = 0.5638	N/A
Symbiont APX	F <sub>1,15</sub> = 375.748, p < 0.0001*	F <sub>1,15</sub> = 0.010, p = 0.9210	F <sub>1,15</sub> = 6.276, p = 0.0243*	F <sub>1,433, 21.488</sub> = 0.063, p = 0.8849	F <sub>1,433, 21.488</sub> = 10.229, p = 0.0019*	F <sub>1,433, 21.488</sub> = 0.529, p = 0.5381	F <sub>1,433, 21.488</sub> = 0.027, p = 0.9361
Symbiont KatG	F <sub>1,16</sub> = 120.166, p < 0.0001*	F <sub>1,16</sub> = 1.972, p = 0.1794	N/A	F <sub>2,15</sub> = 7.623, p = 0.0052*	F <sub>2,15</sub> = 0.471, p = 0.6335	F <sub>2,15</sub> = 2.5687, p = 0.1098	N/A

\*  $p \leq 0.05$



**Fig. 3.** Coral photobiology. Overview of photobiological variables for *Acropora millepora* and *Montipora digitata* at 28 °C (white) and 33 °C (black), over 9 days of treatment. Shown are dark adapted quantum yields of PSII ( $F_v/F_m$ , a, b), light pressure ( $Q_m$ , c, d, circles, left axis), daily mean photosynthetically active radiation (PAR) under shading cloth during daylight hours (c, d, bars, right axis), and chlorophyll *a* content of the coral holobiont (e, f). Values are mean  $\pm$  SE,  $N = 5$ , except for Days 8, 9 in *A. millepora* at 33 °C ( $N = 3, 2$ ). Range of interpolation indicates timeframe used for the statistical analysis (Table 2).

with a particular focus on the regulation of antioxidant enzymes in the symbiont and host compartments. When exposed to elevated temperatures, bleaching and photophysiological stress was evident in the thermally susceptible coral *Acropora millepora*. However, no significant upregulation of enzymatic antioxidant defences in its symbionts was observed at least until after the onset of bleaching (and host mortality in some replicates). Independent of bleaching susceptibility, increased scavenging of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in the host compartment at high temperatures was observed in both coral species, and this preceded apparent algal PSII dysfunction and overall bleaching in *A. millepora*.

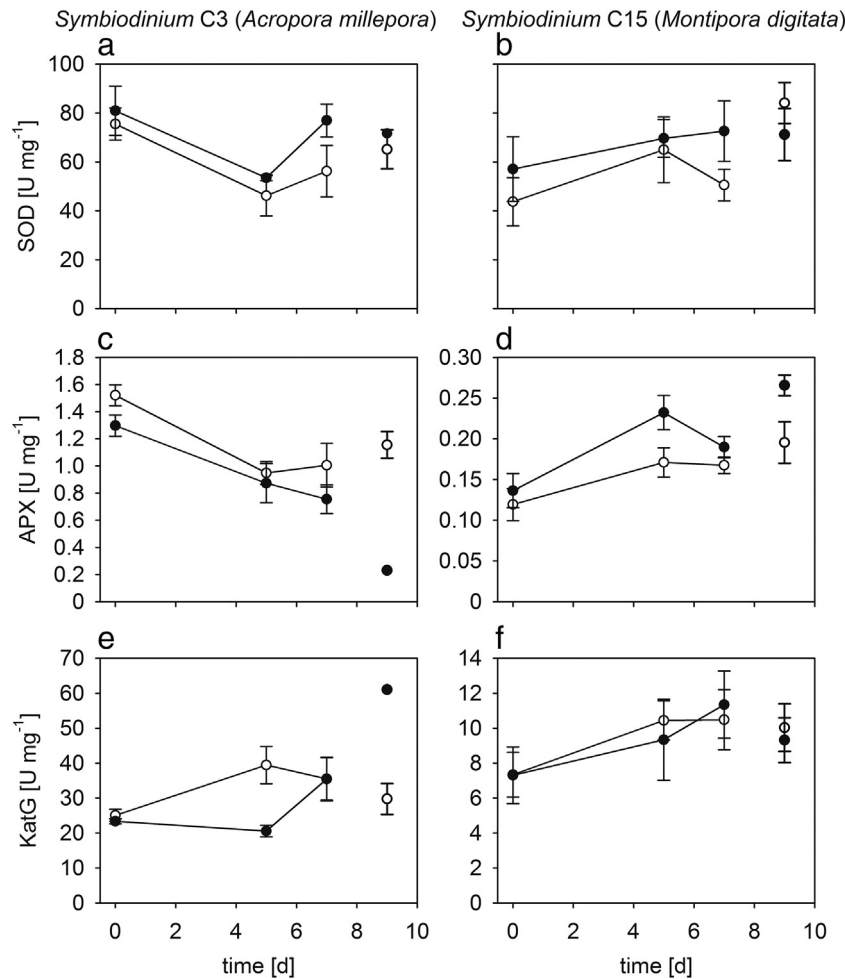
#### 4.1. Oxidative stress as a light and temperature response in host and symbiont

A visual bleaching response in *A. millepora* was not apparent until at least after 7 days of exposure to heating. Substantial changes in the photobiological parameters  $F_v/F_m$  and  $Q_m$  coincided with this bleaching response, an observation that is consistent with previous findings (Hoegh-Guldberg and Jones, 1999; Warner et al., 1999; Ferrier-Pagès et al., 2007). High values of  $Q_m$  show that, under full daylight, some of the PSII reaction centres were inactivated, indicating dynamic or chronic photoinhibition (Maxwell and Johnson, 2000). However, for advanced bleaching states (Day 9), increases in  $Q_m$  might also be related to a brighter light environment for the remaining symbionts, as a result of a decrease in light absorption and increase in light scattering by the coral skeleton (Enríquez et al., 2005). In contrast to *A. millepora*, *Montipora digitata* was thermally robust and moderate reductions in

$F_v/F_m$  and increases in  $Q_m$  suggest that the coral was within an acceptable range for its protective or photoacclimatory mechanisms, e.g., thermal dissipation of light energy (Gorbunov et al., 2001; Fisher et al., 2012).

While not actively manipulated in the experiment, changing ambient irradiance was positively correlated with the activity of the SOD-APX pathway in the symbionts of *A. millepora*, but not in *M. digitata*, at 28 °C. This pathway is commonly associated with photosynthesis as part of the water–water–cycle (Asada, 1999) and the distinct correlative response is most likely a result of different photosynthetic rates in the two coral species and the generation of superoxide as a side-product (e.g. Mehler reaction) in *A. millepora*. A stronger influence of light in *A. millepora* as result of a brighter symbiont microenvironment in response to less overall pigmentation (e.g., significantly lower holobiont Chl *a* content) would be consistent with the observations of higher symbiont baseline activities in *Symbiodinium* C3 and the marked drop in symbiont SOD and APX activity between Days 0 and 5 that coincided with decreasing light levels. The correlation between host and symbiont SOD at 28 °C in *A. millepora* further supports the idea of a stronger influence of light in this coral, since varying tissue oxygen tensions due to daily fluctuations in light and photosynthesis would affect SOD in both compartments. Similarly, a positive correlation between tissue chlorophyll content and host SOD activity has been demonstrated for a range of symbiotic marine invertebrates (Shick and Dykens, 1985).

The photophysiological responses of *A. millepora* symbionts and the observed bleaching of the coral holobiont clearly demonstrate the adverse effect of high temperature on the stability of the symbiosis.



**Fig. 4.** Symbiont enzymatic antioxidants. Symbiont antioxidant activity for superoxide dismutase (SOD, a, b) catalase peroxidase (KatG, c, d), and ascorbate peroxidase (APX, e, f) for the corals *Acropora millepora* and *Montipora digitata* at 28 °C (white) and 33 °C (black), over 9 days of exposure. Range of interpolation indicates timeframe used for the statistical analysis (Table 2). All enzyme activities are expressed as units per milligramme of protein. Values are mean  $\pm$  SE, N = 5, except for Day 9 in *A. millepora* at 33 °C (N = 2). Note different scaling of axis in c, d and e, f.

Surprisingly, however, the photophysiological impact of temperature was not reflected in the enzymatic antioxidant responses that we expected to accompany photo-oxidative stress in the symbiont as assumed by the Oxidative Theory of Coral Bleaching and demonstrated for thermally-challenged *Symbiodinium* in culture (Lesser, 1996; Krueger et al., 2014). Furthermore, we observed that increased CAT activity in both coral hosts was largely independent of the enzymatic removal of ROS in the symbiont or changes in host SOD. Thus, elevated CAT activity in both corals might result from increased metabolic activity in the host at higher temperatures (e.g., increased peroxisomal activity as a result of the mobilisation of reserves) and was in this case not indicative of bleaching susceptibility. The Oxidative Theory of Coral Bleaching suggests that hydrogen peroxide derived from photo-oxidative stress in the symbiont might leak into the host tissue, when symbiont defences are overwhelmed in bleaching corals (Downs et al., 2002). However, our observations show that when temperature is the dominant stressor, coral bleaching with compromised photosynthesis and loss of symbiont cells does not necessarily involve a connection of ROS fluxes between host and symbiont, since increases in host CAT activity at high temperatures can be independent of processes in the symbiont, and can occur in a temperature-tolerant and -sensitive coral. Furthermore, no photosynthetic dysfunction or significant changes in the symbiont SOD-APX pathway that would indicate enzymatic removal of ROS was observed within the first five days of thermal stress in *A. millepora*, whereas host CAT activity at 33 °C was already elevated by Day 5. The study of Hawkins et al. (2014), which was run in tandem with the

current study, adds to this observation by illustrating a transient upregulation of host caspase-like enzyme activity – indicative of apoptotic-like cell death – in *A. millepora* at 33 °C, that coincided with higher catalase-mediated hydrogen peroxide turnover in the host (this study) prior to photosynthetic breakdown in the symbionts. A causal relationship between the induction of nitric oxide synthesis and apoptosis under thermal stress in the host has been suggested (Hawkins et al., 2013; Hawkins et al., 2014), and the patterns seen in *A. millepora* highlight the role that ROS generally might play in the activation of apoptotic-like pathways in susceptible corals (Tchernov et al., 2011). Nevertheless, the point at which ROS production triggers apoptosis in the coral host appears to be species-specific, as a similar response with respect to host CAT in the bleaching-resistant *M. digitata* (this study) did not coincide with an upregulation of caspases or NO production (Hawkins et al., 2014).

While our study does not provide any further insights into the well-known synergistic effects of light and temperature on bleaching physiology (Lesser et al., 1990; Lesser and Farrell, 2004; Downs et al., 2013), because we did not actively manipulate irradiance, it is notable that Day 5 in our experiment had the lowest mean irradiance, highlighting that elevated temperature was the primary stressor to induce the bleaching cascade in this experiment. From an ecological perspective, mass bleaching events, affecting multiple species across latitudinal and depth gradients, are primarily driven by anomalies in temperature rather than light (McWilliams et al., 2005; Sammarco et al., 2006; Eakin et al., 2009). With regard to the role of light and temperature in inducing





the applied heating rate in the experimental setup. Low heating rates profoundly delay the physiological response of the coral holobiont (Middlebrook et al., 2010) and the ramping rate of  $1\text{ }^{\circ}\text{C day}^{-1}$  used in the current study is considerably slower than in previous studies of antioxidant responses under thermal stress; these applied heating rates ranging from near-instantaneous to  $4\text{ }^{\circ}\text{C hour}^{-1}$  (Yakovleva et al., 2004; Richier et al., 2005; Higuchi et al., 2008; Yakovleva et al., 2009; Higuchi et al., 2012; Downs et al., 2013; T. Higuchi and I. Yakovleva, personal communication). Coral reef-flat communities can experience temperature changes of  $1\text{ }^{\circ}\text{C hour}^{-1}$  during spring tides (Berkelmans and Willis, 1999), however temperature changes relevant to mass bleaching are usually slower, and therefore heating rates of  $1\text{ }^{\circ}\text{C day}^{-1}$  were applied in this study. Rapid temperature increases are not typically representative of bleaching conditions in the field. Moreover, they limit the capacity to measure and distinguish short- and long-term responses. For example, applying a  $4\text{ }^{\circ}\text{C hour}^{-1}$  temperature increase from  $25\text{ }^{\circ}\text{C}$  to  $33\text{ }^{\circ}\text{C}$  to the corals *Acropora intermedia* and *Montipora digitata* from the South China Sea resulted in a 90% symbiont loss within 48 h (Imbs and Yakovleva, 2012). It is unknown whether or how heating rate affects the specific mechanism by which bleaching occurs, i.e., the spectrum of responses from symbiont *in situ* degradation to host cell apoptosis and necrosis (reviewed in Weis, 2008), but it seems plausible that slow- vs. rapid heating should induce different cellular pathologies. Although not always feasible, the most realistic and ecologically relevant stress scenario should mimic the time scale and temperature profile of a natural bleaching event.

Photosynthetically-derived  $\text{H}_2\text{O}_2$  has been suggested as the most likely ROS to move between both partners due to the fact that it has a longer lifetime than other ROS, a large diffusion radius, and no net charge (Winterbourn, 2008), potentially enabling it to move through the three chloroplastic membranes, the symbiont cell membrane and the symbiosome membrane complex (Wakefield et al., 2000) to enter the host cytosol (Lesser et al., 1990; Downs et al., 2002). Potential leakage of hydrogen peroxide from *Symbiodinium* and other dinoflagellates (but also extracellular generation of ROS) has indeed been shown experimentally (Palenik and Morel, 1990; Sandeman, 2006; Suggest et al., 2008; Saragosti et al., 2010). Our findings agree with previous studies that, depending on the coral species, catalase activity *per unit protein* is usually three to ten times higher (up to twenty-three times in *M. digitata*) in the host than in the symbiont (Yakovleva et al., 2004; Levy et al., 2006; Higuchi et al., 2008). Although never fully acknowledged before, this observation implies that the host has sufficient scavenging capacity to deal with symbiont-derived hydrogen peroxide. Using symbiont densities and biomass values of the two coral species investigated here, in combination with values of maximum released ROS *per symbiont cell* (as  $\text{H}_2\text{O}_2$  equivalents) due to an oxidative burst (Mydlarz and Jacobs, 2004) or combined high light and temperature stress (Suggest et al., 2008; Roberty et al., 2015), the maximum release rates of hydrogen peroxide as simultaneous release from the whole symbiont population would only amount to 0.07–0.39% of the host's scavenging capacity *via* CAT (assuming saturating hydrogen peroxide levels for host CAT; Table 4). This number does not even include the activity of the host's other enzymatic and non-enzymatic  $\text{H}_2\text{O}_2$ -scavenging systems. Clearly, measurements of the amount and direction of ROS fluxes between both partners under stress are needed to confirm the notion that leaking  $\text{H}_2\text{O}_2$  from the symbiont can indeed significantly challenge or harm the host (Lesser, 1997; Downs et al., 2002).

It is clear that we still have much to learn about the fluxes of ROS between host and symbiont. Admittedly, the absence of an antioxidant upregulation in the symbiont during the onset of thermal bleaching in our experiment does not preclude the potential occurrence of oxidative damage or increases in steady-state ROS levels *per se*, because neither was monitored. Furthermore, the role of other important photoprotective mechanisms that might have prevented ROS formation upstream of antioxidant enzymes, such as xanthophyll cycling (Brown

**Table 4**

Estimated maximum release of symbiont reactive oxygen species (ROS; as hydrogen peroxide equivalents) based on an oxidative burst (Mydlarz and Jacobs, 2004)<sup>1</sup> and high light and high temperature ( $\geq 1000\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ;  $32\text{--}33\text{ }^{\circ}\text{C}$ ) in *Symbiodinium* B1 (Suggest et al., 2008)<sup>2</sup> and A1 (Roberty et al., 2015)<sup>3</sup> contrasted with host hydrogen peroxide scavenging capacity *via* catalase (CAT) for *Acropora millepora* and *Montipora digitata* at  $28\text{ }^{\circ}\text{C}$  (this study). Calculations are based on measured values for cell densities, protein content, and CAT activity at  $28\text{ }^{\circ}\text{C}$  on Day 0. Values from McGinty et al. (2012) were not considered, since the applied probe concentration for dichlorofluorescein diacetate (0.67 mM; DCFDA) was considerably higher than the recommended concentration ( $10\text{ }\mu\text{M}$ ; Halliwell and Gutteridge, 2007), and therefore may have been cytotoxic. Note that host CAT activities represent the maximum activity as measured under saturating substrate concentrations.

	<i>Acropora millepora</i>	<i>Montipora digitata</i>
$\text{H}_2\text{O}_2$ release <i>per symbiont cell</i> [pmol $\text{min}^{-1}$ cell $^{-1}$ ]	~0.042–0.11 <sup>1</sup> /0.061 <sup>2</sup> /0.21 <sup>3</sup>	
Symbiont density [cells $\text{cm}^{-2}$ ]	~1,800,000	~2,000,000
Symbiont $\text{H}_2\text{O}_2$ release <i>per unit surface area</i> [ $\mu\text{mol min}^{-1}\text{ cm}^{-2}$ ]	~0.076–0.378	~0.084–0.420
Host protein <i>per unit surface area</i> [mg $\text{cm}^{-2}$ ]	~0.8	~0.8
Host CAT activity <i>per unit host protein</i> [U $\text{mg}^{-1}$ = $\mu\text{mol min}^{-1}\text{ mg}^{-1}$ ]	~120	~160
Host CAT activity <i>per unit surface area</i> [ $\mu\text{mol min}^{-1}\text{ cm}^{-2}$ ]	~96	~128
Maximum symbiont $\text{H}_2\text{O}_2$ release/host CAT activity	~0.08–0.39%	~0.07–0.33%

et al., 1999; Brown et al., 2002b; but also see Venn et al., 2006), must be considered. Future studies should combine measurements of ROS levels, oxidative damage, and the activities of enzymatic and non-enzymatic antioxidants. Monitoring the degree of oxidative damage to the symbiosome membrane complex as the interface between host and symbiont would also be valuable, as it could provide information on the extent of ROS flow between both partners, regardless of the origin.

#### 4.3. A third role for ROS and NO in coral bleaching?

The observed trends of antioxidant and apoptosis-regulating enzymes and NO accumulation (Hawkins et al., 2014) in the coral holobiont point towards the importance of early physiological events in the host that may precede the photosynthetic dysfunction of the symbionts. Although highly speculative at this point, hydrogen peroxide might have an active role in the host's antipathogenic response towards its symbionts under bleaching conditions, particularly when the host has sufficient catalase activity to attenuate any self-harm. *Symbiodinium* cells are located within arrested phagosomes and a breakdown of host-symbiont recognition mechanisms under stress might lead to their maturation to phagolysosomes. Antipathogenic responses in mature phagolysosomes involve the generation of ROS and nitric oxide (Wink et al., 2011) and thermal stress-induced production of nitric oxide in symbiotic cnidarians has been associated with the apoptotic-like cell death pathways, that themselves are significant cellular mechanisms of bleaching (Perez and Weis, 2006; Dunn and Weis, 2009; Hawkins et al., 2013). The potential importance of host-produced hydrogen peroxide is highlighted in the current study, where host catalase activity in *A. millepora* at  $33\text{ }^{\circ}\text{C}$  remained high until the end of the experiment, despite photosynthetic breakdown (and loss of pigment) that might be expected to limit ROS synthesis as a result of lower rates of oxygen evolution and tissue  $p\text{O}_2$  in advanced bleaching states (Fig. 1; Nii and Muscatine, 1997; Richier et al., 2005). The presence of a high hydrogen peroxide concentration of host origin could also explain the KatG increase and APX inhibition in the symbionts of *A. millepora* on the final day of observation, since neither was related to changes in symbiont SOD levels. While the specific role of ROS in host immune responses has not been investigated in scleractinian corals, Mydlarz and Harvell (2007) have demonstrated an increased peroxidase activity (measured

as holobiont response) to a fungal infection in the symbiotic sea fan *Gorgonia ventalina*.

## 5. Conclusions

The interspecific variability of the coral holobiont to environmental stress is the result of the interplay of many different physiological levels, as well as acclimatory (environmental history) and adaptive processes that modulate the specific stress impact. However, at the basic physiological level, cellular function and the ability to maintain homeostasis (e.g., redox, pH, Ca<sup>2+</sup>) are essential for the stability of this primarily nutritional symbiotic relationship (Fig. 1). This study shows that, under the thermal and light scenario tested here, no significant changes in enzymatic antioxidant defences occurred in the dinoflagellate symbiont. Host defences increased in the form of enhanced scavenging of hydrogen peroxide, which preceded photosynthetic dysfunction and bleaching in the susceptible corals. These observations illustrate that host redox mechanisms under thermal stress are not always dictated by symbiont photophysiology. On the other hand, they also show that host catalase activity, if viewed as an isolated proxy, may not be protective or predictive of bleaching, even though a connection between ROS scavenging and activation of apoptotic-like pathways was evident in the thermally susceptible coral. This interspecific variability in ROS production-apoptosis regulation (Richier et al., 2006; Tchernov et al., 2011) is worthy of further attention.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cbpa.2015.08.012>.

## Acknowledgements

This work was funded by a Royal Society of New Zealand Marsden Fund grant (contract number VUW0902) to SKD, PLF, SD, OH-G, and WL and supported by a Marsden-funded PhD scholarship to TK, and a Commonwealth Scholarship awarded to TDH. We would like to thank the HIRS staff and in particular Dr. Kyra Hay and Dr. Liz Perkins for logistic support and Dr. Olga Pantos (University of Queensland) for assistance with transportation and storage of coral samples. This manuscript benefited greatly from the helpful comments and suggestions of two anonymous reviewers.

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