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Variations in Reactive Oxygen Release and Antioxidant Activity in Multiple *Symbiodinium* Types in Response to Elevated Temperature

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Abstract As ocean temperatures rise, investigations into what the physiological effects will be on the symbiotic microalga *Symbiodinium*, and how these may play into the cnidarian bleaching response, have highlighted the contribution of reactive oxygen species (ROS). Previous studies have laid this groundwork using a limited number of *Symbiodinium* phylotypes, and so this study aims to expand this understanding by exploring the effects of sub-lethal elevated temperatures on the physiological response of seven genetically distinct types of *Symbiodinium*, including A1, B1, B2, C1, D, E1, and F2. The production of ROS (at 26 °C, 29 °C, 30 °C, and 31 °C) and activity of the antioxidants catalase (CAT) and superoxide dismutase (SOD) (at 26 °C and 31 °C) were measured as indicators of sensitivity or tolerance to heat stress. *Symbiodinium* types B1 and C1 were the most thermally sensitive, with C1 producing the highest amount of ROS at elevated temperatures. Types A1 and F2 were tolerant, having no increase in ROS production, and were the only types to increase both CAT and SOD activity with temperature stress. Type B2 had decreased ROS production and elevation of CAT activity, while type E1 had decreased levels of ROS production at elevated temperatures. Type D was the only *Symbiodinium* type to remain unaffected by elevated temperatures. These results are consistent with previous findings of relative sensitivity or tolerance to elevated temperatures, specifically with regards to types A1, B1, and F2. The inclusion of types B2, C1, D, and E1 provides further new evidence of how types differ in their thermal responses, suggesting differing mechanisms exist in the *Symbiodnium* response to higher temperature and highlighting the importance of establishing symbiont identity when exploring the response of intact associations to this type of stress.

Abbreviations

CAT	Catalase
H_2O_2	Hydrogen peroxide
ITS-2	Internal transcribed spacer-2
O_2^-	Superoxide anion
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Photosynthetic microorganisms experience unique challenges in the face of global climate change, and rising sea surface temperatures are a particular concern for marine microalgae [1–4]. Temperature stress can result in changes to the ecology of these organisms through a variety of direct and indirect factors affecting physiological parameters including photosynthetic, metabolic and growth rates [1–3]. Microalgae are the foundation of ocean ecosystems, and these changes pose a significant problem to the many organisms that rely on or are in close association with them [5]. Coral reefs are of particular concern as many of the organisms present, particularly the reef-building scleractinian corals, form a mutualistic relationship with dinoflagellates in the genus *Symbiodinium* enabling them to thrive in oligotrophic tropical habitats [6]. Elevated temperatures have been linked to a loss of the algal symbiont and/or its pigments, causing the phenomenon of coral bleaching [7]. While corals regularly expel their symbionts during stress associated with the peak summer months without discernable long-term adverse affects, extreme cases can be detrimental to coral survival, potentially leading to increased incidence of disease and mortality [8, 9]. Coral bleaching

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has become a focus of attention over the last three decades [10, 11], as events leading to mass mortality and significant die offs are increasing in frequency, and have become a significant factor in worldwide decline of these ecosystems [12–17].

A contributing factor in the bleaching phenomenon is the algal production of damaging reactive oxygen molecules (ROS) [18, 19]. These molecules are a by-product of photosynthesis and cellular respiration, and their highly reactive properties can mutate DNA, denature proteins, and oxidize lipids and cellular membranes [5, 18, 20]. As ROS are produced under normal conditions by the chloroplasts, mitochondria and peroxisosmes [21, 22] cells apply various strategies to mitigate their detrimental effects. ROS produced by the cell can be used as signals for cellular defensive responses as well as apoptosis [18, 20, 22–24] or eliminated or scavenged by various cellular products, such as antioxidants including catalase (CAT) and superoxide dismutase (SOD) [18, 23, 25–27]. Exposure to elevated temperatures can disrupt the photosynthetic apparatus, leading to a build-up of electrons that may react with O_2 molecules and increase the production of ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) [19, 23, 28, 29]. During prolonged periods of stress, ROS are generated in quantities large enough that the algal cells cannot mitigate their production and damage is sustained by cellular components. This damage is not constrained to the algal cell as one of the ROS', H_2O_2, is permeable to cellular membranes, and can move out of the symbiont and into host cells, and potentially trigger the host to expel the algal symbiont [18, 19, 23, 29, 30].

Investigations of the symbiont have discovered a great amount of genetic variability within the *Symbiodinium* genus, which is now classified into nine different clades, lettered A–I [31]. Further identification within each clade can be determined, for example by sequencing of the internal transcribed spacer (ITS) region, into what will be referred to as "types" in this paper [32, 33]. Research into *Symbiodinium* physiology using in hospite and culture techniques has led to the understanding that physiological variability exists both between and within *Symbiodinium* clades, and this variability extends to their tolerance of stress [28, 34–38]. For example, previous work with cultured *Symbiodinium* has identified types A1, E1, and F2 as tolerant to elevated temperature stress and type B1 as sensitive [34, 36, 39]. Work by Thornhill et al. [40] found type B2 to be tolerant of lowered temperatures, contributing to its distribution in sub-tropical waters. It is clear that differences exist in the thermal tolerance of *Symbiodinium* types, but the extent of this and the factors conveying tolerance or sensitivity remain unclear.

This study addresses the need for more knowledge from empirical data concerning physiological differences among *Symbiodinium* types in the context of elevated temperatures as an environmental stressor. As such, seven different types of cultured *Symbiodinium* (A1, B1, B2, C1, D, E1, and F2) were exposed to temperatures between 26 °C and 31 °C, and the production of ROS, and the antioxidants CAT and SOD were measured. *Symbiodinium* types found to be tolerant in previous studies, such as A1, were predicted to produce more antioxidants at elevated temperatures, and/or less ROS than more sensitive types, such as B1.

Methods

Materials

Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise noted, and all assays read with a Bio-Tek Synergy 2 Multi-detection microplate reader using the Gen-5 software package (Bio-Tek Instruments, Inc. Winooski, VT, USA). Assay plates were manufactured by Corning, Inc. (Lowell, MA, USA).

Symbiodinium Cultures

Symbiodinium [41] cultures are identified based on the ITS-2 region as A1, B1, B2, C1, D (ITS type unknown), E1, and F2, and were generous gifts from the collections of Todd LaJeunesse (Pennsylvania State University) and Scott Santos (Auburn University). For a list of host species and geographic origins of all types, see Table 1. Once received, all *Symbiodinium* cultures were grown in 250 mL polycarbonate culture flasks with 0.2 μm filter tops (IscBioExpress, Kaysville, UT, USA) in ASP-8A media [42], and 10 mL were used to seed new subcultures every 3 to 4 weeks. Cultures were maintained in a diurnal growth chamber (Powers Scientific, Inc, Pipersville, PA, USA) at 25 °C with 20-W fluorescent plant growth bulbs under a 14:10 h photoperiod at approximately 55 μmol quanta m^{-2} s^{-1} irradiance (measured with a LI-COR model LI-250 light meter, LI-COR Environmental, Lincoln, NE, USA).

All experiments used *Symbiodinium* cells in stationary phase that were 3 to 4 weeks of age to minimize the effects of senescence [42]. To normalize algal cells per experiment, cells were pelleted by centrifugation for 18 min at 2,530×*g* on an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany) and then standardized to specific concentrations using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA, USA).

Temperature Stress Experiments

All experiments were run in water baths in a temperature and light controlled room. One bath was maintained as a

Table 1 The host species and geographic origins for each type of *Symbiodinium* used in these studies

Identification number	Clade	ITS-2 Type	Geographic origin	Host origin
61	A	1	Caribbean, FL	*Cassiopeia xamachana*
147	B	1	Caribbean, Jamaica	*Pseudoterogorgia bipinnata*
141	B	2.1	Western Atlantic, Bermuda	*Oculina diffusa*
152	C	1	Caribbean, Jamaica	*Discosoma sancti-thomae*
10.8A	D	[a]	Caribbean, FL	*Montastraea faveolata*
383	E	1	East Pacific, CA	*Anthopleura elegantissima*
133	F	2	Caribbean, Jamaica	*Meandrina meandrites*

For all clades but D, information is found in 33. For clade D, see the *Symbiodinium* cultures database hosted by Dr. Scott Santos at http://www.auburn.edu/~santosr/phplabware.htm

[a]Unknown ITS type

control environment while the temperature of a second bath was manipulated. Water temperatures were maintained or manipulated using Techne Tempette Junior TE-8J (Cambridge, UK) circulating water heaters and monitored with a total immersion thermometer. Irradiance levels were the same as the culture conditions and provided by the same bulb-type as in the growth chamber.

For ROS and antioxidant experiments, each *Symbiodinium* type was standardized to 500,000 cells mL^{-1} by dilution in fresh sterile media, and 15 mL were transferred into clean and sterile polypropylene test tubes (BD Biosciences, San Jose, CA, USA). For ROS studies, three replicate test tubes of each *Symbiodinium* type were placed randomly in each water bath and were rotated every other day to minimize the effect of position. Control baths were maintained at 26 °C±0.5 throughout the experiment. Treatment baths started at 26 °C±0.5, and then were raised by 1 °C every 48 h until 31 °C was reached to avoid acute temperature stress. After 48 h of exposure to each temperature, 1.3 mL of samples were taken from each test tube on days 8, 10, and 12, at treatment temperatures of 29 °C, 30 °C, and 31 °C. To prevent interference with the DCFH-DA assay (see below) and immediate scavenging of ROS, cells were maintained in ASP-8A media lacking trace minerals, metals, and vitamins. Cell concentration, viability, and ROS production were established from cells in each aliquot. For antioxidant studies the design was the same, except that there were five replicate test tubes of each *Symbiodinium* type and the entire tube contents were sampled (see below) only on day 12 after 48 h at 31 °C.

ROS Analysis

Prior to the assay, samples were incubated in the dark for 20 min to account for normal ROS production during photosynthesis, as cells from different *Symbiodinium* types may photosynthesize at different rates and contain different numbers of chloroplasts. ROS production was quantified after a 5-min incubation of 1 mL of sample, 75 μL of 10 mM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) in DMSO and 50 μL of esterase (1 mg mL^{-1}). DCFH-DA is permeable to cellular membranes and fluoresces in the presence of esterase and ROS (except singlet oxygen), allowing the quantification of extra- and intra-cellular ROS concentrations [43, 44]. The fluorescence of the samples was measured (485 nm excitation, 528 nm emission) in a Costar 12 well plate, and concentration of ROS was determined from calibration to a standard curve of 30 % high purity H_2O_2. To adjust for the effect of time each culture had spent in the water baths, net ROS production was calculated by subtracting the amount of ROS produced in the cells maintained at the control temperature (26 °C±0.5) measured that same day from ROS produced by the cells in the treatment tubes.

Antioxidant Assays

All *Symbiodinium* cells in each replicate were harvested at the end of the experiment (12 days) by centrifugal pelleting and immediately flash freezing in liquid nitrogen. Pellets were then freeze-dried for 24 h on a VirTis Benchtop 6 K freeze drier (SP Industries, Stone Ridge, NY, USA). To break open the cells and extract the soluble protein, phosphate buffer (50 mM, pH 7.8) and a spatula tip of borosilicate glass beads (1 mm) were added to each freeze-dried pellet. Pellets were then vortexed on high for 5 min in 20 s intervals. They were maintained on ice during each interval. Samples were then centrifuged for 2–3 min at 4,300×*g* in a microcentrifuge (Baxter Scientific Products, Deerfield, IL, USA) to remove cellular debris. Supernatants were removed and transferred to clean 0.5 mL microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at −80 °C until analyzed. Total protein for all samples was quantified using the Quick Start™ Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA). Absorbance was read at 595 nm and standardized to BSA. For all antioxidant assays, activity was normalized to the total protein concentration of each sample. CAT activity was determined using the Amplex Red catalase kit (Invitrogen Corporation, Carlsbad, CA, USA, excitation 540 nm, emission 590 nm), which fluorescently detects the presence of H_2O_2. SOD activity was determined using the SOD Assay kit (Fluka BioChemika, Buchsm, Switzerland, absorbance at 450 nm), which colorimetrically detects the scavenging of O_2^-.

Statistical Analysis

Baseline ROS production for each *Symbiodinium* type was calculated by averaging the control (26 °C) sample's ROS production on days 8, 10, and 12 (n=9); comparisons among *Symbiodinium* types were then made using a one-way ANOVA. To calculate net ROS production during temperature stress, ROS values were averaged for all control (26 °C) replicates at that time period, and this averaged value subtracted from each temperature stress replicate of corresponding *Symbiodinium* type (n=3). The effect of *Symbiodinium* type and temperature on net ROS production was then evaluated using repeated-measures ANOVA. Further comparisons within and among *Symbiodinium* types were made using univariate ANOVA and Tukey–Kramer post-hoc tests with α=0.05. SOD and CAT (n=5) were analyzed using two-way ANOVA with *Symbiodinium* type and temperature as factors. For all analyses, data were first tested for normality and homoscedasticity using Shapiro–Wilks and Levene's tests, respectively. Data that did not conform were power transformed using the Box-Cox Y method to meet the parametric criteria of normality and equal variances. All statistical analyses were performed using JMP Statistical Discovery Software version 8.0 (SAS Institute, Cary, NC, USA).

Results

Effects of Temperature on ROS Production

There was a significant difference in baseline ROS production among *Symbiodinium* types (df=6, F=12.48, p<0.001, Fig. 1). Within type, there was no effect of temperature alone (df=2, F=2.88, p=0.092), but there was a significant interaction between temperature and type (df=12, F=7.28, p<0.0001, Table 2). Significant differences were observed among *Symbiodinium* types for each temperature (Table 2). At 29 °C, the net ROS production of type F2 was significantly lowest compared to the rest. Those in A1, C1, and E1 types also produced relatively lower ROS. At 30 °C, A1 produced the most net ROS and was significantly higher than B1 and F2. The greatest differences in net ROS production among types occurred at 31 °C (Table 3; Fig. 2). Type C1 showed a fourfold increase in ROS production over all other *Symbiodinium* types, while A1, B2, and E1 had a negative net production of ROS (Fig. 2).

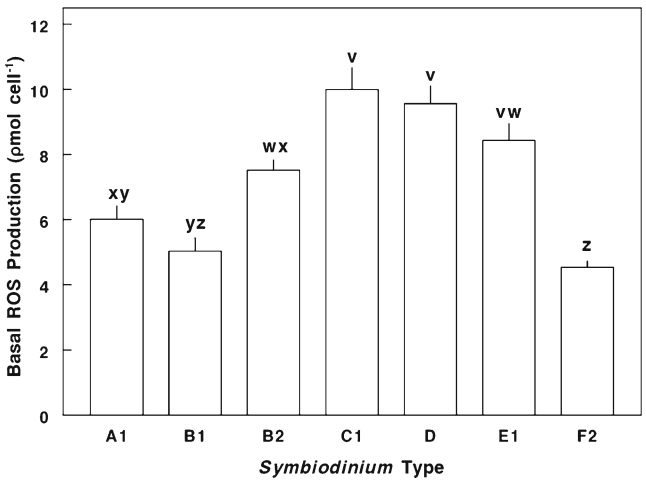

Figure 1 Baseline production of reactive oxygen species (ROS) (mean±s.e, n=9) for all *Symbiodinium* types at 26 °C. Letters *v*, *w*, *x*, *y*, or *z* denote significant differences (α=0.05) in baseline ROS production; values which do not share a letter are statistically different

There was a significant affect of elevated temperature on ROS production in *Symbiodinium* types B2, C1, and E1 (Fig. 2). C1 was the only type to have a higher net production at 31 °C than at lower temperatures and produced four times the amount of ROS than at 29 °C (df=2, F=24.41, p=0.0013, Fig. 2). *Symbiodinium* types B2 and E1 had lower net levels of ROS at 31 °C compared to 29 °C (B2: df=2, F=6.77, p=0.029 and E1: df=2, F=8.68, p=0.017, Fig. 2) and produced less ROS than ambient (26 °C) controls at 31 °C making their net ROS production negative. *Symbiodinium* types A1, B1, D, and F2 showed no significant change at any of the three test temperatures (Fig. 2).

Effects of Temperature on Antioxidant Activity

Both CAT and SOD were significantly affected by temperature (two-way ANOVA, CAT: df=1, F=40.25, p=<0.0001 and SOD: df=1, F=7.74, 0.0074) and *Symbiodinium* type (CAT: df=6, F=22.84, p<0.0001 and SOD: df=6, F=32.29, p<0.0001), while the interaction of temperature and

Table 2 Repeated measures ANOVA results for temperature effects on reactive oxygen species (ROS) production in each *Symbiodinium* type

Repeated Measures			
ANOVA	F	df	p
Within-symbiont type			
Wilks-Lambda	7.28	12	**<0.0001**
Temperature	2.88	2	0.092
Type*Temperature	7.28	12	**<0.0001**
Among-symbiont types			
F test	8.08	6	**0.0007**
Univariate test			
29 °C	4.22	6	**0.013**
30 °C	4.42	6	**0.010**
31 °C	20.26	6	**<0.0001**

Data are for the net production of ROS, i.e., ROS production at elevated temperature—ROS production at basal, 26 °C. p values in bold face lettering are statistically different (α=0.05)

Table 3 Net production of reactive oxygen species (ROS), i.e., ROS production at elevated temperature—ROS production at basal, 26 °C among types at each treatment temperature (29 °C, 30 °C, or 31 °C)

	Net ROS production (ρmol cell^{-1})		
Symbiodinium type	29 °C	30 °C	31 °C
A1	1.67 ± 0.21^{y}	3.05 ± 1.24^{yz}	-0.68 ± 0.84^{xyz}
B1	0.38 ± 0.23^{yz}	-1.05 ± 0.51^{yz}	2.22 ± 0.56^{x}
B2	1.19 ± 0.30^{yz}	0.033 ± 0.83^{z}	-2.65 ± 0.97^{yz}
C1	1.69 ± 0.51^{y}	0.40 ± 0.60^{y}	6.99 ± 0.96^{w}
D	0.46 ± 0.25^{yz}	0.32 ± 0.16^{yz}	0.66 ± 0.14^{xy}
E1	1.64 ± 0.74^{y}	0.70 ± 0.66^{yz}	-2.98 ± 1.03^{z}
F2	-0.43 ± 0.21^{z}	-1.41 ± 0.10^{z}	1.54 ± 0.24^{x}

Significant differences between types within each temperature are denoted by different letters (α=0.05)

type was not significant for either CAT (df=6, F=1.76, p=0.125) or SOD (df=6, F=0.951, p=0.467). CAT activity was the same for all types except E1, which displayed the lowest activity at 26 °C and 31 °C, and F2, which was highest at 26 °C and 31 °C (Fig. 3a). Greater intertype differences were observed in SOD activity (Fig. 3b) with F2 again displaying high values of SOD activity units at 26 °C and at 31 °C, and E1, the lowest values of at 26 °C and at 31 °C.

Antioxidant activity was elevated in response to temperature stress for only a few *Symbiodinium* types. Types A1, B2, and F2 all induced their CAT activity with an increase in temperature from 26 °C to 31 °C (Fig. 3a). For types A1 and F2, SOD activity was positively correlated to elevated temperature as both types elevated their activity of SOD in response to increasing temperatures (Fig. 3b).

Discussion

Microalgal stress responses have the potential to influence ecosystems through organismal level processes, especially in regards to global climate change. This is especially true in microalgae that live symbiotically with ecologically important hosts, such as the *Symbiodinium*-cnidarian relationship. The genetic diversity within the *Symbiodinium* genus has led to questions concerning how variable their physiology may be, particularly in response to factors associated with climate change such as elevated temperatures. Investigations have begun to tease apart the complex responses and have led to the determination that responses are not uniform either within or among clades. Increased production of ROS is an indicator of algal stress [34, 45, 46], while increased antioxidant activity may provide the algae with some level of tolerance to increased ROS output [27, 46]. Our results support previous findings by demonstrating that each *Symbiodinium* type displays a distinct stress response, and the sensitivity to elevated temperatures varies between types.

At ambient temperature and without stress, ROS production and release differed significantly among *Symbiodinium* types. Types C1 and D were among the highest producers of ROS under basal conditions of 26 °C, while types B1 and F2 were among the lowest. This pattern changed as temperatures were raised, and at 29 ° and 30 °C, net ROS production was similar among all *Symbiodinium* types. Differences emerged at 31 °C, with type C1 still among the highest ROS producers. Type B1 was also among the highest, in contrast to being among the lowest at 26 °C. Within each *Symbiodinium* type, little significant variation was observed, suggesting that under these conditions, these cultures may have a higher threshold of tolerance that exceeds 31 °C. *Symbiodinium* type C1 had the

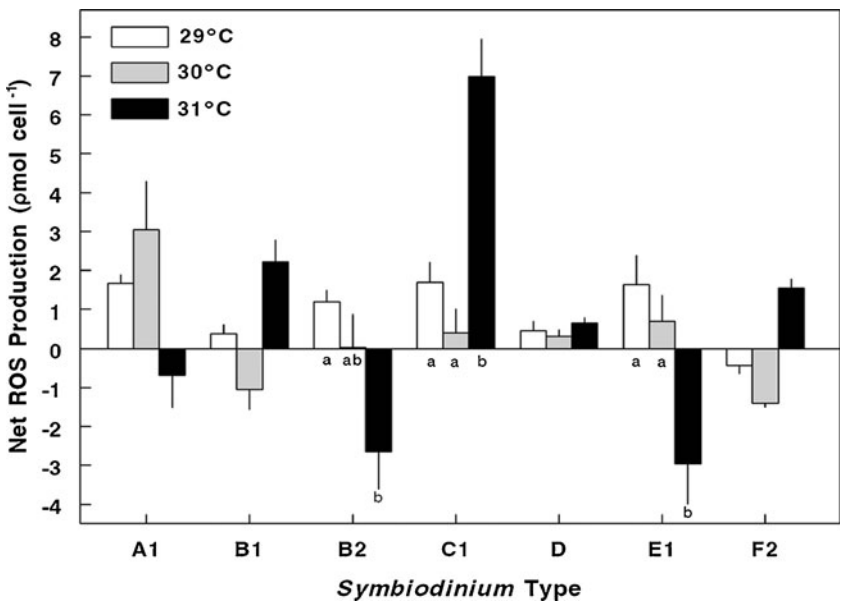

Figure 2 Differences in net production of reactive oxygen species (ROS), i.e., ROS production at elevated temperature—ROS production at basal, 26 °C within *Symbiodinium* types at 29 °C, 30 °C, and 31 °C (mean±s.e, n=3). For those types (B2, C1, and E1) that had significant differences in ROS net production with temperature, letters *a* or *b* below the values indicate significant differences among temperature treatments. For significant differences (α= 0.05) in net ROS production among types at each treatment temperature (29 °C, 30 °C, or 31 °C), see Table 3

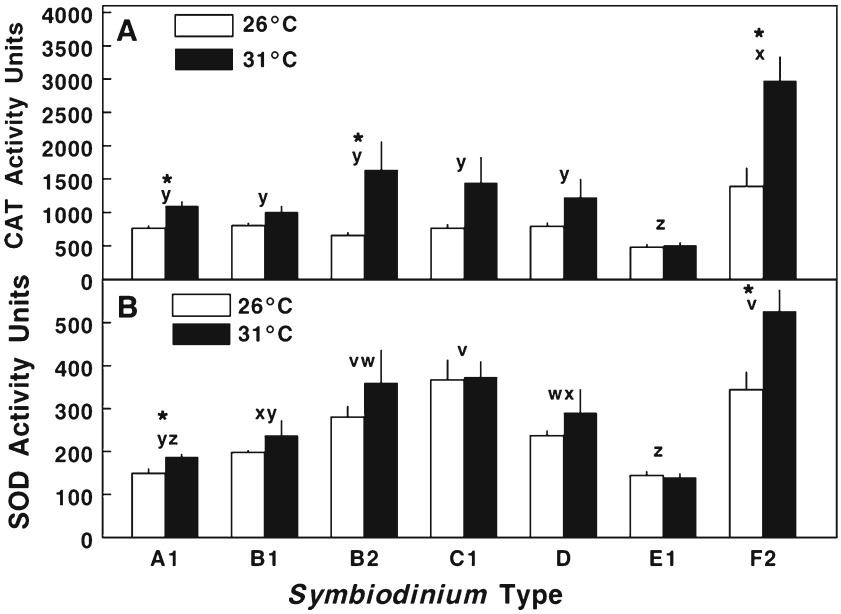

Figure 3 **a** Catalase (CAT) activity units (mean±s.e., $n=5$) and **b** superoxide dismutase (SOD) activity units (mean± s.e., $n=5$). Since the interaction between *Symbiodinium* type and temperature was not significant, the between *Symbiodinium* type significant differences ($\alpha=0.05$) in CAT or SOD activity are indicated by the letters *v*, *w*, *x*, *y*, or *z*. In both panels, *asterisks* above paired bars for any one type indicate that there was a significant difference ($\alpha=0.05$) in CAT or SOD activity between the 27 °C and 32 °C treatment temperatures

greatest response, increasing ROS production fourfold at 31 °C, while types B2 and E1 had surprisingly significant decreases in net ROS production.

The activity of the antioxidants CAT and SOD also varied between algae. CAT activity was the same among types A1, B1, B2, C1, and D. Type E1 had the lowest production of CAT while type F2 had the highest. SOD activity was more variable, with types C1 and F2 having the highest activity and types A1 and E1 the lowest. We predicted that as a mechanism to offset increased ROS production during exposure to elevated temperatures some *Symbiodinium* types would increase antioxidant activity to scavenge ROS shortly after its generation. *Symbiodinium* type B2 did up-regulate the production of CAT and produced less ROS at 31 °C than at 26 °C, while types A1 and F2, which showed no change in ROS production with temperature stress, increased the production of both CAT and SOD. No increases in antioxidants were observed for types B1, C1, D, or E1, and it is possible that a longer exposure to elevated temperatures may be necessary to induce a more discernable response within these *Symbiodinium* types [18]. Alternatively, for types B1, D, and E1, it is possible that at elevated temperatures, the kinetics of the antioxidants was sufficiently increased so that the same amount of antioxidant was sufficient to keep ROS levels from inducing further upregulation. In either case, it may be that the mechanisms responsible for the observed tolerance of some *Symbiodinium* types, like A1 and F2, is due to a rapid active response, preventing or at least delaying the onset of oxidative damage from ROS that can lead to cell death. If this is true, there may be tradeoffs in genetic traits or expression of genetic traits, i.e., phenotypes that were beyond the scope of this study such as growth rates or photosynthetic repair rates [36].

After synthesis of these responses to higher thermal stress, types A1, D, E1, and F2 display attributes of tolerance, while B1 and C1 seem to be sensitive. *Symbiodinium* type C1 demonstrated a distinct sensitivity to thermal stress with the greatest ROS release but no significant increases in antioxidants to mitigate oxidative stress. While the within-type results showed a non-significant increase in net ROS production for type B1, it did produce more ROS among types at 31 °C than 26 °C, and also did not increase the activity of CAT or SOD. The antioxidant activity of both B1 and C1 was not significantly different than most of the other *Symbiodinium* types, suggesting that they may not be capable of sufficiently scavenging the excess ROS, particularly the fourfold higher ROS production in C1. Thus, it is likely that significant oxidative damage is occurring to the algal cells. It can be further hypothesized that if compromised cellular integrity cannot be repaired, acclimatization of these *Symbiodinium* types to high temperature may not be possible.

In contrast, *Symbiodinium* types A1, B2, and F2 all displayed responses that appeared to indicate active responses to mitigate damage due to thermal stress. Type B2 showed a significant decrease in net ROS production in addition to a significant increase in CAT activity, while *Symbiodinium* types A1 and F2 had no change in net ROS production and an increase in CAT and SOD activity. This upregulation may be sufficient to scavenge ROS as it is produced and prevent oxidative damage to the cell. *Symbiodinium* type E1 also had a significant decrease in ROS production, but without a change in antioxidant activity, suggesting effective scavenging of ROS by a different antioxidant such as ascorbate peroxidase [46, 47].

It is notable that *Symbiodinium* type D was the only type where all three physiological measures were unaffected by elevated temperatures. This suggests alternative mechanisms

that prevent ROS up-regulation in the first place, such as a more stable photosynthetic apparatus. If these factors are inherent to the *Symbiodinium* type, such as in the lipid composition of their thylakoid membranes [28], energy and resources may be utilized for growth instead of diverted to the production of compounds for protection or repair.

These responses are consistent with previous findings [34, 36, 39] and expand the knowledge base to a broader comparison of *Symbiodinium* types. The variable responses observed among *Symbiodinium* types indicate a range of sensitivity to elevated temperatures unique to each type. In addition to the factors studied here, many other responses, such as production of mycosporine-like amino acids [46, 48], composition of cellular membranes [28, 44], photosynthetic repair mechanisms [49, 50], and growth rates [39, 51] may contribute to understanding the *Symbiodinium* stress response. It is unlikely that any one of these factors will explain the stress response of all *Symbiodinium*; thus an understanding of how each one contributes as well as the synergy between temperature and increased irradiance [34, 36, 38, 46, 52] is essential. Placing these responses in an ecological context requires a greater understanding of how the stress response is affected by an intact symbiosis, incorporating algal symbiont identity beyond the cladal level and host genotype [30, 37, 53]. Fully characterizing *Symbiodinium* physiology, both in culture and in hospite, is necessary to gain insight into the effects of climate change, e.g., higher temperature, on coral reefs.

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