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ANTIOXIDANT PLASTICITY AND THERMAL SENSITIVITY IN FOUR TYPES OF *SYMBIODINIUM* SP.[1]

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Warmer than average summer sea surface temperature is one of the main drivers for coral bleaching, which describes the loss of endosymbiotic dinoflagellates (genus: *Symbiodinium*) in reef-building corals. Past research has established that oxidative stress in the symbiont plays an important part in the bleaching cascade. Corals hosting different genotypes of *Symbiodinium* may have varying thermal bleaching thresholds, but changes in the symbiont's antioxidant system that may accompany these differences have received less attention. This study shows that constitutive activity and up-regulation of different parts of the antioxidant network under thermal stress differs between four *Symbiodinium* types in culture and that thermal susceptibility can be linked to glutathione redox homeostasis. In *Symbiodinium* B1, C1 and E, declining maximum quantum yield of PSII (F_v/F_m) and death at 33°C were generally associated with elevated superoxide dismutase (SOD) activity and a more oxidized glutathione pool. *Symbiodinium* F1 exhibited no decline in F_v/F_m or growth, but showed proportionally larger increases in ascorbate peroxidase (APX) activity and glutathione content (GSx), while maintaining GSx in a reduced state. Depressed growth in *Symbiodinium* B1 at a sublethal temperature of 29°C was associated with transiently increased APX activity and glutathione pool size, and an overall increase in glutathione reductase (GR) activity. The collapse of GR activity at 33°C, together with increased SOD, APX and glutathione S-transferase activity, contributed to a strong oxidation of the glutathione pool with subsequent death. Integrating responses of multiple components of the antioxidant network highlights the importance of antioxidant plasticity in explaining type-specific temperature responses in *Symbiodinium*.

Key index words: **coral bleaching; dinoflagellate; glutathione; oxidative stress; ROS; temperature stress**

***Abbreviations*: ANOVA, analysis of variance; APX, ascorbate peroxidase; CDNB, 1-chloro-2,4-dinitrobenzene; DFA, discriminant function analysis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); F_v/F_m, maximum quantum yield of photosystem II; GR, glutathione reductase; GSH/GSx, the amount of reduced glutathione relative to the total glutathione content; GSH, glutathione (reduced); GSSG, glutathione (oxidized); GST, glutathione S-transferase; GSx, total glutathione content; M2VP, methyl-2-vinylpyridinium triflate; MANOVA, multivariate analysis of variance; NBT, nitroblue tetrazolium; PCA, principal components analysis; Rf, riboflavin; rmANOVA, repeated measures analysis of variance; ROS, reactive oxygen species; SOD, superoxide dismutase; SST, sea surface temperature**

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Shallow tropical coral reefs worldwide experience severe ecological impoverishment as a result of local stressors and the effects of global climate change (Pandolfi et al. 2003). The success and demise of this ecosystem lies in the endosymbiosis between anthozoan cnidarians and dinoflagellates from the genus *Symbiodinium*. *Symbiodinium* cells supply photosynthetic carbon and enable fast calcification rates, while benefiting from the provision of inorganic nutrients such as ammonium and phosphate (Muscatine and Porter 1977). Localized breakdown of this obligate symbiosis (i.e., coral bleaching), as result of reduced salinity, aerial exposure, high irradiance or disease, occurs naturally on all reefs. However, mass bleaching and mortality events with large-scale impacts on multiple species are mostly driven by increasing sea surface temperatures (Jokiel and Coles 1990, Glynn 1993). Such mass bleaching events are considered a major threat to the survival of coral reefs (Hoegh-Guldberg et al. 2007, Carpenter et al. 2008). Although individual reef systems have been shown to be extremely resilient (Gilmour et al. 2013), the combined effects of local and global environmental pressures may lead to a state, where "coral reefs will no longer be prominent within coastal ecosystems if global average temperatures exceed 2°C above the pre-industrial period" (Frieler et al. 2012).

The genus *Symbiodinium* possesses high genetic diversity (at least nine clades and hundreds of subclades based on the ITS2 phylogeny), and associates with a variety of animal phyla (e.g., Cnidaria, Foraminifera, Porifera; Baker 2003, LaJeunesse 2005, Pochon and Gates 2010). As conspecific *Symbiodinium* types can associate with different hosts, the bleaching sensitivity of corals is determined by the characteristics of both animal host and dinoflagellate symbiont (Loya et al. 2001, Stimson et al. 2002, Abrego et al. 2008, Baird et al. 2009). Excessive light and temperature have been shown to cause physiological stress in *Symbiodinium*, observed as a decline in growth, respiration rate, and photosynthetic rate (Iglesias-Prieto et al. 1992, Lesser 1996, Jones et al. 1998). At the same time, antioxidant enzyme activity has been shown to increase, and the addition of exogenous antioxidants partially improves photosynthetic parameters such as maximum quantum yield of PSII (F_v/F_m) and RUBISCO activity (Lesser 1996, 1997). Concurrent observation of increased antioxidative defense in both partners (Shick et al. 1995, Downs et al. 2000, Yakovleva et al. 2004, Flores-Ramírez and Liñán-Cabello 2007) and detection of reactive oxygen species (ROS; Tchernov et al. 2004, Suggett et al. 2008, McGinty et al. 2012) in *Symbiodinium* in culture under light and/or temperature stress, has established the hypothesis that oxidative stress plays a significant role in coral bleaching. Under environmental stress, a profound redox imbalance in the symbiont might lead to the leakage of ROS to the host (Sandeman 2006, Suggett et al. 2008), potentially overwhelming its antioxidative capacity. Since ROS can act as direct or indirect signaling molecules, leakage of ROS can affect cellular processes in the host cell (Mullineaux et al. 2006, Weis 2008). This "Oxidative Theory of Coral Bleaching" assumes a unidirectional flow of excessive pro-oxidants from symbiont to host and highlights the potential role that the antioxidative capacity of *Symbiodinium* plays in the regulation of coral health (Lesser 1997, Downs et al. 2002).

Oxidative stress is defined as "disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage" (Sies 1991), and has been identified as one key factor in explaining fundamental processes of cellular senescence in organisms (Finkel and Holbrook 2000). A number of oxygen intermediates are generated in the pathways of aerobic metabolism or photosynthesis (Foyer et al. 1994, Halliwell and Gutteridge 2007, Navrot et al. 2007) and a complex antioxidant network, which is comprised of enzymatic and non-enzymatic pathways, maintains the redox balance as a crucial element of cellular homeostasis (Apel and Hirt 2004). The increased production of ROS in photosynthetic systems under environmental stress has been shown to be the result of light-induced processes such as the energy transfer reaction between triplet chl and ground-state oxygen, generating singlet oxygen ($^1O_2$; Krieger-Liszkay 2005), as well as suppressed repair of components of photosystem II by ROS (Takahashi et al. 2004, Nishiyama et al. 2006). While singlet oxygen can be quenched non-enzymatically by carotenoids and tocopherols (Krieger-Liszkay 2005), the enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) primarily scavenge the oxygen intermediates superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Regeneration of ascorbate via the Foyer-Halliwell-Asada cycle is tightly linked to the glutathione sys-

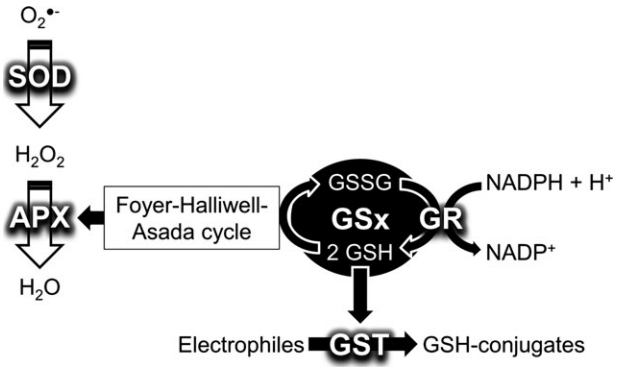

FIG. 1. Connection of elements of the antioxidant network investigated in this study, disregarding subcellular localization. Superoxide dismutase (SOD), ascorbate peroxidase (APX), total glutathione content (GSx), glutathione disulphide (GSSG), reduced glutathione (GSH), glutathione reductase (GR), glutathione S-transferase (GST). Shown are enzymes of the superoxide removal pathway (white arrows) and pathways involving glutathione (black arrows).

tem (Asada 2006, Foyer and Noctor 2011, Fig. 1). The tripeptide glutathione exists in its reduced (GSH) and oxidized forms (GSSG), and through the activity of glutathione reductase (GR), more than 90% of the glutathione pool is usually maintained as GSH (Halliwell and Gutteridge 2007). Glutathione is also involved in the removal of xenobiotics and organic peroxides via the activity of glutathione S-transferase (GST), and plays an important role in protein modification and cell signaling (Foyer and Noctor 2011).

The potential involvement of oxidative stress in *Symbiodinium* stress physiology and coral bleaching has been widely acknowledged (Weis 2008, Lesser 2011), however, few studies have investigated the potential link between genetic diversity in *Symbiodinium*, antioxidant features, and thermal sensitivity (Suggett et al. 2008, McGinty et al. 2012). Here, we tested whether the activity of elements of the antioxidant network fundamentally differs between four *Symbiodinium* types in culture, and whether viability at sublethal and lethal temperatures is connected to the response of the ROS-removal pathway and glutathione system. This is the first study to view the antioxidant response of *Symbiodinium* from a network perspective in order to investigate the relative importance of each antioxidant as part of the dynamic response, and to identify temperature-induced activity shifts within the network.

MATERIALS AND METHODS

Experimental setup. Batch cultures of *Symbiodinium* B1 (culture ID Ap1), C1 (CCMP2466), E (CCMP421) and F1 (Mv), maintained continuously at 25°C, were exposed to temperatures of 25.1°C ± 0.6°C, 29.0°C ± 0.2°C and 33.3°C ± 0.3°C over 14 d in temperature-controlled tanks. Genetic identity of cultures was confirmed via ITS2 sequencing following published protocols (Logan et al. 2010). Since the primary focus of this study was to link varying degrees of thermal sensitivity to antioxidant plasticity, a temperature tolerant *Symbiodinium* type (F1) was compared to three sensitive types, which included two symbiotic members from the major clades of coral symbionts in the Indo-Pacific (C) and Caribbean (B and C), as well as a pre-dominantly non-symbiotic type (E) (sensu *Symbiodinium voratum*; Jeong et al. 2014). The chosen experimental settings were based on preliminary experiments to illustrate the response at sublethal and lethal stress levels covering the immediate (<3 d) and long-term (up to 2 weeks) response.

Cultures were grown in 2 L round-bottom flasks, containing silica-free sterile f/2-medium (pH 8.1; Guillard and Ryther 1962), based on synthetic seawater (salinity 34; Instant Ocean sea salt, Spectrum Brands Inc., Middleton, WI, USA). Cultures were aerated with filter-sterilized air (0.22 μm) under a constant 12:12 light:dark cycle at 40–50 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} PAR (photosynthetically active radiation; cool white fluorescent tubes, Philips 36W/840). Cultures were tested simultaneously, after 10 d of acclimation, and the experiment was replicated four times. After sampling on Day 0, temperatures were increased during the dark phase by 1.67 ± 0.40°C $\cdot$ h^{-1} (29°C tank) and 1.44 ± 0.30°C $\cdot$ h^{-1} (33°C tank), and monitored via HOBO Pendant® data loggers (Onset Computer Corporation, Cape Cod, MA, USA). Initial cell density was ~1 × 10^5 cells $\cdot$ mL^{-1} and cultures were sampled in 50 mL aliquots (N = 9 per culture) on Days 0, 1, 3, 7 and 14. Aliquots were pelleted (5 min 2,000*g*, 25°C) and immediately flash frozen and stored at −80°C. To avoid nutrient limitation and account for removed sample volume, flasks were replenished with 500 mL of f/2-medium after Day 3 (for B1, E and F1) and Day 7 (all cultures). In order to obtain enough biomass for sampling on Day 7, *Symbiodinium* C1 was not replenished on Day 3, because of its smaller size and slower growth.

Cell viability. Growth, F_v/F_m and chl *a* content were monitored as proxies for cell viability. F_v/F_m was measured using an Imaging-PAM chl fluorometer (MI 7-10, SI 10, Gain 1-3, Damp 2-3, saturating width 0.8 s; Heinz Walz GmbH, Effeltrich, Germany). An aliquot of concentrated cell suspension (obtained by centrifugation for 5 min 2,000*g*, 25°C) was measured (technical triplicates) after 20 min dark acclimation in a black flat-bottom microtiter plate. Cell density was assessed by hemocytometer counts (N = 6) and the growth rate (μ) determined. While the chosen light settings might be considered low, obtained growth rates matched previously published values for higher irradiances (e.g., Robison and Warner 2006). Chl *a* of one 50 mL pellet per culture was extracted over 48 h in 1–2 mL *N,N*-dimethylformamide at 4°C in the dark. For spectrophotometric measurements, extracts were centrifuged (3 min, 3,900*g*) and 200 μL of the supernatant measured (technical triplicates) at 646.8, 663.8 and 750 nm in 96-well plates (UVStar, Greiner Bio-One GmbH, Frickenhausen, Germany). Chl *a* concentration was determined after optical path length correction (0.555 cm; Porra et al. 1989).

Cell lysis. For enzyme measurements, five of the frozen pellets per time point were pooled and resuspended in 850 μL cold lysis buffer (50 mM KH_2PO_4/K_2HPO_4, 0.1 mM EDTA, 10% [v/v] glycerol, protease inhibitor [P9599; Sigma-Aldrich, St. Louis, MO, USA], pH 7.0). After addition of 200 mg glass beads (710–1,180 μm; Sigma-Aldrich), cells were lysed in a bead mill (50 Hz, 3 min, 4°C; Qiagen tissue lyser, Qiagen, Hilden, Germany). All lysates were centrifuged (16,000*g*, 5 min, 4°C) and supernatants aliquoted and frozen at −80°C until further analysis. Total aqueous soluble protein content was determined using the improved Bradford assay with BSA as standard (Zor and Selinger 1996). For glutathione determination, samples were processed within a week after sampling. Two frozen 50 mL pellets were washed, pooled in 350 μL 5% [w/v] 5-sulfosalicylic acid and lysed as above. Lysates were centrifuged (16,000*g*, 5 min, 4°C) and the protein-free supernatant used for determination of total glutathione (GSx) and glutathione disulphide (GSSG) content. Renaturation of protein pellets was done in 0.2 M Tris on an orbital shaker (10 min, 4°C). After centrifugation (16,000*g*, 5 min, 4°C), total aqueous soluble protein content was measured as before.

Glutathione content. GSx was assessed using the 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB)-recycling assay in a microtiter format (Baker et al. 1990). For GSx determination 20 μL of sample or glutathione standard (0–500 pmol GSSG/20 μL) were used in a 200 μL reaction mixture containing potassium phosphate (90 mM, pH 7.5), EDTA (0.9 mM), GR (1 U $\cdot$ mL^{-1}), DTNB (0.15 mM) and NADPH (0.2 mM). Change in absorbance after addition of NADPH was monitored at 412 nm for 3 min at 25°C (technical triplicate), using a microtiter plate reader (Enspire® 2300; Perkin-Elmer, Waltham, MA, USA). Glutathione disulphide was determined after derivatization of GSH with 1-methyl-2-vinylpyridinium triflate (M2VP; Shaik and Mehvar 2006). An aliquot (80 μL) of the GSx sample was incubated with 10 μL M2VP (33 mM) and 10 μL 90% (v/v) triethanolamine for 5 min at room temperature. Standard amounts of GSSG (0–150 pmol

GSSG · 20 μL^{-1}) were treated the same way, and samples and standards subjected to the same recycling assay as above.

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). Twenty microliters of lysate or SOD standard (0.5–500 U · mL^{-1}) were measured as technical triplicates in a final reaction mixture of 300 μL potassium phosphate buffer (50 mM, pH 7.8) containing EDTA (0.1 mM), Rf (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 homogenous light field (130 μmol photons · m^{-2} · s^{-1}) at 25°C. Standards and samples were measured using the same reaction mixture and a sigmoidal 5-parameter semi logarithmic standard curve (24 standard levels) used to determine SOD activity of samples. One unit of SOD activity was defined as the amount of enzyme that inhibits the NBT reduction by 50%.

Ascorbate peroxidase. APX activity was assessed by monitoring the oxidation of ascorbate at 290 nm over 3 min at 25°C, 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 H_2O_2 (0.1 mM) (Nakano and Asada 1981) using a temperature-controlled cuvette spectrophotometer (UV-Vis Spectrophotometer UV-2550; Shimadzu Corp., Kyoto, Japan). APX activity was determined with $\varepsilon = 2.8\ mM^{-1} \cdot cm^{-1}$.

Glutathione reductase. GR activity was measured according to the DTNB method, using 20 μL lysate in a final reaction mixture of 200 μL (as technical triplicates), containing sodium phosphate buffer (100 mM, pH 7.5), EDTA (1 mM), DTNB (0.75 mM), NADPH (0.1 mM) and GSSG (1 mM) (Smith et al. 1988, Cribb et al. 1989). Increase in absorbance at 412 nm over 3 min at 25°C was monitored and GR activity calculated ($\varepsilon = 14.151\ mM^{-1} \cdot cm^{-1}$ with d = 0.571 cm).

Glutathione S-transferase. GST activity was determined via GSH conjugation with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm over 3 min at 25°C (Habig et al. 1974). A 20 μL aliquot of lysate was incubated in a final reaction mixture of 200 μL (measured as technical triplicate), containing sodium phosphate buffer (100 mM, pH 6.5), CDNB (1 mM) and GSH (2 mM), and GST activity calculated ($\varepsilon = 9.6\ mM^{-1} \cdot cm^{-1}$ with d = 0.571 cm).

After checking that protein content per cell did not significantly change in any of the treatments, all enzyme activities were normalized to protein content and expressed as specific activity (U · mg^{-1}).

Statistical analysis. PAM data were arcsine square root transformed and analyzed for "time," "type" and "temperature" effects using a repeated measures analysis of variance (rmANOVA). Normality of data was confirmed with the Shapiro–Wilk test and sphericity assumptions tested using Mauchly's sphericity test. In case sphericity was violated, results of Pillai's trace test or the epsilon-adjusted univariate *F*-test (Greenhouse-Geisser) at a confidence level of $P = 0.05$ are reported. For chl and enzyme data, multivariate analysis of variance (MANOVA) on linear slopes over time was performed due to missing data points (death or non-detectable levels). Statistical effects of temperature on antioxidant variables are reported for the complete dataset using the factors "type," "temperature", and "type x temperature," as well as for individual *Symbiodinium* types using ANOVA or Welch ANOVA (with *post hoc* Tukey HSD test). All data sets were checked for autocorrelation using the Durbin–Watson test.

Antioxidant baseline levels at 25°C were compared between types via ANOVA or Welch ANOVA with post hoc Tukey HSD tests for Day 0 data. A linear discriminant function analysis (DFA) was performed to test how well baseline activities of a set of antioxidants would delineate *Symbiodinium* types, using the complete 25°C dataset over 14 d to include the natural variation. Principal components analysis (PCA), using a correlation matrix, was performed to visualize the antioxidant network response for each type. For missing values, the implemented imputation method of JMP was employed and score plots with imputation are shown. For DFA and PCA, only Eigenvalues larger than one were considered. Data were analyzed using JMP 10.0.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Cell viability. Overall, significant differences in F_v/F_m, based on all main factors and their interaction, were found (rmANOVA, $P < 0.001$). While *Symbiodinium* F1 survived at 33°C for 14 d without a decline in growth or F_v/F_m, all other types died within the second week at this temperature, with F_v/F_m values on Day 7 corresponding to 29% (B1), 30% (C1) and 45% (E) of the initial value (Fig. 2). A temperature of 29°C had no effect on F_v/F_m in any of the *Symbiodinium* types, while growth was impacted in just one type, B1, which suffered a significant reduction of 35% during the second week (Wilcoxon test, $\chi^2 = 5.6333$, $P = 0.0176$; Fig. 2A). Chl *a* content (pg · $cell^{-1}$) on Day 0 was significantly different between each type, with *Symbiodinium* E (3.61 ± 0.21) > F1 (2.46 ± 0.25) > B1 (1.14 ± 0.18) > C1 (0.30 ± 0.22) (Welch ANOVA, $F_{3\ 20.934} = 102.499$, $P < 0.0001$). A significant decline in chl *a* concentration was only observed for B1 at 33°C, where levels decreased by 21% over the first week ($F_{2,13} = 15.525$, $P = 0.0004$, data not shown).

Baseline antioxidants levels. Constitutive levels of antioxidants at 25°C varied significantly between all *Symbiodinium* types (Table 1) with the largest differences observed in the glutathione content and GST activity. Type C1 had a five-fold larger glutathione pool than all other types, and GST activity in B1 was an order of magnitude smaller than in E and F1. A DFA based on constitutive antioxidant features identified all four *Symbiodinium* types (Fig. 3; Pillai's trace, $F_{15,159} = 24.207$, $P < 0.0001$). Separation of types was driven by GR, GSx, and GST along discriminant function 1, and GSx and APX along discriminant function 2. While E and F1 overlap within these two functions, the statistical prediction was accurate enough to separate these two types, mainly due to SOD activity, which discriminates along a third function (misclassification of E in predicted F1 was 33%; F1 in predicted E was 7%). Baseline values were most similar for SOD activity, and variability between types increased in the order APX < GR < GST < GSx (based on the ratio between range and mean, Table 1; also visible as relative length of biplot rays, Fig. 3).

Except for GST, changes in all antioxidants were significantly affected by temperature, and these effects were type-specific for the response of

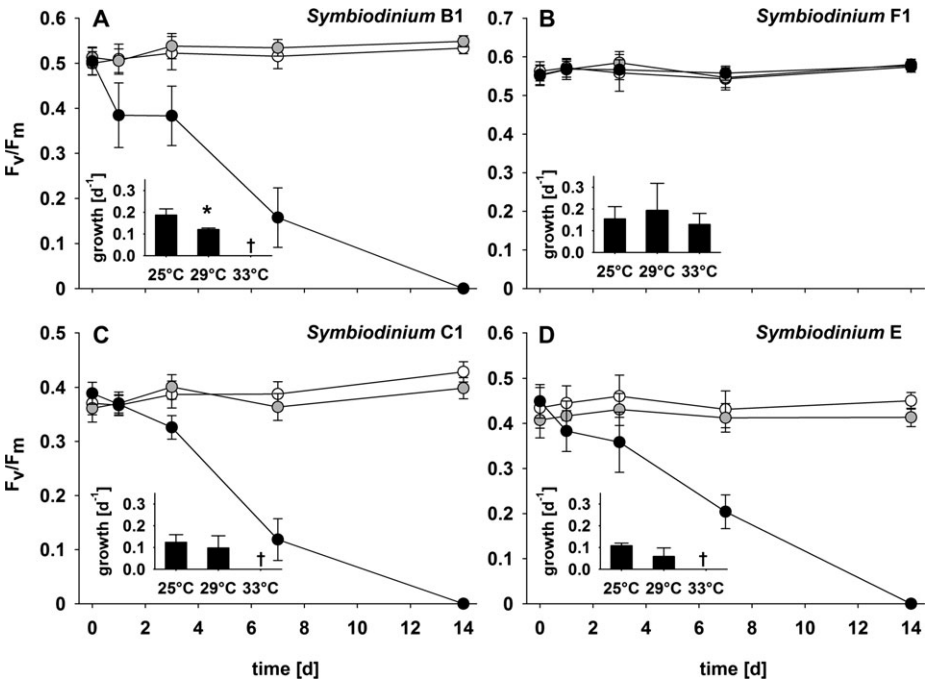

FIG. 2. Viability of *Symbiodinium* types B1 (A), F1 (B), C1 (C), E (D) over time in response to thermal treatments. Maximum quantum yields (F_v/F_m) at 25°C (white), 29°C (gray) and 33°C (black) shown as interpolated scatter plots, with growth rates [d^{-1}] between Days 7 and 14 shown as bar graphs (insert). For growth, significant differences ($P < 0.05$) between treatments are indicated by asterisks, and negative growth rates and subsequent cell death are indicated by daggers. Values are means ± SEM; *Symbiodinium* B1 ($N = 5$–6), C1 ($N = 3$–4), E ($N = 4$), F1 ($N = 4$–5). Note different scaling of axes.

TABLE 1. Constitutive levels and activities of antioxidants in *Symbiodinium* types under control conditions on Day 0 (25°C, 40–50 μmol photons · m^{-2} · s^{-1}).

Symbiodinium strain (ITS2 type)	SOD (U · mg^{-1}) $P < 0.0014$‡	APX (U · mg^{-1}) $P < 0.0001$‡	GSx (nmol · mg^{-1}) $P < 0.0001$	GR (U · mg^{-1}) $P < 0.0001$‡	GST (U · mg^{-1}) $P < 0.0001$‡
Ap1 (B1)	80.31 ± 5.43^{b}	0.50 ± 0.04^{c}	64.62 ± 7.49^{a}	0.40 ± 0.03^{a}	0.037 ± 0.008^{c}
CCMP2466 (C1)	82.85 ± 3.75^{a,b}	0.83 ± 0.08^{b}	319.60 ± 32.77^{b}	0.13 ± 0.03^{b}	0.14 ± 0.03^{b}
CCMP421 (E)	63.14 ± 7.18^{b}	0.77 ± 0.05^{b}	75.30 ± 8.11^{a}	0.13 ± 0.01^{b}	0.48 ± 0.06^{a}
Mv (F1)	106.75 ± 6.91^{a}	1.44 ± 0.05^{a}	68.49 ± 5.80^{a}	0.15 ± 0.02^{b}	0.50 ± 0.06^{a}
Range/mean ratio	0.52	1.05	1.93	1.32	1.61

All values are normalized to protein content and expressed as means ± SEM. Superoxide dismutase (SOD; $N = 9$–16), ascorbate peroxidase (APX; $N = 9$–16), glutathione content (GSx; $N = 10$–13), glutathione reductase (GR; $N = 11$–16), glutathione S-transferase (GST; $N = 12$–15). Probability values are based on ANOVA or Welch ANOVA (‡) tests between types. Types not connected by the same letter within each column are significantly different based on the post hoc Tukey HSD test. Range/mean ratios were calculated from the means shown for each type.

total glutathione content and GR (Table 2). While differing trends in SOD, APX and GSH/GSx (the amount of reduced glutathione relative to the total glutathione pool) for each type and temperature were observed, these effects were marginally non-significant (for GSH/GSx and SOD) or non-significant (APX, GST) in the overall dataset (Table 2).

Antioxidant responses. Temperature had a significant effect on all measured antioxidant variables in *Symbiodinium* B1 (Table 3). It was the only type that responded to a sublethal temperature of 29°C by temporarily (on Day 7) increasing APX activity (Fig. 4B, rmANOVA with 25°C, 29°C, $F_{\text{Temp}(1,9)} = 5.154$, $P = 0.0493$, $F_{\text{Time x Temp}(1.6001,14.401)} = 1.546$, $P = 0.2444$) and GSx content (Fig. 5A; $F_{\text{Temp}(1,7)} = 4.298$, $P = 0.0769$, $F_{\text{Time x Temp}(4,4)} = 9.008$, $P = 0.0280$). While both, APX activity and GSx content, subsequently returned to control levels, GR activity tended to increase over 2 weeks at this temperature with no change in the redox state of glutathione. SOD and APX were significantly correlated at both 29°C ($r = 0.49$, $P = 0.0116$, $N = 26$) and 33°C ($r = 0.67$, $P = 0.0024$, $N = 18$), though a consistently significant up-regulation was only observed at 33°C, with maximum activities for both enzymes observed on Day 7 (165% and 237% of Day 0 value, respectively; Fig. 4, A and B). Glutathione content increased to 198.7 ± 32.5 nmol · mg^{-1} protein after 3 d at 33°C (a 290% increase; note that GSx levels were not detectable beyond Day 3). GR activity, however, declined by 50% from its initial value, and a significant difference between the 29°C and 33°C treatments was observed on Day 7 (ANOVA post hoc Tukey HSD, $F_{2,11} = 8.818$, $P = 0.0052$), though neither was significantly different from the control. *Symbiodinium* B1 was the only type that exhibited elevated GST activity in response to 33°C over 7 d (a 175% increase; Fig. 6B; Table 4).

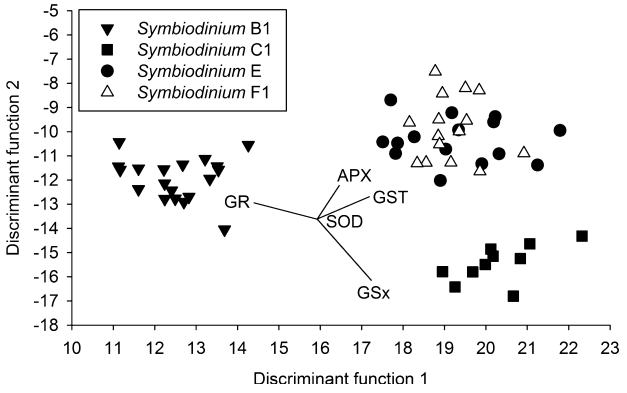

FIG. 3. Linear discriminant function analysis of *Symbiodinium* types based on antioxidant levels at 25°C, integrating enzymatic activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione S-transferase (GST) and the glutathione pool size (GSx) over 2 weeks for *Symbiodinium* B1 (closed triangles), C1 (squares), E (circles) and F1 (open triangles). Values are canonical scores for two discriminant functions that explain 96.4% of the variation. Biplot rays indicate the coordinate direction for each antioxidant. SOD is directed into the plane and mainly discriminates along a third function.

Symbiodinium C1, similarly to B1, showed a significant positive correlation for SOD and APX irrespective of temperature ($r = 0.58$, $P < 0.0001$, $N = 42$). Significantly elevated activities for both enzymes were, however, only observed on Day 7 at 33°C (ANOVA post hoc Tukey HSD, SOD: $F_{2,6} = 9.965$, $P = 0.0124$; APX: $F_{2,6} = 27.182$, $P = 0.0010$; Fig. 4, C and D). While *Symbiodinium* C1 exhibited the largest glutathione pool, its glutathione redox state was much lower (GSH/GSx ~0.50) compared to the other types (~0.90). Statistically, temperature had no significant effect on glutathione content, but levels tended to increase at 33°C and peaked at 553.2 ± 97.5 nmol · mg^{-1} (147%) on Day 7 (Fig. 5C). This increase in total glutathione was accompanied by a significant drop of 0.11 in the redox state (Table 3, Fig. 5D). No significant changes in either GR or GST were observed at any temperature.

Symbiodinium E was the only strain that showed no changes in APX activity or GSx levels at 33°C, despite significantly increasing SOD activities (by 165% on Day 7, Figs. 4, E and F; 5E). The GSH/GSx value declined significantly by 0.30 between Days 0 and 7 at 33°C (Fig. 5F), but no changes in GR or GST were observed (Table 3).

Symbiodinium F1 expressed significant increases in SOD (217%) and APX activity (242%) at 33°C and both enzymes were significantly positively correlated at this temperature ($r = 0.74$, $P < 0.0001$, $N = 25$; Fig. 4, G and H). However, statistical *post hoc* support for higher activities at 33°C was only found for APX. *Symbiodinium* F1 significantly increased its glutathione content at 33°C (471%), with no statistical changes in its glutathione redox ratio (Fig. 5, G and H; Table 3). The GSH/GSx value did, however, drop by 0.08 between Days 0 and 1 and the overall GSH/GSx value over 14 d was significantly reduced at 33°C (rmANOVA, $F_{\mathrm{Temp}(2,7)} = 6.573$, $P = 0.0247$; $F_{\mathrm{Time\ x\ Temp}(8,10)} = 0.862$, $P = 0.575$, Fig. 4H). GR activity did not change during the first week in any treatment, but reached 0.41 ± 0.06 U · mg^{-1} on Day 14 at 33°C. It was therefore about twice the control value (ANOVA post hoc Tukey HSD, $F_{2,10} = 4.824$, $P = 0.0341$, data not shown). No changes in GST activity were observed in any of the treatments.

TABLE 2. Statistical effects of the factors "temperature" and "type" on measured variables of the antioxidant network in *Symbiodinium*. MANOVA test results on changes over time are reported based on the complete dataset over 14 d. Significant effects are indicated by asterisks.

Variable	Type	Temperature	Type x Temperature
SOD	$F_{3,3} = 0.052$, $P = 0.9841$	$F_{2,2} = 18.659$, $P < 0.0001$*	$F_{6,6} = 2.119$, $P = 0.0766$
APX	$F_{3,3} = 1.481$, $P = 0.2351$	$F_{2,2} = 11.265$, $P = 0.0001$*	$F_{6,6} = 1.679$, $P = 0.1530$
GSx	$F_{3,3} = 0.951$, $P = 0.4275$	$F_{2,2} = 15.646$, $P < 0.0001$*	$F_{6,6} = 2.569$, $P = 0.0381$*
GSH/GSx	$F_{3,3} = 0.505$, $P = 0.6818$	$F_{2,2} = 25.172$, $P < 0.0001$*	$F_{6,6} = 2.245$, $P = 0.0614$
GR	$F_{3,3} = 2.488$, $P = 0.0735$	$F_{2,2} = 4.344$, $P = 0.0193$*	$F_{6,6} = 9.479$, $P < 0.0001$*
GST	$F_{3,3} = 2.270$, $P = 0.0951$	$F_{2,2} = 0.044$, $P = 0.9572$	$F_{6,6} = 1.033$, $P = 0.4185$

TABLE 3. Statistical effects of temperature on measured variables of the antioxidant network for individual *Symbiodinium* types (strain designation with ITS2 type). ANOVA/Welch ANOVA test results on changes over time are reported based on the individual type data sets. Significant effects are indicated by asterisks.

	Ap1 (B1)	CCMP2466 (C1)	CCMP421 (E)	Mv (F1)
SOD	$F_{2,12} = 16.678$, $P = 0.0003$*	$F_{2,6} = 0.996$, $P = 0.4332$	$F_{2,9} = 14.782$, $P = 0.0014$*	$F_{2,10} = 6.8259$, $P = 0.0135$*
APX	$F_{2,13} = 12.953$, $P = 0.0008$*	$F_{2,6} = 0.001$, $P = 0.9989$	$F_{2,9} = 2.382$, $P = 0.1479$	$F_{2,10} = 7.4983$, $P = 0.0102$*
GSx	$F_{2,5.2663} = 5.2663$, $P = 0.0047$*	$F_{2,3.931} = 4.9915$, $P = 0.0834$	$F_{2,7} = 0.068$, $P = 0.9350$	$F_{2,7} = 8.368$, $P = 0.0139$*
GSH/GSx	$F_{2,10} = 11.545$, $P = 0.0025$*	$F_{2,8} = 6.1770$, $P = 0.0239$*	$F_{2,3.6873} = 26.542$, $P = 0.0065$*	$F_{2,7} = 0.485$, $P = 0.6350$
GR	$F_{2,13} = 14.941$, $P = 0.0004$*	$F_{2,9} = 0.397$, $P = 0.6836$	$F_{2,9} = 1.596$, $P = 0.2503$	$F_{2,10} = 6.497$, $P = 0.0155$*
GST	$F_{2,11} = 5.438$, $P = 0.0228$*	$F_{2,9} = 0.238$, $P = 0.7930$	$F_{2,9} = 1.847$, $P = 0.2127$	$F_{2,10} = 1.024$, $P = 0.3911$

TABLE 4. Main response of variables related to viability and antioxidant defenses in different *Symbiodinium* types over 14 d at 33°C. Trends are indicated as increase (↑), decrease (↓), or no change (–) relative to the control treatment (25°C). Declining growth rates with subsequent death are indicated by a dagger. Responses to sublethal temperatures (29°C), amplitude of changes, and statistical support are outlined in the text.

	Ap1 (B1)	CCMP2466 (C1)	CCMP421 (E)	Mv (F1)
F_v/F_m	↓	↓	↓	–
Growth	↓(†)	↓(†)	↓(†)	–
Chl *a*	↓	–	–	–
SOD	↑	↑	↑	↑
APX	↑	↑	–	↑
GSx	↑	↑	–	↑
GSH/GSx	↓	↓	↓	–
GR	↓	–	–	↑
GST	↑	–	–	–

Antioxidant network shifts. Integrating the response of all antioxidants into two principal components (PC; two sets of values that account for the majority of the variance) illustrated the main activity shifts in the antioxidant network in response to temperature. Although cumulative variation explained by the first two PC was similar for all types (72.4% for B1, 69.3% for C1, 73.2% for E and 79.8% for F1), a clear thermal or temporal pattern that involved multiple components of the network could only be identified for *Symbiodinium* B1 and F1 (Fig. 7 and Fig. S1 in the Supporting Information). The main shifts in the antioxidant network of *Symbiodinium* B1 at 29°C involved a generally higher GR activity after Day 3 (main vector in PC2). This pattern was reversed at 33°C, where the system showed much lower GR activity compared to 29°C, but expressed

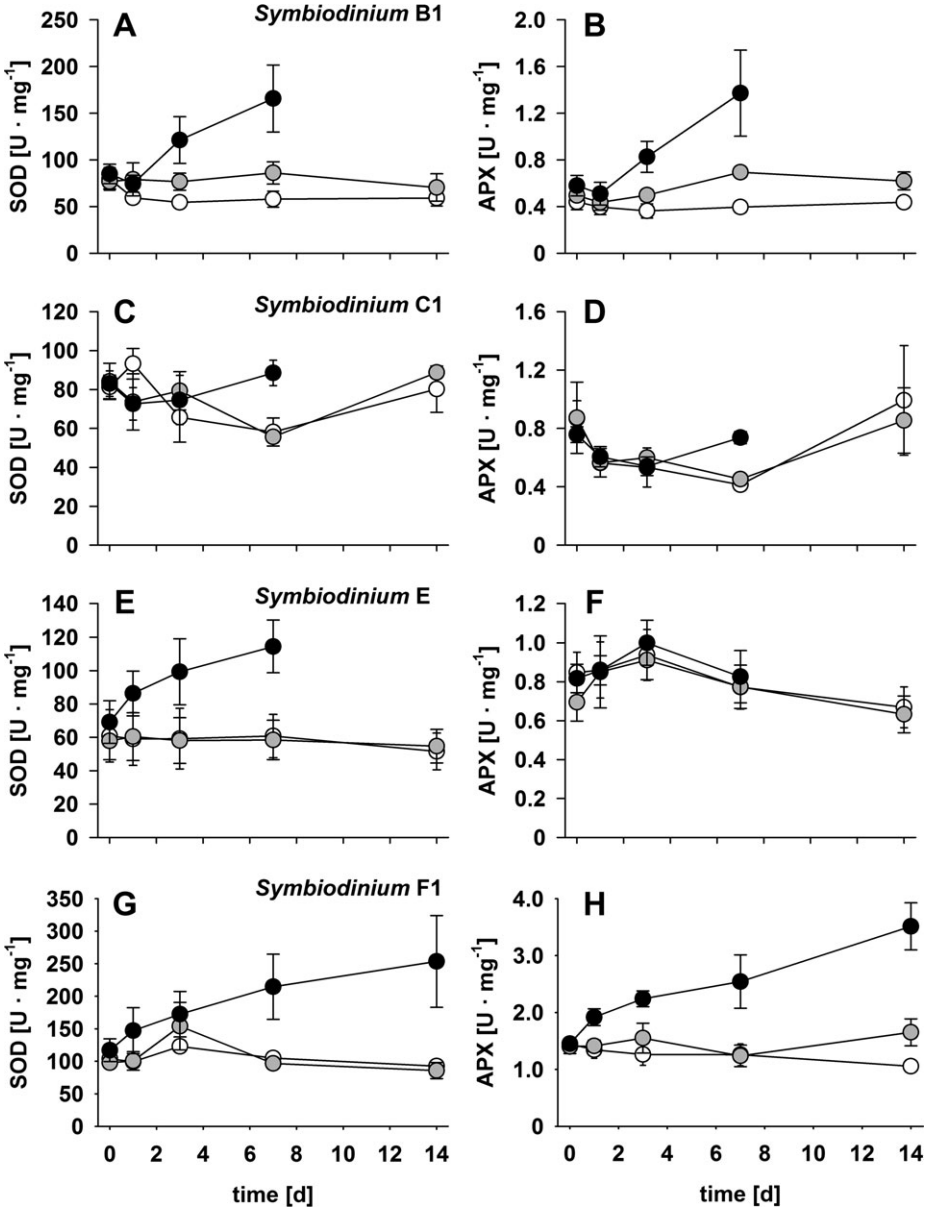

FIG. 4. Superoxide dismutase (SOD, left) and ascorbate peroxidase (APX, right) activity as units per mg protein of *Symbiodinium* types B1 (A and B), C1 (C and D), E (E and F) and F1 (G and H) over 14 d at 25°C (white), 29°C (gray) and 33°C (black). *Symbiodinium* B1, C1 and E died after 7 d at the highest temperature. Values are means ± SEM. *Symbiodinium* B1 ($N = 5$–6), C1 ($N = 3$), E ($N = 4$–5), F1 ($N = 4$–5). Note different scaling of axes.

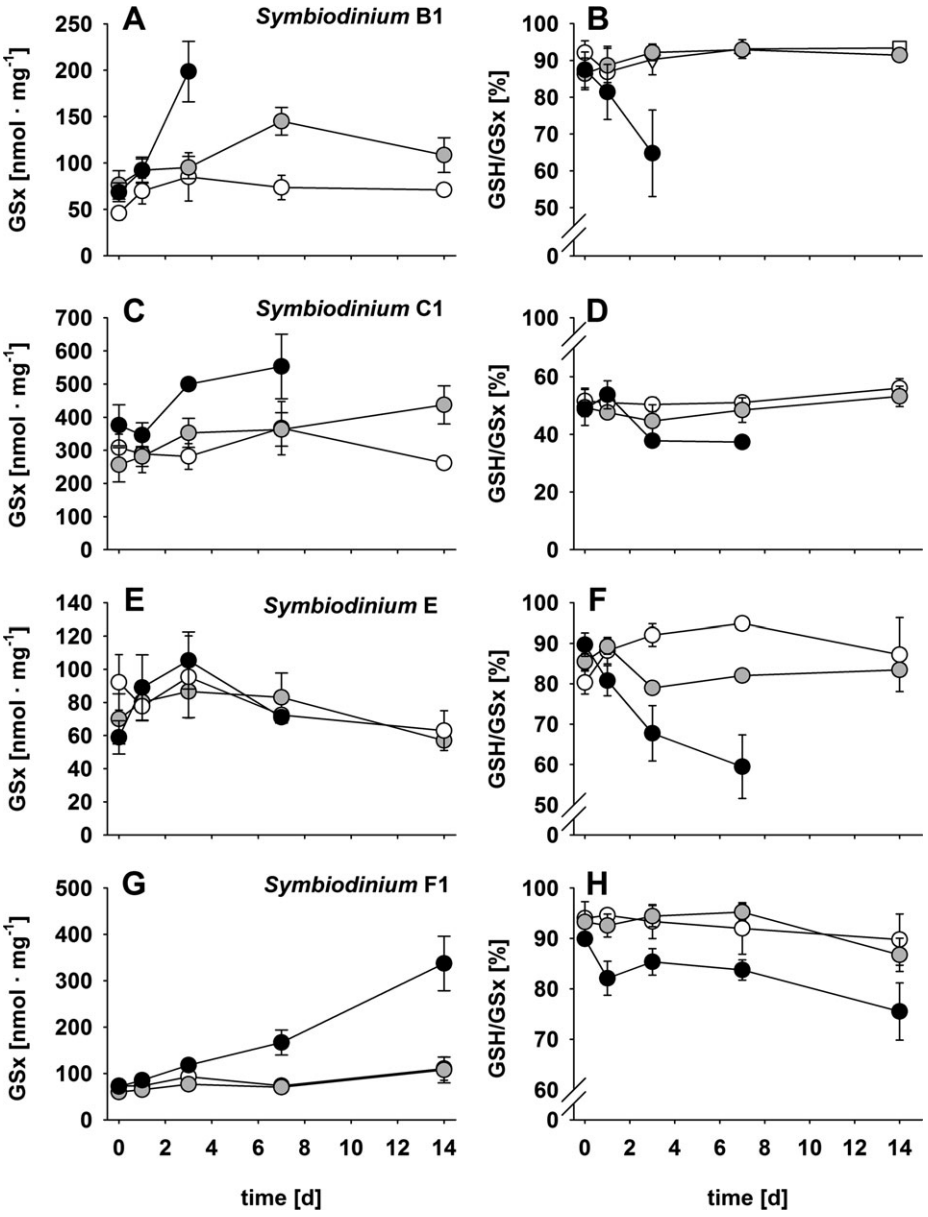

FIG. 5. Glutathione pool size (left; nmol per mg protein) and corresponding redox state (right) for *Symbiodinium* types B1 (A and B), C1 (C and D), E (E and F) and F1 (G and H) at 25°C (white), 29°C (gray) and 33°C (black) over 14 d of exposure. *Symbiodinum* B1, C1 and E died after 7 d at 33°C. Values are means ± SEM. *Symbiodinium* B1 ($N = 4–5$), C1, E and F1 (all $N = 3–4$). Note different scaling of axes.

much higher activity in the SOD/APX pathway as well as the GSx/GST system (correlated vectors in PC1; Fig. 7). For *Symbiodinium* F1, only exposure to 33°C caused an activity shift, which was mainly characterized by increased activity in the SOD/APX pathway and higher glutathione values, rather than any change in the enzymes associated with the glutathione pool (or only a small change in the case of GR). After 14 d at 33°C, a small cluster containing four of the five replicates can be seen in quadrant IV, characterized by high score values for SOD, APX, and GSx (Fig. 7).

DISCUSSION

Integrating the responses of multiple components of the antioxidant network is essential for understanding the specific physiological impact that thermal stress has on different *Symbiodinium* types. This study revealed that constitutive levels of antioxidants and responses of the network differ between types, and that a thermal challenge is not necessarily detected in all parts of the antioxidant system. Activity within different pathways was highly diverse, ranging from solely increased SOD activity in *Symbiodinium* E at 33°C to up- or down-regulation of all investigated components in *Symbiodinium* B1. The most tolerant type, *Symbiodinium* F1, exhibited the largest proportional up-regulation of most antioxidants, whereas the most sensitive type, B1, already exhibited increased glutathione recycling and reduced growth at the sublethal temperature of 29°C. In all three susceptible types, declining F_v/F_m with subsequent cell death was consistently associ-

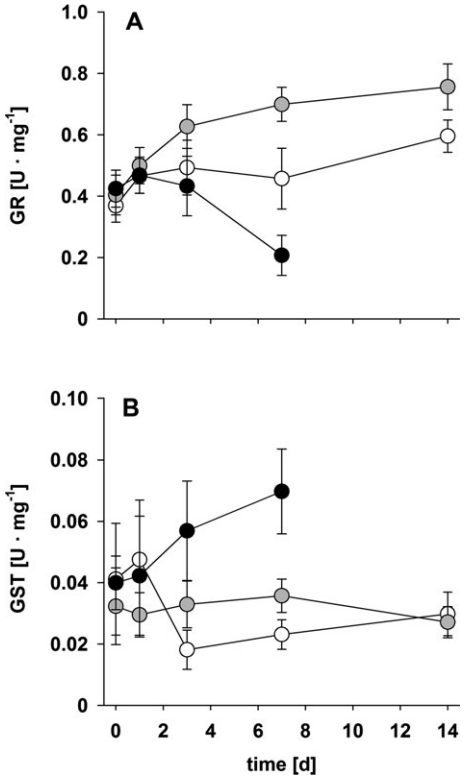

FIG. 6. Activity of glutathione reductase (GR; A) and glutathione S-transferase (GST; B) in units per mg protein at 25°C (white), 29°C (gray) and 33°C (black) in *Symbiodinium* B1 over 14 d. Culture died after 7 d at 33°C. Values are means ± SEM ($N = 5–6$).

ated with an increasing oxidation of the glutathione pool.

Antioxidant profiles are type specific in Symbiodinium. The observed differences in the baseline activities of antioxidants are not surprising, given the high degree of evolutionary radiation in the genus *Symbiodinium* (LaJeunesse 2005). However, the DFA approach shows that features of their antioxidant network were sufficient to distinguish the four *Symbiodinium* types, giving each a unique antioxidant profile. Our analysis confirms previous observations on type-specific differences in constitutive antioxidant activity (Lesser 2011, McGinty et al. 2012), but also highlights that variability between types is stronger in downstream systems such as the glutathione system. The reasons are unclear, but SOD provides the primary line of defense against superoxide and therefore affects the amount of ROS in downstream defense lines, which are connected to other systems such as the ascorbate or glutathione system. In contrast to superoxide scavenging, a number of enzymes can scavenge H_2O_2, which provides functional redundancy and compartmentalized protection from organelle to organelle. Intrinsic genetic redundancy (i.e., two or more genes have the same proteomic function, and modification or inactivation has no or only a small effect on the biological phenotype; Nowak et al. 1997) has been shown for *Arabidopsis* knockout mutants, where other components of the antioxidant network tend to be overexpressed in order to compensate for specific deficiencies due to the suppressed expression of individual antioxidants (Mittler et al. 2004). In tobacco plants, a lack or reduced expression of catalase can be compensated for by increased expression of APX or glutathione peroxidase (GPX; Willekens et al. 1997), whereas APX deficiency results in increased SOD, CAT and GR activity (Rizhsky et al. 2002). Here, processes in downstream system such as the glutathione system were a key difference between the most tolerant and susceptible types, F1 and B1, where relative increases in SOD activity were similar. It has to be acknowledged, however, that glutathione has multiple functions and that, differences in baseline glutathione pool size and the activities of related enzymes could also be the result of other cellular processes.

Photosynthesis and the SOD-APX pathway. Previous studies have shown that increased total SOD activity in thermally sensitive *Symbiodinium* types in vitro and in hospite is generally related to the impairment of photosynthesis (Lesser 1996, Yakovleva et al. 2004, Richier et al. 2005) and declining photosynthetic efficiency as result of thermal stress is a well-known effect in *Symbiodinium* (e.g., Iglesias-Prieto et al. 1992, Robison and Warner 2006). Excessive temperature stress has been associated with Benson–Calvin cycle inhibition, alteration of thylakoid membrane properties with energetic uncoupling, and lower D1 protein turnover and PSII dysfunction in *Symbiodinium* (Jones et al. 1998, Takahashi et al. 2004, Tchernov et al. 2004, Hill and Ralph 2008). We interpret higher SOD activities (though not significant for F1) in conjunction with rapidly declining F_v/F_m values in B1, C1, and E, as indicative of an increased generation of superoxide via the Mehler reaction. In *Symbiodinium* F1, increased metabolic turnover with temperature, though not apparent in its growth rate, might have contributed to the observed increase in SOD activity. Since we did not assess the relative contribution of superoxide from chloroplastic, mitochondrial and cytosolic sources, this remains, however, speculative and requires further investigation. Interestingly, *Symbiodinium* E was the only type where SOD showed no significant correlation with APX at 33°C and no obvious H_2O_2 scavenging via APX and the ascorbate-glutathione pathway. While several other enzymes can scavenge H_2O_2 in dinoflagellates (catalase peroxidase, peroxiredoxins, and potentially Se-containing glutathione peroxidase; Osaka et al. 2003, Foyer and Shigeoka 2011, Bayer et al. 2012), APX has a much smaller K_m-value (μM range) than, for example, eukaryotic catalases (mM) and is regarded as more responsive to small changes in H_2O_2 levels. Since peroxiredoxin-encoding genes have been found in a number of

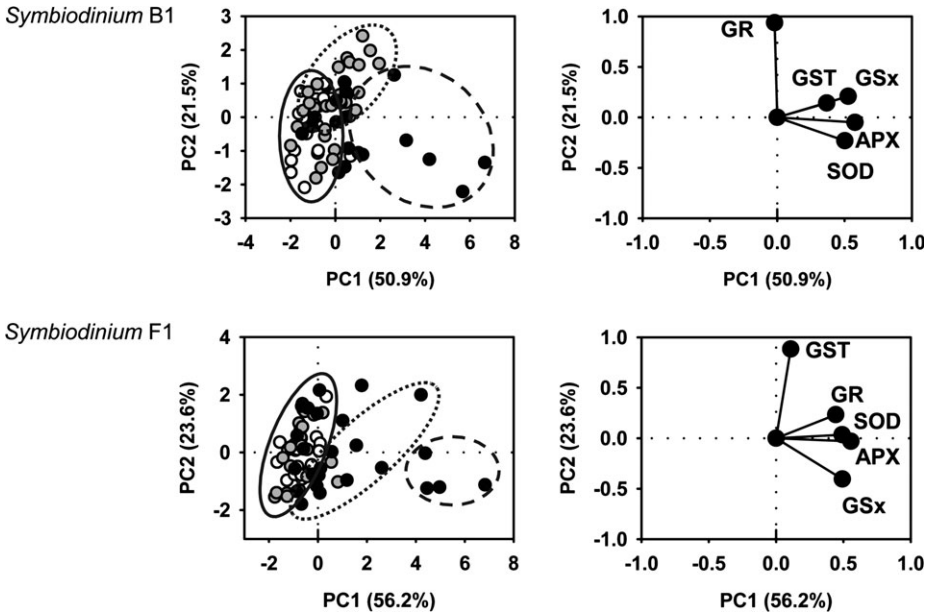

FIG. 7. Principal component analysis of antioxidant responses, integrating 14 d of exposure to 25°C (white), 29°C (gray) and 33°C (black) in *Symbiodinium* B1 and F1. Score plots (left) and corresponding loading plots with Eigenvectors (right) are shown for the first two principal components (PC1 and PC2). Antioxidant abbreviations as outlined in the text. Ovals indicate main network shifts of enzyme activity under treatments, containing all or the majority of replicates. For *Symbiodinium* B1: Replicates for the control treatment and Day 0 and 1 for 29°C and 33°C are enclosed by the solid oval; replicates for Days 3–14 at 29°C (dotted oval); and replicates for Days 3–7 at 33°C (dashed oval). For *Symbiodinium* F1: Replicates for the control and 29°C treatments are enclosed by the solid oval; replicates for Day 3 and Day 7 at 33°C (dotted oval); and replicates for Day 14 at 33°C (dashed oval).

Symbiodinium types, the 2-Cys peroxiredoxin-dependent water/water cycle might be an important alternative chloroplastic scavenging route at low H_2O_2 concentrations (Dietz et al. 2002, Bayer et al. 2012).

The glutathione system as an important part of the antioxidant network under thermal stress. With the exception of *Symbiodinium* E, all types exhibited considerable increases in their glutathione content at the highest temperature, with *Symbiodinium* B1 temporarily doubling its content even at 29°C. For *Symbiodinium* C1, GSx content was an order of magnitude higher than in the other types. Such high, and indeed even higher values have also been published for *Symbiodinium* A1 and Ax (Krämer et al. 2012). The fact, however, that the C1 GSx pool is maintained in a highly oxidized state and baseline GR activity (responsible for GSH regeneration) is not significantly higher than in *Symbiodinium* E and F1, raises the question of whether the large pool-size compensates for the low redox state. The low GSH/GSx values of 0.50 reported here for *Symbiodinium* C1, compared to ~0.90 in the other *Symbiodinium* types, are comparable only to those reported previously for the planktonic diatom *Thallassiosira pseudonana* (Rijstenbil 2002).

Oxidation of the glutathione pool at 33°C was a common response in all *Symbiodinium* types (including the non-symbiotic type). Only *Symbiodinium* F1 was able to stabilize the redox ratio after an initial drop. Up-regulation of GR after 2 weeks, however, indicated that GSH demand was no longer only met by de novo synthesis, but also supported by increased glutathione recycling. These processes were also apparent in the most susceptible type, *Symbiodinium* B1, where they started at sublethal temperatures that did not affect photosynthetic efficiency. While the stress response of *Symbiodinium* B1 at 33°C was similar to that in F1, but more immediate, the glutathione system switched from a reduced state at 29°C to a highly oxidized state at 33°C due to the collapse of the recycling mechanism. This led to a situation where an increased activity of the ascorbate-glutathione pathway under thermal stress caused a progressive oxidation of the glutathione pool, a potential pro-apoptotic signal (Circu and Aw 2010). Moreover, increased GST activity at 33°C consumed GSH from the pool, exacerbating the glutathione redox imbalance. Plant GSTs represent a large family of proteins, and remove peroxides and electrophilic compounds that may potentially attack DNA, RNA and proteins (Noctor et al. 2002). Studies measuring GST in *Symbiodinium* when in hospite have produced mixed results in response to anthropogenic contaminants (Downs et al. 2005, Ramos and García 2007), but this is the first study to show a temperature response at the proteomic level. The reason why we were not able to visualize activity shifts in *Symbiodinium* C1 and E via PCA, was because only few parts of the network responded (e.g., SOD in E) or significant antioxidant up-regulation at 33°C,

despite declining F_v/F_m, was limited to a single time point (e.g., SOD-APX pathway in C1). The responses of these two types highlights that alternative protective measures, including photoprotective measures upstream of antioxidant defenses (type C1), or non-enzymatic scavenging (type E) equally contribute to the onset and impact of thermal stress in some *Symbiodinium* types.

Antioxidant plasticity is key for acclimation. All types investigated here expressed considerable differences in their antioxidant baseline activities. Since the most tolerant F1 type also had the highest activities for the SOD-APX pathway, it seems tempting to link its thermal tolerance to a fundamentally higher scavenging activity in this part of the network. However, one has to consider that this does not necessarily translate to an actual advantage, as it has been shown that steady state fluxes of ROS in some *Symbiodinium* types might simply be higher (McGinty et al. 2012). Irrespective of the type-specific differences found here, the ability to activate and up-regulate the activity of these enzymes under stress is the fundamental mechanism that allows organisms to cope with ROS (Finkel and Holbrook 2000). In terms of proportional up-regulation relative to baseline activity, *Symbiodinium* F1 was superior to all types. This greater capacity to enhance components of its antioxidative defenses probably contributed to the maintenance of cellular viability in a thermal environment that was lethal to all other types. In this context, our study agrees with previous publications on photophysiological responses, which highlighted that the capacity to up-regulate protective measures tends to be a key element of thermally tolerant *Symbiodinium* types (Robison and Warner 2006, Takahashi et al. 2009, Ragni et al. 2010). This physiological plasticity is also observed in the adjustments of the glutathione system in *Symbiodinium* B1, but limited to temperatures up to 29°C. Both cases illustrate that the ability to maintain cellular homeostasis under thermal stress relies on the plasticity of the antioxidant network and its role in defining the "threshold at which a cell or tissue makes the transition from successful acclimation/resistance to oxidative stress-induced cell death" (Mullineaux and Baker 2010).

The data presented here illustrate the complex responses of symbiotic dinoflagellates in culture and highlight the necessity to view thermal stress tolerance in the context of the wider physiological network. The results, however, have to be viewed as an approximation of the processes in the intact symbiosis and emphasize the mechanistic point of view rather than predicting in situ processes. Host-related factors play an important role in defining thermal threshold of coral holobionts and it has been demonstrated that thermal impact sites in *Symbiodinium* can vary between the culture and the symbiotic state (Buxton et al. 2012). From an ecological point of view, our study demonstrates that elevated seawater temperatures, while lethal for most *Symbiodinium* types beyond 30°C, may already have a profound sublethal impact by altering growth rates and energy investment. Such impacts may influence population dynamics in mixed environmental *Symbiodinium* pools and possibly in the intact symbiosis, therefore affecting post-bleaching recovery of corals and other cnidarians.

In summary, we have shown that differential thermal susceptibility in *Symbiodinium* types in culture can be related to the activity of their antioxidant network. Baseline activities, scale of up-regulation in the SOD-APX pathway, and mechanisms to maintain redox homeostasis in the glutathione system, all contributed to overall cell viability and defined the type-specific response to a thermal challenge. Significant network shifts in antioxidant activity were evident in two *Symbiodinium* types and illustrate the importance of antioxidant plasticity in maintaining cellular viability. Future studies should incorporate additional stressors such as high light and expand the range of *Symbiodinium* types (including other thermotolerant types such as D). This study provides an important link between *Symbiodinium* diversity and stress physiology, and delivers insights into the biochemical responses that may determine the fate of *Symbiodinium* types with varying thermal tolerances.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Principal component analysis of antioxidant responses, integrating 14 d of exposure to 25°C (white), 29°C (gray) and 33°C (black) in *Symbiodinium* C1 and E.