A molecular physiology basis for functional diversity of hydrogen peroxide production amongst *Symbiodinium* spp. (Dinophyceae)

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Abstract

Hydrogen peroxide (H2O2) production has been demonstrated to play a pivotal role in the photosynthetic stability of higher plants, corals and algae, and considered a primary Reactive Oxygen Species (ROS) associated with the thermal susceptibility of *Symbiodinium* spp.. Here we simultaneously subjected a large number of *Symbiodinium* isolates (n=16) covering broad phylogenetic diversity (clades A, B, D, F) to heat stress and characterized their photosynthetic response via Fast Repetition Rate fluorometry (FRRf) and parallel measurements of H2O2 emissions. Based on their physiological response, isolates clustered into three novel functional groups: (i) thermally tolerant (unchanged photochemical efficiency (*Fv*/*Fm*), electron turnover (QA) or H2O2 emission), or (ii) thermally susceptible via decreased *Fv*/*Fm*, unchanged QA, but increased H2O2, indicating energetically uncoupled PSII (thylakoid membrane instability), versus (iii) thermally responsive via decreased *Fv*/*Fm*, increased QA and H2O2, indicative of energetically coupled (but downregulated) PSII. There was no correlation between the algal phylogenetic groups and the distribution of isolates amongst these novel functional groups. Two model *Symbiodinium* isolates for functional groups (i) and (ii) (ITS2 type A1, *Symbiodinium microadriaticum*, and type D1-5, Symbiodinium spp., respectively) were selected to further examine how their different thermal responses corresponded with the expression levels of two genes coding for different metalloforms of superoxide dismutase (MnSOD and NiSOD) that potentially regulate production of H2O2. *S. microadriaticum* demonstrated the greatest upregulation of MnSOD gene confirming recent suggestions of a role for this metalloform in the antioxidant network associated with thermal stress protection. Assigning *Symbiodinium* isolates into such functional groups based on coupled molecular-physiological assessment is an important step needed to improve our understanding of *Symbiodinium* diversity relative to its ecological success in nature.

Introduction

Reef building corals sustain immensely productive and biologically diverse ecosystems via a unique symbiosis with dinoflagellate microalgae (genus *Symbiodinium*) housed within the cnidarian tissue. Corals harbour an astonishing diversity of *Symbiodinium* spp., which is divided into nine phylogenetic clades (A-I, Pochon and Gates 2010) that in turn comprise hundreds of genetically distinct types (e.g. La Jeunesse et al. 2012; Pochon et al. 2014; Arif et al. 2014). At relatively broad phylogenetic levels, *Symbiodinium* genetic diversity associates with physiological traits that may influence overall fitness of the coral-symbiont association, for example the capacity to harvest and utilize light energy (Suggett et al. 2015; Warner and Suggett 2016). However, it is increasingly recognized that functional diversity of such traits within *Symbiodinium*’s complex phylogeny is likely a more robust correlate of coral fitness (Parkinson and Baums 2014; Warner and Suggett 2016).

Anomalous light and temperature exposure are integral factors that can destabilize the coral-dinoflagellate symbiosis (Lesser and Farrell 2004) to result in coral bleaching. One of the primary sites of light and thermal stress-induced bleaching is loss of integrity to *Symbiodinium*’s photosynthetic apparatus (Warner et al. 1999; Tchernov et al. 2004; Buxton et al. 2012), caused by and/or leading to excessive production of Reactive Oxygen Species (ROS) (reviewed by Warner and Suggett 2016) including hydrogen peroxide (H2O2) (Tchernov et al. 2004; Smith et al. 2005). H2O2 production can occur via several pathways (Warner and Suggett 2016), notably activity of MAP (Mehler-Ascorbate-Peroxidase) and photorespiration (Lesser 2006; Fahnenstich et al. 2008) within the chloroplasts, as well as mitochondrial alternative oxidase (AOX) (Rhoads et al. 2006). All of these pathways effectively act as energetic sinks by consuming excess electron pressure, intracellular O2, ATP and/or NADPH, with MAP particularly predominating for *Symbiodinium* cells when in culture (Roberty et al. 2014; but see also Roberty et al. 2015). Indeed, MAP-based production of H2O2 has been specifically suggested as a key factor regulating sensitivity to thermal stress in *Symbiodinium* (Smith et al. 2005; Suggett et al. 2008; Krueger et al. 2014; Roberty et al. 2015) via an imbalance between H2O2 produced by superoxide dismutase (SOD) and H2O2 consumed by ascorbate peroxidase (APX). However, enhanced production of H2O2 has been observed for both thermally sensitive (Suggett et al. 2008) and tolerant (Roberty et al. 2015) *Symbiodinium* phylotypes under heat stress, likely reflecting complexities in how these algae differentially regulate SOD (McGinty et al. 2012; Levin et al. 2016) or APX (Krueger et al. 2014) capacity, which in the latter case is further linked to glutathione redox homeostasis (Krueger et al. 2014; Roberty et al. 2015). Such dynamics become further complicated for *Symbiodinium* cells *in hospite* given additional capacity for the host to quench or produce H2O2 (Smith et al. 2005; Armoza-Zvuloni and Shaked 2014).

In efforts to better resolve the complexities underpinning the (MAP-based) ROS anti-oxidant network, researchers have increasingly turned to molecular-, and specifically transcriptional-, profiling based analysis of *Symbiodinium* cultures (Leggat et al. 2007, Bayer et al. 2012, Ladner et al. 2012). Most recently, transcripts of anti-oxidant genes have been successfully amplified for *Symbiodinium* ITS2-types B1, E and F1 (e.g., MnSOD, FeSOD and KatG; Krueger et al. 2015a) and a type C1 (Cu/ZnSOD, MnSOD, FeSOD and NiSOD metalloforms; Levin et al. 2016), to yield evidence for upregulation of the entire anti-oxidant network, including various SOD metalloforms under thermal stress for a thermally tolerant type C1 isolate (Levin et al. 2016). Whilst such studies represent an important step change in how regulation of the anti-oxidant network can be described and therefore understood, the actual link between the transcriptional response (genes of interest) and the production of the ROS of interest is still missing.

Existing evidence for a broad range of responses in H2O2 production and in expression of constituents of antioxidant networks is perhaps not surprising given *Symbiodinium*’s immense phylogenetic diversity, thereby enabling adaptation to very different environmental states (e.g. Hume et al. 2015); however, it is also likely that phylogenetic patterns in mechanisms underpinning stress tolerance have not been established since only a relatively small pool of genetic variants have ever been examined at any one time (see Warner and Suggett 2016). Larger scale taxonomic screening of key photosynthetic traits amongst *Symbiodinium* spp. (n = 18 genetic variants) recently revealed that “functional groups” can be identified that do not exclusively conform to phylogenetic groups (Suggett et al. 2015); however, how such functional groupings apply under thermal stress has not yet been tested. Indeed, Wietheger et al. (2015) recently demonstrated that *Symbiodinium* spp. photosynthetic performance does not always respond to thermal- and H2O2- stress in a consistent manner. However, it should be noted that their study utilised extremely high exogenous H2O2 concentrations that presumably more closely reflect host generated (as opposed to *Symbiodinium* intracellular derived) processes (e.g. Krueger et al. 2015b). Even so, the various responses observed by Wietheger et al. (2015) provided evidence that a single H2O2-based model for thermal susceptibility (*sensu* Tchernov et al. 2004; Smith et al. 2005) unlikely applies across all genetic variants. Here, we build on these recent observations and revisit *Symbiodinium* spp. photochemical competency of photosystem II (PSII) (a key diagnostic of stress susceptibility; e.g. Warner and Suggett 2016) in parallel with H2O2 production under heat stress across multiple (n=16) genotypes; from this, we were able to define for the first time novel functional groups for thermal stress related H2O2 production. Using two model genotypes from these groups (A1, *Symbiodinium microadriaticum*, versus *Symbiodinium* sp. D1-5) we further link genotype-specific differences in H2O2 production with level of expression of genes coding for 2 different metalloforms of superoxide dismutase (MnSOD and NiSOD) in response to thermal stress.

Methods

*Symbiodinium culturing & experimental design*— Sixteen strains from the genus *Symbiodinium* spanning 4 phylogenetic clades (A, B, D, F) were grown at 25oC under a 12:12 light: dark cycle with a photon flux density (PFD) (4π LI-190SA Quantum Sensor, Licor) of ca. 85 µmol photons m-2 s-1. Thirteen of these strains have been previously classified at the intracladal scale by their rDNA internal transcribed spacer 2 regions (ITS2) (as per Robison and Warner 2006; LaJeunesse et al. 2012; Suggett et al. 2015), with several of these now formally classified as species (LaJeunesse et al. 2012, LaJeunesse et al. 2015, Lee et al. 2015); specifically, *S. microadriaticum* (A1, rt61), *S. tridacnidorum* (A3, A3c, CS159 and CCMP2465 respectively), *S. lincheaea* (A4, rt379), *S. necroappetens* (A13, rt80), *S. pseudominutum* (B1, rt12), *Symbiodinium sp.* (B1´, UTSB), *S. psygmophilum* (B2, Mf14.10), *Symbiodinium sp.* (D1-5, PHMSTD4), *S.* kawagutii (F1, CS156 and UTSC) and *Symbiodinium sp. (*F2, rt133); two different strains for each of A3, B1 and F1 were grown and therefore will be referred to here as A3 (CS159) and A3´ (CCMP2465), B1 (rt12) and B1´ (UTSB), and F1 (CS156) and F1´ (UTSC), respectively; it should be noted that these two B1 isolates have been designated two separate species but with UTSB not confirmed as *S. minutum* (see Suggett et al. 2015). Two D-clade strains (DS1, DS2) were also analysed, however, they were identified based on sequence divergence in the chloroplast 23S rDNA and not by ITS2. Full details of these strain identities and original isolation histories are reported in Suggett et al. (2015). An additional alga from clade A of unknown origin, based on the 28S rRNA sequence (99.8% similarity to GenBank accession number AF427455) (see Buxton et al. 2012), was also grown and is referred to here as strain ‘A’. All cultures were maintained semi-continuously in IMK artificial seawater media (Daigo IMK, Nihon Pharmaceutical Co., Ltd.) in exponential growth phase and optically thin with mean growth rates of 0.19-0.26 d-1 over 8-10 sequential generations (see Suggett et al. 2015) and cell densities maintained under 80 x104 cells mL-1.

To assess for physiological thermal stress responses and H2O2 production, 60mL aliquots from each culture under steady state growth were dispensed evenly across triplicate control and treatment vials. For the duration of the experiment, control vials were maintained within an incubator set to the same conditions used for growth (i.e. 85 µmol photons m-2 s-1 on a 12: 12 light: dark cycle, 25ºC). Treatment vials were maintained within an identical incubator and subjected to 72h thermal ramping from 25ºC to 32ºC, as per Robison and Warner (2006). All vials were maintained at 32ºC for a further 72h, to therefore yield a total incubation time of 144h. Samples were taken from all vials for photophysiological analysis and cell counts at 0, 72 and 144h, and for H2O2 production at 144h only. All samples were taken at 09:00 to eliminate any variation due to the natural diel cycle of *Symbiodinium* in the measured physiological parameters and in accordance with incubator light cycles (12:12 cycle, 08:00 local time lights on) (e.g. Sorek et al. 2013). Biomass consistently increased whilst photophysiological parameters were not significantly different for the control vials across the three sampling time points (data not shown) indicating that this set up provided conditions that matched those from the initial steady state culture inoculum, and cultures did not run into nutrient starvation, for the duration of the experiment.

 *Photophysiology, cell counts & H2O2 quantification*— All samples were acclimated to extremely low light (ca. ~5 mol photons m-2 s-1) for at least 20 mins prior to photophysiology measurements. All samples were then analyzed using a *FastOcean* Fast Repetition Rate fluorometer (FRRf) coupled to a *FastAct* base unit (Chelsea Technologies Group, UK). Excitation was delivered using a blue LED excitation source (peak excitation 450 nm) delivering an ST induction protocol of 100 flashlets of 1.1 μs at 2.8 μs (see Suggett et al. 2015). The FRRf was programmed to record the average of 40 sequential ST acquisitions at 150 ms intervals. Photosystem II (PSII) photophysiological characteristics were then determined by fitting acquisitions to the KPF model (Kolber et al. 1998) using FastPRO software (Chelsea Technologies Group, UK) to yield the minimum (*Fo*) and maximum (*Fm*) PSII fluorescence yields (instrument units), PSII absorption cross section (PSII; nm2), and the QA reoxidation rate (QA, s). Cell counts were taken for each sample after FRRf analysis using a haemocytometer as per Hennige et al. (2008). All fluorescence yields were adjusted for baseline fluorescence using sample filtrate (see Suggett et al. 2015).

An Amplex Red-based assay was used to quantify hydrogen peroxide (H2O2) using the protocol provided (Invitrogen, Molecular Probes®) and following modifications for *Symbiodinium* (Suggett et al. 2008; see also Roberty et al. 2015). Amplex red reagent was added to the final reaction mixture made up of a 0.25 M sodium phosphate buffer, pH 7.4, 0.1 U/mL HRP and combined with 1 mL aliquots of sample and no H2O2 controls. Samples were incubated for 1 h at their respective control or treatment temperatures, and under a Rose Pink screening filter (002, Lee Filters, Sydney, Australia) delivering a high PFD of 700 µmol photons m-2 s -1 (Cidly Phantom 300W, Shenzhen ,China) to maximize the H2O2 signal:noise under light saturation (as per Suggett et al. 2008). The Rose Pink filter was placed over the incubation chamber in between the light source and samples to avoid reactions due to blue-green light, ~450-550 nm. Following incubation, samples were centrifuged to pellet the cells and read using a spectrophotometer (Agilent Cary 60 UV-Vis, United States) at 571 nm against a reference blank (distilled water). Concentrations of H2O2 were calculated using the absorbance values of the unknown samples compared to a series of standards of known H2O2 concentrations (0-20 µM) containing the Amplex Red mixture, which were used to create a standard curve. All measures of H2O2 concentration were normalised to corresponding measures of cell concentration for each sample; whilst we report our data throughout as pmol H2O2 cell-1 (as per Suggett et al. 2008), values strictly reflect the H2O2 emitted per cell per hour incubation.

 *Gene expression*—From the sixteen *Symbiodinium* strains initially analysed for physiological characteristics and H2O2 production, two strains, *S. microadriaticum* (A1,rt61) and *Symbiodinium sp.* (D1-5, PHMSTD4), were subsequently selected for expression analysis of two target genes coding for metalloforms of superoxide dismutase (MnSOD and NiSOD) and three reference genes (GADPH, Rp-S4 and psbA). These two target genes were chosen as these metalloforms were the only conserved regions found amongst the types tested. Both strains were again assayed using the exact same heat stress and control procedures as before but with increased replication (n=4) and volume (100mL per replicate); the % change for all photophysiological parameters and for H2O2 production between control and treatment was not statistically different for this second repeat versus initial assay (t-test; not shown) for either strain. All control and treatment replicates were processed as follows:

 (i) RNA extraction: An aliquot of 40 mL (~106-7 cells) was used for the RNA extraction where samples were centrifuged (10min at 2300 x g) and the resulting cell pellets were snap-frozen in liquid nitrogen and stored at -80 o C until analysis (McGinley et al. 2012, Rosic et al. 2011). Total RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Australia). Cell pellets were first homogenized using a bead beater (Biospec, USA) and ~0.2 g of 425-600 microns glass beads with the following cycle: agitation for 25 s at 30 1/s followed by a 5 s interval and a further 25 s at 30 1/s. Samples were then centrifuged for 10 min at 16 000 x g. The aqueous phase was then used for RNA extraction using Qiagen RNeasy Mini Kit (Qiagen, Australia) according to manufacturer’s instructions. RNA yield and purity was checked for each sample using a Nanodrop (Thermo Scientific, USA).

(ii) cDNA synthesis: A total of 100 ng of total RNA was used for cDNA synthesis for each sample, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®) according to manufacturer’s instructions. Briefly, a master mix containing 10 x RT buffer, 25 x dNTP Mix (100 mM), 10 x RT Random Primers, Multiscribe Reverse Transcriptase, nuclease-free water and 100ng of total RNA was added to PCR tubes. PCR was conducted using a Veriti® 96-Well Thermal Cycler, Applied Biosystems® machine with cycling conditions as follows: initial denaturation step at 25 o C for 10 minutes followed by denaturation at 37 o C for 120 minutes, annealing at 85 o C for 5 minutes and extension at 4 o C. RT random primers were used to initiate cDNA synthesis. A DNase step was included to remove contaminating genomic DNA according to manufacturer’s instructions. The cDNA was then diluted 1:50 with nuclease free water and used as the template (i.e. same cDNA quantity used for all samples) in the RT-qPCR analysis. No template control (NTC) and no reverse transcription controls (NRTC) were included for all gene/sample combination to ensure that the cDNA samples were free of DNA contamination. All reactions were performed in triplicate, creating three technical replicates for each biological replicate (4 biological replicates per treatment per strain).

(iii) Primer design and analysis: Target primers, amplifying 100-200 bp PCR products, were designed using Primer3 software based on sequence alignments from multiple *Symbiodinium* types obtained from the National Centre for Biotechnology Information (NCBI) GenBank database ([www.ncbi.nlm.nih.gov)](http://www.ncbi.nlm.nih.gov)); all other primers were sourced from the literature and validated using NCBI Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi> (see Table 1). Standard PCR amplification was conducted for all primers to confirm the reproducibility and specificity of the primers for *Symbiodinium microadiaticum* and *Symbiodinium* sp. (D1-5). PCR conditions were as follows:

An initial denaturing step of 1 cycle at 95°C for 10 mins, followed by 40 cycles of a denaturing step at 95°C for 30 s, an annealing step at 60°C for 30 s, and elongation at 75°C for 30 s. Samples underwent a final elongation step at 72°C for 5 mins and then were held at 10°C. PCR products for all reference genes and genes of interest were visualized after gel electrophoresis ([E-Gel® Imager System](http://www.lifetechnologies.com/au/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/e-gel-electrophoresis-system/e-gel-imager-system.html)**,** Life Technologies, Australia), and sent to the Australian Genome Research Facility (AGRF) for sequencing in order to confirm primer specificity and sequence identity. Primers were further quality controlled with amplification efficiency tests using RT-qPCR (StepOnePlus™ System, Applied Biosystems®) and the creation of a dilution curve using a threefold dilution series to cover a 80-fold range of concentrations (dilution 1:3, 1:9, 1:27, 1:81) and melt curve analysis. Three technical replicates for each cDNA concentration were performed for each primer. For each primer set, technical triplicates were also included for a no template control (NTC) and a noRT control (NRTC) to ensure that the cDNA samples were free of DNA contamination. RT-qPCR settings were as follows: initial step at 95°C for 30 s, cycling stage of 95°C for 30 s, 60°C for 30 s and 68°C for 30 s, all for 50 cycles, melt curve stage of 95°C for 15 s and 60°C for 1 min and 95°C for 15 s. Sample CTs were within the range of tested standard curve for all primers. All primers achieved efficiencies of 90-110% and were subsequently used in gene expression analysis.

 (iv) Gene expression analysis: RT-qPCR assays were conducted for both strains using control and stress treatment cDNA with three technical replicates and 4 biological replicates. A master mix including diluted primers (final concentration 500nM), ultra-pure water and SYBR Green PCR Master Mix (Applied Biosystems, including SYBR green as reporter dye) was made for each primer. 6 µL of each master mix and 4 µL of diluted cDNA (1:50 dilution) were loaded into each well of a 96 well standard microplate making a total reaction volume of 10 µL/well. Cycling conditions as above. Data from RT-qPCR was analysed using Step One Plus Software (Ver. 2.3; Applied Biosystems). Expression levels were determined as the number of cycles needed for the amplification to reach a fixed threshold in the exponential phase of the RT-qPCR reaction (Walker 2002). The cycle threshold (CT) was set at 0.03 for all genes. Replicate variability of the CT values between the 3 technical replicates, which were run on the same plate, was examined for each sample-gene combination. Repeatability of the assay between the technical replicates was consistent across the different genes with the replicate variability falling within the set limit of <0.5 cycles for all the sample-gene combinations tested. To validate changes in target genes expression, CT were imported into the qbase+ software package (Biogazelle).

 *Statistical Analysis*— Independent *t*-tests (MATLABTM) were used to compare FRRf-derived physiological parameters (*Fv*/*Fm*, dimensionless; PSII, nm2; tQA, s) and H2O2 concentration of replicate data (n=3) for each strain between treatments (see supplementary section) Pairwise correlation and Bartlett’s type II model linear regression with a least squares fit (MATLABTM) was used to compare for the general effect of heat treatment compared to control across all strains. Primer + Permanova (ver. 6.1; UK) was used to conduct multivariate analysis of physiological parameters (% change *Fv*/*Fm* and tQA) and % change H2O2 concentration amongst strains using a Euclidian distance clustering and multidimensional scaling (MDS) in order to identify functional groups. A single % change value for each strain was calculated using the equation % change = ([ x̅ t - x̅ c] / x̅ c) x 100, where x̅ represents the mean from the three independent replicates for treatment (t) or control (c), and arcsine transformed and standardized prior to MDS analysis. For RT-qPCR data, the expression level of each target gene (MnSOD or NiSOD) was first normalized against the combination of 3 internal reference genes (Rp-S4, GADPH and psbA, Table 1) using GeNorm algorithm (Hellemans et al. 2007) in order to obtain Normalized Relative Quantity for each treatment, which was then compared across treatment using an independent *t-*test (SPSS Statistics Student Version 19 statistics program, Chicago, IL). The 3 internal reference genes used had a M stability (M) value under 1, reflecting a relative stability in gene expression. The data followed the linear model assumptions of independence, normality (Shapiro-Wilk test) and homoscedasticity (Levene’s homogeneity test).

Results

 *Photophysiological and H2O2 responses*—FRRf-measurements under steady state growth conditions confirmed broad variation of both the PSII maximum photochemical efficiency *Fv*/*Fm*, dimensionless) and effective absorption cross section (, nm2) across all 16 strains, ranging 0.39-0.48 and 3.7-5.3, respectively (Supplementary section; Fig 1); values for both *Fv*/*Fm* and PSII were generally within the range expected (Hennige et al. 2009; Suggett et al. 2015) and were negatively correlated (Fig. 1A) reflecting strain-specific differences in light harvesting efficiency (see Suggett et al. 2015). Subjecting the cells to heat stress, i.e. 72 h thermal ramping and subsequent exposure to 32ºC for a further 72 h, consistently decreased *Fv*/*Fm* (by 2-61%) and increased PSII (by 0-63%) across all strains (except A4, D, and F2 where  was decreased by 3-27%). This general parallel decline of *Fv*/*Fm* and increase of PSII is indicative of a loss of PSII reaction center functionality in *Symbiodinium spp.* (Suggett et al. 2008; Ragni et al. 2010); however, the slope of the linear regression equation describing the covariance between *Fv*/*Fm* and  was lower for the control versus heat stress (-0.036 versus -0.067; see Fig. 1 legend) and indicated a generally larger impact of heat stress on *Fv*/*Fm* than on PSII across strains.

We further compared values of *Fv*/*Fm* with those of the QA re-oxidation rate (τQA, μs) across all strains since two different trends for these parameters have previously been reported for *Symbiodinium* under heat stress; specifically, a reduction of τQA with *Fv*/*Fm* indicative of a loss of thylakoid membrane integrity (Tchernov et al. 2004) and/or increased τQA with decreased *Fv*/*Fm* indicative of a loss of PSII function (Robison and Warner 2006; Suggett et al. 2008). Under control temperatures τQA values were highly variable across strains (280-570 s, see Supplementary section) and generally in the range previously reported (Tchernov et al. 2004; Suggett et al. 2008), but with no overall trend with corresponding values of *Fv*/*Fm* (Fig. 1B). Similarly, under heat stress values of τQA were between 11% lower and 67% higher than the control values, and again with no trend with *Fv*/*Fm*. Thus no consistent response for τQA accompanied the generally reduced values of *Fv*/*Fm* across strains subjected to heat stress (Fig 2B).

 Cellular normalised H2O2 concentrations (pmol cell-1) under steady state ranged from 0.25-0.9 (*S. tridacnidorum* A3, A3c, *S. psygmophilum*, and *Symbiodinium* sp. DS2 and F1) to 1.0-2.64 (all other strains) (Supplementary section) and hence no clear pattern with cladal identity; this range of H2O2 concentrations was again consistent with values previously reported (Suggett et al. 2008; Roberty et al. 2015), and did not correlate with strain-specific differences in cell size (not shown). H2O2 concentrations consistently increased under heat stress (by ca. 25-400%, Fig. 2A), and the % change in H2O2 concentration between steady state control and heat stress treatment was generally (negatively) correlated with the corresponding % change in *Fv*/*Fm* (Fig. 2A) but not % change in τQA (not shown). Further MDS analysis across the 16 strains examining the corresponding % change for H2O2, *Fv*/*Fm*, and τQA, further resolved the presence of three general ‘functional groups’ (Fig. 3):

1. Relatively unchanged *Fv*/*Fm*, QA, i.e. thermally tolerant but with relatively (a) unchanged (*S. microadriaticum* (A1), *S. lincheaea* (A4), *S. necroappetens* (A13), *Symbiodinium* sp. DS2, and *S.* kawagutii (F1´) or (b) enhanced (*Symbiodinium* sp. DS1, *S.* kawagutii (F1), and *Symbiodinium* sp. F2) emission of H2O2;
2. Decreased *Fv*/*Fm*, unchanged QA, and increased H2O2 (*S. tridacnidorum* (A3), *Symbiodinium* sp. B1´, *S. psygmophilum* (B2), *Symbiodinium* sp. D1-5), consistent with patterns described by Tchernov et al. (2004) interpreted as thermal susceptibility via energetically uncoupled PSII, where the transmembrane proton gradient (established by the photochemical reactions in the functional reaction centers) is dissipated without generating ATP;
3. Decreased *Fv*/*Fm*, increased QA and relatively (a) large (*Symbiodinium* sp. A, *S. tridacnidorum* (A3c), *S. pseudominutum* (B1)) or small (*S. tridacnidorum* (A3´)) increases in H2O2, indicative of thermally responsive but via energetically coupled PSII, i.e. transmembrane proton gradient dissipation generates ATP;

Importantly, these three different functional groups do not cluster according to large phylogenetic groupings (i.e. clades), although only clades A and B fell within functional group (iii).

*Molecular responses*— Whilst the number of *Symbiodinium* gene expression studies have increased substantially in the past decade, no universal primer set for references genes are currently available which work across multiple *Symbiodinium* clades thereby limiting the application of RT-qPCR and the relative quantification method (Rosic et al. 2011). Thus, finding primers that successfully amplify reference genes appears problematic given the high level of sequence divergence between *Symbiodinium* clades. For our study, primers sets for the reference genes selected successfully amplified for both isolates of interest, *Symbiodinium* sp. (D1-5) and *S. microadriaticum* cDNA. Real-time dissociation curves further confirmed the presence of a specific PCR product in all amplification reactions.

RT-qPCR analysis revealed that heat stress significantly influenced the transcript abundance of *Symbiodinium* MnSOD; specifically, a ca. 3-fold increase in heat treated cells compared to the control for type A1 (Fig. 4) and a 2-fold increase for type D1-5 (Fig. 4). Whilst NiSOD expression also increased 1.4 fold under heat stress compared to control for type A1, this difference was not statistically significant (see Fig. 4 legend). Similarly, NiSOD was not significantly regulated for type D1-5 between heat stress and control. Overall the transcript abundance for both MnSOD and NiSOD for the A1 isolate (29.595 ± 0.248 Ct and 34.250 ± 0.183 Ct, respectively) was always higher than for D1-5 (28.505 ± 0.141 Ct and 32.895 ± 0.290 Ct, respectively) by ca. two orders of magnitude.

Discussion

A major goal for the past two decades has been to identify how *Symbiodinium* spp. genetic variants respond to different stressors, in particular elevated temperature (e.g. Warner et al. 1996; Suggett et al. 2008; Takahashi et al. 2008; Buxton et al. 2012; McGinty et al. 2012; Krueger et al. 2014), to better understand how environmental variability influences ecological distribution (e.g. LaJeunesse et al. 2010; LaJeunesse et al. 2014) and physiological functioning (e.g. Warner et al. 2006) of these endosymbiont cells in nature. Elevated production of reactive oxygen species (ROS) appears a conspicuous feature of stress susceptibility amongst *Symbiodinium* genotypes (see Warner and Suggett 2016); however, both the form of ROS, and how (or if) it is produced amongst genotypes, appears highly variable across studies examining relatively few isolates (e.g. Tchernov et al. 2004; Takahashi et al. 2008) thereby confounding efforts to reconcile observations and develop a general ROS-based paradigm of heat stress sensitivity. We therefore took the approach here of screening for a key ROS (H2O2, Smith et al. 2005) amongst numerous isolates covering 4 major clades (A, B, D, F) under heat stress to examine for potential phylogenetic patterns. Whilst patterns of photosynthetic physiology and H2O2 emissions could not be reconciled with phylogenetic groupings, we uniquely observed two different functional responses to heat stress. Such a diversity of response is entirely consistent with the recent notion that a single model may be insufficient to explain patterns of heat stress susceptibility amongst the immense genetic diversity inherent to the genus *Symbiodinium* (Buxton et al. 2012; Warner and Suggett 2016).

Several prior studies have reported an increase in H2O2 emission from heat stress sensitive *Symbiodinium* exposed to short-term temperature increases (clades A-C, Tchernov et al. 2004; ITS type B1, Suggett et al. 2008; Roberty et al. 2015). All of these observations correspond with parallel upregulation of light-dependent O2 consumption via photosystem I (notably Mehler Ascorbate Peroxidase activity, Roberty et al. 2016) presumably as excitation pressure as PSII goes into ‘over-drive’ (Lesser and Farrell 2004); however, our observations would in fact suggest that this general response manifests via two alternate modes of operation (at least according to the time scale with which strains were subject to heat stress here):

Our functional group (ii) was consistent with photobiological trends reported by Tchernov et al. (2004), where reduced/unchanged QA (alongside reduced *Fv*/*Fm*) under heat stress was interpreted as H2O2-based lipid peroxidation resulting in higher excitation pressure through PSII; however, heat stress sensitivity was not consistent with phylogenetic groupings. A subsequent study by Hill et al. (2009) examining coral taxa hosting a range of ITS2 types from clades A, C and D similarly could not identify a link between sensitivity of thylakoid membrane stability under heat stress and phylogeny. In contrast, our functional grouping (iii) reflected previous observations (Robison and Warner 2006; Suggett et al. 2008) of high-temperature destabilization of PSII via increased QA.

Fully reconciling the underlying reason for these different functional groups from the present data is not possible. In part, the longer duration of heat stress (244 h) applied by Tchernov et al. (2004) may be required to overwhelm the physiological network that otherwise protects thylakoid stability. This process can clearly be short-circuited where the nature of heat stress is particularly acute relative to the upper thermal thresholds of any given isolate; for example, the relatively heat (32oC) stress tolerant F1 (CS156) can exhibit a functional response (iii) pattern when subjected to rapid acute heating (37oC) (Hill et al. 2009). Some *Symbiodinium* types may in fact have an enhanced capacity for combatting lipid peroxidation (e.g. through volatile gas emissions, see Exton et al. 2015; anti-oxidant networks, Krueger et al. 2014) or are simply so leaky (reduced cell wall thickness) that contact time between H2O2 and the lipid membranes is reduced (Wietheger et al. 2016); the latter process may perhaps also explain why some strains appear to show increased H2O2 emission but no signs of photophysiological sensitivity to heat stress (functional group (ib) (Wietheger et al. 2016). At present, the underlying reason is not entirely clear but regardless our observations of enhanced production of H2O2 but different photobiological patterns is entirely consistent with the notion that *Symbiodinium* isolates do not express a universal response to heat stress (see Buxton et al. 2012; Warner and Suggett 2016).

On balance, our current observations from screening a substantial number of ITS2 types was able to resolve functional diversity that did not correspond with any pattern of phylogenetic diversity. Whether this lack of correspondence may in part reflect some of the challenges that remain in phylogenetically resolving *Symbiodinium* via ITS2 alone (e.g. Hume et al. 2015) versus convergent selection to localised ecological or environmental conditions (Parkinson et al. 2015) from where the various isolates were originally sourced (see Suggett et al. 2015) is unclear. In the latter case, we observed a heat stress tolerant response for our *S. microadiaticum* isolate (A1) CCMP2464 (see also Suggett et al. 2008) that contrasts with a more heat sensitive response recently observed for a different *S. microadiaticum* species isolate (Mediterranean isolate “Avir”; Roberty et al. 2015). How heat stress exposure is conducted relative to differences in thermal history (e.g. isolates from different thermal regimes) is thus clearly important in defining differing physiologies and how isolates fall into their ‘functional groups’.

 An important outcome of our study was subsequent targeted molecular evaluation of heat stress sensitivity. The two model strains selected appeared relatively thermally tolerant and susceptible, respectively, but both had low to moderate H2O2 production (see results section) and hence were likely to have differential molecular control of ROS anti-oxidants (e.g. Krueger et al. 2014; 2015a, Levin et al. 2016). Whilst these two strains did not cover all three emergent functional groups (above), i.e. only thermally tolerant (*S. microadriaticum*; group (i)) versus thermally susceptible via energetically uncoupled PSII (*Symbiodinium* sp. (D1-5); group (ii)), they demonstrate clear differential heat stress sensitivity of SOD metalloform expression. *S. microadriaticum* exhibited greater SOD upregulation (and notably of MnSOD) over *Symbiodinium* sp. D1-5, suggesting that any additional SOD-based H2O2 production be balanced through parallel upregulation of H2O2 antioxidants; indeed, such a notion has recently been demonstrated for a thermally tolerant ITS2 type C1 isolate where upregulation of the entire anti-oxidant network occurred but no elevated ROS was observed under heat stress (Levin et al. 2016). Upregulation of genes (abundance and/or activity) involved in ROS scavenging is well established for *Symbiodinium* isolates under heat stress (e.g. Bayer et al. 2012; McGinty et al. 2012; Krueger et al. 2014). Light-activated ROS production may reflect elevated MAP activity to produce H2O2 in the chloroplast (Suggett et al. 2008; Roberty et al. 2014); although how much H2O2 is produced is ultimately a function of ascorbate peroxidase activity and how it is coupled to glutathione pathway, which are also potential variables amongst isolates (see Krueger et al. 2014).

Our observations overall would suggest a strong role for SOD metalloform MnSOD in heat stress tolerance of our isolates; however, Levin et al. (2016) demonstrate the importance of FeSOD in a thermally tolerant ITS2 type C1 isolate. Increased expression of MnSOD in response to heat stress has been generally well observed for coral hosts of *Symbiodinium* (e.g. Császár et al. 2009) but also *Symbiodinium* (Downs et al. 2013), and reflects upregulation of mitochondrial SOD in eukaryotes. Such a response is entirely consistent with the potential for mitochondria to be significant sources of ROS (Lesser 2006; Warner and Suggett 2016), where mitochondria are also potential targets of heat stress within the host cnidarian (Dunn et al. 2012). Mitochondrial respiration occurs in *Symbiodinium* through the cytochrome c oxidase, Complex IV (COX) or the Alternative Oxidase enzyme (AOX) pathways, both enabling the reduction of O2 to H2O via the oxidation of reduced cytochrome c (cytc) or of ubiquinone respectively (Oakley et al. 2014; Warner and Suggett 2016). The upregulation of MnSOD observed here could therefore point to thermal induced activation of the COX or AOX pathways. Both dark and light-enhanced (oxygen uptake in the light) respiratory rates are relatively high in *Symbiodinium* (Brading et al. 2011; Roberty et al. 2014). Likewise, some (Oakley et al. 2014) but not all (Roberty et al. 2015) studies clearly highlight upregulation of AOX as a mechanism contributing to the light-dependent O2 consumption, leading to direct H2O2 production. Whilst a role for mitochondrial based SOD upregulation is intriguing here, it is important to highlight two critical points at this stage:

Firstly, MnSOD can be assigned to either secretory or mitochondrial pathways (Krueger et al. 2015a). Some *Symbiodinium* strains seem to lack or do not express FeSOD at all (Krueger et al. 2015a; Levin et al. 2016) suggesting that another SOD metalloform may in fact perform the role of FeSOD in the chloroplast. Other free-living microalgae, including cyanobacteria and diatoms, have an evolved localization of MnSOD in their chloroplasts (see Grace et al. 1990; Wolfe-Simon et al. 2006), most likely to overcome Fe limitation and lower cellular Fe quotas. However, Fe requirements of *Symbiodinium* in culture, at least for *S. kawagutii* (Type F1), has recently been shown to be relatively large (especially compared to Mn and Ni) (Rodriguez et al. 2016) suggesting that FeSOD would be unlikely selected against unless *Symbiodinium* persisted in low Fe environments. Alternatively, MnSOD may be located in peroxisomes (see Lin et al. 2015 for *S. kawagutii*) and function well as a quencher of highly diffusible H2O2. In higher plants, peroxisomes generate superoxide and nitric oxide radicals (Schrader and Fahimi 2006) and thus also contain many antioxidant enzymes such as SOD and components of the ascorbate-glutathione cycle. Evaluating any upregulation of SODs specifically within the peroxisomes would therefore clearly enhance our current understanding of H2O2 production within *Symbiodinium*.

Secondly, because sequences for MnSOD and FeSOD are very similar, having diverged from a common ancestor (Miller 2012), it is important to recognize that our RT-qPCR assay could have detected both metalloforms and thus our observations do not necessarily preclude a lack of FeSOD in the strains tested. We have only focused on MnSOD and NiSOD here since these metalloforms were the only conserved regions found amongst the types tested; however, further studies clearly need to consider the transcriptional regulation of other metalloforms such as Fe, Cu and Zn as well as ROS enzymes (or proteins) considering also that dinoflagellates express a high degree of post-translational gene regulation where actual protein amount/activity might not be entirely represented through gene expression studies (Mayfield et al, 2014).

Few studies have yet undertaken a molecular physiology based approach to link genotype-specific differences in ROS production with transcriptional responses of the ROS network under thermal stress (e.g. Levin et al. 2016). However, here we have shown for the first time that production of a key specific ROS (H2O2) appears to correspond with differences in key ROS anti-oxidants/promoters, i.e. SOD that quenches superoxide to produce H2O2, amongst *Symbiodinium* isolates. Fully establishing the link between these two processes is not currently possible and reinforces recent suggestions that fully characterizing mechanisms of heat stress sensitivity ultimately require a complete picture of the physiological (Krueger et al. 2014; Warner and Suggett 2016) and/or molecular (Levin et al. in 2016) network at play. Even so, this work demonstrates how heat stress sensitivity amongst *Symbiodinium* isolates, at least according to the parameters we have examined, can be ascribed to at least three functional groups, (i) tolerant, and sensitive as (ii) energetically uncoupled versus (ii) coupled PSII. Thus, heat stress sensitivity for *Symbiodinium* cannot be explained by a universal mechanistic description.

How *Symbiodinium* isolates fall into functional groups under heat stress sensitivity here does not appear to be the same as under steady state light utilization (Suggett et al. 2015); however, the traits examined in our current study differ from those of Suggett et al. (2015), and thus, perhaps unsurprisingly, the choice of traits(s) will inevitably be an important consideration governing how taxa functionally group together. *Symbiodinium* have clearly evolved a wide range of mechanisms to tolerate heat stress (reviewed by Warner and Suggett 2016); consequently, studies attempting to better reconcile ecological patterns of *Symbiodinium* species diversity with temperature changes over space and time may ultimate need to consider a much broader range of diagnostic traits to temperature sensitivity to best describe functional diversity. Further, given the symbiotic nature of *Symbiodinium*, future studies examining this broader array of traits *in hospite* is clearly needed to confirm if *Symbiodinium* physiological responses and functional grouping observed in culture are still valid when compared to an intact symbiosis.

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**Table I**: Primer sequences, amplicon length, melting temperature and literature source of the primers used as reference genes and genes of interest. The final column indicates studies of previously published sequences and those novel to this study

| **Symbol** | **Forward Primer sequence (5’-3’)** | **Tm (ºC)** | **Reverse Primer sequence (5’-3’)** | **Tm (ºC)** | **Amplicon length (bp)** | **Accession Number/Reference** |
| --- | --- | --- | --- | --- | --- | --- |
| GAPDH | GGTGGTTGATGGCCAGAAGAT | 60 | CACCAGTGGATTCGCAAACA | 59 | 100 | Rosic et al. 2011 |
| Rp-S4 | CCGCACAAACTGCGTGAGT | 59 | CGCTGCATGACGATCATCTT | 58 | 101 | Rosic et al. 2011 |
| psbA | GAGTAGCTGGAGTATTTGGTGGAT | 60 | TGAAGGCTACGAGAGTTATTGAAG | 58 |  | McGinley et al. 2012 |
| MnSOD | TCAACAAGAGGAGGCTGCTT | 60 | GACCTCCCTGACCTTCCCTA | 60 | 149 | This study |
| NiSOD | GAAGACTGCTTGGCCTTCAT | 60 | GACGACCACAGCCGACTACT  | 60 | 129 | This study |

Figure Legends

**Figure 1.** Relationship between corresponding FRRf-based measurements of (a) Maximum PSII photochemical efficiency (*Fv*/*Fm*, dimensionless) and the PSII effective absorption cross-section (σPSII, nm2), (b) *Fv*/*Fm* and the QA reoxidation rate (µs). Data shown is for control (25°C, grey circles) and heat treatment (32°C, black circles), and with each circle representing the mean of replicate measurements (n=3) for each *Symbiodinium* type (n=16; see main text). Error bars are not shown for clarity (but see supplementary Table 1). Statistically significant correlations between corresponding measurements were only observed for (a); the solid and dashed lines (grey shaded section) represent the linear relationship (±95% confidence limits) described by the equation *Fv*/*Fm* = (-0.036 · σPSII) + 0.5954 and *Fv*/*Fm* = (-0.067 · σPSII) + 0.6451 for 25°C and 32°C data, respectively.

**Figure 2**: Relationship between (a) % change H2O2 produced and % change *Fv/Fm* for heat stress relative to control samples (i.e., % change = ([Treatment-Control]/Control) ·100), and (b) % change QA versus % change *Fv/Fm*. Each data point represents the % change calculated from the mean (n=3) control and treatment value for each *Symbiodinium* type (n=16; see main text). Statistically significant correlations between corresponding measurements were only observed for (a); the solid and dashed lines (grey shaded section) represent the linear relationship (±95% confidence limits) described by the equation % change H2O2 = (-3.5475 · % change *Fv/Fm*) + 79.405.

**Figure 3**. Functional groupings based on based on the physiological parameters associated with heat stress sensitivity (% change *Fv/Fm*, QA, H2O2 concentration). Cluster analysis and multidimensional scaling (MDS) were performed on the average of each variable per variant (see the Materials and Methods section); similarity is shown at the 1.1 and 1.9 distance levels and vectors driving the clustering are shown in grey. Note only ITS2 type designation are shown for clarity and full detailed of isolates and taxonomic classification can be found in the material and methods.

**Figure 4**. Mean ± standard deviation (n=4) of normalised relative quantity (NRQ) of the SOD genes (MnSOD and NiSOD) expression level for (a) *S. microadriaticum* (A1) and (b) *Symbiodinium* sp. (D1-5); both under control (25°C, light grey) and treatment (32°C, dark grey) temperatures. Relative quantity of MnSOD and NiSOD gene was normalised to the most stable reference genes as defined by GeNorm analysis (RPS4, GADPH and psbA) (see main text). NRQ was significantly different between control and treatment temperatures (independent t-test) for MnSOD only, but for both ITS2 types A1 (p=0.038) and D1-5 (p=0.047).