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Effects of increased temperature on dimethylsulfoniopropionate (DMSP) concentration and methionine synthase activity in *Symbiodinium microadriaticum*

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Abstract Dinoflagellate algae of the genus *Symbiodinium* occur as endosymbionts in a variety of hosts including coral. The response of *Symbiodinium* spp. to environmental changes could dictate survival of their hosts and the ecological success of coral reef ecosystems. Oxidative stress has been linked to a breakdown in this symbiotic relationship, known as bleaching. Increased temperature is one of the primary environmental changes linked to this phenomenon. Preliminary studies have established high concentrations of the sulfur compound dimethylsulfoniopropionate (DMSP) in *Symbiodinium* spp., with increased temperature. To examine the potential use of DMSP as an antioxidant, a 5 day incubation experiment was conducted at two temperatures with the algae *S. microadriaticum* (CCMP1633) isolated from the cnidarian host *Aiptasia pulchella.* An HPLC assay for the activity of the enzyme B_{12}-dependent methionine synthase was modified and used to determine the link between de novo production of methionine, a precursor to DMSP, and temperature induced oxidative stress. DMSP concentrations per cell increased approximately 38 % in the 33 °C treatment cultures over 120 h. However, these cells also increased more than 2-fold in biovolume (127 ± 43 %), and SYTO-BC stain indicated increased DNA content (approximately 4-fold), suggesting arrested cell division. Normalization of DMSP to biovolume revealed that the concentrations actually decreased approximately 49 % after 2 days in cultures exposed to elevated temperature (33 °C), but were not significantly different from the control treatment at 120 h (27 °C). Concomitant changes in the 33 °C treatment relative to the control (after 120 h) resulted in an approximately 8-fold increase in reactive oxygen species, a 37 % (±7 %) decrease in photosynthetic efficiency of photosystem II, and a 5-fold increase in xanthophyll cycling. Methionine synthase activity (MSA) correlated to the decrease in DMSP concentration ($R^2 = 0.778$), with decreasing activity at the high temperature. Given this decrease in MSA, the increase in DMSP per cell may be due to DMSP production utilizing methionine from protein turnover, and not de novo synthesis via MSA. The findings of this study provide insight into the responses of algal symbionts to environmental changes, shed light on the potential use of DMSP and other known photo-protective mechanisms such as xanthophyll cycling under temperature induced oxidative stress, and support the suspected cessation of cell division under these conditions. This information could be crucial to understanding cellular responses to environmental changes and the ability of these organisms to survive under elevated sea surface temperatures projected for the near future.

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Introduction

The effects of climate change on algal communities are complex. For algae living symbiotically within hosts, as do many *Symbiodinium* spp. (zooxanthellae), these effects may dictate the survival of entire ecosystems such as coral reefs. Increased frequencies of bleaching and mortality of coral reefs haven been attributed to increased temperature and irradiance, and several studies have investigated this relationship (Hoegh-Guldberg 1999; Jones et al. 1998). Bleaching episodes have been associated with ocean 'hot spots', defined by Goreau and Hayes (1994) as positive oceanic anomalies of +1 °C above mean maximum summer sea surface temperatures (SST), and most coral are thought to be within 1–2 °C of their upper temperature threshold at this time of year (Wellington et al. 2001; Coles et al. 1976).

Increased temperature and light can cause oxidative stress in *Symbiodinium* spp., as the reactive oxygen species (ROS) produced during stress add to those produced by normal cell activities and the cell's antioxidant systems are often overwhelmed. Oxidative stress has been implicated as one of the main causes of bleaching, and ROS production has been shown to induce the pro-apoptotic caspase cascade leading to expulsion of the algae from the host (Lesser et al. 1990). Both the host and the symbionts utilize superoxide dismutases (SODs), catalase, and ascorbate peroxidase to bind to or enzymatically breakdown ROS (Dykens and Shick 1982; Fridovich 1995; Lesser et al. 1990).

Another possible physiological mechanism to alleviate cellular oxidative stress involves the production of the sulfur compound dimethylsulfoniopropionate (DMSP) (Sunda et al. 2002; Yost et al. 2010). This tertiary sulfonium compound has sparked interest because it is produced in high concentrations per biovolume in some algae. In addition, the production and conversion, or "turnover", of DMSP can result in four different antioxidant compounds which Sunda et al. (2002) suggest operate as a "highly effective antioxidant system". One of these four sulfur compounds is the climatically active gas dimethylsulfide (DMS) whose formation is catalyzed by DMSP lyase activity.

When released to the atmosphere, DMS can be converted to sulfate aerosols which act as cloud condensation nuclei (Chin et al. 1996) that can potentially impact the radiation budget of the Earth (Charlson et al. 1987). A measurable pulse of DMS has been recorded in the atmosphere above coral reefs during exposure at low tide, which are conditions associated with increased temperature (Jones and Trevena 2005; Jones et al. 2007).

The specific clade B isolate used for this study was chosen due to its ecological relevance. This isolate was extracted from a cnidarian host (*Aiptasia pulchella*; collected near Hawaii) and it has been identified as thermally sensitive (Tchernov et al. 2004). It is an isolate containing high DMSP levels and DMSP-lyase activity, supporting its potential use of the antioxidant cascade (e.g. DMS) in alleviating oxidative stress (Yost and Mitchelmore 2009). Clade B symbionts are found globally and have been used in several previous studies as representative symbionts, although caution is warranted when generalizing to other isolates as within-clade differences can be greater than between clade differences (Karako-Lampert et al. 2005; Tchernov et al. 2004).

Aiptasia spp. have been used for experimental studies of algal-cnidarian symbioses since the 1970s. These anemones are typically found in shallow, tropical and subtropical waters and have symbioses most similar to reef corals (Cook et al. 1988). Dunn et al. (2004) demonstrated bleaching in *Aiptasia* spp. at temperatures of 31.5 and 33.4 °C with loss of zooxanthellae after 48 h of exposure. A recent study also established that the DMSP measured in *A. pulchella* was produced solely by the dinoflagellate symbiont and not the animal host (Van Alsytne et al. 2009). Symbiotic associations appear to be one reason for the success of organisms such as coral, but the roles of potential antioxidant compounds such as DMSP that are transferred to the host, have not been definitively determined.

Preliminary work in our laboratory suggested that DMSP levels increased in this particular isolate (CCMP 1633) at 32 °C, with an even greater increase documented at 34 °C (Miller 2009). However, the cultures exposed to 34 °C did not survive the duration of the 5 day experiment. Therefore, the experiments conducted in the present study employed the

temperature of 33 °C as a potential "threshold" or tipping point when the physiological adaptive mechanisms which determine survival occur in high enough levels to be observed. This acute temperature shift will be used to represent conditions experienced in natural "hot spots" and will test the ability of zooxanthellae to adapt when the duration of exposure is several days. Worldwide, mean summer maximum SST range from approximately 25 to 34 °C, and bleaching thresholds range from 27 to 36 °C (Jokiel and Brown 2004).

Previous studies have established changes in DMSP concentration under various environmental conditions (Miller 2009; Trossat et al. 1998), but because of the potential turnover of DMSP to other compounds, a method to assess production rates is necessary to accurately assess the role(s) of DMSP. For the present study, an HPLC method was adapted for *Symbiodinium* spp. to address the question of DMSP production rates (McLenon 2010). This method was used in the following experiments to examine methionine synthase activity (MSA) and its relation to DMSP concentrations. Methionine synthase is the enzyme responsible for de novo synthesis of methionine from homocysteine and is consequently a key precursor in the DMSP biosynthetic pathway (Gage et al. 1997; Greene 1962).

If DMSP is being used as an antioxidant, it is hypothesized that DMSP production may be upregulated and correspond directly to the physiological changes associated with the oxidative stress response. If MSA is a major point of regulation of DMSP biosynthesis, then its activity will correlate to changes in DMSP concentration induced by oxidative stress.

Materials and methods

Culture conditions

A clade B isolate (CCMP 1633) from *A. pulchella* (symbiotic cnidarian) was used for this study and was obtained from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP at Bigelow Laboratory for Ocean Sciences). The culture was grown in K + copper medium prepared with 0.22 μm filtered Gulf Stream sea water (Sterivex-GP 0.22 μm filter unit; Keller et al. 1987). This medium was designed specifically for oceanic marine phytoplankters that cannot tolerate higher levels of trace metals (includes a 10-fold higher EDTA chelation than other common media). Cultures were kept in a temperature-controlled environmental lab at 27 °C, under illumination of 100 μE m^{-2} s^{-1} and a 14:10 light/dark cycle. Semi-continuous cultures were maintained with dilutions every 10–14 days to maintain cells in log-phase growth.

Experiments

Symbiodinium cultures used in this study have been successfully maintained at 25 °C (±2 °C) for several years. For the temperature shift experiment, external re-circulating water baths and glass aquaria (filled ~ 1/3 with DI water) were used to maintain the cultures at 27 and 33 °C (monitored with a Digi-sense® DualLogR™ Thermocouple Thermometer). An acid-cleaned 20 L Nalgene carboy was inoculated with culture and bubbled with filtered air for about 2 weeks before the start of each experiment, and replicate cultures were separated into 2.5 L Nalgene bottles 48 h prior to commencing the temperature experiment (total $n = 4$ per treatment, two per experiment). Each bottle was bubbled with ambient air through a glass frit attached to an air compressor with a 0.2 μm inline filter.

Experiments were run in separate blocks with inoculates from the same stock culture. To minimize any diel effects all sampling was completed within 2 h after lights first came on (14:10 cycle), and within 1 h of each other. Samples were collected at the start (T-zero), and 24, 48, and 120 h after the temperature shift (0, 24, 48, 120 h).

Physiological indicators

Growth curves were determined using fluorometric detection of extracted chlorophyll a (Turner 10-AU fluorometer) as well as by collecting cell counts. Cell counts were conducted in replicate (3 mL) and stored in plastic vials with formalin (final concentration of 2 %). Fixed cell counts were verified with live counts on the DakoCytomation Mo Flo flow cytometer (FCM). Cell count, average biovolume and average cell diameter were measured from each sample using a Beckman Multisizer III Coulter Counter. Quantum efficiency of photosystem II (PSII) (F_v/F_m) was measured by fast repetition rate fluorometry (FRRF; Fast Tracka® Chelsea Instruments, Inc.), with

12–20 min of dark adaptation. An optimization for the dark adaptation time was previously determined by repeated F_v/F_m readings from the same culture every 2–3 min with incubations of 5–30 min (in triplicate). There was not a significant difference in results from 12–20 min of dark adaptation (data not shown).

Oxidative stress

Reactive oxygen species were estimated via the non-fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Evans et al. 2006). This dye is membrane permeable and is hydrolyzed by esterases once it enters the cell into a polar molecule that becomes trapped, and is subject to oxidation to the fluorescent compound dichlorofluorescein (DCF). Intracellular DCF fluorescence was measured on the FCM using 488 nm excitation and emission at 522 nm (using filtered water sheath and counting 10,000–30,000 cells/run). Because oxidation is required for conversion to this fluorescent compound, increased fluorescence per cell is typically used as a proxy for the presence of ROS. Culture samples (1 mL) were collected and incubated for 60 min at treatment temperature with a 4 μM final concentration of CM-H_2DCFDA. The cellular fluorescence from samples without stain was subtracted as blanks from all timepoint fluorescence readings.

Cell vitality assay

Calcein-AM (Invitrogen®) is a neutral vital dye which can diffuse into cells where it is converted via cell esterase activity into an impermeant fluorescent analog. Healthy cells with intact membranes do not allow this compound to leave the cell. This fluorescence therefore represents esterase activity (which is a recognized parameter for cell health) and membrane integrity and is used as a proxy for cell vitality (Gala and Giesy 1990). Calcein-AM has also shown potential as a detector of intracellular ROS (Uggeri et al. 2000). Culture samples (1 mL each) were collected in plastic tubes in replicate from each culture. A working stock of the stain was prepared fresh each day (0.2 μm filtered seawater, 800 μL to 1 mg vial). Stain was added (100 μL of working stock) and samples were incubated at their treatment temperatures for 45 min prior to analyzing them on the Mo FCM (20,000 cell count; excitation 494 nm, emission 517 nm).

DMSP measurements

Both total and dissolved DMSP samples were collected in 25 mL plastic scintillation vials and were analyzed within 2 weeks of collection. For the determination of dissolved DMSP, samples (25 mL) were gravity filtered through a Whatman GF/F filter, and 200 μL of 50 % H_2SO_4 was added to the first 20 mL of filtrate collected. Another 25 mL of culture was added to a separate vial and 250 μL of 50 % H_2SO_4 was added for total DMSP analysis. All samples were stored in the dark at 4 °C until analysis (Kiene and Slezak 2006). Samples were then base-hydrolyzed to convert the DMSP to DMS, which was measured using a cryogenic purge and trap system coupled to a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a flame photometric detector (White 1982; DiTullio and Smith 1995). Samples were sparged at a flow rate of approximately 125 mL/min with 2 N NaOH for 25 min, and standard calibration curves were created using a DMS permeation device (18 ng S/min; ViciMetronics). Temperature of the GC column (Chromosil 330, Supelco) was maintained at 30 °C, and the limit of detection of the system was approximately 0.5 ng as DMS.

Analytical methods (enzyme activity assay)

The HPLC method of Huang et al. (2001) was adapted and used to assay MSA (McLenon 2010). This method measures concentration of tetrahydrofolate (THF) as opposed to methionine. They are produced in a one-to-one ratio by methionine synthase and therefore THF represents de novo methionine synthesis. Major modifications to the original method included use of a Luna 3 μm C18 column (Phenomenex, 3 μm, 100 Å, 250 × 4.6 mm), sonication of algal cells, incubation at the treatment and control temperatures, and addition of mercaptoethanol to all final samples for accurate measurements of the product.

DNA analysis

SYTO®BC, a green fluorescent nucleic acid stain, was used to determine relative amounts of DNA in the control and treatment cells. Culture samples were collected and preserved in 2 % formalin, stain was added to 1 mL of preserved culture, and they were incubated for 20 min (20 nM final concentration).

All samples were run immediately following the final sampling on the FCM (20,000 cell limit; excitation: 488 nm, emission: 530 nm).

Xanthophyll pigments

Cells were harvested for pigment analysis by filtering aliquots (20–25 mL) of culture through Whatman GF/F glass-fiber filters under gentle vacuum and dim light. Filters were then immediately snap-frozen in liquid nitrogen, wrapped in aluminum foil and stored at −80 °C prior to analyses. Samples were extracted overnight at −20 °C in 1,400 µL of acetone, syringe filtered, and pigments were analyzed by HPLC with a method (DiTullio and Geesey 2002) adapted from Zapata et al. (2000).

Statistical analysis

Statistical analyses were carried out using SigmaStat software (SPSS Inc., Chicago, IL). All data in the figures are expressed as mean ± SE. Unless otherwise noted, two-way repeated-measures ANOVA (TWRMANOVA) were conducted with Holm-Sidak pairwise comparisons for post hoc analyses when appropriate. All data were tested for normality and equal variance, and transformations were used when necessary. ROS and DMSP total per cell data were square root transformed; Calcein-AM results were $\log_{10}$ transformed. Regression analyses were used for ROS versus xanthophyll cycling (represented as the ratio of de-epoxidized DT to the xanthophyll pigment pool (DD + DT)), and DMSP total per biovolume versus MSA per biovolume.

Results

This study demonstrated the stress response of the zooxanthellae isolate CCMP#1633 to increased temperature. Significant cellular physiological changes were observed in the following parameters.

Growth rate

Rates of cell division slowed or stopped (Fig. 1a) in response to 5 days exposure to 33 °C (+6 °C from control, three way ANOVA, $p < 0.001$). Maximum growth rate for the cultures was determined to be between days 2 and 5 (Fig. 1a). Average maximum specific growth rate for the control cultures was 0.34 (±0.02 SE) d^{-1}. These division rates are equivalent to 0.49 doublings per day in the 27 °C cultures. In contrast, cell concentrations in the treatment cultures at 33 °C actually decreased (maximum growth rate- = −0.09 (±0.02 SE) d^{-1}).

Photophysiology

F_v/F_m- Photosynthetic efficiency of PSII as indicated by the ratio of variable fluorescence to maximum fluorescence (F_v/F_m) was 0.39 ± 0.01 for both control and treatment cultures at the beginning of all experiments (Fig. 1b). Control cultures maintained an F_v/F_m of 0.38 ± 0.01. However, the F_v/F_m values in the elevated temperature treatment cultures decreased significantly to 0.24 ± 0.02 after 120 h, a 37 % decrease relative to the control ($p < 0.001$).

ROS

The fluorescent probe DCFH was used to estimate relative amounts of ROS present in each culture. Levels of ROS in the 27 °C cultures did not change over the 5 days, but all timepoints for the 33 °C treatments were significantly different from each other (Fig. 1c). The controls were significantly different from treatment at 24, 48 and 120 h ($p < 0.001$). An approximate 8-fold increase in ROS (based on DCFH fluorescence) was observed in the 33 °C treatment after 120 h relative to the control (Fig. 1c).

Biovolume/cell

Cell size increased significantly in the treatment cultures (Fig. 1d; $p < 0.001$). The 33 °C cells increased 2.3-fold in size from an average biovolume of 150 μm^3 (±18.4 μm^3 SE) to 343 μm^3 (±43.8 μm^3 SE) over 120 h.

DMSP total

It was determined that the dissolved fraction of DMSP comprised a nominal fraction of the total DMSP (<5 %; data not shown). For this reason, only total DMSP results are presented for all analyses. Total DMSP per cell decreased in the controls and increased in the 33 °C cultures over time (Fig. 1e). This change

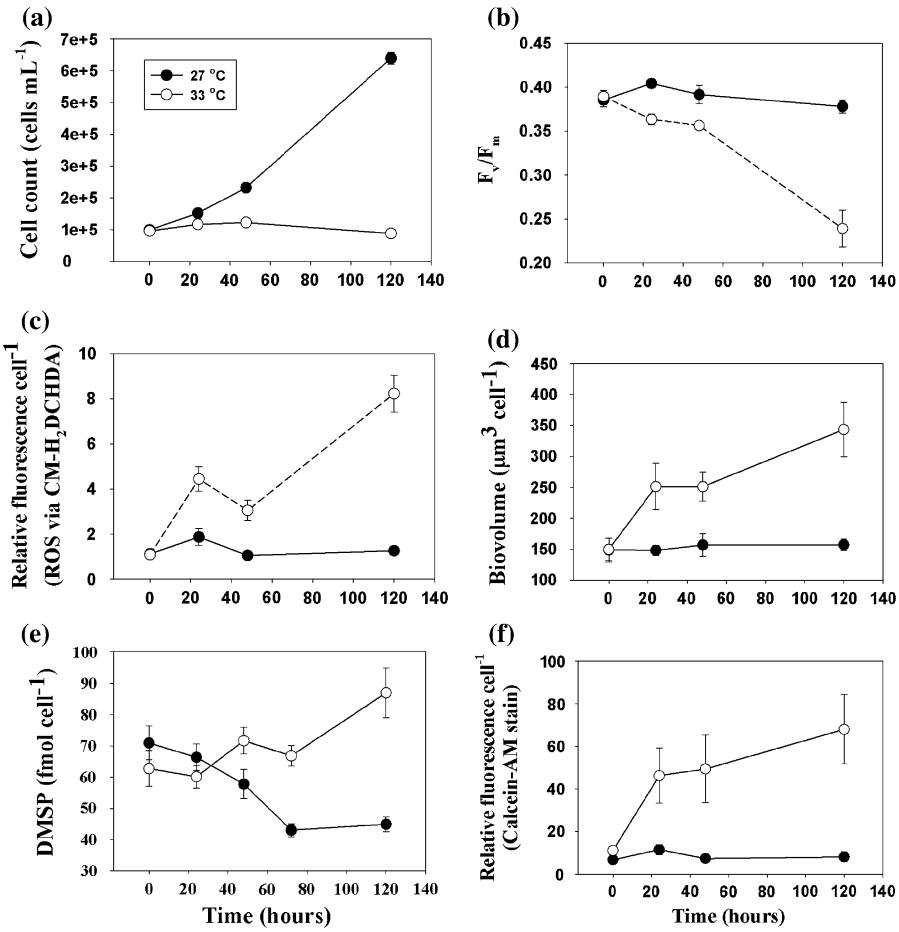

Fig. 1 Results of increased temperature experiments. All results presented as mean ± SE ($n = 4$); solid circles represent control cultures and open circles are high temperature treatment. **a** Cell concentration for control (27 °C) and treatment (33 °C) cultures. Maximum growth rate determined between 48 and 120 h: 27 °C = +0.339 d^{-1}; 33 °C = −0.088 d^{-1}. **b** Photosynthetic efficiency of PSII represented by the ratio of variable fluorescence to maximum fluorescence (F_v/F_m). **c** Estimate of ROS in cultures at two temperatures by CM-H_2DCFDA using a MoFlo flow cytometer. **d** Biovolume normalized to cell count [150 μm^3 (±18.4 μm^3 SE) to 343 μm^3 (±43.8 μm^3 SE)]. **e** Total DMSP per cell. **f** Estimate of vitality by Calcein-AM stain represented as relative fluorescence per cell

is significant between 0 h and 120 for the controls, and all timepoints for the high temperature treatments. Post hoc analyses revealed a significant difference in total DMSP between 27 and 33 °C at T120 h ($p = 0.001$). When normalized to biovolume to account for the changes in cell size, there appears to be a decrease in DMSP in the 33 °C cultures but it is not statistically significant over 120 h (Fig. 2a; $p = 0.123$). However, ratios of DMSP per unit volume were approximately 40 % lower at 33 °C than the control at 24 and 48 h timepoints (Fig. 2a).

Calcein/cell vitality

The Calcein-AM® vitality assay was run concurrently with the ROS samples measured with DCFH. Results of calcein-induced fluorescence (measured by counting 20,000 cells) indicated no significant change in the 27 °C cultures over time, but all times were significantly different from 0 for the 33 °C treatment cultures (Fig. 1f). The treatment cultures had significantly higher fluorescence at 24, 48 and 120 h ($p < 0.001$, $n = 4$).

Methionine synthase activity (MSA)

Due to the significant changes observed in cell biovolume at the higher temperature, methionine synthase was normalized to biovolume as opposed to total protein, which is more typically used in enzyme activity studies. With the change in cell size taken into consideration, there were significant effects of treatment and time, but no interactive effect was observed between time and temperature (Fig. 2b; $p = 0.022, 0.017$). Both the 27 and 33 °C cultures exhibited depressed MSA over time. When plotted as a regression against DMSP per biovolume, there was a linear correlation between these two parameters (Fig. 3; $R^2 = 0.778$).

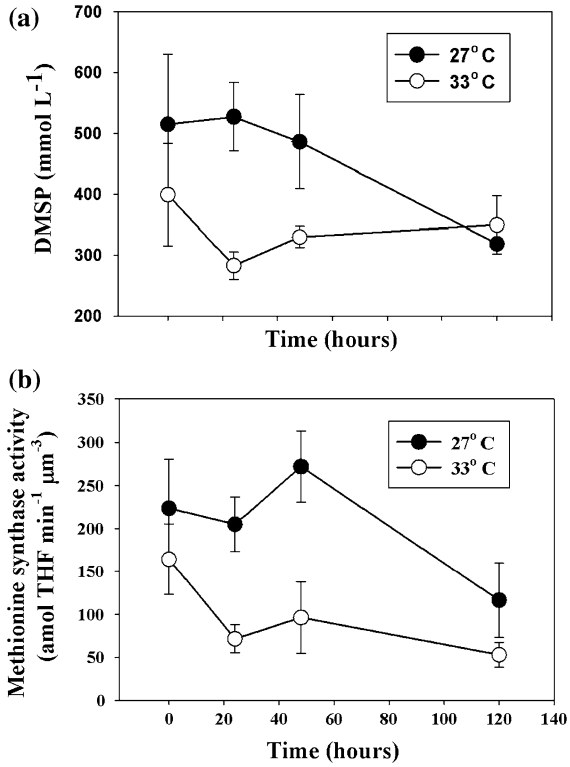

Fig. 2 **a** Total DMSP per biovolume for cultures exposed to 27 and 33 °C. **b** MSA per biovolume

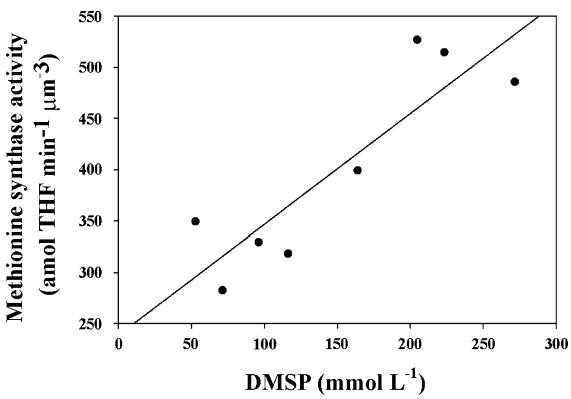

Fig. 3 Regression analysis of DMSP concentration (mmol L^{-1}) and MSA (amol THF min^{-1} μm^{-3}). Adjusted $R^2 = 0.778$

Pigment analyses

The pigment diatoxanthin was normalized to the diadinoxanthin and diatoxanthin (DD + DT) pool. There was a 5-fold increase in the ratio of DT to the pool (DD + DT) in the 33 °C cultures (i.e. from

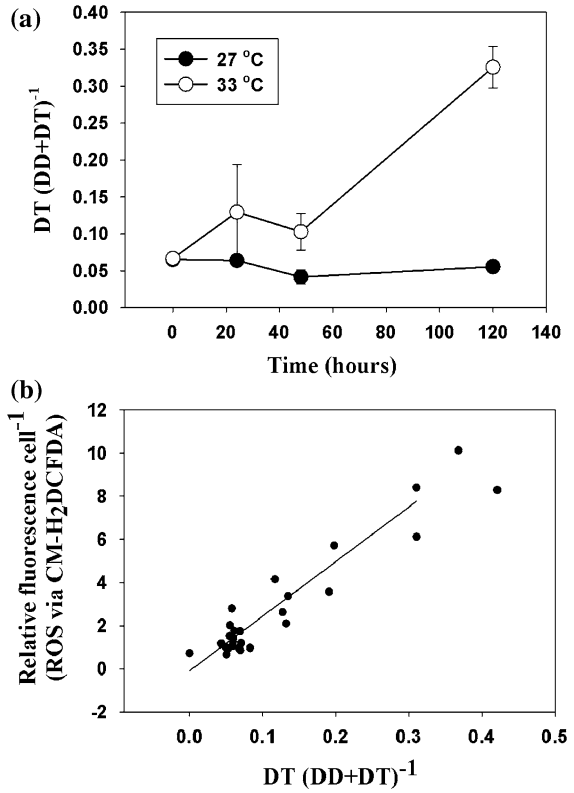

Fig. 4 **a** The pigment diatoxanthin normalized to the diadinoxanthin and diatoxanthin (DD + DT) pool. **b** Regression analysis of ROS (indicated by relative mean fluorescence per cell) and xanthophyll cycling (represented as the ratio of de-epoxidized DT to the xanthophyll pigment pool (DD + DT)). Adjusted $R^2 = 0.905$

0.067 ± 0.003 to 0.326 ± 0.030), by the end of the experiment (Fig. 4a), and a significant effect of time, temperature, and an interactive effect of time and temperature (p values < 0.001, 0.013, <0.001 respectively). Post hoc analyses revealed a difference between control and treatment at 120 h. There was not a significant change in the xanthophyll (DD + DT) pool size when normalized per cell (data not shown), which supports the relative importance of xanthophyll cycling. A regression analysis of ROS and DT/(DD + DT) cycling indicated a strong correlation ($R^2 = 0.905$) between these parameters after 120 h (Fig. 4b).

DNA analyses

Histograms from FCM analyses indicated the relative fluorescence of the stain (y axis) and number of cells

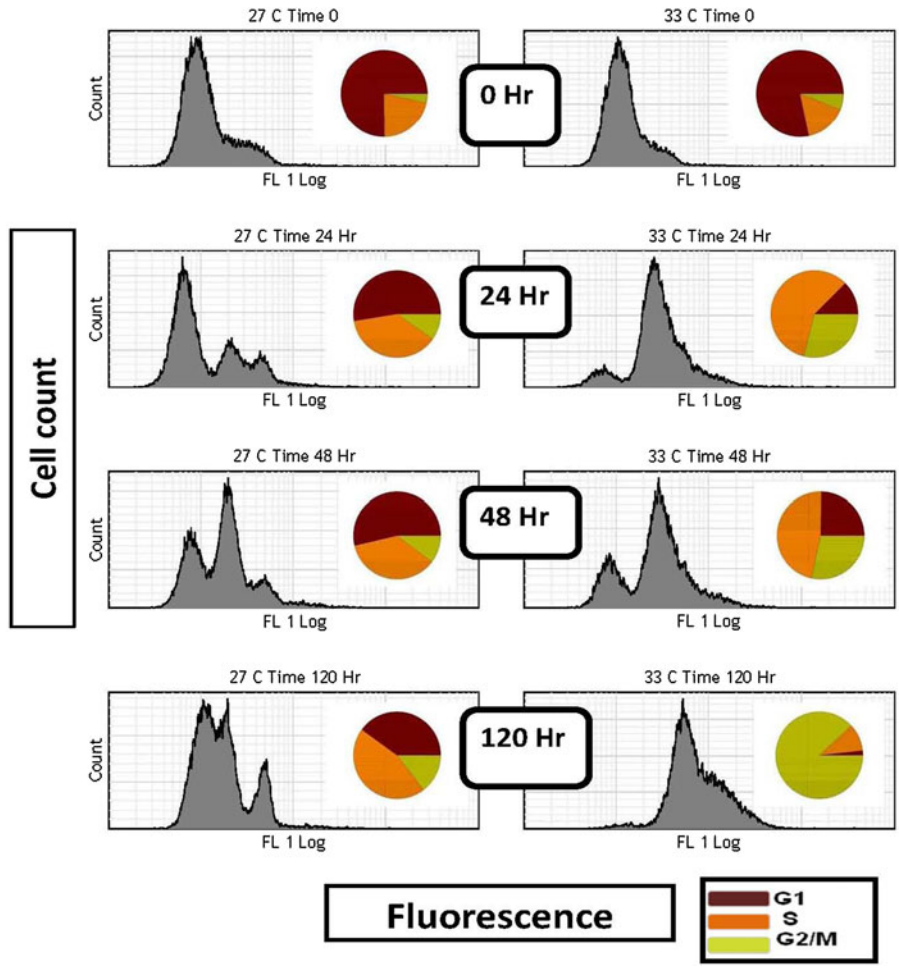

Fig. 5 Flow cytometry analyses of DNA content of cells incubated at 27 and 33 °C. Representative histograms indicate relative fluorescence of the stain (*y* axis) and number of cells (*x* axis). Peaks in the 27 °C results were used to make arbitrary divisions utilizing relative amount of DNA and corresponding phases of the cell cycle. Colored pie charts indicate the proportion of cells in a given stage of cell division at each timepoint. After 120 h exposure 88 % (±2%) of 33 °C treatment cells are in the G_2/M stage with highest DNA content, compared to 15 % (±4.6 %) in controls

(*x* axis) (Fig. 5). Control cultures cycled through the cell division stages, with three apparent peaks which represented cells in each of the three cell cycle phases over time. The colored pie charts indicate the proportion of cells in a given stage of cell division at each timepoint. At 120 h exposure, the DNA content of the 33 °C treatment cultures suggested 88 % (±2 %) of the cells were in the G_2/M stage with the highest DNA content, compared to only 15 % (±4.6 %) in the control cultures.

Discussion

Several physiological indicators suggested that elevated temperature imparted significant cellular oxidative stress on *Symbiodinium microadriaticum*. For example, heat stress caused a significant depression in photosynthetic efficiency of PS II (i.e. F_v/F_m) as well as an increase in ROS production relative to the control treatment in both experiments (Fig. 1b, c). The cessation of cell division suggested by DNA results (Fig. 5) could well be linked to this damage and stress, as previous studies have shown that photosystem impairment affects cell division processes (Wang et al. 2008). Normally, cells enter the growing/DNA synthesis stage from G_1 to S and finally G_2 stage before mitotic division. In *Symbiodinium* spp., darkness is required for progression to these latter stages (Wang et al. 2008). This requirement may reflect a need to reduce ROS in order to progress through cell division. For instance, there are checkpoints between each of

these stages which ultimately determine whether or not cells can progress to the next cell cycle stage. It has been established in other species of algae that arrest in the cell division process resulted in cells that continued to grow in cell size, despite no longer dividing; DNA damage was suspected to be the cause of the cessation (Buma et al. 1996; Mostajir and Demers 1999). The high cellular levels of DNA observed in the cells grown at 33 °, along with the 2.3-fold increase in biovolume per cell (Fig. 1d) suggest that these cells are not able to pass the final checkpoint for cell division. One major criterion for progression to the final stage in cell division is intact DNA; the observed arrest in cell division may therefore be due to DNA damage imparted by heat and oxidative stress (Wang et al. 2008).

In addition to cell division cessation, another possible explanation for the increase in biovolume in the high temperature treatments is vacuolization and disorganization of cell contents prior to cell death (Franklin et al. 2004). However, the conserved levels of SYTO-BC® stain in the DNA samples, and Calcein-AM results for the vitality assay, suggest that the 33 °C cells have relatively intact membranes and high esterase activity after 120 h exposure. The high cellular fluorescence in the 33 °C treatment (Fig. 1f) suggests that the cells were not necrotic since, during necrosis, membranes lose their integrity and the fluorescent stain would leak out of compromised cells (Gatti et al. 1998). This result also suggests that most of these cells were not in the final stages of apoptosis, as cellular fluorescence would rapidly decrease once cells completed programmed cell death (PCD). This result is further support, however, that these cells are under high oxidative stress, as Calcein stain is also indicative of ROS within cells (Uggeri et al. 2000). This stain also reflected the increased biovolume of the heat stressed cells via increased fluorescence over time. Although this result does not indicate high levels of apoptosis or necrosis, some cells within the culture may undergo this fate, as the assay may not detect cells that are in the early stages of PCD.

One type of cellular response to oxidative stress involves non-photochemical quenching (NPQ). NPQ is a photoprotective mechanism which prevents over-reduction of the electron transport chain by dissipating excess absorbed light energy in PSII antennae as heat (Jones et al. 1998). In some unicellular chromophytic algae, de-epoxidation of DD to DT functions in this way, and is a type of xanthophyll cycling. An increase in the DT/(DD + DT) ratio is usually associated with photo-oxidative stress and NPQ and has a relatively fast response time (hours) in algae (Goss et al. 1999).

In the temperature experiment, the ratio of DT/(DD + DT) increased 5-fold after 120 h in the 33 °C cultures in response to elevated temperature (Fig. 4a). This increase in xanthophyll cycling has been proposed as a mechanism zooxanthellae use to adapt to high temperature and irradiance levels before bleaching. To our knowledge, however, the effects of temperature on xanthophyll cycling have not been previously investigated, especially with respect to symbiotic dinoflagellates. It is conceivable that elevated temperature stress causes a similar oxidative stress response within the photosystems as that of excess ROS that accumulates under high irradiance. The largest increase in DT/(DD + DT) in the temperature treatment correlated to the largest increase observed in ROS at 120 h, and the most significant drop in F_v/F_m (Fig. 1b, c). A regression analysis of ROS and DT/(DD + DT) ratio revealed indicated a strong linear correlation ($r^2 = 0.905$) between these parameters for both control and treatment cultures (Fig. 4b). Perhaps this is an antioxidant response which is triggered when heat dissipation is maximal and/or when certain threshold levels of cellular ROS accumulate.

The decrease in intracellular DMSP concentration over the course of the incubation in the control treatment may be related to the increase in cell number causing self-shading in the culture. For instance, light intensity has been shown to significantly affect DMSP concentrations (Matrai et al. 1995). In support of this hypothesis, the largest decrease in MSA in the control treatment (Fig. 2b) occurred at the same time (i.e. between 48 and 120 h) as the major increase in cell density (Fig. 1a) and decrease in cellular DMSP (Fig. 1e).

A significant increase in DMSP per cell occurred at 120 h in the 33 °C cultures (Fig. 1e). This cellular DMSP increase was correlated to changes in photosynthetic efficiency, evidence of xanthophyll cycling, and increased ROS (Figs. 1b, c, 3a). Biosynthesis of DMSP has been shown to vary with environmental conditions (Stefels 2000). However, the increase in cellular DMSP at elevated temperature was observed in cells that were also increasing in size (Fig. 1d). As a result, DMSP normalization to biovolume caused an

actual decrease in total DMSP per unit biovolume presumably due to a faster increase in biovolume compared to the accumulation of cellular DMSP (Fig. 2a). Cells exposed to high temperature may have replenished their cellular DMSP levels in an attempt to maintain osmotic concentrations (as biovolume is increasing) that were optimized for other cellular functions. The decrease in DMSP per unit biovolume, however, is also consistent with an increase in DMSP turnover as an antioxidant response to oxidative stress (Sunda et al. 2002). Potentially, this reflects a dual function (i.e. antioxidant/osmoprotectant) for this compound (Stefels 2000).

Methionine synthase activity (i.e. de novo synthesis of methionine) normalized to biovolume also decreased in the high temperature treatment in a similar fashion as the total DMSP per unit biovolume (Fig 2a, b). These data suggest that MSA may be linked to DMSP biosynthesis when cells are subjected to oxidative stress. It is important to consider, however, that methionine produced by MSA is used for many cell functions in addition to DMSP production. In a study investigating the short-term (18 h) relationship between protein synthesis and DMSP production in algae, labeled protein was observed to be about twice the specific activity of labeled DMSP (Gage et al. 1997). The authors calculated that only approximately one-third of the activity of methionine synthase would correlate to DMSP production. The other two-thirds may correlate to other cell activities such as photosynthesis, cellular repair, and cell division.

It is also possible that other sources of methionine are utilized for DMSP biosynthesis. Gröne and Kirst (1992) observed that methionine availability limited DMSP production, specifically that inhibition of protein turnover delayed DMSP accumulation. This result suggested the potential importance of methionine recycling (via protein turnover) as a potential source of regulation for DMSP production. Increased protein degradation (i.e. turnover) and decreased rates of protein synthesis have been previously measured under oxidative stress conditions in plants (Cooke et al. 1979). Both processes would increase the availability of methionine without increasing MSA. While decreases in MSA correlated to decreases in DMSP per biovolume, the increase in DMSP concentration per cell in the 33 °C cultures could be explained by the relative importance of protein turnover in producing free methionine.

In addition, the initial decrease (e.g. 24 h) in DMSP per unit biovolume observed in the high temperature treatment prior to 120 h may be due to rapid turnover of the DMSP pool. For instance, the proposed function of DMSP serving as an antioxidant in *Symbiodinium* spp. was supported by the presence of relatively high DMSP-lyase activity in the specific isolate (CCMP#1633) used in our study (Yost and Mitchelmore 2009). Conversion of DMSP to DMS by the symbiont may not only provide free radical scavenging equivalents for the alga but may also confer oxidative stress protection to the host. A recent study provided experimental support for the antioxidant function of DMSP within the host: oxidative stress induced by copper additions was found to change the relative levels of total DMSP (i.e. coral tissue) and particulate DMSP (algal only) suggesting a partitioning of DMSP between host and symbiont (Yost et al. 2010).

In addition to protein turnover effects, the relative decrease in the ratio of both MSA/biovolume and DMSP/biovolume in the high temperature treatment could also be a result of enzyme inhibition. For example, acute heat stress in *Symbiodinium* spp. has been found to cause a decline in F_v/F_m and a subsequent increase in production of nitric oxide (NO), a cell signaling molecule (Bouchard and Yamasaki 2008). The increase in NO production was temperature-dependent (Bouchard and Yamasaki 2008). In addition, NO has been implicated ecologically as a signal for bleaching at 33 °C in *Aiptasia* spp. (Bouchard and Yamasaki 2008; Perez and Weis 2006).

Nitric oxide has previously been shown in other species to inhibit MSA and disrupt carbon flow through the folate pathway (Banerjee et al. 1990; Danishpajooh et al. 2001; Fiskerstrand et al. 1997). Nitric oxide interacts with the cobalt of the corrin ring of B_{12} which is structurally similar to the iron of heme-containing proteins which NO usually acts upon. This interaction is irreversible and NO may be competing with cobalamin-dependent methionine synthase for B_{12}, thereby limiting enzyme activity. This mechanism suggests yet another reason for the correlation that we observed between MSA and DMSP concentration. Under oxidative stress, *Symbiodinium* spp. may not be able to use methionine synthase optimally to produce methionine for DMSP synthesis, and may have to rely on other, potentially limiting, sources of this amino acid (i.e. protein degradation). Finally, NO

production may also be responsible for the cessation of cell division rates observed in the high temperature treatment since NO has been shown to damage DNA (Danishpajooh et al. 2001).

Although these lab results are intriguing they should be carefully interpreted with respect to ecological implications. For instance, isolated cultured symbiotic dinoflagellates often represent only a subset of the *in hospite* community of zooxanthellae (Carlos et al. 2000; Santos et al. 2001). In addition, proteins isolated from *Symbiodinium* spp. in the cultured state can be very different with respect to those produced in the endosymbiotic state (Stochaj and Grossman 1997). This may be due to the host, which can impart a large effect on algal gene expression and therefore protein production in the symbiotic condition (Stochaj and Grossman 1997). Consequently, to accurately assess the role of DMSP with respect to the thermal stress response of *S. microadriaticum*, corresponding experiments should be conducted in the host.

However, the findings of this study have several important implications and can contribute to our understanding of the role of the symbiont in observed coral-symbiont, or "holobiont", responses to environmental change. General inhibition of enzymes and the role of this inhibition in both the bleaching phenomenon and the death of algal symbionts could be very important. During development of the MSA enzyme assay, it was determined that MSA decreased significantly from 30 to 37 °C, with an almost complete loss of activity at 37 °C (McLenon 2010). The similar finding that RUBISCO II (the enzyme responsible for carbon fixation and therefore energy acquisition for both the symbiont and host) is almost completely inactive at temperatures above 36 °C adds further support (Lilley et al. 2010). These temperatures coincide with the crucial temperatures determined by numerous studies of coral bleaching thresholds, and they suggest a possible mechanism for the breakdown of the symbiosis.

Further investigation of the potential role of DMSP as an antioxidant will be necessary to determine if temperature-induced oxidative stress is a unique case in which MSA inhibition limits DMSP biosynthesis. Due to the global nature of the current and predicted climate changes, the impacts of such changes on *Symbiodinium* spp. may provide valuable insight into the ability coral reefs to respond and adapt worldwide to rising SST. Coral reefs have been identified as ecosystems of critical importance which are severely threatened and declining in health (Hughes et al. 2003). Our results indicate that at elevated temperatures, the production of DMSP in *Symbiodinium* spp. may be limited due to various cellular regulatory mechanisms which may have an impact on the host's physiology, the reef ecosystem, and potentially local climate.

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