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## Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress

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**Abstract** Elevated seawater temperatures have long been accepted as the principal stressor causing the loss of symbiotic algae in corals and other invertebrates with algal symbionts (i.e., “bleaching”). A secondary factor associated with coral bleaching is solar irradiance, both its visible (PAR: 400–700 nm) and ultraviolet (UVR: 290–400 nm) portions of the spectrum. Here we examined the synergistic role of solar radiation on thermally induced stress and subsequent bleaching in a common Caribbean coral, *Montastraea faveolata*. Active fluorescent measurements show that steady-state quantum yields of photosystem II (PSII) fluorescence in the zooxanthellae are markedly depressed when exposed to high solar radiation and elevated temperatures, and the concentration of D1 protein is significantly lower in high light when compared to low light treatments under the same thermal stress. Both photosynthetic pigments and mycosporine-like amino acids (MAAs) are also depressed after experimental exposure to high solar radiation and thermal stress. Host DNA damage is exacerbated under high light conditions and is correlated with the expression of the cell cycle gene *p 53*, a cellular gatekeeper that modulates the fate of damaged cells between DNA repair processes and apoptotic pathways. These markers of cellular stress in the host and zooxanthellae have in common their response to the enhanced production of reactive oxygen species during exposure to high irradiances of solar radiation and elevated temperatures. Taking these results and previously published data into consideration, we conclude that thermal stress during exposure to high irradiances of solar radiation, or irradiances higher than the current photoacclimatization state, causes damage to both

photochemistry and carbon fixation at the same time in zooxanthellae, while DNA damage, apoptosis, or necrosis are occurring in the host tissues of symbiotic cnidarians.

**Keywords** Coral bleaching · Thermal stress · Solar radiation · PSII damage · DNA damage · Apoptosis · Oxidative stress

**Abbreviations**  $\sigma_{\text{PSII}}$ : Functional absorption cross-section for PSII ·  $F_o$ ,  $F_m$ : Minimum and maximum yields of chlorophyll *a* fluorescence measured after dark acclimation (relative units) ·  $F_v$ : Variable fluorescence after dark acclimation ( $=F_m - F_o$ ), dimensionless ·  $F_v/F_m$ : Maximum quantum yield of photochemistry in PSII measured after dark acclimation, dimensionless ·  $F'$ ,  $F'_m$ : Steady-state and maximum yields of chlorophyll *a* fluorescence measured under ambient light (relative units) ·  $\Delta F/F'_m$ : Quantum yield of photochemistry in PSII measured at steady state under ambient light

### Introduction

Over the past several years, and with increasing regularity, coral reefs around the world have been affected by a phenomenon known as “coral bleaching,” which involves the mass expulsion of their symbiotic dinoflagellates (= zooxanthellae), but can also include the loss of photosynthetic pigments within individual zooxanthellae (Glynn 1991). These events closely follow oceanic warming that results in elevated seawater temperatures of 30–33 °C (Glynn 1991). Field and laboratory studies on bleaching in corals and other symbiotic cnidarians have established a causal link between temperature stress and bleaching (Lasker et al. 1984; Hoegh-Guldberg and Smith 1989; Jokiel and Coles 1990; Lesser et al. 1990; Glynn and D’Croz 1990; Fitt et al. 1993; Lesser 1997; Hoegh-Guldberg 1999).

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Several studies have also implicated photosynthetically active radiation (PAR, 400–700 nm, Hoegh-Guldberg and Smith 1989) and/or ultraviolet radiation (UVR; UVA, 320–400 nm, UVB, 290–320 nm) as a cause of bleaching either alone (Lesser et al. 1990; Gleason and Wellington 1993) or synergistically with elevated temperature (Lesser et al. 1990; Glynn et al. 1992). The direct and indirect effects of UVR may involve damage to DNA, proteins, and membrane lipids (see Shick et al. 1996 for review) that often involves the production of reactive oxygen species (ROS) (Lesser and Shick 1989; Lesser 1996, 1997).

Corals respond to UVR by synthesizing UV-absorbing compounds (mycosporine-like amino acids [MAAs]) and enzymes involved in the protection of both the host and symbiont from oxidative stress (Lesser and Shick 1989; Lesser 1996; Shick et al. 1996; Lesser 2000). For zooxanthellae, damage to PSII following exposure to elevated temperatures and solar radiation has been described as a primary site of damage (Iglesias-Prieto et al. 1992; Lesser 1996; Warner et al. 1996; Warner et al. 1999). Lesser (1996) described damage to PSII in cultures of zooxanthellae after exposure to elevated temperatures and UVR, while Warner et al. (1996) used a pulse-amplitude modulated fluorometer in the laboratory to describe damage to PSII during exposure to elevated temperatures on zooxanthellae *in hospite*. More definitively, using fluorescence measurements and a molecular approach, work by Warner et al. (1999) has shown that damage at the D1 protein of PSII occurs in zooxanthellae exposed to elevated seawater temperatures. Damage to the D1 protein can be caused by ROS (Richter et al. 1990; Macpherson et al. 1993; Lupinková and Komenda 2004) or exposure to UVR (Greenburg et al. 1989).

Exposure to UVR and ROS can act synergistically to cause extensive DNA damage and lead to apoptosis (programmed cell death) or cellular necrosis. Although apoptosis is a well-described regulatory phenomenon during embryonic development and plays a significant role in the biology of cancer, recent interest has focused on its role in the eukaryotic stress response (Hengartner 2000). A key regulatory gene involved in the fate of cells after DNA damage is the cell cycle gene *p 53*. The principal function of *p 53* is to promote either the survival or deletion of cells exposed to agents that cause DNA damage such as ROS (Renzing et al. 1996). The *p 53* protein is therefore involved in a series of complex cellular responses to DNA damage. These responses involve DNA editing and repair followed either by normal cell division (Polyak et al. 1997) or apoptosis (Hale et al. 1996). Cells with DNA damage caused by UVR and oxidative stress can survive but are often retained in the G1/S phase of the cell cycle for long periods of time which involves the expression of *p 53*, *p 21*, and subsequent inhibition of the activity of cyclin-dependent kinases (Evans and Littlewood 1998).

A cellular model of bleaching in symbiotic cnidarians has been developing over the years and includes oxidative stress, PSII damage, DNA damage, and apoptosis

as underlying processes (Lesser et al. 1990; Gates et al. 1992; Lesser 1996, 1997; Warner et al. 1999). This model has been shown to be consistent with a variety of biomarker proteins expressed in cnidarians during thermal stress (Downs et al. 2000, 2002). This paper contributes to that working model by examining the synergistic role of solar radiation on the severity of the thermal stress response in the Caribbean coral, *Montastraea faveolata*.

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## Materials and methods

### Site and experimental design

Experiments were performed at the Caribbean Marine Sciences Center, Lee Stocking Island, Bahamas (23°46'N, 76°05'W) during May 1998. Collections of *Montastraea faveolata* (N = 25, 10–13 cm base diameter) for laboratory bleaching studies were made from Horseshoe Reef, at approximately 8–10 m depth, where maximum *in situ* photosynthetically active radiation (PAR: 400–700 nm) irradiances on clear days at 1300 h are ~ 500–700  $\mu\text{mole quanta m}^{-2} \text{s}^{-1}$ . The coral samples were placed in flowing seawater tables (26–29 °C) and three treatment groups were prepared as follows. Ten corals were placed in flowing seawater tables (0.5 m depth) and ambient irradiance decreased using neutral density screens to adjust the maximum irradiance to approximately ~550  $\mu\text{mole quanta m}^{-2} \text{s}^{-1}$  PAR (low light, LL). Another set of ten corals were initially shielded as just described and subsequently exposed to irradiances that were increased over a 10-day period using neutral density filters to a maximum irradiance of approximately 2,100  $\mu\text{mole quanta m}^{-2} \text{s}^{-1}$  PAR (high light, HL). Irradiances greater than 1,800  $\mu\text{mole quanta m}^{-2} \text{s}^{-1}$  PAR are common in shallow waters (1–2 m) for many coral reef habitats (Dustan 1982; Strömberg 1987; Lesser and Lewis 1996) and 10 days is sufficient time for the photosynthetic apparatus to reorganize and acclimatize to a new irradiance regime (Lesser and Shick 1989; Iglesias-Prieto and Trench 1994, 1997). A final set of corals (N = 5) was immediately placed under full solar irradiances (2,100  $\mu\text{mole quanta m}^{-2} \text{s}^{-1}$  PAR) after collection. They bleached within 3–4 days. These corals were then placed in the flowing seawater table exposed to ~550  $\mu\text{mole quanta m}^{-2} \text{s}^{-1}$  PAR as described above. These corals were designated as the recovery group (Recovery, Re). After an additional week of acclimatization at their respective irradiances, daily measurements (~0900 h) of fluorescence yields were begun on all corals (May 31) with a fast repetition rate fluorometer (FRRF) as described below. Beginning June 3, daily measurements (0800, 1200, 1600, 2000, and 2300 h) of steady-state and dark-adapted chlorophyll fluorescence yields were obtained during elevated temperatures (29–32 °C) as described below. This experiment is therefore separated into two components. First, a test of the effects of different solar irradiances on chlorophyll fluorescence yields after a period of

photoacclimatization followed by a test for the effects of differences in irradiances of solar radiation during exposure to elevated temperatures. At the end of the acclimatization period, subsets of samples and in some cases split samples from randomly chosen individuals from each treatment group were used in the assays described below.

#### Quantification of visible and UV radiation

Visible and UVR (300–700 nm) were measured over several days during May 1998 in the field at Horseshoe Reef (10 m and surface), as well in the experimental sea tables, using a LiCor LI-1800UW scanning spectroradiometer (LiCor, Lincoln, Nebraska) calibrated with National Institute of Standards and Technology (NIST) traceable standards. Hourly readings of three scans were taken and the mean reported in units of  $W\ m^{-2}\ nm^{-1}$  at approximately 1300 h daily. The cosine-corrected collector and sensors were programmed to scan from 300–700 nm in 2-nm intervals. Vertical attenuation coefficients ( $K_d\ m^{-1}$ ) for both visible and UV radiation in the field were calculated for Horseshoe Reef as described by Kirk (1994).

#### Temperature measurements

Temperature measurements in the field and laboratory were conducted using temperature recording thermistors (TempMentors from the CMRC temperature monitoring program and HOBO Temps, Onset Corp.), which are calibrated with NIST traceable standards. Temperature increases in the experimental tanks were to be accomplished using aquarium heaters (80 W) before oceanographic events (see below) intervened to increase seawater temperatures.

#### Photosynthetic pigments

At the end of the experiment, pieces of coral from each treatment ( $N=5$ ), approximately 3.0 to 5.0  $cm^2$ , were placed overnight in 90% acetone at 4 °C. The absorbance of the centrifuged samples was then read at 630, 633, and 750 nm against an acetone blank. The trichromatic equations of Jeffrey and Humphrey (1975) were then used to calculate the concentrations of chlorophyll *a* and *c*<sub>2</sub> normalized to surface area using the aluminum foil technique (Marsh 1970).

#### Mycosporine-like amino acids

For all coral samples, the extraction and analysis of MAAs were performed according to the procedures in Dunlap and Chalker (1986) as modified by Shick et al. (1992). For analysis by high performance liquid chro-

matography (HPLC), coral samples from the laboratory ( $N=5$ ) were cleaned of epiphytes, broken into small pieces, and extracted overnight in 5 ml of 100% HPLC grade methanol at 4 °C. The extracts were centrifuged and the supernatant used for MAA and protein analysis. MAAs were separated by reverse-phase, isocratic HPLC on a Brownlee RP-8 column (Spheri-5, 4.6 mm ID x 250 mm) which was protected with an RP-8 guard column (Spheri-5, 4.6 mm ID x 30 mm). The mobile phase consisted of 40 to 55% methanol (v:v), 0.1% glacial acetic acid (v:v) in water and run at a flow rate of 0.6  $ml\ min^{-1}$ . Detection of MAA peaks was by absorbance at 313 and 340 nm. Standards were available for seven MAAs (mycosporine-glycine, shinorine, porphyra-334, palythine, asterina-330, palythanol, and palythene). Identities of peaks were confirmed by co-chromatography with standards, and the ratios of 313 to 340 nm absorbances. Peaks were integrated and quantification of individual MAAs was accomplished using HPLC peak areas and calibration factors determined by analysis of the standards listed above. All MAAs were normalized to soluble protein from an aliquot of the methanol-extracted sample and concentrations are expressed in  $\mu g\ MAA\ mg\ protein^{-1}$ . Protein measurements were determined using the procedure of Bradford (1976) in kit form (Bio-Rad, Inc.).

#### Fluorescence measurements

Chlorophyll fluorescence yields were measured using a custom-built fast repetition rate (FRR) fluorometer (Gorbunov et al. 2000). FRR fluorometry measures chlorophyll fluorescence transients using a controlled series of sub-saturating flashes that cumulatively saturate PSII within  $\sim 100\ \mu s$ , i.e., a single photochemical turnover (Falkowski and Kolber 1993; Kolber et al. 1998). The FRR technique can therefore measure several fluorescent parameters ( $F_o$ ,  $F_m$ ,  $F_v$ ,  $F'$ , and  $F'_m$ ) and parameters related to photosynthesis ( $F_v/F_m$ ,  $\Delta F/F'_m$ ,  $\sigma_{PSII}$ ) (see Abbreviations section and Gorbunov et al. 2000). All fluorescence measurements reported below were taken from the upper portion of each colony on the surface that provided the greatest planar surface to the incident downwelling irradiance.

The maximum (i.e., potential) quantum yield of chlorophyll fluorescence, or photochemistry, of PSII is determined in a dark-adapted state as the ratio  $F_v/F_m (= (F_m - F_o)/F_m)$  and taken from night-time measurements during this study. Measuring the steady state fluorescence level ( $F'$ ) during a light-acclimated state allows other parameters related to the absorbance and dissipation of energy to be calculated. These include the steady state quantum yield of chlorophyll fluorescence, or photochemistry, in PSII,  $\Delta F/F'_m (= (F'_m - F')/F'_m)$ , and the coefficient of non-photochemical quenching  $qN = 1 - (F'_m - F')/(F_m - F_o)$  (Genty et al. 1989). In addition to these parameters, the FRR fluorometer instantaneously measures the functional absorption

cross section of PSII ( $\sigma_{\text{PSII}}$ ) that characterizes the capacity to absorb and utilize visible radiation (Kolber et al. 1998).

### DNA damage

Cyclobutane pyrimidine dimer (CPD) formation was measured using the procedures and monoclonal antibody (TDM-2) of Mori et al. (1991). The zooxanthellae were separated from host tissue by centrifugation after obtaining a tissue slurry by air brushing using a small volume ( $\sim 1.0$  ml) of 2 mM phosphate buffer (pH 7.5) from individual pieces of coral ( $N=3$ ) from each treatment. Host DNA was isolated using commercially available kits (Easy-DNA, Invitrogen, Inc.). Some samples required re-precipitation of the DNA with 70% ethanol or a wash with 6 M LiCl to improve the quality of the DNA (260/280 nm ratios  $> 1.8$ ). Subsequently, 50 ng of DNA from each sample was used in an enzyme-linked immunoabsorbent assay (ELISA) technique with TDM-2 as the primary antibody. An affinity purified goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase was used and the final color development read in flat-bottomed 96 well microtiter plates using a plate reader (Bio-Rad, Inc.) at 405 nm as described by Mori et al. (1991). This assay measures only DNA damage caused directly by exposure to UVR.

### Western blots

Samples of animal tissue and zooxanthellae ( $N=3$ ) were collected by airbrushing in a small volume of phosphate buffer and separated by centrifugation. The protocol for extraction, polyacrylamide gel electrophoresis (SDS-PAGE), and western blotting is described in Lesser et al. (2001). Briefly, duplicate gels were run; one gel was stained with Coomassie blue for protein and the other electrophoretically transferred to PVDF membranes (0.2  $\mu\text{m}$ ). The membrane was blocked with 5–10% instant milk, immunoblotting of the membrane was completed, and the immunoblot was developed using a secondary antibody at a titer of 1:2000 labeled with horseradish peroxidase. SDS-PAGE gels of animals and zooxanthellae were normalized by loading the gels on an equivalent total protein basis and then probed using polyclonal antibodies to detect Cu-Zn SOD protein (SOD I [cytosolic] 1:1000), and the expressed protein from the cell cycle gene *p 53* (1:100) in host samples while for protein extracted from zooxanthellae samples polyclonal antibodies against the D1 protein (1:100) of PSII were used. In response to the experimental treatments, the differential expression of these proteins was analyzed by measuring the optical density of positive bands using NIH Image software. To remove any differences in the background staining and to quantify each positive band, an internal gray-scale calibration was used and the optical densities compared statistically after log transformation.

### Statistical analysis

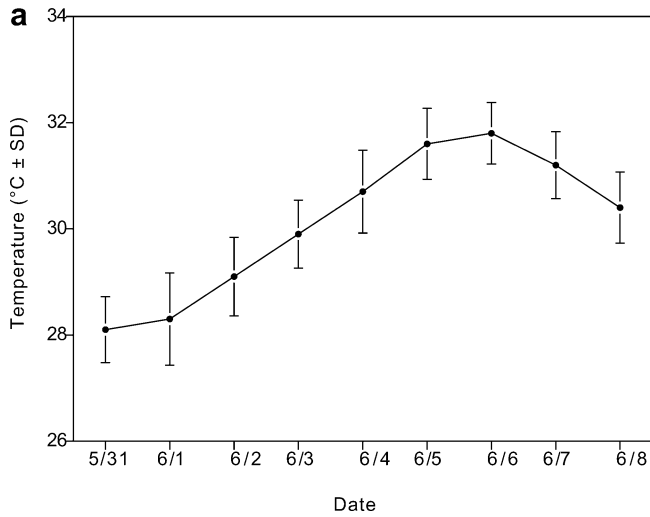
Measurements of the initial (e.g., pre-thermal stress)  $\Delta F/F_m'$ , chlorophyll concentrations, MAAs, and CPD formation were statistically analyzed using a one-way ANOVA at a significance level of 5%. No unequal variances were detected using the  $F_{\text{max}}$  test, and individual treatment differences were assessed using the Student-Newman-Keuls (SNK) multiple-comparison test. Where appropriate, ratios and percentages were arcsine or log transformed for analysis and back transformed for presentation. A repeated-measures ANOVA, with fixed effects of treatment and time of day, and day was performed on the  $F'$ ,  $F_m'$ ,  $\Delta F/F_m'$ ,  $\sigma_{\text{PSII}}$ , and  $qN$  data from the bleaching experiments. Multiple-comparison testing (SNK) could only be done on each independent factor and not on the interaction terms.

## Results

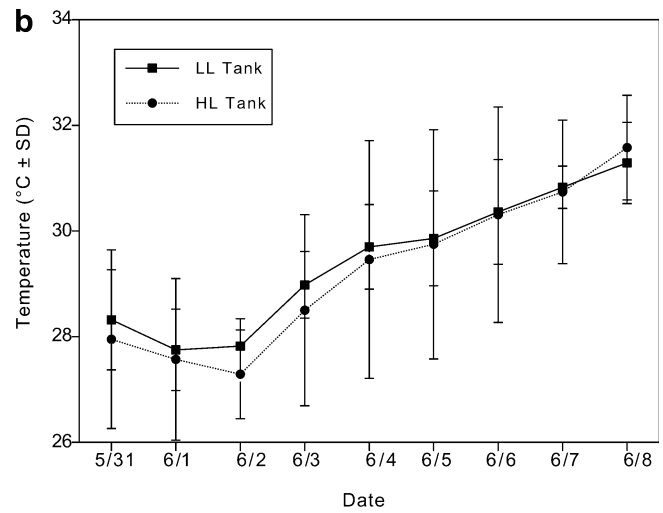
Measurements of visible and UVR on Horseshoe Reef in May 1998 showed typical results for ambient surface and 10-m spectral scans from 300–700 nm on a cloudless day at midday. The maximum surface PAR irradiance (integrated from 400–700 nm) on that day was 2,121  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , while the maximum PAR irradiance at 10 m was 707  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Maximum UVR irradiances were 60.5  $\text{W m}^{-2}$  (UVB: 3.15  $\text{W m}^{-2}$ ) for surface and 9.7  $\text{W m}^{-2}$  (UVB: 0.05  $\text{W m}^{-2}$ ) for 10 m. The average spectral attenuation coefficient for PAR wavelengths ( $K_d=0.12 \text{ m}^{-1}$ ) and UVR wavelengths ( $K_d=0.15 \text{ m}^{-1}$ ) is on the higher end of those observed for Case I waters (Kirk 1994). Integrated measurements of PAR and UVR for the experimental tanks were 2,053  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ : UVB, 3.24  $\text{W m}^{-2}$ ; UVR, 57.1  $\text{W m}^{-2}$  for the HL tank; and 567  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , UVB, 0.83  $\text{W m}^{-2}$ ; UVR, 15.3  $\text{W m}^{-2}$  for the LL and Re tanks.

After 3 days of fluorescence measurements, the temperature of the water surrounding LSI began to rise due to the intrusion of warm Bahama Bank water onto the Exuma Islands (Smith 2001; Fig. 1a). This also led to a rise of seawater temperatures in the seawater tables before planned experimental increases in temperature were carried out. The daily temperature of the seawater tables varied between 26 and 29  $^{\circ}\text{C}$ , and increased to as high as 32  $^{\circ}\text{C}$  during the rest of the experiment (Fig. 1b). There was no statistical difference in temperature for any day during the experiment between experimental seawater tanks. Therefore, the experiments are well controlled for temperature effects and test for the effects of solar irradiance.

At the end of the experiment the aerial values of chl *a* and *c*<sub>2</sub> (Fig. 2) showed a significant treatment effect (ANOVA:  $P=0.0001$ ) with LL values significantly greater than either HL or Re (SNK  $< 0.05$ ). Chl *a/c*<sub>2</sub> ratios, however, showed no significant differences suggesting that the differences were principally due to losses



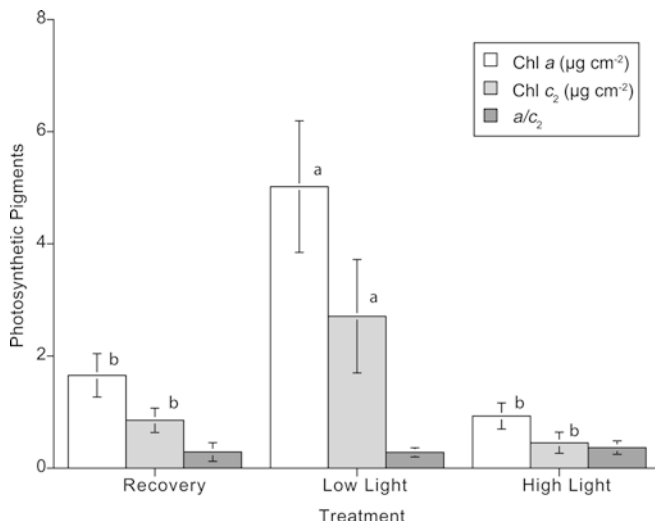
**Fig. 1 a** Eight-day temperature record (Mean  $\pm$  SD) for Rainbow Garden, LSI. **b** Eight-day temperature record (Mean  $\pm$  SD) for same period in experimental sea tables for HL, LL, and Re treatments



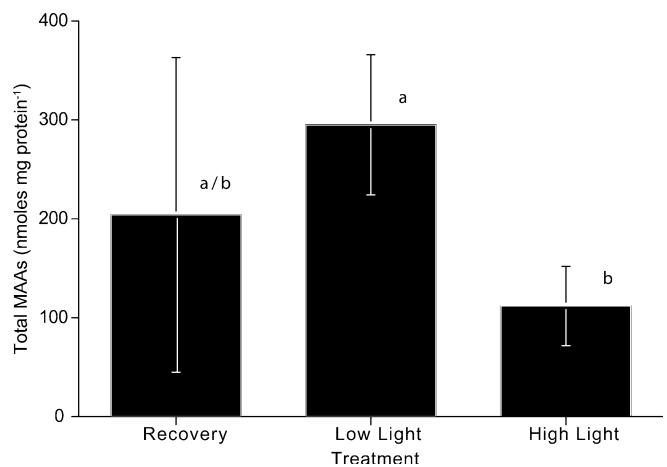
in the number of cells and not pigment per cell. The results from the photosynthetic pigment studies also demonstrate that a sufficient time of acclimatization for the photosynthetic apparatus had occurred between LL and HL treated corals originally collected from the same depth and irradiance regime. Measurements of UVR-absorbing compounds or MAAs at the end of the experiment showed a significant treatment effect (ANOVA:  $P=0.011$ ) with HL MAA concentrations not significantly (SNK:  $P>0.05$ ) different from Re, and LL concentrations not significantly (SNK:  $P>0.05$ ) different from Re. HL MAA concentrations were significantly lower than LL (SNK:  $P<0.05$ ; Fig. 3). Measurements of

CPD formation at the end of the experiment showed a significant treatment effect (ANOVA:  $P=0.002$ ) with HL CPD concentrations significantly (SNK:  $P<0.05$ ) greater than LL and RE treatments that were not significantly (SNK:  $P>0.05$ ) different from each other (Fig. 4).

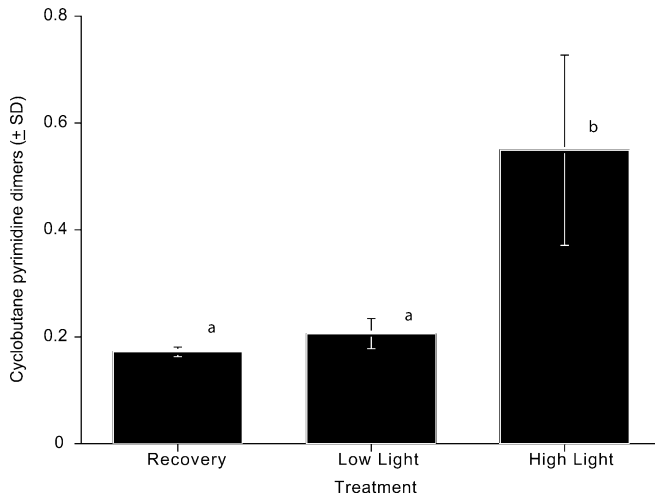
During the first 3 days of the experiment,  $\Delta F/F_m'$  values of the treatment groups (HL, LL, and Re) were significantly different from one another (ANOVA:  $P=0.0001$ , SNK:  $P<0.05$ ). These values reflected their respective photoacclimatization to low and high irradiances (LL and HL) of solar radiation, or recovery from bleaching caused by exposure to elevated temperatures and supraoptimal solar irradiances (Re) and recovering under low light conditions. LL values were the highest ( $0.402 \pm 0.041$ ), followed by Re ( $0.349 \pm 0.062$ ), and HL ( $0.288 \pm 0.044$ ). Subsequently, when increasing seawater temperatures occurred, HL corals began to visibly bleach (e.g., colony paling), while LL



**Fig. 2** Photosynthetic pigment concentrations normalized to surface area at the end of the experiment for *Montastraea faveolata* ( $N=5$ , mean  $\pm$  SD). Superscripts denote groups not statistically different from one another using multiple-comparison testing (SNK)

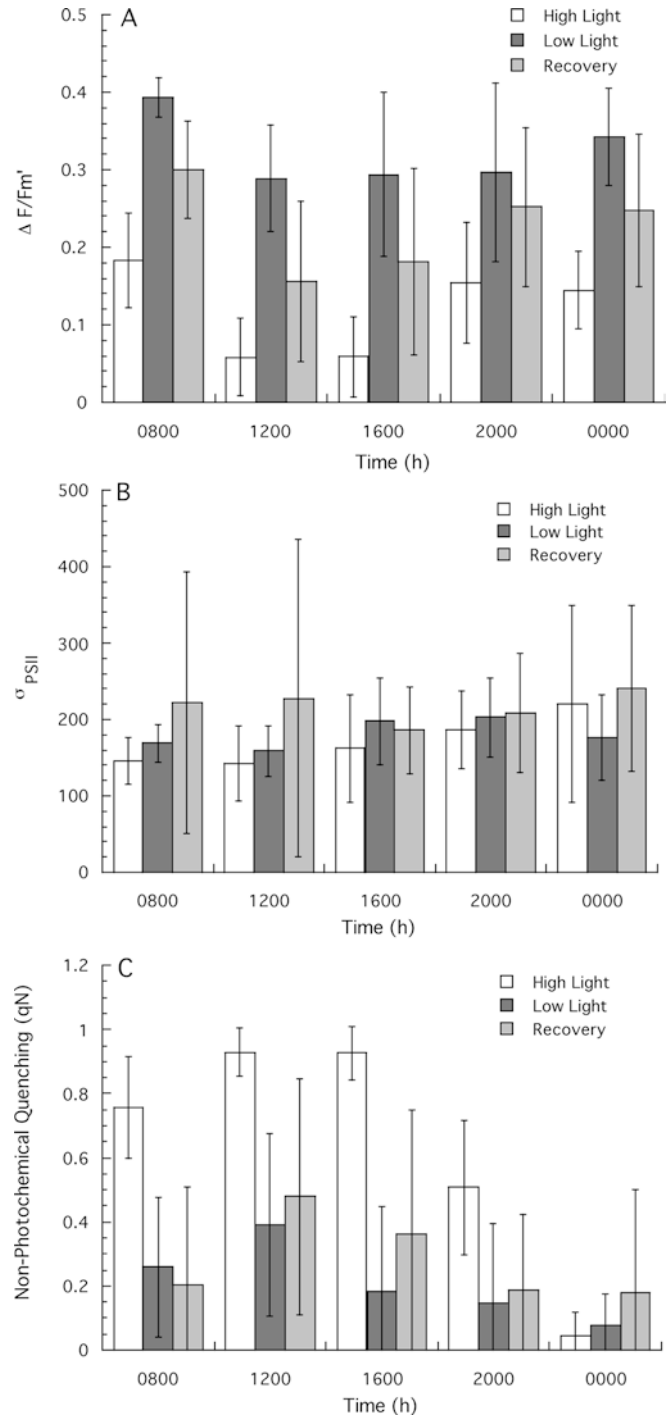


**Fig. 3** Protein-specific mycosporine-like amino acid (MAA) concentrations at the end of the experiment for *Montastraea faveolata* ( $N=5$ , mean  $\pm$  SD). Superscripts denote groups not statistically different from one another using multiple-comparison testing (SNK)



**Fig. 4** Cyclobutane pyrimidine dimer (CPD) concentrations in host tissues at the end of the experiment for *Montastraea faveolata* (N=5, mean±SD). Superscripts denote groups not statistically different from one another using multiple-comparison testing (SNK)

corals did not appear to visibly bleach and Re corals did not change since the colonies were already pale in color. Measurements of  $\Delta F/F_m'$  on corals from June 3 to June 8 reveal diel variability embedded within the synergistic effects of irradiance and elevated seawater temperatures (Fig. 5a). A repeated-measures ANOVA with treatment (HL, LL, Re), time, and day as fixed factors, revealed no significant effects of day for any measurement. Consequently that variability was eliminated from the analysis and all repeated-measures ANOVA were conducted with treatment and time of day as fixed factors. A repeated-measures ANOVA revealed no effect of treatment (ANOVA:  $P=0.30$ ), but time (ANOVA:  $P<0.0001$ ), and the interaction of treatment and time were significant (ANOVA:  $P<0.0001$ ) for  $F'$ . All multiple comparisons except for the 1200 versus the 1600 time period were significant (SNK:  $P<0.05$ ). A repeated-measures ANOVA revealed a significant effect of treatment (ANOVA:  $P<0.0001$ ), time (ANOVA:  $P<0.0001$ ), and the interaction of treatment and time (ANOVA:  $P<0.0001$ ) for  $F_m'$ . All treatment multiple comparisons were significantly different from each other (SNK:  $P<0.05$ ) as were six of ten time comparisons (SNK:  $P<0.05$ ). A repeated-measures ANOVA showed a significant effect of treatment (ANOVA:  $P=0.0001$ ), time (ANOVA:  $P=0.0001$ ), and a significant interaction of treatment and time (ANOVA:  $P=0.03$ ) on  $\Delta F/F_m'$ . Multiple-comparison testing of treatment groups revealed that all treatment groups were significantly different from one another (SNK:  $P<0.05$ ), with HL values lower than LL, and Re lower than LL, but greater than HL (Fig. 5a). All multiple comparisons except for the 2000 versus the 0000 time period were significantly different from one another (SNK:  $P<0.05$ ). For these measurements, the significant effect of time is a function of the diel differences known to occur in corals representing dynamic photoinhibition.



**Fig. 5** A Diel cycle of the steady-state quantum yield of photochemistry in PSII ( $\Delta F/F_m'$ ) and dark acclimated measurement ( $=2,300$  measurements) in the coral *Montastraea faveolata*. B Diel cycle of the functional absorption cross-section ( $\sigma_{PSII}$ ) in the coral *Montastraea faveolata*. C Diel cycle of non-photochemical ( $qN$ ) quenching in the coral *Montastraea faveolata*. All measurements (Mean±SD) for each time of measurement combined for all samples (N=10 corals for HL and LL treatment group, N=5 for Re treatment group, N=25–50 measurements for each coral over the entire experiment) taken during a 5-day period (June 3 to June 7, 1998)

A repeated-measures ANOVA also revealed a significant effect of treatment (ANOVA:  $P=0.031$ ), but not time (ANOVA:  $P=0.117$ ) on  $\sigma_{\text{PSII}}$  with a significant interaction (ANOVA:  $P=0.019$ ) term. Multiple-comparison testing revealed that HL values were not significantly different than LL values (SNK:  $P>0.05$ ), but significantly lower than Re values, while LL values were significantly lower than Re, but greater than HL (Fig. 5b). For  $qN$  a repeated-measures ANOVA of treatment (HL, LL, Re) and time as fixed factors showed a significant effect of treatment (ANOVA:  $P=0.0001$ ), time (ANOVA:  $P=0.0001$ ), and interaction of treatment and time (ANOVA:  $P=0.003$ ). Multiple comparison testing of the treatment groups for  $qN$  revealed that LL and Re treatments were significantly different from the HL treatment (SNK:  $P<0.05$ ), but not from each other (SNK:  $P>0.05$ ), and that for multiple-comparison testing of time, all comparisons were significantly different (SNK:  $P<0.05$ ), except for the 0800 versus 1600 comparison (Fig. 5c).

Immunoblots of host proteins showed a significant effect of solar irradiance on the expression of both SOD (ANOVA:  $P=0.012$ , Fig. 6a) and  $p53$  (ANOVA:  $P=0.02$ , Fig. 6b). Both SOD and  $p53$  multiple-comparison testing showed a significant treatment effect (SNK:  $P<0.05$ ), with the HL treatment exhibiting significantly greater expression for both of these proteins compared to either LL or Re treatments, which were not significantly different from one another (SNK:  $P>0.05$ ). Immunoblots of protein isolated from the zooxanthellae of *M. faveolata* for the 32 kD D1 protein of PSII show a significant treatment effect (ANOVA:  $P=0.003$ ) with all treatment groups significantly different from each other (SNK:  $P<0.05$ ) and the lowest D1 concentration in the HL treatment (Fig. 6c).

## Discussion

The results presented here show that solar radiation is an important environmental factor that significantly affects the degree of stress and bleaching in corals exposed to elevated temperatures. Similar conclusions have been made from field data where during the 1998 El Niño event, coral reefs in French Polynesia avoided significant mortality by the simultaneous occurrence of cloudy weather (Mumby et al. 2001), from the analysis of long-term data sets on the correlation between exposure to high irradiances of solar radiation during thermal stress and bleaching in corals (Dunne and Brown 2001), and from the analysis of experimental data (Jones et al. 1998; Bhagooli and Hidaka 2004).

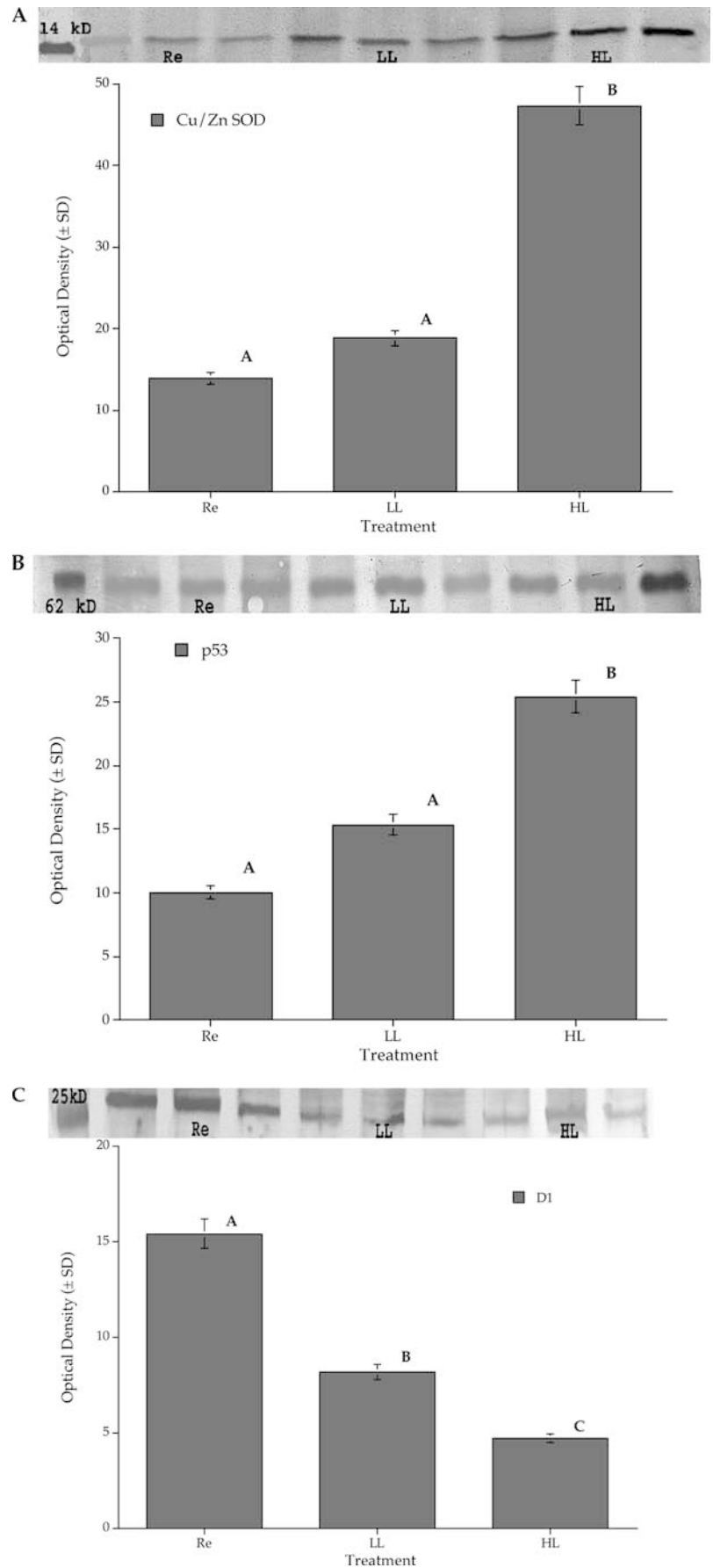
The results from the laboratory bleaching studies described here and elsewhere (Lesser 1996; Bhagooli and Hidaka 2004) clearly show that the underwater light field, both PAR and UVR, play an important synergistic role in the photoinhibition of the photosynthetic apparatus and subsequent bleaching of corals. Corals exposed to supra-optimal solar irradiances and elevated

seawater temperatures exhibit greater damage to the photosynthetic apparatus (e.g., chronic photoinhibition), measured as a depression in the steady-state quantum yield of PSII fluorescence and expression pattern of the D1 protein. Exposure to high irradiances of solar radiation leads to a lower bleaching threshold temperature and an overall shorter time to actually "bleach" compared to corals exposed to lower solar irradiances. Additionally, corals from the experiments presented here show minimal differences in their functional absorption cross-sections across time. This suggests that under these experimental conditions, most of the non-photochemical quenching, or thermal dissipation, occurs by down regulation of reaction centers (Gorbunov et al. 2001). The significant decline of  $\Delta F/F_m'$  over time, especially in the high light treatments, are the result of the significant decrease in  $F_m'$  (data not shown). The significant quenching of  $F_m'$  parallels the decline in  $\Delta F/F_m'$  and the increase in  $qN$  which supports a significant role for dynamic photoinhibition in protecting PSII (Jones et al. 1998; Warner et al. 1999; Gorbunov et al. 2001; Lesser and Gorbunov 2001). Although not statistically significant, we observed lower  $F'$  values which is also consistent with xanthophyll cycling and dynamic photoinhibition (Brown et al. 1999). The decreases of D1 protein concentration we observed, however, is direct evidence that chronic photoinhibition occurred during these experiments.

While declines in chlorophyll  $a$  and  $c_2$  per unit area are consistent with the loss of zooxanthellae, the MAAs of *Montastraea faveolata* were also significantly reduced during exposure to high irradiances of solar radiation and elevated temperatures, a phenomenon which has been previously reported (Lesser et al. 1990; Lesser 1996). Explanations for the decrease in MAAs during this experiment include the possibility that the corals did not increase their concentration of MAAs when transferred to higher irradiances, that the MAAs were broken down because they are also known to have antioxidant activity (Dunlap and Yamamoto 1995; Kim et al. 2001), or that the flux of carbon through the branch of the shikimic acid pathway that produces MAAs was lowered by a decrease in photosynthesis (Jokiel et al. 1997).

Under normal saturating conditions of light and carbon dioxide, photosynthesis is limited by the rate of carbon fixation (= Rubisco activity) and not the rate of photochemistry (Sukenik et al. 1987). Zooxanthellae reside in a hyperoxic environment (Dyken and Shick 1982) and have a Type II Rubisco that has a lower affinity for its substrate,  $\text{CO}_2$  (Rowan et al. 1996) making  $\text{CO}_2$  limitation more likely under certain conditions. Jones et al. (1998) presented a model for damage to PSII leading to bleaching that describes temperature-related changes to Calvin-cycle enzymes, an increase in the non-assimilatory flow of electrons through the Mehler-ascorbate peroxidase (MAP) cycle, and subsequent redox imbalance as a result of carbon sink limitation as the initial perturbation. Quantum yields of PSII fluorescence in zooxanthellae, however, can also be

**Fig. 6** Western Blots of host-associated proteins (N=3 for each treatment) for *Montastraea faveolata*. **A** Optical density ( $\pm$ SD) of immunoblots for SOD from experimental samples of *M. faveolata*. **B** Optical density ( $\pm$ SD) of immunoblots for p 53 from experimental samples of *M. faveolata*. **C** Optical density ( $\pm$ SD) of immunoblots for D1 protein from zooanthellae samples of *M. faveolata*



depressed when exposed to elevated seawater temperatures in the dark (Warner et al. 1996). This suggests that damage to the dark reactions is not necessarily a prerequisite for PSII damage. Lastly, the Jones et al. (1998) experiments eliminated UVR from their experimental protocol, a condition that does not represent shallow water reefs.

We suggest that several important pieces of information can be brought together to show that rather than the dark reactions being effected initially, and then leading to PSII dysfunction (Jones et al. 1998), the available evidence can be integrated to show that both photochemistry and carbon fixation are being affected simultaneously in a feedback loop that greatly enhances damage to PSII. There is substantial evidence that PSII is already affected directly by high solar radiation without any thermal stress (Gorbunov et al. 2001) and that thermal stress without high solar irradiances effects PSII (Warner et al. 1999).

Symbiotic cnidarians routinely experience elevated  $p$  O<sub>2</sub> within the host tissues (Dyken and Shick 1982), and UVR acts synergistically with sublethal temperature perturbations and physiological hyperoxia to produce ROS in host tissues (Dyken et al. 1992). The enzymes superoxide dismutase, catalase, and ascorbate peroxidase, act in concert to inactivate superoxide radicals and hydrogen peroxide, thereby preventing the formation of the most reactive form of reduced oxygen, the hydroxyl radical, and subsequent cellular damage (Fridovich 1986, 1998). Exposure to thermal stress and high irradiances of solar radiation increased the expression of the antioxidant enzyme SOD in *Montastraea faveolata* in our experiments, a direct indication that the production of ROS had increased (Halliwell and Gutteridge 1999). High levels of ROS are a consistent feature of coral physiology, especially during thermal stress or exposure to UVR where the photodynamic production of ROS such as superoxide radicals (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is high (Lesser 1996, 1997). ROS, especially hydrogen peroxide, are well known inhibitors of Rubisco (Asada and Takahashi 1987) and cause damage to PSII (Asada and Takahashi 1987; Richter et al. 1990; Lesser 1996; Lupinková and Komenda 2004). Reactive oxygen species damage the D1 protein of PSII (Lupinková and Komenda 2004) and inhibit the biosynthesis of D1 protein (Nishiyama et al. 2001) and the protein repair machinery in general (Takahashi et al. 2004). The potential for carbon sink limitation to exacerbate ROS production and chronic photoinhibition of PSII in shallow corals with (Warner et al. 1999) or without (Gorbunov et al. 2001) thermal stress was illustrated by Jones et al. (1998). These fluxes of ROS overwhelm host and algal antioxidant defense systems, resulting in damage to both the light reactions (PSII) and dark reactions (carbon fixation).

In addition to damage to the photosynthetic apparatus, the type of DNA damage measured in these experiments, and in other experiments on corals (Anderson et al. 2001), is the result of the direct effects

of solar radiation. DNA damage caused indirectly by exposure to UVR through the photodynamic production of ROS is also likely to have occurred during these experiments (Imlay and Linn 1988). DNA damage can lead to apoptosis or programmed cell death if not repaired. One of the key cell cycle genes activated after DNA damage is *p* 53 that initiates the downstream expression of cell cycle genes that cause cells to arrest in G1/S while DNA damage is being repaired. If DNA repair is not possible, then *p* 53-mediated apoptosis may be initiated. The observed expression pattern of the putative *p* 53 for *M. faveolata* is consistent with the observed pattern of DNA damage. The polyclonal antibody used in the *p* 53 immunoblots was derived from soft shell clam (*Mya arenaria*) full-length *p* 53 sequence, which cross-reacts with recombinant human *p* 53 as well as recombinant clam *p* 53 and *p* 73 (Kelly et al. 2001). Based on the ultrastructural evidence that apoptosis and necrosis do occur in thermally stressed symbiotic cnidarians (Dunn et al. 2002), and that a putative *p* 53 protein is up-regulated in response to DNA damage in the experiments described here, it is reasonable to interpret this data as supportive of the occurrence of apoptosis and possibly cell necrosis mediated by ROS in thermally stressed symbiotic cnidarians. ROS are likely to be involved at both ends of the continuum between damage to the photosynthetic apparatus, DNA damage and apoptosis, and cell necrosis.

It is important to understand the underlying biochemical and molecular mechanisms of bleaching. The results described above point towards the complexity of the stress response in corals and that several cellular mechanisms can be responsible for the observed stress response known as bleaching. Additionally, the variability in the bleaching response that occurs under different environmental conditions (Fitt et al. 2001) is potentially complicated by the genetic diversity of algal symbionts within and between different corals (Rowan et al. 1997; Lajeunesse 2002). The results presented here show that cellular damage in the host and zooxanthellae occurs simultaneously during thermal stress and is exacerbated when exposed to high irradiances of solar radiation.

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