

PHOTOSYNTHESIS AND PRODUCTION OF HYDROGEN PEROXIDE BY *SYMBIODINIUM* (PYRRHOPHYTA) PHYLOTYPES WITH DIFFERENT THERMAL TOLERANCES¹

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Occurrences whereby cnidaria lose their symbiotic dinoflagellate microalgae (*Symbiodinium* spp.) are increasing in frequency and intensity. These so-called bleaching events are most often related to an increase in water temperature, which is thought to limit certain *Symbiodinium* phylotypes from effectively dissipating absorbed excitation energy that is otherwise used for photochemistry. Here, we examined photosynthetic characteristics and hydrogen peroxide (H₂O₂) production, a possible signal involved in bleaching, from two *Symbiodinium* types (a thermally “tolerant” A1 and “sensitive” B1) representative of cnidaria–*Symbiodinium* symbioses of reef-building Caribbean corals. Under steady-state growth at 26°C, a higher efficiency of PSII photochemistry, rate of electron turnover, and rate of O₂ production were observed for type A1 than for B1. The two types responded very differently to a period of elevated temperature (32°C): type A1 increased light-driven O₂ consumption but not the amount of H₂O₂ produced; in contrast, type B1 increased the amount of H₂O₂ produced without an increase in light-driven O₂ consumption. Therefore, our results are consistent with previous suggestions that the thermal tolerance of *Symbiodinium* is related to adaptive constraints associated with photosynthesis and that sensitive phylotypes are more prone to H₂O₂ production. Understanding these adaptive differences in the genus *Symbiodinium* will be crucial if we are to interpret the response of symbiotic associations, including reef-building corals, to environmental change.

Key index words: bleaching; hydrogen peroxide; Mehler reaction; oxygen evolution; photosystem II; reactive oxygen species; *Symbiodinium*

Abbreviations: AOX, mitochondrial alternative oxidase; APX, ascorbate peroxidase; FIRE, fluorescence induction relaxation; ITS2, internal transcribed spacer 2 region; KPF, Kolber-Prasil-Falkowski; MIMS, membrane-inlet mass spectrometer; MT, multiple turnover; PFD, photon flux density; RCII, PSII reaction center; ROS, reactive oxygen species; SOD, superoxide dismutase; ST, single turnover

Occurrences of symbiotic microalgae loss from their cnidarian host in response to extremes of environment are increasing in intensity and frequency (Donner et al. 2005, Lesser 2007). These events ultimately leave the cnidarian with a pale or bleached appearance, but in the worst case, only the white coral skeleton remains. Bleaching itself refers to the loss of cellular abundance and/or pigmentation of microalgae from the host and has been most commonly observed with extremes of temperature (Lesser and Farrell 2004, Smith et al. 2005).

All symbiotic dinoflagellates associated with cnidarians belong to the genus *Symbiodinium* (Coffroth and Santos 2005). Monocultures of *Symbiodinium* spp. isolated from various cnidarian hosts have been used to examine the impact of elevated temperature upon photophysiology (Iglesias-Prieto et al. 1992, Lesser 1996, Robison and Warner 2006). As with other microalgae, *Symbiodinium* spp. not only dissipate excitation energy absorbed by the photosynthetic apparatus via photochemistry, but also employ other mechanisms to prevent complete reduction of quinone acceptor molecules when the light absorbed is in excess of that required for CO₂ assimilation (Falkowski and Raven 1997, Jones et al. 1998, Lesser and Farrell 2004). These pathways include synthesis of quenchers or activation of mechanisms that convert absorbed excitation energy into heat

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(Gorbunov et al. 2001, Warner and Berry-Lowe 2006) and that provide alternate sinks for electrons other than CO₂ assimilation (Tchernov et al. 2004, Levy et al. 2006). Under elevated temperatures, such an ability to limit the reduction of acceptor quinone molecules becomes compromised (Lesser and Farrell 2004, Smith et al. 2005, Lesser 2006).

Increased excitation pressure upon PSII has been shown to correspond with elevated production of the reactive oxygen species (ROS), superoxide (O₂⁻), H₂O₂, and singlet oxygen (¹O₂) for many microalgae, including dinoflagellates (Dykens et al. 1992, Twiner and Trick 2000, Marshall et al. 2002, 2005, Kim et al. 2004, Tchernov et al. 2004). Several investigations have suggested that such an elevation in ROS production by *Symbiodinium* spp. is a primary event that leads to bleaching (Lesser and Shick 1989, reviewed by Smith et al. 2005). However, few have directly observed an increase in ROS production with elevated temperature (Lesser 1996, Franklin et al. 2004, Tchernov et al. 2004) and/or light (Levy et al. 2006). Lesser (1996) reported an increase in both ROS and ROS-scavenging enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) upon exposure of a thermally sensitive strain of *Symbiodinium* to elevated temperature. On the basis of available literature for algae and plants, Smith et al. (2005) have hypothesized that H₂O₂ may be the most important ROS associated with bleaching since it can potentially act as an important signaling molecule between the *Symbiodinium* and its symbiotic host.

Symbiodinium strains exhibit a high genetic diversity (Coffroth and Santos 2005), and initial evidence suggests that susceptibility to elevated temperatures is not the same for all genetic types (Tchernov et al. 2004, Robison and Warner 2006). However, little is currently known of the comparative physiology of different *Symbiodinium* types with varying thermal tolerance, and thus the adaptive basis for bleaching responses observed in nature. Therefore, we examined the photosynthetic physiology (PSII photochemistry, gross O₂ production, and light-driven O₂ consumption) and H₂O₂ production for two types of *Symbiodinium*, a thermally tolerant isolate (ITS2 type-A1) and a thermally sensitive isolate (ITS2 type-B1) (described in Robison and Warner 2006) under steady-state growth (26°C). *Symbiodinium* types A and B are often associated with reef-building cnidaria in Caribbean and Indo-Pacific waters (LaJeunesse 2002, Baker 2003, Visram and Douglas 2006) and are known to exhibit different susceptibilities to elevated water temperatures and light (Savage et al. 2002, Warner et al. 2006). Short-term (24 h) acute heating experiments (to 32°C) were subsequently performed to evaluate the hypothesis that the thermally sensitive *Symbiodinium* isolate produces more H₂O₂ than the thermally tolerant isolate under thermal stress.

MATERIALS AND METHODS

Growth and experimental conditions. *Symbiodinium* cultures designated by their phylogenies based on sequence analysis of the internal transcribed spacer 2 region (ITS2) as A1 and B1 (see Robison and Warner 2006) were grown in climate-controlled cabinets at 26°C and at a photon flux density (PFD; measured using a 4π scalar irradiance sensor, Biospherical Instruments QSL, San Diego, CA, USA; 400–800 nm) of 100 μmol photons · m⁻² · s⁻¹ on a 14:10 light:dark (L:D) cycle. Cultures were maintained semicontinuously in 2 L flasks in artificial seawater media (ASP-8A) (Blank 1987) bubbled gently with air passed via a charcoal column to enrich with CO₂. Preliminary experiments that did not use this enrichment procedure yielded an increase in pH within the cultures of up to 0.3 units during exponential growth. Our CO₂-enrichment approach eliminated this potential source of variability: daily pH measurements using a Ross Series Electrode (Thermo Electron Corporation, Beverly, MA, USA) verified that pH remained constant for the two cultures, 8.24 ± 0.01 and 8.25 ± 0.02 for A1 and B1, respectively. Cell growth was monitored from daily fluorescence measurements, and dilutions were performed where necessary to retain cells in the exponential growth phase. Growth rates were 0.49 ± 0.06 d⁻¹ and 0.26 ± 0.05 d⁻¹ (mean ± SE, *n* = 5) for A1 and B1, respectively. Cultures were optically thin, with chl *a* concentrations of 71 ± 24 mg · m⁻³ and 48 ± 11 mg · m⁻³ for A1 and B1, respectively.

Cells were harvested during exponential growth to characterize photosynthesis and H₂O₂ yield under ambient growth conditions (control). Aliquots of 400–500 mL were drawn from each culture and immediately concentrated by a factor of ~4 using gentle gravity filtration (as in Suggett et al. 2006) to increase the signal for all subsequent photosynthesis and H₂O₂ measurements. Triplicate measurements were obtained from sequential dilutions. On separate days, two 500 mL aliquots were removed from either culture and transferred to separate flasks for the temperature shift experiment. One flask was maintained at 26°C in the climate-controlled cabinet used for culture growth, and the other in a second cabinet of identical setup but at 32°C. Both flasks were left for 24 h before harvesting. Each experiment was performed in triplicate for both clades.

Chl a, cell counts, and light absorption. Known volumes of material were filtered through GF/F filters (Whatman, Maidstone, UK) and stored at -80°C until analysis for chl *a*. Each filter was ground in 5 mL of 100% methanol and refrigerated (4°C) in darkness for 1 h. Supernatants were centrifuged using an Eppendorf model 5403 (Hamburg, Germany), and absorption was read using a U-3000 spectrophotometer (Hitachi High Technologies, Wokingham, UK). Chl *a* was quantified using the equations of Porra et al. (1989). Cells were counted using a Neubauer hemacytometer (Fisher Scientific, Loughborough, UK) following fixation of a small aliquot (160 μL) in 1% glutaraldehyde.

Sample optical density was determined using a U-3000 spectrophotometer with φ-60 integrating sphere (Hitachi). Aliquots of concentrated material (sample) and filtrate (blank) were scanned against a filtrate reference between 380 and 750 nm. Samples were agitated between replicate scans to prevent cells settling. Absorption was calculated from the optical density following normalization at 750 nm where absorption is negligible (Suggett et al. 2007).

Fluorometry. A fluorescence induction relaxation (FIRE) fluorometer (Satlantic Inc., Halifax, Canada) was used to generate single turnover (ST) and multiple turnover (MT) fluorescence transients and quantify various physiological characteristics of PSII photochemistry. Excitation was from a high luminosity blue and green (450 and 500 nm peak heights,

respectively, each with 30 nm bandwidth) light-emitting diode (LED) array with a combined emission of ~ 0.03 mol photons \cdot m $^{-2}$ \cdot s $^{-1}$. Fluorescence emission was detected at 680 nm (20 nm bandwidth) using a sensitive avalanche photodiode. Each acquisition consisted of a four-step transient analogous to the modified fast-repetition-rate (FRR) fluorescence technique of Kolber et al. (1998): (1) ST excitation from a 100 μ s pulse, (2) ST relaxation from a weak modulated light over 500 ms, (3) MT excitation from a 600 ms pulse, and (4) MT relaxation from a weak modulated light over 1 s. Twenty sequential acquisitions of each four-step fluorescence transient were cumulatively averaged to increase the signal-to-noise ratio. A single gain setting was used throughout, and the FIRE maintained in a climate-controlled room.

PSII photochemistry parameters were determined by fitting the biophysical (KPF) model of Kolber et al. (1998) to each fluorescence transient using the FIREPRO software provided by Satlantic Inc. Specifically, minimum fluorescence, maximum ST fluorescence, the functional absorption cross-section of PSII, energy transfer between PSII units (or connectivity, ρ), and the rate of reoxidation of the primary quinone acceptor (Q_A) were determined. Under dark-acclimated conditions, these parameters are termed F_o , F_m^{ST} (dimensionless), σ_{PSII} , ρ (dimensionless), and τ_{QA} (μ s), respectively (Kromkamp and Forster 2003). Both the effective absorption cross-section (σ_{PSII}) and connectivity (ρ) describe the rise from F_o to F_m^{ST} and are output in relative values. Therefore, to adjust σ_{PSII} to absolute values with units of $\text{\AA}^2 \cdot \text{quanta}^{-1}$ we compared our values with those obtained from a calibrated FIRE fluorometer (M. Gorbunov, personal communication). Connectivity remained constant throughout (0.55 ± 0.02). The maximum PSII photochemical efficiency was determined as follows:

$$(F_m^{ST} - F_o)/F_m^{ST} = F_v/F_m \quad (1)$$

Nonlinearities of the instrument response and sample blanks were determined from an extract of chl *a* and the filtrate of each sample, respectively. Both the instrument response and blank were subtracted from the sample fluorescence transient at the time of fitting the KPF model.

Fluorescence-light response curves were generated with the FIRE by using a calibrated actinic light source (ALS; Satlantic Inc.) that comprised a single high output blue LED (450 nm with 30 nm bandwidth, ~ 0.01 mol photons \cdot m $^{-2}$ \cdot s $^{-1}$) interfaced with the FIRE. Following an initial dark-adapted FIRE measurement consisting of 25 acquisitions, actinic PFDs between 5 and 500 μ mol photons \cdot m $^{-2}$ \cdot s $^{-1}$ were delivered each for a period of 4 min (see Suggett et al. 2003). A single FIRE measurement consisting of 12 acquisitions was made throughout the final 1 min of actinic exposure for each PFD. Each sample was periodically stirred to prevent cells from settling. Minimum and maximum (ST) fluorescence yields, the effective absorption cross-section, and connectivity are altered under actinic light from those determined in a dark-adapted state and thus are termed differently as F' , $F_m'^{ST}$, σ'_{PSII} , and ρ' . For each actinic PFD, we determined two fluorescence factors that describe the dissipation of absorbed excitation energy (see Suggett et al. 2006): (i) PSII efficiency factor, an approximation of that dissipated photochemically

$$(F_m'^{ST} - F')/(F_m'^{ST} - F_o') = F'_q/F'_v \quad (2)$$

and (ii) nonphotochemical dissipation in the antenna bed

$$1 - (\sigma'_{PSII}/\sigma_{PSII}) \quad (3)$$

PSII reaction center (RCII) concentration. A custom-made single-turnover O $_2$ -flash yield system was used to determine the

Emerson-Arnold number (mol O $_2$ \cdot mol chl a^{-1} , as described by Suggett et al. 2003, 2007) for concentrates of each algal type under steady-state growth (26°C). Cell-normalized RCII concentrations were determined from the product of the Emerson-Arnold number, the number of RCII required to reduce 1 mol of O $_2$ (a theoretical constant of 4 mol RCII \cdot mol O $_2^{-1}$) and chl $a \cdot$ cell $^{-1}$.

O $_2$ exchange. Gross O $_2$ production and light-dependent consumption of O $_2$ were determined by a membrane-inlet mass spectrometer (MIMS, QMG 422 Pfeiffer Vacuum, Asslar, Germany) using a modified $^{18}\text{O}_2$ dilution technique (Kana 1990) on discrete samples (Kana et al. 1994). Prior to incubation and measurement, the ratio of mass 18 to 16 O $_2$ was increased to $>70\%$. To achieve this, nitrogen gas was gently bubbled through 100 mL of sample contained in a gas-tight syringe. After 15 min, any headspace was removed, and 1.5 mL of $^{18}\text{O}_2$ gas (99% purity; CK Gas Products, Hook, UK) was introduced into the sample. The sample was gently mixed using a stir bar and left for a further 15 min before the $^{18}\text{O}_2$ gas was removed. During this procedure, the sample was kept in low PFD (<10 μ mol \cdot m $^{-2}$ \cdot s $^{-1}$) at the control (26°C) or treatment (32°C) temperature. The sample was then rapidly dispensed from the syringe into 6 mL glass cuvettes to overflowing before sealing with glass stoppers.

Immediately after dispensing, 11 cuvettes were placed in a custom-built linear incubator (University of Essex, Colchester, UK) maintained at either the control (26°C) or treatment (32°C) temperature. Three additional cuvettes were immediately sampled by the MIMS to provide the initial concentration of oxygen isotopes. The cuvettes within the linear incubator were exposed to an actinic PFD gradient between 0 and 500 μ mol photons \cdot m $^{-2}$ \cdot s $^{-1}$ via a blue LED of peak emission of ~ 470 nm. Incubation lasted between 30 and 60 min, during which samples were immediately and sequentially analyzed using the MIMS.

Changes in isotopic oxygen concentrations were measured using a quadrupole mass spectrometer (QMG 422; Pfeiffer Vacuum, Asslar, Germany) with Faraday collection. Multiple ion detection of masses 28 (N $_2$), 32 ($^{16}\text{O}_2$), and 36 ($^{18}\text{O}_2$) were repeatedly measured every 1.2 s. The membrane inlet was kept at 29°C (i.e., the mean of the two treatment temperatures). This midpoint temperature allowed both control and treatment temperature samples to be measured in quick succession over a stable temperature. A stainless-steel coil surrounded by thermostatic water preceded the membrane, which ensured that the sample had equilibrated to 29°C before reaching the membrane inlet. Before and after each set of incubation measurements, three replicate standards were measured. Standards were generated from a flask of air-equilibrated deionized water at known temperature and pressure, with dissolved gas concentrations for O $_2$ and N (see Kana 1990). A zero standard for $^{16}\text{O}_2$ was generated by adding sodium dithionite to the filtered sample and measured for each daily set of incubations. The rate of gross O $_2$ production and O $_2$ uptake (and, by difference, net O $_2$ evolution) was calculated from the change in oxygen concentration compared to initial values, as determined by Kana (1990), and normalized to cell concentration (Fig. 1).

H $_2$ O $_2$ production. H $_2$ O $_2$ was detected by using amplex red (Molecular Probes Inc., Eugene, OR, USA) at a final concentration of 100 μ M. When combined with horseradish peroxidase, amplex red reacts with H $_2$ O $_2$ in a 1:1 stoichiometry to produce a fluorescent product, resorufin, with an absorption maximum at 571 nm (Zhou et al. 1997). Aliquots of 1 mL of each culture were placed in a glass scintillation vial and combined with amplex red and horseradish peroxidase in an NaPO $_4$ buffer (0.2 U \cdot mL $^{-1}$, 0.05 M, pH 7.4). Scintillation vials were then placed in a photosynthetron (OHPT Inc., Delaware, DE, USA) at PFDs of 100 and 1,000 μ mol

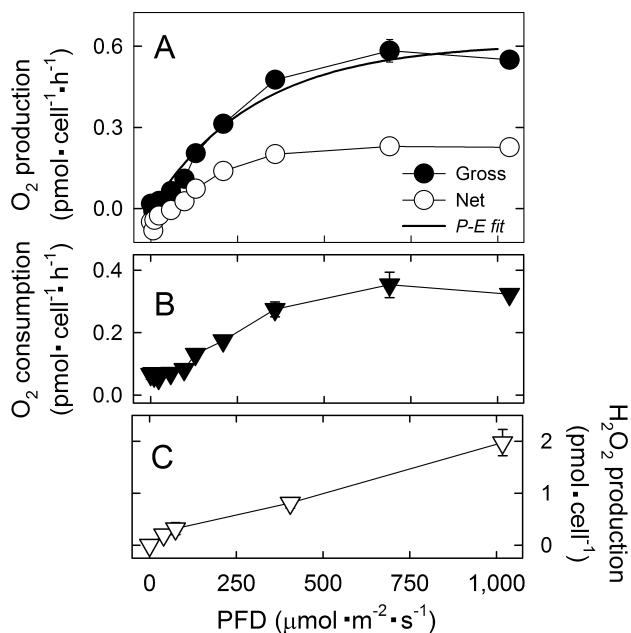


FIG. 1. Example of the photosynthetic light-response of (A) gross and net O_2 production ($\text{pmol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$), (B) O_2 consumption ($\text{pmol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$), and (C) relative H_2O_2 production following one incubation ($\text{pmol } H_2O_2 \cdot \text{cell}^{-1}$) from strain A1 following 24 h at 26°C. All data points are the mean (\pm SE bars) for triplicate experiments, and PFDs are spectrally corrected to that used for growth. Symbols are open and filled circles (gross and net O_2 production, respectively) and open and filled inverted triangles (O_2 consumption and H_2O_2 production, respectively). The solid line (A) is an example of the fit of Jassby and Platt's 1976 model (eq. 6), in this case to gross O_2 ($r^2 = 0.977$). Parameters yielded from the fit were α [$0.00292 (\text{pmol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}) \cdot (\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1})^{-1}$], P^{\max} ($0.639 \text{ pmol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$), and P_0 ($-0.0002 \text{ mol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$) (Table 2). PFD, photon flux density.

photons $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The temperature of the photosynhetron was maintained at the growth or treatment temperature by a water bath. Additional positive and negative dark controls were run in tandem by holding vials of pure growth media or cell suspensions with the amplex red mixture at the same temperature but in darkness. Amplex red reacts with light, and so a Rose Pink screening filter (002, LEE Filters, Andover, UK) was placed over the tungsten-halogen lamps of the photosynhetron to minimize excitation of the amplex red by blue-green light ($\sim 450\text{--}550 \text{ nm}$). Light exposure was terminated after 1 h. Samples were centrifuged briefly (1,200g, 5 min) to pellet the cells, and then absorbance of the supernatant was read on a spectrophotometer at 571 nm. The centrifugation step did not result in any detectable release of H_2O_2 . Concentrations of H_2O_2 were calculated by comparing absorbance of unknown samples to a series of H_2O_2 standards (0–5 μM) treated with the amplex red mixture. This method provides an estimate of relative H_2O_2 production (Fig. 1).

Characterizing the light response of production. Actinic PFDs used for O_2 and relative H_2O_2 production were further adjusted to account for differences in the spectra of the FIRE and MIMS LEDs and the Rose-Pink adjusted photosynhetron lamp. The spectrally resolved PFD for each light source, $\text{PFD}(\lambda)$ ($\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \cdot \text{nm}^{-1}$), was determined using a spectroradiometer (SR-9910; Macam, Livingstone, UK), and an effective rate of light absorption determined as

$$\bar{a} = \left(\sum_{400}^{700} a(\lambda) \cdot \text{PFD}(\lambda) \right) / \sum_{400}^{700} \text{PFD}(\lambda) \quad (4)$$

where $a(\lambda)$ (m^{-1}) is the spectrally resolved optical absorption for either alga. Measured PFDs were then spectrally weighted relative to the spectral quality used for growth, termed $\overline{\text{PFD}}$ ($\mu\text{mol} \cdot \text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), as

$$\overline{\text{PFD}} = (\bar{a}^{\text{actinic}} / \bar{a}^{\text{growth}}) \text{PFD} \quad (5)$$

The initial slope (α) and maximum (P^{\max}) rate of O_2 evolution were determined for each MIMS versus $\overline{\text{PFD}}$ response by fitting a modified version of Jassby and Platt (1976) using least-squares nonlinear regression (Fig. 1)

$$P = P_0 + \{P' \cdot [1 - \exp(-\alpha \cdot \overline{\text{PFD}} / P')]\} \quad (6)$$

where $P^{\max} = P_0 + P'$ and α and P^{\max} are in units of $\text{pmol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$ and ($\text{pmol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$) $\cdot (\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1})^{-1}$, respectively. P_0 , $\text{pmol } O_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$, is an intercept parameter required in the presence of respiration and to improve the distribution of residuals at low PFDs. The so-called light-saturation parameter, E_K ($\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), was calculated as P^{\max} / α .

RESULTS

PSII photochemistry. *Symbiodinium* types A1 and B1 exhibited markedly different dark-adapted PSII photosynthetic characteristics under ambient growth (the control, Table 1). Values of the maximum photochemical efficiency (F_v/F_m) and RCII concentration for type A1 were higher than those for type B1 by $\sim 70\%$ and 175%, respectively (Table 1). In contrast, rates of Q_A reoxidation (τ_{QA}) were higher for type B1 than for type A1 by $\sim 45\%$. These differences suggest that PSII should be capable of dissipating absorbed excitation energy by photochemistry more quickly and efficiently in type A1 compared with type B1. Values of the effective absorption cross-section (σ_{PSII} , $\text{\AA}^2 \cdot \text{quanta}^{-1}$) for type B1 were higher than those for type A1 by $\sim 20\%$.

Actual dissipation of absorbed excitation energy under actinic light can be described by the PSII efficiency factor, F_q'/F_v' , and changes of σ_{PSII}' relative to σ_{PSII} (Fig. 2). Values of F_q'/F_v' decreased by a greater extent for type B1 than for type A1 as actinic PFD was increased; thus, for any given actinic PFD, PSII was more highly reduced for the sensitive type. In contrast, nonphotochemical quenching in the antennae bed, as indicated by $1 - \sigma_{\text{PSII}}'/\sigma_{\text{PSII}}$, increased with PFD in the same manner for both algal types.

As expected, transfer of *Symbiodinium* cultures to experimental flasks for 24 h in the 26°C climate-controlled cabinet exhibited no significant change in PSII photochemistry relative to ambient growth (the control) for either alga (Table 1). PSII photochemistry also remained unchanged when experimental flasks were maintained for 24 h at 32°C for type A1, but not for type B1. At 32°C, F_v/F_m

TABLE 1. Mean values (\pm SE; $n = 3$ replicates) of chl $a \cdot \text{cell}^{-1}$ ($\text{pg} \cdot \text{cell}^{-1}$), PSII maximum photochemical efficiency (F_v/F_m , dimensionless), PSII effective absorption (σ_{PSII} , $\text{\AA}^2 \cdot \text{quanta}^{-1}$), cellular PSII reaction center (RCII) concentration (mol RCII $\cdot \text{cell}^{-1}$), and the rate of reoxidation of the primary acceptor quinone molecule, Q_A (τ_{Q_A} , μs), for the thermally “tolerant” (A1) and thermally “sensitive” (B1) *Symbiodinium* type under ambient growth (control at 26°C) and under experimental treatments after 24 h at both 26°C and 32°C. An analysis of variance (ANOVA) and subsequent Tukey’s test were performed across all treatments and *Symbiodinium* strains for each variable: superscript letters indicate significantly different groupings from Tukey’s test; no letters indicate that the ANOVA test was not significant.

Alga	Treatment	Chl $a \cdot \text{cell}^{-1}$ ($\text{pg} \cdot \text{cell}^{-1}$)	F_v/F_m	σ_{PSII} ($\text{\AA}^2 \cdot \text{quanta}^{-1}$)	τ_{Q_A} (μs)	RCII $\cdot \text{cell}^{-1}$ ($\text{mol cell}^{-1} \cdot 10^{-15}$)
A1	26° Control	1.31 (0.09) ^a	0.53 (0.01) ^a	385 (4) ^a	953 (22) ^a	2.26 (0.21) ^a
	26° +24 h	1.12 (0.12) ^a	0.55 (0.01) ^a	381 (9) ^a	963 (31) ^a	n/a
	32° +24 h	1.17 (0.15) ^a	0.51 (0.02) ^a	395 (8) ^a	978 (35) ^a	n/a
B1	26° Control	0.67 (0.04) ^b	0.31 (0.01) ^b	447 (13) ^b	1369 (32) ^b	0.81 (0.16) ^b
	26° +24 h	0.69 (0.11) ^b	0.30 (0.01) ^b	450 (10) ^b	1310 (45) ^b	n/a
	32° +24 h	0.53 (0.04) ^c	0.25 (0.01) ^c	505 (18) ^c	1622 (35) ^c	n/a

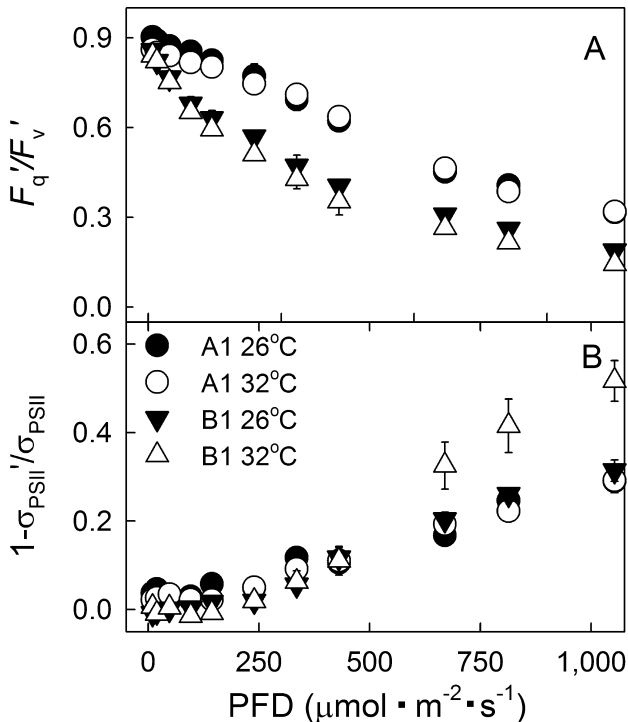


FIG. 2. Fluorescence light-response of (A) the PSII efficiency factor (F_q'/F_v' , dimensionless) and (B) the proportion of absorbed excitation energy dissipated nonphotochemically ($1 - \sigma_{\text{PSII}}'/\sigma_{\text{PSII}}$, dimensionless) for the thermally “tolerant” (A1) and thermally “sensitive” (B1) type of *Symbiodinium* under experimental treatments after 24 h at both 26°C and 32°C. Data from ambient growth conditions (26°C) were not different from experimental treatments for 24 h at 26°C and are not shown for clarity. All data points are the mean (\pm SE bars) for triplicate experiments, and PFDs are spectrally corrected to that used for growth. Symbols are open and filled circles (type A1 at 26°C and 32°C, respectively), and open and filled inverted triangles (type B1 at 26°C and 32°C, respectively). PFD, photon flux density.

decreased by $\sim 20\%$ – 25% , while τ_{Q_A} and σ_{PSII} increased by $\sim 25\%$ and 10% , respectively, compared to values observed at 26°C for type B1 (Table 1). Therefore, dark-adapted fluorescence generally indicated a negative effect of temperature upon algal type B1 but not upon A1. However,

fluorescence quenching light-response characteristics remained unchanged between 26°C and 32°C treatments for either algal type (Fig. 2).

O₂ exchange. Values of P^{max} and α from gross O_2 production for type B1 were lower than those for type A1 by $\sim 70\%$ – 80% (Table 2). Both P^{max} and α for type A1 remained unchanged following 24 h at 26°C and 32°C relative to values observed under ambient growth. In contrast, P^{max} and α for type B1 were decreased by $\sim 20\%$ following 24 h at 32°C (Table 2). Values of E_K for either algal type remained constant throughout.

Light-dependent O_2 consumption increased with actinic PFD for both algal types; however, values plateaued at PFDs where P^{max} was observed for gross O_2 production (e.g., Fig. 1). Rates of light-dependent O_2 consumption for type B1 were always lower than those for type A1 by $\sim 80\%$ – 90% (Table 2). Rates of O_2 consumption in darkness (respiration) were also higher for type A1. These respiration rates were only $\sim 25\%$ – 35% of the maximum light-dependent O_2 consumption (measured at a PFD equivalent to P^{max}) for type A1 but were $\sim 40\%$ – 60% for type B1 (Table 2). Type A1 exhibited an increase in both dark- and light-dependent O_2 consumption by $\sim 40\%$ – 50% at 32°C compared to 26°C. In contrast, O_2 consumption for type B1 remained unchanged regardless of treatment.

Relative H₂O₂ production. For actinic PFDs corresponding to those used for growth ($100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), cell-normalized relative H_2O_2 production was the same for both algal types at 26°C and 32°C (Fig. 3A). For PFDs saturating for both production and light-dependent consumption of O_2 , $1,000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, a significant increase in H_2O_2 production ($\sim 40\%$) was observed for type B1 but not for type A1 (Fig. 3B). H_2O_2 production was increased by a factor of ~ 10 with a 10-fold increase in actinic PFD.

DISCUSSION

Physiology and productivity. Our results are consistent with previous investigations, which suggest that

TABLE 2. Mean values (\pm SE; $n = 3$ replicates) for cell-normalized photosynthetic parameters from the MIMS of P^{\max} (maximum rate of production, $\mu\text{mol O}_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$), α [initial slope, $(\mu\text{mol O}_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}) \cdot (\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1})^{-1}$], E_K (light saturation PFD, $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), O_2 consumption in the dark (respiration, $\mu\text{mol O}_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$) and in the light ($\mu\text{mol O}_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$) for PFDs corresponding to P^{\max} , $\sim 1,000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Values are for the thermally “tolerant” (A1) and thermally “sensitive” (B1) *Symbiodinium* types under ambient growth (control at 26°C) and under experimental treatments after 24 h at both 26°C and 32°C. Superscript letters are as for Table 1.

Alga	Treatment	Gross O_2 production			O_2 consumption	
		P^{\max}	α	E_K	Dark	Light (at P^{\max})
A1	26° Control	0.76 (0.07) ^a	3.69 (0.49) ^a	206 (26)	0.08 (0.01) ^a	0.41 (0.03) ^a
	26° +24 h	0.64 (0.09) ^a	2.92 (0.32) ^a	219 (3)	0.10 (0.02) ^a	0.38 (0.05) ^a
	32° +24 h	0.63 (0.08) ^a	3.05 (0.35) ^a	207 (18)	0.13 (0.02) ^b	0.50 (0.03) ^b
B1	26° Control	0.20 (0.03) ^b	1.04 (0.19) ^b	193 (12)	0.05 (0.01) ^{a,c}	0.10 (0.02) ^c
	26° +24 h	0.20 (0.02) ^b	0.97 (0.11) ^b	206 (20)	0.04 (0.01) ^c	0.11 (0.02) ^c
	32° +24 h	0.12 (0.03) ^c	0.58 (0.06) ^c	209 (4)	0.05 (0.01) ^c	0.07 (0.01) ^c

PFD, photon flux density; MIMS, membrane-inlet mass spectrometer.

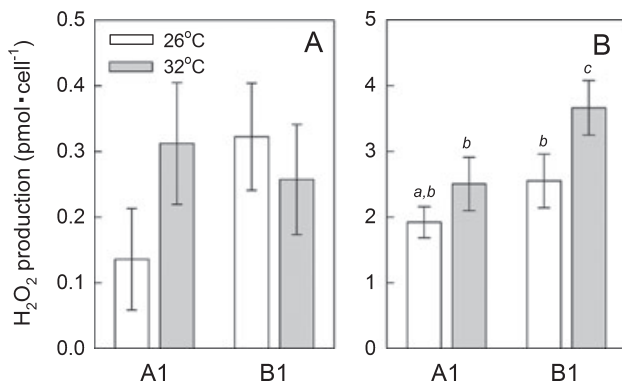


FIG. 3. Mean (\pm SE; $n = 3$ replicates) yields of relative H_2O_2 production normalized per cell following 1 h incubation ($\mu\text{mol H}_2\text{O}_2 \cdot \text{cell}^{-1}$) under (A) $100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and (B) $1,000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for thermally “tolerant” (A1) and thermally “sensitive” (B1) *Symbiodinium* types under experimental treatments after 24 h at both 26°C and 32°C. Note the different scales for the y-axis between panels (A) and (B). An ANOVA and subsequent Tukey’s test were performed across all treatments and *Symbiodinium* types for each PFD. Treatments only induced significantly different H_2O_2 yields under $1,000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (italicized letters indicate Tukey’s test groupings). Data from ambient growth conditions (26°C) were not different from experimental treatments for 24 h at 26°C and are not shown for clarity. PFD, photon flux density.

bleaching phenomena are associated with *Symbiodinium* PSII photochemistry (Iglesias-Prieto et al. 1992, Jones et al. 1998, reviewed by Smith et al. 2005, Lesser 2006). Bleaching in part is attributed to the presence or absence of particular *Symbiodinium* types (Berkelmans and van Oppen 2006, Warner et al. 2006). However, to date, only Tchernov et al. (2004) and Robison and Warner (2006) have measured photophysiological characteristics associated with the plastoquinone (PQ) pool redox state for thermally tolerant or sensitive *Symbiodinium* types. Our results demonstrate that the sensitive B1 type exhibited lower photochemical efficiencies and electron turnover times than the tolerant A1 type under ambient growth of 26°C. On a per cell basis, the sensitive B1 type also exhibited lower rates of gross O_2 production.

Adaptation of the photosynthetic apparatus is known to exist among species of the genus *Symbiodinium* (see Iglesias-Prieto et al. 1992, Iglesias-Prieto and Trench 1994, 1997, Robison and Warner 2006). Differences in P^{\max} for (net) O_2 production are also observed among *Symbiodinium* species (Iglesias-Prieto and Trench 1994, Savage et al. 2002), but of unknown thermal tolerance. Such adaptive regulation between microalgal species is typically characterized by changes in the ratio of functional RCIIIs to the maximum rate of carbon fixation, and hence by τ_{QA} , if light absorption is closely coupled with C fixation (Falkowski and Raven 1997, Moore et al. 2006). Values of τ_{QA} for type B1 were higher than those for type A1 by 45% under ambient growth of 26°C. Our measurements of cellular RCII concentration were lower for B1 by $\sim 65\%$ than for A1, while values of σ_{PSII} , which equate to the absorption per unit RCII (see Suggett et al. 2006), were higher for B1 by $\sim 20\%$ than for A1 (Table 1). As such, the extent to which the light-harvesting apparatus is modified, predominantly from cellular RCII content over σ_{PSII} , would appear to account for the difference in τ_{QA} between these *Symbiodinium* types under ambient growth.

The lower potential efficiency of B1 for processing absorbed excitation energy was further evidenced upon exposure to actinic light (Fig. 2). Type B1 exhibited a more rapid decrease in the PSII efficiency factor, F_q'/F_v' , with increasing PFD exposure, suggesting a more rapid reduction in quinone acceptors with increasing light exposure. However, reoxidation of PSII (“re-opening” of RCIIIs) also becomes limited by the rate of electron turnover “downstream” of Q_A (Bukhov et al. 2003). Here, τ_{QA} becomes decoupled from C fixation (see also Tchernov et al. 2004).

Exposure to elevated temperature (32°C) for 24 h elicited a photophysiological response previously characterized for thermally sensitive *Symbiodinium* types (see Smith et al. 2005, Lesser 2006), specifically, by a decrease in F_v/F_m and $\text{chl } a \cdot \text{cell}^{-1}$ but increase in τ_{QA} (Tchernov et al. 2004, Robison

and Warner 2006) and a decrease in O_2 production (Lesser and Shick 1989, Iglesias-Prieto et al. 1992, Lesser 1996). This response has been documented to accompany an additional loss of enzymatic (RUBISCO) activity, D1 protein turnover, and cellular division rate (Lesser 1996, Jones et al. 1998, Warner et al. 1999, Robison and Warner 2006). Hence, our observations are consistent with the concept that the reoxidation rate through the PQ pool is further limited when temperature-sensitive algal types are heated (Jones et al. 1998, Lesser and Farrell 2004, Smith et al. 2005). The increased turnover time of Q_A (τ_{QA}) and decreased RCII concentration and chl $a \cdot cell^{-1}$ limit the production of O_2 for the sensitive *Symbiodinium* type.

O₂ consumption and H₂O₂ production. Dark respiration in microalgae is predominantly via mitochondrial respiration where cytochrome oxidase is the terminal electron mediator, which provides a source of ATP, and/or from chlororespiration (Lewitus and Kana 1995). While chlororespiration has been implied to operate in *Symbiodinium* from enhanced reduction in PQ and consequently yields lower values of F_v/F_m in the dark (Hill and Ralph 2005), we did not observe values for F_v/F_m that were lower for dark-adapted than for low actinic light conditions from the fluorescence light-response curves (data not shown). This observation confirms that, as expected for eukaryotic algae grown under nutrient-replete conditions (Lewitus and Kana 1995), dark-respiration was most likely the result of mitochondrial respiration.

Values of O_2 consumption in the dark (respiration) were similar for both algal types under steady-state (26°C) growth. However, absolute values of gross O_2 production were lower for type B1 than for A1; as such, these values of dark respiration represented a higher potential metabolic cost of cell maintenance for the thermally sensitive type. Under steady-state growth (26°C), the thermally sensitive B1 type was characterized by a higher light-dependent H_2O_2 production per unit of O_2 consumed compared to the tolerant A1 type (Fig. 4). Adaptive differences between *Symbiodinium* types were also observed following the elevated temperature (Fig. 4): enhanced O_2 consumption but not relative H_2O_2 production for type A1, as opposed to enhanced H_2O_2 production but not O_2 consumption for type B1.

Light-dependent consumption of gross O_2 may be attributed to several pathways, notably the Mehler reaction and mitochondrial alternative oxidase (AOX) respiration (Lewitus and Kana 1995). Operation of either pathway will have different impacts on the pool of energy (ATP) and reductant (NADPH) that is available for all other metabolic processes (Falkowski and Raven 1997) as well as H_2O_2 production. The Mehler reaction elevates H_2O_2 production (Asada 2000, Badger et al. 2000), while AOX activation generally lowers H_2O_2 production (Braidot

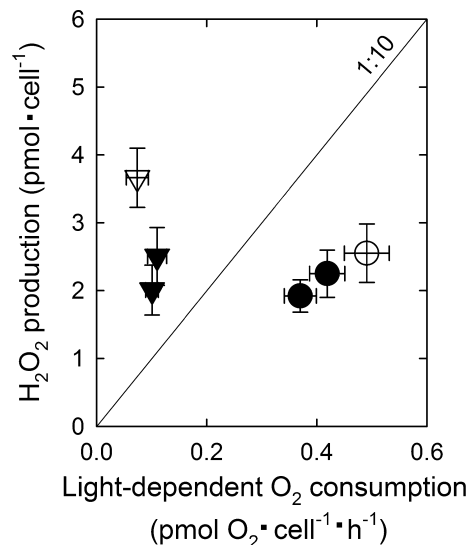


FIG. 4. Relationship between relative H_2O_2 production following 1 h incubation ($pmol H_2O_2 \cdot cell^{-1}$) and light-dependent O_2 consumption ($pmol O_2 \cdot cell^{-1} \cdot h^{-1}$) for both *Symbiodinium* types at a PFD saturating for photosynthesis, $1,000 \mu mol photons \cdot m^{-2} \cdot s^{-1}$, at 26°C (ambient growth and experimental) and 32°C. Data are expressed as the mean ($\pm SE$) for $n = 3$. Symbols are as in Figure 2, except two values for 26°C treatments (the control and +24 h) are shown for both algal types. PFD, photon flux density.

et al. 1999, Czarna and Jarmuszkiewicz 2005). We cannot discount some background AOX; however, in response to the 32°C treatment, elevated light-dependent O_2 consumption was not accompanied by a reduction in H_2O_2 production for type A1 (Fig. 3). Here, an increase in the Mehler reaction must induce the change in ROS production. Dark respiration was elevated for type A1 at 32°C, implying an elevated metabolic cellular demand. In contrast, type B1 did not exhibit increased light-dependent O_2 consumption, and the elevated H_2O_2 production would seem to be associated with a reduced ability to quench H_2O_2 . Such a response to “temperature stress” has been reported for another sensitive *Symbiodinium* type (also a B1, Lesser 1996) and across other microalgal classes (Twiner and Trick 2000, Kim et al. 2004).

In summary, clear adaptive differences in photosynthesis exist for two ecologically relevant *Symbiodinium* types in vivo under steady-state growth and during acute periods of elevated temperature. Thus, the adaptive variability of photophysiology that exists between types must be better accounted for when making future broadscale assessments of PSII activity, photosynthesis, and bleaching in nature. Our results imply that thermally sensitive types would decrease not only the autotrophic contribution to the host metabolism but also result in a net accumulation of H_2O_2 , a potential precursor of the bleaching response, under elevated water temperatures. However, these observations must be placed

within the context of the whole coral, which itself can both produce and quench ROS (Lesser 2006), to ultimately identify the scale with which symbiont-specific responses underlie bleaching, as observed in nature. Only by quantifying the relative composition of each *Symbiodinium* type in nature can we begin to assign the contribution of this genus to the “tolerances” of coral colonies and possibly of entire reef complexes to environmental change.

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