TEMPERATURE INDUCED CHANGES IN THYLAKOID MEMBRANE THERMOSTABILITY OF CULTURED, FRESHLY ISOLATED, AND EXPELLED ZOOXANTHELLAE FROM SCLERACTINIAN CORALS

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ABSTRACT

Coral bleaching events are characterized by a dysfunction between the cnidarian coral host and the symbiotic dinoflagellate algae, known as zooxanthellae (genus *Symbiodinium*). Elevated temperature and intense light induce coral bleaching, where zooxanthellae are expelled from the host tissue. The primary cellular process in zooxanthellae which leads to coral bleaching is unresolved, and here, we investigated the sensitivity of the thylakoid membrane in a *Symbiodinium* culture and in genetically identified freshly isolated and expelled *Symbiodinium* cells. The fluorescence-temperature curve technique was used to measure the critical temperature (T_c) at which irreversible damage to the thylakoid membrane occurred. The accuracy of this technique was confirmed through the collection of scanning transmission electron micrographs which demonstrated the clear relationship between T_c and thylakoid membrane degradation. Analysis of 10 coral species with a diverse range of genetically distinct *Symbiodinium* communities showed a decline in T_c from summer to winter. A *Symbiodinium* culture and fragments of *Pocillopora damicornis* (Linnaeus, 1758) were exposed to a series of light and temperature treatments, where T_c increased from approximately 37 °C to 42 °C upon exposure to elevated temperature. Under bleaching conditions, the thermostability of the thylakoid membrane increased within 4 hrs by 5.1 °C, to a temperature far above bleaching thresholds, in both freshly isolated and photosynthetically competent zooxanthellae expelled from *P. damicornis* under these conditions. It is demonstrated that the thermostability of the thylakoid membrane increases in cultured, freshly isolated, and expelled zooxanthellae exposed to bleaching stress, suggesting it is not the primary site of impact during coral bleaching events.

Unicellular dinoflagellates, known as zooxanthellae (genus *Symbiodinium*) reside within the gastrodermal tissue of scleractinian corals (Titlyanov and Titlyanova, 2002). This obligate symbiotic relationship is sensitive to environmental changes, such as elevated sea temperatures as small as 1–2 °C above average (Hoegh-Guldberg, 1999). Coral bleaching events occur in conjunction with high light intensity, which results in the dysfunction of the coral-algal symbiosis, leading to the expulsion of zooxanthellae, as well as a reduction in photosynthetic pigments (Kleppel et al., 1989). The measurement of chlorophyll *a* fluorescence is a widely used tool for studying the photosynthetic activity of zooxanthellae as it is a rapid and non-invasive method of analysis (Iglesias-Prieto, 1995; Warner et al., 1996; Jones et al., 1998; Fitt et al., 2001; Jones and Hoegh-Guldberg, 2001; Hill et al., 2004). Previous studies have indicated that bleaching causes a decline in the health of *in hospite* zooxanthellae (Jones et al., 2000), and much research has concentrated on identifying the location of bleaching-associated damage within the photosynthetic apparatus. Photosystem II (PSII) has been suggested as the primary site of impact (Warner et al., 1999; Hill et al., 2004; Takahashi et al., 2004; Hill and Ralph, 2006), along with dark-reactions

(Jones et al., 1998) and disruption of the thylakoid membrane (Iglesias-Prieto et al., 1992; Tchernov et al., 2004).

Bound to the thylakoid membrane are chlorophyll molecules, which are the photosynthetic pigments responsible for light absorption. Damage to the membrane's integrity has been shown to cause the degradation of the entire photosynthetic apparatus (Schreiber and Berry, 1977). Tchernov et al. (2004) found thermal damage to occur within the membrane after 168 hrs of exposure to elevated light and temperature, a time period which falls outside the usual definition of bleaching (paling expected within 6 hrs of exposure to bleaching conditions; Dove et al., 2006). In contrast, impacts to the photosynthetic health of zooxanthellae have been shown to occur within several hours by measurements of maximum quantum yield (F_v/F_m) (Warner et al., 1996; Hill et al., 2004, 2005).

Schreiber et al. (1975) and Schreiber and Berry (1977) developed a bioassay which monitors the state of the thylakoid membrane through the measurement of fluorescence-temperature (F-T) curves. This method involves the measurement of minimum fluorescence (F_o) while temperature is ramped at a user-defined speed, generally between 0.5 and 6 °C min^{-1} (Smillie, 1979; Bilger et al., 1984; Seeman et al., 1986; Kuropatwa et al., 1992; Lazár and Ilik, 1997). Analysis of the resulting F-T curve, generates the following parameters: the critical temperature (T_c), the temperature of peak fluorescence (T_p), the ratio of the initial F_o to the maximum F_o ($F_{initial}/F_{maximum}$), the temperature at which F_v/F_m reaches 50% of its initial (T_{50}) and the temperature at which F_v/F_m reaches zero (T_0). These have been shown to correlate well to the heat sensitivity of the thylakoid membrane in numerous studies on higher plants (Schreiber and Berry, 1977; Raison and Berry, 1979; Smillie, 1979; Berry and Bjorkman, 1980; Smillie and Gibbons, 1981; Monson and Williams, 1982; Bilger et al., 1984; Nauš et al., 1992; Havaux, 1993) and in one case, on a green alga (Kouřil et al., 2001). See Figure 1A for graphical explanation of all F-T curve parameters.

The thermal tolerance of the thylakoid membrane (indicated by T_c) has been shown to vary between species of higher plants inhabiting the same environment (Smillie and Nott, 1979; Bilger et al., 1984; Thomas et al., 1986; Havaux et al., 1988). Furthermore, the capacity for rapid acclimation of the thylakoid membranes to changing ambient temperatures has been demonstrated (Berry and Bjorkman, 1980; Smillie and Gibbons, 1981; Raison et al., 1982; Downton et al., 1984; Havaux, 1993; Lazár and Ilik, 1997; Kouřil et al., 2001). In these studies, shifts in the heat tolerance have been reported where the T_c has increased by several degrees Celsius in a matter of hours following exposure to elevated temperatures. Seasonal and spatial variation in thylakoid membrane thermostability, induced by varying temperatures has also been established through measurements of F-T curves. Seeman et al. (1986) and Knight and Ackerly (2002) demonstrated, using the F-T technique, that species inhabiting warmer regions exhibited greater photosynthetic tolerance to elevated temperature, and that seasonal increases in thermostability occurred from winter to summer. This plasticity has been attributed to changes in the composition of the thylakoid membrane lipids and proteins (Pearcy, 1978; Raison et al., 1982; Hugly et al., 1989; Ivanova et al., 1993).

Scleractinian corals have been shown to harbor a number of genetically distinct *Symbiodinium* types (e.g., Baker, 2003; Ulstrup and van Oppen, 2003; Sampayo et al., 2008) and attempts have been made to correlate these with specific physiological traits. The most conclusive evidence of *Symbiodinium*-specific physiologies *in hos-*

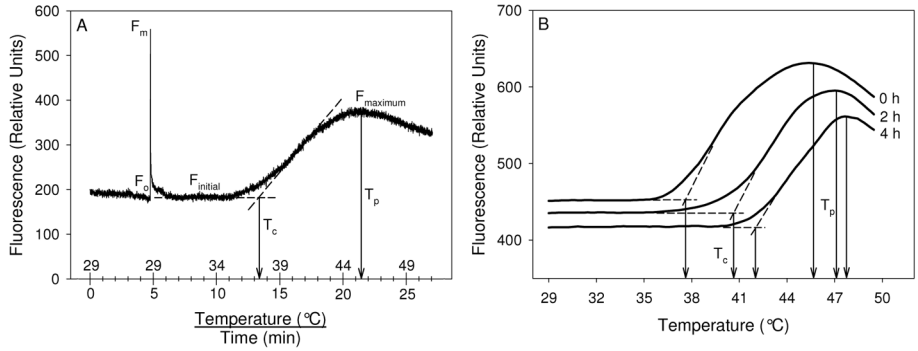

Figure 1. (A) Representative fluorescence-temperature (F-T) curve, where the temperature was increased at a speed of 1 °C min^{-1}. The location of minimum fluorescence (F_o), maximum quantum yield (F_v/F_m), critical temperature (T_c), temperature of maximum fluorescence (T_p), and initial ($F_{initial}$) and maximum fluorescence ($F_{maximum}$) are shown. The temperature (°C) is indicated above the x-axis and time (min) indicated below. This example is of *Acropora millepora* during summer. (B) Average F-T curves of *Pocillopora damicornis* exposed to bleaching conditions (400 µmol photons m^{-2} s^{-1} and 32 °C) at 0, 2, and 4 hrs (n = 4).

pite has been presented for the coral genus *Pocillopora* (Rowan, 2004) and *Acropora millepora* (Ehrenberg, 1834) (Berkelmans and van Oppen, 2006) where associations with a clade D *Symbiodinium* genotype conferred higher thermotolerance than associations with a clade C *Symbiodinium* genotype. However, the ITS1-determined *Symbiodinium* C1 and D infected into juveniles of *Acropora tenuis* (Dana, 1846) showed contrasting results where *Symbiodinium* C1 was found to confer higher thermotolerance of the holobiont than *Symbiodinium* D (Abrego et al., 2008). In combination, these results suggest that holobiont physiologies are not contingent on *Symbiodinium* type alone but also depend on the host examined.

This study explored the thermal tolerance of the thylakoid membrane of a *Symbiodinium* culture as well as genetically identified freshly isolated and expelled zooxanthellae. Further studies were done to evaluate the degree of acclimatization of the thylakoid membrane to light and temperature treatments in the coral, *Pocillopora damicornis* (Linnaeus, 1758). By comparing corals which harbored genetically distinct zooxanthellae, we attempted to identify relationships between genotype and physiology.

Material and Methods

Coral Specimens.—Eight replicate fragments of upper, sun-exposed surfaces of 10 coral species were sampled during the Austral summer (January 2005) and winter (July 2005) from Heron Island reef flat, Great Barrier Reef, Australia (151°55′E, 23°26′S). The species collected were from a depth of 1–2 m and consisted of *A. millepora*, *Acropora nobilis* (Dana, 1846), *Acropora valida* (Dana, 1846), *Cyphastrea serailia* (Forsskål, 1775), *Goniastrea australensis* (Milne-Edwards and Haime, 1857), *Montipora digitata* (Dana, 1846), *Pavona decussata* (Dana, 1846), *P. damicornis*, *Porites cylindrica* Dana, 1846, and *Stylophora pistillata* Esper, 1797. For the temperature and light manipulated experiments, four replicate *P. damicornis* colonies were also collected. Prior to experimentation, all corals were maintained in shaded aquaria (< 100 µmol photons m^{-2} s^{-1}) at ambient lagoon temperature for 2 d.

Fluorescence Measurements.—Coral fragments were air brushed in 15 ml of 0.45 µm filtered seawater to remove zooxanthellae and animal tissue from the skeleton. 10 ml of this

solution was then filtered through a 20 μm mesh to isolate the zooxanthellae which passed through the filter. A freshly isolated zooxanthellae solution was then placed in a Water-PAM fluorometer (Walz GmbH, Effeltrich, Germany) cuvette for analysis. A custom-made heat exchange chamber, with an internal cavity through which water was pumped, was inserted into the Water-PAM cuvette to control the temperature of the sample (2.2 ml). The water flowing through the heat exchange chamber was connected to a temperature regulated water-bath (Julabo Labortechnik, model EC, Germany) and was raised at a user-defined speed. 1 °C min^{-1} was found to be appropriate for these experiments (Table 1), where any faster rise in temperature (i.e., at 2 °C min^{-1}) resulted in an over-estimation of the critical temperature and temperature at maximum fluorescence (Smillie, 1979). A temperature rise of 1 °C min^{-1} was an adequate speed, as decreasing the rate of heating to 0.5 °C min^{-1} did not alter the temperature values obtained (Table 1). The Water-PAM was placed on an oscillating table (set at approximately 90 revolutions min^{-1}) to prevented the settling of zooxanthellae and to eliminate the formation of a temperature gradient in the solution within the cuvette. A K-type thermocouple was inserted into the cuvette to monitor the temperature over time on a digital thermometer (Fluke, 52 Series II, Washington State, USA).

Once a zooxanthellae sample was placed into the Water-PAM, the recording of minimum fluorescence (F_o) began ($\lambda > 710$ nm), and 5 min of dark-adaptation was given at a constant temperature (ambient lagoon temperatures in summer and winter sampling and treatment temperature for the manipulative studies). Following this time, a saturating pulse was applied and the far-red LED (emission peak at 730 nm) was turned on. The far-red light served to re-oxidize the electron transport chain, through the excitation of Photosystem I (PSI) (Schreiber et al., 1975; Bilger et al., 1984; Knight and Ackerly, 2002). This is necessary to suppress the fluorescence rise which can occur under hypoxic conditions in corals in darkness (Bukhov et al., 1990; Bukhov and Mohanty, 1999; Yamane et al., 2000), and has been shown to occur in experiments with *in hospite* zooxanthellae (Ulstrup et al., 2005). No significant difference was detected between the use of far-red and no far-red (Table 1), although this light source was still used to prevent any occurrence of dark-induced reduction of the electron transport chain between PSII and PSI (Hill and Ralph, 2008). The temperature inside the cuvette was then raised at a speed of 1 °C min^{-1} and the minimum fluorescence (F_o) was measured until a down-turn in F_o was observed (Fig. 1A). The critical temperature (T_c) was determined by the intersection of the steady minimum fluorescence (F_o) and the subsequent rise in F_o (Schreiber and Berry, 1977). The temperature of peak fluorescence (T_p) is also indicated on Figure 1A (Bilger et al., 1984). Additional parameters suggested to be of physiological importance (Smillie and Gibbons, 1981; Nauš et al., 1992; Lazár and Ilik, 1997; Kouřil et al., 2001; Knight and Ackerly, 2002) include the calculation of the initial fluorescence over the maximum fluorescence ($F_{initial}/F_{maximum}$), the temperature at which F_v/F_m reaches 50% of its initial (T_{50}) and the temperature at which F_v/F_m reaches zero (T_0). For the seasonal study, saturating pulses were applied every 2 min during the measurement of the F-T curve on half of the replicates. This was performed in order to calculate T_{50} and T_0. In the remaining replicates, no saturating pulses were applied following the initial F_v/F_m measurement, in order to calculate T_c and T_p with greater precision and accuracy.

Table 1. Effect of heating rate and presence/absence of far-red light on initial F_v/F_m, T_c, and T_p of fluorescence-temperature curves performed on cultured *Symbiodinium* sp. (CS-156). Averages ± standard error of mean shown (n = 6). P-values (one-way ANOVAs) and Tukey's post hoc comparison tests are shown as superscript letters.

Light exposure	Ramping speed	F_v/F_m	T_c (°C)	T_p (°C)
Darkness	1 °C min^{-1}	0.503 ± 0.006	37.0 ± 0.1 [a]	43.7 ± 0.7 [a]
Far-red	0.5 °C min^{-1}	0.504 ± 0.001	36.9 ± 0.2 [a]	43.8 ± 0.2 [a]
Far-red	1 °C min^{-1}	0.505 ± 0.004	37.0 ± 0.2 [a]	42.7 ± 0.4 [a]
Far-red	2 °C min^{-1}	0.488 ± 0.004	38.9 ± 0.3 [b]	47.1 ± 0.3 [b]
P-value		0.069	< 0.001	< 0.001

Experimental Protocol.—Freshly isolated zooxanthellae from the 10 coral species examined, were raised in temperature from 29 °C during summer and from 22 °C during winter (ambient lagoon temperatures) at a rate of 1 °C min^{-1} until after T_p was reached (Fig. 1A). Measurements were performed on the CS-156 *Symbiodinium* culture from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) microalgae research center in Hobart, Tasmania, Australia. CS-156 was isolated from *Montipora verrucosa* (Lamarck, 1816) in Hawaii, USA, and grown in f/2 media at 25 °C and 40 µmol photons m^{-2} s^{-1}, with a light to dark ratio of 12:12 hrs. A second dinoflagellate culture, *Amphidinium carterae* Hulburt, 1957, was also analyzed to compare its physiological traits to those found in the *Symbiodinium* culture. This strain, CS-21 (also from CSIRO), isolated in Halifax, Canada, was grown in the laboratory under the same conditions as CS-156.

The impact of increased light intensity and elevated temperature on F_v/F_m, T_c, and T_p was investigated in the *Symbiodinium* culture (CS-156) as well as for freshly isolated and expelled *Symbiodinium* cells from *P. damicornis*. *Symbiodinium* culture solutions (n = 4) were exposed to four treatments (see Table 2) for 9 hrs, with hourly determinations (0–9 hrs) of F_v/F_m, T_c, and T_p. The four treatments were, (i) control (low light and low temperature), (ii) high light and low temperature, (iii) low light and elevated temperature, and (iv) high light and elevated temperature (Table 2). Similarly, freshly isolated zooxanthellae from fragments of *P. damicornis* (n = 4) were exposed to four treatments (Table 2) for 8 hrs, with F_v/F_m, T_c, and T_p determined at 0, 1, 2, 4, and 8 hrs. Figure 1B shows the average F-T curves of *P. damicornis* exposed to high light and elevated temperature (conditions which initiated a bleaching response; see Hill and Ralph, 2006) at 0, 2, and 4 hrs. The rapid and acute stress imposed by the high light and elevated temperature treatment on *P. damicornis* fragments was sufficient to induce a bleaching response where symbionts were expelled from the host. However, it should be noted that this treatment may not directly reflect the conditions experienced by corals during bleaching episodes in nature.

In addition to the measurements on freshly isolated zooxanthellae, F_v/F_m, T_c, and T_p of zooxanthellae which were expelled under bleaching conditions of high light and elevated temperature (400 µmol photons m^{-2} s^{-1} and 32 °C) after the time periods of 0–1 hr, 1–2 hrs, 2–4 hrs, and 4–8 hrs were measured. Coral fragments were placed in filtered seawater and the expelled zooxanthellae were collected by filtering the aquaria seawater through 0.45 µm filter paper after each time interval. Zooxanthellae were isolated by gently brushing the surface of the filter paper into 10 ml of filtered seawater. 2.2 ml of the solution was then placed into the Water-PAM for analysis. The same fragments were then returned to the experimental aquaria with filtered seawater to collect expelled zooxanthellae for the next time interval.

Genetic Identification of Symbiodinium.—Upon completion of fluorescence measurements, a sub-sample of the original isolated zooxanthellae solution were centrifuged to a pellet and stored frozen (–80 °C) for subsequent genetic identification. Zooxanthella DNA was extracted using the DNeasy tissue extraction kit (Qiagen, USA) according to the manufacturer's protocol. In order to distinguish between *Symbiodinium* genotypes the ribosomal DNA internal transcribed spacer 1 (ITS1) region was amplified as described in van Oppen et

Table 2. Photon flux density (μmol photons m^{-2} s^{-1}) and temperature (°C) of the four experimental treatments [control (low light and low temperature)], high light + low temperature, low light + elevated temperature, and high light + elevated temperate) for the *Symbiodinium* culture and freshly isolated *Symbiodinium* samples.

	Cultured zooxanthellae		Freshly isolated zooxanthellae	
Treatment	Photon flux density (μmol photons m^{-2} s^{-1})	Temp. (°C)	Photon flux density (μmol photons m^{-2} s^{-1})	Temp. (°C)
Control	40	25	100	22
High light + low temp.	400	25	400	22
Low light + elevated temp.	40	32	100	32
High light + elevated temp.	400	32	400	32

al. (2001). A forward primer fluorescently labelled with TET enabled detection of sequence variation using single stranded conformation polymorphism (SSCP) (Sunnucks et al., 2000) and the GelScan2000 system (Corbett Research, Australia). PCR (polymerase chain reaction) products mixed with formamide gel-loading dye (Sambrook et al., 1989) were denatured for 3 min at 95 °C and immediately snap-cooled on ice. 1 µL of each sample was loaded onto a 4% non-denaturing TBE-polyacrylamide gel and run on the Gelscan2000 (Corbett Research). PCR products that resulted in different SSCP profiles were re-amplified using a non-labelled forward primer and purified using ExoSAP-IT (USB, USA) and subsequently sequenced. Samples containing multiple DNA templates were cloned using the TOPO® Cloning Kit (Invitrogen). Colony inserts were re-amplified using rDNA ITS1 primers for SSCP analysis and subsequent purification was performed on products with matching SSCP profiles to those of the original sample. Colony PCRs that yielded new SSCP profiles were omitted from the analysis. Samples were sent to Macrogen (Korea) for sequencing and results were compared to existing sequences stored in GenBank (www.ncbi.nlm.nih.gov). In samples where multiple DNA templates were present, the genotype corresponding to the brightest band on the SSCP gel was classified as the dominant genotype.

Following *Symbiodinium* identification, correlations between clusters of corals harboring the same *Symbiodinium* genotype and derived fluorometric parameters were tested.

Scanning Transmission Electron Microscopy.—*Symbiodinium* culture suspensions (CS-156) of 18 ml were ramped from 25 °C to 45 °C at 1 °C min^{-1} with 2 ml aliquots extracted at 25, 32, 35, 36, 37, 38, 39, 40, and 45 °C. Upon extraction, samples were immediately centrifuged at 2000 × *g* for 1 min and fixed in 0.1M sodium cacodylate buffer (in 0.22 µm filtered seawater, pH = 8.2) and 4% glutaraldehyde for 1 hr at room temperature. Pellets were rinsed in a series of three, 10 min, 0.1M sodium cacodylate buffer solutions, followed by secondary fixing in 2% OsO_4 for 1 hr at room temperature. After rinsing in two, 10 min, distilled water solutions, samples were dehydrated in a series of ascending acetone solutions, of 50, 70, 80, 90, 95, and 100%. Samples were then infiltrated overnight in a 1:1 solution of Spurr's resin:acetone and subsequently infiltration by 100% Spurr's resin for 24 hrs. A microtome (Ultracut T, Leica, Vienna, Austria) sectioned samples at 130 nm in thickness and these were viewed on a scanning transmission electron microscope (Quanta 200, FEI Company, Oregon, USA).

Statistical Analysis.—One-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparison tests ($\alpha = 0.05$) were used to detect significant differences between variables and to identify the location of these difference. The Kolmogorov-Smirnov normality test and Levene's homogeneity of variance test were used to identify if the assumptions of the parametric one-way ANOVAs were satisfied. If these assumptions were not met, arcsine (F_v/F_m and $F_{initial}/F_{maximum}$) and $\log_{10}$ (T_c, T_p, T_{50}, and T_0) transformations were performed. All analyses were carried out using the SPSS statistical software package (version 11.0.0, 2001, Chicago, Illinois, USA).

Results

During both summer and winter, significant differences were found between the 10 coral species studied in each of the six calculated parameters F_v/F_m, T_c, T_p, $F_{initial}/F_{maximum}$, T_{50}, and T_0 (Table 3; Tukey's post hoc multiple comparisons test). Three distinct groups were identified from the F_v/F_m data in both summer and winter seasons, where *A. millepora* and *A. valida* were grouped with the highest values, *M. digitata* was placed in the group with the lowest F_v/F_m, and the other species generally fell between these two groups (Table 3). In summer, the 10 coral species fell into two distinct T_c groups (one-way ANOVA: $P < 0.001$), the first with an average of 37.3 °C and the second with an average of 39.5 °C. In winter, this distinct grouping was less apparent with the presence of three groups with overlap between them (one-way

ANOVA: $P < 0.001$; Table 3). The parameter T_p, separated the 10 species into three groups in summer and four groups in winter (one-way ANOVA: $P < 0.001$). No common trend in these delineations could be detected in both seasons. The final three parameters of $F_{initial}/F_{maximum}$, T_{50}, and T_0 also separated the 10 species into several different groups, none of which was constant between the two seasons (Table 3). The T_0 values were further analyzed to determine if they differed from the T_p values. In 80% of all cases there was no significant difference between T_0 and T_p for each species in summer and winter. The 20% which did not follow this trend were *P. cylindrica* in summer and *G. australensis*, *P. damicornis*, and *P. cylindrica* in winter (one-way ANOVAs: where $P < 0.05$).

Variation for each species between summer and winter was also investigated and significant differences are indicated by an asterisk (*), which is shown next to the highest value, whether it occurred in winter or summer for that species (Table 3). Five of the 10 species studied (*A. valida*, *P. decussata*, *P. damicornis*, *S. pistillata*, and *P. cylindrica*) showed a higher F_v/F_m in summer compared to winter. T_c was higher in all species in summer than in winter and this same trend was reflected in five of the 10 species with respect to T_p. The parameter $F_{initial}/F_{maximum}$ showed little variation between summer and winter sampling, with the only difference being higher values in *G. australensis* and *M. digitata* in winter. Significantly higher T_{50} and T_0 were only found in summer for several species examined (Table 3).

The DNA templates of 76 samples out of a total of 81 samples were successfully amplified. Five samples did not yield adequate PCR amplification product for further SSCP analysis (Table 4). Genetic identification of zooxanthellae revealed a highly diverse assemblage across the 10 species examined. Zooxanthellae encompassing three clades (A, C, and D) were observed. Within clade A and D there was one representative genotype (GenBank Accession nos. AF380513 and AY457958, respectively) and within clade C there was three representative genotypes, C1, C2, and C·, *sensu* van Oppen et al. (2001) and van Oppen et al. (2005) (GenBank Accession nos. AF380551, AY758477, and AY327054, respectively). Six out of 10 species of corals examined harbored only one strain, where *C. serailia*, *P. damicornis*, *P. decussata*, and *S. pistillata* harbored C1 and *P. cylindrica* and *M. digitata* harbored C·. *Acropora nobilis* harbored C· in all samples but also clade A in six of eight samples. In four of these samples, clade A dominated. In *A. valida*, all samples harbored C2 but again, also clade A (dominantly) in three samples. *Acropora millepora* harbored C1 and C2 simultaneously in all samples. Bands corresponding to the two genotypes appeared equally strong in all samples. Finally, present in *G. australensis* were all three clade C genotypes as well as clade D. C1, C2 and D co-dominated in four samples whereas C· and C1+C2 dominated in two samples each, respectively (Table 4). The *Symbiodinium* culture (CS-156) exhibited an identical sequence match with *Symbiodinium kawagutii* (Genbank Accession no. AF333517) described in Carlos et al. (1999) and LaJeunesse (2001). Table 3 includes the results from the *Symbiodinium* culture and *A. carterae*. Both cultures were grown under the same conditions, although all parameters were significantly higher (one-way ANOVAs: $P < 0.05$) for *A. carterae*.

Scanning transmission electron micrographs of the CS-156 culture were obtained during a temperature ramp of 1 °C min^{-1} (speed of the F-T curves) at 25, 30, 35, 36, 37, 38, 39, 40, and 45 °C (Fig. 2). The micrographs displayed in Figure 2 are representative of the general trends observed in all random images taken. The organized stacking pattern of the thylakoid membrane was found in zooxanthella cells from 25–36 °C

Table 3. The $F_v/_{Fm}$, T_c, T_p, $F_{initial}/F_{maximum}$, T_{50}, and T_0 parameters for each of the 10 coral species studied during summer and winter, as well as for cultured *Symbiodinium* sp. and *Amphidinium carterae*. Coral species are grouped by *Symbiodinium* genotype. Superscript letters indicate variation between species and show the groups into which they fall within each season for each parameter (determined by Tukey's post hoc test where $\alpha = 0.05$. The P-value (one-way ANOVA) is shown at the bottom of each list of species). Asterisk (*) indicates differences between summer and winter for each species and each parameter and is shown on the significantly higher value ($\alpha = 0.05$). Averages ± standard error of mean shown (n = 8).

Species	F_v/F_m	T_c (°C)	T_p (°C)	$F_{initial}/F_{maximum}$	T_{50} (°C)	T_0 (°C)
Freshly isolated zooxanthellae during summer 2005						
Genotype: A+C2						
Acropora nobilis	0.627 ± 0.01 ^{bc}	37.3 ± 0.1 ^{a} *	47.0 ± 0.2 ^{abc}	1.48 ± 0.03 ^{def}	39.9 ± 0.4 ^{a}	46.7 ± 0.5 ^{abc}
Acropora valida	0.663 ± 0.00 ^{c} *	37.3 ± 0.1 ^{a} *	46.8 ± 0.5 ^{abc}	1.45 ± 0.05 ^{de}	40.5 ± 0.3 ^{a}	47.2 ± 0.7 ^{abc}
Genotype: C1						
Cyphastrea serailia	0.593 ± 0.02 ^{ab}	39.7 ± 0.1 ^{b} *	48.4 ± 0.3 ^{bc} *	1.25 ± 0.05 ^{abc}	42.0 ± 0.2 ^{abc} *	48.0 ± 0.5 ^{abc}
Pavona decussata	0.654 ± 0.01 ^{c} *	39.7 ± 0.1 ^{b} *	48.8 ± 0.8 ^{c}	1.36 ± 0.04 ^{bcde}	43.9 ± 0.2 ^{c} *	49.1 ± 0.2 ^{c}
Pocillopora damicornis	0.627 ± 0.01 ^{bc} *	39.7 ± 0.3 ^{b} *	48.2 ± 0.3 ^{bc} *	1.33 ± 0.05 ^{abcd}	42.4 ± 0.5 ^{abc} *	48.7 ± 0.5 ^{bc} *
Stylophora pistillata	0.634 ± 0.01 ^{bc} *	37.4 ± 0.1 ^{a} *	47.8 ± 0.7 ^{bc}	1.65 ± 0.02 ^{f}	41.5 ± 0.6 ^{abc} *	47.9 ± 0.5 ^{abc}
Genotype: C•						
Montipora digitata	0.575 ± 0.01 ^{a}	36.9 ± 0.4 ^{a} *	48.2 ± 0.3 ^{bc} *	1.43 ± 0.02 ^{cde}	41.3 ± 1.0 ^{ab} *	48.4 ± 0.6 ^{abc} *
Porites cylindrica	0.597 ± 0.00 ^{ab} *	39.4 ± 0.1 ^{b} *	46.7 ± 0.2 ^{ab} *	1.17 ± 0.04 ^{a}	43.3 ± 0.3 ^{bc} *	48.8 ± 0.2 ^{bc} *
Genotype: C1+C2						
Acropora millepora	0.662 ± 0.01 ^{c}	37.4 ± 0.1 ^{a} *	45.7 ± 0.4 ^{a}	1.54 ± 0.04 ^{ef}	41.0 ± 0.6 ^{ab}	45.8 ± 0.8 ^{a}
Genotype: C1+C2+C•+D						
Goniastrea australensis	0.603 ± 0.01 ^{ab}	39.2 ± 0.3 ^{b} *	45.7 ± 0.3 ^{a} *	1.20 ± 0.03 ^{ab}	41.7 ± 0.6 ^{abc} *	46.3 ± 0.8 ^{ab}
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001

Table 3. Continued.

Species	F_v/F_m	T_c (°C)	T_p (°C)	$F_{initial}/F_{maximum}$	T_{50} (°C)	T_0 (°C)
Freshly isolated zooxanthellae during winter 2005						
Genotype: A+C2						
Acropora nobilis	0.623 ± 0.01 ^{bc}	33.3 ± 0.2 ^{ab}	46.4 ± 0.5 ^{cd}	1.47 ± 0.04 ^{bc}	37.6 ± 0.7 ^{ab}	47.3 ± 0.6 ^{b}
Acropora valida	0.639 ± 0.01 ^{c}	34.2 ± 0.3 ^{b}	45.7 ± 0.4 ^{cd}	1.45 ± 0.03 ^{bc}	39.4 ± 0.3 ^{b}	46.3 ± 0.1 ^{b}
Genotype: C1						
Cyphastrea serailia	0.595 ± 0.01 ^{bc}	34.2 ± 0.2 ^{b}	44.9 ± 0.8 ^{cd}	1.30 ± 0.04 ^{ab}	38.5 ± 0.7 ^{ab}	46.2 ± 0.5 ^{b}
Pavona decussata	0.604 ± 0.01 ^{bc}	34.3 ± 0.5 ^{b}	47.6 ± 0.5 ^{d}	1.34 ± 0.03 ^{ab}	42.1 ± 0.1 ^{b}	48.1 ± 0.5 ^{b}
Pocillopora damicornis	0.592 ± 0.01 ^{bc}	38.2 ± 0.4 ^{c}	46.6 ± 0.5 ^{cd}	1.31 ± 0.02 ^{ab}	38.0 ± 0.5 ^{ab}	45.1 ± 0.6 ^{b}
Stylophora pistillata	0.607 ± 0.00 ^{bc}	32.7 ± 0.3 ^{ab}	47.8 ± 0.6 ^{d}	1.64 ± 0.04 ^{c}	38.2 ± 0.5 ^{ab}	46.5 ± 0.8 ^{b}
Genotype: C•						
Montipora digitata	0.524 ± 0.02 ^{a}	32.5 ± 0.5 ^{a}	40.1 ± 1.0 ^{a}	1.87 ± 0.11 ^{c} *	33.8 ± 2.4 ^{a}	39.4 ± 3.2 ^{a}
Porites cylindrica	0.579 ± 0.01 ^{b}	34.1 ± 0.2 ^{b}	41.2 ± 1.0 ^{ab}	1.14 ± 0.01 ^{a}	39.3 ± 0.6 ^{b}	45.6 ± 0.1 ^{b}
Genotype: C1+C2						
Acropora millepora	0.637 ± 0.01 ^{c}	34.2 ± 0.2 ^{b}	45.8 ± 0.2 ^{cd}	1.41 ± 0.04 ^{b}	39.0 ± 0.6 ^{b}	46.5 ± 0.3 ^{b}
Genotype: C1+C2+C•+D						
Goniastrea australensis	0.604 ± 0.01 ^{bc}	33.0 ± 0.5 ^{ab}	44.0 ± 0.3 ^{bc}	1.30 ± 0.03 ^{ab} *	37.5 ± 0.9 ^{ab}	46.1 ± 0.7 ^{b}
P value	< 0.001	< 0.001	< 0.001	< 0.001	0.002	0.003
Cultured zooxanthellae (CS-156) Genotype: C						
Symbiodinium sp.	0.505 ± 0.00	37.1 ± 0.1	43.4 ± 0.3	1.33 ± 0.03	40.7 ± 0.4	47.7 ± 0.3
Cultured *Amphidinium carterae* (CS-21)						
Amphidinium carterae	0.652 ± 0.00	39.4 ± 0.2	48.5 ± 0.9	1.60 ± 0.08	42.5 ± 0.0	49.3 ± 0.1

Table 4. Coral species, sample number (n) and observed *Symbiodinium* genotype frequency (f) of clade A, C (C1, C2, and C•), and D (f_A, f_{C1}, f_{C2}, $f_{C•}$, and f_D, respectively). Dominance is given where multiple types were harbored simultaneously.

	n	f_A	f_{C1}	f_{C2}	$f_{C•}$	f_D	Dominance (n)
Acropora nobilis	8	6	–	8	–	–	A(4), C2(4)
Acropora valida	7	3	–	7	–	–	A(3), C2(4)
Cyphastrea serailia	8	–	8	–	–	–	C1
Pavona decussata	8	–	8	–	–	–	C1
Pocillopora damicornis	6	–	6	–	–	–	C1
Stylophora pistillata	7	–	7	–	–	–	C1
Montipora digitata	8	–	–	–	8	–	C•
Porites cylindrica	7	–	–	–	7	–	C•
Acropora millepora	8	–	8	8	–	–	C1+C2(8)
Goniastrea australensis	8	–	6	6	3	5	C•(2), C1+C2(2), C1+C2+D(4)

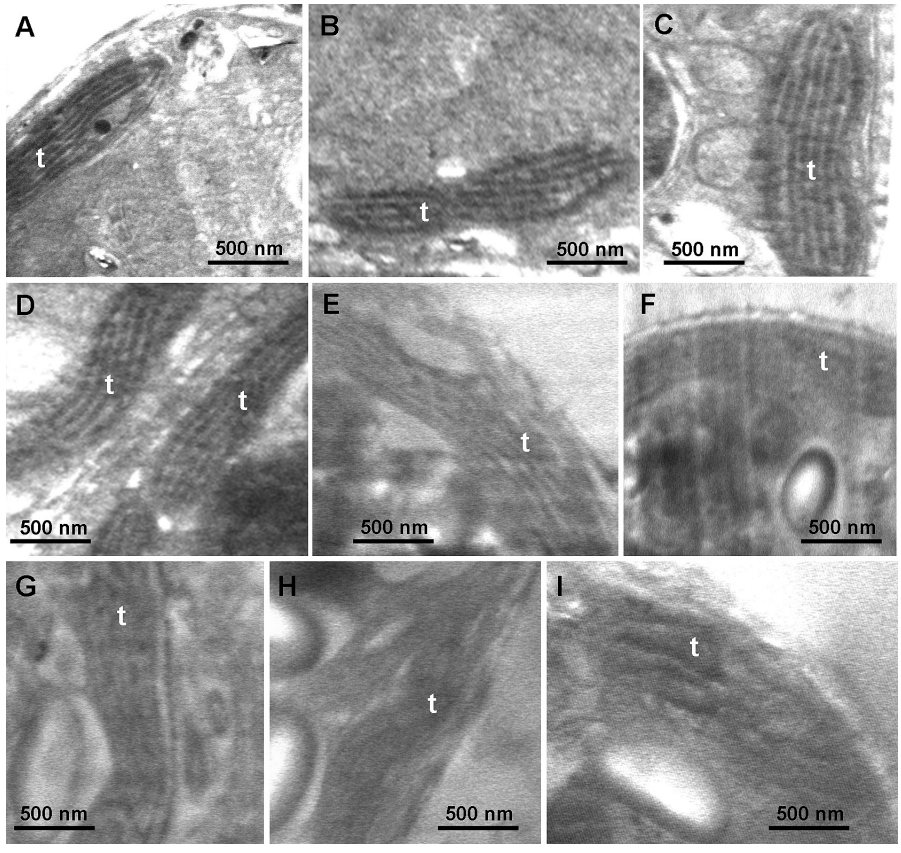

Figure 2. Representative scanning transmission electron micrographs of cultured *Symbiodinium* cells (CS-156) at (A) 25 °C, (B) 30 °C, (C) 35 °C, (D) 36 °C, (E) 37 °C, (F) 38 °C, (G) 39 °C, (H) 40 °C, and (I) 45 °C during a temperature ramp of 1 °C min^{-1}. Scale bars of 500 nm shown on each micrograph. Thylakoid membrane indicated by the symbol, "t".

(Fig. 2A–D). Some disruption to this membrane occurred at 37 °C (Fig. 2E) as distinguished by a loss of the clear stacking pattern. At temperatures above this, from 38–45 °C (Figs. 2F–I), a complete loss of structural integrity was found.

Under control conditions (Fig. 3A), no significant changes were observed in F_v/F_m, T_c, and T_p over the 9 hr exposure (one-way ANOVAs: $P > 0.96$ in all cases) with averages of F_v/F_m remaining at 0.509, T_c at 37.3 °C, and T_p at 43.9 °C. Similar results were obtained for T_c and T_p in the high light and low temperature treatment (Fig. 3B), although in this case, the F_v/F_m significantly dropped from 0.526 at 0 hrs to a minimum of 0.391 over the 9 hr period (one-way ANOVA: $P < 0.001$). During the low light and elevated temperature treatment, F_v/F_m declined from 0.510 at 0 hrs to a minimum of 0.419 over the experimental period (one-way ANOVA: $P < 0.001$), although no further significant decreases were found after 4 hrs (Fig. 3C). T_c increased sharply by 5.1 °C over the first 4 hrs from 37.4 °C at 0 hrs to a maximum of 42.5 °C in this treatment (one-way ANOVA: $P < 0.001$), and T_p showed a similar response, with a rise of 2.4 °C from 44.1 °C to 46.5 °C (one-way ANOVA: $P = 0.021$), becoming constant after 3 hrs. In the high light and elevated temperature treatment (Fig. 3D), F_v/F_m showed the greatest decline, with a significant decrease (one-way ANOVA: $P < 0.001$) occurring from 0 hrs (0.512) to 5 hrs (0.372), after which time it remained steady. For T_c and T_p, significant rises occurred from 36.6 °C and 44.1 °C at 0 hrs to 42.0 °C and 47.2 °C, respectively (two one-way ANOVAs: $P < 0.001$). For both parameters, no significant changes occurred following the 4 h sampling period and the increases equate to 5.4 °C for T_c and 3.1 °C for T_p (Fig. 3D).

A similar experiment was performed on freshly isolated zooxanthellae from *P. damicornis* (Fig. 4). Under control conditions (Fig. 4A) no changes in F_v/F_m, T_c or T_p were detected over time (three one-way ANOVAs: $P = 0.581$, 0.979, and 0.614, respectively). Under high light and low temperature conditions (Fig. 4B), the T_c and T_p parameters remained unchanged over time (two one-way ANOVAs: $P = 0.981$ and 0.748, respectively), although F_v/F_m significantly decreased (one-way ANOVA: $P = 0.008$) from the 0 hrs time interval (0.608) to a plateau from 4 to 8 hrs (minimum $F_v/F_m = 0.465$). In contrast, no significant change in F_v/F_m was found in the low light and elevated temperature treatment (one-way ANOVA: $P = 0.197$; Fig. 4C). Under these experimental conditions T_c increased by 4.5 °C from 0 hrs (where $T_c = 37.5$ °C) to 4 and 8 hrs (where $T_c = 42.0$ °C). A parallel rise in T_p (one-way ANOVA: $P = 0.049$) was also seen from 46.6 °C at 0 hrs to a constant value of 48.2 °C at 4 and 8 hrs (Fig. 4C). Upon exposure to high light and elevated temperature, the F_v/F_m, T_c and T_p of freshly isolated zooxanthellae from *P. damicornis* all significantly changed (one-way ANOVAs: $P < 0.001$ in all cases; Fig. 4D). Figure 1B illustrates the average F-T curves at 0, 2, and 4 hrs of this treatment. F_v/F_m declined from 0 h (where $F_v/F_m = 0.598$) to 2 hrs (where $F_v/F_m = 0.452$), after which there was no further significant drop, although the minimum F_v/F_m was 0.395 at 8 hrs. Both T_c and T_p showed a similar trend over the 8 hr exposure period, and increased from 37.6 °C and 45.8 °C, respectively, at 0 hrs to reach a maximum by 4 and 8 hrs (42.7 °C and 48.2 °C, respectively). Under these bleaching conditions, the three parameters showed the greatest change over the experimental time period for freshly isolated zooxanthellae, with F_v/F_m decreasing by 0.203 units, T_c increasing by 5.1 °C, and T_p increasing by 2.2 °C (Fig. 4D).

In a subsequent experiment, zooxanthellae expelled from *P. damicornis* corals exposed to bleaching conditions, were collected and analysed to determine their F_v/F_m, T_c, and T_p for the time intervals 0–1 hr, 1–2 hrs, 2–4 hrs, and 4–8 hrs (Fig. 5).

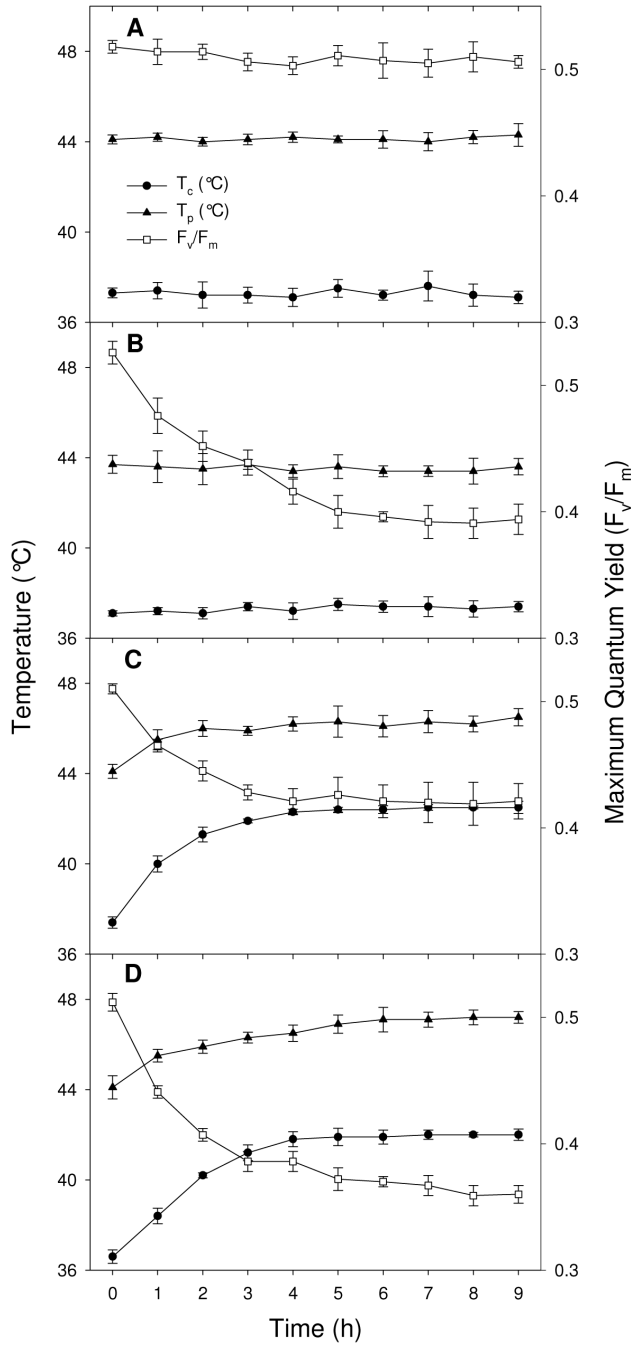

Figure 3. Maximum quantum field (F_v/F_m; open squares), critical temperature (T_c; closed circles), and temperature of peak fluorescence (T_p; closed triangles) of cultured zooxanthellae exposed to control conditions of (A) low light (40 µmol photons m^{-2} s^{-1}) and low temperature (25 °C), (B) high light and low temperature (400 µmol photons m^{-2} s^{-1} and 25 °C), (C) low light and elevated temperature (40 µmol photons m^{-2} s^{-1} and 32 °C), and (D) high light and elevated temperature (400 µmol photons m^{-2} s^{-1} and 32 °C) from 0 to 9 hrs. Temperature (°C) is indicated on the left y-axis and F_v/F_m on the right y-axis. Averages ± standard error of mean shown (n = 4).

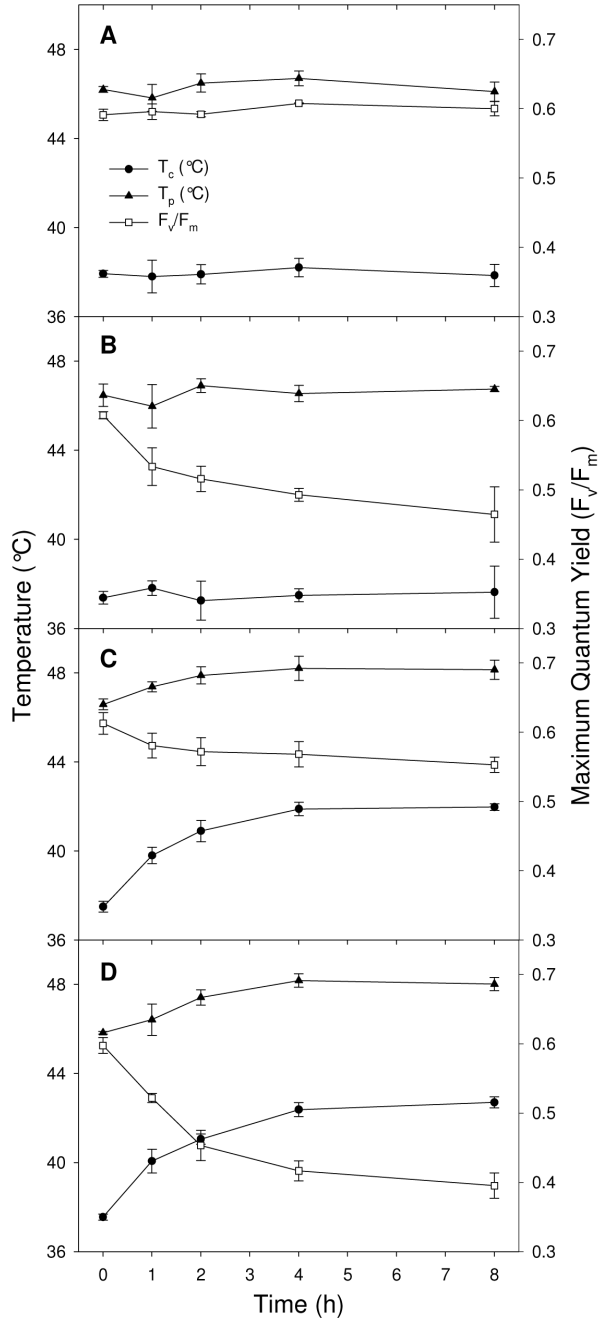

Figure 4. Maximum quantum yield (F_v/F_m; open squares), critical temperature (T_c; closed circles), and temperature of peak fluorescence (T_p; closed triangles) of freshly isolated zooxanthellae from *Pocillopora damicornis* exposed to control conditions of (A) low light (100 µmol photons m^{-2} s^{-1}) and low temperature (22 °C), (B) high light and low temperature (400 µmol photons m^{-2} s^{-1} and 22 °C), (C) low light and elevated temperature (100 µmol photons m^{-2} s^{-1} and 32 °C), and (D) high light and elevated temperature (400 µmol photons m^{-2} s^{-1} and 32 °C) at time intervals of 0, 1, 2, 4, and 8 hrs. Temperature (°C) is indicated on the left y-axis and F_v/F_m on the right y-axis. Averages ± standard error of mean shown (n = 4).

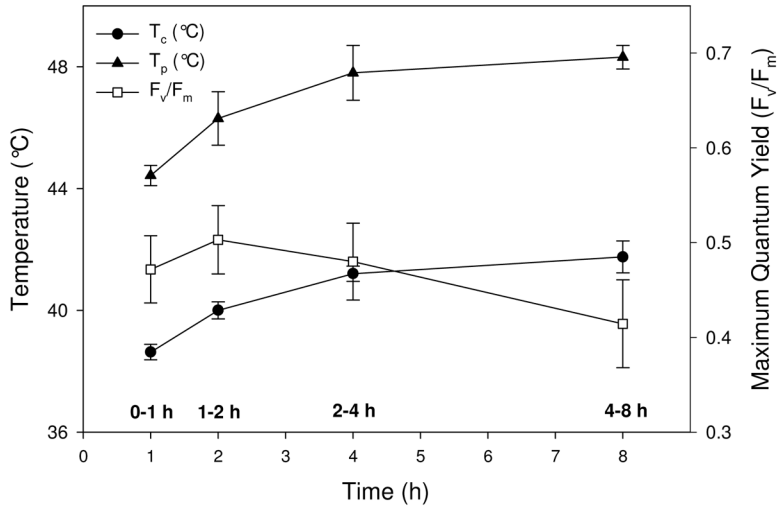

Figure 5. Maximum quantum yield (F_v/F_m; open squares), critical temperature (T_c; closed circles), and temperature of peak fluorescence (T_p; closed triangles) of expelled zooxanthellae from *Pocillopora damicornis* exposed to the bleaching conditions of 400 µmol photons m^{-2} s^{-1} and 32 °C. Measurements were taken at the time intervals of 0–1 hr, 1–2 hrs, 2–4 hrs, and 4–8 hrs. Temperature (°C) is indicated on the left y-axis and F_v/F_m on the right y-axis. Averages ± standard error of mean shown (n = 4).

At these four time intervals F_v/F_m was not found to significantly change (one-way ANOVA: P = 0.474) and the overall F_v/F_m average was 0.467. The T_c and T_p parameters showed corresponding trends and significantly increased (two one-way ANOVAs: P < 0.001 and = 0.007, respectively) from the 0–1 hr time interval (38.6 °C and 44.4 °C, respectively) up until the 2–4 hrs and 4–8 hrs time intervals which were the same. T_c increased by 3.2 °C to reach a maximum of 41.8 °C and T_p increased by 3.9 °C to reach a maximum of 48.3 °C (Fig. 5).

Discussion

The thermostability of zooxanthellae thylakoid membranes was determined through measurements of F-T curves, which monitor the response of chlorophyll *a* fluorescence upon rapid heating of the cells (Schreiber et al., 1975; Schreiber and Berry, 1977). Since chlorophyll molecules are embedded in this membrane, the measurement of fluorescence emissions allows for an assessment of thylakoid integrity. Calculation of the critical temperature (T_c) provided a sensitive indicator to the temperature at which permanent membrane breakdown occurred, as supported by scanning transmission electron micrographs of the CS-156 culture. This demonstrated that the structural integrity of the thylakoid membrane remained intact until temperatures above 37 °C. Higher temperatures resulted in the loss of the organized stacking pattern in this membrane, which is essential for efficient photosynthetic function. This provided clear evidence of the correlation between T_c (found to be 37.1 °C for CS-156) and thylakoid membrane integrity.

The seasonal study revealed variation in thylakoid thermostability between coral species and a shift in each species' tolerance throughout the year. In summer, two distinct groups were identified in the 10 coral species, based on T_c. Havaux et al. (1988)

attributed differences in thermostability to the species being studied, although the biochemical basis for inter-species variation when a group of species all exist in the same environment has not been found (Hugly et al., 1989).

Here, the symbiont population within each coral host was genetically identified. Significant differences in T_c, T_p, $F_{initial}/F_{maximum}$, T_{50}, and T_0 were found between the 10 coral species studied in both seasons and associations with *Symbiodinium* genotype were investigated. Considerable genetic variation in the *Symbiodinium* communities examined was found between and within coral species. However, this study investigated coral colonies from a very narrow environmental range (i.e., Heron Island reef flat) and by examining a wider environmental gradient (Sampayo et al., 2008) or using more variable genetic markers (e.g., Magalon et al., 2006) and more sensitive sequence detection methods (Sampayo et al., 2009) an even higher genetic diversity of *Symbiodinium* may be found. In this study, it was not possible to conclusively correlate *Symbiodinium* genotypes to any parameter of thylakoid thermostability using SSCP-ITS1 detection. We therefore concur with previous studies (Abrego et al., 2008; Baird et al., 2009) that the host may play a lead role in influencing thylakoid membrane thermostability and acclimation of *Symbiodinium*.

Tissue thickness and mass-transfer efficiency were suggested by Loya et al. (2001) to explain mortality patterns following a bleaching event. In our study, the two T_c groups in summer can be divided by host susceptibility, as defined by Loya et al. (2001), where those species in the higher T_c group are more bleaching tolerant and those in the lower T_c group, more sensitive, with the exception of *P. damicornis*.

In order to assess the similarity of zooxanthellae to other dinoflagellates, the thermal properties of the thylakoid membrane were compared between a culture of *Symbiodinium* and *A. carterae*. These two dinoflagellate cultures were grown under the same conditions, but had large variations in thylakoid thermostability. The results from this comparison revealed that zooxanthella thylakoid membranes are significantly less thermo-tolerant than those of *A. carterae*. Nevertheless, the critical temperature of *Symbiodinium* sp. and *A. carterae* were 12.1 °C and 14.4 °C, respectively, above the growth temperature of 25 °C, highlighting the robustness of the photosynthetic membrane of both dinoflagellate species.

In all coral species, a higher T_c value was obtained in summer and this can be attributed to acclimation of the corals to the higher sea temperatures at that time of year. Similar seasonal shifts in thylakoid membrane sensitivity have been reported in a number of higher plants (Seeman et al., 1986; Knight and Ackerly, 2002). The T_c found for both winter and summer were several degrees Celsius above the mean sea temperatures for both seasons, respectively. In summer, the sea temperature was approximately 29 °C for the period of the study, although the T_c for the 10 species ranged from 36.9 °C to 39.7 °C. In winter, the sea temperature was around 22 °C, although the T_c varied between 32.5 °C and 38.2 °C. This clearly demonstrates the plasticity of the photosynthetic membrane and it highlights the degree of tolerance which the thylakoid can withstand under stressful conditions. On Heron Island reef, situated in the southern Great Barrier Reef, bleaching threshold of corals may be below 30 °C (Hoegh-Guldberg, 1999), although it is important to note that this threshold does vary geographically (Berkelmans and Willis, 1999; Hoegh-Guldberg, 1999; Berkelmans, 2002; Ulstrup et al., 2006). During summer, when coral bleaching can potentially affect large areas of Heron Island reef (Jones et al., 2000), the thylakoid membrane will have increased its thermostability, therefore it is unlikely that this

membrane is the primary site of bleaching impact within the photosynthetic apparatus of zooxanthellae.

The other parameters of heat sensitivity from the F-T curves also showed similar responses between seasons, although their sensitivity was less apparent than T_c. In some species, T_p, T_{50}, and T_0 showed an increase in heat tolerance during summer, a response also observed in higher plants (Seeman et al., 1986; Knight and Ackerly, 2002). In two species studied, *G. australensis* and *M. digitata*, $F_{initial}/F_{maximum}$ was lower in summer compared to winter. Nauš et al. (1992) and Lazár and Ilik (1997) showed that this parameter declines with an increase in thermostability, thus confirming the seasonal shift observed towards a more tolerant threshold in corals during summer.

Half of the species studied (*A. valida*, *P. decussata*, *P. damicornis*, *S. pistillata*, and *P. cylindrica*) showed significantly higher F_v/F_m values during summer compared to winter (Table 3). This result is in contrast to Warner et al. (2002), who showed an opposite trend, where F_v/F_m was lower in three species of *Montastraea* during summer months. Here, it is proposed that this disparity is due to the different pre-sampling conditions of the corals. Warner et al. (2002) conducted PAM fluorometry measurements within a matter of hours following collection. In this case, the light history of the coral branches would still be reflected in the F_v/F_m results. A lower F_v/F_m would be expected due to the higher light intensities encountered during summer. In comparison, our study provided the corals with two days of acclimation to low light intensities (< 100 μmol photons m^{-2} s^{-1}) thus allowing the zooxanthellae adequate time for recovery from stressful field light intensities during summer. The higher F_v/F_m in five of the 10 species in this study during summer may be a result of more optimal water temperatures of 29 °C rather than potentially sub-optimal temperatures around 22 °C during the winter survey (Coles and Jokiel, 1977; Iglesias-Prieto et al., 1992).

To further explore the capacity for rapid acclimation of the thylakoid membrane to elevated temperatures, the *Symbiodinium* culture and *P. damicornis* fragments were exposed to bleaching conditions, and changes in F_v/F_m, T_c, and T_p were monitored over time. This allowed for an analysis of the vulnerability of this membrane to acute and rapid bleaching stress. The high light and elevated temperature conditions which induced the bleaching response in the coral fragments may not reflect the conditions experienced by corals in the field where changes in water temperature are likely to be slower. Nevertheless, our results concurred with previous studies indicating the large capacity for plastic acclimation of thylakoid thermo-tolerance (Berry and Bjorkman, 1980; Smillie and Gibbons, 1981; Raison et al., 1982; Downton et al., 1984; Havaux, 1993; Lazár and Ilik, 1997; Kouřil et al., 2001). These three parameters were chosen for analysis as T_p and T_0 were shown to be the same in 80% of cases and therefore both parameters were unnecessary, and $F_{initial}/F_{maximum}$ and T_{50} showed low sensitivity between species and season. Similarly, Smillie and Nott (1979), Havaux et al. (1988) and Havaux (1993) also found T_c and T_p to be sensitive measures of thylakoid thermal tolerance and were used consistently in their comprehensive studies.

In cultured and freshly isolated zooxanthellae from *P. damicornis*, we measured similar responses in the thermostability of the thylakoid membrane. In both cases, an increase in thermal tolerance (in the elevated temperature treatments) occurred from 0 hrs to 4 hrs, after which no further change occurred. This result highlights the plasticity and rapid capacity for acclimation of the membrane (by 4 hrs) in response to rising ambient temperatures (Berry and Bjorkman, 1980; Smillie and Gibbons, 1981; Raison et al., 1982; Downton et al., 1984; Havaux, 1993; Lazár and Ilik,

1997; Kouřil et al., 2001). It also suggests that alterations in the membranes' sensitivity to heat is temperature dependent, but light independent, as no changes were found in the high light and low temperature treatments. Thus, it can be concluded that zooxanthellae have the ability to increase the tolerance of their thylakoid membranes when exposed to stressful temperatures. In comparison, Schreiber and Berry (1977) found light-induced changes to the state of the thylakoid membrane between treatments where light intensity varied by up to four orders of magnitude. However, the light intensities used in our study varied by less than one order of magnitude, and may have been insufficient to cause similar irradiance-based alterations in T_c.

The high light and elevated temperature treatment was effective in producing a bleaching response in the *P. damicornis* fragments, and the freshly isolated zooxanthellae had a significantly reduced F_v/F_m, indicating a loss of photochemical efficiency in PSII (Schreiber, 2004). In contrast to this decline in PSII function, which has been well documented in corals under bleaching stress (e.g., Warner et al., 1996; Hill et al., 2004; Hill and Ralph, 2006), the T_c and T_p parameters showed an increase, indicating an enhancement of thylakoid thermostability. This suggests that zooxanthellae respond to bleaching conditions by enhancing the thylakoid membrane thermostability, although loss of photochemical efficiency does occur, as evidenced by the decline in F_v/F_m. Our results are inconsistent with Tchernov et al. (2004), who suggested that coral bleaching is initiated due to a breakdown of the thylakoid membrane. However, it should be noted that this disparity may be due to variations in experimental design and/or *Symbiodinium* genotypes. In the present study, we propose that the thylakoid membrane is not the primary site of impact during coral bleaching events, and that instead, an increase in thylakoid heat tolerance occurs in zooxanthellae under elevated temperature conditions.

In addition to monitoring freshly isolated zooxanthellae, those that were expelled under bleaching conditions were also collected and analyzed. Previous studies have shown expelled zooxanthellae to be photosynthetically competent (Ralph et al., 2001, 2005; Bhagooli and Hidaka, 2004) and here we aimed to determine the thermostability of these expelled zooxanthellae in order to compare them with freshly isolated zooxanthellae. It is important to note that the freshly isolated zooxanthellae in our study were physiologically and genetically representative of the zooxanthellae within the host tissue (Santos et al., 2001). No change in F_v/F_m occurred over time (0–8 hrs) for expelled zooxanthellae, which had an average value of 0.467 (comparable to the findings of Hill and Ralph, 2007). Similar responses were found for T_c and T_p in the expelled zooxanthellae over time, and those found in the cultured and freshly isolated zooxanthellae exposed to high light and elevated temperature. This further demonstrates that the plasticity and capacity for thermal acclimation of the thylakoid membrane in expelled zooxanthellae is equally as efficient to those which remain *in hospite*.

The variation observed between summer and winter measurements of thylakoid thermo-tolerance, as well as inter-species differences and changes in response to altering ambient temperature, have been attributed to changes in the biochemical composition (specifically proteins, lipids and the specific fatty acids which comprise the lipids) as well as ultrastructural changes in the thylakoid membrane which increase its resistance to thermal stress (Pearcy, 1978; Santarius and Müller, 1979; Raison et al., 1982; Hugly et al., 1989; Ivanova et al., 1993). Future research is needed to

investigate the changes in lipid and protein content of the thylakoid membranes of zooxanthellae during exposure to elevated temperature conditions.

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