Response of holosymbiont pigments from the scleractinian coral *Montipora monasteriata* to short-term heat stress

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Abstract

Heating the scleractinian coral, *Montipora monasteriata* (Forskål 1775) to 32°C under <650 μmol quanta m^{-2} s^{-1} led to bleaching in the form of a reduction in Peridinin, xanthophyll pool, chlorophyll c_2 and chlorophyll a, but areal dinoflagellates densities did not decline. Associated with this bleaching, chlorophyll (Chl) allomerization and dinoflagellate xanthophyll cycling increased. Chl allomerization is believed to result from the interaction of Chl with singlet oxygen ($^1O_2$) or other reactive oxygen species. Thermally induced increases in Chl allomerization are consistent with other studies that have demonstrated that thermal stress generates reactive oxygen species in symbiotic dinoflagellates. Xanthophyll cycling requires the establishment of a pH gradient across the thylakoid membrane. Our results indicate that, during the early stages of thermal stress, thylakoid membranes are intact. Different morphs of *M. monasteriata* responded differently to the heat stress applied: heavily pigmented coral hosts taken from a high-light environment showed significant reductions in green fluorescent protein (GFP)-like homologues, whereas nonhost pigmented high-light morphs experienced a significant reduction in water-soluble protein content. Paradoxically, the more shade acclimated cave morph were, based on Chl fluorescence data, less thermally stressed than either of the high-light morphs. These results support the importance of coral pigments for the regulation of the light environment within the host tissue.

Mass coral bleaching events witnessed over the last 20 years are characterized by drastic losses of dinoflagellate pigmentation from scleractinian corals in response to anomalous increases in sea surface temperature (Hoegh-Guldberg 1999). A statement similar to this occurs in almost every manuscript written about the effect of thermal stress on coral bleaching. However, no publication has yet comprehensively investigated the effect of heat on all of the individual pigments (inclusive of carotenoids) that give these endosymbiotic dinoflagellates their characteristic coloration. The effect of these bleaching events on host or coral-synthesized pigmentation is even less documented, extending merely to anecdotal statements commenting on the brilliant blues, pinks, and greens observed in some corals at recently bleached sites.

The aim of this study was to investigate the effect a short-term heat stress (6 h at 32°C at a relatively low-light inten-

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sity) has on all of the individual pigments that give rise to coloration in the scleractinian coral *Montipora monasteriata.* At Heron Island on the Great Barrier Reef of Australia, *M. monasteriata* is found at a depth of 3–5 m in a variety of external light environments ranging from out in the open (maximum irradiance, $E_{max} < 1000$ μmol quanta m^{-2} s^{-1}) to the shaded mouths of caves ($E_{max} < 600$ μmol quanta m^{-2} s^{-1}). In the open, *M. monasteriata* occurs as a range of color morphs inclusive of a purple and a brown morph. In caves, *M. monasteriata* takes on a brown or red-brown coloration (Anthony and Hoegh-Guldberg 2003). The experiment was conducted under a thick cloud and provided a light regime very similar to that reported by Anthony and Hoegh-Guldberg (2003) for cave *M. monasteriata.* The open morphs were therefore subjected to heat under lower than previous daily photon flux densities (PFD), with the cave morph being subjected to heat under similar PFD profile. The central question of the study was to do a close inspection of all of the pigments involved and to provide any clues as to how heat stress rapidly develops into chronic photoinhibition.

Seasonally, corals lose up to 50% of their areal-dinoflagellate densities (Warner et al. 2002). This response appears to co-occur with reductions in host tissue dry weight and/or host protein concentration and has been attributed to a reduction in dinoflagellates carrying capacity by the host (Fitt et al. 2000); seasonal shifts in light; temperature (Fitt et al. 2000; Warner et al. 2002); and even nutrient availability (Fagonee et al. 1999). However, corals tend to reduce chlorophyll (Chl) *a* densities in response to changes in PFD (achieved experimentally or as a result of transplantation) by reducing dinoflagellate Chl concentration as opposed to altering areal dinoflagellate densities (Falkowski and Dubinsky 1981; Hoegh-Guldberg & Smith 1989; Fitt and Cook 2001). This loss of pigmentation is usually attributed to an overhaul to the structure of the dinoflagellate light-harvesting complexes analogous to the photo-acclimation response observed in all photosynthetic organisms as they gently photo-acclimate to a change in photon flux (photo-adaptation; Iglesias-Prieto and Trench 1997).

Algae and plants that are deficient in carotenoids (e.g., mutated to disable carotenoid biosynthesis) undergo rapid photobleaching when they experience increases in PFD that can lead to plant mortality (Niyogi 1999). This rapid photobleaching is typically attributed to an excessive build-up of singlet oxygen ($^1O_2$) within the light-harvesting antennae due to the extended life of singlet-excited state chlorophyll (^{1}Chl) that are unable to immediately dissipate energy to similarly energized neighbors (Telfer et al. 1994; Vicenti et al. 1995). Bleaching in this case is thought to follow from the self-destruction of the antennae as $^1O_2$ interacts with the histidine ligands that bind chlorophyll to protein, allowing proteolysis of apoproteins (Adamski et al. 1993; Thomas 1997). The interaction of Chl with $^1O_2$ results in the formation of chlorophyll allomers that can be chromatographically quantitated (Hynninen 1991; Wang et al. 2000; Zapata et al. 2000).

An increase in PFD results not only in an increase in xanthophyll cycling pigments in particular and carotenoids in general (Montané et al. 1998), but also increases the conversion rate of this pool to de-epoxidated forms of zeaxan-

thin in higher plants and algae (Hagar and Stansky 1970) and diatoxanthin (Dt) in dinoflagellates (Ambarsari et al. 1997; Brown et al. 1999). A conversion that is controlled by ΔpH across the thylakoid membrane generated as a result of a photochemical flow of electrons between the donor site of photosystem II (PSII) and the acceptor site of photosystem I (PSI) supported by either direct reduction of O_2 in the Mehler pathway (Neubauer and Yamamoto 1992) or by cyclic electron flow through PSI (Munekage et al. 2004). These and other membrane-bound carotenoids perform essential functions, quenching ^{1}Chl, ^{3}Chl, and $^1O_2$. They have also been found to inhibit to some degree lipid peroxidation (Havaux and Niyogi 1999) and stabilize membranes (Tardy and Havaux 1997). Although these latter roles are more usually associated with α-tocopherols (vitamin E) that are not only able to move freely within the membrane, but are also able to scavenge O_2^- and OH in addition to $^1O_2$ (Havaux and Niyogi 1999; Nigoyi 1999).

A variety of mechanisms for bleaching in response to thermal stress have been offered. At relatively elevated temperatures (32–34°C), host cells appear to detach from the mesoglea, leading not only to a loss of the endodermal cell but also of the resident dinoflagellates (Gates et al. 1992). Bleaching at lower temperatures (30–34°C) has predominantly been attributed to the expulsion of the photosynthetically damaged dinoflagellates from intact host cells (Iglesias-Prieto et al. 1992; Fitt et al. 2001). The susceptibility of photosynthesis to thermal stress explains the compounding effect of light on bleaching observed by many researchers (Iglesias-Prieto 1997; Jones et al. 1998). Aspects of the molecular mechanisms that result in increased photosynthetic inhibition and damage to the resident photoautotrophs are a subject of much debate. Some of these explanations place the initial target of thermal stress firmly within the dinoflagellate. In line with this suggestion are studies that support the notions that bleaching is initiated with a thermal disruption to dinoflagellate thylakoid membrane integrity (Iglesias-Prieto et al. 1992; Tchernov et al. 2004); that the primary site of thermal damage is located around PSII (Iglesias-Prieto 1997; Warner et al. 1999); or that damage to PSII is secondary to damage that occurs downstream within the dark reactions of photosynthesis (Jones et al. 1998). A minority of researchers are open to the suggestion that the primary target may lie outside the photosynthesizing endosymbiont, either associated with necrotic host cells limiting the supply of key photosynthetic cofactors (e.g., Ca^{2+}; Bumann and Oesterhelt 1995); substrates (e.g., CO_2; Dunn et al. 2002); or heat-associated disruptions to host pigment leading to an elevation in symbiont illumination and/or a loss of any photoprotective functions provided by these host pigments (Dove 2004).

A detailed analysis of the fate of holosymbiont pigmentation after a short-term heat stress can assist in sorting among these diverse mechanisms presented in the literature. Bleaching resulting from a loss of Chl, but not from a loss of dinoflagellate cells, suggests that it is not, at least initially, driven by a loss of host endodermal cells. The existence of a proportionate increase in the de-epoxidated form of xanthophyll despite significant visual paling would suggest that bleaching did not follow from a total loss of membrane in-

tegrity. Based on an analogy with unicellular algae, thermal effects that result in an increase in excitation pressure at PSII should seemingly be accompanied by an increase, rather than a decrease, in the xanthophylls pool. A significant increase in Chl allomerization is suggestive of photo-oxidation by the interaction of Chl with $^1O_2$ and other reactive oxygen species. Finally, we can investigate the stability of host pigments under thermal stress.

Materials and methods

Collection of samples—Fragments from *M. monasteriata* colonies were collected from the spur-and-groove reef environment (Wistari Reef, GBR, Australia) described by Anthony and Hoegh-Guldberg (2003). In April 2003, purple-high light (HL) (purple) and brown-HL (brown) morphs were collected from open habitats that were free from shading by the walls of the grooves (defined as ''open habitats'' by Anthony and Hoegh-Guldberg, 2003); brown low light (LL) (cave) morphs were collected from under the edge of the shelf-like overhangs formed by the groove walls (defined as ''overhangs'' by Anthony and Hoegh-Guldberg 2003). Three fragments from distinct colonies were collected from each morph. Fragments were cut into 2×1 cm rectangles and left to recover in sheltered aquaria under running sea water for 3 days.

Experimental design—The experiment was undertaken in the open at Heron Research Station (southern GBR, 23°33′S, 151°54′E, a 10-min boat trip from the site at which specimens were collected) during a week of dense cloud in the tanks described by Dove (2004). Light levels were monitored with an ODYSSEY PAR cosine recorder (DATA-FLOW Systems Pty Ltd.), did not exceed 630 μmol quanta m^{-2} s^{-1}, and averaged 60 μmol quanta m^{-2} s^{-1} over the experimental period. Equal numbers of fragments from each morph were randomly assigned to either a control or a heated temperature treatment. The water heater was turned on at 07:00 h the following day, attained 32°C by 10:00 h, and held at that temperature until 16:00 h. Chlorophyll fluorescence measurements were taken 2 h after sunset. Five fragments from each morph and treatment were frozen at −20°C for subsequent dinoflagellate density, protein content, and dinoflagellate subclade analyses. Three fragments from each morph were divided into three equal portions and placed in liquid nitrogen followed by storage at −70°C in preparation for pigment analysis.

Chlorophyll fluorescence measurements—Measurements were taken using a DIVING-PAM (pulse amplitude modulated) underwater fluorometer (WALZ) fitted with a blue LED measuring light. Determination of surface area, total soluble protein concentrations, and dinoflagellate densities was made. The surface area was calculated using the software MATROX Inspector 2.1 (Build 17) from a digital photograph of the sample.

Samples were waterpiked with seawater and homogenized prior to centrifugation at $4000 \times$ g for 5 min. The supernatant was transferred to a clean tube and absorbance values were determined at 280 and 235 nm in a SHIMATZU UV 2450 spectrophotometer. Protein concentration was estimated using the equation proposed by Whitaker and Granum (1980).

The number of dinoflagellates was estimated by counting six independent subsamples of the dinoflagellate pellet resuspended in seawater. Samples were diluted or concentrated to maintain between 20 and 60 cells in the field of view. A digital camera (model 3.2.0, Diagnostic, Inc.) attached to a fluorescent microscope (model BX 41, Olympus) was use to take photograph subsamples. The software MATROX Inspector 2.1 (Build 17) was used to count the number of cells present in each subsample. The count excluded dead cells (A. Lawton pers. comm.).

Analysis of pigmentation— Dinoflagellate (and endolithic) pigment extraction: Frozen whole coral samples (stored at −70°C) were crushed in 90% methanol using a mortar and pestle. The solution was sonicated in iced water for 5 min, then centrifuged at $4000 \times$ g for 3 min. Supernatant was collected and transferred to a clean tube. This protocol was repeated three times to ensure a complete extraction. Next, 0.5 mL aliquots were immediately placed in a vacuum dryer at 25°C and stored at −70°C.

Preparation of standards: Diadinoxanthin from bacillariophyceae, peridinin from dinophyceae, diatoxanthin from bacillariophyceae, β-carotene from Cyanophyceae, Chl c_2 from Cryptophyceae, Chl a from Cyanophyceae, and fucoxanthin from bacillariophyceae were obtained from DHI Water and Environment (Denmark). Standards were resuspended in 90% methanol and placed immediately in a vacuum dryer at 25°C prior to storage at −70°C.

Dinoflagellate (and endolithic) pigment separation: Pigments were separated with a Shimadzu SCL-10 HPLC linked to a Shimadzu SPD-M10A photodiode array detector using the column and method proposed by Zapata et al. (2000). Pigment separation was achieved using solution A (methanol : acetonitrile : aquose pyridine) and solution B1 (methanol : acetonitrile : acetone solution) options (Zapata et al. 2000). Dried samples and standards were resuspended in 60% methanol with 0.2 mL of water added to 1 mL of the resuspended sample immediately prior to injection (Zapata et al. 2000).

Extraction and separation of (host) water-soluble pigments: Samples were air-brushed in 5 mL of 0.06 mol L^{-1} potassium phosphate pH 6.65 and centrifuged at $4000 \times$ g for 5 min to remove the dinoflagellate pellet. Protein concentration of the supernatant was determined as before using the method of Whitaker and Granum (1980). Pigments were separated by gel filtration chromatography as described by Dove (2004) on the Shimadzu system described above.

Dinoflagellate identification—Dinoflagellate DNA was prepared and typed by denaturing-gradient gel electrophoresis (DGGE) using the methods described in LaJeunesse et al. (2003). Profiles of the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal RNA genes obtained from spec-
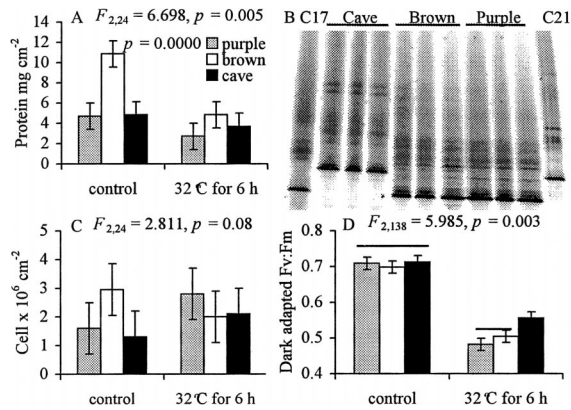
Fig. 1. (A) Summary of results obtained for two-factorial AN-OVA and the interactive effect of morph and temperature on the water-soluble protein concentration of host homogenates normalized to coral surface area. *p*-values for SNK post hoc analyses distinguishing brown-HL control from other groups is shown. (B) DGGE profile comparison of dinoflagellates residing in distinct morphs to standard C17 and C21 dinoflagellate subclades. (C) Interactive effect of morph and temperature on symbiotic dinoflagellate density normalized to surface area. (D) Interactive effect of morph and temperature on dark-adapted Fv:Fm. Lines link groups that do not significantly differ from each other in an SNK analysis. Error bars represent 95% confidence intervals. *Cave,* brown-LL; *brown,* brown-HL; *purple,* purple-HL.

imens were compared with the profiles of standards generated by T. LaJeunesse (University of Georgia).

Statistical analysis of the data—Data were analyzed in STATISTICA 6.0 (Statsoft Inc.) using univariate and multivariate 2 factorial analysis of variance (ANOVA). Assumptions were tested using the Cochran C test. The Student Newman-Keuls (SNK) test was used for post hoc comparisons of group means. In general, we have adopted the protocol of reporting summary statistics for ANOVA with significant main effects in the figures, with SNK statistics reported in the text. Summary statistics for ANOVA with nonsignificant main effects are reported in the text.

Results

M. monasteriata held at 32°C for 6 h showed no reductions in dinoflagellate densities with the application of heat and no significant differences in dinoflagellate densities between morphs (morph, $F_{2,24} = 2.97$, $p = 0.07$; temperature, $F_{1,24} = 0.42$, $p = 0.52$; Fig. 1C). Total animal soluble protein in brown-HL controls only was significantly reduced by heating (SNK, $p = 0.000$; Fig. 1A). The relatively high protein content in brown-HL controls drove the significant interaction observed between temperature and morph (Fig. 1A). The maximum quantum yield of PSII (dark-adapted Fv:Fm) gave a significant effect for temperature, with all morphs from the heated treatment experiencing significant chronic photoinhibition (SNK, control >0.0003 heated; Fig. 1D). There was also a significant interaction between morphs and temperature, with the cave (brown-LL) morph showing lower reductions in Fv:Fm than the open-dwelling brown
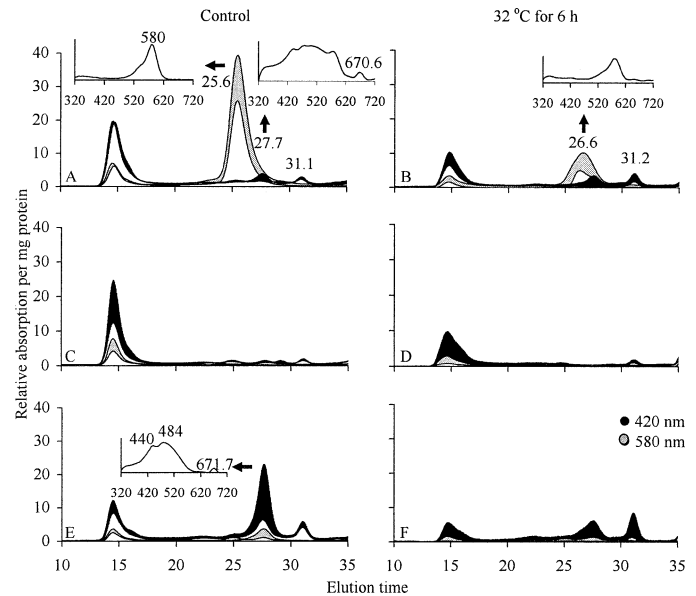

Fig. 2. Gel filtration chromatograms of water-soluble homogenate monitored at 420 and 580 nm. Line width corresponds to mean (±95% confidence interval) absorption normalized to total soluble protein concentration. Arrows point to inset absorption spectra determined by photodiode array at elution times identified for chromatogram peaks. (A,B) Control and heated purple-HL, open-dwelling morph, respectively. (C,D) Control and heated brown-HL, open-dwelling morph, respectively. (E,F) Control and heated, cave-dwelling (brown-LL) morph, respectively.

and purple morphs (SNK, cave-heated >0.0003, brown-HL heated = purple-HL heated; Fig. 1D). Significantly, the cave-dwelling morph was dominated by a different dinoflagellate subclade, C21, to that of the open-dwelling brown and purple morphs. The open-dwelling brown-HL and purple-HL morphs were dominated by dinoflagellate subclade C17 (Fig. 1B). Short-term heating led to a visual change in the coloration of all morphs (data not shown).

The open-dwelling purple, open-dwelling brown, and cave-dwelling brown morphs were distinguishable by distinct gel filtration chromatogram profiles of host homogenates determined at 420 and 580 nm. Chromatograms were normalized to total protein to investigate whether loss of host pigmentation attributable to GFP homologues was occurring at a greater rate than that of observed total protein reductions. Fractions containing pigments absorbing at 420 and 580 nm eluted in the void volume at 25.6, 26.6, 27.7, and 31.1 min. The 25.6 and 26.6 min fractions correspond to distinct oligomeric states of GFP homologues (Dove et al. 2001). Spectra at each elution time point as determined by photodiode array match the profile of pocilloporins (Dove et al. 1995; Dove et al. 2002). The 27.7 min peak is spectrally identical to peridinin-Chl *a* protein (PCP; http//www.prozyme.com/technical/spectra/percp.html). In the purple-HL morph, this PCP peak is contaminated by the tail end of the dominant pocilloporin peak and absorbs maximally in the red at 670.6 nm. In the brown-LL morph this maxima occurs at 671.7 nm (Fig. 2).

A temperature of 32°C for 6 h led to a decrease in pocil-

Table 1. Multivariate Wilk's lambda two-factorial analysis of variance (ANOVA) testing the effect of elevated temperature (temp) and morph on PCP and pocilloporin pigmentation determined by the area under 580 and 420 nm gel filtration chromatograms.

Factor	Value	F	Effect (df)	Error (df)	p	Post hoc (SNK)
Temp	0.297	13.033	2	11	0.00125	PCP: control > 0.007 heated; Pocilloporin: control > 0.0006 heated
Morph	0.044	20.666	4	22	0.00000	PCP: cave > 0.03 others; Pocilloporin: purple > 0.0002 others; PCP: control cave > 0.015 others
Temp × morph	0.185	7.277	4	22	0.00068	Pocilloporin: control purple > 0.0002 others

loporin absorbance (measured as the area under 580 nm chromatogram between 20 and 35 min) per milligram protein for purple-HL morphs (Table 1) and also included a shift toward smaller oligomeric forms of the protein (from 25.6 to 26.6 min; Fig. 2). Elevated temperature also led to a decrease in PCP absorbance (measured as area under 420 nm chromatogram between 20 and 35 min) per milligram protein for the cave morph (Table 1). Purple-HL controls contained more pocilloporin than brown-LL or brown-HL morphs (Table 1), and the water-soluble homogenates of cave controls contained more PCP than all other groups (Table 1).

A complex profile of methanol-soluble pigments from tissue and skeletal material were separated by C8 reverse-phase chromatography. Pigments were identified by comparison to co-eluted standards and/or by photodiode array spectra. Pigments identified included Chl a, b, and c_2; xanthophylls cycling pigments diadinoxanthin; diatoxanthin, the dinoflagellate-specific pigment peridinin; and the siphonous green algae–specific pigment siphonein (Fig. 3). Chl a resulting from both dinoflagellates and endolithic algae was distinguished into three peaks: Chl a allomer, Chl a, and Chl a epimer. Each of these three peaks had characteristic Chl a spectra and was named according to the elution sequence observed in Zapata et al. (2000). A Chl a standard treated similarly to the biological samples eluted as a single peak (data not shown).

Chl c_2 and peridinin measured as peak area normalized to surface are at significantly lower concentrations in corals heated to 32°C in comparison with controls maintained at ambient temperature (Fig. 4B, D). Peridinin concentrations were greatest in the cave-dwelling morph (SNK, p = 0.003; Fig. 4A). In the case of peridinin, there was no interactive

effect between temperature and color morph (temperature × morph, $F_{2, 12}$ = 0.81, p = 0.47). There was, however, a significant interaction between temperature and morph for Chl c_2 (Fig. 4C). This interaction was due to a greater concentration of Chl c_2 in cave-dwelling control corals (SNK, p = 0.000; Fig. 4C) with no difference among cave-dwelling heated corals and purple or brown corals from either the controls or heated treatment.

Chl b and siphonein are pigments associated with coral endolithic algae, rather than dinoflagellates (Highsmith 1981). When normalized to surface area, neither of these pigments was affected by temperature (Chl b, $F_{1,1}$ = 1.13, p = 0.31; siphonein, $F_{1,12}$ = 0.15, p = 0.71); morph (Chl b, $F_{2,12}$ = 0.70, p = 0.52; siphonein, $F_{2,12}$ = 0.72, p = 0.51); or the interactive effect of these factors (Chl b, $F_{2,12}$ = 0.25, p = 0.78; siphonein, $F_{2,12}$ = 0.14, p = 0.87). Based on these results we feel justified in assuming that endolithic algae provide a morph and temperature invariant background con-
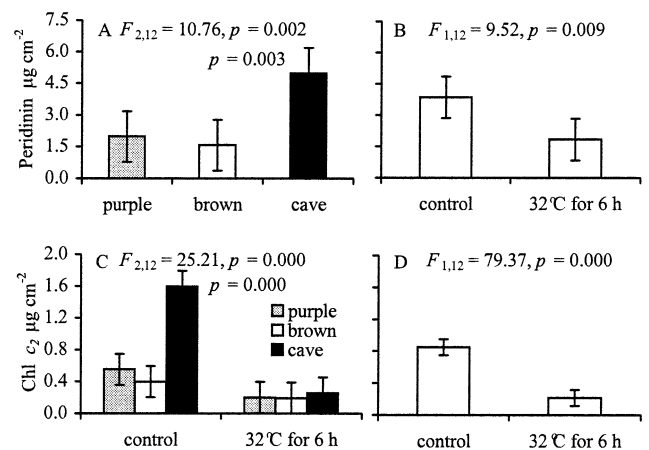
Fig. 4. Summary of significant results obtained for a two-factorial ANOVA determining the effects of morph and temperature treatment on dinoflagellates peridinin and Chl c_2 concentrations normalized to surface area. (A) Effect of morph on peridinin concentration (p-values for SNK post hoc analyses distinguishing cave from other morphs is shown). (B) Effect of temperature on peridinin concentration. (C) Effect of the interaction between morph and temperature on Chl c_2 content (p-values for SNK post hoc analyses distinguishing cave control from all other groups of morphs is shown). (D) Effect of temperature on Chl c_2 content. Error bars represent 95% confidence intervals. *Cave,* brown-LL; *brown,* brown-HL; *purple,* purple-HL.
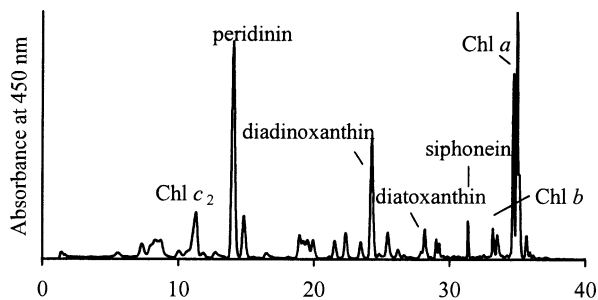
Fig. 3. Typical C-8 elution profile monitored at 450 nm for methanol-soluble pigments extracted from whole coral fragments using the methodology of Zapata et al. (2000).
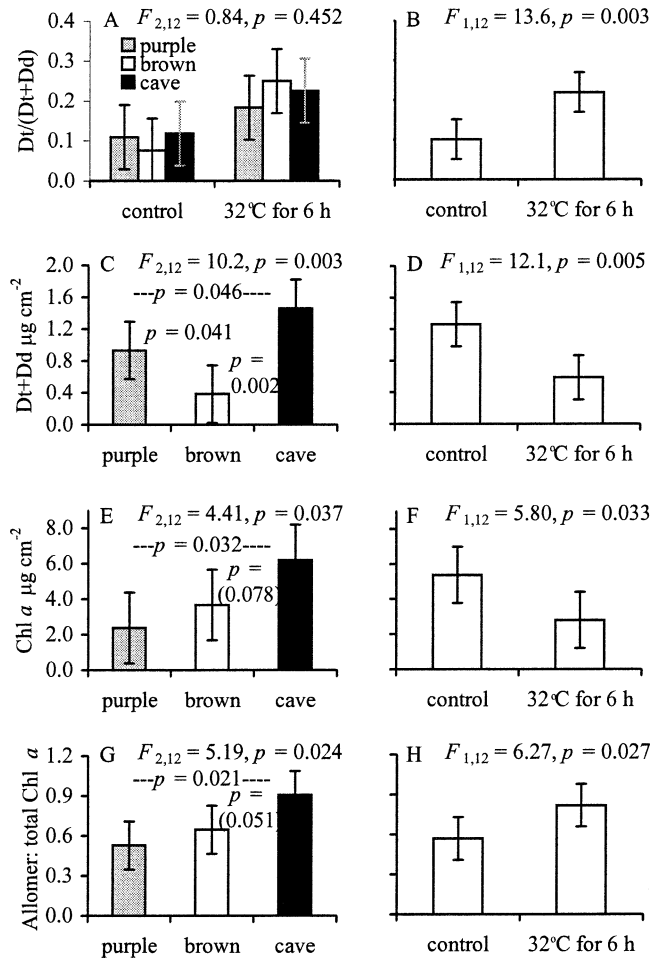
Fig. 5. (A–D) Summary results obtained for a two-factorial ANOVA determining the effects of morph and temperature treatment on dinoflagellates xanthophyll content and (E–H) on algal Chl *a* content. Xanthophyll cycling Dt (Dd + Dt) was not significantly affected by morph or by the interaction of (A) morph and temperature, but was significantly defected by (B) temperature. Dd + Dt normalized to surface area gave no interactive effect, but was significantly affected (C) by morph and (D) by temperature. Total Chl *a* content normalized to surface area gave no interactive effect, but was significantly affected by (E) morph and (F) temperature. The ratio of allomerised Chl *a* to total Chl *a* gave no interactive effect, but was significantly affected by (G) morph and (H) temperature. SNK *p*-values distinguishing morphs are shown; bracketed values are not significant. Error bars represent 95% confidence intervals. *Cave,* brown-LL; *brown,* brown-HL; *purple,* purple-HL.

tamination to dinoflagellate Chl *a* measurements. Changes in Chl *a* are therefore attributed to dinoflagellates.

Diadinoxanthin (Dd) is de-epoxidated to become diatoxanthin (Dt) in dinoflagellates. Diatoxanthin quenches excitation energy within the antenna of PSII. The dinoflagellates in the cave-dwelling morph had a significantly greater xanthophyll pool (Dd + Dt) than those in the purple-HL morph (SNK $p = 0.046$; Fig. 5C). Similarly, dinoflagellates in the purple-HL morph had more Dd + Dt than the brown-HL morph (SNK, $p = 0.041$; Fig. 5C). Heating corals to 32°C for 6 h resulted in a significant decrease in the concentration of Dd + Dt (Fig. 5D), and the rate of decrease across all

morphs was constant as there was no significant interaction between treatment and morph (temperature × morph: $F_{2,12} = 0.60$, $p = 0.57$). Although the pool of xanthophylls decreased with heat, a higher proportion of the remaining pool was in the de-epoxidated form of diatoxanthin (Fig. 5B). No significant interaction between morph and temperature was observed (temperature × morph, $F_{2,12} = 0.84$, $p = 0.47$). The brown-HL morph, however, had the lowest mean proportion of diatoxanthin in the control and the highest mean proportion of this pigment in the heated treatment (Fig. 5A).

Total chlorophyll was determined by adding the area under chromatogram peaks for Chl *a* allomer, Chl *a,* and Chl *a* epimer. The cave-dwelling morph was found to have more total Chl *a* than the purple-HL morph (SNK, $p = 0.021$; Fig. 5E). The brown-HL morph contained an intermediate, but not significantly distinct, concentration of total Chl *a* (Fig. 5E). Again, temperature was found to negatively effect the concentration of total Chl *a* (Fig. 5F), and the lack of interaction between temperature and morph suggested that the rate of decrease was constant across all morphs (temperature × morph, $F_{2,12} = 1.82$, $p = 0.20$). The proportion of Chl *a* allomer to total Chl *a* was also analyzed. A significantly greater proportion of Chl *a* allomer was extracted from corals held at an elevated temperature (Fig. 5H). Again the cave-dwelling morph contained significantly more of this oxidized form of Chl *a* than the purple-HL morph (SNK, $p = 0.021$; Fig. 5G), with the brown-HL morph containing an intermediate, but not significantly different, concentration of Chl *a* allomer (Fig. 5G).

The relative size of the xanthophylls pool was less for the heated treatment than for controls (32°C, [Dt + Dd]: Chl $a^{-1} = 0.21$; control, [Dt + Dd]: Chl $a^{-1} = 0.23$). These results show that there was no increase in the relative xanthophyll pool in response to a short-term temperature stress. The relative size of the xanthophyll pool was greater for the purple-HL morph (= 0.39) than for the brown-LL morph (= 0.24); the brown-HL morph (= 0.10) had the lowest xanthophyll pool size relative to Chl *a.* Suggesting that Dt + Dd normalized to Chl *a* did not relate to the light habitat from which the corals were obtained. Interactive relative xanthophyll pool means were not compared as there was no interactive effect for either Dt + Dd or Chl *a* concentrations.

Discussion

Why did Montipora monasteriata look bleached after heating to 32°C for 6 h?—More often than not, bleaching is reported and discussed only in terms of an actual loss of dinoflagellates (e.g., Coles and Brown 2003; Tchernov et al. 2004). This has led many studies to assume that visible losses of pigmentation in corals are necessarily caused by a loss of resident dinoflagellates (e.g., Baker 2001). The sometimes untested assumption is then used to spearhead the argument that bleaching is required to open up residential space within the coral house for occupation by a "new" dinoflagellate population (Buddermeir and Fautin 1993; Baker 2001). In this study, a reduction in chlorophyll concentration associated with heat stress was observed across all morphs under relatively low external light intensities (<650 μmol quanta

m^{-2} s^{-1}, but averaging only 50 μmol quanta m^{-2} s^{-1}) in the absence of significant dinoflagellate loss. Decreases in Chl *a* concentrations without dinoflagellate reductions have previously been reported by a number of studies investigating the effects of increased photon flux (e.g., Falkowski and Dubinsky 1981; Hoegh-Guldberg and Smith 1989). Experimental studies on thermal bleaching have tended to investigate the effect of heat on dinoflagellate populations; Chl fluorescence parameters; and only occasionally Chl concentrations, mostly Chl *a* as opposed to Chl c_2, and never carotenoids. The results of these studies are varied, even for so-called bleaching sensitive corals, with some reporting 50% or greater losses of dinoflagellates for visibly bleached corals (e.g., Hoegh-Guldberg and Smith 1989, after 4 d of heating to 32°C) and others reporting losses of Chl *a* as opposed to dinoflagellates (e.g., Takahashi et al. 2004; after 12 h of heating to 32 °C). These experiments, for the most part, are performed with different coral species, differently pigmented morph and under different light conditions. Takahashi et al. (2004) results were obtained under a light regime that was unnatural, at a constant 1000 μmol quanta m^{-2} s^{-1} PAR for 12 h, even for shallow-water corals (1–3 m). The data presented in this study, however, show that loss of Chl *a* without loss of symbionts also occurs when corals are subjected to elevated temperature in conjunction with PAR regimes equivalent to or less than the PFD experienced in their natural habitat.

In higher plants, the major light-harvesting Chl proteins of PSII (LHCP II) and early light-inducible proteins (ELIPs), a subgroup of LHCPs that bind xanthophylls-cycle carotenoids in addition to Chl, have a reciprocal response to changes in the excitation pressure on PSII (Krol et al. 1995). ELIP mRNAs are expressed within 2 h under increased PFD (Krol et al. 1995), with increases in ELIP protein noted within a single day (Montané et al. 1998). The rapid response is partially owed to ELIPs scavenging components from degrading LHCP II. In the present study, Chl loss (Chl *a* and Chl c_2) does not appear to be a rapid acclimation to an increase in PSII excitation pressure because the abundance of xanthophyll-cyclic carotenoids were simultaneously reduced by the application of heat. Dinoflagellates are photosynthetically unique insofar as their light-harvesting antennae is formed from water-soluble PCPs and Chl *a*–Chl c_2–peridinin-protein complexes (acpPCs, or *i*PCPs). The peripheral antennae (PCP) are unrelated to the family of known LHCP. The membrane-bound acpPCs, on the other hand, are related to the LHCP family of proteins (Green and Durnford 1996). Iglesias-Prieto and Trench (1997) demonstrated that some, but not all, cultured dinoflagellates belonging to the genus *Symbiodinium* are able to respond to increases in PFD by increasing their xanthophylls pool. This ability is thought to be associated with a differential ability to express appropriate acpPC genes (Iglesias-Prieto and Trench 1997).

M. monasteriata look bleached after heating to 32°C for 6 h because they have lost 50% or more of their total chlorophyll, peridinin, and xanthophyll pools. The reduction of the xanthophyll pool is of particular interest as it does not conform to typical photoacclimatory behavior among photosynthezing organisms, inclusive of some *Symbiodinium* spp. Is the lack of a relative increase in xanthophylls pool

due to (1) temperature stress providing an inappropriate signal for photo-acclimation; (2) the presence of inappropriate dinoflagellates; and/or (3) the photosynthetic units of these dinoflagellates undergoing rampant photo-oxidative damage after 6 h of heat?

Does a temperature–initiated excitation pressure on PSII provide an inappropriate signal for xanthophylls accumulation?—Excitation pressure on PSII due to elevated thermal stress can, as discussed in the introduction, eventuate for a number of different reasons other than increasing PFD. Excitation pressure on PSII will increase as a result of interference in electron transport and limited access to final electron transport acceptors. In unicellular algae a non-PFD–driven increase in excitation pressure is capable of stimulating structural charges to the antennae (Maxwell et al. 1995). Studies have shown, however, that for cultured unicellular *Symbiodinium* spp., tolerance to high PFD does not automatically confer tolerance to high temperature (e.g., *Symbiodinium microadriaticum* from the Scyphozoa, *Cassiopeia* spp.; Iglesias-Prieto et al. 1992; Iglesias-Prieto and Trench 1997). Results such as these would seem to suggest that free-living *Symbiodinium* respond distinctly to different environmental cues, unlike unicellular colleagues such as green algae from the genus *Chlorella*. Perhaps the distinction is attributable to the dominant lifestyle of each organism. In contrast to *Chlorella, Symbiodinium* spp. predominantly occur in symbiosis with a wide variety of phyla (Taylor 1973; Trench 1979). The sheer physiological complexity of living in symbiosis may drive the retention of an ability to discriminate among an array of intercellular signals (Walters 2004). The complexity of the holosymbiont is amply demonstrated by the present study where no single uniform host response to temperature was observed for either of the two parameters measured. As a result of heating to 32°C, host pigmentation in purple-HL morphs was reduced by 75%, whereas the brown-HL morph, which lacked host pigmentation, experienced a 50% reduction in host protein concentration, and the brown-LL morph that also lacked host pigmentation experienced no significant reduction in host protein. Significantly, dinoflagellates residing within the least host-affected brown-LL morph were less photoinhibited, according to dark-adapted F_v/F_m measurements, than more host-affected purple-HL and brown-HL morphs. These are counterintuitive results given the indication based on elevated control of Chl c_2 and PCP concentrations that this cave-dwelling morph was initially more shade-acclimated than either the purple-HL or the brown-LL morphs. Typically, shade-acclimated corals have lower saturation irradiances, E_k, and photosynthetic maxima, P_{max} (Falkowski and Dubinsky 1981; Anthony and Hoegh-Guldberg 2003), with the consequence that brown-LL morphs should be nearer to their excitation pressure threshold than either purple-HL or brown-HL morphs. A potential solution to the paradox driven by differential changes in tissue thickness (protein content), pocilloporin, and dinoflagellate pigmentation is that the higher algal pigmentation of the cave-dwelling morph may have contributed to reduce the radiation fields within the host tissue, decreasing the light-dose per unit Chl *a* experienced by the endosymbiotic population of these morphs in comparison with that

experienced by the algae harbored by the open-dwelling morphs (Enríquez et al. 2005). Alternatively, it may be due to the fact that the C21 *Symbiodinium* spp. found to be residing in the brown-LL morph are more heat tolerant than the C17 *Symbiodinium* spp. residing in open-dwelling morphs.

How flexible are these different morphs at handing either high PFD or elevated temperature?—In contrast to the results of this study, Anthony and Hoegh-Guldberg (2003) found no difference in the resident symbiont type—all were C17 for *M. monasteriata* collected from open or cave habitats. In Anthony and Hoegh-Guldberg (2003), C17 dinoflagellates showed a high capacity to acclimate (in 3 weeks) to downshifts or upshifts in irradiance. In cultured *Symbiodinium* spp., this flexibility to accommodate variable PFDs has been found to correlate with the ability to express both monomeric and dimeric forms of PCP; enrichment in the monomeric form occurs under high PFD and coincides with an increase in the xanthophyll pool (Iglesias-Prieto and Trench 1997). In this study, PCP from both C17 and C21 eluted with red absorption maxima at 670.6 and 671.7 nm, respectively, which is indicative of a mixing of monomeric and dimeric forms and suggests that both C17 and C21 dinoflagellates are able to flexibly acclimate to different light regimes (Iglesias-Prieto and Trench 1997). Based on this circumstantial evidence it does not seem as though either of these dinoflagellates was incapable of responding to elevated PFD by increasing their xanthophylls pool.

Recently Tchernov et al (2004) supported the view that *Symbiodinium* spp. thermostability is dictated by the polyunsaturated fatty acid composition of the thylakoid membrane (Iglesias-Prieto et al. 1992; Tchernov 2004). In this context, sensitivity to thermal bleaching was based on the predisposition of corals to shed dinoflagellate cells. Corals containing sensitive dinoflagellates shed their symbionts after only 3 days at 32°C, whereas bleaching insensitive species retain symbionts for greater than 2 months at this temperature. Based on a comparison of cultured thermally tolerant and intolerant *Symbiodinium* spp., bleaching was argued not to eventuate because of excess excitation pressure on PSII, but rather as the result of a thermal meltdown in the integrity of the thylakoid membrane that is then unable to form the necessary proton gradient to drive both assimilatory photochemical quenching and nonphotochemical quenching, thereby forcing excitation energy through the water-water cycle and risking the generation of super oxide anions (O_2^-) and hydroxyl radicals (OH). According to the model presented, it is the leaking of these reactive oxygen species to the host that leads to their expulsion (Tchernov et al. 2004). This nonphotoinhibition model of bleaching is very elegant and attractive insofar as it efficiently captures the notion that dinoflagellates can handle high PFD but fail to handle elevated temperature. However, its consistency with other studies conducted on thermally sensitive *Symbiodinium* cultures at 32°C under similar PFD is questionable. For example, Iglesias et al. (1992) noted a subtle decrease in the rate of electron transfer between the Q_A and Q_B, and hence increase in excitation pressure at PSII after just 45 min at 32°C for *S. microadriaticum*. Warner et al.

(1999) noted increasing nonphotochemical quenching for a cultured, thermally sensitive dinoflagellate 7 d into heating to 32°C. This latter study is particularly significant because the Tchernov et al. (2004) Chl fluorescence data, upon which the conclusion was based, was also collected 7 d into heating.

The significant visual paling observed to occur 6 h into heating to 32°C in this study falls outside the definition for bleaching assumed by Tchernov et al. (2004). In the present study, the visual paling observed mirrors the rapid photobleaching observed to occur in algae and plants that are exposed to increased PFD but deficient in carotenoids (Niyogi 1999). The increase in Chl *a* allomerization witnessed for thermally stressed corals is consistent with a photoinhibition model of thermal bleaching where antennal Chl is unable to pass on excitation energy to similarly energized neighbors. Allomerization of Chl *a* occurs when the enolate anion of Chl *a* is oxidized by free radicals (Hynninen 1991). In vitro, it is inhibited by the presence of β-carotene (Hynninen 1991), which is believed to primarily scavenge $^1O_2$ (Bumann and Oesterhelt 1995). Although alternative models for allomerization have been presented, it is thought to be principally driven by $^1O_2$ as opposed to other reactive oxygen species. Also consistent with a photoinhibition model of thermal bleaching is the relative increase in nonphotochemical quenching observed as an increase in Dt/(Dd + Dt) for the heated treatment. Increased ΔpH across the thylakoid membrane is required for the stimulation of Dd de-epoxidase activity and a down-regulation of Dt epoxidase activity. Increases in Dt/(Dd + Dt) cannot occur over a leaky thylakoid membrane (Mewes and Richter 2002). In higher plants, it has been demonstrated that zeaxanthin, the analog of dinoflagellate Dt, incorporates into the lipid bilayer, stabilizing increased thylakoid membrane fluidity induced by thermal stress (Havaux and Tardy 1996). An active xanthophyll cycle therefore performs at least two essential functions under heat stress: a nonphotochemical quenching and a membrane stabilization role.

However, membranes can also be destabilized through the action of reactive oxygen species: $^1O_2$, H_2O_2, and OH radicals. Liu et al. (2004) have recently argued that under high PFD, O_2^- is generated at the reaction center of PSII defensively in preference to the more harmful $^1O_2$. This is a high-risk defense mechanisms that in the presence of an inadequate antioxidant system can lead to the formation of H_2O_2 and OH radicals and a subsequent peroxidation of membrane lipids.

Under our experimental conditions, thermally induced bleaching (loss of pigmentation) in *M. monasteriata* clearly preceded a total loss of thylakoid membrane integrity and clearly occurred as the result of an increase in the excitation pressure on PSII. The lack of an increase in the relative size of the xanthophylls pool may have played a significant additional role in the observed photo-oxidation of the light-harvesting antennae; also, via a loss of their stabilizing role, may eventually have led to thylakoid membrane meltdown. The heating experiment was terminated at 6 h, and therefore we have no idea as to whether bleaching (loss of dinoflagellates) would eventuate for any of the experimental morphs under investigation. Typically, loss of dinoflagellates

occurs 3–4 days after the onset of thermal stress (Hoegh-Guldberg and Smith 1989; Tchernov et al. 2004). Enríquez et al. (2005) have recently demonstrated that significant loss of Chl can lead to an exponential increase in the light environment within coral tissue because of the high reflectivity of coral skeleton. Under such a scenario, an efficient capacity to photo-acclimate to high PFD, as well as high thermal tolerance, may be required of resident dinoflagellates.

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