PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2008) **275**, 2273–2282
doi:10.1098/rspb.2008.0180
Published online 24 June 2008

Species–specific interactions between algal endosymbionts and coral hosts define their bleaching response to heat and light stress

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The impacts of warming seas on the frequency and severity of bleaching events are well documented, but the potential for different *Symbiodinium* types to enhance the physiological tolerance of reef corals is not well understood. Here we compare the functionality and physiological properties of juvenile corals when experimentally infected with one of two homologous *Symbiodinium* types and exposed to combined heat and light stress. A suite of physiological indicators including chlorophyll *a* fluorescence, oxygen production and respiration, as well as pigment concentration consistently demonstrated lower metabolic costs and enhanced physiological tolerance of *Acropora tenuis* juveniles when hosting *Symbiodinium* type C1 compared with type D. In other studies, the same D-type has been shown to confer higher thermal tolerance than both C2 in adults and C1 in juveniles of the closely related species *Acropora millepora*. Our results challenge speculations that associations with type D are universally most robust to thermal stress. Although the heat tolerance of corals may be contingent on the *Symbiodinium* strain *in hospite*, our results highlight the complexity of interactions between symbiotic partners and a potential role for host factors in determining the physiological performance of reef corals.

Keywords: *Symbiodinium*; coral bleaching; heat stress; photosystem II; oxygen consumption and respiration; xanthophylls

1. INTRODUCTION

The obligate symbiosis between reef-building corals and dinoflagellates of the genus *Symbiodinium* has been fundamental to the evolution of reef corals. However, over the last few decades, this relationship has been disrupted on global scales by mass bleaching events, which render corals white through the loss of symbionts or pigments within them. The main triggers for these events are elevated sea surface temperatures acting synergistically with high irradiance levels (Brown 1997; Fitt *et al.* 2001; Lesser & Farrell 2004). Predicted increases in the frequency and severity of anomalously warm summers present a significant threat to coral reefs worldwide and to the goods and services they provide (Hoegh-Guldberg 1999; Hughes *et al.* 2003).

Recent studies demonstrating high genetic diversity within the genus *Symbiodinium* raise new possibilities regarding their potential role in the resilience of reef corals to climate stress. The genus consists of eight lineages or clades (A–H), each of which comprises multiple types (Baker 2003; Coffroth & Santos 2005). Although some coral colonies appear to harbour only a single symbiont type (Goulet 2006), others harbour two or more types simultaneously (Rowan & Knowlton 1995; Ulstrup & van Oppen 2003), which may include a dominant type and background levels of other types (Mieog *et al.* 2007).

It has been proposed that corals may adapt to warmer oceans by changing their symbiotic partners for new, heat-tolerant types (Buddemeier & Fautin 1993; Baker 2001), and form novel host–symbiont combinations, either by acquiring a new symbiont type (switching) or by increasing the relative abundance of a symbiont type already present within the host (shuffling; Baker 2001). Among coral endosymbionts, clade D *Symbiodinium* has been characterized as heat- or stress- tolerant based on increased prevalence of types within this clade in Caribbean and Indo-Pacific corals after bleaching events (Glynn *et al.* 2001; Toller *et al.* 2001; Baker *et al.* 2004; van Oppen *et al.* 2005; Jones *et al.* 2008), or in corals living in reef lagoons exposed to higher temperature regimes than surrounding waters (Fabricius *et al.* 2004). However, only a few published studies have tested and compared the physiological response with heat stress among corals hosting D types versus types in other *Symbiodinium* clades. In one study (Rowan 2004), adult corals hosting clade D *Symbiodinium* had higher rates of photochemical efficiency of photosystem II (PSII) and higher ratios of maximum net photosynthesis to respiration than corals hosting clade C. In a second study (Berkelmans & van Oppen 2006), adult corals that had shuffled their dominant endosymbiont from C2 to D (ITS1 defined types) following bleaching had

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2008.0180 or via http://journals.royalsociety.org.

Received 7 February 2008
Accepted 6 June 2008

higher photochemical efficiency and higher symbiont densities than C2-dominated colonies when subsequently tested in a heat stress experiment. These studies are consistent with field observations and support the notion that the upper thermal tolerance of corals is enhanced when hosting clade D *Symbiodinium*. Nevertheless, observations of both thermally robust and thermally sensitive types within *Symbiodinium* clades caution against making clade-wide generalizations (Tchernov *et al.* 2004) and highlight the need for comparative physiological studies of types within *Symbiodinium* clades. Moreover, because clade D is relatively uncommon in Indo-Pacific corals, in contrast to the ubiquity of clade C (LaJeunesse 2001; Baker 2003; LaJeunesse *et al.* 2003), knowledge of the influence of symbiont type on holobiont physiology has important implications for understanding the impact that warming oceans may have on coral communities.

Recent studies showing that symbiont stress responses differ between freshly isolated and *in hospite* cells suggest that the host may play a significant role in regulating the response of the holobiont (host–symbiont combination) to heat/light stress (Bhagooli & Hidaka 2003; Goulet *et al.* 2005). Host-driven protective mechanisms that could contribute to regulation of the holobiont's bleaching response include the production of anti-oxidant enzymes (Lesser *et al.* 1990), mycosporine-like amino acids (Dunlap & Shick 1998) and fluorescent pigments (Salih *et al.* 2000). Greater understanding of the host–symbiont interactions that govern holobiont physiology in intact coral–algal endosymbioses would provide fresh insights into the resilience of reef-building corals.

Here we use physiological indicators to compare bleaching tolerance between corals hosting *Symbiodinium* type C1 or D to test the hypothesis that Indo-Pacific corals achieve optimal bleaching tolerance when dominated by *Symbiodinium* clade D. Hereafter we use the term 'clade' to denote the sub-generic level of *Symbiodinium* classification; the term 'type' to denote genetic types within a clade and 'C1' and 'D' to denote specific ITS1 types (*sensu* van Oppen *et al.* 2001) when discussing our study species. Contrary to expectations, we show that C1-corals have higher thermal/light tolerance than D-corals in juveniles of the common Indo-Pacific coral *Acropora tenuis*. Our results challenge the view that clade D is universally associated with thermal robustness and provide evidence that the heat/light tolerance of *Symbiodinium* types differs with host species.

2. MATERIAL AND METHODS

Three independent heat stress experiments were carried out using *A. tenuis* juveniles raised following spawning events between 2003 and 2005 at Magnetic Island (19°10′ S, 146°50′ E) in the central section of the Great Barrier Reef (GBR). This coral was selected for study because it naturally associates with types C1 and D during early ontogeny at our study site (Little *et al.* 2004), thus both types are homologous in juveniles of this species. The first experiment was a pilot study to identify appropriate temperature ranges and time-spans required to elicit a bleaching response in corals that had been experimentally inoculated with either ITS1 types C1 (GenBank accession no. AF380551) or D (GenBank accession no. EU024793) *Symbiodinium*. We refer to each association as C1- or D-corals. Two independent, full-scale stress experiments (experiments 1 and 2) were then completed to incorporate light as an additional stress factor and to evaluate the generality of the holobiont response at different juvenile ages. Experimental procedures used to develop and raise juveniles, experimentally infect them with different *Symbiodinium* types, and verification of clade type followed those of Little *et al.* (2004). Four temperatures were selected for the pilot study and experiment 1 (28, 30, 31 and 32°C) and three for experiment 2 (26, 29, and 32°C). Irradiance levels were 130 μmol photon m^{-2} s^{-1} for the pilot study, 160 and 360 μmol photon m^{-2} s^{-1} for experiment 1, and 250 μmol photon m^{-2} s^{-1} for experiment 2. Further details on experimental design and set-up for temperature and light treatments are given as supplementary material.

(a) *Bleaching condition of corals: pilot study and experiment 1*

Bleaching was quantified every other day by visual scoring of all experimental colonies. Colonies were scored as normal (normally pigmented), pale (including moderately bleached colonies), bleached (completely translucent tissue) or dead (bare skeleton in various stages of overgrowth by other organisms).

(b) *Photochemistry of heat-stressed corals*

The maximum quantum yield of PSII (F_v/F_m), a proxy for photochemical efficiency, was measured using a pulse amplitude modulated fluorometer (PAM). For the pilot study and experiment 1, we used a Mini PAM (Walz, Germany) fitted with a 2 mm diameter fibre optic probe. For experiment 2, we used an Imaging-PAM (I-PAM, Walz) that allowed us to haphazardly select three 'areas of interest' within each replicate colony using the Imaging-PAM software (ImagingWin v. 2.12a). Dark-adapted colonies were measured every morning before the lights went on.

To better characterize the physiological performance of the symbionts, we calculated the maximum excitation pressure over PSII(Q_m) as described in Iglesias-Prieto *et al.* (2004). Excitation pressure was calculated using the equation,

$$Q_m = 1 - [(\Delta F/F'_m)/(F_v/F_m)], \quad (2.1)$$

where $\Delta F/F'_m$ is the effective quantum yield of fluorescence in light-saturated conditions and F_v/F_m is the maximum quantum yield in a dark-adapted state. Excitation pressure was calculated based on a $\Delta F/F'_m$ measurement after 1 hour of exposure to lights, following observations that Q_m did not change significantly after 1, 4 and 7 hours of exposure to light in a pilot study preceding this experiment. Fluorescence measurements were taken every third day.

(c) *O_2 microelectrode characterization of photosynthesis and respiration*

To further characterize the physiological impact of heat- and light stress on the juvenile holobiont, rates of gross and net photosynthesis as well as respiration were measured for four colonies per *Symbiodinium* type per temperature during the second experiment. Measurements were performed on days 1, 8 and 15 of heating using oxygen microelectrodes (approx. 50 μm in diameter; see the electronic supplementary material for set-up details).

Gross photosynthesis rate (P_g) was determined using the light–dark shift technique in units of nmol O_2 cm^{-3} s^{-1} (Revsbech & Jorgensen 1983; see the electronic supplementary material). After attaining physiological steady state,

oxygen microprofiles were measured through the diffusion boundary layer (DBL) in darkness and at 50, 150 and 250 μmol photons $m^{-2}\,s^{-1}$. Oxygen flux measurements were then calculated from the oxygen concentration profiles as described in Kühl *et al.* (1995).

The ratio ($P_n : R_D$) of the resulting flux in the dark (R_D) and at 250 μmol photons $m^{-2}\,s^{-1}$ (P_n) was calculated as a measure of metabolic cost incurred during stress. In order to estimate the irradiance above which the tissue exhibited net oxygen production, known as the compensation irradiance (E_c), oxygen flux estimates obtained at 0, 50, 150 and 250 μmol photons $m^{-2}\,s^{-1}$ were linearly integrated and the E_c was estimated as the irradiance where the oxygen flux was zero.

(d) *Chlorophyll* a *content and xanthophyll pigments*

The concentrations of chlorophyll *a* (Chl *a*) and xanthophyll pigments in each coral juvenile were determined by reverse-phase high performance liquid chromatography (HPLC) using an integrated PC-interfaced Waters HPLC system in the second experiment. Small branch fragments (approx. 0.5 cm long) were sub-sampled on days 1, 8 and 15 of heating ($n=3$ per *Symbiodinium* type in each temperature treatment). After measurement of their reflectance spectra (see §2*e*), fragments were snap-frozen in liquid nitrogen and stored at −80°C. Following pigment extraction, the surface area of each fragment was calculated using photogrammetry and digital model construction as described in Jones *et al.* (2008).

Pigments were extracted in 1 ml of methanol for 2 hours in the dark at −20°C. Detailed extraction and analytical methods are provided in the electronic supplementary material. Chl *a* and xanthophylls (diadinoxanthin and diatoxanthin) were detected by photodiode array spectroscopy (350–750 nm) and by fluorescence (excitation: 440 nm, emission: 650 nm). Absorbance chromatograms were extracted at 440 nm. Pigment identity was confirmed by co-chromatography with authentic standards (Sigma Aldrich, and DHI, Denmark). The xanthophyll ratio was calculated as the ratio of diatoxanthin to the total xanthophyll pool [diadinoxanthin plus diatoxanthin] (Ambarsari *et al.* 1997). Due to the small size of fragments (38–66 mm^2) and the limited number of colonies available for sub-sampling, it was not possible to collect samples for quantification of algal cells. While this restricted the interpretation of pigment data (i.e. whether differences were due to changes in the presence of pigment, or the number of algal cells), standardizing pigment concentrations to coral surface area allowed for comparison of general patterns among treatments.

(e) *Reflectance spectra of corals and calculation of the chlorophyll* a *specific absorption coefficient* ($a^*_{\mathrm{Chl}\,a}$)

To quantify changes in the light absorption efficiency of chlorophyll *a* in the symbionts, reflectance spectra of the corals and skeletons used in the second experiment were measured between 400 and 750 nm with 0.3 nm resolution using a USB2000 Fiber Optic Spectrometer (25 μm optical slit with grating #3 installed, Ocean Optics). The method was adapted from Enriquez *et al.* (2005). Coral colonies were sub-sampled by taking a small branch fragment and placing it on a black, non-reflecting surface in a small container filled with seawater. The fragment was positioned so that the side that received more downwelling light while attached to the colony was facing up. Illumination was provided by a metal halide lamp approximately 40 cm above the sample. Reflected light was collected with a 200 μm diameter waveguide attached to the spectrometer. The waveguide was placed underwater 0.5 cm away from the sample at a 45° angle. To avoid complications due to morphological variance, the waveguide was always pointed to the coenosarc (tissue that joins adjacent polyps). The field of view of the waveguide was approximately 0.1 cm^2. Reflectance was calculated as the ratio of the radiance measured from the coral surface relative to the radiance obtained from a reference white diffusing surface. The specific absorption coefficient of chlorophyll *a* ($a^*_{\mathrm{Chl}\,a}$) was calculated as described in Enriquez *et al.* (2005).

(f) *Statistical analysis*

Physiological parameters measured for C1- and D-corals, including F_v/F_m, Q_m, P_g, $P_n : R_D$, E_c, Chl *a*, $a^*_{\mathrm{Chl}\,a}$ and $D_t/(D_t+D_n)$ were compared among temperature treatments at the end of the three experimental exposures. In addition, values for each parameter were compared between the first and last day of each experiment for each *Symbiodinium* type to examine changes within each association over the period of heat stress. Non-parametric tests (Mann–Whitney *U*) were used in all cases as transformation of the data did not satisfy the assumption of homogeneous variances required by ANOVA.

3. RESULTS

(a) *Bleaching condition of corals*

Elevated temperatures had a much greater impact on juvenile corals of *A. tenuis* when they hosted ITS1 type D compared with type C1 *Symbiodinium*, both in terms of bleaching intensity and mortality. In the pilot study, all D-corals exposed to 32°C bleached or died after 29 days, whereas the proportion of C1-corals that bleached under the same conditions was only 5% (electronic supplementary material, figure 1*a*). Results from the two light level treatments in experiment 1 emphasize the importance of light dose and intensity on the bleaching response of corals. Corals exposed to elevated light levels bleached more rapidly than those exposed to lower light levels. The proportion of colonies that bleached or died in the 32°C treatment was 3–4 times greater for D-corals compared with C1-corals (70% at low light and 94% at high light for D-corals, compared with 13 and 33% for C1-corals, electronic supplementary material, figure 1*a*). In both the pilot study and experiment 1, the response was diminished at lower temperatures, but the pattern of bleaching by type association was consistent (electronic supplementary material, figure 1*b*).

(b) *Photochemistry of heat-stressed coral juveniles*

C1-corals had consistently greater maximum quantum yields (F_v/F_m) than D-corals in our three independent experiments (figures 1 and 2; electronic supplementary material, figure 2). At the end of the pilot study (33 days after heating began), F_v/F_m in C1-corals at 32°C was only 5% lower than the same association at 28°C (control temperature). In contrast, F_v/F_m for D-corals was 65% lower at 32°C than at the control temperature and almost threefold lower than in C1-corals ($p<0.001$, Mann–Whitney *U*, figure 1*a*,*b*). Both associations showed a significant decline in F_v/F_m over time at 32°C ($p<0.001$, Mann–Whitney *U*). However, the decline for D-corals was over 70% from initial values, compared with only 20.4% for C1-corals (figure 1*b*). At 31°C, the extent of the response was smaller, but F_v/F_m was still significantly higher for

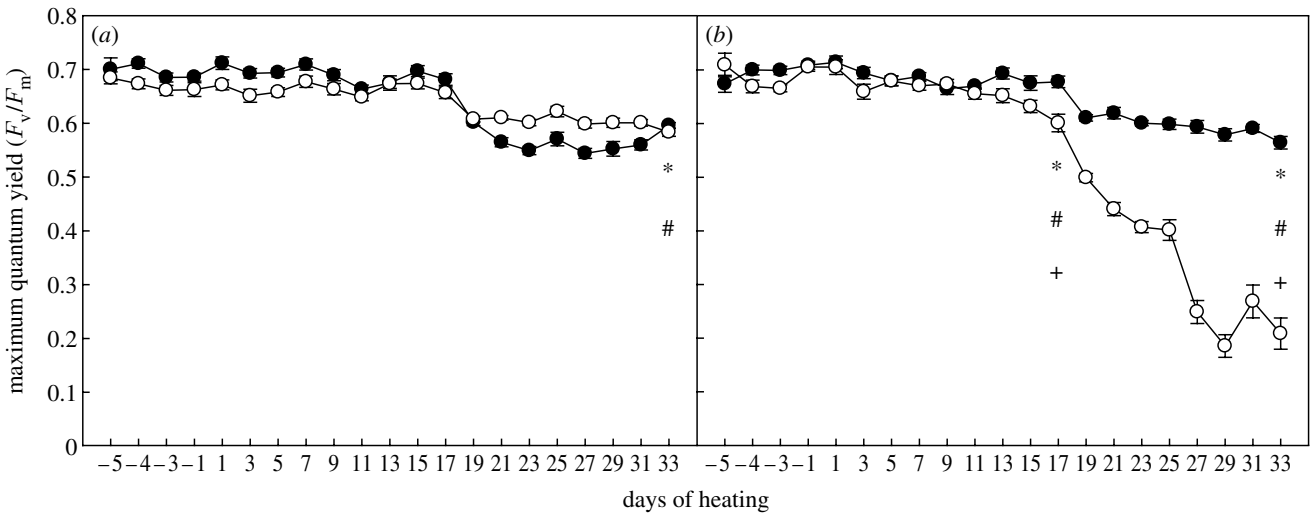

Figure 1. Maximum quantum yield (F_v/F_m) of corals hosting either *Symbiodinium* C1 (filled circles) or D (open circles) at (*a*) 28 and (*b*) 32°C. Values are means ± s.e. for each *Symbiodinium* type ($n=24$). * and # notations refer to significant differences over time within C1- and D-corals, respectively. + denotes a difference between C1- and D-corals. Comparisons are by Mann–Whitney *U*-test.

C1- than for D-corals ($p<0.001$, Mann–Whitney *U*, electronic supplementary material, figure 2*b*). Declines in F_v/F_m of both associations after day 17 (figure 1*a*,*b*; electronic supplementary material, figure 2*a*,*b*) coincided with an increase in photoperiod (from 7.5 to 10 hours) that was initiated on this day. The much more rapid decline of F_v/F_m for D-corals in the 32°C treatment after this point (figure 1*b*) emphasizes the additive and potentially synergistic interaction of light and temperature to substantially increase the sensitivity of this clade to heat stress.

In the first full-scale experiment, which incorporated two light levels, we again found that F_v/F_m of heat-stressed C1-corals was higher compared with D-corals. F_v/F_m declined significantly in all corals under high light in both the 31 and 32°C treatments ($p<0.05$, Mann–Whitney *U*, figure 3*c–d* in the electronic supplementary material). Changes in the photochemistry of corals were smaller in the low-light treatments and significant differences in F_v/F_m were only detected at 32°C (figure 3*h* in the electronic supplementary material).

In the second full-scale experiment, we monitored the maximum excitation pressure of PSII (Q_m) in addition to F_v/F_m to further analyse the response of these coral–algal associations to heat stress. At 32°C, Q_m was always significantly higher in D-corals than in C1-corals ($p<0.001$, Mann–Whitney *U*, figure 2*a*). In general, there was a significant increase in Q_m by the end of heat exposure (day 17) for D-corals but not for C1-corals ($p<0.001$, Mann–Whitney *U*, figure 2*a*). At the intermediate temperature, Q_m was always around 25% higher in D-corals but levels did not differ significantly between the start and end of the experiment for either association (figure 2*a*). Colonies at the control temperature showed a small but significant ($p<0.05$, Mann–Whitney *U*) decline in Q_m for both associations throughout the experiment, but by the end there was no significant difference between them (figure 2*a*). As in the previous experiments, corals hosting C1-symbionts at 32°C had significantly higher F_v/F_m compared with D-corals ($p<0.001$, Mann–Whitney *U*, figure 2*b*). This was in spite of lower initial F_v/F_m in the C1-corals than in the D-corals. At the intermediate temperature (29°C), there was a small but non-significant drop in F_v/F_m for both associations (figure 2*b*). At the control temperature, F_v/F_m in C1-corals increased initially but levels did not differ significantly between the start and end of the experiment (figure 2*b*). In contrast, D-corals showed a sustained and significant decline in F_v/F_m in the control temperature treatment ($p<0.05$, Mann–Whitney *U*, figure 2*b*), but had slightly higher F_v/F_m than C1-corals by the end of the experiment.

(c) *O_2 microelectrode characterization of photosynthesis and respiration*

Comparisons of gross photosynthesis rate (P_g) confirm results of reduced photochemical efficiency of D-corals at elevated temperatures obtained using chlorophyll *a* fluorescence. In the highest temperature treatment (32°C), P_g in C1-corals was significantly higher ($p<0.05$, Mann–Whitney *U*, figure 3*a*) after 15 days of heating than in D-corals, which exhibited significant declines in P_g over time ($p<0.05$, Mann–Whitney *U*, figure 3*a*). Rates of gross photosynthesis of corals in the intermediate temperature treatment (29°C) were not significantly different from rates of corals in the control (26°C) treatment. P_g was similar for both coral–algal associations throughout the duration of the experiment in both the control and intermediate temperature treatments (figure 3*a*).

The ratio between rates of net photosynthesis and dark respiration ($P_n : R_D$) decreased significantly during heating in D-corals at 32°C, but not in C1-corals ($p<0.05$, Mann–Whitney *U*, figure 3*b*). There was no significant difference in $P_n : R_D$ between C1- and D-corals at the start of heating (day 1, figure 3*b*). However, at day 15, $P_n : R_D$ of C1-corals was significantly higher at 32°C ($p<0.05$, Mann–Whitney *U*, figure 3*b*). At day 15, E_c in D-corals was significantly higher than in C1-corals in the 32°C treatment ($p<0.05$, Mann–Whitney *U*, figure 3*c*).

(d) *Chlorophyll a content, absorption coefficient ($a^*_{\mathrm{Chl}\,a}$) and xanthophyll pigments*

After 15 days of heating at 32°C, the Chl *a* content in both associations was significantly lower relative to initial levels ($p<0.05$, Mann–Whitney *U*). However, the Chl *a*

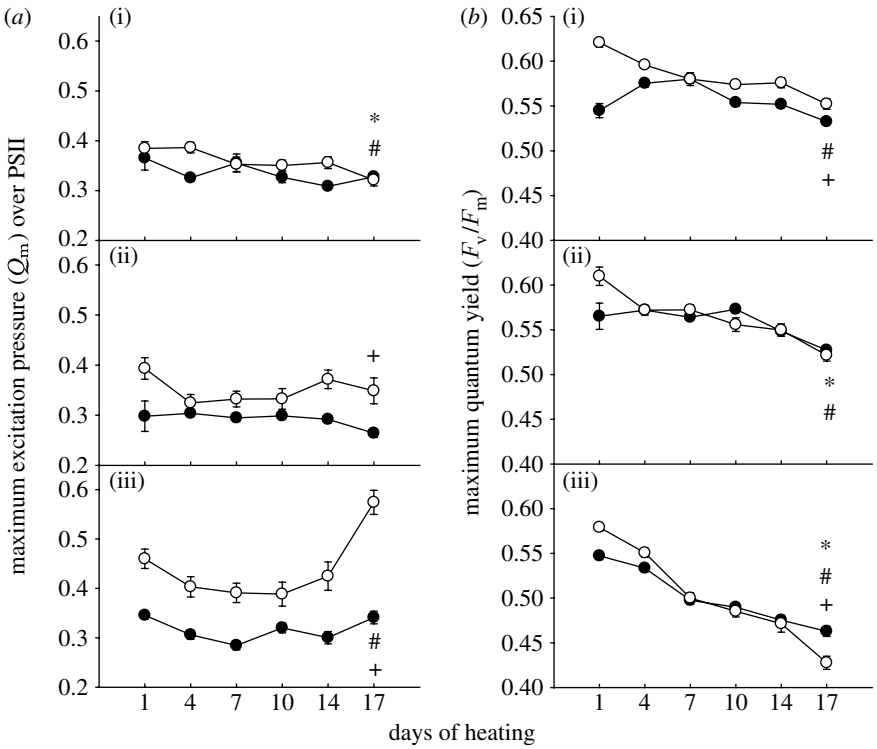

Figure 2. (*a*) Maximum excitation pressure over PSII (Q_m) of C1- (filled circles) or D-corals (open circles) at (*a*(i),*b*(i)) 26°C, (*a*(ii),*b*(ii)) 29°C and (*a*(iii),*b*(iii)) 32°C. (*b*) Maximum quantum yield (F_v/F_m) of the same corals. Values are means ± s.e. for each *Symbiodinium* type ($n=7$–13). * and # notations refer to significant differences over time within C1- and D-corals, respectively. + denotes a difference between C1- and D-corals. Comparisons are by Mann–Whitney *U*-test.

content of C1-corals was 1.8 times higher ($p<0.05$, Mann–Whitney *U*) compared with that of D-corals, even though the latter had slightly higher initial Chl *a* concentrations (figure 4*a*). No significant differences were found within or between associations at the lower temperatures (26 and 29°C).

The absorption coefficient of Chl *a* in D-corals was 2.4 times higher compared with C1-corals ($p<0.05$, Mann–Whitney *U*, figure 4*b*) after 15 days of heating at 32°C. Despite having similar initial levels, $a^*_{\text{Chl }a}$ increased significantly over time in both associations, but in D-corals this increase was more than fourfold by day 15 ($p<0.05$, Mann–Whitney *U*, figure 4*b*). No significant differences were found within or between associations at the lower temperatures.

There was a significant decline in the total pool of xanthophylls in both associations at 29 and 32°C ($p<0.05$, Mann–Whitney *U*; data not shown). The change in the xanthophyll pool was driven by a significant decline in diadinoxanthin ($p<0.05$, Mann–Whitney *U*). However, when normalized to the amount of Chl *a*, the differences in xanthophyll pigments over time or between D and C1 types were not significant. The xanthophyll ratio of both associations at 32°C increased during the experiment; but by day 15, it was significantly higher in D-corals than in C1-corals ($p<0.05$, Mann–Whitney *U*, figure 5). Although changes in the ratio of xanthophyll pigments at 32°C suggested the activation of xanthophyll cycling as a photo-protective mechanism, there was no correlation between this elevated ratio and the bleaching response of the corals. The xanthophyll ratio of corals at 29°C was not significantly different between associations; but it showed a small but significant decline in C1-corals relative to initial levels ($p<0.05$, Mann–Whitney *U*, figure 5). At 26°C, the xanthophyll pool in C1-corals did not change significantly during the course of the experiment, whereas there was a significant drop in both pigments during the experiment for D-corals ($p<0.05$, Mann–Whitney *U*; data not shown). For both associations at this temperature, the xanthophyll ratio changed significantly from initial values and was significantly different between associations by the end of the experiment ($p<0.05$, Mann–Whitney *U*, figure 5).

4. DISCUSSION

Our results demonstrate that the bleaching response of corals can vary dramatically depending on the *Symbiodinium* type with which they associate. Juveniles of the common coral *A. tenuis*, which naturally establish symbioses with both type C1- and D-*Symbiodinium* in field uptake studies (Little *et al.* 2004), were found to have much greater thermal tolerance when associated with type C1. The greater robustness of C1-juveniles to temperature and light stress was supported by all physiological parameters measured in each of three independent heat/light stress experiments. The only exception was the increased xanthophyll ratio in D-juveniles, which may have been induced to counter stress. However, it is possible that the xanthophyll pigments of these juveniles were still overwhelmed and therefore no improvement in the overall physiological state was observed. Moreover, the proportion of D-corals that bleached and/or died at 31 and 32°C was higher than C1-corals in all three experiments. Although a number of studies have suggested that corals associated with clade D have greater thermal tolerance (Glynn *et al.* 2001; Toller *et al.* 2001; Baker *et al.* 2004; Fabricius *et al.* 2004; Rowan 2004; Berkelmans & van Oppen 2006), our results

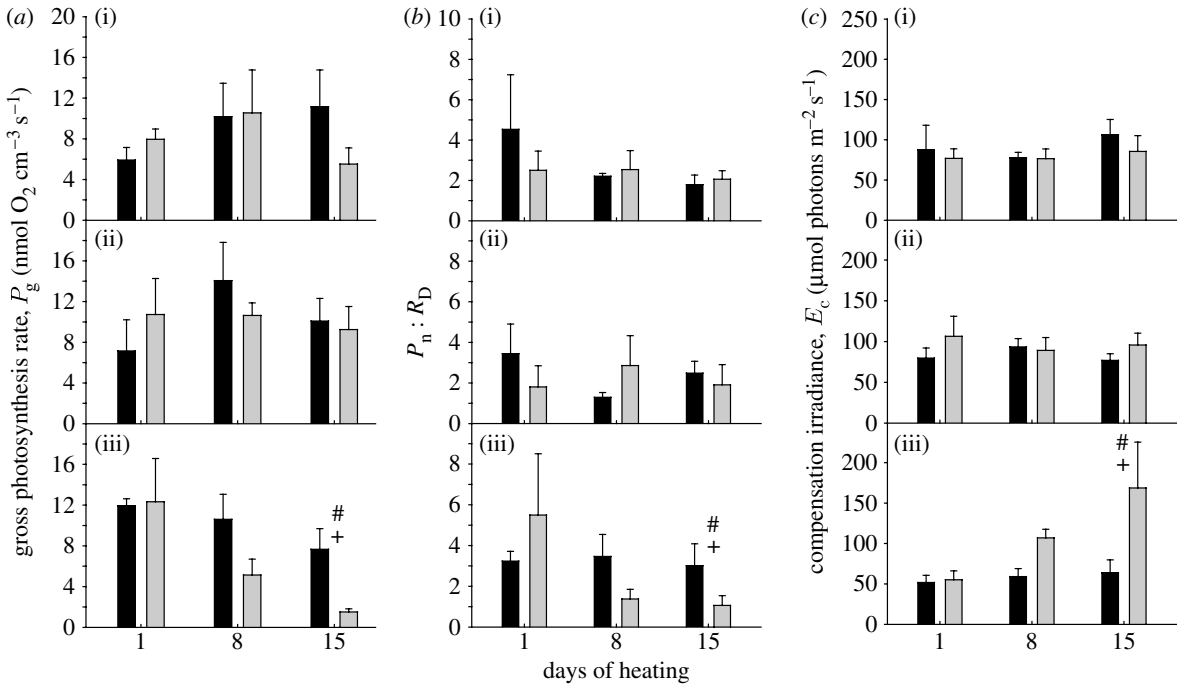

Figure 3. O_2 microelectrode measurement of photosynthesis in C1- (black columns) or D-corals (grey columns). (*a*) Gross photosynthesis rate, P_g (nmol O_2 cm^{-3} coral surface s^{-1}), (*b*) net photosynthesis rate versus dark respiration rate ($P_n : R_D$) and (*c*) compensation irradiance, E_c, at (i) 26°C, (ii) 29°C and (iii) 32°C. Values are means ± s.e. ($n = 4$ for each *Symbiodinium* type). * and # notations refer to significant differences over time within C1- and D-corals, respectively. + denotes a difference between C1- and D-corals. Comparisons are by Mann–Whitney *U*-test.

demonstrate that enhanced bleaching tolerance of corals is not universally associated with *Symbiodinium* types within clade D. Moreover, our conclusion that type C1 is thermally robust is consistent with recent field observations showing a dramatic shift in the symbiont community of *Acropora millepora* from type C2 to types D, C1, or a mix of C1/D after bleaching (Jones *et al.* 2008). Although it is possible that clade D includes algal types that differ in thermal tolerance (see Tchernov *et al.* 2004), the specific D-type associated with lower bleaching tolerance in juveniles of *A. tenuis* in our study was shown to confer higher thermal tolerance in adults of *A. millepora* (Berkelmans & van Oppen 2006). Thus, caution must be exercised in making generalizations about the performance of *Symbiodinium* clades *in hospite*, and there is need for further studies to explore host–symbiont interactions and their impact on the physiology of the coral holobiont.

(a) *Photochemical confirmation of enhanced thermal tolerance of C1-juveniles*

In addition to macroscopic indicators of holobiont health, photochemical measures clearly demonstrate enhanced thermal tolerance of coral juveniles when associated with *Symbiodinium* type C1. The steady and up to threefold greater decline in photochemical efficiency of PSII in D-corals well into the period when they started to bleach suggests that these corals were experiencing chronic photo-inhibition (Brown *et al.* 1999; Gorbunov *et al.* 2001). In contrast, the smaller decline of F_v/F_m and lack of substantial bleaching in C1-corals is consistent with photo-acclimation (Robison & Warner 2006). Moreover, measurements of maximum excitation pressure over PSII (Q_m) corroborated the reduced photochemical efficiency of clade D *Symbiodinium* when associated with *A. tenuis*. Q_m takes into account the induction of photochemical and non-photochemical processes competing within the reaction centres of PSII for de-activation of chlorophyll *a* excited states (Maxwell *et al.* 1995; Iglesias-Prieto *et al.* 2004) and hence enables the distinction between photo-acclimation and photo-inhibition. Values close to 1 indicate photo-inhibition, whereas values close to 0 indicate light limitation. Our conclusion that the smaller decline of F_v/F_m and lack of bleaching in C1-corals in the high-temperature treatment represented photo-acclimation is supported by the lack of change in excitation pressures of PSII (Q_m) at 32°C for C1-corals, in contrast to the significant increase found for D-corals. Under normal conditions, photosynthetic marine organisms can regulate Q_m by changing the concentration of chlorophyll *a*, thereby modifying the light absorption efficiency of this pigment ($a^*_{\mathrm{Chl}\,a}$; Enriquez 2005; Robison & Warner 2006), and thus decrease the probability of damage to PSII by chronic photo-inhibition. However, under thermal stress conditions, when the chain of degradation events leading to coral bleaching is activated by damage to PSII (Iglesias-Prieto & Trench 1994, 1997; Warner *et al.* 1996, 1999; Takahashi *et al.* 2004) or downstream from PSII (Jones *et al.* 1998), this photo-acclimation mechanism can break down. The higher values of Q_m at 32°C in D-corals (figure 2), combined with lower amounts of chlorophyll *a* and higher absorption efficiency of this pigment (figure 4), provide strong evidence of decreased physiological performance of *A. tenuis* at high temperatures when associated with *Symbiodinium* D.

(b) *The role of light in the bleaching response of heat-stressed corals*

The rapid decline in photochemical efficiency in the pilot study when the photoperiod was increased highlights the importance of light in the bleaching response of corals.

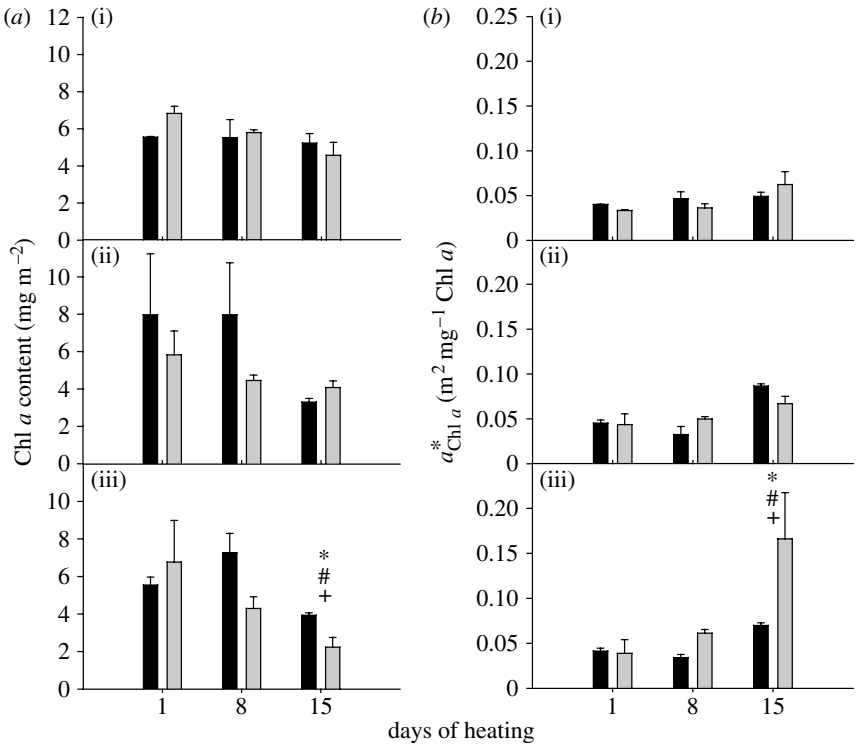

Figure 4. (*a*) Chl *a* content in sub-samples of C1- (black bars) or D-corals (grey bars). (*b*) Specific absorption coefficient of Chl *a* ($a^*_{\mathrm{Chl}\,a}$) in the same samples as in (*a*). (i) 26°C, (ii) 29°C and (iii) 32°C. Values are means ± s.e. ($n=3$ for each *Symbiodinium* type). Where error bars are not visible, they are small and hidden by the columns. * and # notations refer to significant differences over time within C1- and D-corals, respectively. + denotes a difference between C1- and D-corals. Comparisons are by Mann–Whitney *U*-test.

In D-corals in particular, rapid declines in photochemical efficiency correlated with rapid increases in the proportion of bleached colonies. Such inverse correlations may be explained by feedback loops that magnify photo-inhibition as coral tissues become more translucent. The highly reflective nature of coral skeletons (Kühl *et al.* 1995), particularly when the number of symbionts and/or amount of pigment is reduced during bleaching, results in a magnified light field within the host tissue and further exacerbates damage to remaining cells (Enriquez *et al.* 2005). Ironically, the rise in absorption efficiency of Chl *a* (a^*) in the high-temperature treatment during the second experiment would imply that the efficiency of light capture increases with temperature. However, due to the loss of reaction centre integrity, this light becomes a liability and contributes to further degradation of PSII. In combination with the more rapid onset of bleaching in the first experiment in the high-light treatment, our results underscore the enormous role that light prehistory and dose can have on the bleaching response of heat-stressed corals (Brown *et al.* 2002).

(c) *Contribution of symbiont to metabolic costs incurred during heat stress*

Our oxygen microelectrode measurements add important insights into the photosynthetic performance of *Symbiodinium* types when associated with *A. tenuis* juveniles and further corroborate our conclusion that *A. tenuis* juveniles are more tolerant to combined heat- and light stress when associated with *Symbiodinium* C1. The reduced rate of photosynthesis (P_g) found for D-juveniles indicates a reduced capacity for carbon fixation (Li *et al.* 1984; Jones *et al.* 1998) that is consistent with photo-inhibition. Moreover, the decreased ratio of net photosynthesis to dark respiration ($P_n : R_D$) for D-corals, but not C1-corals (figure 3), suggests that when associated with D *Symbiodinium*, the holobiont invests more heavily in maintenance and repair processes associated with metabolic costs incurred during heat stress (Warner *et al.* 1996; Takahashi *et al.* 2004). Such energetic costs are likely to impact other important parameters such as growth and reproduction of the holobiont (Michalek-Wagner & Willis 2001; Baird & Marshall 2002).

In addition, the lower efficiency of light utilization in photosynthesis found for heat- and light-stressed D-juveniles, as indicated by their increased compensation irradiance (E_c; Epping & Kühl 2000), suggests that greater energetic costs contributed to their poorer performance. The linear integration by which E_c was calculated may skew the irradiance intensity at which net energy acquisition occurs due to the normal shape of the photosynthesis–irradiance curve (Platt *et al.* 1980). However, the significant increase in E_c observed in D-corals at 32°C after 15 days (figure 3*c*) corresponds well with our other estimates of photosynthetic activity. Furthermore, due to the highly reflective nature of the coral skeleton, the light field around the symbionts was amplified as the experiment progressed and corals bleached (Enriquez *et al.* 2005). Therefore, the effects of underestimating E_c can be considered negligible. While every method has its limitations, the use of microsensors permits minimally invasive and accurate mapping of oxygen and photosynthesis activity at high spatial resolution. It has not been shown whether reactive oxygen species (ROS) could influence these measurements or by how much, however, the cleavage of ROS molecules to form free oxygen would be required to bias our results.

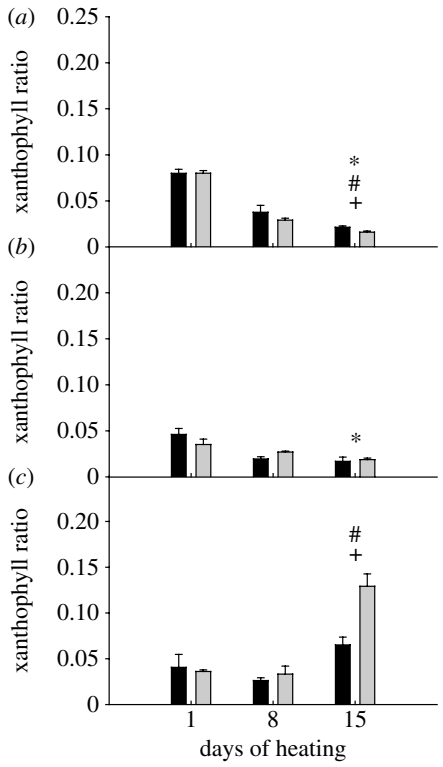

Figure 5. Changes in xanthophyll ratio (ratio of diatoxanthin to the sum of diatoxanthin and diadinoxanthin) of C1- (black bars) or D-corals (grey bars). (*a*) 26°C, (*b*) 29°C, (*c*) 32°C. Values are means±s.e. ($n=3$ for each *Symbiodinium* type). Where error bars are not visible, they are small and hidden by the columns. * and # notations refer to significant differences over time within C1- and D-corals, respectively. + denotes a difference between C1- and D-corals. Comparisons are by Mann–Whitney *U*-test.

(d) *Potential role of host factors in the heat stress response*

Differences in the tolerances of C1-corals versus D-corals to heat and light stress between ours and previous studies may be explained partially by host factors, or interactions between hosts and symbionts that may modify the physiological response of the holobiont. For example, each partner in the symbioses is capable of producing protective enzymes involved in protein regeneration and/or anti-oxidant defence pathways (Shick *et al.* 1995; Downs *et al.* 2000; Brown *et al.* 2002). Synthesis of one or more of these enzymes in one partner may elicit a response in the other which differs according to its identity. Previous studies, which have shown that corals associated with type D are more thermally tolerant, have involved species in which type D is homologous, highlighting a potential role for host factors. Berkelmans & van Oppen (2006) showed that adult corals of *A. millepora* that had shuffled their dominant symbiont population after bleaching, from type C2 to D, were more thermally tolerant in a subsequent heat stress experiment. Similarly, juvenile *A. millepora* achieved superior thermal tolerance when associated with type D (J. C. Mieog 2007, personal communication), the type normally hosted by adults of this species at Magnetic Island. In both of these studies, the D-type was the same as those used in our study (GenBank accession no. EU024793). Although juveniles of *A. tenuis* host *Symbiodinium* type D at this location (Little *et al.* 2004), adult colonies do not (van Oppen *et al.* 2001), thus host factors required to maintain this association past an initial flexible stage may not have evolved.

Interestingly, *A. tenuis* juveniles initially establish a symbiosis with a mix of type D and C1 at this location, and although they rapidly become dominated by type D during early ontogeny (less than 1 year old), they grow much faster when hosting *Symbiodinium* type C1 (Little *et al.* 2004). Why juveniles of *A. tenuis* should establish and maintain a symbiosis with a *Symbiodinium* type not found in adults remains to be investigated. Possible explanations for the change from D to C1 dominance include (i) onset of as yet undescribed host factors in early ontogeny that may regulate the symbiosis and favour type C1-symbionts (Rodriguez-Lanetty *et al.* 2004), (ii) accumulation of deleterious impacts arising from associating with type D *Symbiodinium* that increases mortality of D-juveniles through time (Little *et al.* 2004), (iii) superior competitive ability of type C1-symbionts within host cells (Fitt 1985), (iv) changing physiological needs associated with life-history stage and/or (v) changing micro-environmental conditions associated with the growth of the host which differentially favour one type over the other through time.

In summary, juvenile *A. tenuis* achieved superior thermal tolerance when associated with *Symbiodinium* C1, the type normally hosted by adults at the study location. Type C1 is a very common and widespread symbiont in *A. tenuis* on the GBR, but the most common type found associated with this coral throughout the GBR is C2 (van Oppen *et al.* 2005). This and the fact that C is the most common and diverse clade in Indo-Pacific corals (LaJeunesse 2005) call for further exploration of how genetic diversity within clade C correlates to physiological diversity. Along with this, continued efforts to understand the cellular mechanisms underlying host–symbiont interactions will provide insights into how corals and the reefs they build may respond to environmental change.

We thank Andrew Negri for his time and technical help during analysis of pigments and protocol development for HPLC. Luke O'Donnell kindly provided us with the script used for estimating the surface area of coral fragments and valuable advice using the software for this purpose. We also thank the Orpheus Island Research Station staff and the many volunteers who provided assistance during fieldwork associated with coral spawning and raising of coral juveniles. Prof. Michael Kühl took part in valuable intellectual discussion, which improved earlier versions of this manuscript. This work was supported by the Australian Research Council, JCU and AIMS. D.A. received financial support from CONACYT (Mexico) and Brockmann/State of Jalisco Scholarship Foundation. K.E.U. was supported by an ISRS Fellowship.

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