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Heating rate and symbiont productivity are key factors determining thermal stress in the reef-building coral *Acropora formosa*

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SUMMARY

The onset of large-scale coral bleaching events is routinely estimated on the basis of the duration and intensity of thermal anomalies determined as degree heating weeks. Degree heating weeks, however, do not account for differential rates of heating. This study aimed to explore the relationship between different rates of heating above the documented regional winter threshold, and resultant bleaching of the reef-building coral *Acropora formosa*. Under a relatively low light field, rapid heating of 1°C day^{-1} from 29°C to 32°C lead to a 17.6% decline in F_v/F_m, concurrent with a rapid increase in xanthophyll de-epoxidation sustained into the dark, whereas slower heating rates of 0.5°C day^{-1} lead to no decline in F_v/F_m and no change in dark-adapted xanthophyll cycling. At the winter bleaching threshold of 30°C, areal net O_2 evolution exceeded the control values for rapidly heated corals, but was lower than the controls for slowly heated corals. At the maximum temperature of 33°C, however, both treatments had net O_2 fluxes that were 50% of control values. At 30°C, only symbiont densities in the slowly heated controls were reduced relative to controls values. By 33°C, however, symbiont densities were 55% less than the controls in both treatments. The rate of heat accumulation was found to be an important variable, with rapidly heated corals attaining the same bleaching status and loss of areal O_2 production for half the degree heating week exposure as slowly heated corals. The study revealed that it is incorrect to assume that significant dark acclimation disables non-photochemical quenching, because 75% of an increased xanthophyll pool was found to be in the de-epoxidated state following rapid heat accumulation. This has important ramifications for the interpretation of chlorophyll fluorescence data such as dark adapted F_v/F_m.

Key words: acclimation, coral bleaching, heating rate, photosynthetic productivity, *Symbiodinium* sp.

INTRODUCTION

The projected increase in global sea surface temperatures is expected to cause widespread damage of coral reefs. Thermal anomaly events during the past decade have already led to several large-scale coral bleaching and mortality events throughout the tropics (Hoegh-Guldberg, 1999; Hughes et al., 2003; Donner et al., 2005). Global temperatures under climate change scenarios are predicted to rise at least by 2°C by 2050 to 2100 (IPCC, 2007) due to rising carbon dioxide levels that are currently higher than anything seen in the past 420,000 years, during which most extant marine organisms evolved (Hoegh-Guldberg et al., 2007). The onset of coral bleaching, however, varies among comparable thermal anomalies (Berkelmans et al., 2004; McClanahan et al., 2007), suggesting an influence of environmental history, adaptation or acclimatisation. Importantly, the severity of thermal stress has generally been based on the accumulated difference between the historical maximum monthly mean temperature and the current temperature profile (Strong et al., 2006; Eakin et al., 2008). Currently, thermal anomalies are monitored using sea surface temperature (SST) products such as NOAA's (National Oceanic and Atmospheric Administration) HotSpot and Degree Heating Weeks, which are measures of the current and recent-accumulation of heat stress and which provide early warning systems for bleaching events across the globe (Eakin et al., 2008). Recently, it has been suggested from large-scale surveys on the Great Barrier Reef (GBR) that the rate of heat accumulation is more indicative of bleaching response than the size of the thermal anomaly (Maynard et al., 2008). However, the processes involved have not yet been examined experimentally.

Thermal stress leads to the loss of symbionts (*Symbiodinium* sp.) and/or pigment, causing coral bleaching (Hoegh-Guldberg and Smith, 1989; Hoegh-Guldberg, 1999). Higher than normal SST and/or high UV penetration, often in conjunction with other environmental stresses are the most common cause of symbiont loss in the coral holobiont (Jones et al., 1998; Hoegh-Guldberg, 1999; Lesser, 2004), with elevated light leading to the loss of pigment in the symbiont (Hoegh-Guldberg and Smith, 1987; Falkowski and Dubinski, 1981). Losses of symbiotic dinoflagellates of greater than 50–70% have been linked to energy (or carbon)-deficient corals that require heterotrophically obtained supplements to survive (Anthony et al., 2009). Death follows from prolonged stress when the host succumbs to starvation or disease as a result of reduced carbon (energy) flow from their endosymbionts (Glynn, 1996; Anthony et al., 2007).

Differential rates of heating can have dramatically different outcomes for the cnidarian physiology at the cellular level. Schmid et al. (Schmid et al., 1981) demonstrated that hydrozoans (*Podocoryne carnea*) immersed in 30–40°C water, dependent on life stage, detached host gastrodermal cells from the mesoglea. This phenomenon became less and less reversible with incubation time at the elevated temperature. Other studies however, have found a range of different mechanisms in operation with the loss of *Symbiodinium* resultant from symbiont and/or host cell apoptosis or necrosis (Dunn et al., 2002; Dunn et al., 2004; Gates et al., 1992;

Glynn et al., 1985). These cell-death pathways are possibly stimulated by starvation, by ROS (reactive oxygen species) generated from electron transport in either photosynthesis or host mitochondria (Nii and Muscatine, 1997), singlet O_2 generated by photosensitisers (Adamska et al., 1993), or by UV-inflicted conformational changes in DNA (Lesser and Barry, 2003; Lesser, Kruse and Barry, 2003).

Here, we test the hypothesis that a slower heating rate, above the regionally determined threshold, will be less detrimental to the coral–algal symbiosis than a more rapid heating. The study was conducted under low light and in winter to lessen the effects of either heat or high light on the controls. The threshold temperature was, therefore, reduced by 1°C from the stipulated 31°C to 32°C, 5-day bleaching threshold for *Acropora formosa* from Orpheus Island, GBR as described by Berklemans and Willis (Berklemans and Willis, 1999). Furthermore, 30°C is also almost 1°C lower than the 30.8°C regional summer 1-day summer threshold in Berklemans (Berklemans, 1999), with the implication that, under the selected heating rates, no significant bleaching is predicted to occur as corals are heated from 23°C to 30°C. We hypothesised that for the same degree heating weeks, slow heated coral would perform better than rapidly heated coral, on the grounds that slow heating allows time for structural acclimation (Edmunds and Gates, 2008) also known as genotype-independent phenotypic changes (Prosser, 1991). It is important to also acknowledge, however, that sometimes stress, such as incurred excitation pressure in photosynthesis, is required to stimulate structural changes (Pocock et al., 2007). As part of our study, we investigated the efficacy of heating rate as a predictor of thermal bleaching in *A. formosa*, a widespread species of reef-building coral found on reefs in the Indo-Pacific (Veron, 2000). The taxon name is in accordance with Veron (Veron, 2000). This species is also referred to as *Acropora muricata* in accordance with Wallace (Wallace, 1999). We also explore the response characteristics of symbiont (*Symbiodinium*) primary productivity under two differential heating rates. Importantly, in understanding the response of corals to thermal anomalies, we use an integrated coral–*Symbiodinium* holobiont approach to assessing a variety of responses including rates of primary productivity, lipid stores and tissue protein content. This approach enables a deeper mechanistic understanding of the role of thermal history in the stress physiology of the coral host and symbionts, potentially improving the precision of predictive models for coral bleaching and associated mortality.

MATERIALS AND METHODS

Study site, study species and coral collecting

Fragments (sub-colonies) of *Acropora formosa* Dana 1846 were collected from 3 m below lowest astronomical tides, at three sites on the leeward side of Orpheus Island, Central GBR in July 2007. Fragments were transferred to indoor aquaria supplied with filtered flow-through seawater at Orpheus Island Research Station. Fragments were allocated to experimental tanks in preparation for a repeated measures analysis of the data with site (colony) as a within subject factor. *A. formosa* were treated to a dark:light (12 h:12 h) regime at constant sub-saturating light intensity of 140 µmol quanta m^{-2} s^{-1} (140 PAR – photosynthetically active radiation) and were allowed to acclimatise to aquaria conditions at 23°C for 4 days prior to treatment. The experimental population was then divided into two treatments; a rapid (1°C per day) and slow progressive (0.5°C per day) heating rate from 23°C to 33°C, and untreated controls (Fig. 1A). Each treatment was separately replicated in two tanks haphazardly distributed within the set-up. Temperatures in both treatments were increased linearly so that the accumulated amount of heat [e.g. NOAA degree heating days (Strong et al., 2006)] above the nominal conservative winter thermal threshold for Orpheus Island (30°C) would be approximately double in the slower heating rate compared with the rapid heating rate. When the treatments reached temperatures of 30, 31, 32 and 33°C, three coral fragments

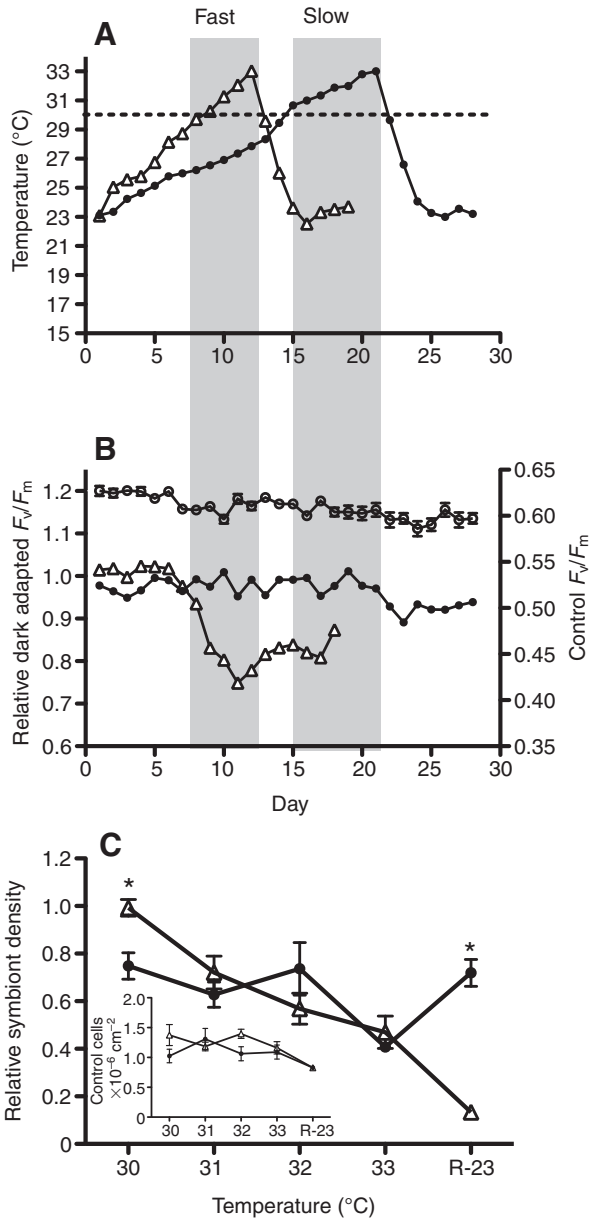

Fig. 1. The effect of heating rate on the efficiency of *Symbiodinium* photosystem II and *Symbiodinium* density. (A) Thermal profiles indicating rapid heating rate of 1°C per day (white triangle) and slow heating rate of 0.5°C per day (black circles) from 23 to 33°C. Dashed line indicates predicted thermal threshold. (B) Dark adapted F_v/F_m relative to control populations for *A. formosa* corals exposed to rapid (white triangles) and slow (black circles) heating rates; N=12. Secondary y-axis shows raw data for control corals (white circles). Grey shading indicates time spent above the nominated thermal threshold for the respective treatments. (C) Changes in *Symbiodinium* cell density within *A. formosa* colonies exposed to rapid heating or slow heating relative to control populations. Raw control (cell density, 10^{-6} cm^{-2}) values are shown in inset graph (white triangles, control for rapid heating; black circles, control for slow heating); N=6. *Significant difference (*post-hoc* LSD analysis, P<0.05) between treatments on the same day. Error bars indicate ±s.e.m.

(one from each sample site, inclusive of controls) per treatment per tank (total of 12) were used to evaluate O_2 flux. Fragments were subsequently snap-frozen, following dark acclimation of 70 min, in liquid nitrogen for analysis of symbiont and pigment density, total lipid and protein concentrations. Once temperatures of 33°C had been attained, three fragments from each tank were returned to ambient (23°C) over 4 days, where their recovery was monitored through daily sampling of F_v/F_m (a measure of the maximum quantum efficiency of photosystem II, based on the fluorescence of chlorophyll) and biochemical sampling after 1 week in the local ambient temperature. Owing to differential heating rates, corals were sampled at set temperature points rather than equal time points, and hence the majority of the measurements were normalised to the response of control corals held at the ambient winter temperature of 23°C.

Chlorophyll fluorescence

A MAXI imaging pulse amplitude modulation (IPAM) chlorophyll fluorometer (Walz, Effeltrich, Germany) was used to analyse the efficiency with which absorbed energy is converted to photochemical energy at PSII, at 18:30 h each day. Six coral branches were measured per treatment per tank (totalling 12 branches per treatment). Corals were dark acclimated for 30 min prior to measuring dark adapted F_v/F_m (Warner et al., 1996). Changes in dark adapted F_v/F_m were then correlated with changes in symbiont density and/or pigments, O_2 flux per cell and the extent to which xanthophyll de-epoxidation was maintained into the dark.

Respirometry rate

Respiration and light-saturated rate of net photosynthesis (P_{net}) were measured directly on individual coral fragments by recording changes in the seawater O_2 concentrations (Iglesias-Prieto et al., 1992). Three coral fragments per treatment, per tank (12 in total) were used to measure photosynthetic and respiration rate at 18:30 h at 30, 31, 32, 33°C and then after 5 days of recovery at ambient temperature. Fragments were placed in small custom-built cylindrical Perspex chambers and filled with filtered (15 μm) seawater from the respective treatments and sealed to exclude all air. An oxygen probe was carefully inserted through a small opening at the top of the chamber and positioned approximately 1 cm from the top of the coral branch (Oxy4 v2 sensor, PreSens, Germany). Chambers were equipped with a magnetic stirrer at the base to simulate natural convection and were collectively placed in a temperature-controlled water jacket to maintain constant treatment temperatures. To characterise photosynthesis *versus* irradiance curves (P–E curves) by measuring O_2 flux at low irradiances (α) and O_2 flux at high irradiances (net P_{max}), corals were exposed to actinic light (within the photosynthetically active range, 400–700 nm) at levels of 20, 55, 110 μmol photons m^{-2} s^{-1} for 10 min each, followed by 925, 1075, 1250 μmol photons m^{-2} s^{-1} for 5 min, respectively. Dark respiration was measured before each P–E curve and for 10 min after to measure light enhanced dark respiration. After completion, fragments were dark adapted for 1 h before being snap-frozen in liquid nitrogen and maintained at –80°C for later biochemical analyses.

Extraction of water-insoluble pigments

Coral tissue from frozen coral branches was removed using an airbrush and 5 ml filtered phosphate buffer. Algal symbionts were separated from the coral tissue by centrifugation at 4500 ***g*** (4°C) for 5 min. The supernatant was removed and frozen for host protein analysis and each pellet was resuspended in 1 ml of phosphate buffer and partitioned evenly into three Eppendorf tubes. A single aliquot was spun down and the pellet resuspended in HPLC-grade methanol (1 ml methanol for every 750 μl of sample) for pigment analysis. Supernatants were filtered through a 0.22 μm membrane filter (GSWP04700, Millipore) and 50 μl sterilised milli-Q was then added to a 250 μl aliquot prior to use in high performance liquid chromatography (HPLC). Samples were then separated and analysed using the methods of Dove et al. (Dove et al., 2006) and Zapata et al. (Zapata et al., 2000) using a SHIMADZU (Japan) SCL-10 HPLC attached to a SHIMADZU SPD-M10A photodiode array detector.

Cell densities of *Symbiodinium*

Dinoflagellate density was determined using a Sedgewick (ProSciTech s8050, Kirwin, Queensland, Australia) rafter cell 550. Ten 1 μl cells were counted within a 1 ml slide, and averaged per sample. Coral surface area was determined using the melted paraffin technique of Stimson and Kinzie III (Stimson and Kinzie, 1991).

Protein concentration

Host protein was measured using the difference in absorbance method adapted from Whitaker and Granum (Whitaker and Granum, 1980). Host tissue suspended in filtered phosphate buffer was diluted and absorbance measured at 235 and 280 nm. Total host protein was standardised to surface area (cm^{-2}) for each fragment.

Lipid content

For each sample time point, a snap frozen fragment of 4 cm of coral was used for the analysis of total lipid content. Surface areas were determined by foil wrapping (Marsh, 1970). The sample was then ground to a powder and total lipid extracted using the technique of Leuzinger et al. (Leuzinger et al., 2003) and Folch et al. (Folch et al., 1956). Lipid was extracted three times in chloroform–methanol (2:1 v/v), and the slurry filtered through a Whatman GF/C filter. The extract was washed with 0.88% KCl and then three sets of methanol:H_2O (1:1 v/v). The chloroform phase was poured into aluminium weighing pans of predetermined weight and allowed to evaporate to constant weight. Lipid content was standardised to surface area (cm^{-2}) for each fragment.

Statistical analysis

Changes in photosynthesis, xanthophyll ratios, lipid and protein concentrations, and *Symbiodinium* density data were analysed using a multivariate repeated measures ANOVA with STATISTICA 7.0 (Statsoft Inc.). Tank, temperature and treatment (heating rate) were treated as within factors. Following a significant 'treatment × temperature' interaction and a non-significant 'treatment × tank' interaction, differences were determined between treatments using Fishers least significant difference (LSD) test for *post-hoc* comparisons. Both significant and non-significant ANOVA statistics are reported in the text.

RESULTS

Data provided below show the response of the organisms in the treatment tanks relative to the response of the controls. Data were analysed in this way given that differential heating rates meant that treatments could not be made to coincide in time. Raw starting values (at 30°C) for comparisons with other studies are given in Table 1 and raw control values are given separately in the relevant figure. Statistical analysis was also conducted on all control populations

Table 1. Physiological parameters of *Acropora formosa* measured at 30°C for both rapid and slow treatments and control measurements at equal time points for the purpose of comparison with values from other studies on *A. formosa*

Parameter	Rapid 30°C	Control	Slow 30°C	Control
Symbiont cell density ($\times 10^6$ cm^{-2})	1.65±0.05	1.36±0.17	0.77±0.06	1.02±0.11
F_v/F_m	0.507±0.005	0.61±0.003	0.608±0.002	0.613±0.004
net P_{max} cm^{-2}	1.56±0.02	1.32±0.16	0.96±0.13	1.32±0.15
net P_{max} cell^{-1}	1.37±0.18	0.96±0.1	1.31±0.01	1.4±0.01
Total lipids (cm^{-2})	1.79±0.57	2.28±0.72	1.42±0.15	1.94±0.24
Host protein (mg cm^{-2})	3.13±0.49	3.76±0.49	3.01±0.21	3.09±0.48

Rapid heating, 1°C day^{-1}; slow heating, 0.5°C day^{-1}.
Values are means ± s.e.m.

and revealed that there was no significant change throughout the experiment, all P values >0.100.

Symbiodinium photosystem II dynamics

Corals exposed to the rapid heating treatment (temperature increases of 1°C per day) showed a 17.6% decrease (relative to control population) in the quantum yield of fluorescence of photosystem II (PSII) from 29°C to 32°C (Fig. 1B, day 8). By contrast, dark-adapted F_v/F_m did not decrease for corals that were heated at the slower rate of 0.5°C per day during the heating period, however, a 5.1% decline was observed upon re-introduction to the winter temperature of 23°C.

Symbiodinium density

Relative to controls, there was no effect of treatment on symbiont density ($F_{1,2}$=7.39, P=0.112); there was an effect with time ($F_{4,8}$=48.46, P=0.00001), and there was an interactive effect (treatment × temperature, $F_{4,8}$=17.471, P=0.0005; Fig. 1C). At 30°C, *Symbiodinium* density in slowly heated corals was approximately 76% that of the controls, however, in rapidly heated corals the *Symbiodinium* density was no different to that of the controls. At 31°C, rapidly heated corals were on a par with slowly heated corals, with approximately 75% of the population densities present in controls (*post-hoc* test: rapid 30°C *vs* 32°C, P=0.007; slow 30°C *vs* 31°C, P=0.725). At 33°C, both treatment groups had further reduced population densities to approximately 45–50% of controls (*post-hoc* test: rapid 30°C *vs* 33°C, P=0.0013; slow 30°C *vs* 33°C, P=0.002). In recovery, rapid pre-treatment *vs* slow pre-treatment had opposing effects, with slow pre-treatment groups returning to population densities observed at 33°C, at approximately 80% of that observed in controls. Rapid pre-treatment led to further reductions in symbiont densities to approximately 15% of controls (*post-hoc* test: R-23 slow *vs* rapid P=0.00006).

Photosynthetic and respiratory rates

Relative net P_{max} was evaluated per unit of surface area and per remnant *Symbiodinium* cell. Measurements of relative respiration yielded no significant changes over the course of the experiment (per cell, $F_{4,8}$=1.0875, P=0.424; per cm^2, $F_{4,8}$=1.5458, P=0.2777; Table 1). Since respiration does not change, changes in net O_2 indicate changes in the amount of O_2 evolved and not changes in animal respiration. A significant 'time × treatment' interaction was found for relative net P_{max} cm^{-2} ($F_{4,8}$=12.836, P<0.002) but not for net P_{max} cell^{-1}. Notably at 30°C, the areal productivity of rapidly heated corals was approximately 1.4 times that of control corals maintained at 23°C, whereas slowly heated corals produced at 0.7 times the rate observed for controls (*post-hoc* test: rapid *vs* slow 30°C, P=0.0013; Fig. 2A). The photosynthetic areal productivity (net P_{max} per cm^{-2}) of corals decreased under both heating regimes, with the rapid heating rate leading to a significant decline in net photosynthetic rates between 30 and 31°C (*post-hoc* test: rapid heat 30°C *vs* 31°C, P=0.0086; Fig. 2A). Both treatment groups experienced a significant decline in net photosynthetic rates between 31°C and 33°C (*post-hoc* test: slow 31°C *vs* 33°C, P=0.001, rapid 30°C *vs* 33°C, P=0.008), but were not significantly different from each other ,with both heat treatments 70% less productive than controls. One week post re-introduction to ambient winter temperatures, areal productivities were reversed, with previously slowly heated corals functioning 1.4 times better than controls (*post-hoc* test: slow 33°C *vs* R-23, P=0.002), and previously rapidly heated corals functioning at half the rate of controls, so showing no significant improvement from 33°C (*post-hoc* test: rapid 33°C *vs* R-23, P=0.25).

O_2 evolution per symbiont

Interestingly, O_2 evolution per symbiont cell (Fig. 2B) did not relate well within the current data sets to measurements of dark adapted F_v/F_m. Significant reductions in F_v/F_m observed between 30°C and 32°C for rapidly heated corals, and between 33°C and 23°C for previously slowly heated corals are not indicative of reductions in net O_2 evolution per cell. Across the full treatment inclusive of heating and re-introductions to ambient temperatures, there was no effect of treatment ($F_{1,2}$=2.620, P=0.247) or an interactive effect of treatment and time ($F_{4,8}$=2.175, P=0.1624), but there was a significant effect of time ($F_{4,8}$ =10.024, P=0.003) on the photosynthetic performance of remnant symbionts. Between 30°C and 33°C, O_2 evolution per cell in rapidly heated corals declined from 1.5 to 0.02 times the net O_2 produced by control corals, whereas slowly heated corals maintained 0.8 times net O_2 produced of controls (*post-hoc* test: time 30°C *vs* 33°C, P=0.004). The return to cold water resulted in a significant increase in net P_{max} per cell with remnant symbionts, within corals that had previously been heated, evolving O_2 at twice the rate of the control that was maintained throughout at 23°C (*post-hoc* test: time R-23 vs all other time points P<0.006; Fig. 2B).

Data across all treatments and controls were combined to investigate the relationship between net P_{max} per algal cell and symbiont population density in the coral tissues. When all net P_{max} and symbiont densities across all treatments and controls were combined (Fig. 2C), there was a loss of symbionts of up to 50% in *A. formosa* did not coincide with a pronounced decline in symbiont-specific O_2 evolution. In fact, a reduction in symbionts per unit of surface area below 0.5 million lead to a 2–6 fold increase in net P_{max} per cell.

Photosynthetic pigments in *Symbiodinium* sp.

Although no significant effect of heating rate was found for chlorophyll *a* (Chl *a*) per cm^{-2} ($F_{4,8}$=3.86, P=0.075, average 0.069±0.004 μg cm^{-2}; mean ± s.e.m.), there was a significant

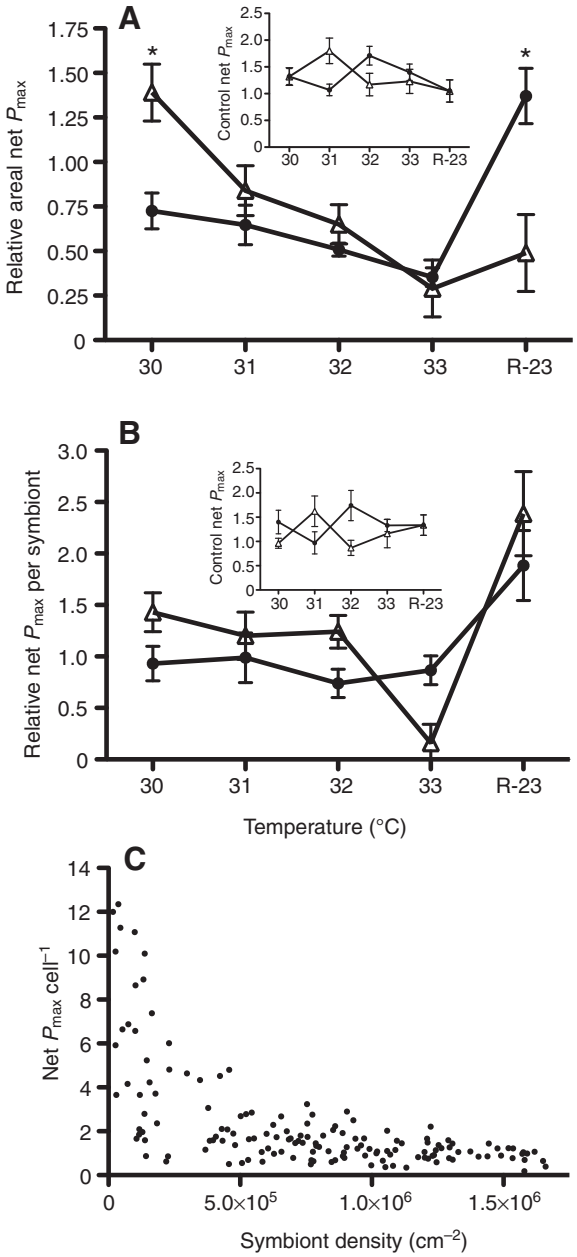

Fig. 2. Changes in photosynthetic efficiency (net P_{max}) between 30 and 33°C and after 1 week of recovery at local ambient temperature (23°C). (A) net P_{max} per unit of surface area in corals exposed to the rapid heating rate (white triangles) and the slow heating rate (black circles) standardised to control populations. Raw control values are shown in the inset graph (white triangles, control for rapid heating; black circles, control for slow heating). (B) Net P_{max} per symbiont cell standardised to control populations, N=6. (C) The relationship between photosynthetic capacity (net P_{max} per cell) and symbiont density per unit of surface area. *Significant differences (*post-hoc* LSD analysis, P<0.05) between treatments at same temperatures.

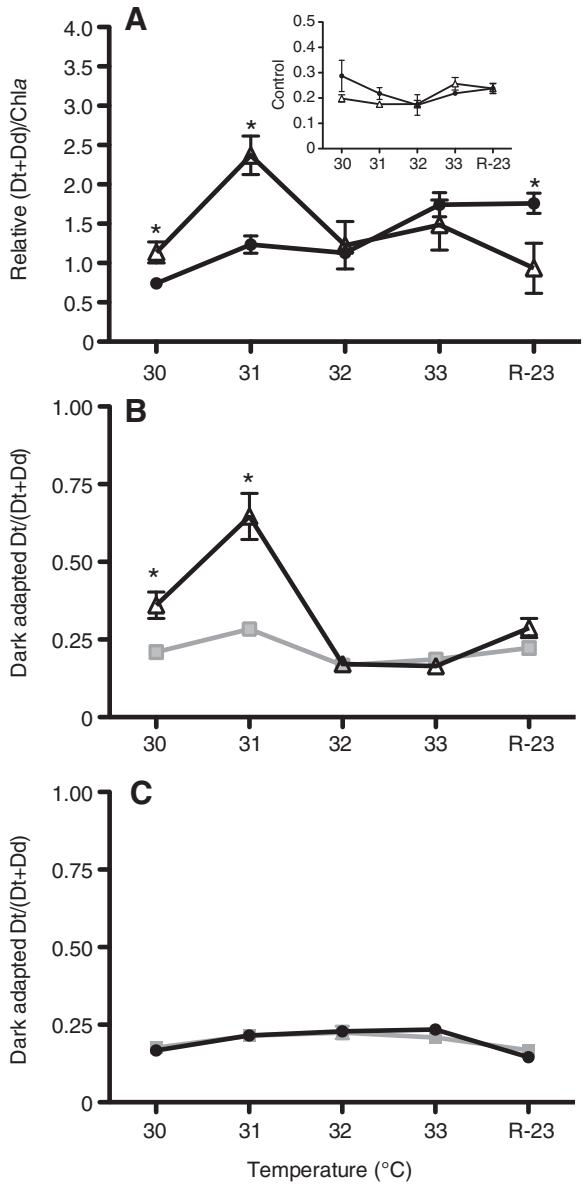

Fig. 3. Effect of heating rates on xanthophylls pool size and xanthophylls cycling in *Symbiodinium* within the tissues of the coral *Acropora formosa* following 30 min in the dark, expressed as the ratio between treatment and control (23°C) concentrations with xanthophylls pool size determined relative to chlorophyll concentrations and xanthophylls cycling determined as the concentration of Dt relative to the concentration of Dt + Dd. (A) Relative xanthophyll pool size in corals exposed to the rapid heating rate (white triangles) or the slow heating rate (black circles); N=6. *Significant difference (*post-hoc* LSD analysis, P<0.05) between treatments on the same day. Raw control values are shown in the inset graph (white triangles, control for rapid heating; black circles, control for slow heating). (B,C) Xanthophyll cycling during (B) rapid heating (white triangles) and (C) slow heating (black circles), and control (grey square). N=6; *significant difference (*post-hoc* LSD analysis, P<0.05) between treatment and control on the same day. Error bars indicate ±s.e.m.

interaction for the ratio of the xanthophyll pool [diatoxanthin (Dt) and diadinoxanthin (Dd)] to Chl *a* (time×treatment, $F_{4,8}$=20.82, P=0.0002; Fig. 3A). Corals heated at 0.5°C per day to 33°C showed a gradual increase in concentration of the relative xanthophyll pool from 30°C to 33°C relative to control corals, stabilising while in recovery (*post-hoc* test: slow 30°C *vs* R-23, P=0.0002). By contrast, corals heated at 1°C per day to 33°C experienced a rapid increase in the relative xanthophyll pool size to 31°C (*post-hoc* test: rapid 30°C *vs* 31°C P=0.00006) followed by an equally rapid decrease in the course of one day (*post-hoc* test: rapid 31°C *vs* 32°C, P=0.0001). The

size of the total xanthophyll pool continued to decrease in rapidly heated corals throughout the remainder of the treatment, including recovery stages.

A significant interaction for time × treatment was found for xanthophyll de-epoxidation, Dt/(Dt+Dd), between the rapid heating rate and the control (time×treatment $F_{4,8}$=8.247, P=0.006) despite being sampled after a 45 min dark acclimation (Fig. 3B). Xanthophyll cycling at 30°C and 31°C was greater than in control populations (rapid *vs* control 30°C, P=0.01; 31°C, P=0.002) and increased rapidly from 30°C to 31°C (*post-hoc* test: 30°C *vs* 31°C, P=0.001), followed by a rapid decrease from 31°C to 32°C (*post-hoc* test: 31°C *vs* 32°C, P=0.0002) before maintaining ratios similar to the control populations. No significant differences in dark-adapted xanthophyll cycling were found under slow heating (Fig. 3C).

Biochemical composition of coral tissues

Lipid

There were significant effects of treatment ($F_{1,2}$=193.64, P=0.005), time ($F_{4,8}$=5.0973, P=0.024) as well as an interactive effect (time×treatment $F_{4,8}$=3.906, P=0.047) for total lipid concentrations (Fig. 4A). By 30°C both slow and rapid heating treatments resulted in approximately 60% of the lipids present in control populations. By 33°C there was a significant divergence between the heated treatments, with rapidly heated corals having further reduced lipid concentrations (*post-hoc* test: rapid, 30°C *vs* 33°C, P=0.038; 33°C rapid *vs* slow, P=00394). In recovery, only slowly heated corals returned to lipid concentrations similar to those of controls (*post-hoc* test: R-23 slow *vs* rapid P=0.0003).

Protein

A significant difference for treatment was found for protein (ANOVA $F_{1,2}$=32.604, P=0.03) as well for the within subject factor time ($F_{4,8}$=7.107, P=0.0006). No interactive effect between time and treatment was found ($F_{4,8}$=2.18, P=0.16). *Post-hoc* analysis showed that irrespective of treatment, 32°C, 33°C and recovery, protein concentrations were significantly lower than at 30°C. Protein concentrations improved at the recovery temperature, increasing from concentrations observed at 33°C, but did not re-establish concentrations observed at 30°C. At 30°C, slowly heated corals had a protein concentration relative to the controls of approximately 1, however, rapidly heated corals had a protein concentration relative to controls of approximately 0.8. This difference in protein concentrations was essentially maintained throughout the experiment (Fig. 4B).

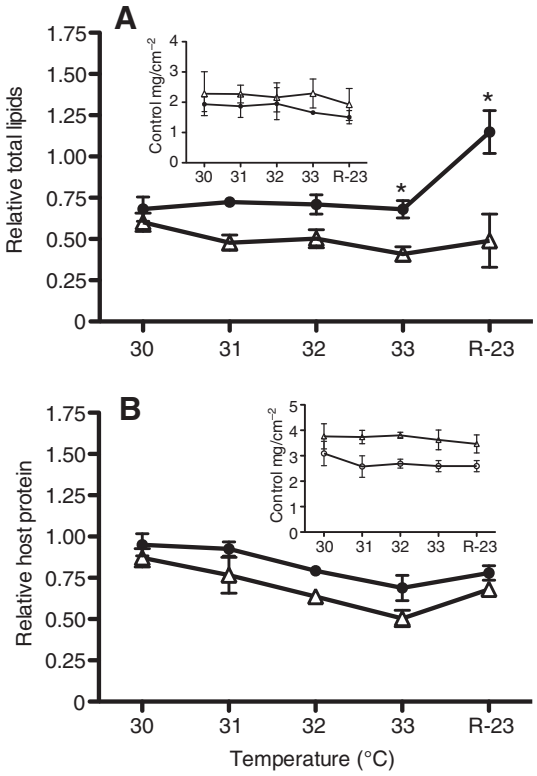

Fig. 4. Effect of heating rate on lipid and protein concentrations expressed as the ratio of treatment concentration to control concentration (control = 1 at 23°C). (A) Relative total lipids (host and symbiont), (B) relative host water soluble protein; in A and B, rapid heating rate (white triangles), slow heating rate (black circles). *Significant difference (*post-hoc* LSD analysis, P<0.05) between treatments at the same temperature; N=6. Error bars indicate ±s.e.m. Raw control values (mg cm^{-2}) are shown in the inset graphs (white triangles, control for rapid heating; black circles, control for slow heating).

DISCUSSION

Mass bleaching events such as those reported in 1998, 2002 and 2005 in the Caribbean and 1998 and 2002 in the Western Pacific describe a scenario in which entire coral communities bleach, followed by a high percentage of these corals dying. However, the bleaching of corals also occurs on a seasonal basis when many corals lose at least half of their symbionts, sometimes without showing visible signs of bleaching to the human eye (Fitt et al., 2001). Often, when a coral does bleach, the remaining symbionts can be found to be in good health (Hoegh-Guldberg and Smith, 1987; Porter et al., 1989; Brown et al., 1995; Dove at al., 2008), suggesting that care must be taken when assessing the event of bleaching and implications for the health of the coral holobiont. Thus, when identifying threshold temperatures, we need to further identify the temperature or scenario that mark the *distinct* downturn in coral viability rather than focus solely on visible signs of bleaching (Dove et al., 2008). Acclimation may alter the bleaching threshold (Coles and Jokiel, 1978; Middlebrook et al., 2008), but not necessarily alter mortality thresholds (Berkelmans and van Oppen, 2006), or, perhaps more importantly for coral cover on reefs, net annual growth rates (Diaz-Pulido et al., 2009; Little et al., 2004). In this study, we therefore, focussed not only upon the timing of the onset of bleaching, but also upon whether or not there is a consistent response in a range of other physiological parameters that have previously been used as proxies for the health status of corals.

Current methods for predicting the bleaching and mortality of reef building corals are based on the length and intensity of a thermal anomaly (Hoegh-Guldberg, 1999; Strong et al., 2006; Maynard et al., 2008). Our present study showed that degree heating weeks do not adequately predict bleaching fate. Corals heated twice as fast reached the same bleaching status at 33°C with half of the degree heating rate exposure. At 30°C, symbiont densities in rapidly heated corals were similar to densities observed in controls. By contrast, symbiont densities in slowly heated corals were slightly reduced. In both treatments, however, symbiont densities declined to 45–50% of control values as the hottest temperature, of 33°C, was attained and held for 24 h (Fig. 1C).

Reductions of dark-adapted F_v/F_m have previously been defined as reductions in 'zooxanthellar fitness' (Berkelmans and van Oppen, 2006), and have been used as an indicator of the ability of the host to maintain its autotrophic status (Rowan, 2004). Here, we

demonstrate that while perturbation of PSII can underlie the physiological collapse of *Symbiodinium* in corals (Iglesias-Prieto et al., 1993; Warner et al., 1996; Warner et al., 1999; Jones and Hoegh-Guldberg, 1999), an observation of reduced F_v/F_m is not itself sufficient to demonstrate photosynthetic dysfunction at either the level of the symbiont or the host. Under rapid heating between 29°C and 32°C, a significant decline occurred in the photosynthetic efficiency of PSII as determined from the quantum yield of chlorophyll fluorescence. A decline also occurred in net productivity per unit of surface area, and a decline in symbiont density, but rather than showing a decline in net O_2 evolution per cell, symbiont specific productivity tended to exceed that observed in controls. Between 32°C and 33°C, the decline in F_v/F_m ceased, but simultaneously, productivity per symbiont rapidly declined. Again, on conclusion of the experiment when corals were returned to ambient winter temperature, observed reduction in F_v/F_m of slowly (0.5°C day^{-1}) heated corals, coincided with both a significant increase in net O_2 evolution per symbiont, and over the coral surface area. Reducing F_v/F_m is typically interpreted as representative of situations in which the rate of damage to the reaction centres of photosynthesis exceeds the rates of D1 repair (Takahashi et al., 2005). The interpretation assumes that non-photochemical quenching is absent after dark acclimation, with typical suggested timeframes of 30 min to achieve this end (Jones et al., 1998). The present study, however, suggests that this assumption is not warranted, as even after 70 min of dark acclimation, 75% of an increased xanthophyll pool was in the de-epoxidated state at 31°C under rapid heating. De-epoxidation decreased to control levels by 32°C, despite the maintenance of high productivity per symbiont cell and low F_v/F_m. Similar scenarios have been observed in algae, with high excitation pressures at PSII leading to the initial maintenance of ΔpH into the dark, followed by a subsequent restructuring of the thylakoid membrane to allow closed (damaged) reaction centres (RC) to direct acquired energy to heat, and act as heat quenching units for open RCs (Matsubara and Chow, 2004; Pocock et al., 2007). Potentially, at 32°C RC quenching is taking over from xanthophyll de-epoxidation, with extremely low symbiont productivity at 33°C resultant from excessive heat dissipation of harvested energy at RCs. Relaxation of links between open and closed RCs, and/or repair of closed RCs, could then account for the rapid recovery in symbiont specific productivity following the return to colder waters. Increased internal light fields as a result of reductions in self-shading because of reduced symbiont densities may then explain the two fold increase in symbiont specific productivity post treatment, with the relationship between symbiont specific production and cell number (Fig. 2C) highly similar to that found by Enriquez et al. (Enriquez et al., 2005) for coral absorbance and chlorophyll density.

Heat driven photosynthetic dysfunction only becomes evident when the photosynthetic unit is illuminated, with resultant damage rapidly accumulating with increasing light intensities (Iglesias-Prieto et al., 1992). However, corals, for the most part, acclimate to high light through a decrease in the concentration of chlorophyll per symbiont cell (Anthony and Hoegh-Guldberg, 2003; Falkowski and Dubinsky, 1981), potentially confounding the extent to which visual observations of bleaching are based on reductions in symbiont densities, as opposed to pigment per cell. The principal concern of this study was with the effect of anomalous heat as opposed to light. Corals were therefore exposed to 12 h per day of a continuous, but low light regime (140 μmol quanta m^{-2} s^{-1}), and were found not to vary in chlorophyll concentration per cell over 28 experimental days. Similar light regimes have been use for long-term studies (40 days) of *Acropora intermidians*, a staghorn coral very similar to *A. formosa* that is co-located on the reef surrounding Orpheus Island. In that study, light intensity (high light, 400 μmol quanta m^{-2} s^{-1}; low light, 120 μmol quanta m^{-2} s^{-1}) was found to have no effect on the rates of coral mortality in either the presence or absence of anomalous heating (Anthony et al., 2007). That is, whilst the light levels used in the experiment were low, they were not too low to maintain coral health, as further exemplified by the non-significant changes observed in controls over the same time course in the present study.

Overall, corals exposed to both heating rates experienced a negative trend in net areal O_2 production with increases in temperature (Fig. 2A). This trend was also observed in areal protein concentrations for both heating rates in comparison with controls over the treatment period, whereas only a minor decline was observed in the total lipid content in corals rapidly heated. Although increases in temperatures can lead to protein dysfunction (Somero, 1995), the measurement of protein concentration used in this study is based on the absorption properties of amino acids and hence does not differentiate functional from dysfunctional proteins (Whittaker and Granum, 1980). Certainly, acclimation to elevated temperature can increase the temperature required for the induction of isotopic forms of heat shock proteins, thereby potentially extending the temperature range for which these proteins can aid in the maintenance of cellular integrity (Dietz and Somero, 1992). Determination, however, of the significance of slight reductions in areal protein and lipid concentrations are complicated by the possibility that actively growing corals are likely to be reduced in these aspects, whereas they may be enriched in sexually reproducing corals prior to spawning (Ward, 1995; Oku et al., 2003).

A final, yet important, part of this study explored the physiological flexibility (acclimation) of corals and their *Symbiodinium* when conditions were reverted back to pre-experimental conditions. Generally, organisms that have acclimated to a new environment frequently require a similar period in which to acclimate if they are to cope when returned to their initial environment. For instance, the acclimation of some corals to low and high light frequently takes approximately 5–7 days in both directions (Anthony and Hoegh-Guldberg, 2003). Corals, especially those located in reef flat environments, typically experience large daily variations in temperatures as a result of tidal, light and weather conditions, suggesting that corals possess a high degree of flexibility in the way they respond to these natural environmental stressors. Interestingly, with respect to symbiont densities, areal net O_2 evolution and lipid concentrations, slowly heated corals coped with the re-introduction to winter temperature better than the rapidly heated corals. Potentially, rapidly heated corals experienced cold stress due to the completion of a successful acclimation to elevated temperatures that may be absent in slowly heated corals. Alternatively, rapid chilling may compound any additional, but unknown, negative effects associated with rapid heating. What is clear, however, is that minor differences in heating rate can have very distinct effects on both areal and symbiont specific photosynthetic productivity, symbiont density and biochemical composition over time. This study further provides clear evidence that *Symbiodinium* like many higher plants are adept at altering their physiology and metabolism in response to thermal experience (Bruce et al., 2007). Much detail, however, still remains to be understood about the mechanisms by which *Symbiodinium* alters its physiology and the costs associated with these changes, such as alterations to carbon transfer, growth and reproductive fitness, which is necessary if we are to understand how these small changes in heating rate affect coral populations long term (Abrego et al., 2008). This study further highlights the reality that coral bleaching is an extremely dynamic event with many

different and confounding triggers, responses and consequences to holobiont health. Understanding these factors will become evermore important as we try to predict and model bleaching events and coral mortality into the future under a changing climate.

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