Colony-specific investigations reveal highly variable responses among individual corals to ocean acidification and warming

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ABSTRACT

As anthropogenic climate change is an ongoing concern, scientific investigations on its impacts on coral reefs are increasing. Although impacts of combined ocean acidification (OA) and temperature stress (T) on reef-building scleractinian corals have been studied at the genus, species and population levels, there are little data available on how individual corals respond to combined OA and anomalous temperatures. In this study, we exposed individual colonies of *Acropora digitifera*, *Montipora digitata* and *Porites cylindrica* to four $p\text{CO}_2$ -temperature treatments including 400 μatm -28°C, 400 μatm -31°C, 1000 μatm -28°C and 1000 μatm -31°C for 26 days. Physiological parameters including calcification, protein content, photosynthetic efficiency, *Symbiodinium* density, and chlorophyll content along with *Symbiodinium* type of each colony were examined. Along with intercolonial responses, responses of individual colonies versus pooled data to the treatments were investigated. The main results were: 1) responses to either OA or T or their combination were different between individual colonies when considering physiological functions; 2) tolerance to either OA or T was not synonymous with tolerance to the other parameter; 3) tolerance to both OA and T did not necessarily lead to tolerance of OA and T combined (OAT) at the same time; 4) OAT had negative, positive or no impacts on physiological functions of coral colonies; and 5) pooled data were not representative of responses of all individual colonies. Indeed, the pooled data obscured actual responses of individual colonies or presented a response that was not observed in any individual. From the results of this study we recommend improving experimental designs of studies investigating physiological responses of corals to climate change by complementing them with colony-specific examinations.

Keywords: Scleractinian corals, colony-specific responses, ocean acidification, global warming, zooxanthellae

1. Introduction

Unprecedented anthropogenic CO₂ emissions are considered a serious threat to global coral reefs due to resulting global warming and ocean acidification (Hoegh-Guldberg et al., 2007). Global sea surface temperatures increased approximately 0.6°C in the last century and are predicted to increase another 2-3°C by the end of this century (Meehl et al., 2007). Absorbance of approximately one third of the emitted CO₂ by oceans in the past century has resulted in ocean acidification (OA), which includes declines in pH and aragonite saturation state. Ocean pH has already declined by 0.1 units (Solomon et al., 2007) and it is predicted to drop another 0.3-0.4 units by 2100 (Orr et al., 2005). Aragonite, which has already decreased by 0.6 (from 4.6) units in tropical regions in the past hundred years, will drop to less than 2.8 by the end of this century (Kleypas et al., 1999).

Since reef-building scleractinian corals are calcifiers (producing CaCO₃) and aragonite is their precipitated calcium carbonate, the above-mentioned changes in ocean carbonate chemistry can result in less calcium carbonate precipitation and higher rates of dissolution of calcium carbonate structures of corals and reefs (Kleypas et al., 1999). Moreover, other physiological damage, including declined productivity and increased bleaching under high *p*CO₂ conditions, may also occur (Anthony et al., 2008). Increases in temperatures also can have substantial impacts on reef-building corals. There have been several reports of declines in calcification rates of corals due to increases in temperature in the last decades (Tanzil et al., 2007; Cooper et al., 2008; Cantin et al.,

2010). Increased temperatures can result in mass coral bleaching, loss of endosymbiotic *Symbiodinium* spp. and/or photosynthetic pigments, and ultimately massive coral mortality (Goreau et al., 2000). Indeed, the expected changes in temperature and OA may cause several coral species to go extinct and also cause the decay of coral reefs worldwide (Hoegh-Guldberg et al., 2007; Carpenter et al., 2008). In spite of these predictions, unlike the effects of both thermal stress and ocean acidification, which have been generally thoroughly investigated, the impacts of combined OA and temperature on corals' physiology still requires more experiments to obtain a clearer understanding of the responses of corals.

Corals' responses to thermal stress and OA have been suggested to be genera- and species-specific (Goreau et al., 2000; Anthony et al., 2008; Ries et al., 2009; Edmunds et al., 2012). Responses of corals to high temperature stress at the colony-level, especially in natural conditions, are frequently being investigated (Berkelmans and Oliver 1999; Ban et al., 2013; Depczynski et al., 2013). Responses of individual coral colonies under OA, however, have not been well studied with only a few studies having tested the effects of acidified waters on calcification of individual colonies (Marubini et al., 2003; Iguchi et al., 2012, Ohki et al., 2013). Furthermore, other physiological parameters including photosynthetic efficiency, chlorophyll content of *Symbiodinium*, and *Symbiodinium* density of individual colonies have only been investigated for three colonies of *Porites australiensis* (Iguchi et al., 2012). Clearly, further investigations on how the physiological functions of coral colonies besides from calcification are affected by OA, and in coral species other than *P. australiensis*, are needed. Furthermore, how individual coral colonies and their symbiotic *Symbiodinium* respond to the combination of OA and high temperatures remains unexamined. Moreover, whether responses of individual coral

colonies to the combined effects of OA and temperature can be predicted based on their responses to OA or temperature alone remains unknown.

Almost all physiological studies on calcifying corals have used pooled data from a few colonies of designated species to infer how most coral taxa will respond to either temperature or OA or their combination. However, our recent study on colony-specific responses of *Montipora digitata* to OA shows that pooled data are not necessarily representative of individuals (Kavousi et al., unpublished data). Although pooled data suggested a significant decline in calcification of coral colonies compared to control colonies, individual colonies showed different responses. For example, two of 15 colonies were not affected by OA, and one showed decalcification (Kavousi et al., unpublished data). Whether pooled data show different results compared to those from responses of individuals under OA, temperature stress, and their combination, and whether such differences can be observed when studying other physiological functions rather than calcification are other questions that need to be investigated. Answering these questions will provide opportunities to assess different methodological and experimental designs that will in turn affect experiments and predictions on future of corals and coral reefs.

In this study, we exposed three individual colonies each of *Acropora digitifera*, *Montipora digitata*, and *Porites cylindrica* to four $p\text{CO}_2$ -temperature treatments including 400 μatm - 28°C, 400 μatm -31°C, 1000 μatm -28°C and 1000 μatm -31°C for 26 days. Physiological parameters including calcification, protein content, photosynthetic efficiency, *Symbiodinium* density, and chlorophyll content along with *Symbiodinium* type of each colony were examined. The main questions that this study tried to answer were; 1) how each individual coral colony responds to OA, thermal stress, and OA + thermal stress; 2) if there are intercolonial differences in

physiological responses of corals and their *Symbiodinium* under combined OA + thermal stress;
and 3) whether pooled data are representative for responses of individual coral colonies.

2. Material and methods

2.1. Coral collection

Three healthy colonies each of *Montipora digitata*, *Porites cylindrica* and *Acropora digitifera* (nine colonies in total) were sampled from the shallow reef lagoon at Bise (26°42'34"N, 127°52'46"E), Okinawa, Japan in June 2014. Nubbins of similar sizes and shapes were attached to plastic screws using super-glue. The coral nubbins were kept in an outdoor tank with running ambient seawater for 5 weeks before starting the experiment. All coral nubbins were apparently healthy with no signs of disease or external algal growth prior to the experiment.

2.2. Experimental set-up

The experiment was conducted at the AICAL (Acidification Impact on CALcifiers) lab of Tropical Biosphere Research Center Sesoko Station, University of the Ryukyus, Motobu, Okinawa. A pH-stat system through CO₂ bubbling was used in our study (Tanaka et al., 2014). Briefly, the system was charged with running seawater filtered through 10 and 1 µm filters respectively. Pure CO₂ from compressed CO₂ tanks was bubbled into the seawater to provide the desired *p*CO₂ conditions. The computer program CO₂SYS was used to estimate the pH, HCO₃⁻, CO₃²⁻ and aragonite saturation state (Ω_{arg}) from xCO₂, temperature, total alkalinity of 2267 µmol/kg, and salinity of 34.5 (Lewis and Wallace, 1998). The acidified waters were ejected to 4 L tanks with a flow rate for 150 ml min⁻¹. Four treatments including *p*CO₂-temperatures 400

μatm -28°C, 400 μatm -31°C, 1000 μatm -28°C and 1000 μatm -31°C (Table 1) with two tank replications were used. $p\text{CO}_2$ conditions of 400 μatm and 1000 μatm were chosen as present day and near-future scenarios (IPCC, 2013), respectively. 28°C was chosen as ambient temperature, which was close to natural sea surface temperatures in Okinawa during the experiment, and 31°C was chosen as the stress temperature. Similar 12:12 light: dark photoperiods (120-140 $\mu\text{mol m}^{-2} \text{s}^{-1}$) under metal-halide lamps (Funnel2 150W, Kamihata, Japan) were used for all tanks. Heaters and cooling systems were used to keep the temperature conditions in the aquaria stable.

2.3. Physiological analyses

Six nubbins of each colony were put in each $p\text{CO}_2$ -temperature treatment (three in each tank replication) for 26 days. All nubbins were used to measure the following parameters. Buoyant weight was measured based on Davies (1989) as a representative of calcification rate. We represented calcification rate as percent change in final weight relative to the initial weight over 26 days. Soluble protein content was calculated using the Bradford Protein Assay (Bradford, 1976). Protein content then was converted to coral surface area and was presented as $\text{mg}^{-1} \text{cm}^{-2}$ of coral surface. *Symbiodinium* density was counted for each nubbin using a Thoma hemocytometer (Erma, Japan). To convert the counted *Symbiodinium* cells and protein content per cm^{-2} of coral surface, the aluminum foil method (Marsh, 1970) was used to measure nubbin surface area. Maximum photosynthetic efficiency of the PSII of zooxanthellae, F_v/F_m , was measured by a pulse-amplitude modulated (Diving PAM; Walz, Effeltrich, Germany) fluorometer (Schreiber et al., 1986). Coral nubbins were kept in darkness for 30-40 min prior to measurements. Since the initial measured F_v/F_m of coral nubbins of colonies showed variations, we presented the data on F_v/F_m as the ration of final/initial measurements for each nubbin instead of using only the final measurements. This way of presenting F_v/F_m correctly shows changes in photosynthetic

efficiency of *Symbiodinium* in each nubbin. Chlorophyll content (including chlorophyll *a* + *c*₂) was calculated based on Jeffery and Humphrey (1975). Chlorophyll content and *Symbiodinium* density were then presented as pg^{-1} *Symbiodinium* cell^{-1} and $\text{cell}^{-1} \text{ cm}^{-2}$ of coral surface respectively.

2.4. *Symbiodinium* DNA extraction, PCR, phylogenetic analyses

Symbiodinium types of each colony were identified at the beginning of experiment using three nubbins of each colony (n=27 nubbins total). A spin-column DNeasy Animal DNA Extraction kit (QIAGEN, Tokyo, Japan) was used to extract the DNA. PCR amplification was performed using Hot-StarTaq DNA polymerase (QIAGEN) according to the manufacturer's instructions. The internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was amplified using the dinoflagellate-specific primer pair zITSf (5'-CCG GTG AAT TAT TCG GAC TGA CGC AGT-3') (Rowan and Powers, 1992; Hunter et al., 1997) and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990) as mentioned in Reimer et al., (2006).

PCR reactions were checked using 1.5% agarose gel electrophoresis, and positive amplicons were purified by using shrimp alkaline phosphate (SAP) and Exonuclease I (Takara Bio Inc., Shiga, Japan). PCR products were then sequenced by Fasmac Co., Ltd. (Kanagawa, Japan).

ITS-rDNA sequences obtained were aligned with the most-closely matching and previously published *Symbiodinium* ITS2 sequences from GenBank (National Center for Biotechnology Information). Alignment was performed manually as all sequences belonged to *Symbiodinium* 'clade C', using Se-Al v2.0a11 Carbon (Rambaut, 2002). Alignments were manually inspected in the same software. The *Symbiodinium* ITS2 alignment consisted of 41 taxa and 253 base pairs, and is available upon request from the second author.

Phylogenetic trees were reconstructed by maximum likelihood (ML) and neighbor-joining (NJ) methods. ML trees were made by PhyML Online (Guindon et al., 2010) with the GTR (general time reversible) substitution model. The number of rate substitution categories was set to eight, and bootstraps calculated for 1000 replicates. NJ trees were constructed using CLC Free Workbench 3 (Aarhus, Denmark) with 1000 replicates.

2.5. Statistical analyses

A two-way ANOVA test was applied followed by the Bonferroni post hoc test to compare each physiological function of nubbins from each colony and also the pooled data from all three colonies of each species, as well as intercolonial differences among treatments while colonies and treatments were fixed factors. Wherever the data sets did not meet the ANOVA assumptions, the non-parametric Kruskal-Wallis H test was applied. After that, the Mann-Whitney U test was used for pairwise comparisons if the Kruskal-Wallis test showed any significant difference. To compare temperature and chemical characteristics of tank replications, an independent T test was used. Statistical analyses were performed using the software SPSS (version 22). The significance level, α , was set as 0.05.

3. Results

Pairwise comparisons between all two tank combinations showed no significant differences for any treatment in temperature, pH, $p\text{CO}_2$, and Ω_{arg} (Table 1, S1). Therefore we pooled the nubbins of two replications as one $p\text{CO}_2$ -temperature treatment.

3.1. Calcification rate

3.1.1 Inter-treatment responses of each colony

Acropora digitifera: Colonies 1 and 2 of *A. digitifera* only showed significant decline with a combination of OA and temperature stress (OAT treatment) and not with other treatments (Fig. 1a). Colony 3 showed significant decreases in calcification due to thermal stress (treatment T) and OAT compared to rates under OA and control conditions (Fig. 1a, Table S2).

Montipora digitata: Colony 1 of *M. digitata* had significantly lower calcification rates under T and OAT treatments compared to control; however under OA results were not different from any other treatment (Fig. 1b). Colony 2 did not show any significant differences among treatments (Fig. 1b). Colony 3, however, was negatively affected under OA and OAT treatments compared to the C treatment (Fig. 1b, Table S2).

Porites cylindrica: Colony 1 of *P. cylindrica* had a significant decline in calcification under OA when compared to under C (Fig. 1c). The calcification rate of colonies 2 and 3 were only affected in the OAT treatment, which was significantly lower than those under C and T (Fig. 1c, Table S2).

3.1.2. Responses of individual colonies vs. pooled data (of three colonies)

Pooled data of all three colonies showed differences in calcification rates compared to individuals (Fig. 1a). Pooled data for *A. digitifera* showed a significant decline in calcification in OAT and T treatments compared to those under C, which were similar to the responses of colony 3. However, colonies 1 and 2 were not affected under T (Fig. 1a). Pooled data of *Montipora*

digitata showed significant declines in calcification in OA and OAT treatments compared to under C, similar to responses of colony 3). Pooled data, however, were not representative of colony 2, which was not affected by any treatment, or of colony 1, which showed significant declines in calcification under OAT and T conditions (Fig. 1b). Pooled data of *Porites cylindrica* were also partly similar to colony 1 and partly similar to colonies 2 and 3 (for detailed information see Fig. 1c).

3.1.3. Intercolonial responses

Colony 3 of *A. digitifera* had significant declines in calcification rates under OAT and T compared to those of colonies 1 and 2, with no other differences observed (Table 2). Colonies 1 and 2 of *M. digitata* had lower calcification rates than colony 3 under the C treatment, but there were not any significant differences between colonies in any treatment except between colonies 1 and 3 under the T treatment (Table 2). *P. cylindrica* did not show any differences between colonies under any treatment (Table 2).

3.2. Protein content

3.2.1. Inter-treatment responses of each colony

Acropora digitifera: Colonies 1 and 2 did not show any significant changes in protein content (Fig. 2a). However, colony 3 showed a decrease in protein content under treatment T compared to under C (Fig. 2a, Table S2).

Montipora digitata: Colony 1 had significantly higher protein contents under OA and OAT treatments compared to under C (Fig. 2b). However, colonies 2 and 3 did not show any changes in protein content among treatments (Fig. 2b, Table S2).

Porites cylindrica: Protein content in colonies 1 and 3 of *P. cylindrica* did not differ among treatments (Fig. 2c), but colony 2 showed significant increases in protein content under OAT and T conditions compared to under C (Fig. 2c, Table S2).

3.2.2. Responses of individual colonies vs. pooled data (of three colonies)

The pooled data of *A. digitifera* did not show any significant changes in protein content among treatments, which was similar to the results of colonies 1 and 2 but different from colony 3 (Fig. 2a). The pooled data of *Montipora digitata* showed only a significant increase in protein content under OA treatment compared to that under C (Fig. 2b). However, colony 1 showed increases under the OA and OAT treatments while colonies 2 and 3 did not show any changes under any treatment (Fig. 2b). The pooled data of *Porites cylindrica* did not show any differences in protein content under any treatment (Fig. 2c), which reflected the results for colonies 1 and 3 (Fig. 2c). However, this was different from colony 2, which showed increased protein content under OAT and T treatments compared to the C treatment (Fig. 2c).

3.2.3. Intercolonial responses

Protein content of *A. digitifera* showed changes only under the OA treatment in which colony 1 had significantly lower protein content than colonies 2 and 3 (Table 3). For *M. digitata*, significant differences were recorded only between colony 1 and the other colonies under the C treatment (Table 3). Intercolonial comparisons for *P. cylindrica* revealed that colony 3 had significantly lower protein content than the other colonies under all treatments, except under the OA treatment, with results showing no difference among colonies (Table 3).

3.3. *Symbiodinium* density

3.3.1. Inter-treatment responses of each colony

Acropora digitifera: *Symbiodinium* density of *A. digitifera* colony 1 showed significant decreases under OA and OAT compared to C (Fig. 3a). However, there was no difference in *Symbiodinium* density between treatments T and C for colony 1 (Fig. 3a). There were no significant changes for colonies 2 and 3 among treatments (Fig. 3a, Table S2).

Montipora digitata: Colony 1 did not show any changes under any treatment (Fig. 3b). Colony 2, however, showed significant decreases in numbers of *Symbiodinium* in OAT and T treatments compared to C (Fig. 3b). The *Symbiodinium* density of colony 3 significantly decreased in OAT treatment compared to other treatments (Fig. 3b, Table S2).

Porites cylindrica: *Symbiodinium* density of colonies 1 and 3 of *P. cylindrica* did not show any changes among treatments (Fig. 3b). However, the *Symbiodinium* density of colony 2 was significantly lower in OAT than those in OA and T treatments (Fig. 3b, Table S2).

3.3.2. Responses of individual colonies vs. pooled data (of three colonies)

Pooled data for *A. digitifera* did not show any significant changes in numbers of *Symbiodinium* among treatments, which was different from the results of colony 1 (Fig. 3a). Pooled data for *M. digitata* showed only a significant decrease in *Symbiodinium* density in the OAT treatment compared to C (Fig. 3b). However, this change was similar to that of colony 2 and 3, it was different from that of colony 1, which did not show any significant changes (Fig. 3b). Moreover, the pooled data did not resemble the significant decrease in *Symbiodinium* density in colony 2

under treatment T compared to C (Fig. 3b). Pooled data of *P. cylindrica* did not show any differences in *Symbiodinium* density in any treatment, resembling the results for colonies 1 and 3 (Fig. 3c). However, it was different from colony 2, which showed a decrease in OAT compared to OA and T (Fig. 3c).

3.3.3. Intercolonial responses

Symbiodinium density in *A. digitifera* was significantly lower in colony 1 compared to colonies 2 and 3 only in the OA and OAT treatments (Table 4). Intercolonial comparisons for *M. digitata* revealed that colony 1 had a significantly lower *Symbiodinium* density than the other colonies in C treatment, and had a significantly higher number of *Symbiodinium* than colony 3 under OAT (Table 4). Results from T treatment showed that *M. digitata* colony 3 had a significantly higher *Symbiodinium* density than colonies 1 and 2 (Table 4). *P. cylindrica* showed significantly higher *Symbiodinium* density in colonies 2 and 3 than colony 1 in T treatment (Table 4).

3.4. Photosynthetic efficiency

3.4.1. Inter-treatment responses of each colony

Acropora digitifera: Data on *Fv/Fm* from colonies 1 and 2 of *A. digitifera* revealed that the only change was a significant decrease under OAT compared to other treatments (Fig. 4a). In colony 3, however, *Fv/Fm* significantly decreased under OAT and T treatments compared to under C and OA (Fig. 4a, Table S2).

Montipora digitata: F_v/F_m of colony 1 of *M. digitata* showed significant decreases in all treatments compared to C (Fig. 4b). However, no changes were observed for colonies 2 and 3 (Fig. 4b, Table S2).

Porites cylindrica: F_v/F_m for all colonies of *P. cylindrica* did not show any significant differences (Fig. 4c, Table S2).

3.4.2. Responses of individual colonies vs. pooled data (of three colonies)

Pooled data of F_v/F_m for *A. digitifera* was exactly resembled the results from colonies 1 and 2 (Fig. 4a). However, unlike the pooled data, colony 3 showed significantly lower F_v/F_m in the T treatment than C and OA (Fig. 4a). Pooled data of *M. digitata* was not representative of any colony's reactions (Fig. 4b). Pooled data showed significantly higher F_v/F_m in OA than OAT, which was not observed in any single colony (Fig. 4b). Pooled data for *P. cylindrica* only showed a significant decrease in F_v/F_m in T treatment compared to C, yet such a trend was not observed in any individual colony (Fig. 4c).

3.4.3. Intercolonial responses

Intercolonial comparisons of F_v/F_m did not show any significant changes in any coral species except between colony 1 and the others in C treatment for *P. cylindrica* (Table 5).

3.5. Chlorophyll content (chl cell⁻¹)

3.5.1. Inter-treatment responses of each colony

Acropora digitifera: Chl cell⁻¹ of *A. digitifera* colony 1 significantly increased under OAT compared to under C or OA treatments (Fig. 5a). Colony 2 did not show any significant changes among treatments (Fig. 5a). However, colony 3 showed significant lower chl cell⁻¹ in OA and OAT compared to T treatment (Fig. 5a, Table S2).

Montipora digitata: Although chl cell⁻¹ of *M. digitata* did not differ among treatments for colony 1, it showed significant increases in OAT and T treatments compared to under C in colony 2, and a significant increase under OAT treatment compared to in C and T for colony 3 (Fig. 5b, Table S2).

Porites cylindrica: Chl cell⁻¹ of *P. cylindrica* colonies did not show significant changes under any treatment compared to C (Fig. 5c, Table S2).

3.5.2. Responses of individual colonies vs. pooled data (of three colonies)

Similar to results for colonies 2 and 3, pooled data of *A. digitifera* did not show any significant changes in chl cell⁻¹ under any treatment (Fig. 5a). However, pooled data could not show the changes seen in colony 1 (Fig. 5a). Pooled data of chl cell⁻¹ for *M. digitata* only showed significant increases under OA and OAT compared to that under C (Fig. 5b). Although colonies 2 and 3 also showed the same differences between OAT and C, they also showed additional changes, not observed in the pooled data (Fig. 5b). Furthermore, colony 1 was not affected under any treatment (Fig. 5b). Pooled data of chl cell⁻¹ of *P. cylindrica* did not show any significant

changes, similar to results for colonies 2 and 3 (Fig. 5c). However, the pooled data did not show significant changes in colony 1 (Fig. 5c).

3.5.3. Intercolonial responses

Intercolonial comparisons of chl cell⁻¹ of *A. digitifera* only showed significantly higher amounts in colonies 2 and 3 than colony 1 under C treatment (Table 6). Chl cell⁻¹ of *M. digitata* was significantly lower in colony 3 compared to colonies 1 and 2 under C, OA and T treatments (Table 6). Significant increases in chl cell⁻¹ in colony 3 compared to colonies 1 and 2 under OA treatment were the only differences for *P. cylindrica* (Table 6).

3.6. *Symbiodinium* identification and phylogenetic analyses

Symbiodinium ITS-rDNA sequences were successfully amplified from all three colonies of each coral species examined in this study, and from all nubbins except one nubbin from colony 3 of *M. digitata* (n=26 novel sequences). Novel sequences were deposited in GenBank (Accession Numbers XXXX-XXXX).

Symbiodinium ITS2 sequences from *A. digitifera* were almost all identical, with one base pair difference among some sequences from colonies 2 and 3 (sequences 5-8 in Fig. S1). All sequences from the three nubbins of colony 1 and one sequence from colony 2 were identical to each other (sequences 1 to 4 in Fig. S1), and were identical to previously reported sequences corresponding to subclade C3cc (LaJeunesse *et al.*, 2010) and various *Symbiodinium* reported from *Acropora* spp. as well as to water column environmental samples (Fig. S1). These sequences together formed a well-supported clade (ML=84%, NJ=85%) separate from other sequences (Fig. S1).

All *Symbiodinium* ITS2 sequences from *P. cylindrica* were identical to each other (sequences 10-18), and to previously reported sequences of subclade C15 from various locations, including the Great Barrier Reef and Yakushima, Japan (Fig. S1). These sequences together formed a very well-supported clade separate from other sequences (ML=98%, NJ=97%) (Fig. S1).

For *Symbiodinium* ITS2 from *M. digitata*, sequences from colonies 1 and 2 (sequences 19-24) were identical to each other, and distinct from all other previously reported sequences in GenBank (ML=63%, NJ=66%), being most closely related to a group of sequences reported from a variety of hosts (Fig. S1), including some *Montipora* and *Stylophora*. These *M. digitata* sequences formed a clade separate from other sequences (Fig. S1). Unusually, the two sequences of ITS2 from colony 3 (sequences 25, 26) were identical to those acquired from *P. cylindrica* colonies in this study.

4. Discussion

In this study, colony-specific responses of three scleractinian coral species to combined effects of thermal stress (31°C) and ocean acidification (OA; 1000 μ atm) were investigated for 26 days. Physiological functions including calcification rate, protein content, *Symbiodinium* density, *Fv/Fm* of *Symbiodinium* and chl cell⁻¹ were studied. Moreover, *Symbiodinium* type of each colony was identified. The results are discussed in the following.

4.1. Inter-treatment responses of each colony

4.1.1. Calcification rate

There were no two colonies of any species that responded to OA, T and OAT treatments in the same way from the viewpoint of calcification rate (Fig. 1). For example, the calcification rate of *M. digitata* colony 2 was not significantly affected by any OA, T or OAT treatment (Fig. 1a), suggesting that this colony was tolerant of the experimental conditions at least over period of the experiment. The majority of the examined colonies (regardless of species) showed tolerance to either T or OA (Fig. 1). Previous studies have suggested that tolerant species to OA may be negatively affected by temperature and vice versa (Rodolfo-Metalpa et al., 2011; Okazaki et al., 2013). However, this generalization did not appear to be true in our study as there were colonies tolerant to both OA and T (e.g. *A. digitifera* colonies 1 and 2). Moreover, there was no colony with a calcification rate that was significantly lower than C in both OA and T treatments. On the other hand, except for *P. cylindrica* colony 1 and *M. digitata* colony 2, colonies that were tolerant to both OA and T (i.e. colonies 1 and 2 of *A. digitifera*, colonies 2 and 3 of *P. cylindrica*) were negatively and significantly affected by OAT compared to the C treatment (Fig. 1). Unlike previous studies that have shown a combination of OA and T may mediate the OA-induced decline in calcification in some coral species (Anthony et al., 2008), we did not record such a response. However, this could be due to the limited number of examined colonies in this study.

4.1.2. Protein content

Six of the examined nine colonies (*A. digitifera* colonies 1 and 2, *M. digitata* colonies 2 and 3, *P. cylindrica* colonies 1 and 3) did not show any significant differences in protein content among treatments (Fig. 2). However, for the remaining three colonies, opposite responses were observed. When the T treatment was compared to the C treatment, only *A. digitifera* colony 3 and *P. cylindrica* colony 2 showed significantly different responses, which were a decrease and increase in protein content, respectively (Fig. 2). A decrease in protein content of corals under

thermal stress has been previously reported (Colombo-Pallotta et al., 2010; Grottoli et al., 2004; Porter et al., 1989). Protein content along with lipid and carbohydrates are considered to be potential short-term energy sources of corals in the absence of sufficient photosynthates (Porter et al., 1989; Szmant and Gassman, 1990; Grottoli et al., 2004). Therefore, under thermal stress when photosynthetic interchanges between *Symbiodinium* and corals are decreased or ceased, protein and carbohydrates can be used by corals to survive and recover (Grottoli et al., 2004, Fitt et al., 2000). The significant increase in protein content observed in *P. cylindrica* colony 2 is similar to results of a study by Schoepf et al., (2013) in which *A. millepora* under ambient $p\text{CO}_2$ levels and high temperatures (29°C) for approximately 1 month showed a significant increase in protein content when compared to colonies at ambient temperatures (26.5°). This increased protein content could be a defense mechanism under moderate and short-term temperature stress that results in thicker tissues, which may help corals in tolerating further thermal stress. Previous studies have shown that corals with thicker tissue have more energy resources and can be more resistant to thermal stress (Loya et al., 2001). Thick tissues also provide more photoprotection for *Symbiodinium* (Loya et al., 2001; Dimond et al., 2012).

OA and OAT did not negatively affect the protein content of any colony in this study. In fact, protein content significantly increased under both the OA and OAT treatments for *M. digitata* colony 1 (Fig. 2b) and in the OAT treatment for *P. cylindrica* colony 2 (Fig. 2c). Previous studies have also reported enhanced protein content for some corals under OA (Fine and Tchernov, 2007, Krief et al., 2010) and under a combination of OA and T (Schoepf et al., 2013 in *Montipora monasteriata*). However, Reynaud et al., (2003) did not find any changes in the protein content of *Stylophora pistillata* under $p\text{CO}_2$ -temperature treatments (450-480 μatm and 730-800 μatm and 25 and 28°C) after 5 weeks.

427

428 4.1.3. *Symbiodinium* density

429 Although thermal stress is known as one of the major reasons for the breakdown of the symbiotic
430 coral-*Symbiodinium* relationship, in this study only the significant decrease in *Symbiodinium*
431 density was observed in *M. digitata* colony 2 (Fig. 3). Therefore, it appears that the high
432 temperature treatments of 31°C for slightly less than one month used in this study were not
433 enough stress for the coral species. *Symbiodinium* density was affected in *A. digitifera* colony 1
434 under OA, which showed a significant decrease compared to density under C (Fig. 3a), similar to
435 results seen by Krief et al., (2010), who examined two coral species under three different $p\text{CO}_2$
436 conditions for 14 months. Our results, however, contradict some other studies that have reported
437 no changes (e.g. Iguchi et al., 2012; Takahashi and Kurihara, 2013) or even increases in the
438 number of *Symbiodinium* under acidified conditions (Reynaud et al., 2003). Krief et al., (2010)
439 suggested that relatively short studies could be a cause for lack of observed changes in
440 *Symbiodinium* density and chlorophyll content among $p\text{CO}_2$ treatments. Although short-term
441 studies may not be representative of longer-term experiments (e.g. Form and Riebesell, 2012),
442 our results reject the general suggestion by Krief et al., (2010), and we suggest that the reasons
443 for such differences may also be caused by differences among host corals and their associations
444 with *Symbiodinium*.

445 The combination of T and OA (OAT) significantly reduced the number of *Symbiodinium* in some
446 colonies, including *M. digitata* colony 3, even though it was resistant to OA and T treatments
447 alone (Fig. 3b), confirming that resistance to OA and T alone does not necessarily provide
448 resistance to their combined effects.

449

450 4.1.4. *Fv/Fm*

451 *Fv/Fm* is a direct indicator of *Symbiodinium* responses to stress, and in this study results differed
452 among treatments for the majority of the colonies. T negatively affected *Fv/Fm* in one colony of
453 each species (Fig. 4). OA only reduced the *Fv/Fm* of *M. digitata* colony 1, similar to the results
454 of Iguchi *et al.*, (2012). The other colonies in this study were not affected by OA, also similar to
455 other previous studies (e.g. Takahashi and Kurihara, 2013). On the other hand, OAT reduced the
456 *Fv/Fm* of all *A. digitifera* colonies and colony 1 of both *M. digitata* and *P. cylindrica* (Fig. 4),
457 which suggests the combination of OA and T may be more detrimental to *Fv/Fm* of some
458 scleractinian corals (e.g. Sinutok *et al.*, 2011) but not all (Rodolfo-Metalpa *et al.*, 2010).
459 Moreover, it should be noted that we used very low light intensities ($120\text{-}140\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) in
460 this study that were not as high as found in nature. Natural high light intensities accompanied by
461 OAT have been shown to increase negative responses of corals and their associated
462 *Symbiodinium* (Anthony *et al.*, 2008).

463 4.1.5. Chl cell⁻¹

464 Chl cell⁻¹ of *Symbiodinium* of all colonies except for *M. digitata* colony 2 were unaffected by T
465 (Fig. 5). *M. digitata* colony 2 showed a significant increase in chl cell⁻¹ in treatment T, which is
466 similar to as reported by Reynaud *et al.* (2003, reported as chl a). OAT had either no effect or a
467 positive effect on chl cell⁻¹ of the colonies except for *A. digitifera* colony 3, which showed lower
468 chl cell⁻¹ in OAT compared to T (Fig. 5). All colonies showing increases in chl cell⁻¹ also showed
469 decreases in *Symbiodinium* density, which may suggest an acclimation response to stress. OA
470 had no significant effects on chl cell⁻¹ of any colony compared to C (Fig. 5), which is similar to

many previous studies (Reynaud et al., 2003, Iguchi et al., 2012; Takahashi and Kurihara, 2013) but opposite to others (e.g. Krief et al., 2010).

4.1.6. Relationship between *Symbiodinium* type and host colonies' responses

Although all *A. digitifera* colonies (C3cc) and *P. cylindrica* colonies (C15) had their own distinct *Symbiodinium* type, *M. digitata* colony 3 had a different *Symbiodinium* type than as was found in *M. digitata* colonies 1 and 2. These data show that there is a possibility for individuals from a given population to have different *Symbiodinium* type and therefore, potentially different tolerances to stresses as *Symbiodinium* types have different stress thresholds (Sampayo et al., 2008; Brading et al., 2011). However, in this study, we also recorded significant differences in responses to treatments not only between host corals with different *Symbiodinium* type, but also between similar *Symbiodinium* types in colonies of each examined species (Figs. 4, 5). Therefore, it seems that different host corals have different stress thresholds regardless of their symbionts. These differences could be due to genetic differences or phenotypic plasticity.

Previous studies suggest that fine-scale type differences in *Symbiodinium* spp. (Sampayo et al., 2008) and the vulnerability of *Symbiodinium* to high light intensities and temperatures, which results in producing reactive oxygen species, are causes of the breakage of the mutualistic relationship between host corals and their *Symbiodinium* (e.g. Franklin et al., 2004). However, we hypothesize that vulnerability of host corals and their negative (or positive) responses can be another reason of susceptibility of the holobiont (Figs. 1-5). Negative responses of host corals may be one reason why similar *Symbiodinium* types in different hosts show different F_v/F_m and

chl cell⁻¹ (Figs. 4, 5). Therefore, we suggest that to understand stress responses of coral host colonies and *Symbiodinium* types should both be considered.

4.2. Individual corals vs pooled data

Comparing data on any of the measured physiological parameters of individual coral colonies with each species' pooled data (of three colonies), it is apparent that pooled data were not representative of most colonies. We measured five physiological functions for three different coral species, which gave us 15 comparisons between pooled data and individual coral colonies. In none of these comparisons were the pooled data exact representatives of all three colonies. For example, pooled data on the calcification rate of *M. digitata* showed a significant decrease under OA and OAT but not under T compared to C. However, *M. digitata* colony 1 showed decreases of the calcification rate under T and OAT, and colony 2 showed tolerance to all stress treatments (Fig. 1b).

Pooled data in some cases hid the actual impacts of stressors on target physiological functions. For example, significant changes in protein content of *A. digitifera* colony 3 and *P. cylindrica* colony 2 were not reflected in the pooled data (Figs. 2a, c). In our previous work, we examined the calcification rate of 15 colonies of *M. digitata* under ambient (~400µatm) and high (800µatm) *p*CO₂ for about 4 months (Kavousi *et al.*, unpublished data). Although pooled data showed significantly lower calcification rates in high *p*CO₂ treatment than at ambient treatment, some colonies were not affected by acidified conditions despite one such colony suffering from decalcification (Kavousi *et al.*, unpublished data).

Moreover, in the present study, in some cases pooled data were not only different from single colonies, but also indicated a response not observed in any individual colonies. For example, whereas F_v/F_m of none of the *P. cylindrica* colonies was affected by any stress treatments, the pooled data showed significant decline in F_v/F_m in T treatment compared to C (Fig. 4c). Moreover, the pooled data for chl cell⁻¹ of *M. digitata* showed significant increases in OA, more than in C. However, none of the colonies actually showed such a change of chl cell⁻¹ under the OA treatment (Fig. 5b).

Therefore, we recommend researchers to also investigate colony-specific responses when considering physiological functions of corals due to the potentially problematic nature of pooled data. Indeed, presenting data on individual coral colonies, especially in studies on adaptive responses of corals, may be a more accurate way to understand how corals interact with climate change.

4.3. Colony specific responses

The results of this study confirmed that there are intercolonial differences in target physiological functions in both C treatment and stress treatments (Tables 3-7). Colony-specific responses of coral populations to bleaching have been known for a long time (see Glynn, 1996). However, information on the responses of individual colonies to OA is much more limited. Marubini et al., (2003) studied the calcification rate of three colonies of *Acropora verweyi* under acidified conditions ($pCO_2 = > 850 \mu atm$) for 8 days and reported significant differences among colonies. Iguchi et al., (2012) also found decreases in the calcification rate and F_v/F_m of symbiont *Symbiodinium* in three colonies of *Porites australiensis* with significant differences between

535 colonies after an 8-week exposure to three acidified conditions despite reduced calcification in
536 all colonies. They, however, did not record any changes in *Symbiodinium* density or chl cell⁻¹. In
537 our previous work (Kavousi et al., unpublished data), we showed that more diverse responses in
538 a population of *M. digitata* can be observed if a larger number of colonies are investigated over
539 longer time frames. The recorded responses included reduced and stable calcification and
540 decalcification of a colony, whereas the maximum difference in calcification rates of two
541 colonies under OA was 44% (Kavousi et al., unpublished data).

542 In present study, for the first time we demonstrated various responses of individual coral
543 colonies to the combined impacts of OA and temperature stress. However, in some cases no
544 significant intercolonial differences were recorded. For example, individual colonies of *P.*
545 *cylindrica* showed stable calcification in all treatments, which may be due to the fact that the
546 number of colonies investigated was limited. On the other hand, the same species showed
547 significant intercolonial differences regarding other physiological functions (see Tables 4-7).

548 In conclusion, we investigated the responses of three colonies each of three scleractinian coral
549 species. Results clearly showed intercolonial differences. However, the reasons why coral
550 colonies of a given species have different responses still need to be investigated in more detail.
551 Responses to either OA or T or OAT may be different between two individual colonies of the
552 same species. Results of this study confirm that tolerance to either OA or T is not synonymous
553 with tolerance to the other. Moreover, tolerance to both OA and T does not necessarily lead to
554 tolerance of their combination (OAT). Furthermore, OAT may have negative, positive or no
555 impacts on coral colonies under short-term time frames. The results of this study may call into
556 question the utility of generalizing physiological responses of corals at the genus, species and
557 population level.

Although in ecological and physiological studies pooling data is an important and inevitable tool to investigate populations and ecosystems, pooling data in studies on coral responses to climate change may easily lead to incorrect and/or inaccurate assumptions and interpretations. Pooled data may not be representative of individuals and hide the true range of responses of individuals. As was shown in this study, pooled data may sometimes represent a response(s) that is not observed in any single individual. Therefore, we recommend modifying experimental designs by investigating colony-specific responses accompanied by pooling individuals to better understand physiological processes in reef-building scleractinian corals.

Acknowledgements

This study was supported by an AICAL project funded by the Global Environment Research Fund A-0804 from the Ministry of the Environment of Japan and KAKENHI (No. 23241017 and No. 26220102) to YN. This research was also supported by JST/JICA, SATREPS. Protein analyses were performed in Dr. Masaya Morita's laboratory with Mr. Ugwu Stanley Ifeanyi's help.

References

- Anthony, K.R.N., Kline, D.I., Díaz-Pulido, G., Dove, S., Hoegh-Guldberg, O., 2008. Ocean acidification causes bleaching and productivity loss in coral reef builders. *Proceedings of the National Academy of Sciences* 105, 17442–17446.
- Ban, S.S., Graham, N.A.J., Connolly, S.R., 2013. Relationships between temperature, bleaching and white syndrome on the Great Barrier Reef. *Coral Reefs* 32, 1-12.
- Berkelmans R, Oliver JK (1999) Large-scale bleaching of corals on the Great Barrier Reef. *Coral Reefs* 18, 55-60.
- Blois, J.L., Zarnetske, P.L., Fitzpatrick, M.C., Finnegan, S., 2013. Climate change and the past, present, and future of biotic interactions. *Science* 341, 499-504.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.
- Brading, P., Warner, M.E., Davey, P., Smith, D.J., Achterberg, E.P., Suggett, D.J., 2011. Differential effects of ocean acidification on growth and photosynthesis among phylotypes of *Symbiodinium* (Dinophyceae). *Limnology and Oceanography* 56, 927-938.
- Cantin, N.E., Cohen, A.L., Karnauskas, K.B., Tarrant, A.M., McCorkle, D.C., 2010. Ocean warming slows coral growth in the central Red Sea. *Science* 329, 322-325.

- Cantin, N.E., Cohen, A.L., Karnauskas, K.B., Tarrant, A.M., McCorkle, D.C., 2010. Ocean warming slows coral growth in the central Red Sea. *Science* 329, 322-325.
- Carpenter, K.E., Abrar, M., Aeby, G., Aronson, R.B., Banks, S., Bruckner, A., Chiriboga, A., Cortés, J., Delbeek, J.C., DeVantier, L., Edgar, G.J., Edwards, A.J., Fenner, D., Guzmán, H.M., Hoeksema, B.W., Hodgson, G., Johan, O., Licuanan, W.Y., Livingstone, S.R., Lovell, E.R., Moore, J.A., Obura, D.O., Ochavillo, D., Polidoro, B.A., Precht, W.F., Quibilan, M.C., Reboton, C., Richards, Z.T., Rogers, A.D., Sanciangco, J., Sheppard, A., Sheppard, C., Smith, J., Stuart, S., Turak, E., Veron, J.E.N., Wallace, C., Weil, E., Wood, E., 2008. One-third of reef-building corals face elevated extinction risk from climate change and local impacts. *Science* 321, 560-563.
- Chan, N., Connolly, S.R., 2013. Sensitivity of coral calcification to ocean acidification: a meta-analysis. *Global Change Biology* 19, 282-290.
- Colombo-Pallotta, M.F., Rodríguez-Román, A., Iglesias-Prieto, R., 2010. Calcification in bleached and unbleached *Montastraea faveolata*: evaluating the role of oxygen and glycerol. *Coral Reefs* 29, 899-907.
- Comeau, S., Carpenter, R.C., Nojiri, Y., Putnam, H.M., Sakai, K., Edmunds, P.J., 2014. Pacific-wide contrast highlights resistance of reef calcifiers to ocean acidification. *Proceedings of the Royal Society B: Biological Sciences* 281, 20141339.
- Comeau, S., Edmunds, P.J., Spindel, N.B., Carpenter, R.C., 2013. The responses of eight coral reef calcifiers to increasing partial pressure of CO₂ do not exhibit a tipping point. *Limnology and Oceanography* 58, 388–398.

- Cooper, T.F., De'Ath, G., Fabricius, K.E., Lough, J.M., 2008. Declining coral calcification in massive *Porites* in two nearshore regions of the northern Great Barrier Reef. *Global Change Biology* 14, 529-538.
- Crook, E.D., Potts, D., Rebolledo-Vieyra, M., Hernandez, L., Paytan, A., 2012. Calcifying coral abundance near low-pH springs: implications for future ocean acidification. *Coral Reefs* 31, 239-245.
- Davies, P.S., 1989. Short-term growth measurements of corals using an accurate buoyant weighing technique. *Marine Biology* 101, 389-395.
- Depczynski, M., Gilmour, J.P., Ridgway, T., Barnes, H., Heyward, A.J., Holmes, T.H., Moore, J.A.Y., Radford, B.T., Thomson, D.P., Tinkler, P., Wilson S.K., 2013. Bleaching, coral mortality and subsequent survivorship on a West Australian fringing reef. *Coral Reefs* 32, 233-238.
- Dimond, J.L., Holzman, B.J., Bingham, B.L., 2012. Thicker host tissues moderate light stress in a cnidarian endosymbiont. *The Journal of Experimental Biology* 215, 2247-2254.
- Edmunds, P.J., Brown, D., Moriarty, V., 2012. Interactive effects of ocean acidification and temperature on two scleractinian corals from Moorea, French Polynesia. *Global Change Biology* 18, 2173-2183.
- Fine, M., Tchernov, D., 2007. Scleractinian coral species survive and recover from decalcification. *Science* 315, 1811-1811.
- Fitt, W.K., McFarland, F.K., Warner, M.E., Chilcoat, G.C., 2000. Seasonal patterns of tissue biomass and densities of symbiotic dinoflagellates in reef corals and relation to coral bleaching. *Limnology and Oceanography* 45, 677-685.

- Form, A.U., Riebesell, U., 2012. Acclimation to ocean acidification during long-term CO₂ exposure in the cold-water coral *Lophelia pertusa*. *Global Change Biology* 18, 843-853.
- Franklin, D.J., Hoegh-Guldberg, O., Jones, R.J., Berges, J.A., 2004. Cell death and degeneration in the symbiotic dinoflagellates of the coral *Stylophora pistillata* during bleaching. *Marine Ecology Progress Series* 272, 117-130.
- Glynn, P.W., 1996. Coral reef bleaching: facts, hypotheses and implications. *Global Change Biology* 2, 495-509.
- Goreau, T., McClanahan, T., Hayes, R., Strong, A.L., 2000. Conservation of coral reefs after the 1998 global bleaching event. *Conservation Biology* 14, 5-15.
- Grottoli, A.G., Rodrigues, L.J., Juarez, C., 2004. Lipids and stable carbon isotopes in two species of Hawaiian corals, *Porites compressa* and *Montipora verrucosa*, following a bleaching event. *Marine Biology* 145, 621-631.
- Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* 59, 307-321.
- Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., Gomez, E., Harvell, C.D., Sale, P.F., Edwards, A.J., Caldeira, K., Knowlton, N., Eakin, C.M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R.H., Dubi, A., Hatziolos, M.E., 2007. Coral reefs under rapid climate change and ocean acidification. *Science* 318, 1737-1742.

Hunter, C.L., Morden, C.W., Smith, C.M., 1997. The utility of ITS sequences in assessing relationships among zooxanthellae and corals, in: Proceedings of the 8th International Coral Reef Symposium. 2, 1599–1602.

Iguchi, A., Ozaki, S., Nakamura, T., Inoue, M., Tanaka, Y., Suzuki, A., Kawahata, H., Sakai, K., 2012. Effects of acidified seawater on coral calcification and symbiotic algae on the massive coral *Porites australiensis*. Marine Environmental Research 73, 32-36.

IPCC., 2013. Climate change 2013: the physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change.

Jeffrey, S.T., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls a, b, c₁ and c₂ in higher plants, algae and natural phytoplankton. Biochemie und Physiologie der Pflanzen 167, 191–194.

Kleypas, J., Buddemeier, R.W., Archer, D., Gattuso, J.P., Langdon, C., Opdyke, B.N., 1999. Geochemical consequences of increased atmospheric carbon dioxide on coral reefs. Science 284, 118–120.

Krief, S., Hendy, E.J., Fine, M., Yam, R., Meibom, A., Foster, G.L., Shemesh, A., 2010. Physiological and isotopic responses of scleractinian corals to ocean acidification. Geochimica et Cosmochimica Acta 74, 4988-5001.

LaJeunesse, T.C., Pettay, D.T., Sampayo, E.M., Pongsuwan, N., Brown, B., Obura, D.O., Hoegh-Guldberg, O., Fitt, W.K., 2010. Long-standing environmental conditions, geographic isolation and host-symbiont specific influence the relative ecological dominance and genetic diversification of coral endosymbionts in the genus *Symbiodinium*. Journal of Biogeography 37, 785-800.

- Langdon, C., Takahashi, T., Sweeney, C., Chipman, D., Goddard, J., Marubini, F., Aceves, H., Barnett, H., Atkinson, M.J., 2000. Effect of calcium carbonate saturation state on the calcification rate of an experimental coral reef. *Global Biogeochemical Cycles* 14, 639-654.
- Lewis, E., Wallace, D.W.R., 1998. Program Developed for CO₂ System Calculations. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Dept. of Energy, Oak Ridge, TN, ORNL/CDIAC-105.
- Loya, Y., Sakai, K., Yamazato, K., Nakano, Y., Sambali, H., van Woesik, R., 2001. Coral bleaching: the winners and the losers. *Ecology Letters* 4, 122-131.
- Marsh, J., 1970. Primary productivity of reef-building calcareous red algae. *Ecology* 51, 255–263.
- Marubini, F., Ferrier–Pages, C., Cuif, J.P., 2003. Suppression of skeletal growth in scleractinian corals by decreasing ambient carbonate-ion concentration: a cross-family comparison. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270, 179-184.
- Meehl, G.A., Stocker, T.F., Collins, W.D., Friedlingstein, P., Gaye, A.T., Gregory, J.M., Kitoh, A., Knutti, R., Murphy, J.M., Noda, A., Raper, S.C.B., Watterson, I.G., Weaver, A.J., Zhao, Z.C., 2007. Global climate projections. *Climate Change*, 283.
- Ohki, S., Irie, T., Inoue, M., Shinmen, K., Kawahata, H., Nakamura, T., Kato, A., Nojiri, Y., Suzuki, A., Sakai, K., van Woesik, R., 2013. Calcification responses of symbiotic and aposymbiotic corals to near-future levels of ocean acidification. *Biogeosciences* 10, 6807-6814.
- Okazaki, R.R., Swart, P.K., Langdon, C., 2013. Stress-tolerant corals of Florida Bay are

vulnerable to ocean acidification. *Coral Reefs* 32, 671-683.

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G-K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M-F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* 437, 681-686.

Porter, J.W., Fitt, W.K., Spero, H.J., Rogers, C.S., White, M.W., 1989. Bleaching in reef corals: physiological and stable isotopic responses. *Proceedings of the National Academy of Sciences* 86, 9342-9346.

Rambaut, A., 2002. Se-Al: Sequence alignment editor, Version 2.0a11. Available at <<http://tree.bio.ed.ac.uk/software/seal/>>

Reimer, J.D., Takishita, K., Ono, S., Maruyama, T., Tsukahara, J., 2006. Latitudinal and intracolony ITS-rDNA sequence variation in the symbiotic dinoflagellate genus *Symbiodinium* (Dinophyceae) in *Zoanthus sansibaricus* (Anthozoa: Hexacorallia). *Phycological Research* 54, 122-132.

Reynaud, S., Leclercq, N., Romaine-Lioud, S., Ferrier-Pagés, C., Jaubert, J., Gattuso, J.P., 2003. Interacting effects of CO₂ partial pressure and temperature on photosynthesis and calcification in a scleractinian coral. *Global Change Biology* 9, 1660-1668.

Ries, J.B., Cohen, A.L., McCorkle, D.C., 2009. Marine calcifiers exhibit mixed responses to CO₂-induced ocean acidification. *Geology* 37, 1131-1134.

- Rodolfo-Metalpa, R., Hoogenboom, M.O., Rottier, C., Ramos-Esplá, A., Baker, A.C., Fine, M., Ferrier-Pagès, C., 2014. Thermally tolerant corals have limited capacity to acclimatize to future warming. *Global Change Biology* 20, 3036–3049.
- Rodolfo-Metalpa, R., Houlbrèque, F., Tambutté, É., Boisson, F., Baggini, C., Patti, F.P., Jeffree, R., Fine, M., Foggo, A., Gattuso, J-P., Hall-Spencer, J. M., 2011. Coral and mollusc resistance to ocean acidification adversely affected by warming. *Nature Climate Change* 1, 308–312.
- Rodolfo-Metalpa, R., Martin, S., Ferrier-Pagès, C., Gattuso, J.P., 2010. Response of the temperate coral *Cladocora caespitosa* to mid-and long-term exposure to pCO₂ and temperature levels projected for the year 2100 AD. *Biogeosciences* 7, 289-300.
- Rowan, R., Powers, D.A., 1992. Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae). *Proceedings of the National Academy of Sciences* 89, 3639–3643.
- Sabine, C.L., Feely, R.A., Gruber, N., Key, R.M., Lee, K., Bullister, J.L., Wanninkhof, R., Wong, C.S., Wallace, D.W.R., Tilbrook, B., Millero, F.J., Peng, T-H., Kozyr, A., Ono, T., Rios, A.F., 2004. The oceanic sink for anthropogenic CO₂. *Science* 305, 367-371.
- Sampayo, E.M., Ridgway, T., Bongaerts, P., Hoegh-Guldberg, O., 2008. Bleaching susceptibility and mortality of corals are determined by fine-scale differences in symbiont type. *Proceedings of the National Academy of Sciences* 105, 10444-10449.
- Schoepf, V., Grottoli, A.G., Warner, M.E., Cai, W.J., Melman, T.F., Hoadley, K.D., Pettay, D.T., Hu, X., Li, Q., Xu, H., Wang, Y., Matsui, Y., Baumann, J.H., 2013. Coral

energy reserves and calcification in a high-CO₂ world at two temperatures. PLoS ONE 8, e75049.

Schreiber, U., Schliwa, U., Bilger, W., 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynthesis Research 10, 51-62.

Shamberger, K.E., Cohen, A.L., Golbuu, Y., McCorkle, D.C., Lentz, S.J., Barkley, H.C. (2014) Diverse coral communities in naturally acidified waters of a Western Pacific Reef. Geophysical Research Letters 41, 499-504.

Sinutok, S., Hill, R., Doblin, M.A., Wuhrer, R., Ralph, P.J., 2011. Warmer more acidic conditions cause decreased productivity and calcification in subtropical coral reef sediment-dwelling calcifiers. Limnology and Oceanography 56, 1200-1212.

Solomon, S., 2007. Climate change 2007- the physical science basis: Working group I contribution to the fourth assessment report of the IPCC (Vol. 4). Cambridge University Press.

Szmant, A., Gassman, N.J., 1990. The effects of prolonged “bleaching” on the tissue biomass and reproduction of the reef coral *Montastrea annularis*. Coral Reefs 8, 217-224.

Takahashi, A., Kurihara, H., 2013. Ocean acidification does not affect the physiology of the tropical coral *Acropora digitifera* during a 5-week experiment. Coral Reefs 32, 305-314.

Tanaka, Y., Iguchi, A., Nishida, K., Inoue, M., Nakamura, T., Suzuki, A., Sakai, K., 2014. Nutrient availability affects the response of juvenile corals and the endosymbionts to ocean acidification. Limnology and Oceanography 59, 1468-1476.

Tanzil, J.T.I., Brown, B.E., Tudhope, A.W., Dunne, R.P., 2009. Decline in skeletal growth of the coral *Porites lutea* from the Andaman Sea, South Thailand between 1984 and 2005. *Coral Reefs* 28, 519-528.

van Woesik, R., Sakai, K., Ganase, A., Loya, Y., 2011. Revisiting the winners and the losers a decade after coral bleaching. *Marine Ecology Progress Series* 434, 67-76.

White, T.J., Bruns, T., Lee, S., Taylor, W.J., 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics, in: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.) *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, pp. 315–322.

809 **Table 1** Chemical characteristics of experimental conditions

Treatments	Tank	Temperature (°C)	pH	$p\text{CO}_2$ (μatm)	HCO_3^- (μmol / kg SW)	CO_3^{2-} (μmol / kg SW)	Ω_{arg}
400 μatm- 28°C	1	27.99±0.13	8.15±0.01	442.22±14.06	1749.75±10.53	208.88±04.25	3.38±0.07
	2	27.96±0.16	8.15±0.01	442.22±14.06	1749.93±10.61	208.81±04.29	3.37±0.07
400 μatm- 31°C	1	31.05±0.13	8.15±0.01	442.22±14.06	1709.82±11.30	225.20±04.55	3.70±0.08
	2	30.97±0.11	8.15±0.01	442.22±14.06	1709.40±10.32	225.38±04.16	3.71±0.07
1000 μatm- 28°C	1	28.00±0.18	7.83±0.01	1038.62±26.57	1982.80±05.00	114.41±02.03	1.85±0.03
	2	27.97±0.15	7.84±0.00	1009.49±08.57	1976.81±02.34	116.84±00.96	1.89±0.02
1000 μatm- 31°C	1	30.98±0.18	7.84±0.01	1021.71±16.45	1951.68±03.63	127.26±01.46	2.09±0.02
	2	31.02±0.12	7.84±0.01	1021.71±16.44	1952.42±03.91	126.96±01.59	2.09±0.03

Table 2 Summary of statistical analyses on intercolonial comparisons of calcification rate of colonies 1, 2 and 3 of each species in $p\text{CO}_2$ -temperature treatments including C= (400 μatm -28°C), T= (400 μatm -31°C), OA= (1000 μatm -28°C) and OAT= (1000 μatm -31°C) after 26 days. Significant differences are shown in bold. “na” means post-hoc test was not applicable because of no significant differences.

ANOVA	Treatment	Sum of Squares	df	F	P	Pairwise comparisons
<i>Acropora digitifera</i>						
	C	13.946	2	0.712	0.495	na
	OA	33.233	2	1.696	0.192	na
	OAT	72.584	2	3.704	0.30	(1=2)>3
	T	272.753	2	13.919	<0.001	(1=2)>3
<i>Porites cylindrica</i>						
	C	3.305	2	0.214	0.808	na
	OA	33.722	2	2.188	0.121	na
	OAT	12.160	2	0.789	0.459	na
	T	9.553	2	0.620	0.541	na
Kruskal-Wallis		Mean Rank	df	Chi-Square	p	Pairwise comparisons
<i>Montipora digitata</i>						
	C	5.33, 8.17, 15.00	2	10.398	0.006	(1=2)<3
	OA	7.50, 9.00, 12.00	2	2.211	0.331	na
	OAT	5.50, 11.83, 11.17	2	5.099	0.78	na
	T	4.83, 9.40, 12.83	2	7.574	0.023	1<3; 1=2; 2=3

Table 3 Summary of statistical analyses on intercolonial comparisons of protein content of colonies 1, 2 and 3 of each species in $p\text{CO}_2$ -temperature treatments including C= (400 μatm -28°C), T= (400 μatm -31°C), OA= (1000 μatm -28°C) and OAT= (1000 μatm -31°C) after 26 days. Significant differences are shown in bold. “na” means post-hoc test was not applicable because of no significant differences.

ANOVA	Treatment	Sum of squares	df	F	p	Pairwise comparisons
<i>Acropora digitifera</i>						
	C	559.016	2	2.312	0.108	na
	OA	1859.639	2	7.692	0.001	1<(2=3)
	OAT	103.833	2	0.429	0.653	na
	T	104.395	2	0.432	0.651	na
<i>Montipora digitata</i>						
	C	2543.050	2	4.348	0.017	1<(2=3)
	OA	811.872	2	1.388	0.257	na
	OAT	952.752	2	1.629	0.205	na
	T	672.374	2	1.150	0.324	na
Kruskal-Wallis		Mean Rank	df	Chi-Square	p	Pairwise comparisons
<i>Porites cylindrica</i>						
	C	13.33, 10.50, 4.67	2	8.222	0.16	(1=2)>3
	OA	12.50, 8.33, 7.67	2	2.889	0.239	na
	OAT	13.83, 11.17, 3.50	2	12.117	0.002	(1=2)>3
	T	14.50, 10.50, 3.50	2	13.053	0.001	(1=2)>3

Table 4 Summary of statistical analyses on intercolonial comparisons of *Symbiodinium* density of colonies 1, 2 and 3 of each species in $p\text{CO}_2$ -temperature treatments including C= (400 μatm -28°C), T= (400 μatm -31°C), OA= (1000 μatm -28°C) and OAT= (1000 μatm -31°C) after 26 days. Significant differences are shown in bold. “na” means post-hoc test was not applicable because of no significant differences.

ANOVA	Treatment	Sum of squares	df	F	p	Pairwise comparisons
<i>Acropora digitifera</i>						
	C	0.005	2	0.130	0.878	na
	OA	0.378	2	9.815	<0.001	1< (2=3)
	OAT	0.315	2	8.164	0.001	1< (2=3)
	T	0.051	2	1.319	0.275	na
<i>Montipora digitata</i>						
	C	0.801	2	9.932	<0.001	1< (2=3)
	OA	0.154	2	1.913	0.157	na
	OAT	0.264	2	3.280	0.044	1>3; 2=3
	T	0.940	2	11.662	<0.001	(1=2) < 3
<i>Porites cylindrica</i>						
	C	0.070	2	1.066	0.351	na
	OA	0.147	2	2.243	0.115	na
	OAT	0.032	2	0.491	0.615	na
	T	0.572	2	8.715	<0.001	1< (2=3)

881 **Table 5** Summary of statistical analyses on *Fv/Fm* of colonies 1, 2 and 3 of each species in
882 *p*CO₂-temperature treatments including C= (400 µatm- 28°C), T= (400 µatm-31°C), OA= (1000
883 µatm-28°C) and OAT= (1000 µatm-31°C) after 26 days. Significant differences are shown in
884 bold. “na” means post-hoc test was not applicable because of no significant differences.

ANOVA	Treatment	Sum of squares	df	<i>F</i>	<i>p</i>	Pairwise comparisons
<i>Porites cylindrica</i>						
	C	0.012	2	4.764	0.012	1> (2=3)
	OA	0.004	2	1.708	0.190	na
	OAT	0.003	2	1.377	0.260	na
	T	0.004	2	1.799	0.174	na
Kruskal-Wallis		Mean Rank	df	Chi-Square	<i>p</i>	Pairwise comparisons
<i>Acropora digitifera</i>						
	C	10.17, 7.50, 10.83	2	1.310	0.519	na
	OA	11.83, 5.83, 10.83	2	4.351	0.114	na
	OAT	11.50, 8.83, 8.17	2	1.310	0.519	na
	T	12.50, 8.67, 7.33	2	3.029	0.220	na
<i>Montipora digitata</i>						
	C	11.17, 7.00, 10.33	2	2.047	0.359	na
	OA	9.00, 11.17, 8.33	2	0.924	0.630	na
	OAT	10.17, 10.17, 8.17	2	0.561	0.755	na
	T	11.00, 8.83, 8.67	2	0.713	0.700	na

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Table 6 Summary of statistical analyses on chl⁻¹ cell⁻¹ of colonies 1, 2 and 3 of each species in pCO₂-temperature treatments including C= (400 µatm- 28°C), T= (400 µatm-31°C), OA= (1000 µatm-28°C) and OAT= (1000 µatm-31°C) after 26 days. Significant differences are shown in bold. “na” means post-hoc test was not applicable because of no significant differences.

	Kruskal-Wallis	Mean Rank	df	Chi-Square	p	Pairwise comparisons
<i>Acropora digitifera</i>						
	C	3.83, 11.17, 13.50	2	10.713	0.005	1<(2=3)
	OA	8.67, 10.00, 9.83	2	0.222	0.895	na
	OAT	13.67, 8.17, 6.67	2	5.719	0.057	na
	T	7.50, 8.33, 12.67	2	3.240	0.198	na
<i>Montipora digitata</i>						
	C	14.17, 10.33, 4.00	2	11.099	0.004	(1=2) > 3
	OA	11.83, 11.83, 4.83	2	6.877	0.032	(1=2)>3
	OAT	6.00, 13.17, 9.33	2	5.415	0.067	na
	T	11.67, 13.00, 3.83	2	10.327	0.006	(1=2) > 3
<i>Porites cylindrica</i>						
	C	7.17, 11.17, 10.17	2	1.825	0.402	na
	OA	5.67, 8.00, 14.83	2	9.556	0.008	(1=2) < 3
	OAT	10.33, 8.67, 9.50	2	0.292	0.864	na
	T	12.17, 8.83, 7.50	2	2.433	0.296	na

Fig. 1 Calcification rate of the coral colonies and pooled data of **a** *Acropora digitifera*, **b** *Montipora digitata*, **c** *Porites cylindrica* under $p\text{CO}_2$ -temperature treatments including C= (400 μatm - 28°C), T= (400 μatm -31°C), OA= (1000 μatm -28°C) and OAT= (1000 μatm -31°C) after 26 days. Bars show standard deviation. Treatments without any letter (a, b, c) in common show significant differences (see also Table S2).

Fig. 2 Protein content of the coral colonies and pooled data of **a** *Acropora digitifera*, **b** *Montipora digitata*, **c** *Porites cylindrica* under $p\text{CO}_2$ -temperature treatments including C= (400 μatm - 28°C), T= (400 μatm -31°C), OA= (1000 μatm -28°C) and OAT= (1000 μatm -31°C) after 26 days. Bars show standard deviation. Treatments without any letter (a, b, c) in common show significant differences (see also Table S2).

Fig. 3 *Symbiodinium* density of the coral colonies and pooled data of **a** *Acropora digitifera*, **b** *Montipora digitata*, **c** *Porites cylindrica* under $p\text{CO}_2$ -temperature treatments including C= (400 μatm - 28°C), T= (400 μatm -31°C), OA= (1000 μatm -28°C) and OAT= (1000 μatm -31°C) after 26 days. Bars show standard deviation. Treatments without any letter (a, b, c) in common show significant differences (see also Table S2).

Fig. 4 Photosynthetic efficiency, F_v/F_m , (ratio of final/initial measurements) of the coral colonies and pooled data of **a** *Acropora digitifera*, **b** *Montipora digitata*, **c** *Porites cylindrica* under $p\text{CO}_2$ -temperature treatments including C= (400 μatm - 28°C), T= (400 μatm -31°C), OA= (1000 μatm -28°C) and OAT= (1000 μatm -31°C) after 26 days. Bars show standard deviation.

Treatments without any letter (a, b, c) in common show significant differences (see also Table S2).

Fig. 5 Chl cell⁻¹ of the coral colonies and pooled data of **a** *Acropora digitifera*, **b** *Montipora digitata*, **c** *Porites cylindrica* under *p*CO₂-temperature treatments including C= (400 µatm-28°C), T= (400 µatm-31°C), OA= (1000 µatm-28°C) and OAT= (1000 µatm-31°C) after 26 days. Bars show standard deviation. Treatments without any letter (a, b, c) in common show significant differences (see also Table S2).

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4

Figure 5