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ORIGINAL PAPER

# High-temperature acclimation strategies within the thermally tolerant endosymbiont *Symbiodinium trenchii* and its coral host, *Turbinaria reniformis*, differ with changing $pCO_2$ and nutrients

**Kenneth D. Hoadley[1] · D. Tye Pettay[1] · Andréa G. Grottoli[2] · Wei-Jun Cai[1,3] · Todd F. Melman[4] · Stephen Levas[2,8] · Verena Schoepf[2,7] · Qian Ding[3,9] · Xiangchen Yuan[1] · Yongchen Wang[5] · Yohei Matsui[2] · Justin H. Baumann[2,6] · Mark E. Warner[1]**

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**Abstract** The dinoflagellate *Symbiodinium trenchii* associates with a wide array of host corals throughout the world, and its thermal tolerance has made it of particular interest within the context of reef coral resilience to a warming climate. However, future reefs are increasingly likely to face combined environmental stressors, further complicating our understanding of how *S. trenchii* will possibly acclimatize to future climate scenarios. Over a 33-day period, we characterized the individual and combined affects of high temperature (26.5 vs. 31.5 °C), $pCO_2$ (400 vs. 760 µatm), and elevated nutrients (0.4 and 0.2 vs. 3.5 and 0.3 µmol of $NO_3^-/NO_2$ and $PO_4^{3-}$, respectively) on *S. trenchii* within the host coral species *Turbinaria reniformis*. Global analysis across all treatments found temperature to be the largest driver of physiological change. However, exposure to elevated temperature led to changes in symbiont physiology that differed across $pCO_2$ concentrations. Net photosynthesis and cellular chlorophyll a increased with temperature under ambient $pCO_2$, whereas temperature-related differences in cellular volume and its affect on pigment packaging were more pronounced under elevated $pCO_2$. Furthermore, increased nutrients mitigated the physiological response to high temperature under both ambient and elevated $pCO_2$ conditions and represented a significant interaction between all three physical parameters. Individual responses to temperature and $pCO_2$ were also observed as cellular density declined with elevated temperature and calcification along with respiration rates declined with increased $pCO_2$. *Symbiodinium trenchii* remained the dominant symbiont population within the host across all treatment combinations. Our results reveal distinct physiological changes in response to high temperature within the *S.*

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* Kenneth D. Hoadley
khoadley@udel.edu

1 Present Address: School of Marine Science and Policy, University of Delaware, Lewes, DE, USA

2 School of Earth Sciences, The Ohio State University, Columbus, OH, USA

3 Department of Marine Sciences, University of Georgia, Athens, GA, USA

4 Reef Systems Coral Farm, New Albany, OH, USA

5 Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China

6 Present Address: Department of Marine Sciences, University of North Carolina, Chapel Hill, NC, USA

7 Present Address: ARC Centre of Excellence for Coral Reef Studies, UWA Oceans Institute and School of Earth and Environment, University of Western Australia, Crawley, WA, Australia

8 Present Address: Department of Geography and Environmental Science, Villanova University, Villanova, PA, USA

9 Present Address: Ocean College, Zhejiang University, Hangzhou, China

*trenchii*/*T. reniformis* symbioses that are dependent on $pCO_2$ and nutrient concentration, and represent important interactive effects to consider as we consider how corals will respond under future climate change scenarios.

## Introduction

For scleractinian corals, tolerance to high-temperature stress is influenced in part by the type of dinoflagellate algal symbiont (*Symbiodinium* spp.) living within the hosts' gastrodermal cells (e.g., Fitt et al. 2001; Hennige et al. 2011). Association with thermally tolerant symbionts is relevant, as corals face an ever-greater frequency of high-temperature events (Hoegh-Guldberg and Bruno 2010; Anthony et al. 2011; Manzello 2015). Although several *Symbiodinium* species have been described as thermally tolerant, *S. trenchii* is perhaps best known, with both global distribution and multiple host associations (LaJeunesse et al. 2009, 2014; Hennige et al. 2011). However, future climate projections suggest coral reefs will face the combined stress of increased temperature, high $pCO_2$ and nutrient levels (Hughes and Connell 1999; Hughes et al. 2003; Hoegh-Guldberg et al. 2007). Whether or not different genotypes of *S. trenchii* and their respective hosts are robust, stress-tolerant species under a combination of environmental stressors requires further investigation.

Previous studies report both positive and negative interactions between elevated temperature and $pCO_2$, which are likely dependent on the specific host/symbiont combination in question (Reynaud et al. 2003; Anthony et al. 2008; Rodolfo-Metalpa et al. 2010; Schoepf et al. 2013; Wall et al. 2014; Kwiatkowski et al. 2015). Enhanced carbon availability due to high $pCO_2$ can stimulate greater carbon fixation rates, thereby altering electron transport through the photosynthetic apparatus (Suggett et al. 2012; Brading et al. 2013), a common site for thermal damage (Warner et al. 1999). Additionally, higher carbon fixation may also provide greater photosynthate to the host (Hoadley et al. 2015b), which may be advantageous during high-temperature stress as an additional source of carbon. However, most beneficial effects of elevated $pCO_2$ such as increased photosynthetic rates, carbon uptake, growth rates, and asexual reproduction have only been observed within symbiotic anemones (Suggett et al. 2012; Towanda and Thuesen 2012; Gibbin and Davy 2014; Hoadley et al. 2015b). Effects of elevated $pCO_2$ appear more varied within scleractinian coral species (Comeau et al. 2009, 2013; Edmunds et al. 2013; Schoepf et al. 2013), with reductions, no change, and increases in calcification rates observed across various coral species.

High rates of PSII reaction center repair are critical for maintaining PSII maximum quantum yields during thermal stress (Takahashi et al. 2004, 2009; Smith et al. 2005). However, Symbiodinium living *in hospite* are thought to be nitrogen and phosphorus limited (Cook et al. 1994). Increased nutrient concentrations may mitigate thermal stress by improving rates of repair to the photosynthetic apparatus. Similarly, for certain host/symbiont combinations, increased heterotrophy, and hence nutrient delivery, during thermal stress can minimize reductions in PSII maximum quantum yields by improving nitrogen availability (Borell and Bischof 2008; Borell et al. 2008). Improved nutrient availability may also be beneficial under high $pCO_2$ conditions as elevated nitrate and phosphate concentrations ameliorated $CO_2$ induced reductions in calcification within the temperate coral species *Astrangia poculata* (Holcomb et al. 2010). Similarly, increased heterotrophy enabled the massive *Porites* spp. to resist $CO_2$ induced reductions in calcification (Edmunds 2011). Despite these examples of positive impacts of increased nutrient availability on coral physiology, negative effects from elevated nutrient concentrations may also be present. For symbionts *in hospite,* nutrient availability is largely influenced by the host (Rands et al. 1993; Yellowlees et al. 2008). Therefore, increased environmental nutrient concentrations may disrupt the carefully balanced host/algal symbioses (Cook et al. 1994), potentially leading to a loss in coral growth and/or resilience (Marubini and Atkinson 1999). Increased dissolved inorganic nutrients have been linked to increased disease prevalence (Vega Thurber et al. 2014) and reductions in the bleaching threshold for certain coral species (Wooldridge 2009; Wiedenmann et al. 2013). Increased nutrient concentrations may also lead to greater symbiont cell density within certain coral species (D'Angelo and Wiedenmann 2014). On an ecosystem level, increased nitrogen concentrations can benefit spatially competitive macroalgal species, impeding recovery of corals during and after bleaching events through physical contact and encroachment onto damaged tissue (Aronson and Precht 2000; Furman and Heck 2008; Smith et al. 2010).

Previous studies characterized the coral *Turbinaria reniformis* as having relatively high biomass and total energy reserves as compared to other Pacific coral species (Schoepf et al. 2013). Energy stores in the form of lipids, carbohydrates and protein content form the majority of a coral's tissue biomass and species with greater energetic reserves are more likely to recover from thermal bleaching events, when energy-rich symbiont photosynthate is reduced (Grottoli et al. 2004, 2014; Schoepf et al. 2015). Additionally, greater tissue biomass may also be advantageous during thermal stress as thicker tissue provides better photoprotection to the symbionts (Loya et al. 2001; Dimond et al. 2012).

This study tests for the presence of interactive effects between elevated temperature, $pCO_2$, and nutrients on the

photobiology and physiology of *S. trenchii* and its host coral species *Turbinaria reniformis* over a 33-day period. Specifically, we were interested in whether the physiological response to elevated temperature differs when combined with either elevated $pCO_2$ and/or nutrient concentrations. Overall, physiological changes in response to elevated temperature were observed primarily within the symbiont. However, temperature-induced changes in symbiont physiology differed across $pCO_2$ concentrations and were mitigated under elevated nutrient conditions. We confirm the presence of interactive effects across all three parameters (temperature, $pCO_2$ and nutrients) within this host/symbiont combination and discuss potential implications of these results in the context of future climate change scenarios.

## Materials and methods

The experimental systems, along with many of the methodologies used in this current study are the same as those utilized in an earlier companion study which are described in greater detail within (Schoepf et al. 2013; Hoadley et al. 2015a). In brief, six colonies of *T. reniformis* were collected at a depth of between 3 and 10 m in northwest Fiji, and transported to a coral aquaculture facility in New Albany, Ohio (Reef Systems Coral Farm). Coral colonies were maintained within the aquaculture facility for over 16-month prior to the start of this experiment in October, 2012. Eight fragments from each colony were removed and mounted on 2-in. plastic tiles using coral glue (Eco Tech). After 1 month of recovery, coral fragments were transferred into the experimental systems and slowly acclimated over 10 days to synthetic seawater closely resembling natural seawater chemistry with regards to dissolved inorganic carbon and total alkalinity (ESV Aquarium Products Inc.). Corals were further acclimated to the experimental systems for an additional month prior to the start of the experiment. The experimental system was comprised of eight separate seawater treatment systems, each consisting of six, 57-L aquaria connected to a central 905-L sump. For each of the eight treatment systems, one fragment from each of the six colonies was placed into each treatment system, with a separate colony fragment in each replicate tank for a sample size of six per experimental system. Corals were maintained under a 12:12-h light/dark cycle with fluorescent lights (Tek Light T5), providing 275 μmol quanta $m^{-2}$ $s^{-1}$ at the base of the filled aquaria. After initial acclimation to the experimental systems, each treatment ran for 33 days. A 25 % volume water change was completed every 3 days on each system, and aquaria were kept free of any bio-fouling by periodic cleaning. Salinity was maintained at 35 ppt through daily top-offs with reverse-osmosis-filtered fresh water. Corals were fed freshly hatched *Artemia nauplii* twice a week.

The experimental treatments consisted of all combinations of ambient (26.5 °C) and high (31.5 °C) temperature, ambient (400-μatm) and high $pCO_2$ (760-μatm), and ambient (0.4-μmol $NO_3$/$NO_2$ and 0.2-μmol $PO_4$) and high (3.5-μmol $NO_3$ and 0.3-μmol $PO_4$) nutrient concentrations, for a total of eight separate treatment conditions. Temperature and $pCO_2$ levels were selected as described in Schoepf et al. (2013). Briefly, ambient temperatures represent average annual temperatures for Fiji and elevated temperatures reflect the bleaching threshold for the area (www.ospo.noaa.gov/Products/ocean/index.html). $CO_2$ conditions reflect the current global average, whereas the elevated $CO_2$ conditions reflect conditions predicted by the end of this century (IPCC 2013). Nutrient concentrations were deliberately chosen to only be slightly higher than ambient conditions. Temperature within the high-temperature treatment tanks was slowly increased (0.5 °C $day^{-1}$) to a maximum of 31.5 °C. Temperature was maintained using titanium heaters housed in each sump and regulated with a digital controller (Apex AquaController, Neptune Sys).

$pCO_2$ within each sump was controlled by a pH stat system for precise control of air and $CO_2$ gas input into each sump (KSgrowstat, University of Essex). For elevated $pCO_2$ treatments, $CO_2$ was increased 100 μatm $day^{-1}$ until the desired $pCO_2$ concentration was met. Within each treatment, pH measurements were taken every 30-s with a glass microelectrode (Thermos Scientific Orion Ross Ultra pH glass electrode) which then controlled a series of solenoids for delivery of $CO_2$ gas, air, or $CO_2$-free air (provided by a soda lime scrubber). All pH electrodes were recalibrated daily to NBS standards and independent measurements of pH and alkalinity were made using an AS-ALK2 (Apollo SciTech Inc) titrator according to published protocols (Cai et al. 2010). Seawater carbonate chemistry based on pH and alkalinity measurements was calculated using the CO2SYS program (Lewis et al. 1998) and is reported in Table 1. TA values were compared against known seawater standards provided by the laboratory of Dr. A Dickson (San Diego, CA, USA).

During the experiment, daily concentrations of $NO_3$ and $PO_4$ in each treatment were measured spectrophotometrically using the Hach Nitrate (Method 8192) and Phosphate (Method 8084) assay kits. Based on these measurements, appropriate additions of $KNO_3$ and $KH_2PO_4$ from 1 M stock solutions were made in order to bring nutrient treatment concentrations back up to 3.5 μmol $NO_3$ and 0.3 μmol $PO_4$, respectively. One hour after the addition of nutrients, concentrations of $NO_3$ and $PO_4$ were again spectrophotometrically measured in order to verify the correct amount of nutrients was added. Because nutrient levels would decrease throughout the following 24-h, these nutrient additions can be described as pulses as opposed to a stable nutrient concentration. In addition to the spectrophotometric

**Table 1** Mean (±1 SE) carbonate chemistry parameters for the eight treatments representing two $pCO_2$ levels (400 vs. 760 µatm), nutrient concentrations (low vs. high) and two temperatures (26.5 vs. 31C) ($n = 20–26$)

| | Temp (°C) | pH (NBS scale) | TA (µmol $kg^{-1}$) | $pCO_2$ (µatm) | $xCO_2$ (ppm) | $Sl_{arag}$ |
|---|---|---|---|---|---|---|
| LT + LC + LN | 26.06 ± 0.05 | 8.19 ± 0.01 | 2350.87 ± 18.12 | 410.63 ± 17.42 | 424.50 ± 18.01 | 3.59 ± 0.09 |
| HT + LC + LN | 29.34 ± 0.41 | 8.18 ± 0.01 | 2302.01 ± 16.34 | 414.56 ± 10.34 | 432.27 ± 10.90 | 3.81 ± 0.08 |
| LT + HC + LN | 26.60 ± 0.16 | 7.97 ± 0.02 | 2333.18 ± 11.15 | 752.82 ± 30.49 | 778.95 ± 31.50 | 2.41 ± 0.09 |
| HT + HC + LN | 30.13 ± 0.30 | 7.98 ± 0.01 | 2338.55 ± 12.31 | 741.93 ± 25.56 | 774.34 ± 26.90 | 2.74 ± 0.07 |
| LT + LC + HN | 26.51 ± 0.03 | 8.22 ± 0.01 | 2293.55 ± 14.16 | 358.34 ± 6.50 | 370.76 ± 6.71 | 3.74 ± 0.06 |
| HT + LC + HN | 30.01 ± 0.30 | 8.17 ± 0.01 | 2319.94 ± 12.85 | 423.89 ± 10.38 | 442.26 ± 10.95 | 3.84 ± 0.06 |
| LT + HC + HN | 26.33 ± 0.04 | 7.97 ± 0.01 | 2377.92 ± 8.78 | 752.10 ± 28.14 | 777.88 ± 29.10 | 2.45 ± 0.07 |
| HT + HC + HN | 29.59 ± 0.33 | 7.96 ± 0.02 | 2365.49 ± 11.51 | 794.31 ± 32.51 | 828.10 ± 34.20 | 2.64 ± 0.08 |

*HT* High temperature, *LT* low temperature, *HC* high $pCO_2$, *LC* low $pCO_2$, *HN* high nutrients and *LN* low nutrients

**Table 2** Mean (±1 SE) nutrient concentrations for the eight treatments representing two $pCO_2$ levels (400 vs. 760 µatm), nutrient concentrations (low vs. high) and two temperatures (26.5 vs. 31C) ($n = 20–26$)

| | TN (µmol N/L) | $NO_3/NO_2$ (µmol N/L) | $NH_3$ (µmol N/L) | TP (µmol P/L) | Ortho $PO_4$ (µg P/L) |
|---|---|---|---|---|---|
| LT + LC + LN | 3.81 ± 1 | 0.39 ± 0.08 | 0.26 ± 0.19 | 0.24 ± 0.02 | 6.54 ± 0.29 |
| HT + LC + LN | 3.97 ± 1.45 | 0.35 ± 0.05 | 0.49 ± 0.65 | 0.27 ± 0.02 | 6.57 ± 0.35 |
| LT + HC + LN | 5.67 ± 2.24 | 0.49 ± 0.19 | 0.22 ± 0.09 | 0.25 ± 0.02 | 6.84 ± 0.28 |
| HT + HC + LN | 3.88 ± 0.79 | 0.41 ± 0.22 | 0.24 ± 0.11 | 0.25 ± 0.03 | 7.41 ± 1.33 |
| LT + LC + HN | 7.40 ± 1.58 | 3.48 ± 0.64 | 0.55 ± 0.67 | 0.32 ± 0.02 | 9.97 ± 1.85 |
| HT + LC + HN | 7.10 ± 1.55 | 3.52 ± 1.21 | 0.32 ± 0.47 | 0.32 ± 0.09 | 9.81 ± 1.85 |
| LT + HC + HN | 7.62 ± 2.67 | 3.68 ± 1.60 | 0.16 ± 0.17 | 0.29 ± 0.04 | 9.04 ± 1.89 |
| HT + HC + HN | 7.19 ± 0.88 | 3.56 ± 0.96 | 0.19 ± 0.09 | 0.32 ± 0.07 | 9.23 ± 2.35 |

*HT* High temperature, *LT* low temperature, *HC* high $pCO_2$, *LC* low $pCO_2$, *HN* high nutrients and *LN* low nutrients

measurements mentioned above, water samples were collected into a 10 % acid-cleaned 50-mL syringe approximately 1 h after each nutrient addition and filtered through a GF/F filter (0.7-µm nominal pore size) into 10 % acid-cleaned 30-mL polycarbonate bottles. These samples were immediately frozen until nutrient concentration measurements were made on a three-channel Lachat QuikChem 8500 in order to quantify total nitrogen (TN), nitrate/nitrite ($NO_3/NO_2$), ammonia ($NH_3$), total phosphorus (TP), and orthophosphate ($PO_4$) (Table 2). Inorganic nutrient fractions were analyzed simultaneously using the following chemical methods from Lachat instruments: 10-107-06-1-M ($NH_3$); 10-107-04-1-C/J ($NO_3/NO_2$); 10-115-01-1-M ($PO_4$). TN and TP methods were performed using in-line digestions (K2S2O8 oxidation): 10-107-04-3-A (TN); 10-115-01-3-F (TP). The sample loops used with this instrument offered sensitivity to 0.002 mg $L^{-1}$ $PO_4$, 0.005 mg $L^{-1}$ $NO_2$, 0.01 mg $L^{-1}$ $NH_3$, 0.002 mg $L^{-1}$ TP and 0.01 mg $L^{-1}$ TN.

## Symbiont identification

Symbionts were identified through amplification of the internal transcribed spacer 2 region (ITS2) of the ribosomal array and analysis by previously published protocols for denaturing gradient gel electrophoresis (DGGE) and cycle sequencing (LaJeunesse et al. 2003).

## Symbiont photophysiology

Dark acclimated maximum quantum yield of photosystem II ($Fv/Fm^{MT}$) was measured every other day, 1 h after the end of the light period by pulse amplitude modulation fluorometry (Diving PAM, Waltz, Germany). Fragments were probed in three separate locations using a 0.6-s saturation pulse (saturation intensity >8000-µmol quanta $m^{-2}$ $s^{-1}$) and then averaged together in order to calculate a colony mean maximum quantum yield of PSII. In addition, the single turnover maximum quantum yield of PSII ($Fv/Fm^{ST}$), functional absorption cross section of PSII ($\sigma PSII$), and the maximum electron transport rate between $Q_A$ and $Q_B$ on the acceptor side of the PSII reaction center were collected with a Fluorescence Induction and Relaxation (FIRe) fluorometer (Satlantic Inc., Halifax) (Gorbunov and Falkowski 2004) on the final night of the experiment. Measurements were taken 1 h after the start of the dark period and consisted of five iterations of a 120-µs single turnover flash

followed by a 2000-μs relaxation phase consisting of 1-μs flashes of a weaker light spaced 59 μs apart. Each of the five iterations was spaced 5 seconds apart and provided by an external blue (455 nm) LED light source (Satlantic Inc, Halifax). All photochemical parameters listed above were calculated by fitting each fluorescence transient curve using the FIREPRO software (Kolber and Falkowski 1998). Excitation via a single turnover fluorometer (such as the FIRe flurometer) only reduces the primary electron acceptor ($Q_A$) within the PSII reaction center, whereas the longer saturation flash of PAM fluorometer reduces both primary and secondary acceptors within the PSII reaction center and the plastoquinone pool (Suggett et al. 2003).

On the final day, maximal photosynthetic rates and light acclimated dark respiration ($R_L$) were measured via oxygen evolution and consumption with galvanic electrodes (Qubit systems) housed in clear acrylic chambers (350-mL). Chambers were surrounded by a water bath to maintain the control and experimental temperatures. Constant circulation was provided by a stirbar in each chamber. Illumination was supplied by a customized 24 LED array (Cree Cool White XP-G R5). In order to ensure maximal photosynthetic rates, light intensity was set to 600 μmol quanta $m^{-2}$ $s^{-1}$, and pilot experiments at this light intensity showed no signs of photoinhibition (not shown). Maximal net photosynthesis $_{(Pmaxnet)}$ was recorded for 15–20 min, followed by a 10-min dark incubation after the lights were switched off to record the light acclimated dark respiration ($R_L$). After incubation, coral fragments were returned to their respective treatments tanks. The photosynthesis to respiration ratio was calculated as $_{(Pmaxgross)/(RL)}$ where $Pmax_{gross} = (Pmax_{net} + R_L)$. Gross photosynthesis and light acclimated dark respiration ($R_L$) were normalized to total surface area ($cm^2$) for each coral fragment (described below) and net photosynthesis was normalized to algal cell number.

### Host and symbiont physiology

At the end of the 33-day treatment, samples were frozen in liquid $N_2$ and stored at −80 °C until further processing. Coral tissue was removed with a water pick (Johannes and Wiebe 1970) in 40 mL of recirculating synthetic seawater. The resulting slurry was homogenized with a TissueTearorTM (BioSpec products, Inc), and then centrifuged for 5 min (5000g) to separate the algal symbiont and coral fractions. Pelleted symbionts were resuspended in synthetic seawater and divided into 1 mL aliquots. For algal cell density and volume calculations, an aliquot was preserved with 10 μL of 1 % glutaraldehyde. Six independent replicate counts were performed for each algal sample on a hemocytometer. Samples were photographed using a Nikon microphot-FXA epifluorescent microscope (×100 magnification). Photographs were then analyzed by computer using Image J (NIH) with the analyze particles function. Pixel size of each cell was converted to $\mu m^2$ using a calibrated scale micrometer and then used to calculate cell diameter and volume based on calculations for a sphere.

For symbiont cell protein concentration, 1-mL samples were homogenized with a bead-beater (BioSpec) for 2 min and then analyzed using the BCA method by absorbance at 595 nm (Smith et al. 1985) (Thermo Scientific Pierce), with bovine serum albumin standards. For determination of chlorophyll a, a second 1 mL aliquot of pelleted symbionts was resuspended in 90 % methanol and then homogenized via bead beating for 2 min. Samples were then incubated at −20 °C for 2 h and then centrifuged at 2300g for 5 min. Absorbance of the resulting supernatant was measured at 665, 652 and 750 nm and chlorophyll a calculated by published equations (Porra et al. 1989). All absorbance measurements were recorded by a FLUOstar Omega plate reader (BMG labtech). Coral skeletal surface area was determined by the foil method (Marsh 1970).

### Calcification rates

Net calcification was determined using the buoyant weight technique (Jokiel et al. 1978) and then converted to dry weight. Each coral fragment was buoyantly weighed at the beginning and end of the experiment. Daily calcification rates were calculated as the difference between initial and final weights, divided by number of experimental days, and standardized to coral surface area.

### Statistical analysis

Physiological variables were split into symbiont specific ($FvFm^{ST}$, $\sigma_{PSII}$, chlorophyll a cell$^{-1}$, symbiont cellular volume, photosynthesis cell$^{-1}$, and symbiont protein cell$^{-1}$) and holobiont (Cellular density, PR ratio, LEDR and Calcification) physiological parameters. Symbiont-specific and holobiont physiological parameters were analyzed using an ANalyses Of SIMilarities test (ANOSIM) with 9999 permutations to test for significant separation between temperature, $CO_2$, and nutrient treatments. Because only temperature revealed any significant separation, ANOSIM was again utilized to test for significant temperature affects within each $CO_2$ and nutrient combination. Non-metric multidimensional scaling (nMDS) on Euclidean distances after log(x + 1) transformation (Ziegler et al. 2014) for each set of physiological variables was also utilized to visualize separation across treatment groups. Multivariate analysis was followed up with univariate analysis in order to better elucidate small-scale differences observed among individual variables. A three-factor analysis of variance (ANOVA) was utilized to test for significant effects

and any possible interactive effects between $pCO_2$, temperature, and nutrients. When significant differences were observed, a Tukey's post hoc test was performed for analysis between different factor combinations. If there was a significant interaction between all three factors, a pairwise analysis between all eight treatments was performed, and the main effects were ignored if interactive effects were observed. All data sets were tested for homogeneity of variance and normality of distribution using the Levene and Shapiro Wilks tests, respectively. If either test was significant ($P < 0.05$), the data were log-transformed and retested prior to further analysis. For PAM-based measurement of the maximum quantum yield of PSII ($Fv/Fm^{MTT}$), a generalized linear mixed model (GLMM) was used to test for the effects of time, temperature, $pCO_2$, and nutrients (Supplementary Table 3). All statistical analyses were performed using R software with 'ez', 'car' and 'pgirmess' packages installed. Resulting statistical tables can be found within the supplementary materials (Table S1-5).

## Results

### Symbiont identification

*Symbiodinium trenchii* (ITS2-type *D1a*) was the only symbiont detected within all fragments of *T. reniformis* throughout the duration of the experiment.

### Multivariate analysis

Global analysis across all treatments found only temperature-induced significant separation (ANOSIM: $R = 0.220$, $P = 1e{-}04$), and only with respect to symbiont-specific physiology (Fig. 1). However, dissimilarity between ambient and elevated temperature treatments was only significant under low nutrient conditions as elevated nutrient concentrations mitigated the thermal response for both ambient and elevated $pCO_2$ treatments. In addition, the nMDS analysis shows the direction of thermal separation within the low nutrient treatments differs between ambient and elevated $pCO_2$ conditions, suggesting that the physiological changes in response to temperature also differ (Fig. 1).

### Symbiont physiology

Net photosynthesis $cell^{-1}$ increased with temperature by an average of 47 % but only at low $pCO_2$ levels ($P = 0.03839$) (Fig. 2a; Table S1). Cellular volume increased with temperature ($P < 0.0001$) and decreased with $pCO_2$ ($P = 0.0138$) (Fig. 2b; Table S1). Chlorophyll *a* cell$^{-1}$ significantly increased (av. 38 %) with temperature but only within the ambient $pCO_2$ treatments ($P = 0.0109$) (Fig. 2c; Table S1). Cell protein concentration increased significantly with temperature ($P = 0.0087$) on average by 21 % (Fig. 2d; Table S1).

### Holobiont physiology

Cell density declined by an average of 34 % with increasing temperature ($P = 0.00857$), irrespective of $pCO_2$ or nutrient concentration (Fig. 3a; Table S2). Likewise, calcification rates decreased significantly by 35 % with elevated $pCO_2$ ($P = 0.01$) (Fig. 3b; Table S2). Light enhanced dark respiration decreased significantly by 37 % ($P = 0.0189$) with elevated $pCO_2$ (Fig. 3c; Table S2). The ratio of photosynthesis to respiration (P:R) increased 37 % with elevated $pCO_2$ but only under low nutrient concentrations ($P = 0.0210$) (Fig. 3d; Table S2).

### Photochemistry

On the final day of the experiment, $Fv/Fm^{ST}$, as measured by single turnover chlorophyll fluorometry, was significantly reduced by 20 % with elevated temperature and nutrients but only within the high $pCO_2$ treatments ($P = 0.015$) (Fig. 4a; Table S4). A significant interactive effect among all three factors ($P = 0.0129$) was observed for the functional absorption cross section of PSII ($\sigma_{PSII}$), which was 40 % higher in the high $pCO_2$ treatment compared to the ambient $pCO_2$ treatment but only under ambient temperature and nutrient conditions (Fig. 4b; Table S4).

Although no significant four-way interaction between time, temperature, $pCO_2$ and nutrient concentration was noted for $Fv/Fm^{MTT}$, minimal yet significant interactions ($P < 0.0001$) between temperature, $pCO_2$ and nutrient concentrations were noted (Table S3). Under elevated nutrient conditions, $Fv/Fm^{MTT}$ declined due to either increased $pCO_2$ and/or elevated temperature, whereas under low nutrients, $Fv/Fm^{MTT}$ declined only when both $pCO_2$ and temperature were elevated (Fig. 5). Elevated $pCO_2$ also decreased $Fv/Fm^{MTT}$ but only under elevated nutrient conditions (Fig. 5).

## Discussion

### Symbiont physiology

Although previous studies have observed interactive effects between elevated temperature and $pCO_2$ or nutrient concentrations on coral physiology (Holcomb et al. 2010; Edmunds 2011; Schoepf et al. 2013; Wall et al. 2014; Kwiatkowski et al. 2015), few studies have tested interactive effects between all three factors. In this study, temperature was the main factor driving physiological change.

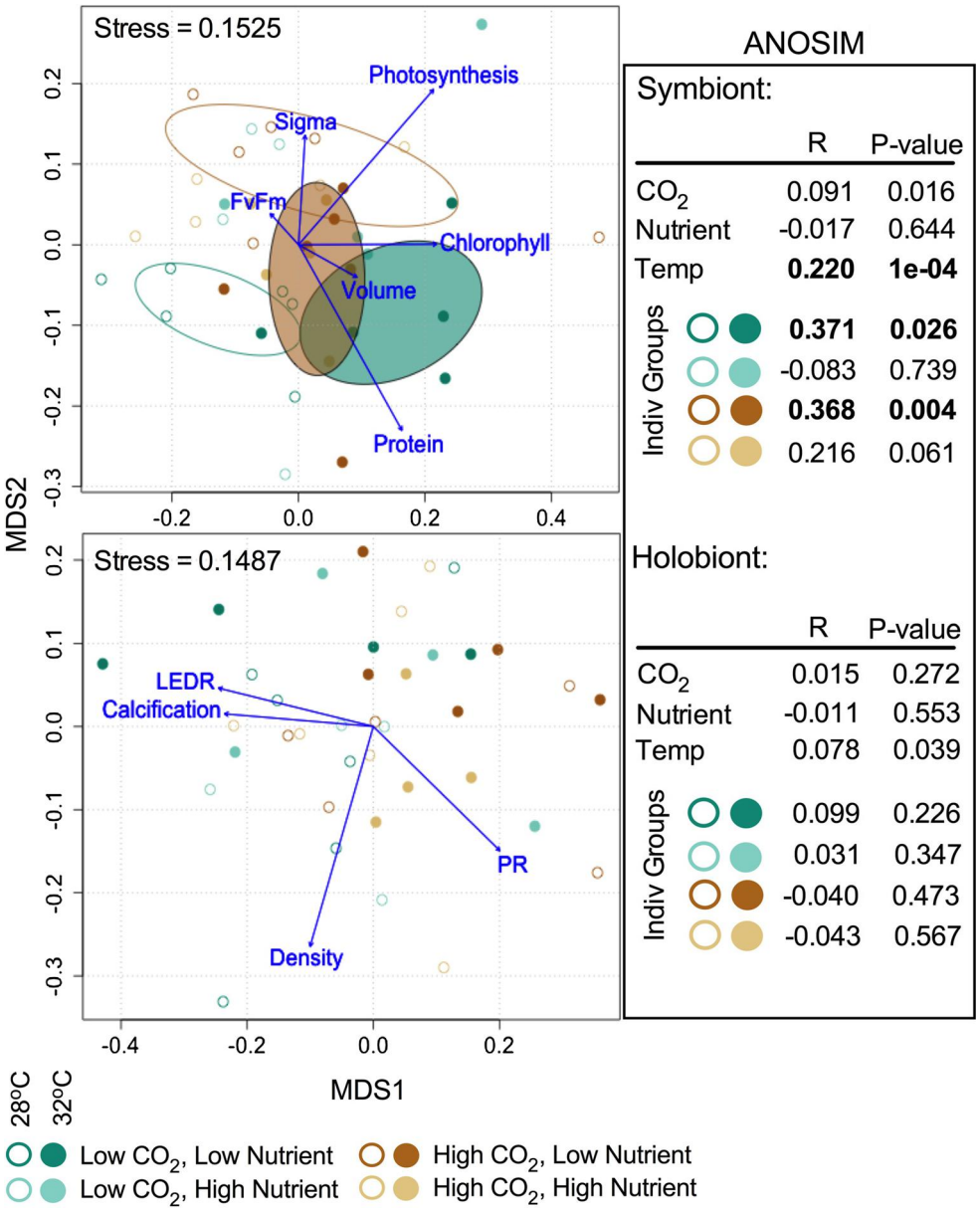


**Fig. 1** Multivariate analysis: non-metric multidimensional scaling (nMDS) plot. *Top*: symbiont physiology and photobiology and *bottom*: holobiont physiology. *Colors* depict each of the four $pCO_2$ and nutrient treatments. *Closed circles* represent low-temperature treatments and *open circles* represent high-temperature treatments. Because global analysis via ANOSIM only found significant separation with respect to temperature, subsequent multivariate analysis test for temperature effects within each $pCO_2$ and nutrient treatment and are included within the figure (Table). Ellipses represent a 95 % confidence bubble around the mean for low-temperature (*open ellipse*) and high-temperature (*closed ellipse*) treatments and are displayed only for groups with significant separation as observed using ANOSIM

However, there was a clear difference in how *S. trenchii* responded to elevated temperature while under ambient or elevated $pCO_2$ treatments. Under ambient $pCO_2$ conditions, increased net photosynthesis (as normalized algal cell $^{-1}$) and chlorophyll content accompanied reduced cell density at elevated temperatures (Figs. 1a, 2c). Symbiont reductions increase the availability of dissolved inorganic carbon (DIC) for remaining symbionts (Wooldridge, 2009), potentially explaining the increase in productivity rates observed under ambient $pCO_2$ conditions (Weis et al. 1989; Weis 1993; Weis and Reynolds 1999). In addition, no high-temperature-induced change in Fv/Fm or functional cross section of PSII is observed under ambient $pCO_2$ and nutrient conditions, suggesting the physiological changes observed in *S. trenchii* indicate minimal, if any, thermal stress. In contrast, under high $pCO_2$ conditions, the drop in cell density with high temperature was not accompanied by increased chlorophyll a or net photosynthesis (Figs. 2a, c, 3c). Interestingly, while cell density decreased with elevated temperature, cellular volume of the remaining *Symbiodinium* increased (Figs. 2c, 3a). Increased cell volume in free-living phytoplankton is typically associated with a higher 'package effect' where greater chlorophyll concentrations within larger cells increase self-shading and thereby attenuate the light intensity within the cell (Finkel 2001; Key et al. 2010). Increased chlorophyll concentration with temperature may have increased the package effect under ambient $pCO_2$; however, no increase was observed within the elevated $pCO_2$ treatments. Static chlorophyll content, combined with increased cellular volume, would

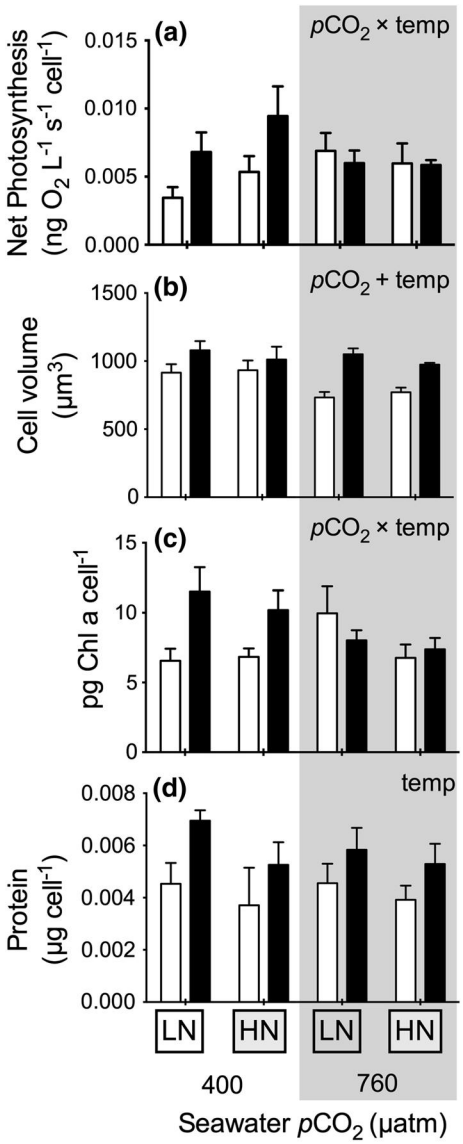


**Fig. 2** Cell physiology: average (±1 SE) net photosynthesis cell$^{-1}$ (**a**), cell volume (**b**), chlorophyll *a* cell$^{-1}$ (**c**) protein cell$^{-1}$ (**d**) at two $pCO_2$ levels, nutrient concentrations (*LN* low nutrients, *HN* high nutrients) and 26.5 °C (*light bars*) or 31.5 °C (*dark bars*). For each *panel*, the designations 'temp', '$pCO_2$' and 'nutr' indicate significant temperature, $pCO_2$, nutrient concentration, or their interactive effects (multifactorial ANOVA results in Table S1). $n$ = 5–6 per average

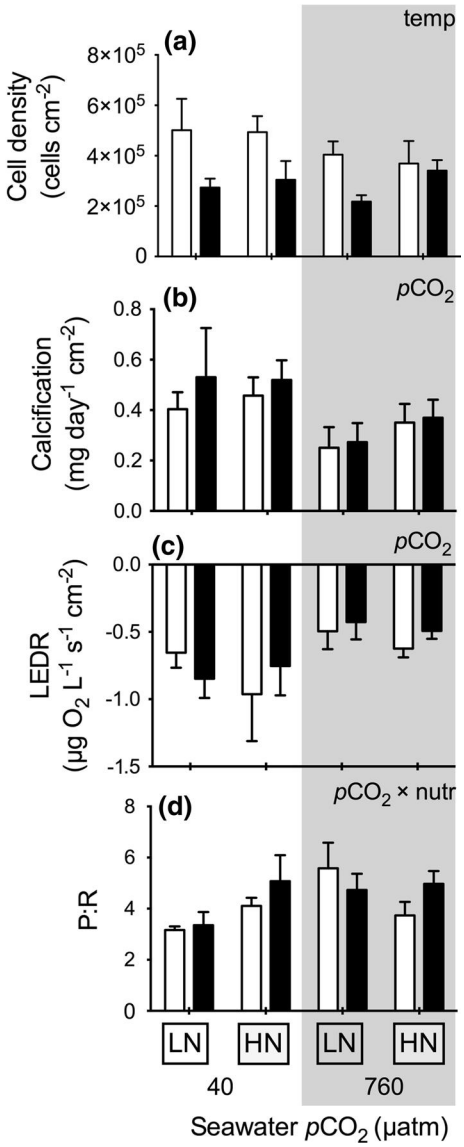


**Fig. 3** Holobiont physiology: average (±1 SE) cell density (**a**), calcification rates (**b**), light enhanced dark respiration cm$^{-2}$ (LEDR) (**c**), photosynthesis to respiration (P:R) (**d**) at two $pCO_2$ levels, nutrient concentrations (*LN* low nutrients, *HN* high nutrients) and 26.5 °C (*light bars*) or 31.5 °C (*dark bars*). For each *panel*, the designations 'temp', '$pCO_2$' and 'nutr' indicate significant temperature, $pCO_2$, nutrient concentration, or their interactive effects (multifactorial ANOVA results in Table S2). $n$ = 5–6 per average

decrease pigment packaging and enhance light intensity within the cell. This in turn may have increased the excess excitation energy within the symbionts subjected to high temperature, thus leading to the decline in PSII maximum quantum yield (for both $Fv/Fm_{MI}$ and $Fv/Fm_{SI}$) observed under elevated $pCO_2$ and ambient nutrient levels.

Similar to that observed in another study that examined the physiological impacts of ocean acidification and temperature in four Pacific coral species (Hoadley et al. 2015a), algal cellular protein levels increased with temperature and were unaffected by $pCO_2$ in this study (Fig. 2d). However, increased symbiont protein content at low $pCO_2$ was most likely due to the coinciding increase in chlorophyll *a*, whereas chlorophyll *a* did not change in the high $pCO_2$ treatments. Although algal proteins increased with temperature at both $pCO_2$ concentrations, the type of proteins synthesized may have differed. Host and symbiont heat-shock protein expression are commonly unregulated in response to thermal stress (Leggat et al. 2011; Rosic

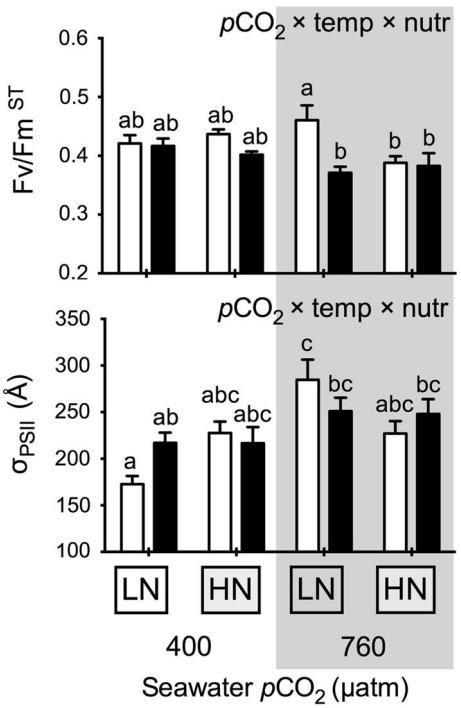


**Fig. 4** Single turnover fluorometry: average (±1 SE). $Fv/Fm^{ST}$ (**a**), functional absorption cross section of PSII (**b**) at two $pCO_2$ levels, nutrient concentrations (*LN* low nutrients, *HN* high nutrients) and 26.5 °C (*light bars*) or 31.5 °C (*dark bars*). For each *panel*, the designations 'temp', '$pCO_2$' and 'nutr' indicate significant temperature, $pCO_2$, nutrient concentration, or their interactive effects (multifactorial ANOVA results in Table S4). If an interactive effect between all three factors was observed, *letters* above each *bar* indicate significant differences among the 8 treatments. *n* = 5–6 per average

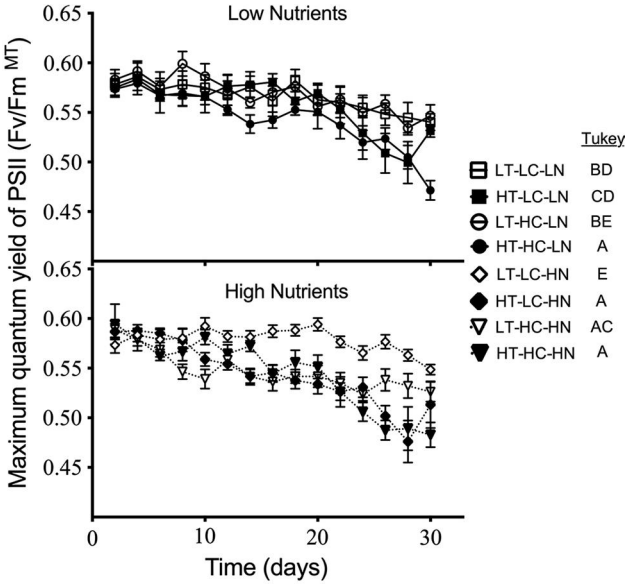


**Fig. 5** Maximum photosynthetic efficiency of PSII ($Fv/Fm^{MT}$): average (±1 SE) in *S. trenchii* (*T. reniformis*) at low nutrients (*top panel*) and high nutrients (*bottom panel*). Treatment abbreviations are *LT* low temperature, *HT* high temperature, *LN* low nutrients, *HN* high nutrients, *LC* low $CO_2$ and *HC* high $CO_2$. *Letters* next to treatment abbreviations indicate significant differences among the eight treatments (Tukey's post hoc). *n* = 5–6 per average

et al. 2011), and under high $pCO_2$ and temperature, heat-shock protein synthesis may have been higher relative to ambient conditions. Additionally, elevated Rubisco content may have also accounted to the increased protein content at high temperature. Greater photosynthetic carbon acquisition has been observed in response to elevated temperature within certain *Symbiodinium* strains (Oakley et al. 2014). Similarly, Rubisco activity and gene expression were both elevated with temperature in the marine Diatom *Thalassiosira weissflogii* (Helbling et al. 2011). Such different patterns in cellular protein content highlight another important interactive effect between environmental stressors that warrants further investigation.

Despite the differences observed in response to elevated temperature between ambient and elevated $pCO_2$ conditions, the multivariate analysis showed dissimilarity between ambient and elevated temperature groups is minimized under elevated nutrient conditions (Fig. 1). Additional nutrients to the host and symbiont provided through increased feeding rates or food availability can lead to lower rates of both bleaching and photosynthetic damage during high-temperature events (Grottoli et al. 2006; Ferrier-Pagès et al. 2010; Tolosa et al. 2011; Béraud et al. 2013). For cultured *Symbiodinium* grown under nutrient replete conditions, enhanced nitrate reductase activity can provide an important electron sink within the photosynthetic apparatus, thereby helping to reduce partial pressure over the PSII reaction center (Lomas and Glibert 1999; Rodríguez-Román and Iglesias-Prieto 2005). In addition, greater nutrient availability within the alga can also increase repair rates of the D1 protein in PSII, as nitrogen is no longer a limiting factor in protein synthesis (Steglich et al. 2001). This is of particular interest under elevated temperature conditions since the D1 protein is especially susceptible to thermal damage within certain symbionts (Warner et al. 1999). High nutrient conditions may increase cellular densities within certain scleractinian coral species (Falkowski et al. 1993; Fabricius 2005), and recent work has suggested that environmental stressors that increase cellular density may also increase susceptibility to thermal bleaching (Cunning and Baker 2013). For *T. reniformis* housing *S. trenchii,* no difference in cellular density due to elevated nutrient concentrations is observed and may suggest that nutrient dependent changes in cellular density may be species specific.

## Holobiont physiology

Although the interactive effects observed within this experiment were predominantly symbiont driven, holobiont

physiology also changed in response to temperature and $pCO_2$. Symbiont cellular density can be highly dynamic and seasonal and environmental differences in algal density have been noted across many species (Fitt et al. 2000; Cunning et al. 2015). Many corals lose *Symbiodinium* during high-temperature stress, and while the precise cellular triggers for expulsion are still unknown, one predominant hypothesis is that cell loss is due to a host response to increased reactive oxygen species produced by photodamaged algal symbionts (Lesser 1997; Fitt et al. 2001; Smith et al. 2005). However, for certain thermally tolerant host/symbiont combinations, a loss of symbionts during high-temperature exposure may also reflect acclimation (Jones and Berkelmans 2010; Takahashi et al. 2013) or be driven by a host-derived stress response alone. Ulstrup et al., (2006) demonstrated symbiont loss with elevated temperature in *T. reniformis* harboring thermally sensitive *Symbiodinium C1* as well as colonies harboring more thermally tolerant clade D symbionts. Since *S. trenchii* is often considered a thermally tolerant symbiont (Grottoli et al. 2014; Silverstein et al. 2015) and Fv/Fm did not decline under elevated temperature alone (Fig. 4), symbiont loss with high temperature (Fig. 1a) may indicate a host response independent of symbiont stress level. Because algal cell loss with elevated temperature may occur independently of major reductions in PSII photochemistry (Ulstrup et al. 2006; Tolosa et al. 2011), it is likely that reduced cell density is a host response of *T. reniformis* to high temperature and not necessarily in response thermal damage occurring within the symbionts.

Although minimal, the lower rates of LEDR noted at higher $pCO_2$ likely reflected a lower metabolic demand in *T. reniformis* (Fig. 2d). However, as respiration rates incorporate simultaneous host and symbiont $O_2$ consumption and the relative proportion from each can vary (Hawkins et al. 2016), we are unable to pinpoint how this decline is partitioned between the symbiont and host. Reduced respiration was also observed for *A. millepora* under elevated $pCO_2$, and corresponded with transcriptional down regulation of metabolic activity within the host (Kaniewska et al. 2012). It is possible that lower calcification rates in corals under elevated $pCO_2$ reduce metabolic demand thereby driving down respiration further. However, lower respiration and calcification rates with elevated $pCO_2$ were not observed for *T. reniformis* in previous work by Hoadley et al. (2015a) and Schoepf et al. (2013), and this difference may have resulted from the longer duration of the current experiment. From this study and others, it is clear that the metabolic demand of the symbioses can be significantly influenced by changing $pCO_2$ (Kaniewska et al. 2012, 2015). Interestingly, respiration rates increased with high $pCO_2$ for the anemones *E. pallida* and *Anemonia viridis*, possibly reflecting fundamental differences in how ocean acidification impacts calcifying versus non-calcifying cnidarian/alga symbioses (Suggett et al. 2012; Gibbin and Davy 2014).

### Symbiont photophysiology

Although minimal, $Fv/Fm^{MT}$ declined with high temperature within both the 'ambient $pCO_2$ and high nutrient' treatment as well as within the 'elevated $pCO_2$ and ambient nutrient' treatments (Fig. 5). However, when active chlorophyll a fluorescence was measured with the FIRe fluorometer, significant declines in $Fv/Fm^{ST}$ due to high temperature were only observed within the elevated $pCO_2$ and ambient nutrient treatment (Fig. 5a). Because $Fv/Fm^{ST}$ is insensitive to changes occurring downstream of the $Q_A$ site in the PSII reaction center, it is likely that the site of thermal stress within the high $pCO_2$ and low nutrient treatment resided within the PSII reaction center. In contrast, the absence of a significant decline in $Fv/Fm^{ST}$, while $Fv/Fm^{MT}$ was reduced within the ambient $pCO_2$ and high nutrient treatment likely reflected changes occurring within the plastoquinone pool or even further downstream the electron transport chain or other locations within the chloroplast (Buxton et al. 2012). These differences provide an additional example of how the *S. trenchii* response to high temperature differs with respect to $pCO_2$ and nutrient concentrations and suggests that the mechanism responsible for high-temperature-induced reductions in Fv/Fm likely differ between the two groups.

Enhanced PSII repair with high N availability is of particular importance, as high temperature or high $pCO_2$ may increase rates of D1 protein degradation (Warner et al. 1999; Gao et al. 2012). For these reasons, high-temperature-induced declines in $Fv/Fm^{MT}$ are contrary to that expected for symbionts under high temperature and elevated nutrient conditions. However, for the high nutrients and ambient $pCO_2$ treatment, the decline in $Fv/Fm^{MT}$ observed under high temperature occurred early in the experiment and was then maintained throughout the remainder of the experiment, likely reflecting a different acclimation state and not thermal stress and photoinactivation per se (Rodríguez-Román and Iglesias-Prieto 2005). In contrast, small yet significant high-temperature-induced reductions in $Fv/Fm^{MT}$ were observed only within the last days of the elevated $pCO_2$ and low nutrient treatment. It is unclear whether these represent a threshold where PSII protein repair rates could no longer keep up with damage caused by compounding temperature and/or $pCO_2$ stress, or a change in acclimation state similar to that observed early on in the experiment within the low $pCO_2$ and high nutrients treatment.

## Conclusion

Although previous studies found few interactive effects between temperature and $pCO_2$ within the *T. reniformis/S. trenchii* symbiosis (Schoepf et al. 2013; Hoadley et al. 2015a; Levas et al. 2015), the longer experimental duration

of this study [24 days in Schoepf et al. (2013), Levas et al. (2015) and Hoadley et al. (2015a) vs. 33 days in this study] may account for the greater number of interactive effects observed here. Overall, temperature was the largest factor in driving physiological change. However, interactive effects are also present as under ambient $pCO_2$, cellular density declined with elevated temperature, allowing remaining symbionts to possibly take advantage of an increase in DIC availability, resulting in increased photosynthetic productivity on a per cell basis and increases in chlorophyll a. Under elevated $pCO_2$ conditions, reduced cell density due to high temperature was not accompanied by increases in chlorophyll a or net photosynthesis. Instead a larger difference in cellular volume between ambient and elevated temperature was observed, potentially altering the package effect and increasing light intensity within the cell. Differences in the ratio of chlorophyll *a* to protein also suggest that symbiont protein expression during thermal stress is also $pCO_2$ dependent. Decreased holobiont respiration and coral calcification rates confirm previously reported changes in metabolism and growth rates that are dependent on $pCO_2$ concentration (Kaniewska et al. 2012; Comeau et al. 2013). Despite $pCO_2$ based differences in the physiological response to high temperature, our multivariate analysis shows that elevated nutrient concentrations minimize the thermal response under both ambient and elevated $pCO_2$ conditions. This is of particular importance given the global distribution of *S. trenchii* and its association with multiple host species. However, our study utilized only minor nutrient pulses and results may differ under higher nutrient loads. Additionally, such interactive effects will almost certainly vary across host/symbiont combinations and future research is needed to incorporate additional species responses from both Pacific and Caribbean coral reefs.

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