Methodology from C. B. Wall, T.-Y. Fan, P. J. Edmunds (2014) Ocean acidification has no effect on thermal bleaching in the coral. Coral Reefs 33:119-130

Experimental design

Four treatments contrasted ambient temperature-ambient pCO2 (AT-ACO2), ambient temperature-high pCO2 (AT-HCO2), high temperature-ambient pCO2 (HT-ACO2), and high temperature-high pCO2 (HT-HCO2). Ambient temperature referred to the seawater on shallow reefs in Nanwan Bay when the experiment was conducted in July and August 2011 (28.0 ± 0.2 oC at 3 m depth [mean ± SE, n = 21 days]) and therefore was set at 27.5 oC. Ambient pCO2 reflected ambient CO2 conditions, although this routinely was above the global mean atmospheric value of ~39.0 Pa. High temperature was relative to the maximum seawater temperature recorded at 3 m depth on the study reef in summer (31.0 oC) (T-Y Fan pers comm) and was 30.5 oC. High pCO2 represented conditions projected to occur by the year 2100 (~86 Pa) under the high CO2 emission scenario RCP 6.0 (van Vuuren et al. 2011). We hypothesized that OA would cause bleaching as defined by decreased photochemical efficiency, reduced photosynthetic capacity and efficiency, depressed chlorophyll a content, and lowered Symbiodinium densities and that these effects would be exacerbated with high temperature. To test this hypothesis, corals were exposed to treatments in 8 tanks (n = 2 tanks treatment-1) filled with 130 L of filtered (1.0 um) seawater. Treatments were maintained at a salinity of 33.4 (measured with a YSI 3100 Conductivity Meter, YSI Inc., USA) with 20 % water changes each evening. Temperatures were maintained independently by microsensor-based regulators (Aqua- Controller, Neptune Systems, USA) connected to a 300-W heater (Taikong Corp.), and chiller (Aquatek, Aquasystems, Taiwan), and the seawater was mixed with a pump (1,451 L h-1). Light was provided to each tank by two 18-W fluorescent bulbs (TL-D Blue, Phillips, USA) and two 150-W metal-halide bulbs on a 12 h light: 12 h dark cycle that created mean irradiances ranging from 251 to 279 umol photons m-2 s-1 as measured beneath the seawater using a spherical light sensor (LI-193, Li-Cor, USA). Treatments of pCO2 were maintained by bubbling ambient air (A-CO2) or CO2-enriched air into the tanks (H-CO2). To prepare high pCO2 treatments, CO2 was mixed with ambient air by solenoid-controlled gas mixing technology (Model A352, Qubit Systems, Canada). CO2 and ambient air were mixed in a chamber, and the pCO2 measured using an infrared (IR) gas analyzer (S151, Qubit Systems) calibrated against certified reference gas (1,793 ppm CO2, San Ying Gas Co., Taiwan). The pCO2 treatments were maintained dynamically by the IR gas analyzer, which regulated a solenoid valve controlling the flow of CO2 gas. The final pCO2 was logged in ppm on a PC using LabPro software (Vernier Software and Technology, USA), and a pump delivered the gas mixture to the high-pCO2 tanks at ~15 L min-1. Ambient pCO2 tanks received ambient, non-CO2-enriched air at a similar flow rate.

Treatments were monitored daily at 0900, 1200, and 1700 hrs for temperature and salinity; irradiance was measured at 1200 hrs; pH and carbonate chemistry of the treatments were determined daily on seawater samples (~250 mL) taken from all tanks at 0900 hrs. Temperature was measured using a certified digital thermometer (Fisher Scientific 15-077-8, ± 0.05 oC), and seawater was assessed for total alkalinity (TA, umol kg-1) and pCO2 by potentiometric titrations following standard operating procedures (SOP) 3 (Dickson et al. 2007); pHT was determined spectrophotometrically using m-cresol purple (SOP 6B,

Dickson et al. 2007). Seawater samples were titrated using an open-cell autotitrator (Model DL50, Mettler- Toledo, USA) filled with certified acid titrant (from A. Dickson, Scripps Institution of Oceanography) and equipped with a DG115-SC pH probe (Mettler-Toledo). TA was evaluated for precision and accuracy using certified reference materials (CRM) of known TA (from A. Dickson, Scripps Institution of Oceanography) with our analyses differing <0.9 % from certified values. pHT, salinity, temperature, and TA were used in CO2SYS software in Microsoft Excel (Fangue et al. 2010) to calculate the components of the dissolved inorganic carbon (DIC) system in seawater.

Coral collection

Sixty juvenile S. caliendrum (<4 cm diam.) were collected on July 22, 2011, from Hobihu Reef (21o56.799'N, 120o44.968'E), Nanwan Bay. Colonies were collected from 3 to 4 m depth and transported to a flow-through aquarium at the National Museum of Marine Biology and Aquarium (NMMBA) where they were allowed to recover from collection for 24 h. The recovery tank was filled with flowing, filtered seawater (50 um) and mixed with a pump (1,451 L h-1). Temperature was maintained at ambient conditions (28.07 ± 0.10 oC, ±SE, n = 24) and light was supplied at 164 ± 4 umol photons m-2 s-1 on a 12 h light: 12 h dark cycle.

One day following collection, the colonies of S. caliendrum were suspended in the recovery tank using nylon line and left to recover for 5 days. On July 28, 2011, they were placed randomly into the treatment tanks (n = 7 tank-1) for incubations lasting 14 days. On August 11, 2011, corals were processed over 3 days for the dependent variables described below and retained in experimental conditions during this time. To maintain comparable exposure periods among like-temperature treatments, corals from the HT treatments were processed first, followed by corals in the AT treatments, with corals randomly selected for processing within each temperature treatments.

Photochemical efficiency

The effects of temperature and pCO2 on photochemical efficiency were tested by measuring the maximum photochemical efficiency of open RCIIs in the dark (Fv/Fm) and the effective photochemical efficiency of RCII in the light (deltaF/Fm') using pulse amplitude modulation (PAM) fluorometry. PAM fluorometry is an effective tool to assess noninvasively the photophysiology of Symbiodinium in hospite (Warner et al. 1996, 2010) and provides an indication of PSII photochemical activity and the transport of electrons through PSII (Cosgrove and Borowitzka 2010). Fv/Fm provides a measure of photochemical quenching (qP) reflecting the rate of charge separation across PSII in the open (i.e., dark-adapted) state, while deltaF/Fm' accounts for photochemical and nonphotochemical quenching (NPQ), including mechanisms for the dissipation of excess absorbed light energy as heat through the PSII antennae complex (Hill et al. 2005). NPQ is of particular biological importance as a mechanism of photoprotection and avoidance of photoinhibition under peak daily irradiance and under conditions causing bleaching (Warner et al. 1996; Jones et al. 1998; Hoegh-Guldberg and Jones 1999).

Photochemical efficiency was assessed using a Diving- PAM (Waltz, GmbH, Effeltrich, Germany) operated at a gain of 6, intensity of 9, and a slit width of 0.8. Prior to the start of the experiment, PAM settings were adjusted to obtain a range of minimum fluorescence yield (Fo) between 200 and 400 (arbitrary units) and stabile maximum fluorescence yield (Fm). deltaF/Fm' was measured to quantify changes in quantum yield relative to the dark-adapted state due to excess thermal energy dissipation and NPQ, and Fv/Fm was measured to quantify the maximum efficiency of open RCIIs in the dark-adapted state. Photochemical efficiency was measured using a 5-mm-diameter fiberoptic probe held ~5 mm above the tissue and ~1 cm behind branch tips. deltaF/Fm' was measured under actinic irradiance (~265 umol photons m-2 s-1) and Fv/Fm under weak indirect red lighting (<=2.0 umol photons m-2 s-1). deltaF/Fm' was measured every second day of the incubation at 1230 hrs, and Fv/Fm was measured every second day at 1730 hrs. A pilot study was used to determine the duration of dark adaptation necessary to stabilize values of Fv/Fm, to identify effects of prolonged darkness on Fv/Fm (i.e., darkinduced reduction in the PQ pool; Hill and Ralph 2008), and to test whether weak indirect red light (as produced from small lamps used during nocturnal PAM measurements) affected Fv/Fm. Results indicated Fo stabilized after <0.5 h of darkness, and Fv/Fm was statistically indistinguishable when measured following dark adaptation lasting 0.5, 1.0, or 2.0 h ($F_{2,27} = 0.137$, $P = 0.872$), or measured with and without weak red light ($F_{1,18} = 0.352$, $P = 0.561$).

Photosynthesis-irradiance (P/I) curves

To test for the effects of pCO2 and temperature on the ability for Symbiodinium to utilize light and perform photosynthesis, net photosynthesis (P^net), determined from changes in O2 concentrations in seawater, was measured under different irradiances using three corals selected randomly from each treatment tank (n = 6 treatment-1). Two respirometers were used to measure P^net, and each housed a single coral in trials lasting ~1.5-2.0 h. Measurements of P^net began on the 14th day of incubations, and 3 days were required to process all corals in the experiment. Temperatures were maintained by placing the respirometers in a water bath. Water motion in each chamber was provided by a stir-bar, and the flow rate quantified by photographing hydrated Artemia spp. eggs (Sebens and Johnson 1991), revealing the mean flow rate near the center of the respirometer to be 5.43 ± 0.32 cm s-1 (±SE, n = 20). Prior to each trial, corals were maintained in darkness for 1 h to allow the stimulatory effect of light on respiration to abate (Edmunds and Davies 1988). O2 flux then was measured at ten irradiances supplied in an ascending sequence between 0 and 747 umol photons m-2 s-1. Light intensities were created by adjusting the height of a 400-W metal-halide lamp (Osram Sylvania, USA) above the respirometer, and measuring the irradiance using a cosine-corrected light sensor recording photosynthetically active radiation (PAR). The light sensor was 1.0 mm diameter and attached to a Diving-PAM (Waltz, GmbH, Effeltrich, Germany) and was calibrated using a Li-Cor LI-192 quantum sensor. O2 fluxes were adjusted for changes in O2 concentrations in control chambers filled with seawater alone, and controls were run at each combination of temperature and pCO2 for each irradiance, and during darkness (n = 3 treatment-1).

The O2 saturation of seawater was measured using an optrode (FOXY-R, 1.58 mm diameter, Ocean Optics, USA) connected to a spectrophotometer (USB2000, Ocean Optics), which logged O2

concentrations on a PC running Ocean Optics software (OOISensors, version 1.00.08, Ocean Optics). The optrode was calibrated using watersaturated air at the measurement temperature and a zero solution of sodium sulfite (Na2SO3) and 0.01 mol L-1 sodium tetraborate (Na2B4O7). O2 saturation during the trials was maintained between 80 and 100 % by replenishing chambers with filtered (1.0 um) seawater from respective temperature and pCO2 treatments. Changes in O2 saturation were converted to O2 concentrations (umol L-1) using tabulated gas solubility at a known temperature and salinity [N. Ramsing and J. Gundersen at www.unisense.com, based on Garcia and Gordon 1992]. Rates of change in O2 concentrations were determined by regressing O2 concentration against time, and standardizing to the surface area of the coral tissue (cm2) as determined by wax dipping (Stimson and Kinzie 1991). The relationship between P^net and irradiance was described with a hyperbolic tangent function (Jassby and Platt 1976) that included an exponent for photoinhibition (e.g., b) at high irradiances (Platt et al. 1980) to account for photoinhibitory effects of high-light or high-temperature exposure known to occur in phytoplankton (Platt et al. 1980) and Symbiodinium (Smith et al. 2005):

P^beta = Ps^beta(1 - (e^-a))e^b

where a = alphaI/Ps^beta,b = betaI/Ps^beta, P^beta is the rate of net primary productivity (P^net), Ps^beta is the maximum rate of net photosynthesis accounting for photoinhibition, a is the initial slope of the light-limited portion of the curve, and I is irradiance in umol photons m-2 s-1. Hyperbolic tangent functions were fit to the productivity data by nonlinear regression using Systat 11 software (Systat, Inc., USA) and used to characterize the photosynthetic efficiency (alpha, a) and P^net under high-light conditions. The aforementioned curves describe the full biological relationship between P^net and I for a large range of irradiances supplied in the laboratory, with these intensities exceeding maximum intensities found on the study reef. Mean PAR at 3 m depth on Hobihu reef (measured between March 6 and 10, 2011, using a 4p spherical quantum sensor (MkV-L, JFE Advantech Co., Kobe, Japan) between 0900-1500 hrs was 660 ± 30 umol photons m-2 s-1 (±SE, n = 148). To compare the photosynthetic performance of corals under ecologically relevant conditions and following exposure to treatments, best-fit curves were used to calculate P^net at 660 umol photons m-2 s-1 (hereafter P660^net), and this metric, along with alpha, and dark respiration, was compared among treatments. While inclusion of an exponent for photoinhibition (beta) improved the fit of the response curves to the empirical data, in situ irradiances were not sufficiently high to elicit photoinhibition on the reef, and therefore, beta was not employed as a dependent variable in the analysis.

Chlorophyll a concentration and Symbiodinium density

Chlorophyll a concentration and Symbiodinium density were quantified by removing coral tissue from the skeleton using an airbrush filled with filtered seawater (1.0 um). Colonies were airbrushed into a plastic bag, producing 8-40 mL of slurry that was homogenized (Polytron PT2100, Kinematica, USA) prior to separating the Symbiodinium by centrifugation (1,5009g). The Symbiodinium pellet was resuspended by vortexing in filtered seawater and used to measure chlorophyll a concentration and symbiont density.

Symbiodinium used for chlorophyll determinations were frozen (-4 oC for 24 h) and subsequently thawed (4 oC for 24 h) and filtered onto a cellulose acetate membrane filter (3.0 um pore size, Critical Process Filtration, USA) to which 3 mL of 90 % acetone was added. Samples were refrigerated (4 oC) in darkness for 36 h, centrifuged (1,5009g for 3 min), and absorbances at 630 and 636 nm measured and used to calculate chlorophyll a concentration using the trichromatic equations of Jeffrey and Humphrey (1975) for dinoflagellates. Chlorophyll a concentration was standardized by algal cell (pg cell-1) and surface area (lg cm-2) of the coral. Symbiodinium density (cells cm-2) was determined by counting Symbiodinium in the homogenized slurry stripped from the coral colonies, with the counts completed using a hemocytometer (n = 4 counts). Preliminary data showed that the mean and standard deviation of replicate determinations of Symbiodinium density stabilized after four counts.