Reduced expression of the rate-limiting carbon fixation enzyme RuBisCO in the benthic foraminifer *Baculogypsina sphaerulata* holobiont in response to heat shock

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ABSTRACT

Baculogypsina sphaerulata (Parker and Jones, 1860) is a common large benthic foraminifer (LBF) and is an important calcifier in coral reef ecosystems. As there are concerns that global increasing temperatures may compromise the survival of this species, which forms a symbiotic relationship with the diatom *Nitzschia* sp., we investigated the response of the *B. sphaerulata* holobiont from the intertidal algal flats of Xiao Liu Chiu Island, Taiwan to heat shock. *B. sphaerulata* specimens were incubated at 26 (ambient), 28, 30, 32, or 34 °C for 5 h designed to simulate short pulses of elevated temperature that occur in situ from subaerial exposure at low tide. To assess the molecular-level response, we measured the expression of the ribulose 1-5-bisphosphate carboxylase/oxygenase (RuBisCO) protein in the diatom symbiont. There was a significant decrease in expression of this rate-limiting carbon fixation enzyme in *B. sphaerulata* holobionts incubated at 34 °C/8 °C above ambient (~50% decline relative to controls), but expression was resilient to levels of warming up to 6 °C above ambient. This suggests that exposure to high temperatures occasionally experienced in nature may diminish the capacity for carbon fixation of the diatom symbiont. Given the importance of photosynthesis and carbon fixation in these marine calcifiers, these data suggest that climate-driven ocean warming may exert deleterious effects on the *B. sphaerulata* holobiont.

1. Introduction

Symbiotic associations between marine animals and algae are common in the phyla Cnidaria, Mollusca, and Foraminifera, many of which reside in shallow, tropical waters (Trench, 1993). Recently, there have been major concerns as to how calcifying organisms with symbiotic algal associations, in particular corals and Foraminifera, will fare in a warming and acidifying ocean (Hoegh-Guldberg, 1999; Sinutok et al., 2011; Uthicke et al., 2012). In corals, increased temperature can compromise dinoflagellate (genus *Symbiodinium*) photosynthesis (e.g. Warner et al., 1999), leading to increases in reactive oxygen species production (Lesser, 1997), and, in severe cases, symbiotic disintegration (i.e., bleaching) and host mortality (Gates, 1990; Hoegh-Guldberg, 1999). Acute (<48 h) thermal stress decreases photosynthetic efficiency and expression of the photosystem II protein D1 (Jones et al., 2000; Warner et al., 1999). Although photoinhibition due to thermal stress is well documented in corals (reviewed in Smith et al., 2005), little is known about how other marine symbioses, such as the foraminiferan-algae association, will respond to increased temperature in the context of climate change.

Large benthic Foraminifera (LBF) are major contributors to calcium carbonate production in coral reef ecosystems and perform a crucial role in buffering against changes in ocean chemistry (Hohenegger, 2006; Langer et al., 1997; Scoffin and Tudhope, 1985). They host a variety of algal symbionts that are deleteriously affected by prolonged exposure to increased temperature, as evidenced by decreased photosynthetic efficiency and calcification (Hallock, 1981; Lee, 2006; Schmidt et al., 2011; Sinutok et al., 2011). For the diatom-hosting species, *Amphistegina radiata*, *Heterostegina depressa* and *Calcarina hispida*, incubation at 8 °C above ambient for 30 days led to decreased photosynthetic efficiency and growth, resulting in bleaching (Schmidt et al., 2011). Similar experiments with *Marginopora vertebralis*, a *Symbiodinium*-bearing species found that incubation at +5 °C above ambient for 7 days resulted in significant morality (Uthicke et al., 2012), as well as decreases in calcification. These changes were suggested to be due to a decrease in physiological performance of the dinoflagellate symbionts (Sinutok et al., 2011).

While previous long-term elevated temperature studies have enhanced our understanding of the foraminiferal response to environmental changes, no studies have been conducted on their response

to the acute increases in temperature that they may encounter in the field at low tide from subaerial or near subaerial exposure. Furthermore, only one other study to date has used molecular tools to gauge the sub-cellular response of these critically important, and potentially threatened, calcifiers (Heinz et al., 2012). To address these knowledge gaps, we investigated the heat shock response of the *Baculogypsina sphaerulata* holobiont from the intertidal algal flats of Xiao Liu Chiu Island, Taiwan. Samples were exposed for 5 h to temperatures aimed to simulate pulses of warming experienced during summer day-time low tides in their intertidal environment; 26 (ambient), 28, 30, 32, and 34 °C. The upper level of warming (34 °C) potentially represents a "tipping point" that results in photosystem degradation, as observed in other foraminiferan species exposed to long-term (weeks) increases in temperature (Schmidt et al., 2011).

The physiological response of the *B. sphaerulata* holobiont was documented by measuring protein expression of ribulose 1-5-bisphosphate carboxylase/oxygenase (RuBisCO), a highly conserved, rate-limiting Calvin cycle (carbon fixation) enzyme in the diatom symbionts *Nitzschia* sp. (Lee and Correia, 2005) that are commonly associated with this foraminifer host. Given the well-documented photoinhibition and decreased carbon fixation in both corals and Foraminifera exposed to elevated temperature (Schmidt et al., 2011; Warner et al., 1999), we hypothesized that heat shock treatments would lead to a decrease in expression of RuBisCO. Previous studies of heat shock response in marine species along a thermal gradient have found higher thermotolerance of intertidal species compared to subtidal, although many intertidal species may be living near their maximum thermal tolerance (Somero, 2010). Therefore, by investigating the thermal biology of an intertidal foraminifer holobiont already adapted to life with significant temperature fluctuations, we provide insights into how these organisms may respond to future climate-driven ocean warming.

2. Methods

2.1. Field collection and heat shock experiment

Specimens of *B. sphaerulata* were collected from the intertidal algal flat during low tide in an exposed cove on the coral reef island Xiao Liu Chiu (22° 21′ 3″N, 120° 23′ 17″E), off the southwest coast of Taiwan (Fig. 1A–B). In the field, *B. sphaerulata*, which typically attach firmly to algal substrates, were located on macroalgae on rocky reef flats that were regularly or nearly exposed during spring tides. To characterize the thermal regime of the field site, a HOBO® Pendant (Onset, Pocasset, MA, USA) temperature logger was deployed nearby (22° 20′ 53.4″N, 120° 23′ 26.6″E) at 8 m depth for two months (June–July 2011) around our sampling date with measurements every 6 min. In addition, because this species typically has minimal water cover during summer low tides, air temperature data were also collected from the Central Weather Bureau (22° 19′ 55.4″N, 120° 21′ 44.0″E) (Fig. 1C–E).

Specimens were collected in July 2011 and transported to the National Museum of Marine Biology and Aquarium (NMMBA) in Pingtung County, Taiwan. Samples were placed in aquaria set to ambient (26 °C) temperature for two days prior to use in the experiments. Thirty *B. sphaerulata* were placed haphazardly into 20 ml glass scintillation vials with 8 replicates per treatment. The vials were sealed to prevent evaporation and immersed in ~20 l temperature-controlled water baths with recirculating water set at either 26 (control), 28, 30, 32, or 34 °C for 5 h. Temperature was measured with a digital thermometer (model 15-077-8, Fisher Scientific, Pittsburg, PA, USA), and photosynthetically active radiation (PAR) was measured with a Li-Cor meter (LI-1400, Li-Cor Biosciences, Lincoln, NE, USA). After the treatment exposure for 5 h, the specimens were quickly transferred into 1.5 ml microcentrifuge tubes, frozen in liquid nitrogen and stored at –80 °C until protein extraction.

2.2. Protein extraction, SDS-PAGE, and western blotting

Soluble protein was extracted from all 30 individuals in each of the 40 samples with 60 μl RIPA buffer (50 mM Tris–HCl pH [7.4], 1% Nonidet-P40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1× complete protease inhibitor cocktail, [Roche, Basel, Switzerland]). All 30 *B. sphaerulata* in each sample were mechanically ground with metal tweezers and frozen at −20 °C for 12 h. They were then centrifuged for 5 min at 12,000×g, and the supernatant with the total solubilized protein was transferred to a new microcentrifuge tube. Protein content was quantified using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Soluble protein (5 μg) was dissolved in 1× Laemmli sample buffer (Laemmli, 1970), boiled for 5 min at 95 °C, and spun at 12,000×g for 10 min at 4 °C to pellet insoluble material. Forty microliters of the supernatant were loaded into SDS-PAGE gels, which were electrophoresed on ice at 70 V for 30 min followed by 120 V for 1 h through the 5% stacking and 12% separating gels, respectively. Each gel was also loaded with protein extracted from a stock homogenate of *B. sphaerulata* incubated at 26 °C as a positive control to normalize samples across gels.

Following electrophoresis, proteins were transferred to PVDF membranes at 4 °C at 100 V for 90 min in transfer buffer (25 mM Tris–HCl, pH [6.8], 192 mM glycine and 20% methanol). To determine efficacy of transfer, SDS-PAGE gels were stained with SYPRO® Ruby (Invitrogen) after transfer according to the manufacturer's recommendations and visualized on a Typhoon Trio™ Variable Mode Imager (Amersham Biosciences, Little Chalfont, United Kingdom) at 532 nm. In certain cases, the PVDF membranes were stained with Ponceau S (Sigma, St. Louis, MO, USA) according to the manufacturer's recommendations to further visualize degree of protein transfer.

Membranes were blocked in 5% skim milk (w/v) in Tris-buffered saline with Tween-20 (TBST, 100 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature (RT). The blocking buffer was decanted, and 10 ml of a 1:2000 dilution of a RuBisCO large subunit (RBCL) primary antibody (forms I and II, Agrisera, Vännas, Sweden) in 5% skim milk (w/v) in TBST was added to the membranes, which were then incubated for 2 h with gentle agitation at RT. Samples were washed 3 times (10 min each) with TBST and then incubated with a 1:5000 dilution of goat anti-rabbit secondary antibody (Millipore, Billerica, MA, USA) for 7 min and washed with TBST as above. Samples were then stained with SuperSignal® West Pico Chemiluminescent Substrate Kit chemiluminescent reagent (Pierce, 34082 Amersham Biosciences), and the chemiluminescent signal immediately visualized on a Fusion FX7 (Vilber Lourmat, Marne-la-Vallée, France).

2.3. Image and statistical analysis

Densitometry measurements were made with ImageJ (NIH), and the data were normalized to the intensity of the positive control RuBisCO band from the same gel to compare expression across blots, as five gels/blots were required to process all 40 samples. Data were analyzed by 1-way analysis of variance (ANOVA) with temperature as the fixed factor after a square root-transformation to meet assumptions of ANOVA (normality and homoscedasticity). Post-hoc analysis was performed using Tukey's Honestly Significant Difference (HSD) tests. A visual inspection of residuals indicated five outliers, which were removed from further analysis. All statistical analyses were performed with JMP® (version 9, Cary, NC, USA).

3. Results

3.1. Field and experimental conditions

Mean seawater temperature values between June and August 2011 were 26.8 °C, with a 2.0 °C mean daily variation around the collection time (range: 24.5–29.8 °C) (Fig. 1C). Mean air temperature was 28.0 °C,
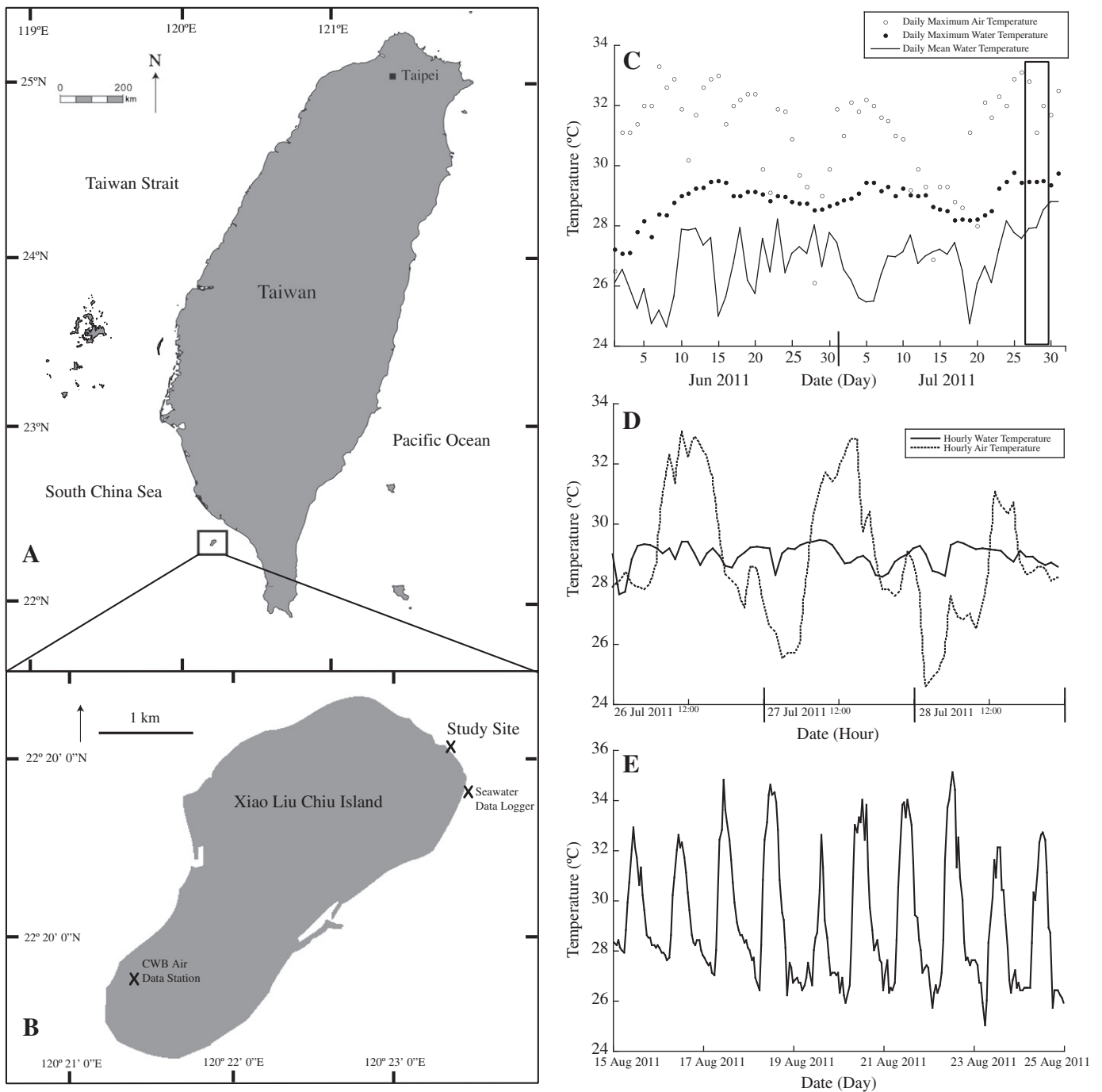
Fig. 1. A. Map of Taiwan with B. Xiao Liu Chiu Island. The study site, deployment of the water HOBO data temperature logger, and CWB Air Data Station are all marked with an X. C. Daily maximum air and seawater temperature values from Xiao Liu Chiu Island with mean seawater values for two months surrounding our sampling period (June–July 2011). Daily maximum air temperature values are considerably higher (>+4 °C) than mean seawater temperature values. The enclosed box represents D. hourly seawater and air temperature values over a representative tidal cycle. E. A representative graph of daily variation in air temperature that reaches +34 °C during summer months.

but exhibited a much higher daily flux (~5.3 °C mean daily variation, range: 24.1–33.3 °C) compared to seawater conditions. The control to +6 °C temperature values used in this study were similar to conditions experienced in situ, with the +8 °C treatment potentially representing temperatures during low tide subaerial or near subaerial exposure with increased thermal stress from air temperature (Fig. 1C–E).

Experimental temperature and PAR remained relatively stable within treatments during the incubation period and PAR did not differ significantly between treatments ($F_{4,5} = 0.0831$, $p = 0.984$) (Table 1). Temperature varied significantly between all treatments ($F_{4,15} =$ 39.253, $p < 0.0001$) (Table 1). At the end of the 5 h temperature treatments, all specimens were alive, and none exhibited obvious signs of bleaching (loss in color of the foraminifer test due to loss of symbionts).

3.2. RuBisCO protein expression

Protein yield ranged from 0.724 µg/µl to 1.226 µg/µl and was similar across treatments ($F_{4,35} = 0.218$, $p = 0.927$). This equates to 1.95 ± 0.04 µg of total protein per individual *B. sphaerulata* (n = 40). Positive RuBisCO signals were detected in 39/40 samples (~98%), and the one sample that failed was removed from further analysis.

Table 1
Temperature and photosynthetically active radiation (PAR) conditions of aquaria during heat shock treatments. Temperature was measured hourly for the first 4 h, and PAR measured at the beginning and mid-way through the heat shock treatments. Values are means ± SE (temperature, n = 4, PAR, n = 2).

Treatment	Temperature (°C)	PAR ($\mu mol\ m^{-2}\ s^{-1}$)
26 °C (control)	26.11 ± 0.06	258.1 ± 46.5
28 °C	28.31 ± 0.02	252.7 ± 18.9
30 °C	30.34 ± 0.05	240.7 ± 27.8
32 °C	32.02 ± 0.12	256.3 ± 16.8
34 °C	34.16 ± 0.03	241.3 ± 24.0

There was a significant temperature effect on RuBisCO protein expression ($F_{4,30} = 3.386$, p = 0.021) (Fig. 2). Tukey's HSD post hoc tests indicated a significant decrease in RuBisCO in the +8 °C (34 °C) treatment compared to the controls (26 °C), but no other pairwise differences (see Tukey's HSD groups of Fig. 2). There was an approximate two-fold decrease (~50%) in RuBisCO expression in samples incubated at +8 °C (34 °C) relative to controls.

4. Discussion

This is one of the first heat shock studies for a foraminiferan species and represents the first to document decreased RuBisCO protein expression in the diatom symbionts (*Nitzschia* sp.) of *B. sphaerulata* in response to increased temperature. This species was resilient to increases up to 6 °C above ambient SST, levels of warming experienced in nature during summer at low tide. Thus, *B. sphaerulata* appears to be thermotolerant to conditions it is likely to experience in the field. It may, however, be living close to its thermal maximum, as evidenced by the decrease in RuBisCO expression at +8 °C (34 °C). This is of note, as air temperature values rise to >34 °C during summer months, and the intertidal populations of *B. sphaerulata* in this study are routinely subaerially exposed in the field during spring tides. As RuBisCO catalyzes the initial carboxylation reaction in the photosynthetic carbon fixation pathway, these data suggest that overall photosynthetic activity of the diatom symbiont may be compromised during periods of warming during low tides. The ~50% reduction in RuBisCO protein expression at 34 °C (8 °C above ambient) indicates that this temperature may serve as a thermal threshold above which the diatom symbionts are unable to fix carbon at optimal levels.
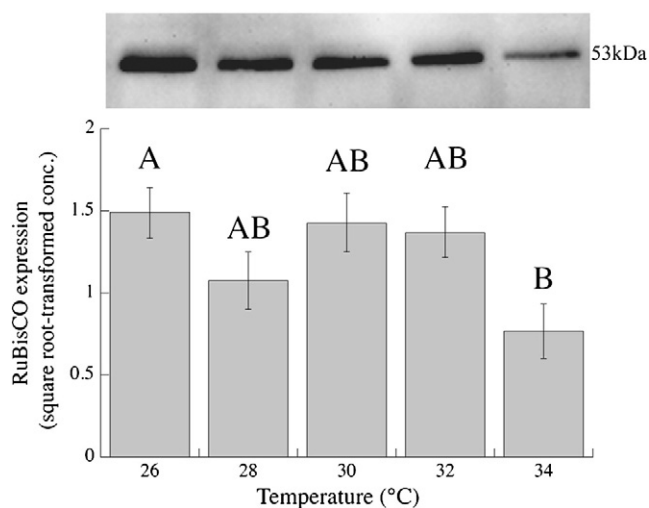

Fig. 2. Mean values of square root-transformed RuBisCO protein expression normalized to positive control band intensity in *Baculogypsina sphaerulata* in experimental treatments after incubation for 5 h. Letters indicate Tukey's HSD post-hoc groups. Representative RuBisCO bands are shown above their respective treatments.

While there are no comparative studies of protein expression of symbiotic algae associated with Foraminifera, a recent heat shock study with larvae from the coral *Pocillopora damicornis* reported a decrease in RuBisCO expression at +4 °C above ambient, possibly resulting in the metabolic suppression observed in samples of the same treatment (Putnam et al., in review). In response to heat shock, adult corals (*Montastrea annularis* and *Plesiastrea versipora*) exhibited a decrease in expression of the photosystem II protein D1 as well as decreased efficiency of photosystem II within 48 h of thermal stress (+6 °C), determined through the use of pulse amplitude modulation (PAM) fluorometry (Jones et al., 2000; Warner et al., 1999). While PAM fluorometry was not conducted in this study, it is possible that photoinhibition is a general response of algal photosymbionts to elevated temperatures.

Several studies have investigated the photosynthetic response of foraminiferal symbionts to thermal stress (2–8 °C above ambient) in long-term (28–37 days) experiments using PAM and concentrations of chlorophyll-a (*chl-a*) and other pigments as indicators of symbiont performance (Schmidt et al., 2011; Uthicke et al., 2012). In *A. radiata*, *H. depressa*, and *C. hispida* (all diatom-bearing Foraminifera), a decrease in photosynthetic efficiency was observed, and bleaching was documented at temperatures 9 °C above ambient (Schmidt et al., 2011). Studies of growth and calcification of *M. vertebralis* incubated at 5–6 °C above ambient for several weeks also indicated significant decreases and changes in calcification as determined by weight and crystal structure formation (Schmidt et al., 2011; Sinutok et al., 2011; Uthicke et al., 2012). Decreased photosynthetic efficiency was also reported for *M. vertebralis* within 2 days of immersion at elevated temperature of +6 °C above ambient (Uthicke et al., 2012).

Our observation of decreased RuBisCO expression in the diatom *Nitzschia* sp. associated with *B. sphaerulata* potentially indicates a decrease in photosynthetic efficiency as documented in longer-term exposures (days–weeks) to elevated temperature (Schmidt et al., 2011; Uthicke et al., 2012). We observed sub-lethal effects of thermal treatments within 5 h of incubation, but how this relates to the response seen in chronic thermal stress experiments is not known, although Uthicke et al. (2012) indicated that deleterious effects of thermal stress in *M. vertebralis* were nearly instantaneous, as determined by respiration and carbon production rates. Further work involving parallel RuBisCO, PAM, and *chl-a* measurements in the *B. sphaerulata* holobiont sampled across longer exposure to stress-inducing temperatures would be useful to understand how these indicators of photosynthesis can be compared.

Understanding the effects of acute thermal stress on the molecular response may provide insight into how climate-driven increases in temperature will affect mechanistic processes in foraminiferal biochemistry. A previous study examining heat stress on the RuBisCO protein in cotton plants suggested that deactivation of RuBisCO results from a dual response of increased catalytic misfire, causing increased misprotonation at the active site and decreased efficiency of RuBisCO activase, an enzyme that increases efficiency of reactivation of RuBisCO (Salvucci and Crafts-Brandner, 2004). It is currently unclear whether RuBisCO enzymes in the *B. sphaerulata* holobiont observed in this study were compromised in such a way, as only expression itself, not enzymatic activity/efficiency, was measured. Alternatively, simple heat denaturation of the RuBisCO protein could also account for the decrease in activity, as RuBisCO has been shown to be highly susceptible to thermal stress (Whitney and Yellowlees, 1995).

While the exact mechanism that led to decreased RuBisCO expression in *B. sphaerulata* is not known, our results suggest that an overall decrease in photosynthetic capacity could have an effect on pH near the site of calcification. In *Orbulina universa*, *M. vertebralis*, *Amphistegina lobifera* and *Amphisorus hemprichii*, reduced calcification and photosynthetic activity at night were associated with large decreases in pH near the shell surfaces, as determined by microsensor studies (Köhler-Rink and Kühl, 2000; Rink et al., 1998). Decreased RuBisCO expression resulting from thermal stress in *B. sphaerulata* may be associated with decreased photosynthetic activity, which in turn may reduce the pH at

the site of calcification (Köhler-Rink and Kühl, 2000; Rink et al., 1998). These conditions have the potential to cause a reduction in the calcite saturation state at the site of calcification (Erez, 2003). In addition, an increase in seawater temperature will reduce available soluble inorganic CO_2 available for uptake in photosynthetic pathways (Weiss, 1974), potentially compounding natural oscillations in calcification due to biological effects of the foraminiferal holobiont. Further work into mechanistic causes of decreased calcification in response to thermal stress will aid in developing a clearer picture of the effects of both acute and prolonged warming on this process.

By studying populations of intertidal Foraminifera adapted to life in dramatically fluctuating temperature environments, we can better understand how populations of these organisms may be affected by climate change. Previous studies have reported increased thermotolerance of intertidal taxa to thermal stress as a result of adaptation to rapidly changing conditions (Somero, 2010; Stillman, 2003). Similarly, studies of corals have suggested that previous exposures to heat stress increase the ability to acclimate to future temperature increases (Howells et al., 2012; Middlebrook et al., 2008; Oliver and Palumbi, 2011). In corals, this is suggested to be a direct effect of the ability of the dinoflagellate symbiont *Symbiodinium* to adapt to various thermal regimes (Howells et al., 2012), suggesting that acclimatization to local thermal variation has a larger effect than dinoflagellate identity (Oliver and Palumbi, 2011). The *B. sphaerulata* symbiont may also demonstrate increased resiliency in more thermally variable habitats, implying that intertidal populations would be more resilient than sub-tidal conspecifics. How the thermal biology of foraminiferal populations varies across environmental (e.g. intertidal to sub-tidal) gradients is an important area for future research and may aid in forecasts of future persistence of these ecologically important species.

Acknowledgments

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