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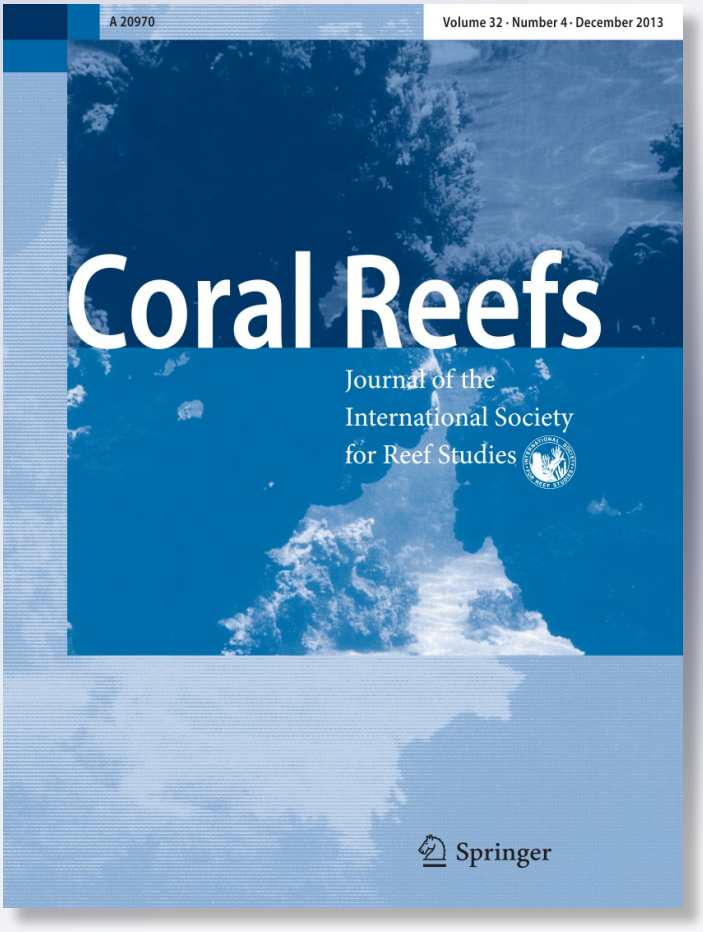

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REPORT

Physiological acclimation to elevated temperature in a reef-building coral from an upwelling environment

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Abstract Recent work has found that pocilloporid corals from regions characterized by unstable temperatures, such as those exposed to periodic upwelling, display a remarkable degree of phenotypic plasticity. In order to understand whether important reef builders from these upwelling reefs remain physiologically uncompromised at temperatures they will experience in the coming decades as a result of global climate change, a long-term elevated temperature experiment was conducted with *Pocillopora damicornis* specimens collected from Houbihu, a small embayment within Nanwan Bay, southern Taiwan that is characterized by 8–9 °C temperature changes during upwelling events. Upon nine months of exposure to nearly 30 °C, all colony (mortality and surface area), polyp (*Symbiodinium* density and chlorophyll *a* content), tissue (total thickness), and molecular (gene expression and molecular composition)-level parameters were documented at similar levels between experimental corals and controls incubated at 26.5 °C, suggesting that this species can readily acclimate to elevated temperatures that cause significant degrees of stress, or even bleaching and mortality, in conspecifics of other regions of the Indo-Pacific. However, the gastrodermal tissue layer was relatively thicker in corals of the high temperature treatment sampled after nine months, possibly as an adaptive response to shade *Symbiodinium* from the higher photosynthetically active radiation levels that they were experiencing at that sampling time. Such shading may have prevented high light and high temperature-induced photoinhibition, and consequent bleaching, in these samples.

Keywords Acclimation · Coral reefs · Endosymbiosis · Gene expression · Thermal stress · Upwelling

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Introduction

Scleractinian corals and the reefs they construct are potentially threatened by rising global temperatures and pCO_2 (Hoegh-Guldberg et al. 2007) due to the fact that coral–dinoflagellate (genus *Symbiodinium*) endosymbioses have typically been shown to disintegrate (i.e., bleach) upon prolonged exposure to changes in seawater quality (Gates 1990; Mayfield and Gates 2007). However, some recent works have shown that not all corals bleach, or even manifest signs of stress, at elevated temperatures predicted to characterize reefs in the coming decades (Barshis et al. 2013). For instance, corals from highly variable

temperature environments of both American Samoa (Oliver and Palumbi 2011) and southern Taiwan (Mayfield et al. 2011, 2013) have previously been shown to withstand exposure to temperatures (e.g., 30–31 °C) that induce bleaching or even mortality in conspecifics from other regions (Jokiel and Coles 1990; Brown 1997), and there has been a growing interest in attempting to understand how these corals acclimate to temperatures that are >1 °C above their mean summer maxima.

In recent years, southern Taiwan has served as a natural laboratory for studying the physiological and molecular basis for reef coral resilience to temperature changes (e.g., Putnam et al. 2010), as there are locations within close proximity that demonstrate dramatically different temperature profiles (Mayfield et al. accepted). For instance, Nanwan Bay, the southernmost embayment in Taiwan, is characterized by episodic, summer, spring tide upwelling (Jan and Chen 2008) in which temperature changes of up to 8–9 °C (typically from 19 to 27 °C or 20 to 28 °C) can occur over a single day. It was previously found that *Seriatopora hystrix* specimens of Houbihu, a reef within Nanwan Bay, do not demonstrate a gene-level molecular chaperone response in response to elevated temperature (29.5 °C) exposure (Mayfield et al. 2011), potentially due to "front-loading" of stress-targeted gene (STG) expression (Barshis et al. 2013).

Several kilometers away from Nanwan Bay are coral reefs (e.g., Houwan) that do not experience upwelling and are hence characterized by relatively stable temperature regimes (Lee et al. 1999). However, *S. hystrix* populations from Houwan can readily acclimate to fluctuating temperature regimes they never experience in situ (Mayfield et al. 2012a), suggesting a marked ability for phenotypic plasticity (Mayfield et al. accepted). *Pocillopora damicornis* specimens from intertidal regions of Houwan have even been shown to acclimate to temperatures up to 31.5 °C, though only when the temperature was reduced to ambient levels at night (Mayfield et al. 2013). These works collectively demonstrate that certain corals of southern Taiwan have the capacity to acclimate to high and/or fluctuating temperatures, though to date, only their short-term (days–weeks) responses have been documented.

To uncover the long-term impacts of elevated temperature exposure to corals from reefs that experience episodic upwelling, a mesocosm-based experiment (sensu Liu et al. 2009) was conducted whereby *P. damicornis* specimens collected from the upwelling coral reef Houbihu were exposed for nine months to nearly 30 °C, a temperature they only encounter in situ for several hours a year (Mayfield et al. 2012a), as well as one expected to characterize their environments in the coming decades (IPCC 2007). It was hypothesized that these samples would be able to acclimate to this temperature, given the results of the aforementioned studies describing the remarkable thermal plasticity of corals of Nanwan Bay, Taiwan. However, it was also hypothesized that cellular and sub-cellular parameters, described in detail below, might differ between treatments and thus yield insight into the molecular mechanisms underlying physiological acclimation to elevated temperature over a long-term timescale in a ubiquitously distributed reef coral (Veron 2000).

Assessing the health of a coral is actually a controversial topic (Weis 2008) because, for instance, vision-based indices, such as degree of pigmentation, do not necessarily reflect what is occurring at the cellular or sub-cellular level. It is possible that a coral may be darkly pigmented and free of disease or macroalgal overgrowth, yet be displaying aberrant gene or protein expression patterns that collectively suggest that the cells are enacting a stress response necessary to attempt to restore homeostasis (Kultz 2005). The approach taken herein was to target response variables across multiple biological scales, from the whole colony down to the molecular level. First, a series of parameters aimed to gauge the response of the coral at the polyp and colony levels of biological organization were measured, including surface area (SA; a proxy for size), *Symbiodinium* density, and chlorophyll *a* (chl-*a*) content. Although these parameters have repeatedly been shown to decrease in thermally challenged corals (e.g., Hoegh-Guldberg and Smith 1989; Fitt et al. 2009), they were expected to remain similar between the corals of the control (26.5 °C) and high temperature treatments herein given that prolonged exposure to this temperature was not hypothesized to cause stress to these corals.

To gauge the cell- and tissue-level response, the thickness of both the epiderm and gastroderm, as well as the total tissue thickness, were measured with either histology or scanning electron microscopy (SEM). Tissue thickness has been hypothesized to serve as an important determinant of bleaching susceptibility (Loya et al. 2001), with species with relatively thinner tissues, such as *P. damicornis*, typically demonstrating greater temperature sensitivity due to their diminished capacity for self-shading of their *Symbiodinium* populations (Hoegh-Guldberg 1999). Thickness of the individual tissue layers, as well as the total thickness and epiderm/gastroderm tissue thickness ratio, were hypothesized to be similar between treatments at the later sampling times (24 and 36 weeks), given that the high temperature specimens were predicted to have acclimated to high temperature by this point in the experiment.

Finally, expression of both *Symbiodinium* and host coral target genes was measured across the experiment, and there was a particular focus on genes encoding proteins involved in photosynthesis, metabolism, and the stress response (sensu Mayfield et al. in press). Photoinhibited *Symbiodinium* might display reductions in photosynthesis-targeted gene (PTG) expression (Doo et al. 2012), as well as corresponding decreases and increases in metabolism-targeted gene (MTG) and STG expression (Downs et al. 2000),

respectively. Therefore, expression of four PTGs; ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*), photosystem I (subunit III, *psI*), phosphoglycolate phosphatase (*pgpase*), and ascorbate peroxidase (*apx1*); two MTGs: nitrate transporter-2 (*nrt2*) and a putative carbonic anhydrase; and one STG: heat shock protein-70 (*hsp70*; also assessed in the host), was measured and, in contrast to the physiological and tissue-level parameters, hypothesized to vary in response to the experimental treatment given that large changes in gene expression have been documented even in physiologically uncompromised corals exposed to elevated temperatures (Barshis et al. 2013). It was further hypothesized that such gene expression differences could help to develop a mechanism by which *P. damicornis* acclimates increases in temperature (sensu Podrabsky and Somero 2004).

Materials and methods

Mesocosm design

Six mesocosms (150 l) were constructed indoors at Taiwan's National Museum of Marine Biology and Aquarium (NMMBA) as in Mayfield et al. (2013) except that natural light was used. Briefly, a porous, black tarp was suspended 15 m above the mesocosms. Although the use of natural light indeed introduces the concern that not all sampling times will be conducted at the same photosynthetically active radiation (PAR) level, it was hypothesized that its use would allow for the corals to survive for a greater length of time relative to those exposed to artificial light based on the results of our previous, unpublished work. The six mesocosms were housed within a larger aquarium system described in Mayfield et al. (2010), and seawater was pumped directly from nearby Houwan (22°01′23.30″N, 120°41′18.29″E) and filtered through sand before entering the mesocosms. As such, the corals and other experimental organisms were able to feed over the duration of the experiment. Crustose coralline algae (CCA), sea urchins, sea cucumbers, gastropods, and a variety of other taxa common to the southern Taiwan reef ecosystems (sensu Mayfield et al. 2013) were collected from NMMBA's husbandry facility and cultured simultaneously in each of the six mesocosms for two months prior to introduction of the *P. damicornis* specimens (described below). The same total mass and density of each species (sensu Mayfield et al. 2013) were used in each mesocosm (data not shown).

Experimental design

Six *P. damicornis* colonies were collected under Kenting National Park permit 0992900842 from approximately 3 m depth on SCUBA from Houbihu, Nanwan Bay, southern Taiwan (21°56′18.01″N, 120°44′45.54″E) in October 2010 and transported to seawater tables at NMMBA characterized by the same conditions as the experimental mesocosms. For more details on the oceanography and ecology of Houbihu, readers are referred to other works (e.g., Dai 1991; Meng et al. 2008). On the day of coral sampling, PAR was measured hourly on SCUBA with a cosine-corrected LI-193 meter attached to a LI-1400 data logger via a 10-m cable (Li-Cor Biosciences, Lincoln, NE, USA) from 06:00 to 20:00 h at the approximate site and depth of coral collection in order to estimate the average hourly PAR (μmol photons m^{-2} s^{-1}) in situ. A similar PAR sampling strategy was conducted at the same site on two other sampling days prior to experimentation. PAR was also measured hourly in each of the six mesocosms at randomly chosen days during the course of the experiment, and typically on the day of coral sampling, as well, in order to ensure that experimental levels were similar to those documented in situ. As natural light was used, relative changes in the light environment in the mesocosms, such as those driven by the lengthening of days in the summer, were expected to parallel those experienced by these coral populations in situ given the close distance (~20 km) between the sampling site and the mesocosm facility. Therefore, the average hourly diel PAR in the mesocosms was expected to demonstrate similar seasonal variation as in situ.

Three days after the *P. damicornis* colonies were brought to NMMBA, 12 nubbins of ~4 cm in maximum length and ~2 g [see Supplemental Fig. 1 of Mayfield et al. (2013)] were generated from each colony with pliers and mixed within the seawater table. Seventy-two nubbins were strung on fishing lines, suspended ~5 cm below the surface, and allowed to acclimate in the six mesocosms ($n = 12$ nubbins per mesocosm) for five weeks at 26.5 °C under shaded, natural light (average hourly PAR = 350 μmol photons m^{-2} s^{-1}). Once tissue was found to have completely overgrown at the site of fracture, the six mesocosms were randomly assigned to one of the two treatments, and one nubbin taken was taken from each mesocosm at 14:00 h ($t = 0$) and processed as described below.

Then, the temperature was increased to 30 °C in the three high temperature mesocosms over the course of 4 h with AquaController Apex units (Neptune Systems, San Jose, CA, USA) connected to both an aquarium heater and a cooler (both from AquaTech, Kaohsiung, Taiwan) as described in Mayfield et al. (2013). The other three mesocosms were maintained at 26.5 °C over the duration of experiment, and additional sampling of one nubbin per mesocosm at 14:00 h was conducted after 2, 4, 8, 24, and 36 weeks ($n = 3$ biological replicates at each sampling time). As such, not all nubbins were sacrificed in the experiment.

Temperature, PAR, and salinity were monitored routinely over the course of the experiment. The former was measured with a digital certified thermometer (15-077-8, accuracy 0.05 °C, resolution 0.001 °C; Control Company, Friendswood, TX, USA) and logged at 10-min intervals with a HOBO Pendant® data logger (Onset Corporation, Bourne, MA, USA). PAR was measured as described above, and salinity was measured as described in Mayfield et al. (2013).

Physiological analyses

Nubbins were visually inspected at least once a day to check for bleaching and mortality (sensu Mayfield et al. 2013). Nubbins that died were removed once all tissues had sloughed off and the skeletons were covered by non-symbiotic macroalgae. These nubbins were not processed. The total number of nubbins that died in each mesocosm was summed within mesocosms of each treatment to calculate the percent mortality after 36 weeks of treatment exposure.

At all sampling times, a ~50 mg branch each from the six sampled nubbins was broken off and submerged in 500 µl TRIzol® (Life Technologies, Grand Island, NY, USA) for later extraction of RNA, DNA, and protein. Nubbins were not buoyantly weighed, as this process takes 5–10 min, a period of time in which gene expression, in particular, could change.

Instead, surface area (SA) was used as a proxy for coral size, as the nubbins were of a similar mass at the beginning of the experiment and similarly sized branches were removed for tissue and molecular analyses. In order to calculate SA, the tissue was removed from the skeleton as described in Mayfield et al. (2012a) from samples collected at 0, 24, and 36 weeks only, and the SA of the skeletons was then calculated as in Stimson and Kinzie (1991). The tissue from these samples was collected and processed for *Symbiodinium* density and chl-*a* concentration as described in Mayfield et al. (2012a), and both of these parameters were normalized to SA. The areal chl-*a* (µg cm^{-2}) was divided by the *Symbiodinium* density (10^6 cells cm^{-2}) of the same sample to yield cell-specific chl-*a* concentration (pg $cell^{-1}$).

Tissue analyses

For nubbins sampled at 24 (to be analyzed by histology) and 36 (to be analyzed by SEM) weeks, an additional ~100 mg branch was removed prior to tissue removal and anesthetized in 3.5 % $MgCl_2$ [in filtered seawater (FSW)] for 10 min at room temperature (RT). Then, the branches were immersed in 7 % $MgCl_2$ (in FSW) and incubated for 10 min at RT, at which point all polyps had extended. Branches were then fixed for 3 h at 4 °C in 3.6 % freshly prepared paraformaldehyde in phosphate-buffered saline (PBS). Then, branches were washed thrice with PBS for 10 min each wash. For a detailed description of the histology and SEM methodologies, please see Electronic Supplemental Material (ESM) Appendix 1.

Molecular analyses

For a detailed description of the molecular analyses employed herein, please see ESM Appendix 2.

Statistical analyses

All statistical analyses were conducted with JMP® (ver. 5.0, SAS Institute, Inc., Cary, NC, USA). For seawater temperature data, a two-way ANOVA was used to test for the effect of treatment, time, and their interaction. To analyze the PAR data acquired at the time of nubbin collection only, a repeated-measures ANOVA with mesocosm nested within temperature treatment was conducted over the 8-, 24-, and 36-week sampling times. For parameters assessed at one sampling time (i.e., histology and SEM-derived parameters), a one-way ANOVA with polyp nested within sample was used to test for an effect of temperature. When the polyp term was not statistically significant, it was dropped from the model. In order to determine changes between treatments over time for all physiological and molecular composition parameters, repeated-measures ANOVAs employing a MANOVA model were used with mesocosm nested within treatment. Finally, a two-way ANOVA was used to test for the effects of temperature, tissue layer, their interaction, and mesocosm nested within temperature for the SEM-derived tissue thickness data. In all cases, the mesocosm term was dropped from the model when not found to be significant ($p > 0.05$).

Univariate tests were used in place of the MANOVA for the gene expression data, as an outlier was identified (see Table 2 for details). Chi-square tests were used to test for differences in the proportions of samples housing *Symbiodinium* of clade A. For all parameters, Shapiro–Wilk W and Levene's tests were used to determine whether datasets were normally distributed and of homogeneous variance, respectively, and when such was not the case, log transformations were conducted. Tukey's honestly significant difference (HSD) tests were used to test for individual mean differences when significant differences were detected in the model ($p < 0.05$). In all cases below, standard errors are presented unless otherwise noted.

Results

Experimental conditions

Seawater temperature (Fig. 1a) differed significantly between treatments and averaged 26.5 ± 0.25 and 29.7 ± 0.06 °C for

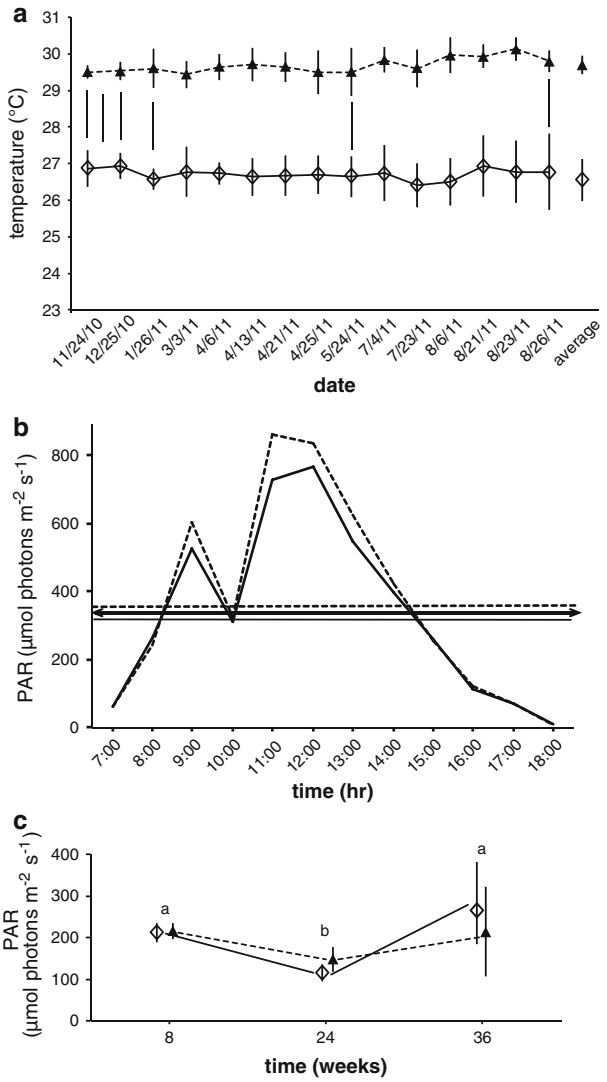

Fig. 1 Seawater quality data. **a** Mean temperature (°C; ± standard deviation) on randomly chosen days was plotted across both the control (*white diamond*) and high temperature (*black triangle*) mesocosms ($n = 3$). *Vertical lines* within the temperature plots indicate the times at which corals were sampled. **b** Photosynthetically active radiation (PAR; µmol photons m^{-2} s^{-1}) was measured hourly as described in the text, and data from a representative day (October 5, 2011) were plotted across both the control (*solid line*) and high temperature (*dotted line*) mesocosms. The *solid, dotted, and arrowed lines plotted horizontally* represent the average diel PAR levels for the control mesocosms, high temperature mesocosms, and Houbihu, respectively. **c** The average PAR at the time of coral sampling (~14:00 h) was plotted at the 8-, 24-, and 36-week sampling times for both the control (26.5 °C; *white diamonds*) and high (29.7 °C; *black triangles*) temperature treatments. Letters denote Tukey's honestly significant difference temporal groups ($p < 0.05$), and *error bars* represent standard error of the mean

the control and high temperature mesocosms, respectively (Table 1). However, temperature did not vary over time or in response to the interaction of temperature and time (Table 1), suggesting that it was stable across the duration of the experiment within treatments. That being said, temperature did vary across tanks within treatments (Table 1), though this may be due to the large number of data points (~40,000).

A representative diel PAR plot is shown in Fig. 1b. The average hourly PAR of the control (338 ± 55 µmol photons m^{-2} s^{-1}) and high temperature mesocosms (370 ± 6.0 µmol photons m^{-2} s^{-1}) during the pre-experimentation period (October 2010) did not differ significantly from the average hourly PAR measured at the depth of coral collection (354 ± 63 µmol photons m^{-2} s^{-1}, $n = 3$ sampling days; Wilcoxon rank-sum test, $Z = 0.0524$, $p = 0.974$). With respect to PAR levels at the time of coral sampling (Fig. 1c), PAR was statistically similar between treatments (Table 1) and, unlike temperature, was also statistically similar across mesocosms within treatments (Table 1). However, the average PAR assessed across mesocosms of both treatments did vary between sampling times (Table 1). Specifically, the PAR at the 24-week sampling time (14:00 h; 132 ± 25.2 µmol photons m^{-2} s^{-1}) was approximately two-fold lower than the PAR measured at weeks 8 and 36 (14:00 h; 214 ± 21.1 and 249 ± 104 µmol photons m^{-2} s^{-1}, respectively).

With respect to the biology of the six mesocosms, there were no notable differences in the density of the cultured organisms (data not shown), and none of the grazing fauna (e.g., sea urchins and gastropods) died over the course of the experiment. CCA was abundant on the surface of all mesocosms, suggesting that the various species of unidentified CCA cultured herein are resilient to exposure to 29.7 °C. While exact density and biomass measurements were not calculated, it does not appear that the ~3 °C temperature differential had a visible impact on the ecology of the mesocosms, though a more quantitative approach should be taken in future mesocosm-based experiments to demonstrate that such is indeed the case.

Physiological response variables

Over the course of the 36-week experiment, four nubbins (11.1 %) died in each of the two treatments (Fig. 2a), and these nubbins were not processed for physiological or molecular analyses. Two of the four control nubbins died within the first 10 days of experimentation, potentially suggesting that they had not yet acclimated to the ex situ environment. On the other hand, the first high temperature treatment nubbin did not die until the fifth month of treatment exposure.

After 24 weeks of treatment exposure, SA was similar between treatments (Fig. 2b). Nubbins sacrificed after 36 weeks (96.0 ± 26.8 cm^2) had 16-fold more SA relative to those sampled at 24 weeks (6.12 ± 2.73; Table 2). However, there was no effect of temperature or the interaction of time and temperature on this parameter (Table 2). *Symbiodinium*

Table 1 Seawater quality parameters

Parameter (source of variation)	Statistical test	*F*/exact *F*	*p*	Fig.
Temperature (°C)				1a
Treatment	Two-way ANOVA	334	**<0.0001**	
Time		1.010	0.455	
Treatment × time		1.070	0.400	
Mesocosm (treatment)		8.98	**<0.001**	
PAR (μmol photons m^{-2} s^{-1})	Repeated-measures ANOVA			1c
Treatment		0.0126	0.916	
Time		12.4	**0.0356**	
Treatment × time		0.465	0.667	
Mesocosm (treatment)		0.621	0.649	
Salinity (psu)	Repeated-measures ANOVA			Not shown
Treatment		21.4	**<0.0001**	
Time		72.2	**<0.0001**	
Treatment × time		1.93	0.0898	
Mesocosm (treatment)		2.29	0.0817	

F and exact *F* statistics correspond to the results of two-way and repeated-measures ANOVAs, respectively. Statistically significant differences are emphasized in bold font. *PAR* photosynthetically active radiation

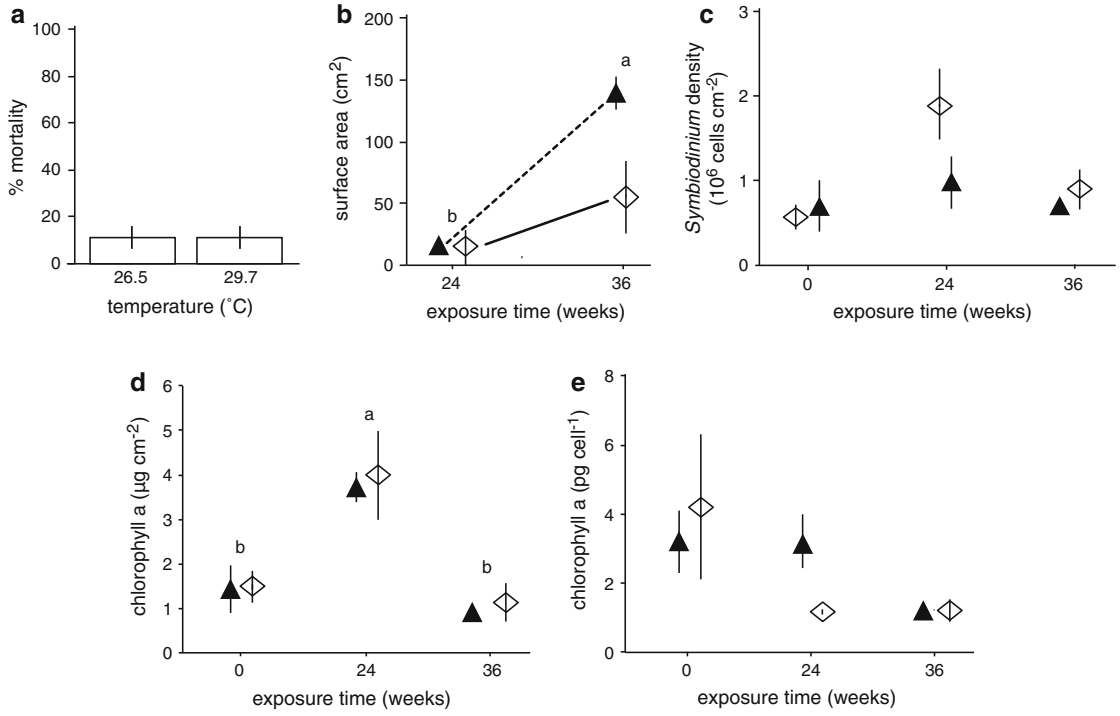

Fig. 2 Physiological response variables. Mortality (**a**), surface area (**b**), *Symbiodinium* density (**c**), and areal (**d**) and per-cell (**e**) chlorophyll *a* (chl-*a*) concentration were assessed in *Pocillopora damicornis* nubbins incubated at either the control (26.5 °C; *white diamonds* in **b–e**) or the high (29.7 °C; *black triangles* in **b–e**) temperature treatments for 36 weeks. In all panels, *error bars* represent standard error of the mean. In **b** and **d**, *letters above icons* represent Tukey's honestly significant difference groups ($p < 0.05$) for the effect of time only

density (Fig. 2c) did not differ over time or between treatments (Table 2) and averaged ~ 10^6 cells cm^{-2}. Areal chl-*a* (Fig. 2d) was temporally variable (Table 2) and was significantly higher in nubbins sampled after 24 weeks of treatment exposure (3.74 ± 0.497 μg cm^{-2}) relative to those sampled at the beginning (1.88 ± 0.392 μg cm^{-2}) and end (0.965 ± 0.222 μg cm^{-2}) of the experiment. Finally, cell-specific chl-*a* (Fig. 2e) did not differ between treatments or over time (Table 2) and typically ranged from 1 to 4 pg $cell^{-1}$ (mean = 2.37 ± 0.543 pg $cell^{-1}$).

Table 2 One-way, repeated-measures ANOVA of physiological parameters

Parameter (source of variation)	Exact F	p	Tukey's groups	Fig.
Surface area (cm^2)				2b
Temperature	4.30	0.1069		
Time	18.1	**0.0127**	36 > 24	
Temperature × time	4.24	0.1085		
Symbiodinium density (10^6 cells cm^{-2})				2c
Temperature	2.44	0.193		
Time	3.56	0.161		
Temperature × time	1.76	0.312		
Chlorophyll *a* (μg cm^{-2})[a]				2d
Temperature	0.358	0.582		
Time	15.3	**0.0266**	24 > 36 = 0	
Temperature × time	0.264	0.784		
Chlorophyll *a* (pg $cell^{-1}$)				2e
Temperature	0.240	0.650		
Time	1.82	0.3033		
Temperature × time	1.71	0.320		

Statistically significant differences are emphasized in bold font

[a] Log-transformed data

Tissue-level response variables

The mean tissue thickness ratio of nubbins sampled after 24 weeks (Fig. 3a, b) was 1.0 ± 0.16 (Fig. 3c), and there was no difference between treatments (one-way ANOVA, $F = 0.140, p = 0.89$). SEM (Fig. 4a–h) was used to assess the thickness of the individual tissue layers (Fig. 4i), as well as the epiderm/gastroderm ratio and total tissue thickness (Fig. 4j). A two-way ANOVA testing the effects of temperature, tissue layer, and their interaction found that the gastroderm (13.9 ± 0.848 μm) was significantly thicker than the epiderm (10.3 ± 0.499 μm, $F = 12.6$, $p < 0.001$). Individual mean (Tukey's HSD) differences can be found in Fig. 4i. Briefly, the epiderm was ~15 % thinner at the high temperature relative to the control temperature, while the gastroderm was ~10 % thicker in corals of the high temperature treatment. This led to a statistically significant ($F = 2.19$, $p = 0.0344$) 25 % reduction in the epiderm/gastroderm ratio (Fig. 4j) in nubbins of the high temperature treatment relative to the controls. In contrast, the total tissue thickness (Fig. 4j) was similar between treatments ($F = 0.171, p = 0.865$).

Molecular composition

RNA/DNA (ESM Fig. 1a; mean = 2.3 ± 0.18) and protein/DNA (ESM Fig. 1b; mean = 75 ± 5.3) ratios were stable between treatments and over time (Table 3). On the other hand, both the *Symbiodinium* (ESM Fig. 1c) and host (ESM Fig. 1d) GCPs changed significantly over time (Table 3). Notably, the real-time PCR-derived *Symbiodinium* DNA content decreased approximately three-fold over the course of the experiment, which led to a corresponding increase in the relative proportion of host DNA.

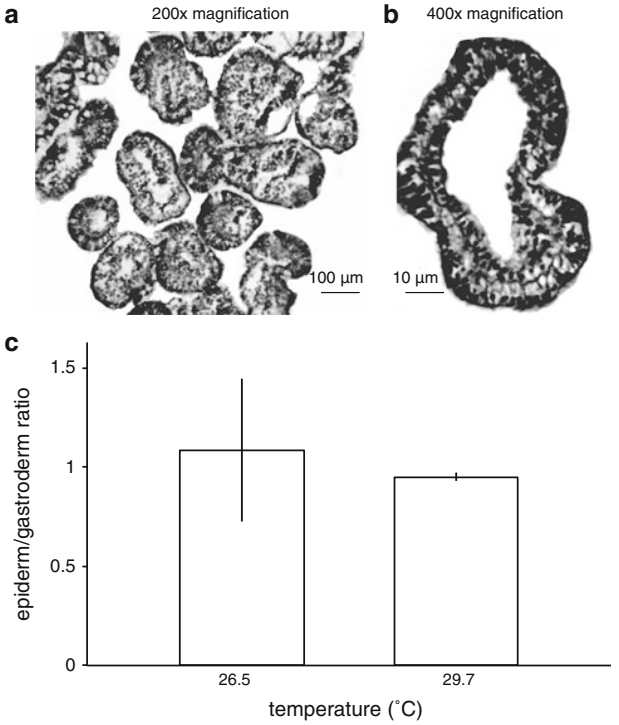

Fig. 3 Histology. *Pocillopora damicornis* tissues were anesthetized, fixed, decalcified, embedded, sectioned, and stained as described in the text for samples exposed to control (26.5 °C) or high (29.7 °C) temperature treatments for 24 weeks. **a** A cross section (6 μm) of a representative polyp. **b** A cross section (6 μm) of a representative tentacle. **c** The ratio of the thickness of the epiderm versus that of the gastroderm was calculated as described in the text, and *error bars* represent standard error of the mean

The *Symbiodinium* DNA was also used for genotyping, and it was found that all nubbins possessed *Symbiodinium* of clade C ($C_t < 30$) but not those of clade B or D. The

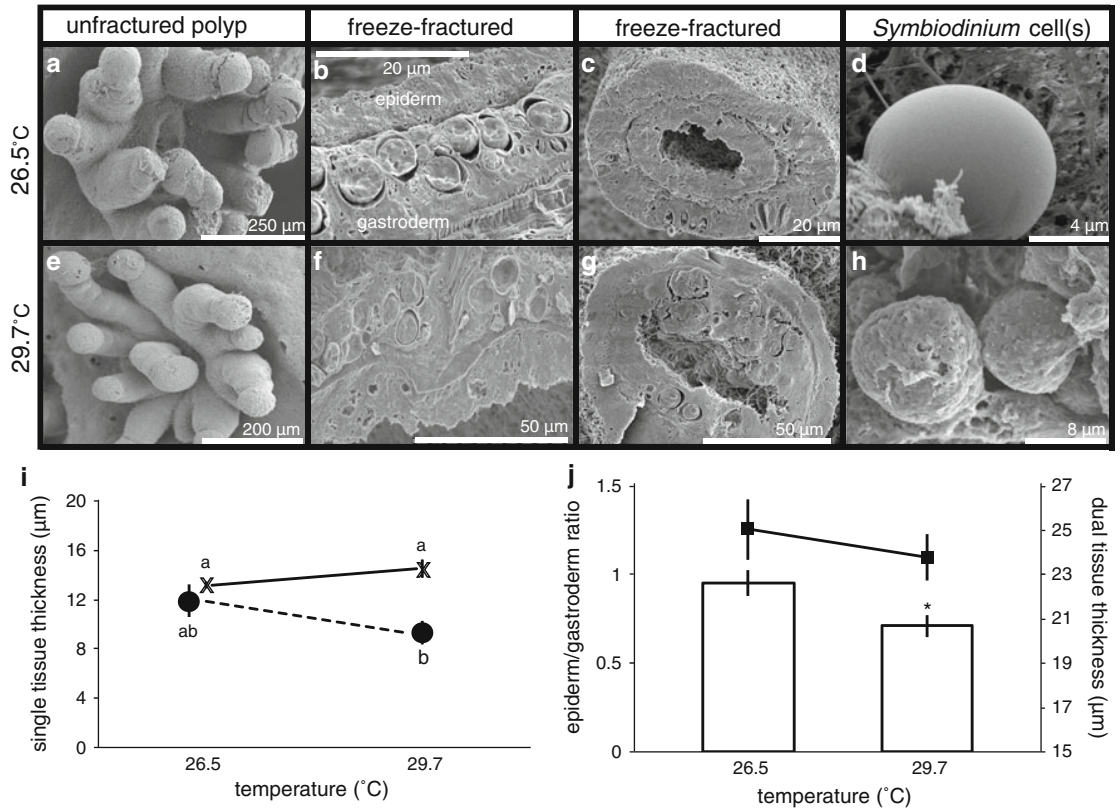

Fig. 4 Scanning electron microscopy (SEM). Representative micrographs of non-freeze-fractured polyps (**a** and **e**), freeze-fractured tissues (**b–c** and **f–g**), and *Symbiodinium* isolated from freeze-fractured tissues (**d** and **h**) are shown for corals of both the control (26.5 °C; **a–d**) and high (29.7 °C; **e–h**) temperature treatments sampled after 36 weeks of treatment exposure. **i** The thickness of both the gastroderm (*black exes*) and epiderm (*black circles*) was measured as described in the text, and *letters adjacent to icons* represent Tukey's honestly significant difference groups ($p < 0.05$). **j** The ratio of the thickness of the epiderm relative to that of the gastroderm (columns and left *y*-axis), as well as the tissue thickness of both layers (*squares connected by a solid line*, right *y*-axis), was calculated as described in the text. The asterisk denotes the significant difference between treatments of the former parameter discussed in the text. *Error bars* represent standard error of the mean for panels **i–j**

presence of clade A *Symbiodinium* (Table 4) was variable over time and between treatments, and there was a significantly higher proportion of control corals hosting clade A *Symbiodinium* at the 4-week sampling time relative to the high temperature nubbins (Table 4). Finally, the proportion of nubbins hosting clade A *Symbiodinium* was variable over time for the control, but not the high temperature treatment (Table 4).

Gene expression

The expression of none of the eight genes targeted herein was affected by temperature, though the majority of the genes demonstrated variable expression over time (Table 3). Specifically, expression of *Symbiodinium rbcL* (Fig. 5a), *psI* (Fig. 5b), *pgpase* (Fig. 5c), *apx1* (Fig. 5d), and *hsp70* (Fig. 6a), as well as host coral *hsp70* (Fig. 6b), varied significantly over time and was, in general, highest in corals sampled after 24 weeks of exposure. Only *Symbiodinium nrt2* (Fig. 5e) and *ntf2*-like (Fig. 5f) were expressed at stable levels over time (Table 3). It is evident from Figs. 5 and 6 that the seven *Symbiodinium* genes tended to covary and, in addition to being expressed at highest levels at sampling time 24, were expressed at the lowest levels at the 36-week sampling time. For instance, *rbcL* was expressed at 6.2-fold higher levels after 24 weeks relative to 36 weeks. Likewise, *Symbiodinium psI*, *pgpase*, *apx1*, and *hsp70* were expressed at 4.4, 3.6, 4.9, and 5.1-fold higher levels, respectively, in corals sampled after 24 weeks relative to those sacrificed at 36 weeks. Although the host *hsp70* expression was also highest in corals sampled after 24 weeks, the lowest levels were instead documented in those sampled after 4 and 8 weeks, and there was a 2.6-fold difference in expression between the highest and lowest expression levels. Finally, there was a weak ($r^2 = 0.20$), positive, statistically significant correlation between *Symbiodinium* and host *hsp70* expression (Fig. 6c).

Table 3 One-way, repeated-measures ANOVA of molecular composition and gene expression parameters

	Exact F	p	Reference	Fig.
RNA/DNA				ESM Fig. 1a
Temperature	0.183	0.710	Mayfield et al. (2011)	
Time	3.71	0.0543		
Temperature × time	1.14	0.4030		
Protein/DNA				ESM Fig. 1b
Temperature	0.714	0.460	Mayfield et al. (2011)	
Time	0.7060	0.6029		
Temperature × time	0.210	0.928		
Symbiodinium/host genome copy proportion[a]				ESM Fig. 1c–d
Temperature	1.14	0.363	Mayfield et al. (2011)	
Time	0.852	0.519		
Temperature × time	1.62	0.234		
Solaris™ RNA spike				Not shown
Temperature	2.89	0.231	Putnam et al. (in press)	
Time	1.093	0.422		
Temperature × time	2.55	0.121		
Symbiodinium rbcL expression[a]				5a
Temperature	0.181	0.712	Mayfield et al. (2012a)	
Time	14.0	**0.00110**		
Temperature × time	3.57	0.0597		
Symbiodinium psI (subunit III) expression[a]				5b
Temperature	0.131	0.753	Mayfield et al. (2012a)	
Time	11.7	**0.00200**		
Temperature × time	3.49	0.0623		
Symbiodinium pgpase expression[a]				5c
Temperature	0.00850	0.935	Crawley et al. (2010)	
Time	7.39	**0.00850**		
Temperature × time	1.709	0.240		
Symbiodinium apx1 expression[a]				5d
Temperature	0.4030	0.591	Mayfield et al. (2012a)	
Time	8.55	**0.00550**		
Temperature × time	5.083	**0.0246**		
Symbiodinium nrt2 expression[a]				5e
Temperature	1.077	0.4084	Mayfield et al. (2013)	
Time	5.78	**0.0173**		
Temperature × time	0.499	0.738		
Symbiodinium ntf2-like[a]				5f
Temperature	1.47	0.350	Vidal-Dupiol et al. (2009)	
Time	7.21	**0.00920**		
Temperature × time	2.10	0.173		
Symbiodinium hsp70 expression[a]				6a
Temperature	0.417	0.585	Mayfield et al. (2009)	
Time	12.5	**0.00160**		
Temperature × time	4.45	0.0348		
P. damicornis hsp70 expression[a]				6b
Temperature	1.206	0.387	Putnam et al. (in press)	
Time	3.95	**0.0466**		
Temperature × time	0.857	0.528		

Univariate tests were used for analysis of all gene expression data since there were only two replicates at sampling time 24 due to having removed an outlier (control tank 2)

[a] Log-transformed data

Table 4 Proportion of samples hosting clade A *Symbiodinium*

Treatment	2 weeks	4 weeks	8 weeks	24 weeks	36 weeks	X^2 p value
Control (26.5 °C)	0.00	1.00	0.33	0.33	0.00	**0.02**
High (29.7 °C)	0.33	0.33	0.33	0.67	0.33	0.90
X^2 p value	0.21	**0.05**	1.00	0.41	0.21	–

Presence was verified when threshold cycle (C_t) values less than 30 were measured in the real-time PCR assay. The X^2 p values in the bottom row correspond to comparisons between treatments at each sampling time, while those in the rightmost column correspond to temporal differences within each treatment. Statistically significant values are emphasized in bold font

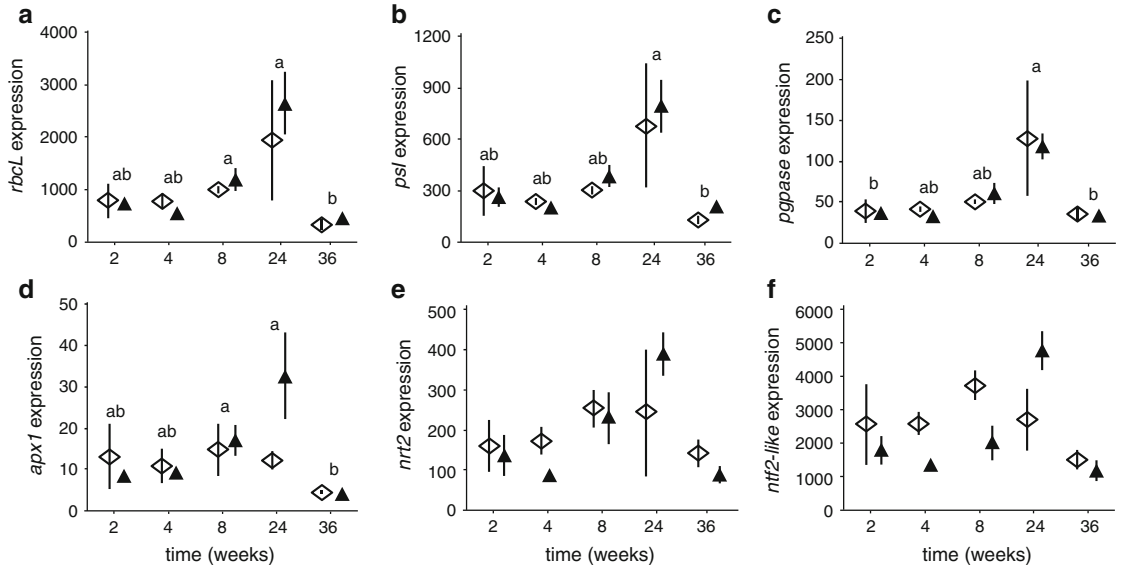

Fig. 5 *Symbiodinium* gene expression. *Symbiodinium* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*; **a**), photosystem I (*psI*, subunit III; **b**), phosphoglycolate phosphatase (*pgpase*; **c**), ascorbate peroxidase (*apx1*; **d**), nitrate transporter-2 (*nrt2*; **e**), and nuclear transport factor-2 (*ntf2*-like; **f**) expression in corals exposed to either control (26.5 °C; *white diamonds*) or high (29.7 °C; *black triangles*) temperatures. In **a–d**, *letters above icons* indicate Tukey's honestly significant difference groups ($p < 0.05$) for the effect of time only, and in all panels, *error bars* represent standard error of the mean

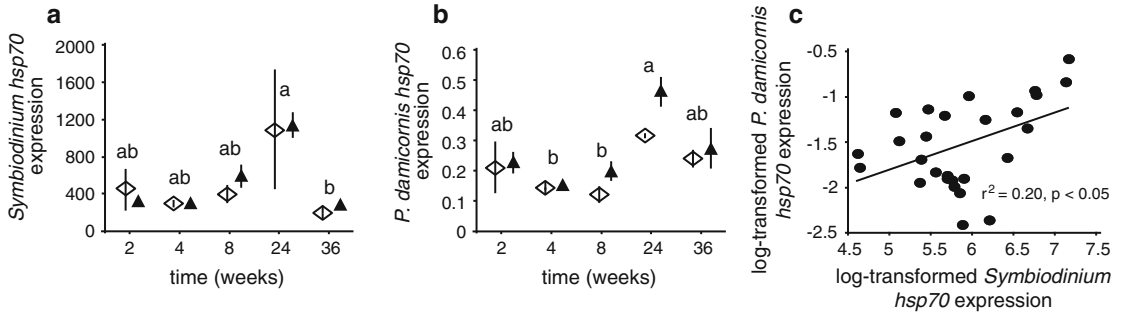

Fig. 6 *Symbiodinium* and host coral heat shock protein-70 (*hsp70*) gene expression. *Symbiodinium* (**a**) and coral (**b**) *hsp70* mRNA expression was measured in samples exposed to either control (26.5 °C; *white diamonds*) or high (29.7 °C; *black triangles*) temperatures. In both panels, *error bars* denote standard error of the mean, and *letters above icons* represent Tukey's honestly significant difference groups ($p < 0.05$) for the effect of time only. **c** The degree of correlation in expression of this gene ortholog within holobionts was assessed by plotting the log-transformed host expression against the log-transformed *Symbiodinium* expression, and the positive association ($r^2 = 0.20$) was found to be statistically significant (linear regression t test, $t = 16.8$, $p < 0.001$)

Discussion

Upon a collective assessment of the colony (Fig. 2), polyp-level (Fig. 2), and sub-cellular (Figs. 5, 6 and ESM Fig. 1) parameters assessed herein, it appears that *P. damicornis* specimens from Houbihu, Nanwan Bay, Taiwan, can acclimate to a temperature approaching 30 °C, as had been hypothesized. Specifically, the SA, *Symbiodinium* density, chl-*a* content, RNA/DNA and protein/DNA ratios, GCPs, and gene expression were markedly similar between treatments at each sampling time. In fact, corals of the high temperature mesocosms tended to have greater SA at the final sampling time, though this difference was not statistically significant. In the future, it will be interesting to conduct an identical study with corals collected from a non-upwelling reef, such as Houwan, in order to prove that this acclimation capacity is indeed driven by thermal history and is not a general property of all *P. damicornis* populations of southern Taiwan. Previously, it was shown that the *S. hystrix* samples from Houwan could acclimate to a fluctuating temperature regime that they would rarely, if ever, experience in situ (Mayfield et al. 2012a). Whether or not this suggests that con-familiars from the same site could acclimate to elevated temperatures remains to be demonstrated.

The average high treatment temperature employed herein, 29.7 °C, is over 1 °C warmer than the maximum daily mean summer temperature of this reef (28.5 °C, Mayfield et al. 2012a). A significant body of prior work (e.g., Glynn 1983) has found that prolonged exposure to a temperature ~1 °C greater than the summer maximum elicits coral stress and can ultimately lead to bleaching and mortality if continued for many days (Jokiel and Coles 1990). In terms of previously documented responses of *P. damicornis* specifically, Mayfield et al. (2013) found that *P. damicornis* specimens from intertidal locations of southern Taiwan could acclimate to temperatures up to 31.5 °C, but only when the temperature was reduced to ambient (27 °C) levels at night. When a sustained temperature of 31.5 °C was employed, all corals died within two weeks, an observation corroborated by microcosm-based studies with this same species (Vidal-Dupiol et al. 2009). Given that the corals from Houbihu, southern Taiwan, used in this study appeared to have acclimated to 29.7 °C, yet those from nearby intertidal regions died upon sustained exposure to 31.5 °C, it stands that the temperature threshold beyond which detectable signs of stress can be documented in this coral is between these two temperatures.

The fact that no corals bleached or, more generally, appeared to be physiologically compromised at the elevated temperature employed herein suggests that a simple bleaching threshold cannot be established in the absence of a thorough understanding of the thermal history of the population in question. Indeed, there is now a growing body of evidence to support the notion that corals inhabiting more thermally unstable habitats outperform conspecifics from reefs characterized by more stable temperatures when exposed to elevated temperatures (Coles 1975; Castillo and Helmuth 2005; Oliver and Palumbi 2011). This differential performance has previously been hypothesized to be driven by preferential acquisition of thermotolerant clade D *Symbiodinium* (Baker 2003), though only *Symbiodinium* of clade A and C were found to be present in the corals sampled herein. Alternatively, a shift in nutrition mode from autotrophy to heterotrophy has been found to be important in the acclimation response of corals exposed to high temperatures in other studies (e.g., Grottoli et al. 2006); such a transition could indeed have occurred herein given that seawater was filtered only through sand before reaching the mesocosms, allowing for the corals to feed over the entire duration of the experiment.

In other systems, provocative gene expression changes, such as the constitutive upregulation of genes involved in thermotolerance (e.g., *hsp*s; Heath et al. 1993; Feder 1996), underlie the capacity for organisms to inhabit high and/or variable temperature environments. Such has, more recently, been documented in corals (Barshis et al. 2013), and, indeed, the expression of both host and *Symbiodinium hsp70* was high in all samples of this study, regardless of treatment. However, there was no evidence for expression differences between treatments for this, or any other, target gene, in contrast to what was hypothesized. This could be due to the targeting of non-responsive genes or, alternatively, those whose respective proteins are regulated post-transcriptionally. It is also possible that the associated pathways (i.e., photosynthesis, metabolism, and the stress response) were not affected by elevated temperature, suggesting that, in general, the coral and *Symbiodinium* cells had also acclimated at the molecular level.

Although gene expression within cells was similar between treatments, the size of these cells was affected by elevated temperature exposure. Specifically, though the total tissue thickness did not change, the epiderm/gastroderm tissue thickness ratio was significantly diminished in high temperature nubbins sampled after 36 weeks relative to controls, and this was due in part to an increase in thickness of the gastroderm. It is possible that this increase in gastrodermal thickness, which was not documented after 24 weeks of treatment exposure (spring), could be due to the relatively higher PAR level documented at the final sampling time, which took place in the summer. Specifically, this gastrodermal expansion could potentially have aided in shielding the *Symbiodinium* from the higher light levels they were experiencing during the summer months by increasing the distance between the coral plasma

membrane and the *Symbiodinium* cell wall. In this way, the potential for scattering and/or absorption of excess and/or harmful radiation (e.g., ultraviolet radiation) by specialized pigments or green fluorescent proteins (GFPs) could be enhanced (Salih et al. 2000; Smith et al. 2013). Indeed, preliminary data from transcriptome sequencing of these same samples (Mayfield pers comm) with Illumina TRU-Seq® (San Diego, CA, USA) technology found 26-fold enriched *gfp*-like chromoprotein gene expression levels in the *P. damicornis* specimens that had been exposed to 29.7 °C for 36 weeks.

Loya et al. (2001) conjectured that coral species with thinner tissues (i.e., the "losers") would have a diminished capacity for self-shading of *Symbiodinium*, and this could contribute to their limited ability to withstand elevated temperatures under high light levels. We have shown herein that thickness of the individual tissue layers is not a static property of *P. damicornis* populations of southern Taiwan; in fact, it is temporally variable and potentially responsive to changes in temperature and/or light and can allow for this "losing" (i.e., sensitive) coral species to acclimate to increases in temperature. Given these results, an investigation of the molecular mechanisms underlying this potentially adaptive increase in gastrodermal thickness, as well as the hypothesized increase in self-shading that such a shift could promote, represents a fruitful avenue for future research on this phenotypically plastic, Indo-Pacific reef builder.

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