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# Short-Term Thermal Acclimation Modifies the Metabolic Condition of the Coral Holobiont

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The nutritional symbiosis between coral hosts and photosynthetic dinoflagellates is fundamental to the functioning of coral reefs. Rising seawater temperatures destabilize this relationship, resulting in drastic declines in world-wide coral cover. Thermal history is thought to play an important role in shaping a coral's response to subsequent thermal stress. Here, we exposed *Pocillopora damicornis* to two thermal acclimation regimes (ambient vs. warm) and compared the effect that acclimation had on the coral holobiont's response to a subsequent seven day heat stress event. We conducted daily physiological measurements at the holobiont level (gross photosynthesis, respiration, host protein content, symbiont density and chlorophyll content) throughout the heating event, as well as cellular-level imaging of <sup>13</sup>C-bicarbonate and <sup>15</sup>N-nitrate assimilation (using NanoSIMS) at the end of the heat stress event. Thermal acclimation history had a negligible effect on the measurements conducted at the holobiont level during the heat stress event. No differences were observed in the O<sub>2</sub>-budget between ambient and warm-acclimated corals and only small fluctuations in host protein, symbiont density and chlorophyll content were detected. In contrast, this lack of differential response, was not mirrored at the cellular level. Warm-acclimated corals had substantially higher <sup>13</sup>C enrichment in the host gastrodermis and lipid bodies, but significantly lower <sup>15</sup>N-nitrate assimilation in the symbionts and the host tissue layers, relative to the ambient-acclimated corals. We discuss potential reasons for the disconnect that occurred between symbiont bicarbonate and nitrate assimilation (in the absence of photosynthetic breakdown) in the warm-acclimated corals. We suggest this represents either a shift in nitrogen utilization, or supply limitation by the host. Our findings raise several interesting hypotheses regarding the role that nitrogen metabolism plays in thermal stress, which will warrant further investigation if we are to understand the acclimatization capacity of the coral holobiont.

**Keywords:** scleractinian corals, *Symbiodinium*, NanoSIMS, global climate change, nitrogen, acclimation

## INTRODUCTION

The mutualistic relationship that exists between reef-building corals, photosynthetic microalgae (genus: *Symbiodinium*) and a consortium of microbial partners (the “coral holobiont”; Rosenberg et al., 2007) is critical for the health and persistence of reefs. The symbiotic partners exist in a closely-regulated, yet highly dynamic metabolic landscape, which is fuelled by the reciprocal exchange of nutrients (Wang and Douglas, 1999). The coral host affords *Symbiodinium* access to inorganic nutrients ( $\text{CO}_2$ ,  $\text{NH}_3$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ ) present in the seawater, as well as metabolic waste products (Muscatine and Porter, 1977; Muller-Parker and D’Elia, 1997), which the *Symbiodinium* cells recycle to synthesize organic compounds such as sugars, alcohols, amino acids, fatty acids and lipids (Burriesci et al., 2012). Most of the photosynthates produced are then translocated back to the coral or lost *via* respiration. The metabolic condition of the holobiont is strongly influenced by light (Falkowski et al., 1990), which imposes limitations on the rate of photosynthetic carbon fixation (Wangpraseurt et al., 2016) and nitrogen availability (Rädecker et al., 2015), which plays a central role in controlling the population density of the symbionts (Falkowski et al., 1993). In ambient conditions, *Symbiodinium* can meet up to 90–100% of the coral’s energetic demands (Falkowski et al., 1984; Muscatine, 1990), but in more extreme conditions (such as elevated temperatures and/or high solar irradiance), the symbiont contribution is greatly reduced (Grottoli et al., 2004). Sustained exposures to such conditions lasting weeks to months initiates a predictable cascade of cellular responses (Weis, 2008), which triggers the progressive loss of photosynthetic pigments and/or the expulsion of symbiont cells (Brown, 1997); a process known as coral bleaching. If a coral is deprived of its principle energy source for prolonged periods it will eventually starve and die. The devastating loss of reefs that followed the mass bleaching events of 1998, 2010, and 2016 (Hughes et al., 2017), are testament to the severe consequences that can arise from this symbiotic breakdown.

With ocean temperatures continuing to rise over the coming century (IPCC, 2014), an increase in the frequency and severity of global mass bleaching events is expected (Hoegh-Guldberg et al., 2007). Reef recovery following such events depends on the capacity of coral holobionts’ for acclimation and/or adaptation (Weis, 2010; Logan et al., 2014; Van Oppen et al., 2015). Reefs that have been subjected to frequent thermal anomalies and/or highly variable thermal regimes over the past century tend to exhibit higher bleaching thresholds than those exposed to stable conditions suggesting a certain degree of plasticity exists in the coral holobionts’ thermal resistance (Carilli et al., 2012). Indeed, some coral species exhibit increased energy reserves (Rodrigues and Grottoli, 2007; Grottoli et al., 2014) and/or changes in the dominant *Symbiodinium* type after bleaching events (Howells et al., 2012; Boulotte et al., 2016), while others modify their microbial community (Ziegler et al., 2017). A recent analysis of temperature fluctuations recorded over 27 years on the Great Barrier Reef, Australia, found that 75% of all thermal stress events were characterized by temperatures greater than the maximum monthly mean temperature (MMM) but less than

the bleaching threshold ( $\text{MMM}+1-2^\circ\text{C}$ ). These events were followed by a recovery period lasting 10 days on average, during which temperatures returned to levels below the MMM. Such fluctuations have been termed “protective” because they change the expression of key thermal stress-related genes and thus confer thermal resilience to the coral (Ainsworth et al., 2016). This could be an additional reason why increased resilience has been observed after consecutive bleaching events on some reefs (Maynard et al., 2008; Guest et al., 2012; Penin et al., 2013).

There is some experimental support for the hypotheses outlined above. For example, *Acropora aspera* acclimated in elevated but sub-lethal temperatures ( $31^\circ\text{C}$ , 2 days) for 1 or 2 weeks before a simulated bleaching event ( $34^\circ\text{C}$ , 6 days), retained more of their symbionts and exhibited enhanced photosynthetic efficiency than those exposed to ambient ( $27^\circ\text{C}$ ) conditions prior to the simulated bleaching event (Middlebrook et al., 2008). Similarly, *A. millepora* that were exposed to elevated temperatures ( $28^\circ\text{C}$ , 10 days) performed better than corals kept in control conditions when subsequently subjected to a simulated bleaching experience event ( $31^\circ\text{C}$ , 8 days) (Bellantuono et al., 2011). However, any benefits derived from short-term thermal acclimation (e.g.,  $33^\circ\text{C}$ , 2 days) were lost if *A. millepora* was transplanted back into the environment before the bleaching event (Middlebrook et al., 2012), showing that the mechanism(s) involved in thermal acclimation function over much shorter time scales than initially expected. A recent transcriptomic study monitored *A. nana* in three acclimation temperatures ( $29^\circ\text{C}$ ,  $31^\circ\text{C}$ , and variable:  $29-33^\circ\text{C}$ ), across four time-points (0, 2, 7, and 11 days) and found that 11 days was sufficient to observe significant decreases in the transcription of genes related to rRNA processing, carbohydrate metabolism and the production of heat shock proteins: a finding that suggests that short-term acclimation reduces heat stress-related protein damage (Bay and Palumbi, 2015).

The studies detailed above provide valuable information about the responses of the host and the symbiont to short-term acclimation at sub-lethal temperatures, but no studies to date have addressed how the metabolic condition of the holobiont (in terms of the nutritional interactions between the two partners) is affected. Nanoscale secondary ion mass spectrometry (NanoSIMS) imaging enables the visualization and quantification of the flux of nutrients between *Symbiodinium* and corals and their utilization in different host compartments at high-spatial resolution (Pernice et al., 2012, 2015; Kopp et al., 2013, 2015). This approach has recently been used to determine how long-term exposure to sub-lethal/sub-bleaching temperatures ( $29^\circ\text{C}$ , 6 weeks) impacts carbon and nitrate utilization in Northern Red Sea coral *Stylophora pistillata* (Krueger et al., 2017). These authors found subtle decreases in the amount of carbon ( $-17\%$ ) and nitrate ( $-6\%$ ) that was assimilated by the symbionts and greater decreases in the amount of carbon and nitrate translocated to the host gastrodermis ( $-31$  and  $-35\%$ , respectively) without visible signs of bleaching, suggesting that this species is able to mount some form of acclimation response. Here we investigated whether these types of sub-cellular changes (which occur during short-term exposure to sub-lethal temperatures) are able to modulate the metabolic condition

of the holobiont (specifically the acquisition and allocation of autotrophic carbon and nitrogen) during a subsequent heat stress event. We chose to use *Pocillopora damicornis* in Kaneohe Bay, Hawaii for this study; a coral living closer to its upper thermal limit (Jokiel and Coles, 1977; Jokiel and Brown, 2004; Bahr et al., 2015) than *S. pistillata* in the Red Sea, as the response of Northern Red Sea corals to thermal stress is not likely to be representative of most corals because of the strong evolutionary selection they have undergone in the past for heat tolerance and their current location in the cooler parts of the Red Sea (Fine et al., 2013; Krueger et al., 2017; Osman et al., 2017).

## MATERIALS AND METHODS

### Coral Collection, Recovery, Acclimation, and Heat Stress Conditions

On the 25th of May 2016, four *Pocillopora damicornis* colonies were collected from a shallow fringing reef in Kaneohe Bay, Hawaii. Each colony was divided into 40 identical fragments ( $3 \times 2$  cm), which were mounted atop plastic plugs using hot glue and placed in a  $\sim 1,300$  L flow-through common garden tank outdoors with natural solar irradiation. Fragments were left to recover in these tanks for 19 days (from 25th May to 14th of June) in flowing seawater pumped directly from Kaneohe Bay. High flow rates ( $\sim 6$  L  $\text{min}^{-1}$ ) were generated within the tank using recirculating pumps (700 gph Magnetic Drive, Danner Manufacturing Inc. Islandia, NY, USA). Temperature and light conditions were recorded every 15 min using underwater temperature (Hobo Water Temp Pro v2, Onset Computer Corporation, Bourne, MA, USA) and light loggers (Odyssey PAR loggers, Dataflow Systems Ltd, Christchurch, NZ). Temperatures and light fluctuated on natural diurnal cycles. Daily temperatures averaged  $26.5 \pm 0.4^\circ\text{C}$  but ranged from  $25.9$  to  $27.2^\circ\text{C}$ . Daytime light (between the hours of 06:00 to 18:00) averaged  $135 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  but ranged from  $10$  to  $564 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Figure 1C).

At the end of this recovery period, fragments were evenly distributed between four acclimation tanks ( $n = 10$  fragments *per* tank, *per* colony), where they were kept for an additional 2 weeks (from 15th of June to 1st of July). Two tanks were maintained at ambient temperatures (daily average:  $26.8 \pm 0.6^\circ\text{C}$ , range:  $26.4$  to  $27.5^\circ\text{C}$ , hereafter referred to as “ambient-acclimated corals”), while two were raised to elevated but sub-lethal conditions (daily average:  $28.3 \pm 0.3^\circ\text{C}$ , range:  $27.9$  to  $28.7^\circ\text{C}$ , hereafter referred to as “warm-acclimated corals”). Desired temperatures were achieved by mixing ambient seawater with seawater pre-heated to  $36^\circ\text{C}$ . Manipulating the proportion of ambient to hot water enabled us to adjust temperature without modifying flow rates in the tanks. Daytime light levels during this time averaged  $180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , but ranged from  $10$  to  $627 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Figure 1C).

All fragments were then transferred to a common garden flow-through tank, where they were exposed to a heat stress event for 7 days (from 1st of July to 7th of July). Temperatures during this experimental period averaged  $\sim 2.5^\circ\text{C}$  above the current 10-year maximum monthly mean (Figure 1; daily average:  $29.6$

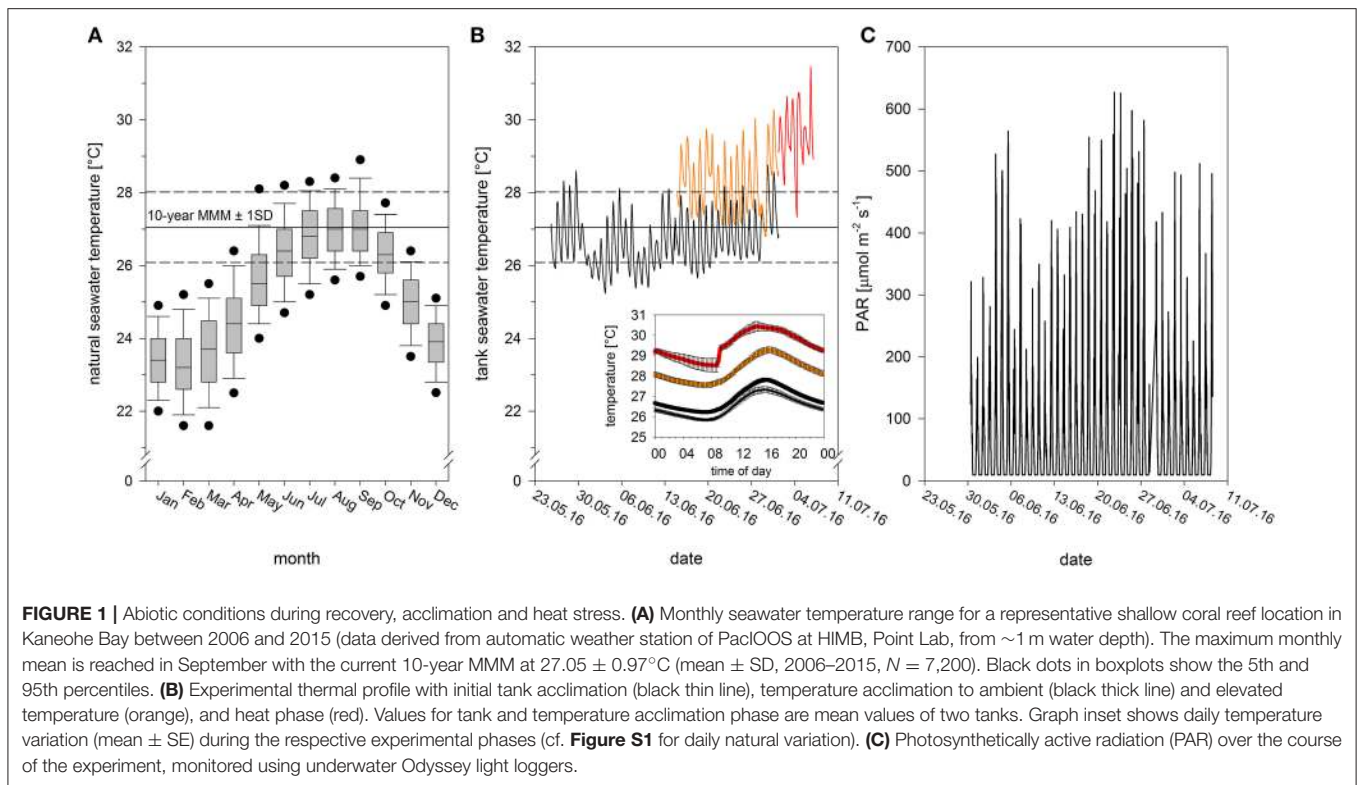
$\pm 0.1^\circ\text{C}$ , range:  $29.4$ – $28.7^\circ\text{C}$ ). By the end of the heat stress event, warm-acclimated corals had experienced cumulative heat stress equivalent to 4.5 degree heating weeks (DHW; Liu et al., 2014), and ambient-acclimated corals had experienced 2.5 DHW (calculations based on daily mean temperatures). The diurnal fluctuation range (ca.  $1.5^\circ\text{C}$  in all phases of the experiment), matched the diurnal natural seawater temperature fluctuation (Figure 1B inset; Figure S1). Daytime light levels during the heat stress averaged  $128 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  but ranged from  $10$  to  $511 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Figure 1C).

### Holobiont Scale Physiological Measurements

One coral fragment from each colony and acclimation treatment was sampled daily and used to measure five physiological parameters: gross photosynthesis ( $P_{\text{gross}}$ ), respiration (R), host protein content, symbiont density and chlorophyll content.  $P_{\text{gross}}$  and R were calculated from the evolution and consumption of oxygen in light and dark conditions, respectively. The measurements were conducted in 250 mL Plexiglas chambers, fitted with specially designed rubber O-ring sealed lids into which temperature and oxygen probes were placed. Each chamber contained identical stands, which held the coral fragment above a stir bar. The chambers were placed in a water bath, maintained at  $29.5 \pm 1^\circ\text{C}$  by aquarium heaters (WON pro-heat IC heaters, Fredericksburg, Virginia, USA) and the bath was placed atop a magnetic stir plate to prevent seawater stratification in the chamber. Measurements were conducted at the same time each day. Fragments were kept in darkness for 20 min before the lights were turned on and oxygen measurements were started to ensure the fragments had time to recover from any stress incurred from handling. Oxygen evolution was measured under  $350 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  of light, provided by an actinic white LED system (AI Sol, 72 W 100 to 240 VAC/50–60 Hz, C2 Development Inc., Ames, Iowa). Oxygen measurements were recorded every second using a ten channel fiber-optic optode oxygen meter (Presens, Regensburg, Germany), pre-calibrated with  $\text{O}_2$ -saturated (air-bubbled) and zero  $\text{O}_2$  ( $10 \text{ mg ml}^{-1}$  sodium sulfite) seawater. Oxygen evolution was recorded until it was linear, a period that lasted a minimum of 20 min. The lights were then switched off, the chambers covered with aluminum foil and oxygen was recorded until a steady-state of respiration (light enhanced dark respiration, LEDR) was observed. Rates of oxygen flux were adjusted to account for the volume of each fragment (via buoyant weighing using Archimedes’ principle) before fragments were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Rates of net photosynthesis ( $P_{\text{net}}$ ) and R were derived from the number of moles of  $\text{O}_2$  that were produced or consumed *per* hour using linear regression controlling for any oxygen flux in the water using rates from blank chambers. R was then subtracted from  $P_{\text{net}}$  to generate  $P_{\text{gross}}$ .

Samples were thawed and tissue was brushed from the skeleton in 10 mL of buffer ( $50 \text{ mM KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ ,  $0.1 \text{ mM EDTA}$ ,  $10\% \text{ [v/v] glycerol}$ ,  $\text{pH } 7.0$ ,  $4^\circ\text{C}$ ) using a waterpik. The resulting slurry was homogenized (electric hand-held homogenizer) and centrifuged ( $5 \text{ min}$ ,  $2,000 \times g$ ,  $4^\circ\text{C}$ ) to form an





algae pellet. The host-tissue containing supernatant was decanted and re-centrifuged (10 min,  $16,000 \times g$ ,  $4^\circ\text{C}$ ). A 1 mL aliquot of this was snap-frozen and stored at  $-20^\circ\text{C}$  for subsequent analysis of the host protein content, using the improved Bradford assay (590/450 nm) with bovine serum albumin as the standard (Zor and Selinger, 1996). The algal pellet was re-suspended in 1.1 mL of the aforementioned buffer, 100  $\mu\text{L}$  of which was fixed in 4% formaldehyde for determination of symbiont density, 1 mL of which was re-pelleted (5 min,  $4,000 \times g$ ,  $4^\circ\text{C}$ ) and stored at  $-20^\circ\text{C}$  for subsequent chlorophyll extraction. Chlorophyll content was quantified after extraction in 3 mL of 90% acetone (48 h at  $4^\circ\text{C}$ ). The absorbance of triplicate aliquots of 200  $\mu\text{L}$  was determined at 663, 630, and 750 nm (to account for seawater turbidity) in a 96-well plate (UV Star, Greiner), using a spectrophotometer (Molecular Devices, Sunnyvale, California, USA). Chlorophyll *a* and *c*<sub>2</sub> content was calculated using modified equations, specific to dinoflagellates (Jeffrey and Humphrey, 1975), which was corrected for the different path length (0.555 cm; Equations 1 and 2).

$$\text{Chl } a : \frac{(11.43 \times (E_{663 \text{ nm}} - E_{750 \text{ nm}})) - 0.64 \times (E_{630 \text{ nm}} - E_{750 \text{ nm}})}{0.555} \quad (1)$$

$$\text{Chl } c_2 : \frac{(27.09 \times (E_{630 \text{ nm}} - E_{750 \text{ nm}})) - 3.63 \times (E_{663 \text{ nm}} - E_{750 \text{ nm}})}{0.555} \quad (2)$$

Finally, symbiont density was calculated from six cell counts, which were performed using an Improved Neubauer

haemocytometer (Boeco, Hamburg, Germany). All physiological measurements are normalized to the surface area of the coral, calculated using the paraffin wax dipping method (Johannes and Wiebe, 1970).

## Microscale Physiological Measurements

On the last day of the heat stress event (i.e., day 7), one coral fragment from each colony and acclimation treatment was incubated with stable isotopes of carbon and nitrogen to assess their assimilation efficiency. The stable isotope experiment was conducted in 500 mL glass beakers containing small stir bars, which were placed in the same water bath (and therefore exposed to the same light and temperature conditions) that was used for the photosynthesis and respiration measurements. Isotopically-labeled seawater was prepared immediately before use. Final concentrations of 2 mM  $\text{NaH}^{13}\text{CO}_3$  and 3  $\mu\text{M}$   $\text{K}^{15}\text{NO}_3$  (both 98% atom, Sigma-Aldrich, Germany) were achieved by acidifying the seawater with a defined amount of 1 M HCl ( $\text{pH} < 3$ ), shifting the carbon equilibrium to  $>99\%$  dissolved  $\text{CO}_2$ , before bubbling with air for 2 h (DIC stripping), and subsequently spiking the water with 2 mM  $\text{NaH}^{13}\text{CO}_3$  before returning the pH to normal values ( $\text{pH} 8$ ) using a defined amount of 1 M NaOH. Fragments were incubated in the isotopically labeled seawater for 6 h. After, samples were preserved in fixative 2.5% [v/v] glutaraldehyde, 0.5% [v/v] formaldehyde in 0.22  $\mu\text{M}$  filtered Sørensen-sucrose phosphate buffer (0.1 M phosphate at  $\text{pH} 7.5$ , 0.6 M sucrose) for 2 h at room temperature followed by 22 h at  $4^\circ\text{C}$ . Fragments were decalcified (0.5 M EDTA) for 3 days at room temperature before being returned to Sørensen

phosphate buffer for storage. Samples were later transferred to the Laboratory for Biological Geochemistry (EPFL, Switzerland) under CITES permit 16US98467B, where they were micro-dissected into segments of connective coenosarc tissue using a binocular microscope. *Post*-fixation lasted for 1 h in 1% osmium tetroxide in distilled water before samples were dehydrated and embedded in Spurr's resin (Kopp et al., 2015). Semi-thin sections (500 nm) were cut using an Ultracut S microtome (Leica Microsystems, Germany), equipped with a 45° diamond knife (Diatome, Hatfield, PA, USA). Sections were then gold-coated and imaged using a NanoSIMS 50 L ion microprobe. NanoSIMS images (40 × 40 μm, 256 × 256 pixels, 5 ms/pixel dwell time, 5 layers) were obtained with a 16 keV Cs<sup>+</sup> primary ion beam, which was focused to a spot size of ca. 150 nm. Secondary ions (<sup>12</sup>C<sub>2</sub><sup>-</sup>, <sup>13</sup>C<sup>12</sup>C<sup>-</sup>, <sup>12</sup>C<sup>14</sup>N<sup>-</sup>, and <sup>12</sup>C<sup>15</sup>N<sup>-</sup> were counted in individual electron-multiplier detectors at a mass resolution power of about 9000 (Cameca definition), sufficient to resolve all potential interferences. A minimum of 30 symbiont cells and their surrounding tissue were imaged from each sample (10–20 images *per* sample). In most cases, this required multiple sections to be imaged. When this arose, sections were taken a minimum of 20 μm apart in the original tissue to ensure imaged symbiont cells were independent replicates. Analysis was conducted using the NanoSIMS software, L'IMAGE (Dr. Larry Nittler, Carnegie Institution of Washington). Drift-corrected maps of <sup>13</sup>C- and <sup>15</sup>N-enrichment were obtained from the ratios of <sup>13</sup>C<sup>12</sup>C<sup>-</sup> to <sup>12</sup>C<sub>2</sub><sup>-</sup> and <sup>15</sup>N<sup>12</sup>C<sup>-</sup> to <sup>14</sup>N<sup>12</sup>C<sup>-</sup>, respectively. Regions of interest (ROI) were then hand-drawn around *Symbiodinium* cells, host gastrodermis (excluding symbionts), host epidermis, and host lipid bodies in each image.

## Statistical Analysis

Holobiont scale physiological measurements ( $P_{\text{gross}}$ , R, host protein content, symbiont density and total chlorophyll content *per* cell) were analyzed by a restricted maximum-likelihood (REML) linear mixed model with acclimation temperature (ambient or warm) and time (days 1, 2, 4, 5, 6, or 7) as fixed factors. Colony was included as a random factor to assess how much variance in the data was explained by the source of each fragment (variance components analysis). A minimal model approach was employed where the interaction term between acclimation and time was removed from the model when it was non-significant. When terms were found to be significant, least square means contrasts were performed to identify where the differences lay.

The microscale physiological measurements were analyzed using a repeated measures analysis of variance (rmANOVA), using acclimation temperature (ambient or warm) and colony (matched pairs) as factors. Carbon and nitrogen enrichment were tested separately for each region of interest in the oral layer of the coenosarc coral tissue (symbiont cells, gastrodermis, host lipid bodies and epidermis). This approach enabled multiple ROI data points to be contained within each replicate, rather than being treated as independent data points.

All dependent variables were tested for normal distribution using the Shapiro–Wilks test and were transformed to achieve

normality if required and all statistical analyses were performed using the software JMP (SAS Institute, Cary, NC, USA).

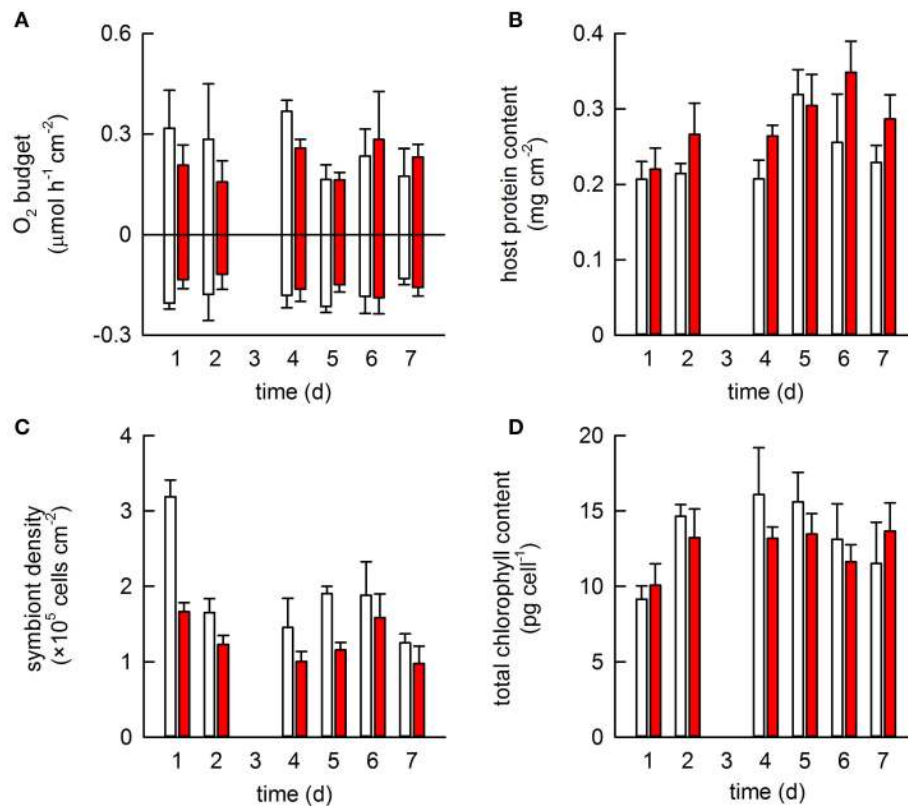
## RESULTS

All colonies from both treatments survived the heat stress event. Acclimation temperature did not significantly affect either oxygen production or consumption and no significant changes were observed throughout the heat stress event (**Table S1**, **Figure 2A**). Acclimation temperature did have a significant effect on host protein content; warm-acclimated corals contained 22% more protein on average than the ambient-acclimated corals (**Figure 2B**). Host protein also varied significantly across the heat stress event, being highest on days 5 and 6. Symbiont density significantly differed between the two acclimation temperatures; warm-acclimated corals contained 3% fewer symbionts than ambient-acclimated corals. Symbiont density also differed across the heat stress event (**Table S1**), although the only significant difference occurred on days 1 and 2; corals on day 2 containing 4% less symbionts on average, than corals on day 1 (**Figure 2C**). In contrast, total chlorophyll content *per* symbiont was lowest on day 1 of the heat stress event and showed no relationship to the acclimation temperature (**Table S1**; **Figure 2D**).

The final measurements at the end of the heat stress event showed there was no statistical effect of acclimation temperature on the density, total chlorophyll content, or oxygen productivity of the symbiont population in the tested colonies (**Figure 3A**). However, three of the four warm-acclimated colonies showed a consistent trend, characterized by a reduction in symbiont density, but increased chlorophyll pigmentation *per* cell and enhanced O<sub>2</sub>-production (**Figure 3A**). Notably, the between colony variance in the specific Chl *a*:Chl *c*<sub>2</sub> ratio was much smaller in warm-acclimated corals.

At the cellular scale, acclimation temperature had no consistent effect on the carbon assimilation in *P. damicornis*, which was highly variable between colonies (**Table 1**, **Figure 3B**). The level of <sup>13</sup>C-carbon enrichment in the symbionts significantly varied between colonies; ranging from 1,700 to 3,000. Significant effects of acclimation temperature were only observed for <sup>13</sup>C-enrichment in the gastrodermis and host lipid bodies, but all regions of interest had significant interactions between acclimation temperature and colony (**Table 1**). In three out of the four colonies, warm-acclimation resulted in greater enrichment of <sup>13</sup>C in the gastrodermis (+22–117%) and similar between-colony variation was evident in the amount of <sup>13</sup>C assimilated into host lipids (**Table S2**).

In contrast to carbon metabolism, acclimation temperature had a strong impact on <sup>15</sup>N-assimilation in symbiont cells and the enrichment in gastrodermis and epidermis (**Table 1**; **Figure 3B**). In general, warm-acclimation decreased <sup>15</sup>N-assimilation, although again, the magnitude of the reduction was colony-specific (**Table 1**; **Figure 3B**). Symbionts in three out of the four warm-acclimated colonies assimilated less <sup>15</sup>N (−69 to −94%) than ambient-acclimated corals, yet those in one colony were unaffected. Similar intra-colony variability was observed in assimilation in the coral gastrodermis: three of the



**FIGURE 2 |** The effect of acclimation temperature on the physiological response of *Pocillopora damicornis* exposed to heat stress. Four coral colonies were exposed to two different acclimation temperatures for 2 weeks: ambient (26.8°C; white) and warm (28.2°C; red) in a paired design. All were then exposed to heat stress (29.5°C) for 1 week. One fragment from each colony and each acclimation treatment was sampled daily (except for day 3, when data was lost) and used to monitor a suite of physiological measurements: **(A)** gross photosynthesis and respiration; **(B)** host protein content; **(C)** symbiont density and **(D)** chlorophyll content *per* symbiont cell. Bars represent mean  $\pm$  SE ( $N = 4$ ).

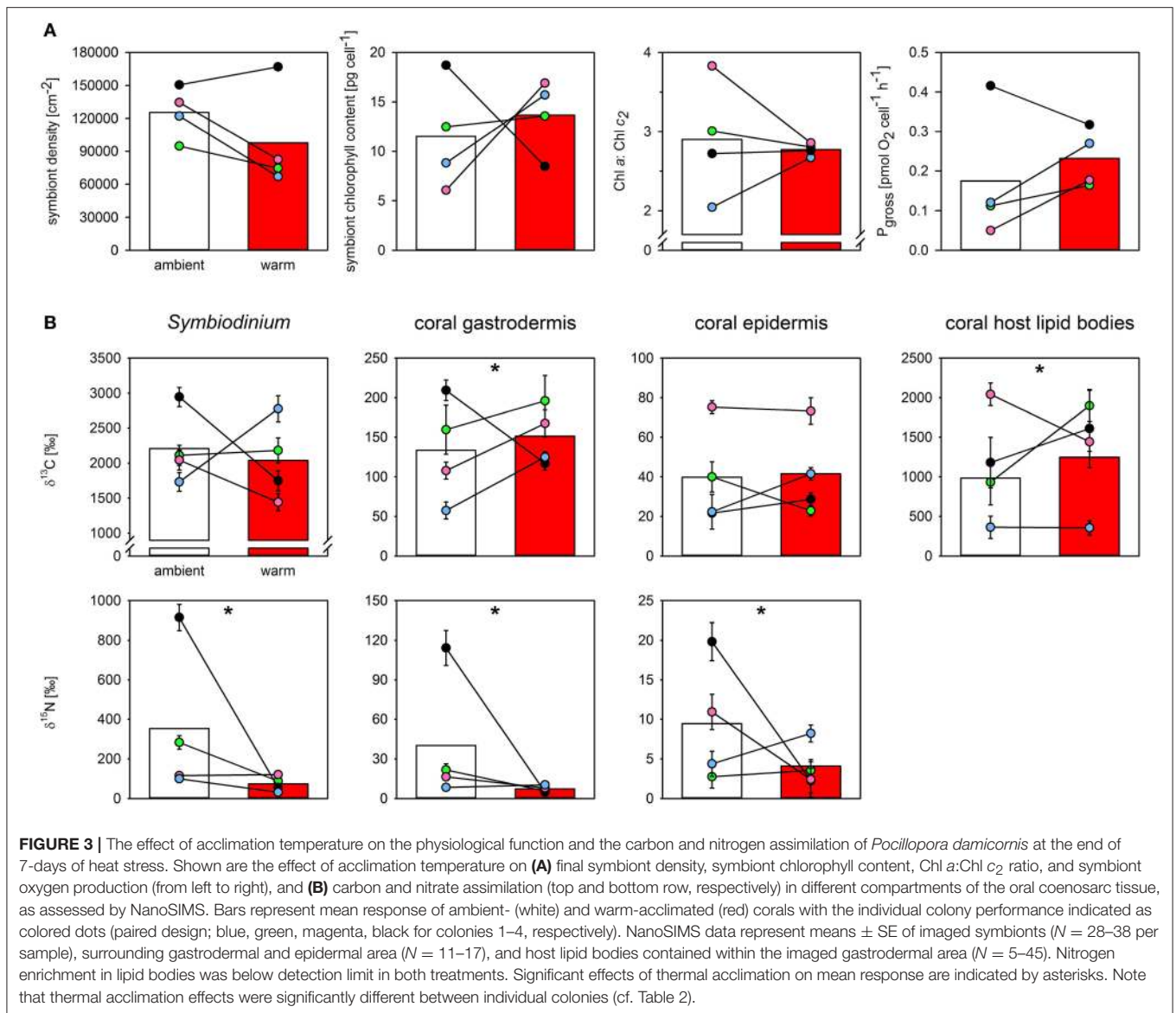
warm-acclimated colonies assimilated less <sup>15</sup>N (−51 to −96%), while one, incorporated more (+23 %). Finally, two warm-acclimated colonies exhibited enhanced <sup>15</sup>N-enrichment in their epidermis (+86 and 33%), relative to ambient-acclimated corals, while two incorporated less (−78 and −89%). <sup>15</sup>N-assimilation into host lipid bodies was not detectable for either acclimation temperature.

## DISCUSSION

Short-term temperature anomalies have been shown in some instances to modify a coral's response to subsequent heat events (Middlebrook et al., 2008; Bellantuono et al., 2011). The coral species that we used in this study, *Pocillopora damicornis*, lives close to its thermal maximum in Kaneohe Bay, Hawaii and is usually among the most bleaching-susceptible species in the region (Jokiel and Brown, 2004; Bahr et al., 2015). Comparison of our values with those reported in previous studies, revealed that our photosynthesis and respiration rates and the symbiont densities were substantially less for  $P_{gross}$  and R; (Lesser et al., 1994) and symbiont density (Stimson and Kinzie, 1991; Wall et al., 2017); than usual for *P. damicornis*, yet we observed no

visible signs of bleaching. Acclimation temperature had little effect on final holobiont level measurements: with no detectable differences between the O<sub>2</sub>-budget (both  $P_{gross}$  and R) of ambient and warm-acclimated corals, and negligible differences in symbiont density and total chlorophyll content *per* symbiont cell. In fact, the only holobiont level variable that differed was host protein, which was 22% higher in warm-acclimated corals (which could represent a change in either tissue thickness and/or protein content *per* cell). The lack of observed differences is likely a reflection of the heat and light conditions that the corals had experienced. By the end of the 7-day heat stress event, ambient corals had experienced 2.5 DHW and warm-acclimated corals had experienced 4.5 DHW (conditions classified as “likely” to cause bleaching by NOAA), yet the light levels during the experiment were relatively low (arising from an abnormal period of cloudy weather; **Figure 1C**). Since temperature and light are synergistic stressors of coral bleaching, it is possible that the low light conditions that prevailed during the experiment alleviated the impact of the 4.5 DHW treatment to some extent (Skirving et al., 2018). While unexpected, such conditions emphasized the impact of heat stress on coral holobiont metabolism, without the interference of excessive light.





Temperature acclimation did not affect carbon incorporation in the symbionts, but it did increase carbon assimilation in the host gastrodermis (+15%) and the host lipid bodies (+13%), of warm-acclimated corals. There are two explanations for this finding: (i) symbionts in warm-acclimated corals are more efficient at fixing carbon, but retain the same amount of carbon for themselves as the symbionts in ambient-acclimated corals, allowing excess carbon to be translocated to their coral host and invested in tissue production or energy storage in lipid bodies; or (ii) the same amount of carbon is translocated to the host, but warm-acclimated corals allocate more to storage and/or have a higher general turnover of carbon in their host tissue reserves (note that soluble compounds are lost in conventional NanoSIMS sample preparation). Since host protein content increased in warm-acclimated corals in the absence of changes in respiration rates we favor the first hypothesis, although this would require a

higher efficiency of carbon capture or enhanced electron cycling because the activity of photosystem II ( $P_{\text{gross}}$ ) was unchanged.

The most striking finding of this study is the effect that acclimation had on nitrogen metabolism. Warm-acclimation reduced nitrogen incorporation from external seawater nitrate on average by 79% in the symbionts, 81% in the gastrodermis, and 55% in the epidermis. Significant reductions in nitrate uptake at elevated temperatures have been observed in *Stylophora pistillata* (Godinot et al., 2011) and *P. damicornis* (Ezzat et al., 2016) but, in both cases the decreases were linked to a decline in photosynthesis. Here, the relative reduction in nitrate uptake in warm-acclimated corals observed at the end of the heat stress event is independent of the photosynthetic performance of the symbionts (i.e.,  $O_2$ -production), suggesting a general impact on nitrate supply to the symbiont and/or specific thermal impact on the enzymes involved in nitrate reduction.

**TABLE 1** | The effect of acclimation temperature on carbon and nitrogen assimilation rates in *Pocillopora damicornis* at the end of a 7-day heat stress event. Statistical output of rmANOVA ( $n = 4$ ), using acclimation temperature (ambient or warm) and colony (matched pairs) as factors.

Isotope	ROI	Acclimation temperature	Colony	Acclimation temperature × Colony
$\delta^{13}\text{C}$	Symbiont	$F_{(1, 265)} = 2.67$ , $p = 0.1032$	$F_{(3, 263)} = 4.07$ , <b><math>p = 0.0075</math></b>	$F_{(7, 259)} = 17.71$ , <b><math>p &lt; 0.0001</math></b>
	Gastrodermis	$F_{(1, 99)} = 4.00$ , <b><math>p = 0.0482</math></b>	$F_{(3, 97)} = 8.19$ , <b><math>p &lt; 0.0001</math></b>	$F_{(7, 93)} = 12.01$ , <b><math>p &lt; 0.0001</math></b>
	Host lipid bodies	$F_{(1, 177)} = 6.03$ , <b><math>p = 0.0151</math></b>	$F_{(3, 175)} = 27.55$ , <b><math>p &lt; 0.0001</math></b>	$F_{(7, 171)} = 5.45$ , <b><math>p = 0.0013</math></b>
	Epidermis	$F_{(1, 96)} = 1.36$ , $p = 0.2467$	$F_{(3, 94)} = 33.77$ , <b><math>p &lt; 0.0001</math></b>	$F_{(7, 90)} = 7.17$ , <b><math>p = 0.0002</math></b>
$\delta^{15}\text{N}$	Symbiont	$F_{(1, 265)} = 123.47$ , <b><math>p &lt; 0.0001</math></b>	$F_{(3, 263)} = 23.85$ , <b><math>p &lt; 0.0001</math></b>	$F_{(7, 259)} = 39.84$ , <b><math>p &lt; 0.0001</math></b>
	Gastrodermis	$F_{(1, 99)} = 23.39$ , <b><math>p &lt; 0.0001</math></b>	$F_{(3, 97)} = 5.64$ , <b><math>p = 0.0013</math></b>	$F_{(7, 93)} = 27.17$ , <b><math>p &lt; 0.0001</math></b>
	Host lipid bodies	N/A	N/A	N/A
	Epidermis	$F_{(1, 96)} = 13.01$ , <b><math>p = 0.0005</math></b>	$F_{(3, 94)} = 3.79$ , <b><math>p = 0.0129</math></b>	$F_{(7, 90)} = 14.56$ , <b><math>p &lt; 0.0001</math></b>

Carbon and nitrogen enrichment were tested separately for regions of interest (ROI) in the oral layer of the coenosarc coral tissue (symbiont cells, gastrodermis, host lipid bodies and epidermis). No  $^{15}\text{N}$ -labeling was detected in host lipid bodies. Statistically significant results are highlighted in bold ( $p < 0.05$ ).

Dissolved inorganic nitrogen (DIN) can be acquired in many forms by the coral holobiont. Measurements for *S. pistillata* show the majority is acquired as ammonia ( $\text{NH}_4^+$ : 42%), but that nitrate ( $\text{NO}_3^-$ : 34%), dissolved free amino acid (DFAA: 21%) and urea (3%) can all be sources of nitrogen for corals (Grover et al., 2008). It is possible that decreases in nitrate assimilation and translocation represent a switch to ammonia rather than a reduction in the overall amount of nitrogen fixed. *Symbiodinium* is known to favor ammonia over nitrate if both are present. The concentration of ambient ammonia also influences nitrate uptake (D'Elia et al., 1983; Domotor and D'Elia, 1984; Wilkerson and Trench, 1985; Taguchi and Kinzie, 2001). In the intact symbiosis, 3–4  $\mu\text{M}$  of ammonia is sufficient to completely inhibit nitrate uptake in *S. pistillata* (Grover et al., 2003). Ammonia concentrations in Kaneohe Bay typically range between 0.6 and 0.8  $\mu\text{M}$  (Drupp et al., 2011; Silbiger et al., 2014), so it seems unlikely that ambient ammonia concentrations induce a substantial shift toward ammonia assimilation. Temperature-dependent nitrate incorporation has been observed in *Acropora tenuis*, but these differences were caused by the genetic identity of the symbiont clade that they hosted (Baker et al., 2013). We did not genotype the symbionts in our study since *P. damicornis* in Hawaii consistently and almost exclusively hosts *Symbiodinium* ITS2 type C1 (Lajeunesse et al., 2004; Magalon et al., 2007). The endodermal location of the symbionts means that DIN acquired from seawater present in the coelenteron must cross a minimum of two host-derived membranes (the plasma membrane and the symbiosome) before it reaches the symbionts, and so it stands to reason that the symbionts' capacity to fix nitrate depends on the uptake capabilities of both the host and the symbiont (Grover et al., 2003). It is possible that the reductions in  $^{15}\text{NO}_3^-$  assimilation and translocation that we observed in the warm-acclimated corals simply reflect changes in the ability of the host to supply nitrate to the symbionts. Nothing

is known about how the host regulates the supply of nitrate, nor its response to thermal stress. Previous studies that monitored the gene expression of nitrate-transporter 2 (*nrt2*) in *Symbiodinium* C under different thermal regimes and in different host species found that temperature had little impact on *nrt2* expression (Mayfield et al., 2013a,b, 2014), but no studies have examined the activity of nitrate reductase of *in hospite Symbiodinium* under thermal stress.

The disconnect that we observed between carbon and nitrate assimilation in the warm-acclimated corals (in the absence of holobiont level changes) can be viewed from two perspectives: either this is a mechanistic response to delay bleaching by the coral host, or the warm-acclimated corals are simply one step closer to their bleaching threshold than their ambient-acclimated counterparts. The first hypothesis relies on the reduction in  $\text{NO}_3^-$  acquisition being caused by limited host supply and not being a direct negative effect on the ability of the symbiont to fix nitrate. If a limited host supply is indeed the case, then the stable symbiont  $\text{O}_2$ -production, reduced nitrate acquisition and enhanced carbon delivery to the host could feasibly represent a host response to limit nitrogen supply to the symbiont population. A number of studies have shown that nitrogen deprivation can enhance lipid formation and modify the amount of carbon outflow from *Symbiodinium* (Muller-Parker et al., 1996; Zhu et al., 2011; Weng et al., 2014; Pasaribu et al., 2016; Suesscún-Bolívar et al., 2016). Such a mechanism could potentially boost the hosts long-term energy reserves (Grottoli et al., 2004, 2014; Rodrigues and Grottoli, 2007), while reducing symbiont population growth. If on the other hand, warm-acclimated corals are simply closer to their bleaching threshold than ambient-acclimated corals, then it is possible that the reduced nitrate assimilation we observed in warm-acclimated corals represents a heightened state of nitrogen-limitation in the holobiont. Changes in nitrogen assimilation are thought to be

critical in determining the transition to a carbon-limited state and thus determining the onset of bleaching (Cunning et al., 2017). Since our corals did not progress into the bleaching phase, we cannot conclude which hypothesis is more likely. However, the consistent response of three of the four colonies showing a reduction in symbiont density but an increase in  $P_{\text{gross}}$  and gastrodermal  $^{13}\text{C}$ -enrichment offers support for a host mechanism, although further investigation is warranted.

All considered, our NanoSIMS data provides a pre-bleaching snap shot of the differences in metabolic condition of differentially-acclimated coral holobionts, exposed to different degrees of sub-lethal thermal stress. We show that sub-lethal temperatures can have a substantial impact on nutrient metabolism and therefore modify the energetic basis of the symbiotic association. It is possible that the modifications play an important role in delaying the onset of coral bleaching. It is equally possible that the changes we observed reflect the transition phase that occurs just before the onset of bleaching. Either way, our study opens many interesting avenues for future research. One particular question requiring attention is the role of nitrogen metabolism under thermal stress.

## AUTHORS CONTRIBUTIONS

EG, TK, and HP designed the experiment. EG, TK, HP, and KB conducted the experiment. EG, TK, and JB analyzed the samples. EG and TK performed data analysis. EG produced the first draft of the manuscript, and all authors contributed to the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00010/full#supplementary-material>

**Figure S1** | Daily temperature variation (mean  $\pm$  95%CI) over the annual cycle for a representative shallow reef location in Kaneohe Bay between 2006 and 2015 (data derived from automatic weather station of PaCOOS at HIMB, Point Lab, from  $\sim$ 1 m water depth). Solid line indicates 10-year MMM  $\pm$  1SD.

**Table S1** | The effect of acclimation on the global scale physiological response of *Pocillopora damicornis* to a simulated bleaching event. Dependent variables were analyzed by a restricted maximum-likelihood (REML) linear mixed model with acclimation and time as fixed factors. Colony was included as a random factor to assess how much of the variance in the data was explained by the source colony that each fragment originated from (variance components analysis). A minimal model approach was employed whereby acclimation and time were initially crossed to assess their combined effect, but removed if their interaction was non-significant (-). Degrees of freedom (d.f.), F-ratios (F) and probability levels (P) are provided and significant effects ( $P < 0.05$ ) are in bold.

**Table S2** | Summary of enrichment values for carbon and nitrogen from NanoSIMS image analysis. Regions of interest (ROI) were based on coenosarc cross sections and refer to the oral tissue layer. Gastrodermis ROIs refer to gastrodermis excluding symbionts, but including host lipids.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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