Changes in physiological performance and protein expression in the larvae of the coral *Pocillopora damicornis* and their symbionts in response to elevated temperature and acidification

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ABSTRACT

Climate change causes ocean warming and acidification, which threaten coral reef ecosystems. Ocean warming and acidification cause bleaching and mortality, and decrease calcification in adult corals, leading to changes in the composition of coral communities; however, their interactive effects on coral larvae are not comprehensively understood. To examine the underlying molecular mechanisms of larval responses to elevated temperature and $p\text{CO}_2$, we examined the physiological performance and protein expression profiles of *Pocillopora damicornis* at two temperatures (29 and 33 °C) and $p\text{CO}_2$ levels (500 and 1,000 μatm) for 5 d. Extensive physiological and proteomic changes were observed in coral larvae. The results indicated a significant decrease in net photosynthesis (P_{NET}) and autotrophic capability ($P_{\text{NET}}/R_{\text{D}}$) of larvae exposed to elevated temperature but a marked increase in P_{NET} and $P_{\text{NET}}/R_{\text{D}}$ of larvae exposed to high $p\text{CO}_2$ levels. Elevated temperature significantly reduced endosymbiont densities (to approximately 70%) and photochemical efficiency, indicating that warming impaired host — symbiont symbiosis. Expression of photosynthesis-related proteins, the photosystem (PS) I reaction center subunits IV and XI as well as oxygen-evolving enhancer 1, was downregulated at higher temperatures in symbionts, whereas expression of the PS I iron-sulfur center protein was increased under high $p\text{CO}_2$ conditions. Furthermore, expression of phosphoribulokinase (involved in the Calvin cycle) and phosphoenolpyruvate carboxylase (related to the C_4 pathway) was downregulated in symbionts under thermal stress; this finding suggests reduced carbon fixation at high

temperatures. The abundance of carbonic anhydrase-associated proteins, which are predicted to exert biochemical roles in dissolved inorganic carbon transport in larvae, was reduced in coral host and symbionts at high temperatures. These results elucidate potential mechanisms underlying the responses of coral larvae exposed to elevated temperature and acidification and suggest an important role of symbionts in the response to warming and acidification.

Keywords: Coral larvae, *Pocillopora damicornis*, symbionts, proteome, elevated temperature, acidification

1. INTRODUCTION

Adverse consequences of ocean warming and acidification, caused by rising anthropogenic atmospheric CO₂, occur globally and are particularly acute regarding reef-building corals and coral reef ecosystems (Hoegh-Guldberg et al., 2017; Hughes et al., 2017). Ocean warming generally causes coral bleaching and even mortality, as evidenced by the increasing frequency and magnitude of mass bleaching events over the past several decades (Hughes et al., 2017), and the effects of acidification have raised concerns regarding disturbance of coral reef ecosystems due to decreased calcification and growth following carbonate depletion (Hoegh-Guldberg et al., 2007).

Understanding the physiological responses of corals to climate change stressors that allow higher stress tolerance is imperative for future coral conservation efforts (Robbins et al., 2019). Significantly reduced physiological performance has been widely reported in adult corals after exposure to warming and/or acidified seawater conditions. For instance, symbiont has been reported to decrease density at elevated $p\text{CO}_2$ (Mason, 2018) and temperature (Wall et al., 2014) or changes Clades in tropical and subtropical regions (Tong et al., 2017). *Pocillopora damicornis* and *Stylophora pistillata* cannot maintain positive calcification rates

under any pH condition when exposed to elevated temperature (31 °C) (Guillermic et al., 2021). Interestingly, exposure to elevated temperatures and/or $p\text{CO}_2$ increases production in *Acropora* by 30% (Anthony et al., 2008), does not change symbiont Clades in the Persian Gulf (Howells et al., 2020), and does not cause bleaching and photophysiological changes in *Seriatopora caliendrum* (Wall et al., 2014). Regarding coral larvae, during 24 h of exposure to elevated temperature and $p\text{CO}_2$, symbiont density of *P. damicornis* larvae was highest at the elevated temperature, and it was significantly affected by temperature and by the interaction of temperature and $p\text{CO}_2$ (Rivest et al., 2018). During five days of such treatments, autotrophic capacity increases in *P. damicornis* larvae under elevated temperature and $p\text{CO}_2$ conditions (29 vs 30.8 °C and 500 vs 1,000 μatm) (Jiang et al., 2020). The performance of *P. damicornis* larvae in response to these two stressors is affected by larval release time (Cumbo et al., 2013a). In *Acropora palmata*, *A. millpora* and *Porites astreoides* larvae, fertilization, settlement, growth, and metabolism are also negatively affected by elevated $p\text{CO}_2$ (Albright and Langdon, 2011; Albright et al., 2010; Doropoulos et al., 2012). Thus, the success of early life stages of coral (including larval availability) is critical for successful coral population recruitment and for the overall recovery of reefs after disturbances (Albright, 2011; Harrison, 2011; Harrison and Wallace, 1990).

Despite increasing efforts to elucidate the physiological responses of reef corals and their symbionts to anthropogenic stressors (Cumbo et al., 2018; Gibbin et al., 2018; Levy et al., 2020; Yuyama et al., 2012), the molecular mechanisms underlying these responses are still poorly understood. A better understanding of these mechanisms is essential for predicting the impacts of future global change on corals and their symbionts and their potential

individual contributions to the stress tolerance of the holobiont.

A few studies have investigated the molecular basis of how coral larvae host-symbiont interactions are affected by elevated temperature and $p\text{CO}_2$. For example, a possible explanation for the effects of thermal and/or acidification stress on coral larvae was proposed in studies using RNA-seq (Meyer et al., 2011; Polato et al., 2010; Rivest et al., 2018), and proteome analysis was used to study individual responses of coral host and free-living species of Symbiodiniaceae to different environmental stressors (Gochfeld et al., 2015; Pollack et al., 2009). Protein expression changes during symbiosis establishment between the sea anemone *Exaiptasia pallida* and two symbiont types (Clades C and D) were quantified previously (Sproles et al., 2019); however, to date, very few studies have addressed coral host-symbiont protein expression changes due to climate change, with the exception of one recent study on the coral *P. acuta* (Mayfield et al., 2018). Thus, studies on the responses of coral hosts and their symbionts in a symbiotic state using proteome analysis are still lacking. Specifically, the role of proteins in the regulation of physiological responses and their potential contribution to increased resilience in response to climate change is poorly understood.

In this study, we quantified essential physiological traits of corals (e.g., net photosynthesis, ratio of net photosynthesis to dark respiration, and symbiont density) and analyzed differential protein expression in *P. damicornis* larvae grown at ambient and elevated temperatures (29 and 33 °C) and $p\text{CO}_2$ levels (500 and 1,000 μatm). Expression was measured over 5 d, which is an ecologically relevant duration and is sufficient to capture the physiological effects of temperature and $p\text{CO}_2$ on brooded coral larvae (Cumbo et al., 2013b). We assessed individual and combined effects of end-of-century predicted temperature and

$p\text{CO}_2$ using four treatments: (i) ambient temperature and $p\text{CO}_2$ (control); (ii) elevated $p\text{CO}_2$ (OA); (iii) elevated temperature (ET); and (iv) elevated temperature and $p\text{CO}_2$ (ETOA). In addition, we hypothesized that increased temperature would induce larval bleaching, whereas increased $p\text{CO}_2$ would improve the photosynthetic health of symbionts. The results provide a detailed analysis of the specific proteins and cellular mechanisms that are altered by elevated temperature and acidification. Our findings also provide insights into the cellular biology of the initial phase of corals under thermal stress and elucidate the interplay between host and symbiont before detectable bleaching.

2. MATERIALS and METHODS

2.1 Collection of Larvae

Ten *P. damicornis* colonies (15–20 cm diameter) were collected on August 15, 2018, from the Luhuitou fringing reef (18°12' N, 109°28' E) in Sanya, Hainan Island, China. The colonies were immediately transferred to the Tropical Marine Biological Research Station in Hainan and were placed in individual 20-L containers with flow-through seawater at ambient temperature (28.6 ± 0.2 °C) within a 200-L tank under partially shaded light conditions (noon irradiance: approximately $300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). The outflow of each container was passed through a cup filter fitted with a 180- μm mesh at the bottom to retain the released larvae, which were collected at 06:30 on August 18, 2018, and were then pooled across colonies.

2.2 Experimental Setup

To test the effects of temperature and $p\text{CO}_2$ levels on coral larvae, we studied four experimental systems: control (ambient temperature, ~ 29 °C; ambient $p\text{CO}_2$, $\sim 500 \mu\text{atm}$), OA (ambient temperature; high $p\text{CO}_2$, $\sim 1,000 \mu\text{atm}$), ET (elevated temperature, ~ 33 °C; ambient $p\text{CO}_2$), and ETOA (elevated temperature, ~ 33 °C; elevated $p\text{CO}_2$, $\sim 1,000 \mu\text{atm}$).

Four replicate 3-L plastic beakers were used for each system. Approximately 600–800 larvae were placed in each beaker containing 2.5 L UV-sterilized seawater that was filtered through a 0.5- μm filter. These beakers were placed in a 40-L freshwater bath tank to control the seawater temperature. We used the recorded extreme temperature (Li et al., 2012) and 4 °C above the summer ambient temperature for corals at the collection site. The treatments were designed based on the predicted oceanic temperature and pH levels of the representative concentration pathway 8.5 by 2100 (IPCC, 2007).

Ambient and elevated $p\text{CO}_2$ levels were produced by bubbling with ambient air and air with elevated $p\text{CO}_2$ (produced by mixing air with CO_2 using a CO_2 enricher (CE100B, Ruihua, China)), respectively. In addition, ambient and elevated temperature levels were controlled using digital temperature regulators (TC-05B, Sieval, China) and 50 W heaters (MX-1019, Weipro, China). Seawater temperatures were monitored at 30-min intervals using HOBO Pendant temperature loggers (Onset, Bourne, MA, USA) throughout the experiment. Freshwater was mixed using a submerged pump (600 L h^{-1}), and seawater in the plastic beakers was partially exchanged (30%) with temperature- and $p\text{CO}_2$ -equilibrated seawater at 11:00 every day throughout the experimental period.

The experimental system was maintained under a 12:12 h light:dark cycle from 06:00 to 18:00 using T5 fluorescent lamps (two aqua blue coral tubes and two actinic-blue tubes; Giesemann, Germany), and the light intensity was $\sim 200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, which approximated the irradiance in crevices where juvenile corals were found at 3–4 m depths at our study site. Temperature, salinity, and pH were continuously monitored and were adjusted twice daily using a pH meter (SevenGo meter, Mettler Toledo, Switzerland), and the pH

probe was calibrated through two-point calibration every other day using standard buffers (pH 7.00 and 10.00, respectively). During the five-day experiment, seawater samples of 50 mL were collected from each beaker every day and were preserved using saturated HgCl_2 solution to assess total alkalinity (TA); 25 g seawater sample was titrated using 0.1 mol/L HCl solution, which was terminated at solution pH 4.0–4.2 for runs with bromocresol green. TA was determined by measuring seawater absorbance at 444 nm and 630 nm using a spectrophotometer (UV-2700, Shimadzu, Japan) (Yao and Byrne, 1998). TA and pH were used to calculate carbonate parameters (i.e., dissolved inorganic carbon (DIC) and $p\text{CO}_2$) using the CO2SYS program (Lewis, 1998). The seawater conditions of each treatment are shown in Table 1.

2.3 Measurement of Carbon Metabolism

A measuring system was established, comprising a 1.75-mL custom-made glass vial, a water bath, a heater, a light source, a magnetic stirrer, and fluorescent quenching sensors (OXY-4 mini, PreSens, Germany). Net photosynthesis (P_{NET}), light-enhanced respiration (R_{L}), and dark respiration (R_{D}) were measured on the last day of the experiment using respirometry. Constant temperature and light at levels, identical to those in the incubation systems, were maintained during all measurements. Thirty larvae were randomly sampled from each plastic beaker and were transferred to a vial containing a miniature stir bar (3×5 mm) and seawater from the corresponding experimental beaker. Briefly, P_{NET} was measured for 10 consecutive minutes, after which R_{L} was measured for 10 min using the same batch of 30 larvae under non-illumination conditions between 08:00–12:00. R_{D} was assessed using the same method between 20:00–22:00 using a different batch of 30 larvae. Measurements were conducted by continuously recording the oxygen concentrations at 10^{-3} intervals over 10 min. P_{NET} , R_{L} , and

R_D were calculated using the least squares linear regression of the oxygen concentration plotted over time and were expressed as nanomoles of oxygen per minute per larva ($\text{nmol O}_2 \text{ larva}^{-1} \text{ min}^{-1}$). P_G was measured by adding P_{NET} to R_L ; P_{NET}/R_D was calculated to assess the potential for autotrophy (Muscatine et al., 1981). A P_{NET}/R_D ratio > 1 indicated that the net production of organic carbon by photosynthesis was sufficient to sustain the metabolic demands at night (i.e., the total capacity for autotrophy) and vice versa.

2.4 Photosynthetic Efficiency, Symbiont Density, and Survivorship

After R_D measurement, the maximum quantum yield (F_V/F_M) of dark-adapted symbionts was measured using a Diving Pulse Amplitude Modulated Fluorometer (Diving PAM, Walz, Germany) between 22:00 and 23:00, which allowed sufficient time for the relaxation of photochemical quenching in coral larvae ($n = 10$). Initial fluorescence (F_0) was determined by applying a weakly modulated pulse of red light. A 0.8-s saturating pulse of actinic light was then applied to measure the maximum fluorescence level (F_M). F_V/F_M values were calculated as $(F_M - F_0)/F_M$ (Hoogenboom et al., 2012). The larvae were then preserved in liquid nitrogen for symbiont density measurements. To separate symbionts, larvae were ground using a handheld mortar and pestle. The homogenate was centrifuged at $10,600 \times g$ and 4°C for 10 min, and the pellet was re-suspended in sterilized and $0.45\text{-}\mu\text{m}$ filtered seawater. The number of symbionts was counted using a hemocytometer with six replicate counts per sample, and symbiont density was expressed as the number of cells per larvae. Regarding survivorship, larvae were placed in transparent tubes with both ends covered using a $180\text{-}\mu\text{m}$ mesh to ensure sufficient ventilation through the surrounding seawater. One 100-mL tube containing 20 larvae was examined per beaker to assess survivorship. Larvae in the 100-mL tubes were examined daily. At the end of the experiment, 12 larvae samples (three biological replicates

per treatment) were preserved in liquid nitrogen for protein extraction.

2.5 Protein Isolation, Digestion, and Quantification

Protein extraction and digestion were performed as previously described (Wiśniewski et al., 2009). Approximately 0.1 g (~ 100 larvae) of larvae samples were ground to powder using liquid nitrogen and 1 mL SDT buffer (pH 7.6) containing 4% sodium dodecyl sulphate (SDS), 100 mM Tris-HCl, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonylfluoride. The 12 samples were transferred to 2-mL centrifuge tubes containing quartz sand (MP 6910–050) and a 1.25-inch ceramic bead (MP 6540–424) and were homogenized using an MP homogenizer (MP Biomedicals, Solon, USA) (24×2 , 6.0 M/S, 60 s, twice). The samples were then sonicated and boiled at 100 °C for 15 min, followed by centrifugation at $14,000 \times g$ at room temperature for 40 min. The supernatant was filtered through a 0.22- μ m filter and was used as a protein extract. Protein concentrations were quantified using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA).

A 20 μ g aliquot of protein per sample was mixed with 5X loading buffer, and the mixture was boiled for 5 min. Proteins were separated on a 12.5% SDS-PAGE gel (constant current 14 mA, 90 min), and protein bands were visualized by Coomassie Blue R-250 staining.

For each sample, protein (200 μ g) was added to 30 μ L SDT buffer (4% SDS, 100 mM Tris-HCl, and 1 mM dithiothreitol), and the mixture was boiled for 5 min and then cooled to room temperature. To remove detergent, dithiothreitol, and other low-molecular-weight components, each sample was loaded on an ultrafiltration filter (10 kDa cut-off, Sartorius, Germany) containing 200 μ L UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0) followed by centrifugation at $14,000 \times g$ for 15 min, and the filtrate was discarded. This step was repeated

once. Following this, 100 μ L of 100 mM iodoacetamide in UA buffer was added to the filter to block the reduced cysteine residues. The samples were incubated for 30 min at room temperature in the dark, followed by centrifugation at $14,000 \times g$ for 15 min. The filters were washed with 100 μ L UA buffer and centrifuged twice at $14,000 \times g$ for 15 min. Following this, the samples were washed with 100 μ L of 100 mM triethylammonium bicarbonate (TEAB) buffer twice and centrifuged twice at $14,000 \times g$ for 15 min. The protein suspensions were digested with 40 μ L trypsin buffer (4 μ g trypsin in 40 μ L TEAB buffer) at 37 °C for 16–18 h. The filter unit was then transferred to a new tube and was centrifuged at $14,000 \times g$ for 15 min. The resulting peptides were collected as filtrates, and the peptide concentration was determined at OD₂₈₀.

2.6 Tandem Mass Tag Labelling

For each sample, 100 μ g of the peptide mixture was labeled using a tandem mass tag reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.7 High pH Reversed-Phase Peptide Fractionation

A Pierce High pH Reversed-Phase Fractionation peptide Kit (Thermo Fisher Scientific) was used to fractionate the tandem mass tag-labeled and digested samples into 15 fractions using an increasing acetonitrile step-gradient elution according to the manufacturer's instructions.

2.8 Mass Spectrometry

Each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture was loaded onto a reverse-phase trap column (Acclaim Pepmap_100, 100 μ m $\times$ 2 cm, nanoViper C18, Thermo Fisher Scientific) connected to the C18-reversed-phase analytical column (Thermo Fisher Scientific easy column, 10 cm long, 75 μ m inner diameter, 3 μ m resin) in

buffer A (0.1% formic acid). The mixture was separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min controlled by IntelliFlow technology. The 1-h gradient was as follows: 0%–55% buffer B for 50 min, 50%–100% buffer B for 5 min, and held in 100% buffer B for 5 min.

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an Easy nLC (Thermo Fisher Scientific) for 60 min. The mass spectrometer was operated in the positive ion mode. MS data were acquired using a data-dependent top 10 method that dynamically selects the most abundant precursor ions from the survey scan (300–1,800 m/z) for high-energy collisional dissociation (HCD) fragmentation. The automatic gain control target was set to $1e^{-6}$, maximum injection time was set to 50 ms, and the dynamic exclusion duration was 30 s. Survey scans were acquired at a resolution of 70,000 at 200 m/z, and the resolution for HCD spectra was set to 17,500 at 200 m/z with an isolation width of 2 m/z. Normalized collision energy was 30 eV, and the underfill ratio was defined as 0.1%, which specifies the minimum percentage of the target value likely to be reached at maximum fill time. The instrument was operated with the peptide recognition mode enabled.

2.9 Bioinformatics Analyses

The MS/MS spectra were searched using MASCOT engine (version 2.2, Matrix Science, UK) embedded in Proteome Discoverer 1.4 (Thermo Fisher Scientific) to search against *P. damicornis* database (NCBI_pocillopora_damicornis_52137_20190313) and *Symbiodinium microadriaticum* database (NCBI_Symbiodinium_microadriaticum_43448_20190313) (accession number PX-submission 419395). The following search parameters were used: monoisotopic mass, peptide mass tolerance at ± 20 ppm, fragment mass tolerance at 0.1 Da,

trypsin as the cleavage enzyme, two missed cleavages, TMT labelling, carbamidomethylation of cysteine as fixed modifications, and the oxidation of methionine as a variable modification. The results were filtered based on a false discovery rate of < 0.01 . Only unique peptides were used for protein identification. In addition, three comparisons were made to examine changes in coral and symbiont protein expression between the OA, ET, and ETOA treatments and the control: OA vs. control, ET vs. control, and ETOA vs. control. Differentially expressed proteins (DEPs) were detected based on a 1.2-fold change threshold (with a fold change > 1.2 or < 0.83 , $P < 0.05$) (Yu et al., 2017) using the limma package (Ritchie et al., 2015).

To express the characteristics of coral hosts and symbionts among different treatments, Bray-Curtis dissimilarity-based principal coordinates analysis (PCoA), permutational multivariate analysis of variance using distance matrices (PERMANOVA), and clustering of DEPs based on Bray-Curtis distance were performed using the Vegan package (Oksanen et al., 2013) with R software. In addition, DEPs in the three treatments were retrieved and were plotted in a Venn diagram.

The protein sequences of DEPs were retrieved in batches from the UniprotKB database (release 2016_10) in FASTA format. The retrieved sequences were locally searched against the SwissProt database using the NCBI BLAST + client software to find homologue sequences, from which the functional annotations could be transferred to the studied sequences. The top 10 blast hits with E-values $< 1e^{-3}$ for each query sequence were retrieved and loaded into Blast2GO (version 3.3.5) for gene ontology (GO) mapping and annotation. An annotation configuration with an E-value filter of $1e^{-6}$, default gradual EC weights, a GO weight of 5, and an annotation cut-off of 75 were chosen. The un-annotated sequences were

then re-annotated with more permissive parameters. The sequences without BLAST hits and un-annotated sequences were then selected for an InterProScan search against the European Bioinformatics Institute databases to retrieve functional annotations of protein motifs and to add the InterProScan GO terms to the annotation set.

To further explore the impact of DEPs in cell physiological processes and to examine the internal relations between DEPs, enrichment analysis was performed. GO enrichment on three ontologies (biological process [BP], molecular function [MF], and cellular component [CC]) was applied based on Fisher's exact test, using the quantified protein annotations as the background dataset. Benjamini-Hochberg correction for multiple testing was applied to adjust derived *P*-values, and only functional categories with *P*-values under a threshold of 0.05 were considered significant.

2.10 Data Analysis

To test for the effects of temperature and pH on P_{NET} , R_L , P_G , R_D , P_{NET}/R_D , F_V/F_M , and larval symbiont density, data were analyzed using two-way ANOVAs. Temperature (29 and 33 °C) and pH (7.85 and 8.15) were used as fixed effects, and 'tank' was a random effect nested in temperature and $p\text{CO}_2$. Statistical significance is reported at $P < 0.05$. These analyses were conducted using SPSS version 22.0 (IBM, Armonk, NY, USA) and Microsoft Excel (Microsoft Corporation, Redmont, WA, USA).

3. RESULTS

3.1 Physiological Responses to Warming and Acidification

High $p\text{CO}_2$ ($F_{1,12} = 100.015$, $P < 0.001$) significantly increased P_{NET} , whereas high temperature ($F_{1,12} = 31.746$, $P < 0.001$) significantly reduced it (Table S7). However, temperature and $p\text{CO}_2$ together did not affect P_{NET} . Specifically, P_{NET} was -0.011 ± 0.006 nmol O₂ larva⁻¹ min⁻¹ under elevated temperature treatment and 0.2 ± 0.017 nmol O₂ larva⁻¹

min⁻¹ under elevated $p\text{CO}_2$ treatment (Fig. 1A). In addition, temperature, $p\text{CO}_2$, and their interaction had no significant effect on R_L and R_D (Table S7), which ranged from 0.15 ± 0.011 to 0.235 ± 0.042 nmol O₂ larva⁻¹ min⁻¹ and from 0.12 ± 0.011 to 0.195 ± 0.025 nmol O₂ larva⁻¹ min⁻¹ across all treatments, respectively (Fig. 1B, E).

In contrast to R_L and R_D , P_G and P_{NET}/R_D were significantly affected by temperature elevation ($F_{1,12} = 23.445$, $P < 0.001$; $F_{1,12} = 22.042$, $P < 0.001$, respectively) and CO₂ enrichment ($F_{1,12} = 10.272$, $P = 0.008$; $F_{1,12} = 14.888$, $P = 0.002$, respectively). There was no significant effect of interaction between temperature and $p\text{CO}_2$ (Table S7). Regarding P_{NET}/R_D , the ratio in the OA treatment was 1.37 ± 0.29 , indicating that the net organic carbon fixed by photosynthesis by the symbiont was sufficient to sustain the amount consumed by respiration. However, the ratio was below 1 in the other three treatments (Fig. 1D).

Elevated temperature reduced F_V/F_M at both ambient and elevated $p\text{CO}_2$ conditions ($F_{1,12} = 11.761$, $P = 0.005$), whereas $p\text{CO}_2$ alone had no effect. Under ambient temperatures, F_V/F_M was ~ 0.6 , whereas under elevated temperature treatments, F_V/F_M was 0.42 ± 0.076 at ambient $p\text{CO}_2$ and 0.51 ± 0.055 at elevated $p\text{CO}_2$ (Fig. 1F). Symbiont density decreased significantly with increased temperature ($F_{1,12} = 46.953$, $P < 0.001$), leading to a reduction in algal counts from 20,000 cells larva⁻¹ at ambient temperature treatments to 5,000 cells larva⁻¹ under elevated temperature treatments (Fig. 2A). However, no effect was found in response to CO₂ enrichment or its interaction with temperature. Larval survival rates ranged from 85% to 96% across all treatments and did not differ significantly between treatments (Fig. 2B). Supplementary Table 7 lists all statistical results with regard to P_{NET} , R_L , P_G , R_D , P_{NET}/R_D , F_V/F_M , symbiont density, and survivorship.

3.2 Assembly of the Proteome Data and Differential Protein Expression

In total, 191,726 spectra were identified from TMT experiments. Based on the Mascot search results, 33,857 matched peptides, 30,109 matched unique peptides, and 4959 matched proteins were identified. In total, 346 DEPs were identified in coral holobiont, 178 (3.58%) were from the coral host across all comparisons, with 168 (3.39%) from the symbionts across all comparisons.

In the coral host, 15, 76, and 87 proteins were differentially expressed in the OA vs. control, ET vs. control, and ETOA vs. control treatment comparisons, respectively (Figure S2 and Table S1, S2, S3, and S8), whereas in the symbionts, 16, 90, and 62 proteins were differentially expressed in the OA vs. control, ET vs. control, and ETOA vs. control treatment comparisons, respectively (Figure S2 and Table S4, S5, S6 and S8). In addition, the Bray-Curtis dissimilarity-based PCoA and clustering of DEPs based on the Bray-Curtis distance revealed that the DEPs in coral host (PERMANOVA: $R^2 = 0.51$, $P = 0.037$) and symbionts (PERMANOVA: $R^2 = 0.54$, $P = 0.017$) under ambient temperature treatments were clearly distinguished from those in warming treatments (Fig. 4, Table S10).

3.3 GO Classification and Major DEPs Analysis

Based on the three GO categories, 117, 13, and 59 GO terms were identified in coral host in BP, CC, and MF respectively, under the three treatments, whereas 17, 3, and 11 GO terms were classified in symbionts in BP, CC, and MF, respectively (Table S9). To present the functions of DEPs in the coral host and symbionts, the top 20 GO terms and GO enrichment ($P < 0.05$) analysis of DEPs are shown in Supplementary Figure 1.

Among the symbionts, although 168 DEPs were obtained across all comparisons, the function of several of these DEPs was known. Most DEPs related to photosynthesis, the

Calvin cycle, glycolysis, and the C₄ pathway were significantly downregulated across all comparisons. For instance, thirteen DEPs in the photosynthesis pathway exhibited changes in expression levels in the three treatments compared to the control. Under ET and ETOA treatments, fucoxanthin-chlorophyll a-c binding protein F, chloroplast soluble peridinin-chlorophyll a-binding protein precursor, photosystem II 12 kDa extrinsic protein, phosphoenolpyruvate carboxylase, fucoxanthin-chlorophyll a-c binding protein, phosphoribulokinase, sedoheptulose-1,7-bisphosphatase, photosystem I reaction center subunit XI, fructose-1,6-bisphosphatase, photosystem I reaction center subunit IV, oxygen-evolving enhancer protein 1, ribulose biphosphate carboxylase, and glyceraldehyde-3-phosphate dehydrogenase were downregulated (Table S5 and S6), whereas photosystem I iron-sulfur center protein was upregulated under the OA treatment (Table S4). In addition, the four proteins of the Calvin cycle, phosphoribulokinase, sedoheptulose-1,7-bisphosphatase, fructose-1,6-bisphosphatase, and glyceraldehyde-3-phosphate dehydrogenase were downregulated under ET and ETOA treatments (Table S5 and S6). DEPs related to the Calvin cycle, including phosphoribulokinase, were also downregulated in the ET and ETOA treatments (Table S5 and S6).

Furthermore, glycolysis was also affected in the symbionts. Five DEPs, including pyruvate kinase (PK), phosphoglycerate kinase, putative phosphoglycerate kinase (PGK), triosephosphate isomerase-like protein, and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase-like protein, were downregulated in response to warming (Fig. 4). Interestingly, PK was also downregulated under the OA treatment (Fig. 4). In addition,

phosphoenolpyruvate carboxylase (PEPCase), which is involved in the C₄ pathway, was also significantly downregulated in the ET and ETOA treatments (Table S5 and S6). Carbonic anhydrase (CA) 2-like was downregulated more than 0.6-fold in symbionts under ET and ETOA treatments (Figure 4).

In the coral host, 178 DEPs were obtained across all comparisons, however, the function of several of these DEPs was known. Expression of CA 2-like, which facilitates the interconversion of CO₂ and HCO₃²⁻, was downregulated more than 0.6-fold in the host (ETOA treatment) (Figure 4). In addition, two DEPs related to glycolysis, triosephosphate isomerase-like and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase-like, were downregulated in the ET and ETOA treatments (Figure 4).

4. DISCUSSION

Here, we studied the physiological and molecular responses in *P. damicornis* larvae to evaluate the potential responses of corals to extreme environmental conditions that could occur in future. The results of this study not only demonstrated that net photosynthesis declined with elevated temperature and increased with acidification in larvae, but also showed that proteins associated with photosynthesis and inorganic carbon acquisition of symbionts were downregulated due to warming. Temperature had a greater adverse effect on symbionts than on the coral host, with a large number of differentially expressed proteins related to important functional processes in symbionts, including light reaction, the Calvin cycle, and the C₄ pathway. Taken together, our results not only demonstrate that excess thermal stress significantly reduces photosynthesis in Luhuitou reef, but also outline the key proteins involved in important molecular pathways.

4.1 Thermal Stress Decreases Photosynthesis, Unlike Acidification

Temperature, $p\text{CO}_2$, and their interaction had no significant effect on R_D , whereas temperature significantly reduced P_{NET} and P_{NET}/R_D , and $p\text{CO}_2$ had the opposite effect. There are two possible explanations of our results. The most likely mechanism is that R_D of *P. damicornis* larvae in Luhuitou fringing reef is relatively high. Larval dark respiration ranged from 0.12 ± 0.011 to 0.195 ± 0.025 $\text{nmol O}_2 \text{ larva}^{-1} \text{ min}^{-1}$ in all treatments, and P_{NET}/R_D was below 1 in all treatments except OA. The results agree with those of Jiang et al. (2020), who found that R_D and P_{NET} for the freshly released *P. damicornis* larvae from the same study site were 0.201 ± 0.007 and 0.084 ± 0.008 $\text{nmol O}_2 \text{ larva}^{-1} \text{ min}^{-1}$, respectively, resulting in low rates of P_{NET}/R_D (below 1). A high rate of R_D was also observed in *P. damicornis* larvae, with 0.262 $\text{nmol O}_2 \text{ larva}^{-1} \text{ min}^{-1}$ under elevated temperature and ambient $p\text{CO}_2$ conditions (Cumbo et al., 2013a).

An alternative, albeit not mutually exclusive, explanation is that P_{NET} should be expected to show a dome-shaped relationship with temperature elevation, and the temperature threshold of the larvae in the same site was between 32 and 33 °C (Jiang et al., 2021). The decreased P_{NET} and P_{NET}/R_D found in this study may indicate a sharp response to the extreme temperature (33 °C) recorded in Luhuitou reef. It is important to note that this extreme temperature is above the thermal optimum of the species and would adversely affect larval primary productivity (Jiang et al., 2021; Samiei et al., 2015). A strong negative response to elevated temperatures has also been observed in other studies, where P_{NET} of *A. downingi* (Samiei et al., 2015) and *P. damicornis* (Kuanui et al., 2015) and P_{NET}/R_D (also below 0) of other corals (Samiei et al., 2015; Van der Zande et al., 2020) were largely decreased at high temperatures (Kuanui et al., 2015; Samiei et al., 2015), thus demonstrating that

photosynthetic performance could not support basal metabolic needs.

Elevated P_{NET} due to acidification may be attributed to high $p\text{CO}_2$ levels, indicating that high CO_2 may exert a fertilizing effect on larval P_{NET} . This explanation is consistent with a recent report on the impacts of elevated $p\text{CO}_2$ on pocilloporid coral (Guillermic et al., 2021). Conversely, high $p\text{CO}_2$ (560 and 800 μatm) has the potential to negatively affect *Porites astreoides* larval recruitment by impacting larval respiration and settlement (Albright and Langdon, 2011). Similar results were also observed in *A. digitifera* larvae performance under acidification (pH 7.6 and 7.3) (Nakamura et al., 2011).

Although significant physiological changes were observed, no significant variation in larval mortality was detected (Fig. 2B), possibly indicating that the negative effects on survivorship may not be evident before 5 days of exposure to stress conditions. It is often inferred that coral larvae are more vulnerable to environmental changes than adults (Albright, 2011) and therefore are more strongly affected by climate changes. However, according to this data recorded during extreme temperature treatments, metabolic suppression has been shown in *P. damicornis* larvae, and similar responses occur in adult corals (Kuanui et al., 2015; Samiei et al., 2015). Development of coral larvae is generally a good predictor of larval dispersal patterns and reef connectivity (Albright, 2011); thus, future studies should elucidate whether the effects observed in our study affect the connectivity to other reefs, using biophysical models.

4.2 Warming and Acidification Affected the Light Reaction in Symbionts

A suite of light reaction proteins was downregulated, which was consistent with low P_{NET} under elevated temperature (Fig. 4c). As PS I reaction center subunits IV and XI, and oxygen-evolving enhancer 1 (OEE 1) levels were downregulated under ET and ETOA (Table

S5, 6), light reaction was likely reduced under elevated temperature. The function of the subunits is to convert light energy into chemical energy during the light reaction (Orf et al., 2018). A similar response was found in the transcripts of the free-living *Fugacium kawagutii* and the endosymbiont *Symbiodinium* in the coral *A. palmata*, which exhibited downregulation following thermal stress (DeSalvo et al., 2012; Lin et al., 2019), suggesting that the energy transfer process may be inhibited by thermal stress.

Consistently, the oxygen evolving enhancers are auxiliary components of the PS II manganese cluster, whose function is essential for oxygen evolving activity and PS II stability (Momonoki et al., 2009). Impairment of the oxygen-evolving complex is likely to be the rate-limiting step in the overall process of photodamage to PS II (Takahashi and Murata, 2008). Moreover, the result regarding endosymbionts coincided with photosynthetically evolved oxygen in cultured symbionts, suggesting that O₂ evolution may be mediated by OEE 1 (Mayfield, 1991; Sorek et al., 2013).

In contrast, the PS I iron-sulfur center protein was upregulated in the OA treatment (Fig. 4c, Table S3), which may indicate increased capacity of ATP synthesis. One possible explanation is that high CO₂ may have a fertilizing effect on larval net photosynthesis. Thus, P_{NET} was significantly promoted by elevated CO₂, which is consistent with the physiological performance under high $p\text{CO}_2$.

4.3 Warming Decreased the Carbon Fixation in Symbionts

In the coral holobiont, up to 95% of the photosynthetically fixed carbon (glucose and amino acids) produced by symbionts is transferred to the coral host (Muscatine, 1990), and unavailability of this energy source over a long period of time may result in mortality. The Calvin cycle is fueled by, and dependent on, ATP and NADPH from the light reactions, which

is an important pathway to produce glucose. However, several DEPs involved in the Calvin cycle were downregulated under elevated temperature conditions (e.g., phosphoribulokinase, sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase) (Fig. 4c), potentially suggesting that coral larvae may produce less glucose under thermal stress. This would result in a reduction in the amount of the fixed carbon available to coral host.

PEPCase was significantly downregulated in the context of the C₄ pathway in ET and ETOA (Fig. 4a), suggesting that it may influence carbon fixation under elevated temperature. Genomic studies suggest that PEPCase is involved in the C₄ metabolism in *F. kawagutii* (Lin et al., 2015) and catalyzes the carboxylation of phosphoenolpyruvic acid in the C₄ pathway that uses HCO₃⁻ as an inorganic carbon substrate in oxaloacetic acid, which is catalyzed by phosphoenolpyruvate carboxykinase into CO₂ for fixation by RuBisCo (Fig. 5) (Reinfelder et al., 2000). PEPCase inhibition in the diatom *Thalassiosira weissflogii* (Reinfelder et al., 2004) and the macroalga *Udotea flabellum* (Reiskind and Bowes, 1991) results in a 70%–90% decrease in photosynthesis, highlighting the important role of PEPCase in carbon fixation.

CA homologues were downregulated in the coral host (ETOA treatment) and in symbionts (OA and ETOA treatments) (Fig. 4a). Downregulated expression of CA homologues suggests that the DIC transport function is inhibited in both coral host and symbionts, leading to low CO₂ in symbionts. DIC is present in the ocean mainly as HCO₃⁻ (92%), which can be interconverted to CO₂ through the activity of CA—an integral part of the carbon concentrating mechanisms utilized by symbiotic cnidarians in the photosynthetic process (Bertucci et al., 2013; Ip et al., 2017). Interestingly, Graham et al. (2015) observed that downregulation of CA occurred in symbionts but not regarding host CA in *Zoanthus* sp.,

indicating that CA activity in symbionts may be affected by elevated temperature. In addition, two cnidarian CA genes in the coral *A. viridis* were found to be significantly downregulated due to thermal stress (Moya et al., 2012). Thermal stress may thus have caused impairment of CA activity in both host tissue and symbionts, such that DIC transport to symbionts was severely limited, leading to lower levels of carbon fixation.

4.4 Warming Decreased Glycolysis-Related Protein Levels in Coral Hosts and Symbionts

The temperature increase was accompanied by a downregulation of glycolytic enzymes such as PGK and PK in symbionts, and triosephosphate isomerase-like protein, and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase-like protein in the host (Fig. 4b), indicating that coral host and symbionts consume less glucose to cope with thermal stress. For example, PGK plays a major role in ATP production during glycolysis as it catalyzes the first step of ATP production, and 3-phosphoglycerate participates in the Calvin cycle (Joshi et al., 2016). PGK downregulation was suggested to occur so as to adjust the metabolism and to optimize growth (Rosa-Téllez et al., 2018). This result is in line with results of previous studies on other corals (Gust et al., 2014), and it suggests that corals may decrease metabolic potential due to environmental stress. Furthermore, PK is the final rate-limiting enzyme of glycolysis and plays a role in regulating cell metabolism (Israelsen and Vander Heiden, 2015). Lower expression of PK leads to lower levels of pyruvate and ATP fueling the tricarboxylic acid cycle, which affects the production of acetyl-CoA. Together, these results suggest reduced potential for carbon fixation and cellular energy production through carbohydrate metabolism in coral larvae.

5. CONCLUSION

This study identified physiological changes and protein expression (summarized in Fig.

5) affected by elevated temperature and $p\text{CO}_2$ conditions, and the predicted functions of these proteins give rise to hypotheses regarding the regulation of larval energy metabolism. Net photosynthesis was significantly decreased under extreme temperature and increased by acidification. Under thermal stress, the light reaction and DIC transport were repressed, and Calvin cycle arrest was evidenced by the proteome expression profile, suggesting that carbon fixation in symbionts was inhibited. The glycolysis pathway in both coral host and symbionts was reduced, indicating that the energy supply in the coral holobiont was decreased. Metabolic suppression is considered an adaptive strategy for the survival of short-term stress; however, decelerated metabolism is generally achieved by reducing such important processes, and therefore, if sustained, may lead to reductions in growth and recruitment potential. Further studies should focus on the differentially expressed proteins, which may be candidate reference proteins in them. Overall, our results provide novel and valuable insights into the biochemical regulation and physiological functions in *P. damicornis* larvae in response to important environmental factors associated with global climate change.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA ACCESSIBILITY

All raw data from this study are available in the ProteomeXchange under the accession number PX-submission 419395.

AUTHOR CONTRIBUTIONS

Youfang Sun, Lei Jiang and Hui Huang conceived the study. Youfang Sun and Lei Jiang conducted the experiments. Lintao Huang, Guillermo Diaz-Pulido, Yuyang Zhang, Haoya

Tong, Xiangcheng Yuan, Guowei Zhou, and Hui Huang contributed to interpretation of the results. Youfang Sun and Hui Huang drafted the manuscript. All authors commented on the draft and gave final consent for publication.

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TABLES

Table 1. Experimental seawater conditions in each treatment (mean $\pm$ se).

Treatment	pH _T	T (°C)	Salinity	TA	pCO ₂ (μ atm)	DIC	Ω_{Arag}
Control	8.13 $\pm$ 0.003	29.10 $\pm$ 0.006	33.50 $\pm$ 0.012	2252 $\pm$ 8.5	477 $\pm$ 7.7	1980 $\pm$ 6.4	3.23 $\pm$ 0.04
OA	7.86 $\pm$ 0.002	28.90 $\pm$ 0.006	32.50 $\pm$ 0.011	2245 $\pm$ 5.2	963 $\pm$ 9.6	2102 $\pm$ 3.6	1.93 $\pm$ 0.02
ET	8.15 $\pm$ 0.003	33.02 $\pm$ 0.007	33.60 $\pm$ 0.011	2244 $\pm$ 5.7	471 $\pm$ 8.3	1941 $\pm$ 4.8	3.67 $\pm$ 0.05
ETOA	7.88 $\pm$ 0.002	33.04 $\pm$ 0.008	33.50 $\pm$ 0.012	2258 $\pm$ 4.3	957 $\pm$ 8.4	2087 $\pm$ 4.2	2.28 $\pm$ 0.02

Carbonate chemistry parameters: pH total scale (pH_T), temperature (T), salinity, total alkalinity (TA), partial pressure of carbonate dioxide (pCO₂), and aragonite saturation (Ω_{Arag}).

The treatment conditions were as follows: control (29 °C and pCO₂: ~ 500 μ atm), OA (29 °C and pCO₂: ~ 1,000 μ atm), ET (33 °C and pCO₂: ~ 500 μ atm), ETOA (33 °C and pCO₂: ~ 1,000 μ atm).

Figure 1. Net photosynthesis (A), light respiration (B), gross photosynthesis (C), ratio of net photosynthesis to dark respiration (D), dark respiration (E) (n =30), and maximum photochemical efficiency F_V/F_M (F) (n =10) of *Pocillopora damicornis* larvae under combinations of two temperatures and pCO₂ levels. Different lowercase letters indicate

significant differences as determined using a Bonferroni test ($P < 0.05$). Error bars indicate standard errors. Control (29 °C and $p\text{CO}_2$: ~ 500 μatm), OA (29 °C and $p\text{CO}_2$: ~ 1,000 μatm), ET (33 °C and $p\text{CO}_2$: ~ 500 μatm), ETOA (33 °C and $p\text{CO}_2$: ~ 1,000 μatm).

Figure 2. Symbiont density (A) and survivorship (B) ($n=10$) of *Pocillopora damicornis* larvae under combinations of two temperatures and $p\text{CO}_2$ levels. Different lowercase letters indicate significant differences as determined using a Bonferroni test ($P < 0.05$). Error bars indicate standard errors. Control (29 °C and $p\text{CO}_2$: ~ 500 μatm), OA (29 °C and $p\text{CO}_2$: ~ 1,000 μatm), ET (33 °C and $p\text{CO}_2$: ~ 500 μatm), ETOA (33 °C and $p\text{CO}_2$: ~ 1,000 μatm).

Figure 3. Principal coordinates analysis plots (A, C) and cluster dendrograms (B, D) of all detected differential expression proteins in the coral host (A, B) and symbionts (C, D) based on the Bray-Curtis distance. OA 1, 2, and 3 represent elevated $p\text{CO}_2$ treatments; ET 1, 2, and 3 represent elevated temperature treatments; ETOA 1, 2, and 3 represent elevated temperature and $p\text{CO}_2$ treatments. Control (29 °C and $p\text{CO}_2$: ~ 500 μatm), OA (29 °C and $p\text{CO}_2$: ~ 1,000

μatm), ET (33 °C and $p\text{CO}_2$: $\sim 500 \mu\text{atm}$), ETOA (33 °C and $p\text{CO}_2$: $\sim 1,000 \mu\text{atm}$).

Figure 4. Selected proteins that are differentially abundant in DIC transport (a), Glycolysis (b), and photosynthesis (c). Differentially expressed proteins (DEPs, fold change > 1.2 or < 0.83 , $P < 0.05$) shown are those discussed in the text. Error bars indicate standard errors of these proteins' fold change. The full list of DEPs in both the coral host and symbionts under different treatments is available in Supplementary Tables 1–6. Abbreviations: A: carbonic anhydrase 2-like [symbiont]; B: carbonic anhydrase 2-like [*Pocillopora damicornis*]; C: pyruvate kinase [symbiont]; D: phosphoglycerate kinase [symbiont]; E: putative phosphoglycerate kinase [symbiont]; F: triosephosphate isomerase-like [*Pocillopora damicornis*]; G: 2,3-bisphosphoglycerate-independent phosphoglycerate mutase-like [*Pocillopora damicornis*]; H: fucoxanthin-chlorophyll a-c binding protein; F: chloroplastic [symbiont]; I: chloroplast soluble peridinin-chlorophyll a-binding protein precursor

chloroplast; and mt: mitochondrion. Pink, red, and blue colors represent OA, ET, and ETOA treatments, respectively. The red arrow represents upregulation. ISC: iron-sulfur center; CPA: cytosolic phospholipase A2-like; AC: acid ceramidase-like; NA: alpha-N-acetylgalactosaminidase-like; HS: beta-hexosaminidase subunit beta-like β ; CCB2: cytochrome b2, mitochondrial; SCL: succinyl-CoA ligase ADP-forming subunit beta; ATPS: ATP synthase subunit beta; NADT: NAD(P) transhydrogenase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PGK: phosphoglycerate kinase; TPI: triose-phosphate isomerase; PGM: phosphoglycerate mutase; FEPCCK: phosphoenolpyruvate carboxykinase; PEPC: phosphoenolpyruvate carboxylase; SBPase: sedoheptulose-1, 7-bisphosphatase; FBPase: fructose 1,6-bisphosphatase; PGK: phosphoglycerate kinase; PK: pyruvate kinase; PSI: photosynthesis I; PSII: photosynthesis II; RCS IV: photosystem I reaction center subunit IV; RCS VI: photosystem I reaction center subunit VI; OEC: oxygen-evolving complex center; EP: photosystem II 12 kDa extrinsic protein; BP: chloroplast soluble peridinin-chlorophyll a-binding protein precursor; FCPF: fucoxanthin-chlorophyll a-c binding protein F; FCP: fucoxanthin-chlorophyll a-c binding protein; PsbO: oxygen-evolving enhancer protein 1; PsbP: oxygen-evolving enhancer protein 2; PsbQ: oxygen-evolving enhancer protein 3; B6F: cytochrome *b6/f* complex; pCA: periplasmic (extracellular) carbonic anhydrase; NAD(P)H: nitrate reductase. Solid arrows indicate direct steps in a pathway and dashed arrows indicate omission of multiple steps in a pathway.

Graphical Abstract

Highlights

- Thermal stress reduced and acidification increased larval photosynthesis in *P. damicornis*.
- Thermal stress decreased light reaction and carbon fixation in endosymbionts.
- Thermal stress decreased dissolved inorganic carbon transport in coral host and symbionts.
- Acidification upregulated photosystem I iron-sulfur center protein levels in endosymbionts.