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Research Article

The Impacts of *Ex Situ* Transplantation on the Physiology of the Taiwanese Reef-Building Coral *Seriatopora hystrix*

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We sought to determine whether the Indo-Pacific reef-building coral *Seriatopora hystrix* performs in a similar manner in the laboratory as it does *in situ* by measuring *Symbiodinium* density, chlorophyll a (chl-*a*) concentration, and the maximum quantum yield of photosystem II (F_V/F_M) at the time of field sampling (*in situ*), as well as after three weeks of acclimation and one week of experimentation (*ex situ*). *Symbiodinium* density was similar between corals of the two study sites, Houbihu (an upwelling reef) and Houwan (a nonupwelling reef), and also remained at similar levels *ex situ* as *in situ*. On the other hand, both areal and cell-specific chl-*a* concentrations approximately doubled *ex situ* relative to *in situ*, an increase that may be due to having employed a light regime that differed from that experienced by these corals on the reefs of southern Taiwan from which they were collected. As this change in *Symbiodinium* chl-*a* content was documented in corals of both sites, the experiment itself was not biased by this difference. Furthermore, F_V/F_M increased by only 1% *ex situ* relative to *in situ*, indicating that the corals maintained a similar level of photosynthetic performance as displayed *in situ* even after one month in captivity.

1. Introduction

Molecular biology promises to yield insight into the subcellular mechanisms underlying the stable mutualism between reef-building scleractinians and dinoflagellates of the genus *Symbiodinium* [1, 2], as well as their macromolecular responses to changes in their environment [3–5]. The latter topic is of particular interest given that global climate change (GCC)-driven temperature and pCO_2 increases have been hypothesized to lead to more frequent coral bleaching events in the years to come [6]. Alongside other anthropogenic pressures, such GCC-derived threats have generated an urgent need to shift the monitoring of coral reef health from a retroactive process to a proactive one [7]. Assessment of reef health is currently conducted by visual surveys in which the number of dying or dead corals is quantified (e.g., [8]). However, such late-stage manifestations of health decline likely occurred well after the initial insult. An analysis of the expression or activity of subcellular biomarkers, such as stress genes and proteins, may allow for the determination of which corals are at risk from anthropogenic impacts on a proactive timescale. Such a monitoring approach could potentially allow for scientists and managers to work together to mitigate local-scale insults to reef stability, such as water pollution [9], prior to extensive loss of coral.

In order to validate the efficacy of certain gene and protein-level biomarkers for proactive coral health assessment, their behavior in response to environmental stress must first be determined in the laboratory. Unfortunately, little attention has been given to ensure that laboratory-borne results do not carry experimental artifacts emerging from the transplantation of corals out of the field and into aquaria. For instance, corals are routinely fragmented with chisels or pneumatic drills, removed from the ocean, transported to a laboratory, and then used in experiments within several hours or days (e.g., [10–12]). Their physiological responses

to the experimental treatment could therefore be masked by their recovery response to fragmentation and subsequent incubation in an environment that may differ greatly from the reef from which they were sampled.

If molecular biomarkers are to ultimately gauge the health of corals on a proactive timescale, not only must laboratory-reared corals be shown to behave in a similar manner as *in situ*, but the expression and/or activity of these macromolecules must also be shown to either correlate with or predict a certain phenotype. To date, there have been few attempts to document correlations between a particular physiological response and the expression of the underlying genes and proteins. Putnam et al. [13] found no correlation between ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene and RBCL protein expression in larvae of the scleractinian coral *Pocillopora damicornis*, in contrast to what they had hypothesized. However, Putnam et al. [13] did not attempt to draw inferences from correlations between physiological parameters, such as *Symbiodinium* density, and subcellular response variables, such as gene expression. Such covariation analyses could ultimately allow for the development of molecular or cellular biomarkers (*sensu* [14]) that could be used for reef coral health assessment provided that strong correlations are documented between indices of physiological performance, such as growth rate, and expression of certain macromolecules.

In order to address these two deficiencies in the coral biology field, specimens of the Indo-Pacific reef-building coral *Seriatopora hystrix* were collected from two reefs nearby Taiwan's National Museum of Marine Biology and Aquarium (NMMBA), and their physiology was assessed both *in situ* and after a previously published experiment [4] to determine whether this coral demonstrates a similar physiology in the laboratory (i.e., *ex situ*) as it does in the field (i.e., *in situ*). Then, a previously published dataset was explored again in order to document the degree of correlation between four physiological parameters: growth, *Symbiodinium* density, chlorophyll a (chl-*a*) concentration (both areal and cell specific), and the maximum quantum yield of photosystem II (F_V/F_M) [4], and the expression of four *Symbiodinium* genes (Table 1). It was hypothesized that expression of *Symbiodinium* photosynthesis genes, such as *rbcL*, photosystem I (*psI*, subunit III), and phosphoglycolate phosphatase (*pgpase*), would be associated with increases in F_V/F_M, which is often used as a proxy for photosynthetic performance in *Symbiodinium* populations within other reef-building corals [15]. The ultimate goal of this work was to begin to develop a standard operating procedure for manipulative experiments with molecular endpoints with *S. hystrix*, which has recently emerged as a model for understanding the impacts of GCC on coral physiology [3, 14, 16].

2. Materials and Methods

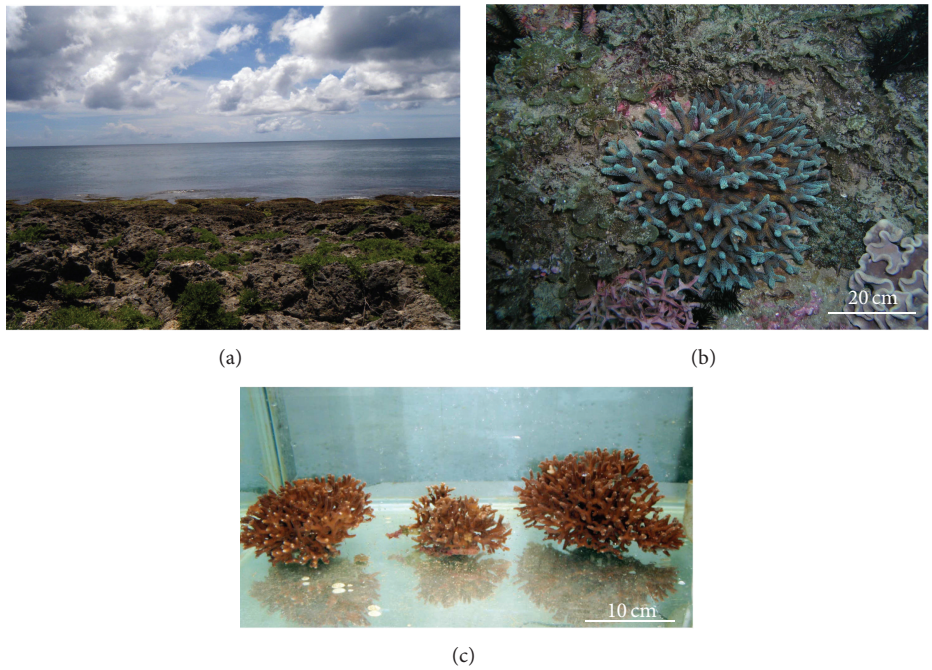
2.1. Coral Collection, Manipulative Experiment, and Physiological Parameter Analysis. A reciprocal transplant was previously conducted in the laboratory whereby *S. hystrix* specimens from Houbihu, a reef characterized by extensive upwelling [17], were exposed to either a fluctuating temperature treatment (23–29°C over a 5 hr period) or a stable one (26°C) for 7 d [4]. Conspecifics from Houwan (Figure 1(a)), a nonupwelling reef, were simultaneously exposed to the same two treatments, and sampling was conducted at the end of the experiment only. Coral nubbins (~2 g) from both sites of origin were acclimated at constant seawater conditions (described in [4]) for three weeks prior to the experiment to allow for recovery from the fragmentation, transplantation, and husbandry processes. However, from analysis of the published results alone [4], it was unclear whether the experimental nubbins performed in a similar manner as *in situ* after four weeks of husbandry.

On the evening prior to collecting the six *S. hystrix* colonies from their respective field sites, a diving pulse amplitude modulating (PAM) fluorometer (Walz, Germany) was used to calculate the maximum dark-adapted F_V/F_M of each sampled colony at 18 : 30 as described in [5]. It was hypothesized that corals of the two sites would demonstrate similar F_V/F_M values *in situ*. Furthermore, although the *ex situ* F_V/F_M values of nontransplanted corals (see [4] for details) were hypothesized to be similar to those documented *in situ*, the *ex situ* F_V/F_M values of transplanted corals were hypothesized to be lower than those documented *in situ*.

The following day, six colonies (Figure 1(b)) from each site were removed from the ocean, and three 50 mg biopsies were fragmented with pliers from each ($n = 18$ biopsies per site) prior to transportation of the colonies to the laboratory (a 2 km distance away). One biopsy was submerged in 500 μL of TRIzol (Life Technologies, Grand Island, NY, USA), while the other two were immersed in 1-2 mL of RNALater (Ambion, Austin, TX, USA). Coral biopsies within one of the two tubes containing RNALater were stored at 4°C for one month. One hour after returning to the laboratory, both the coral samples and RNALater in the other six tubes were decanted into a mortar and homogenized with a pestle for several minutes. The RNALater-coral tissue slurry was then frozen at −20°C for one month.

We have previously found that RNALater does not preserve the integrity of coral RNA to an extent that is suitable for downstream analyses, such as real-time PCR. It was hypothesized that this poor capacity for fixation could be due to the inability of the salts within the RNALater solution to penetrate the coral tissue-skeleton interface. Therefore, homogenization in RNALater could potentially lead to better protection of RNA against ribonucleases, as this would allow for the fixative to come into contact with and permeate a greater proportion of both the coral and *Symbiodinium* cells. If this method led to RNA of similar quantity and quality to that of samples stored in TRIzol, this would represent a preferable means for preserving corals sampled *in situ* since it would not require the transport of liquid nitrogen or toxic chemicals (i.e., TRIzol) into the field. Only the 18 biopsies generated from the six coral colonies collected from Houwan were processed for this fixation strategy comparison.

Upon arriving at NMMBA, branches (~2 g) were removed from each of the 12 colonies (Figure 1(c)), wrapped in aluminum foil, and frozen at −20°C. The tissue was removed from the branches as in [4] the next day, and *Symbiodinium*



(a) (b)

(c)

Figure 1: Field site and model coral. Corals were collected from two field sites in southern Taiwan, Houbihu (see Figure 1 of [4]) and Houwan (a). *Seriatopora hystrix* colonies (b) were collected *in situ* and transported to aquaria (50 l; c) at Taiwan's National Museum of Marine Biology and Aquarium (NMMBA). Ten "nubbins" of approximately 2 g were generated from each of 12 such colonies (6 from Houbihu and 6 from Houwan) for use in the experiment [4].

Table 1: Expression of *Symbiodinium* ribulose,1-5,bisphosphate carboxylase/oxygenase large subunit (*rbcL*), photosystem I (*psI*, subunit III), phosphoglycolate phosphatase (*pgpase*), and ascorbate peroxidase (*apx1*) was measured in samples from either Houbihu (an upwelling reef) or Houwan (a nonupwelling reef) exposed to either a variable or stable temperature regime for seven days.

Gene	Function	Results from prior works	Results discussed herein
rbcL	Photosynthesis	Higher at variable temperature[a]	
psI	Photosynthesis	Higher at variable temperature[a] Higher at Houbihu	Negatively correlated with F_V/F_M[b]
pgpase	Photosynthesis	Higher at variable temperature[a]	Positively correlated with *psI*[b]
apx1	Stress response		Positively correlated with *pgpase*[b]

[a]See [4]. [b]See Figure 4.

density and chl-*a* concentration were determined as in [18]. Surface area (SA) was calculated with the wax dipping method developed by [19]; 12 wooden dowels of a range of known dimensions and SAs were created and dipped twice in vats containing molten paraffin wax in order to generate a standard curve of SA versus the difference in wax mass accumulated between the 1st and 2nd wax dips. The wax mass differences of the branches were then compared against the standard curve to calculate SA.

Both *Symbiodinium* density and chl-*a* content were normalized to SA and reported as cells cm^{-2} and $\mu g\,cm^{-2}$, respectively. The areal chl-*a* concentration was divided by the *Symbiodinium* density of the same sample to calculate the cell-specific chl-*a* concentration, which was reported as pg $cell^{-1}$. The *in situ* values for these four physiological response variables (including F_V/F_M) were compared to those obtained after a three-week acclimation period and a one-week experimental period [4], and it was hypothesized that there might be increases in chl-*a* concentration (both areal and per cell) due to the use of stable, rather than fluctuating (as occurs *in situ*), photosynthetically active radiation (PAR) levels during the experiment itself.

During the three-week acclimation period, the experimentally fragmented coral nubbins were exposed to shaded, natural light (~90 μmol photons $m^{-2}\,s^{-1}$). Artificial lights were utilized and set to a 12 hr light (~90 μmol photons $m^{-2}\,s^{-1}$)-12 hr dark cycle during the one-week experiment that followed [4], meaning that the light profile did not vary over the daytime portion of the diel cycle during the experiment, as it would *in situ* (see Figure 1 of [20]). Despite this utilization of a stable light regime characterized by the same average hourly PAR level that these corals experience

in situ [4], corals from each of the two sites were expected to be affected similarly by this transplant from a fluctuating to a stable light regime.

2.2. RNA, DNA, and Protein Extractions. RNA, DNA, and protein were extracted in sequential fashion from the 18 Houwan samples stored in either TRIzol or RNALater (either uncrushed and stored at 4°C or homogenized and stored at −20°C). For the six samples already stored in 500 μL of TRIzol, an additional 1 mL of TRIzol was added to the samples, and the tissues were homogenized in the 1.5 mL of TRIzol with a mortar and pestle. The TRIzol-tissue mixture (1 mL) was then transferred to a new 1.5-mL microcentrifuge tube. For the 12 samples stored in RNALater, the tubes were pulse spun for 1 s to bring the coral skeletons to the bottom of the tubes, and the RNALater was decanted. The six samples that had not been previously homogenized were ground into a powder in 1.5 mL of TRIzol with a mortar and pestle, and 1 mL of TRIzol-tissue slurry homogenate was transferred to a new 1.5-mL microcentrifuge tube. The previously homogenized samples ($n = 6$) were vigorously vortexed with 1 mL of TRIzol for several seconds after having decanted the RNALater. For all 18 samples, tissues in TRIzol were pulse spun for 1 s to sediment the residual skeleton, and approximately 1 mL of TRIzol-tissue mixture was transferred to a new 1.5-mL microcentrifuge tube.

RNA and DNA were then extracted as in [13] from all the 18 samples. RNAs were precipitated with a high salt solution (250 μL) and 250 μL of isopropanol at room temperature for 10 min as in [21], and precipitated pellets were further purified with a commercial spin column kit (Plant RNA Miniprep kit, Hopegen Biotechnology, Inc., Taipei, Taiwan) as in [13]. DNAs were separated from the protein phase as in [22], and the precipitated DNAs were further purified with the AxyPrep PCR clean-up kit (Axygen Biosciences, Union City, CA, USA) as directed by the manufacturer. RNA and DNA quantity and quality were assessed as described in [23] after eluting into DEPC-treated water (30 μL) and manufacturer's eluent (30 μL), respectively. Proteins were extracted from the organic phase of the samples fixed in TRIzol and RNALater (4°C only) as described in [3], and their quantity and quality were assessed as described therein.

2.3. Statistical Analyses. All statistics were calculated with JMP (ver. 5.0, SAS Institute Inc., Cary, NC, USA). For *in situ* physiological parameter comparisons between the two sites, Student's *t*-tests were used when the data were normally distributed and of homogenous variance (determined by Shapiro-Wilk *W* tests and Levine's tests, resp.). When log or square root transformations did not generate datasets suitable for parametric analyses, either the Mann-Whitney median test or the Wilcoxon rank-sum test was used instead. To compare the effects of sampling time (*in situ* versus *ex situ*) across the two temperature regimes (stable versus variable) and two sites (Houbihu versus Houwan), a two-way, repeated-measures ANOVA was used to test for the effects of site, temperature, their interaction, tank nested within temperature, site × time, temperature × time, and time. When the tank term was not statistically significant ($P > 0.05$), it was dropped from the model. One-way ANOVAs were used to assess the impact of fixation/homogenization strategy on [RNA], [DNA], and [protein], as well as the respective 260/280 and 260/230 ratios for both RNA and DNA. Tukey's honestly significant difference (HSD) tests were used to determine individual mean differences when the model detected an overall treatment effect ($P < 0.05$). In all instances, error terms presented later and in figures represent standard error of the mean (SEM).

Expression of four *Symbiodinium* genes (Table 1) was measured previously in the same samples from which the physiological data were obtained after seven days of exposure to either the stable or variable temperature regime [4]. In order to determine whether expression of any of these genes correlated significantly with any of the four physiological response variables or amongst each other, analysis of covariance (ANCOVA) was conducted after identifying the four most significant correlations with JMP's multivariate correlation analysis program. After plotting the global trend lines across all data points, those of the individual temperature regimes within each of the two sites were plotted and assessed against each other, and slopes were considered to differ significantly at $P < 0.05$. It was hypothesized that *Symbiodinium* density and chl-*a* content might demonstrate a negative relationship due to the fact that corals with higher densities of *Symbiodinium* would potentially maintain lower chl-*a* content in response to self-shading by other *Symbiodinium*. Regarding the comparisons between physiological and molecular parameters, it was hypothesized that expression of the *Symbiodinium* photosynthesis genes may display strong degrees of correlation with chl-*a* content and F_V/F_M. Furthermore, high expression of photosynthesis genes might be associated with an increase in oxygen production *in vivo*, which would potentially necessitate the translation of higher concentrations of reactive oxygen species (ROS)-detoxifying proteins, such as APX1. Expression of the photosynthesis genes—*psI*, *rbcL*, and *pgpase*—was therefore hypothesized to correlate positively with *apx1* gene expression.

3. Results

3.1. Physiological Parameters. Symbiodinium density *in situ* (Figure 2(a)) did not differ significantly between the two sites (Wilcoxon rank-sum test, $Z = -0.52$, $P = 0.60$) and was approximately $3.1 \pm 0.20 \times 10^6$ and $3.0 \pm 0.070 \times 10^6$ cells cm^{-2} for the corals from Houbihu and Houwan, respectively ($n = 6$). There was no temporal change in *Symbiodinium* densities between the values measured *in situ* (Figures 2(b) and 2(c)) and those documented *ex situ* (Table 2).

Areal chl-*a* concentration (Figure 2(d)) increased from ~3 to ~6 μg cm^{-2} between the *in situ* and *ex situ* sampling times (Table 2), and this increase was statistically significant for corals of both Houbihu (Figure 2(e)) and Houwan (Figure 2(f)). When normalized per cell (Figure 2(g)), chl-*a* concentration also increased significantly (~2-fold; Table 2) from the *in situ* sampling time (Figure 2(g)) to the *ex situ* sampling time in *Symbiodinium* within corals of both Houbihu (Figure 2(h)) and Houwan (Figure 2(i)). A significant ~1% increase in F_V/F_M (Figure 2(j)) was observed between

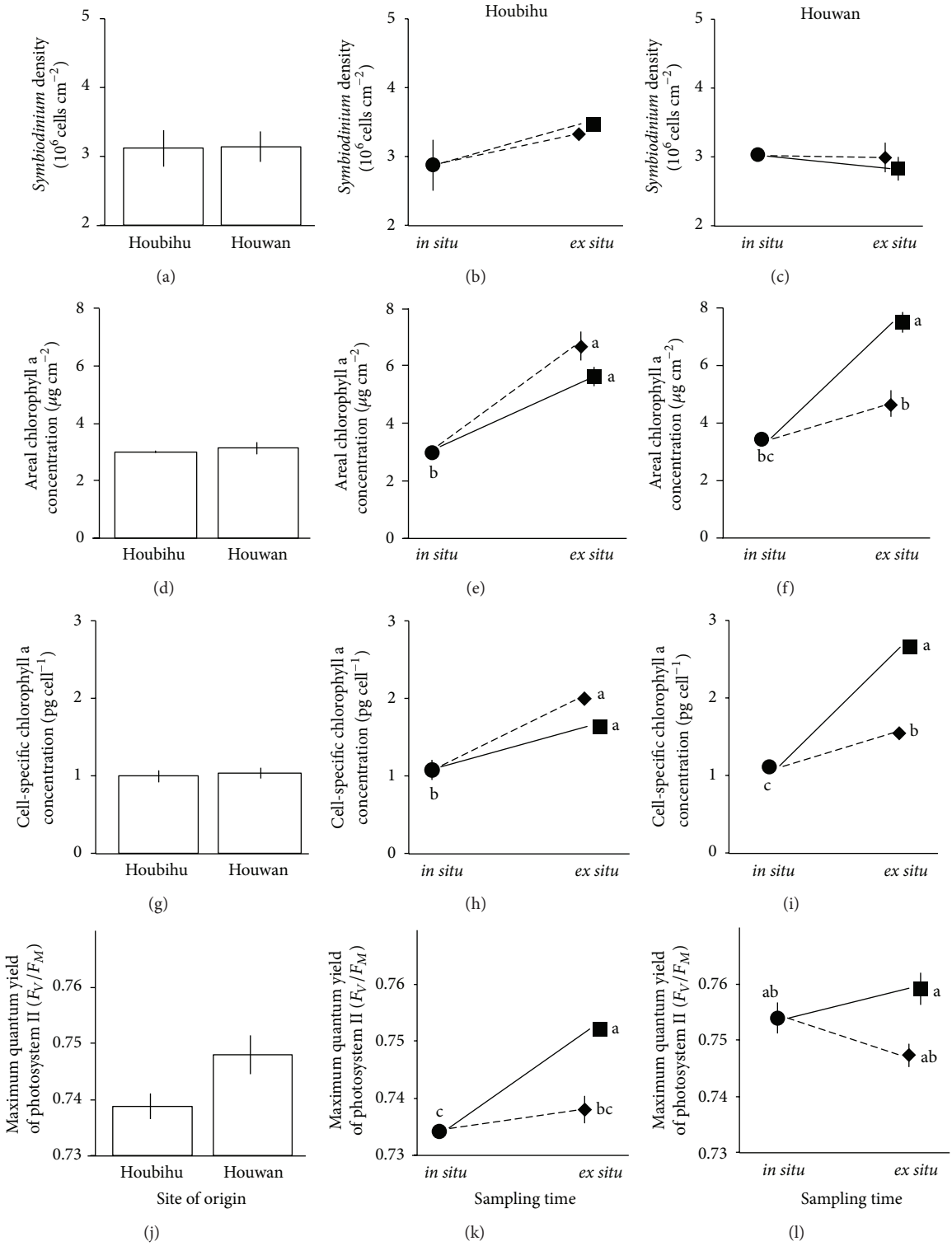


Figure 2: *In situ* versus *ex situ* physiology. Biopsies were removed from each of six *Seriatopora hystrix* colonies collected from the upwelling (Houbihu) and nonupwelling (Houwan) sites and analyzed for *Symbiodinium* density ((a)–(c)), areal chlorophyll a (chl-*a*) concentration ((d)–(f)), cell-specific chl-*a* concentration ((g)–(i)), and maximum dark-adapted quantum yield of photosystem II (F_V/F_M; (j)–(l)) as described in the text. Comparisons between sites of origin *in situ* are shown in (a), (d), (g), and (j), while comparisons over time (*in situ* versus *ex situ*) are shown across both temperature treatments (stable (black circles connected to black squares with straight lines) and variable (black circles connected to black diamonds by dotted lines)) for Houbihu ((b), (e), (h), and (k)) and Houwan ((c), (f), (i), and (l)). In all panels, error bars represent standard error of the mean. Tukey's honestly significant difference groups in (e), (f), (h), (i), (k), and (l) represent significant differences ($P < 0.05$) in response to the interaction of sampling time and temperature treatment.

Table 2: Two-way, repeated-measures ANOVA of physiological parameter data. Four physiological response variables were measured both *in situ* and *ex situ* (after three weeks of acclimation and one week of experimentation) in corals from either Houbihu (HBH) or Houwan (HWN). "Stab": stable temperature treatment (TT). "Var": variable TT. Letters trailing treatment groups in the "Post hoc tests" column represent Tukey's honestly significant difference groupings ($P < 0.05$). Site of origin (SO) comparisons were conducted across all data, regardless of TT and sampling time and so do not represent the *in situ* SO differences, whose values were compared with Student's *t*-tests or Wilcoxon rank-sum tests and are discussed in the text and displayed in Figures 2(a), 2(d), 2(g), and 2(j). "Time" corresponds to the *ex situ* versus *in situ* comparison, as there was a four-week gap between the two sampling times. Time × SO × TT test statistics are not shown.

Source of variation	Exact F	P	Post hoc tests
	Symbiodinium density (cells cm^{-2})[a]		
SO	2.047	0.190	
TT	0.0940	0.767	
SO × TT	0.0104	0.921	
Time	0.000	1.000	
Time × SO	3.31	0.1064	
Time × TT	0.422	0.534	
All within	1.52	0.282	
	Areal chlorophyll a concentration ($\mu g\ cm^{-2}$)		
SO	0.0353	0.856	
TT	8.96	**0.0172**	stab > var
SO × TT	34.0	<**0.001**	HWN stab (A), HBH var (AB), HBH stab (BC), HWN var (C)
Time	113	<**0.001**	*ex situ* > *in situ*
Time × SO	0.114	0.744	
Time × TT	1.28	0.290	
All within	3.48	0.0704	
	Cell-specific chlorophyll a concentration (pg $cell^{-1}$)		
SO	7.56	**0.0251**	HWN > HBH
TT	9.30	**0.0158**	stab > var
SO × TT	54.9	<**0.001**	HWN stab (A), HBH var (B), HBH stab (C), HWN var (C)
Time	211	<**0.001**	*ex situ* > *in situ*
Time × SO	3.61	0.0940	
Time × TT	8.46	**0.0196**	stab *ex situ* (A), var *ex situ* (A), var *in situ* (B), stab *in situ* (B)
All within	10.53	**0.0038**	
	Maximum (dark-adapted) quantum yield of photosystem II (F_V/F_M)		
SO	19.1	**0.0024**	HWN > HBH
TT	34.7	<**0.001**	stab > var
SO × TT	0.0078	0.932	
Time	12.0	**0.0084**	*ex situ* > *in situ*
Time × SO	0.0844	0.779	
Time × TT	0.6079	0.458	
All within	0.423	0.742	

[a]Rank-transformed data.

the *in situ* and *ex situ* sampling times (Table 2), and this effect of time was similar for *Symbiodinium* within corals from Houbihu (Figure 2(k)) and Houwan (Figure 2(l)). There were no significant interaction effects of site and sampling time (Table 2) on areal chl-*a* concentration, cell-specific chl-*a* concentration, or F_V/F_M, indicating that the fragmentation, transplantation, and husbandry processes did not affect corals from one site more than the other.

3.2. Fixation Strategy Comparisons. There was a statistically significant effect of tissue storage/homogenization strategy on [RNA] (Figure 3(a); one-way ANOVA, $F = 4.1$, $P = 0.038$). Although there were no post hoc differences, it does appear that the difference detected by the model is due to the higher [RNA] generated by samples fixed in TRIzol (mean = 130 ± 65 ng μL^{-1}). When looking at the quality of the RNA with respect to protein contamination (i.e., the 260/280 ratio;

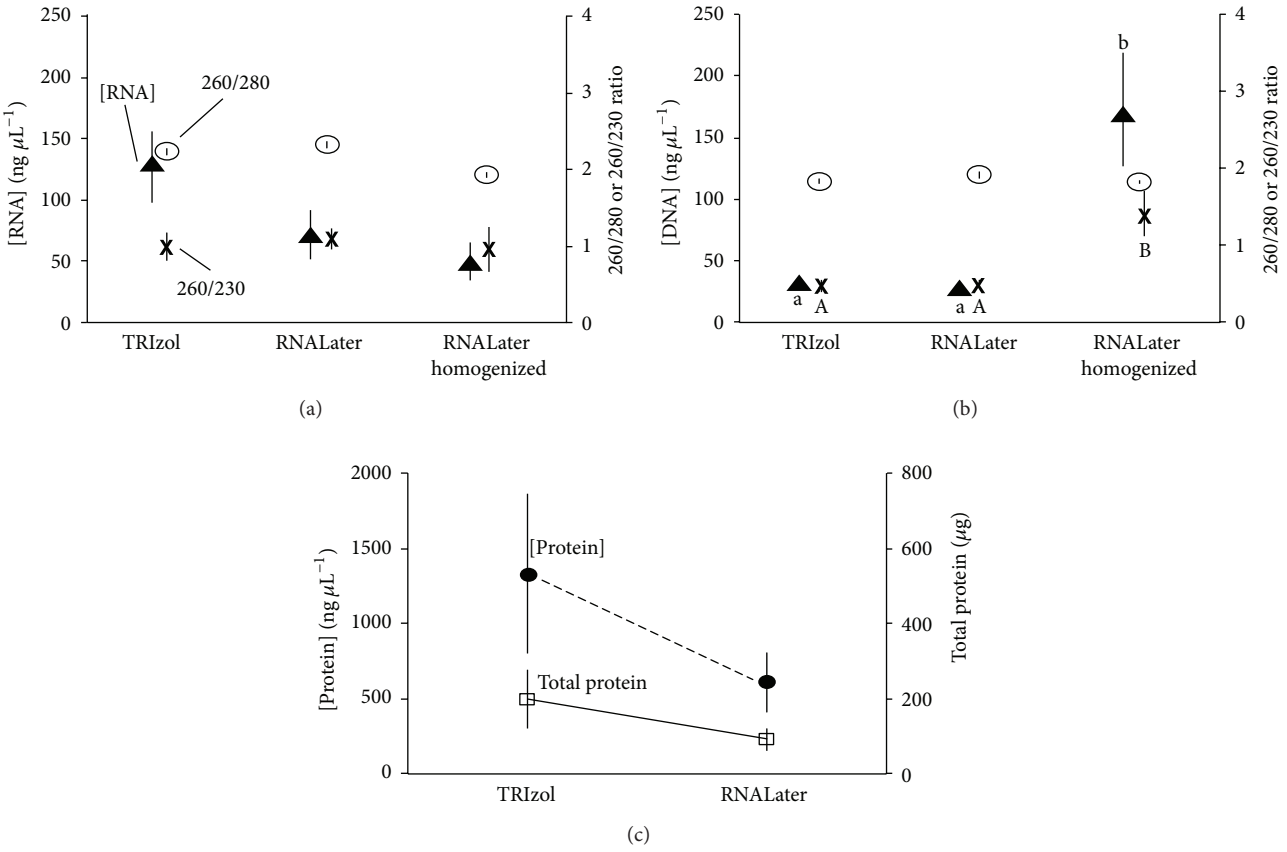


Figure 3: TRIzol versus RNALater. Six *Seriatopora hystrix* samples were collected from Houwan, and three biopsies (~50 mg) were removed from each and immersed in either TRIzol or RNALater as described in the text. Half of the samples stored in RNALater were homogenized and stored at −20°C, while the other half of the samples were simply kept at 4°C. RNA, DNA, and, in certain cases, protein were extracted from the six biopsies of each fixation strategy treatment. RNA concentration ([RNA]; black triangles and left y-axis) and 260/280 (hollow circles and right y-axis) and 260/230 (exes and right y-axis) ratios were compared between the three fixation strategies (a). Likewise, the DNA concentration ([DNA]; black triangles and left y-axis) and 260/280 (hollow circles and right y-axis) and 260/230 (exes and right y-axis) ratios were also compared (b). In panel b, the lower case letters adjacent to icons represent Tukey's honestly significant difference (HSD) groups ($P < 0.05$) for the [DNA] data, while upper case letters represent HSD groups for the 260/230 ratio data. Both protein concentration ([protein]; black circles connected by a dotted line and left y-axis) and total protein (hollow squares connected by a solid line and right y-axis) were compared between only two fixation strategies, immersion in either TRIzol or RNALater (without homogenization) (c). In all panels, error bars represent standard error of the mean.

Figure 3(a)), there was also a statistically significant effect of fixation strategy (Wilcoxon rank-sum test, $Z = 8.2$, $P = 0.017$), and this appears to be driven by a significantly lower ratio in samples homogenized in RNALater (mean = 1.9 ± 0.14); however, there were no post hoc differences. The 260/230 ratio (Figure 3(a)), which reflects the degree of contamination of the purified RNAs with phenol or alcohol, did not vary across the three fixation strategies (one-way ANOVA, $F = 0.19$, $P = 0.83$).

There was also a statistically significant effect of fixation strategy on [DNA] (Figure 3(b); Wilcoxon rank-sum test, $Z = 9.7$, $P = 0.0078$) due to the approximately 5-fold higher [DNA] (mean = 170 ± 31 ng μL^{-1}) emerging from samples homogenized in RNALater relative to those fixed in TRIzol and RNALater (without homogenization). The 260/280 ratios (Figure 3(b)) were similar between the three fixation strategies (one-way ANOVA, $F = 1.7$, $P = 0.22$) and averaged 1.9 ± 0.023 across all 18 samples. The 260/230 ratio was significantly different across the three fixation techniques (Wilcoxon rank-sum test, $Z = 9.7$, $P = 0.0079$) due to this parameter being significantly higher in samples homogenized in RNALater (mean = 1.4 ± 0.202). This signifies that the DNAs from samples homogenized in RNALater had relatively less organic solvent contamination. Whether this increase in purity can be attributed more to the homogenization step itself, or merely the difference in storage temperatures (4 versus 20°C), remains to be determined.

Despite notably higher protein concentrations (Figure 3(c)) from samples fixed in TRIzol (mean = 1300 ± 380 ng μL^{-1}) relative to those fixed in RNALater (non-homogenized, mean = 610 ± 320 ng μL^{-1}), this difference was not statistically significant (Mann-Whitney median test, $Z = 1.7$, $P = 0.093$). Given that the total protein values (Figure 3(c)) were derived directly from the protein

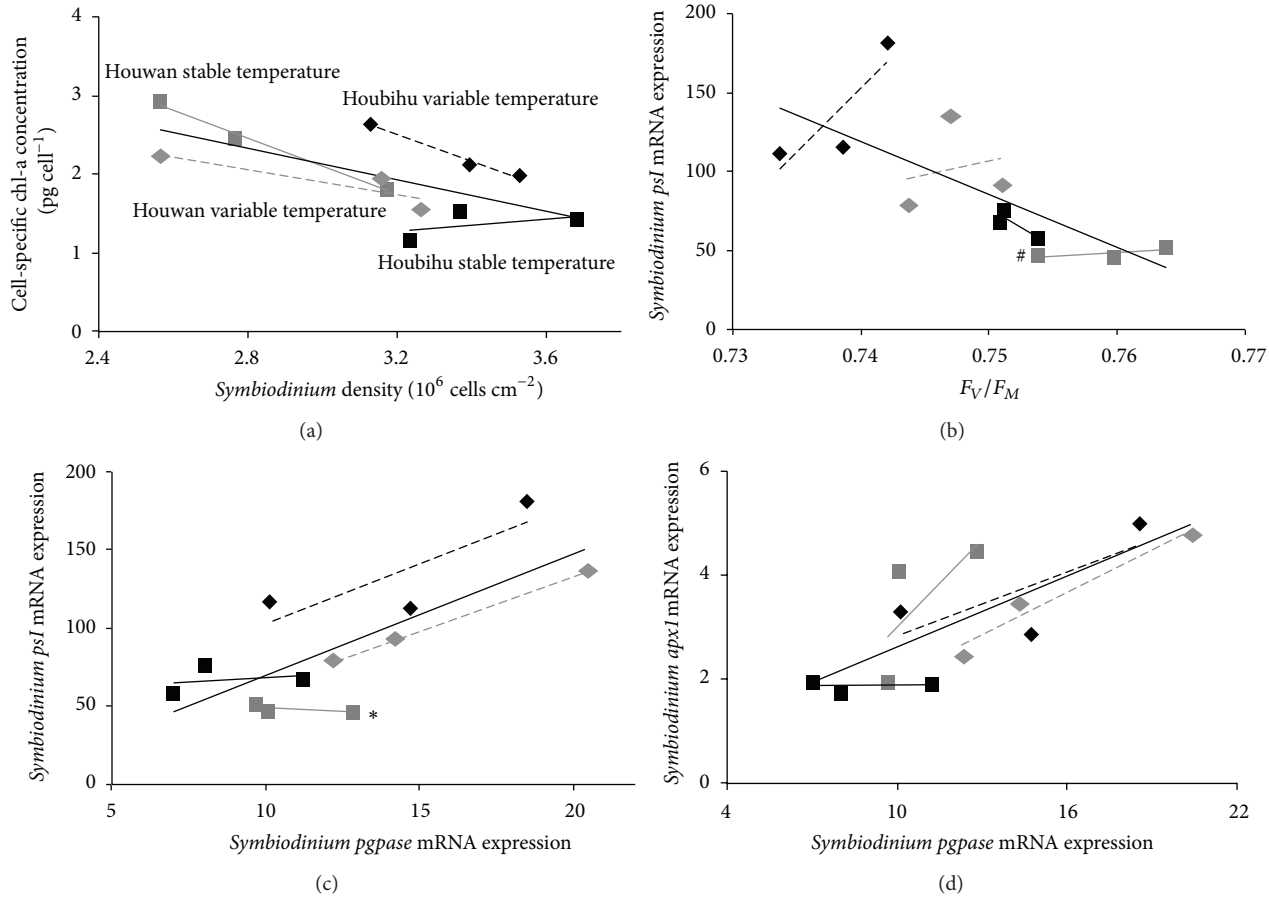


Figure 4: Correlations. Multivariate correlation analyses were run on both the physiological and molecular data, and the four strongest associations were selected for plotting. There were statistically significant, negative correlations between cell-specific chlorophyll a (chl-*a*) concentration and *Symbiodinium* density (a), as well as between *Symbiodinium* photosystem I (*psI*, subunit III) mRNA expression and the maximum dark-adapted quantum yield of photosystem II (F_V/F_M; (b)). There were statistically significant, globally positive correlations between *Symbiodinium psI* and phosphoglycolate phosphatase (*pgpase*) gene expression (c), as well as between *Symbiodinium* ascorbate peroxidase (*apx1*) and *pgpase* gene expression (d). In all cases, correlations were assessed across the dataset as a whole, and the global correlations are represented by solid black lines that span the entire panel. Individual correlations were then assessed within each site of origin and temperature treatment, and the corals from Houbihu exposed to a variable and stable temperature regime are denoted by black diamonds and squares, respectively. The Houbihu variable and stable temperature regime trend lines are dotted and solid black, respectively. Similarly, the corals from Houwan exposed to a variable and stable temperature regime are denoted by grey diamonds and squares, respectively, and are associated with grey dotted and grey solid trend lines, respectively. When a statistically significant difference between slopes of two temperature treatments within a site was documented with ANCOVA ($P < 0.05$), an asterisk (∗) was placed next to one of the two trend lines. When a statistically significant difference between slopes of two sites within a temperature treatment was documented with ANCOVA ($P < 0.05$), a "#" was placed next to one of the two trend lines.

concentrations, the statistical test results were identical. This signifies that total protein yields were similar between the two fixation strategies.

3.3. Correlations. The four most statistically significant correlations within the *ex situ* dataset were found to be between cell-specific chl-*a* concentration and *Symbiodinium* density (Figure 4(a)), *Symbiodinium psI* mRNA expression and F_V/F_M (Figure 4(b)), *psI* and *pgpase* mRNA expression (Figure 4(c)), and *apx1* and *pgpase* mRNA expression (Figure 4(d)). Regarding the former, there was a statistically significant (linear regression *t*-test, $t = -2.9$, $P = 0.017$), negative correlation ($r^2 = 0.45$) between these two parameters. There was also a statistically significant ($r^2 = 0.49$, linear regression *t*-test, $t = -3.1$, $P = 0.011$), negative association between *psI* mRNA expression and F_V/F_M (Figure 4(b)). ANCOVA revealed a significant difference between the two field sites within the stable temperature treatment ($F = 20$, $P = 0.021$) for the latter association. There was a slight, positive relationship between these two parameters in Houwan samples exposed to a stable temperature regime, whereas there was a slight negative correlation between them in Houbihu samples exposed to a stable temperature regime.

A statistically significant ($r^2 = 0.56$, linear regression *t*-test, $t = 3.6$, $P = 0.0049$), positive correlation was observed between *Symbiodinium pgpase* and *psI* mRNA expression (Figure 4(c)). For the samples from Houwan exposed to a stable temperature regime, the slope was significantly lower

than that of all other interaction groups, and, specifically, was significantly lower than the slope of the regression line corresponding to data from coral samples from Houwan exposed to a variable temperature regime (ANCOVA, $F = 57$, $P = 0.017$). Finally, there was a statistically significant ($r^2 = 0.58$, linear regression t-test, $t = 3.7$, $P = 0.0042$), positive association between *Symbiodinium pgpase* and *apx1* gene expression (Figure 4(d)).

4. Discussion

Previous researchers have rarely ensured that their experimental corals behaved in a similar fashion *ex situ* as *in situ*. In fact, it is likely that both the transportation and fragmentation processes are stressful to corals, and, for that reason, a three-week recovery period was utilized herein in order to provide specimens with sufficient time to heal and acclimate to the laboratory environment. Tissues of all nubbins overgrew the fishing lines used for suspension prior to the initiation of the experiment and some parameters, such as the *Symbiodinium* density, did not change between the time of collection (i.e., *in situ*) and the termination of the experiment (i.e., *ex situ*). However, chl-*a* cell^{-1} approximately doubled over the four weeks of husbandry, and the light regime utilized in the experimental aquaria may have accounted for this difference.

The use of subsaturating PAR levels (e.g., 80–100 μmol m^{-2} s^{-1}) has become a hallmark of manipulative experiments in the coral biology field (e.g., [24]), possibly because older studies (e.g., [25]) found that corals bleached in captivity when *in situ* PAR levels were employed. The ~90 μmol m^{-2} s^{-1} PAR level employed herein was chosen because this is the approximate level this coral experiences *in situ* in the reefs of southern Taiwan from which the experimental colonies were collected [4]. That being said, *S. hystrix* populations in southern Taiwan are routinely exposed to saturating PAR levels (e.g., ~400 μmol m^{-2} s^{-1}) for at least several hours each day (Mayfield, unpublished findings), and these higher PAR levels may partially determine the standing chl-*a* concentrations within the *Symbiodinium* cells. The reason for this is because *Symbiodinium*, as well as all other photosynthetic organisms, must establish chl-*a* levels that are associated with a degree of electron harvesting which results in a balance between high levels of carbon fixation and an acceptable amount of ROS production [26]. While the same average hourly PAR was experienced by the corals *ex situ* as *in situ*, the lower diel maximum PAR experienced by the corals *ex situ* may have contributed to the ability for their *Symbiodinium* populations to accumulate high cellular chl-*a* levels without a contingent increase in risk for ROS production. Although low expression levels of the *Symbiodinium* oxidative stress marker *apx1* were documented in these corals, direct measurements of ROS levels are needed to confirm this relationship between chl-*a* levels and ROS production in *Symbiodinium*.

Despite the documented *ex situ* increase in both areal chl-*a*, as well as chl-*a* cell^{-1}, relative to *in situ* values, corals from Houbihu and Houwan responded similarly in terms of this increase in *Symbiodinium* chl-*a* content. Furthermore, dark-adapted F_V/F_M values measured after three weeks of acclimation and one week of experimentation were similar to those measured on the evening before field sampling. This suggests that, although chl-*a* cell^{-1} doubled, the efficiency of the photosystem II complexes of the *Symbiodinium* populations was similar to field levels. As a final comment on chl-*a* effects, *Symbiodinium* populations tended to have higher chl-*a* concentrations when they were present at lower densities (Figure 4(a)). This tendency may have allowed corals with lower *Symbiodinium* densities to photosynthesize at a similar level as those with higher densities and could be related to a decrease in self-shading of *Symbiodinium* in these coral tissues.

Our small-scale correlation analysis identified other sets of parameters whose degree of correlation did not conform to our expectations. This is evident in the negative association between *Symbiodinium psI* gene expression and F_V/F_M (Figure 4(b)). Whereas *Symbiodinium* with more photosystem mRNA transcripts present in their chloroplasts would appear to have a higher capacity for photosystem protein expression, and thereby electron capture, such does not appear to be the case in the *Symbiodinium* populations within the corals sampled herein. This could be because the respective protein is regulated posttranscriptionally. Putnam et al. [13] found a negative relationship between *rbcL* gene and RBCL protein expression, and so it is possible that a negative relationship between *psI* gene and PSI protein expression also exists. The extent to which *Symbiodinium* proteins involved in photosynthesis are regulated posttranscriptionally would therefore represent a promising question for future study.

In corals exposed to the variable temperature treatment only, a positive relationship was observed between expression of two *Symbiodinium* photosynthesis genes, *pgpase* and *psI*. Corals exposed to a variable temperature regime could be hypothesized to necessitate a more concerted regulation of photosynthesis gene expression in order to quickly modify expression levels of the respective proteins in response to rapid and dramatic changes in temperature. This hypothesis is based on the idea that, all else being equal, the rate of translation of a protein will be higher if the intracellular concentration of its respective mRNA is also higher. If *Symbiodinium* populations express high levels of both genes, then they may be able to more quickly adjust expression of the respective proteins during the acclimation response to abrupt temperature changes, such as those associated with upwelling events. This may explain why *Symbiodinium* populations in coral samples of the variable temperature treatment that expressed high levels of *pgpase* were also likely to express high levels of *psI*. This hypothesis could also account for the significant correlation between expression of *Symbiodinium pgpase* and *apx1* in samples from Houbihu exposed to a variable temperature regime, though this correlation might simply be due to the increase in ROS generated at times at which photosynthesis is occurring at high levels [4, 14]. Future efforts should seek to verify whether changes in expression of these photosynthesis genes, as well as their respective proteins, actually drive increases in carbon fixation in order

to validate their capacity to serve as molecular biomarkers of the photosynthetic performance of *Symbiodinium*.

5. Conclusions

As a potential acclimation response to a light regime that differed from the *in situ* condition, the *Symbiodinium* populations within the *S. hystrix* specimens studied herein were found to undergo a doubling of their chl-*a* concentration after four weeks of husbandry. Although the results of a previously published work [4] are not discredited by these results given that these concentration changes were similar in corals of both study sites, we nevertheless recommend that researchers track the recovery of corals transported to the laboratory on a more finely tuned timescale, such as within several hours of their fragmentation and consequent incubation in laboratory aquaria, in order to demonstrate that the experimental samples have recovered and acclimated to their *ex situ* environment. A conservative approach was taken herein by utilizing a lengthy acclimation time. However, too extensive an acclimation period could begin to influence the physiology of corals in a manner that causes them to perform differently *ex situ* compared to *in situ*. Although corals herein appeared to be physiologically competent after four weeks of husbandry, the fact that their chl-*a* content doubled demonstrates the sensitivity of *S. hystrix* to changes in its environment. Indeed, this coral is amongst the most environmentally sensitive species studied to date [27] and may require a longer acclimation time than more robust species, such as massive poritids.

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