

Article

Environmentally-Driven Variation in the Physiology of a New Caledonian Reef Coral

Anderson B. Mayfield ^{1,2,3,*}  and Alexandra C. Dempsey ⁴¹ Cooperative Institute for Marine and Atmospheric Studies, University of Miami, Miami, FL 33149, USA² Atlantic Oceanographic and Meteorological Laboratory, National Oceanic and Atmospheric Administration, Miami, FL 33149, USA³ Coral Reef Diagnostics, Miami, FL 33129, USA⁴ Khaled bin Sultan Living Oceans Foundation, Annapolis, MD 21403, USA; dempsey@livingoceansfoundation.org* Correspondence: andersonblairmayfield@gmail.com; Tel.: +1-(337)-501-1976

Abstract: Given the widespread threats to coral reefs, scientists have lost the opportunity to understand the basic biology of “pristine” corals whose physiologies have not been markedly perturbed by human activity. For instance, high temperature-induced bleaching has been occurring annually since 2014 in New Caledonia. Because most corals cannot withstand repeated years when bleaching occurs, an analysis was undertaken to showcase coral behavior in a period just before the onset of “annual severe bleaching” (ASB; November 2013) such that future generations might know how these corals functioned in their last bleaching-free year. *Pocillopora damicornis* colonies were sampled across a variety of environmental gradients, and a subset was sampled during both day and night to understand how their molecular biology changes upon cessation of dinoflagellate photosynthesis. Of the 13 environmental parameters tested, sampling time (i.e., light) most significantly affected coral molecular physiology, and expression levels of a number of both host and Symbiodiniaceae genes demonstrated significant diel variation; endosymbiont mRNA expression was more temporally variable than that of their anthozoan hosts. Furthermore, expression of all stress-targeted genes in both eukaryotic compartments of the holobiont was high, even in isolated, uninhabited, federally protected atolls of the country’s far northwest. Whether this degree of sub-cellular stress reflects cumulative climate change impacts or, instead, a stress-hardened phenotype, will be unveiled through assessing the fates of these corals in the wake of increasingly frequent marine heatwaves.

Keywords: cnidarians; coral reefs; dinoflagellates; gene expression; global climate change; invertebrate physiology; molecular biology; New Caledonia; photosynthesis; South Pacific



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1. Introduction

Climate change threatens reefs across the globe [1,2], from locales once thought to be “pristine” (e.g., Chagos [3]) to those abutting high human population density areas such as Taiwan [4], Mexico’s Pacific Coast [5], and South Florida (USA) [6]. Unfortunately, many of Earth’s (potentially) most precious, high-biodiversity coral reefs are unknown to science (and perhaps only to local fisherfolk); see Darling et al. [7] for a thorough (albeit incomplete) depiction of areas from which coral reef data have been collected as of 2018. This lack of data unfortunately means that we may not even know or understand what will be lost in the coming decades as ocean temperatures continue to rise [8]. To this end, the Khaled bin Sultan Living Oceans Foundation (LOF) began its “Global Reef Expedition” (GRE) in 2010 with the intent of surveying, sampling, and, more generally, providing data for Earth’s most difficult-to-access, poorly studied, and presumably least-impacted reefs. Although we may never know what these coral reefs were like in their pre-industrial states, such research missions nevertheless permitted the generation of a dataset against which further change can be assessed, and the results from a number of

research missions (typically divided by country) can be found on the LOF website as field reports (<https://www.livingoceansfoundation.org/publications/field-reports/>, accessed on 1 December 2021), as well as in the peer-reviewed literature (e.g., [9]).

In many cases, LOF scientists had the opportunity to survey coral reefs never before studied, and this was the case in November 2013 when the LOF research ship *M.Y. Golden Shadow* visited reefs at the southern and uninhabited northern reaches of New Caledonia. For general information on the cruise (e.g., goals, itinerary, personnel, site meta-data, and maps), readers are referred to the field report: <https://www.livingoceansfoundation.org/publication/new-caledonia-field-report/>, accessed on 1 December 2021. Although reefs around New Caledonia's main island, Grand Terre, have featured in prior works [10], the northern reefs and atolls (NRE) have not, and, given their remote and governmentally protected nature, we found that the physiology of the model scleractinian coral *Pocillopora damicornis* [11] was distinct from conspecifics of relatively more impacted reefs of the south (namely around the nickel mine at Prony Bay, Grand Terre) [12]. In an attempt to develop a more rigorous understanding of the environmental drivers of variability [13–15] in the molecular physiology of this model coral species [16], we performed an additional analysis on previously published data [12] whereby we focused not only on spatial heterogeneity (among locations and across environmental gradients), but also intraspecific (inter-genotype) and even intra-individual (colony) variation within locations and over time. By relating intraspecific variability in 10 key response variables (RV) across 13 environmental parameters (EP), we also sought to outline a baseline of coral performance and physiological plasticity [17] prior to the occurrence of “annual severe bleaching” (ASB; [18]) in 2014 [19]. These baseline data will be critical for developing models capable of accurately forecasting coral resilience; failure to incorporate natural sources of physiological variation could instead lead to unsubstantiated attribution of behavioral shifts to climate change and other stressors (i.e., type I statistical errors). In the event that the local corals cannot adapt or acclimatize, this dataset may instead serve as the last record of how these animal–plant symbioses behaved in the period before their cellular biology was fundamentally and constitutively perturbed by anthropogenic impact.

2. Materials and Methods

2.1. Overview of Research Expedition and Analytical Goals

For static maps of the 79 reef sites surveyed across both the southern and northern regions of New Caledonia, please see Mayfield et al. [12]; detailed, interactive habitat maps can instead be found at <https://maps.lof.org/lof> (accessed on 1 December 2021) (see prior works, e.g., [20], for map generation details). *P. damicornis* colonies were sampled (~50-mg biopsies/colony) across numerous environmental gradients (Table 1), with a series of RV assessed in each (described in detail previously [12] but with pertinent information provided below, Table 1, and in the Supplementary Materials). Originally, 15 EP were hypothesized to influence coral physiology: the 13 listed in Table 1 plus sampling date and reef site. Since sample sizes on any given date or for any given reef were generally small, only those 13 EP listed in Tables 1 and 2 featured in model building. Please note that, although Symbiodiniaceae assemblage and colony color are both technically properties of the coral colonies themselves, they were considered EP herein given that they were hypothesized to influence the RV discussed below.

2.2. Night Coral Sampling

Prior work with *P. damicornis* revealed extensive temporal variation in gene expression [21], even in genes known to exhibit temporally stable expression in other eukaryotes (e.g., cytoskeleton genes, such as beta-actin and alpha-tubulin). This temporal variation has generally invalidated the use of housekeeping genes in real-time PCR-based target mRNA analyses of corals [22] and was actually a methodological impetus for this project. To document natural temporal variation in coral molecular biology in the relative absence of localized anthropogenic stressors, *P. damicornis* colonies (n = 4–9, dependent on local abundance) in the vicinity of one another (but with >10-m separation) at 10 ± 1 m depth

(std. dev. for this and all other error terms unless stated otherwise) were tagged with glow sticks at three southern and one NRE reefs (all physically protected/sheltered reefs due to the complexity of night diving operations in remote locations), sampled within one hour of solar noon (typically 11:30–13:00), and then re-sampled (ensuring that a branch >20 cm away from the initial lesion was sampled) 30–60 min after sunset (typically 18:00–19:00). Photosynthetically active radiation (PAR) was measured next to each colony at the time of sampling with a Diving-PAM fluorometer (Walz) calibrated against a LiCor 193SA “4-pi” sensor.

Across the entire, month-long mission, 104 unique pocilloporid colonies were sampled, 36 of which were re-sampled at night; this resulted in 140 coral biopsies. Each biopsy was frozen at $-150\text{ }^{\circ}\text{C}$ in a liquid nitrogen vapor shipper (MVE Chart) upon return to the small craft vessel, and RNAs, DNAs, and proteins were extracted from these coral biopsies as described previously [12] after export under a CITES permit to A.B.M. to Taiwan. Specifically, 30 colonies sampled during both day and night ($n = 60$ biopsies), as well as 33 additional biopsies sampled only during the day, underwent extraction ($n = 93$). Of these, four of the daytime biopsies yielded degraded RNA and were not analyzed, resulting in a final sample size of 30 night and 59 daytime samples (of which 52 represented matched-pair light–dark sampling of the same 26 colonies). Please see: (1) the online supplemental data file (OSDF) for meta-data associated with all sampled colonies/biopsies and (2) the Supplementary Materials for genotyping and data filtering details.

Table 1. Environmental parameters (EP) and coral response variables (RV) measured. Please note that the first two RV were not generally featured in the statistical models. PAR = photosynthetically active radiation.

EP (n = 13)	Number and Description of Categories/Bins
Region	2: south vs. north
Island/atoll	6: see Figures 1 and 2 of Mayfield et al. [12] ^a
Reef exposure	3: protected, intermediate, exposed
Reef zone	4: backreef, forereef, lagoon, channel ^b
Reef type	4: patch reef, fringing reef, barrier reef, atoll
Time	3: <10:00, 10:00–14:00, >18:00
Light (PAR)	2: >0 (light) vs. 0 (dark) $\mu\text{mol}/\text{photons m}^2/\text{s}$
Depth	6: 5–10, 10–15, 15–20, 20–25, 25–30, >30 m
Coral cover	3: 15–30, 30–45%, undetermined
Temperature	5: 23–24, 24–25, 25–26, >26 $^{\circ}\text{C}$, undetermined
Salinity	4: 34.9, 35.1, 35.2, 35.3
Colony color	5: normal, pale, very pale, green, purple
Symbiodiniaceae assemblage	2: <i>Cladocopium</i> only vs. <i>Symbiodinium</i> + <i>Cladocopium</i>
RV (n = 12)	Function/Notes
Maximum colony length (cm)	colony size
Planar surface area (cm ²)	colony size
RNA/DNA ratio	proxy for total gene expression level
Symbiodiniaceae genome copy proportion	proxy for endosymbiont density
Symbiodiniaceae <i>rbcL</i> mRNA expression	ribulose-1,5-bisphosphate carboxylase/oxygenase (photosynthesis)
Symbiodiniaceae <i>ubiq-lig</i> mRNA expression	ubiquitin ligase (stress response)
Symbiodiniaceae <i>hsp90</i> mRNA expression	heat shock protein 90 (stress response)
Symbiodiniaceae <i>zif11</i> mRNA expression	zinc-induced facilitator-like 1-like (unknown/osmoregulation)
Host coral <i>ca</i> mRNA expression	carbonic anhydrase (metabolism)
Host coral <i>lectin</i> mRNA expression	cell adhesion
Host coral <i>cu-zn-sod</i> mRNA expression	copper-zinc superoxide dismutase (stress response)
Host coral <i>gfp-like cp</i> mRNA expression	green fluorescent protein-like chromoprotein (light regulation)

^a No *P. damicornis* colonies were found at Prony Bay or Pelotas Atoll. ^b No *P. damicornis* colonies were found within sheltered bays (a 5th reef zone considered previously).

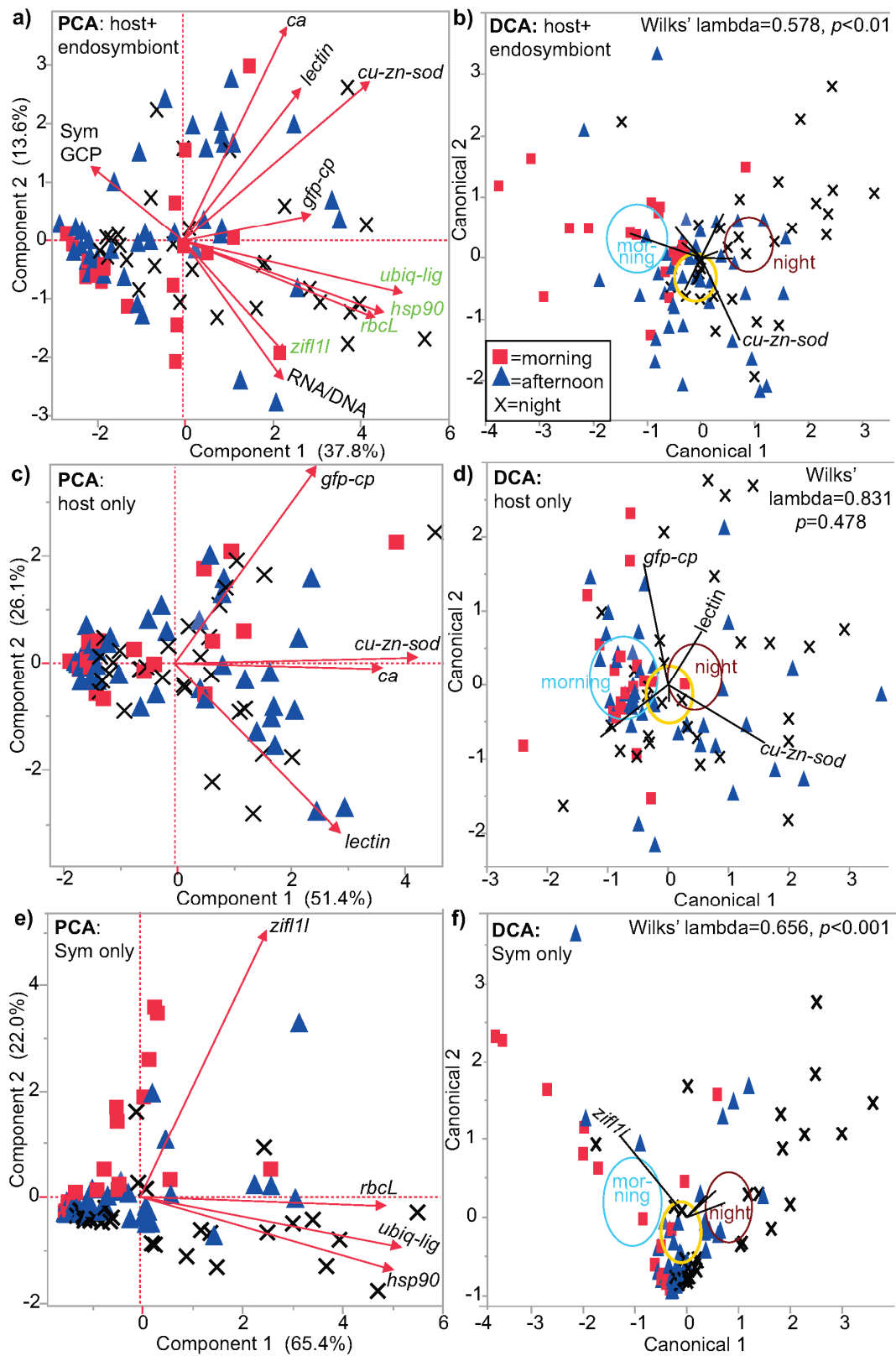


Figure 1. Multivariate effects of sampling time on coral physiology. Both PCA (on correlations; (a,c,e)) and DCA (on standardized data; (b,d,f)) were used to depict molecular-physiological data in two dimensions such that relationships among corals sampled at different times—morning (<10:00; red squares), afternoon (10:00–14:00; blue triangles), and night (18:00–20:00; black exes)—could be explored. All 10 response variables (both compartments; (a,b)), as well as the four host coral (c,d) and

four Symbiodiniaceae (Sym) genes ((e,f); highlighted in green in the holobiont plots (a,b)) were included in the PCA and DCA, respectively. The results of multivariate ANOVA (as Wilks' lambda), which is calculated as part of DCA, are shown in (b,d,f); these results were corroborated with the more conservative and robust PERMANOVA (Table 2). Not all vectors are labeled in the DCA plots due to spatial constraints, and the blue, yellow, and brown ellipses signify 95% confidence for the morning, afternoon, and night multivariate means, respectively. Multi-dimensional scaling of standardized data yielded a similar solution to the PCAs (data not shown).

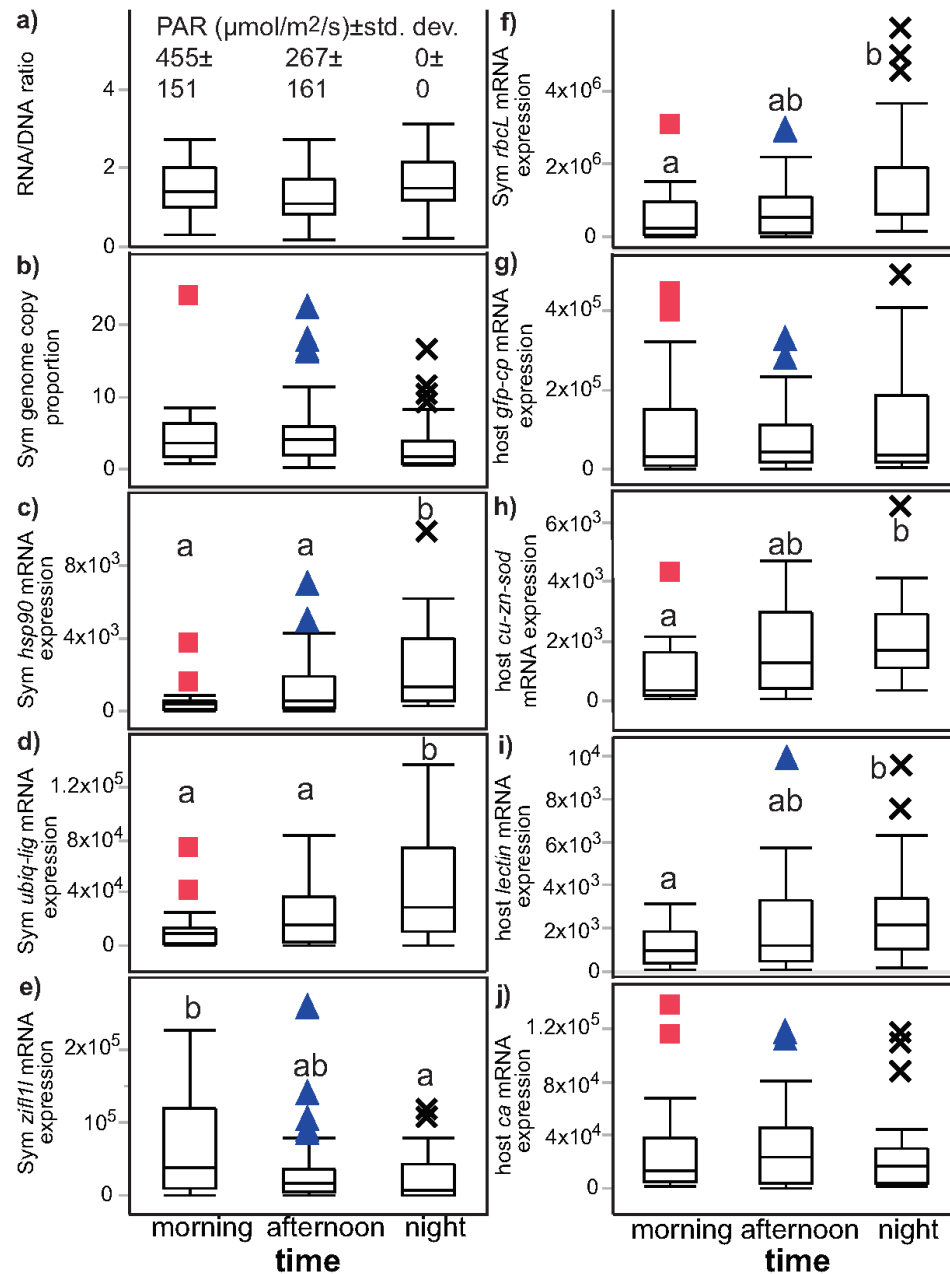


Figure 2. Sampling time effects on the 10 pocilloporid coral-Symbiodiniaceae response variables. When the Wilcoxon test determined a statistically significant effect of time ($df = 2$; squares, triangles, and exes for morning (before 10:00), afternoon (10:00–14:00), and night (after 18:00), respectively) for any of the 10 response variables (a–j), rank-based *post-hoc* tests (i.e., Dunn’s joint-ranking tests) were carried out among individual sampling time means ($p < 0.05$; as lowercase letters). Mean PAR levels for each sampling bin are included at the top of panel (a).

Table 2. Comparison of methods on the *Pocillopora damicornis* multivariate response (10 response variables). Permutational multivariate ANOVA (PERMANOVA; similarity (Euclidean distances) among standardized data) and partial least squares (PLS) were carried out with the exclusion of eight outliers and two samples collected in the late afternoon (n = 89). Environmental parameters with the same PERMANOVA *p*-values are ranked based on their “predictor score,” defined as: accuracy (1-PLS % misclassified) + PLS % variation explained. Unmeasured EP contributed ~10% of the residual variation. NS = not statistically significant ($p > 0.045$). Sym = Symbiodiniaceae.

Environmental Parameter	PERMA-NOVA	PLS-% Variation Explained	PLS-% Misclassified	Predictor Score
light	$p < 0.001$	7.0	26	81
time	$p < 0.01$	7.4	40	67
coral cover	$p < 0.01$	9.0	45	64
temperature	$p < 0.01$	11.6	48	64
colony color	$p < 0.01$	7.3	51	56
region	$p = 0.01$	4.0	24	80
reef type	$p < 0.045$	9.9	40	70
reef exposure	$p < 0.045$	4.4	36	68
island/atoll	$p < 0.045$	9.6	52	58
depth	$p < 0.045$	7.7	51	57
salinity	NS	2.8	33	70
Sym assemblage	NS	1.1	35	66
reef zone	NS	7.4	49	58

2.3. Response Variables (RV)

Ten RV were analyzed in each of 89 coral biopsies, including the RNA/DNA ratio (a proxy for total gene expression), the Symbiodiniaceae genome copy proportion (GCP; a molecular proxy for endosymbiont density [22]), and the expression of four host coral and four Symbiodiniaceae mRNAs. Based on prior transcriptomic analyses [23,24], these mRNAs were hypothesized to be environmentally sensitive and useful in physiology-based resilience model building. The eight mRNAs encode proteins involved in metabolism, photosynthesis, the stress response, and other key cellular processes (Table 1). It is worth noting here, though, that endosymbiosis-tailored protocols developed in our prior works [22,23] were adopted to ensure that spatio-temporally variable host/endosymbiont nucleic acid ratios did not bias gene expression analyses. After first normalizing gene expression (inverse-log raw threshold cycle [Ct] values) to recovery of an exogenous RNA spike (Solaris[®], Thermo-Fisher Scientific, Waltham, MA, USA), we then normalized the dinoflagellate spike-normalized gene expression values to the aforementioned Symbiodiniaceae GCP to control for the fact that samples with high endosymbiont densities would inherently demonstrate higher dinoflagellate gene expression levels. Raw, spike-normalized only, and spike- and GCP-normalized data for all eight target genes can be found in the OSDF.

2.4. Multivariate Data Analysis

A series of multivariate approaches were undertaken with JMP[®] Pro (ver. 16) to determine which EP drove the greatest proportion of the variation in the molecular biology of *P. damicornis* (assessed from the 10 RV) in New Caledonia. Briefly, principal components analysis (PCA), discriminant analysis (DCA), permutational multivariate ANOVA (PERMANOVA; instead undertaken with PRIMER (ver. 6)), stepwise regression (SRA), and partial least squares (PLS) were used to uncover relationships among coral samples across the 13 EP (Table 1), as well as to identify the RV(s) most impacted by environmental heterogeneity (multivariate alpha = 0.045). PCA was also used to depict relationships among RV, while DCA was used to quantify the differences evident from PCA in two ways: calculation of (1) Wilks' lambda (i.e., multivariate ANOVA (MANOVA)) and (2) a PLS model misclassification rate (% of samples misclassified by the associated predictive model). The former

was presented in the figures but not interpreted since, unlike PLS, MANOVA is poorly suited for highly collinear datasets featuring non-normally distributed data (Shapiro–Wilk $p < 0.01$) such as the one produced herein. In addition to PLS, PERMANOVA is also robust against non-normally distributed datasets featuring high degrees of multicollinearity since the similarities (i.e., distances) among samples, rather than absolute differences in levels of individual RVs, are instead tested in response to the EP(s) of interest. In a separate PLS, in which the first four coordinates from a multi-dimensional scaling (MDS) analysis of standardized data from the 10 coral RV were the model Y terms, each EP was tested as a predictor, and the percent variation in the multivariate trait space was calculated (NIPALS fit with k-fold validation of 7). SRA was only used to assess multivariate effects for light and time (see Supplementary Materials) and served mainly to corroborate findings from the non-parametric univariate analyses of the 10 RV (next section).

2.5. Univariate Data Analysis

Since the multivariate analyses pointed to an effect of sampling time and light on coral physiology (Table 2), several univariate statistical approaches were used to model light vs. dark differences in individual RV. First, rank-based Wilcoxon tests were utilized to determine the effect of PAR ($n = 2$ bins: 0 = night vs. >0 = day) with the entire, 89-sample dataset for each of the 10 RV. Similarly, Kruskal–Wallis tests were carried out across three sampling time windows: morning (before 10:00), midday (10:00–14:00), and night (18:00–19:00). In another two analyses, the differences (light minus dark, i.e., matched pairs) and relative differences (light/dark) between light and dark values were calculated for each RV for the 26 night dive samples only. The goals of these latter two analyses were to test the null hypotheses that the mean differences were significantly different from 0 and 1, respectively, and this was undertaken with non-parametric sign-rank tests ($\alpha = 0.02$ for all univariate tests). Log₂-transformed ratio data for each colony can be found in Figure S1.

3. Results

3.1. Multivariate Analysis

PERMANOVA revealed a number of EP that significantly affected similarity among coral colonies (Tables 2 and S3). Only salinity, reef zone, and the endosymbiont assemblage did *not* significantly influence multivariate similarity among sample groups. Despite highly significant PERMANOVA p -values, the PLS models were not characterized by particularly high predictive power, and misclassification rates ranged from ~25 to ~50% (Table 2); this is partially a testament to the high inter-colony variation associated with the dataset (see OSDF for raw values). Furthermore, no EP in isolation explained more than 15% of the variation in the multivariate coral trait space (Table 2).

Because sampling time (three bins) and light (two bins: PAR = 0 vs. >0 $\mu\text{mol}/\text{photons m}^2/\text{s}$) both significantly affected similarity among corals and were characterized by modestly low PLS misclassification rates (Table 2), a detailed look was taken at temporal variation in the multivariate trait space. First, PCA and DCA were performed with all 10 RV (Figures 1a and 1b, respectively), the host genes only (Figures 1c and 1d, respectively), and the four Symbiodiniaceae genes only (Figures 1e and 1f, respectively). When looking at the holobiont and host coral data with PCA (Figures 1a and 1c, respectively), the temporal effects are less obvious; only when the endosymbiont genes were assessed in isolation was there more noted separation between samples of the three sampling times (morning, afternoon, and night; Figure 1e). This observation was recapitulated by DCA; although the multivariate effect was not significant for the coral response only (Figure 1d; MANOVA $p > 0.40$), the endosymbiont DCA of time (Figure 1f) was highly significant.

3.2. Univariate Effects of Time and Light on Coral Response Variables

Given these multivariate temporal differences, temporal and light/dark effects on the individual RV were assessed, and the univariate data were analyzed in several different ways. First, the mean values of each of the 10 RV were compared over the three time

windows: morning, afternoon, and night (Figure 2 and Table 3). Wilcoxon tests were used to assess the temporal variation for each since the data were non-normally distributed, and six of the RV demonstrated significant temporal variation. Symbiodiniaceae *hsp90* (Figure 2c), *ubiq-lig* (Figure 2d), and *rbcL* (Figure 2f) mRNA expression were all significantly higher at night; *zifl11* expression (Figure 2e) instead peaked in the morning. Two host coral genes varied in expression over time, *cu-zn-sod* (Figure 2h) and *lectin* (Figure 2i), and both were instead higher at night relative to morning.

As a secondary means of assessing light vs. dark differences, the morning and afternoon data were pooled, and one-way, day ($n = 59$) vs. night ($n = 30$) comparisons on ranks were made with Wilcoxon tests (also known as Kruskal–Wallis tests). When analyzing data in this manner (Figure 3 and Table 3), seven of the ten RV varied significantly between day and night. These generally included those identified in Figure 2, with the exception of Symbiodiniaceae *zifl11* mRNA expression (Figure 3e), which was not found by this secondary approach to differ between light and dark sampling times. However, two RV that did *not* differ in Figure 2, RNA/DNA and endosymbiont density (GCP), were found to differ significantly between day and night (Figures 3a and 3b, respectively) using this pooled, less conservative statistical approach. Specifically, the RNA/DNA ratio was 50% higher at night, and the Symbiodiniaceae GCP was 50% higher in the day.

Table 3. Effects of light and time on coral response variables (RV). Rank-based tests were used for all RV due to lack of normality. When considering the entire dataset ($n = 59$ light vs. 30 dark), time was tested as three bins (“Time (df = 2):” morning, afternoon, or night), with light as two (“Light (df = 1):” PAR = 0 vs. $>0 \mu\text{mol}/\text{photons m}^2/\text{s}$). When analyzing only the 26 colonies that were sampled twice (approximately solar noon and 1-hr post-sunset), both non-parametric matched-pairs tests (i.e., signed-rank of “Light minus dark”), or one-sample signed-rank tests of the mean “Light/dark” ratio against a null hypothesis of 1 were instead undertaken. Sample sizes for the three southern and lone northern night dive sites were 9, 4, 7, and 6, respectively ($n = 26$ total). NS = not statistically significant ($p > 0.02$).

Effect type	Time (df = 2)	Light (df = 1)	Light minus dark	Light/dark
Sample size	89	89	26	26
Type of test	Wilcoxon test	Wilcoxon test	Matched pairs (signed-rank)	Signed-rank vs. 1
Figure	Figure 2	Figure 3	Not plotted	Figures 4 and S1
Response variable				
RNA/DNA	NS ($p = 0.055$)	night > day ($p = 0.012$) ^a	NS ($p = 0.853$)	NS ($p = 0.824$)
Sym GCP	NS ($p = 0.044$)	day > night ($p = 0.010$)	NS ($p = 0.261$)	NS ($p = 0.108$)
Sym <i>hsp90</i>	night > day ($p < 0.001$) ^a	night > day ($p < 0.001$) ^a	NS ($p = 0.683$)	NS ($p = 0.890$)
Sym <i>ubiq-lig</i>	night > day ($p = 0.002$)	night > day ($p = 0.001$)	NS ($p = 0.612$)	NS ($p = 0.351$)
Sym <i>zifl11</i>	day > night ($p = 0.019$) ^a	NS ($p = 0.222$) ^b	day > night ($p = 0.001$)	day > night ($p < 0.001$)
Sym <i>rbcL</i>	night > day ($p = 0.008$)	night > day ($p = 0.002$)	NS ($p = 0.971$)	NS ($p = 0.931$)
host <i>ca</i>	NS ($p = 0.724$)	NS ($p = 0.585$)	day > night ($p = 0.012$)	day > night ($p = 0.003$)
host <i>lectin</i>	night > day ($p = 0.018$)	night > day ($p = 0.014$)	NS ($p = 0.053$)	NS ($p = 0.831$)
host <i>cu-zn-sod</i>	night > day ($p = 0.006$)	night > day ($p = 0.006$)	NS ($p = 0.170$)	NS ($p = 0.107$)
host <i>gfp-cp</i>	NS ($p = 0.874$)	NS ($p = 0.863$)	day > night ($p = 0.007$)	day > night ($p = 0.009$)

^a supported by stepwise regression. ^b contradicted by stepwise regression.

As a more conservative approach for assessing temporal variation for each of the 10 coral RV, both the difference between light and dark (assessed by non-parametric matched-pairs tests; Table 3) and the light/dark ratio were compared for a subset of 26 corals that were sampled in both the day and night. In this analysis (Figure 4), only three RV demonstrated significant variation between day and night (i.e., light minus dark > 0 and light/dark > 1): Symbiodiniaceae *zifl11* mRNA expression (light > dark; Figure 4f) and host coral *gfp-cp* (light > dark; Figure 4h) and *ca* mRNA expression (light > dark; Figure 4k). These univariate data are summarized in Table 3.

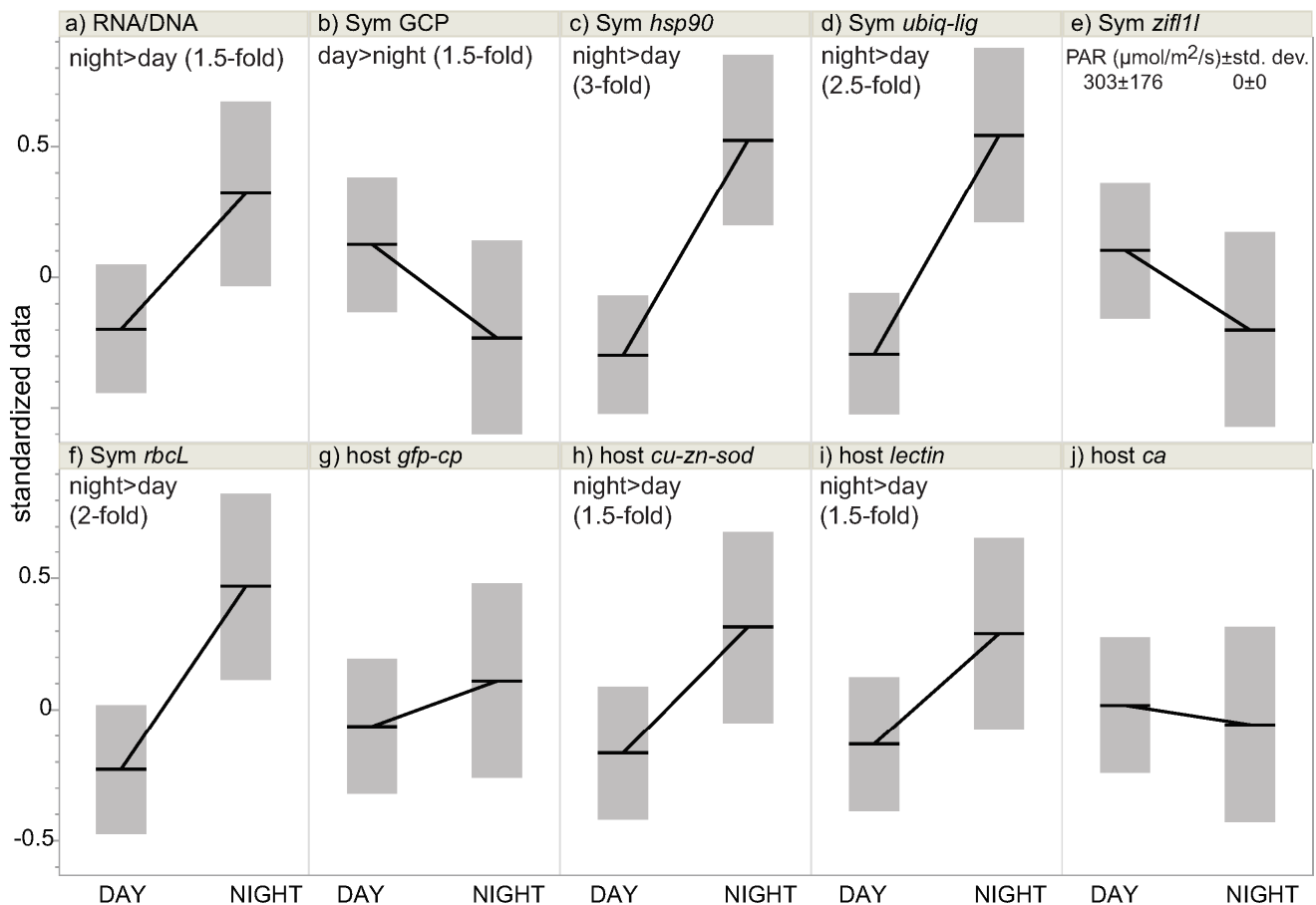


Figure 3. Light effects on 10 coral response variables. In contrast to Figure 2, data were pooled across the two light sampling windows (before 10:00 and 10:00–14:00; PAR = 455 ± 151 and 267 ± 161 μmol/m²/s, respectively) in this figure, with the differences vs. dark-sampled colonies tested with Wilcoxon tests (on ranks) for each of the 10 response variables (a–j). Grey bands depict 95% confidence, and the trend and fold difference included in the upper left-hand corner of the plot represent statistical significance according to the Wilcoxon test ($p < 0.02$). Mean day and night PAR levels at the position of the sampled coral colonies are included at the top of panel (e).

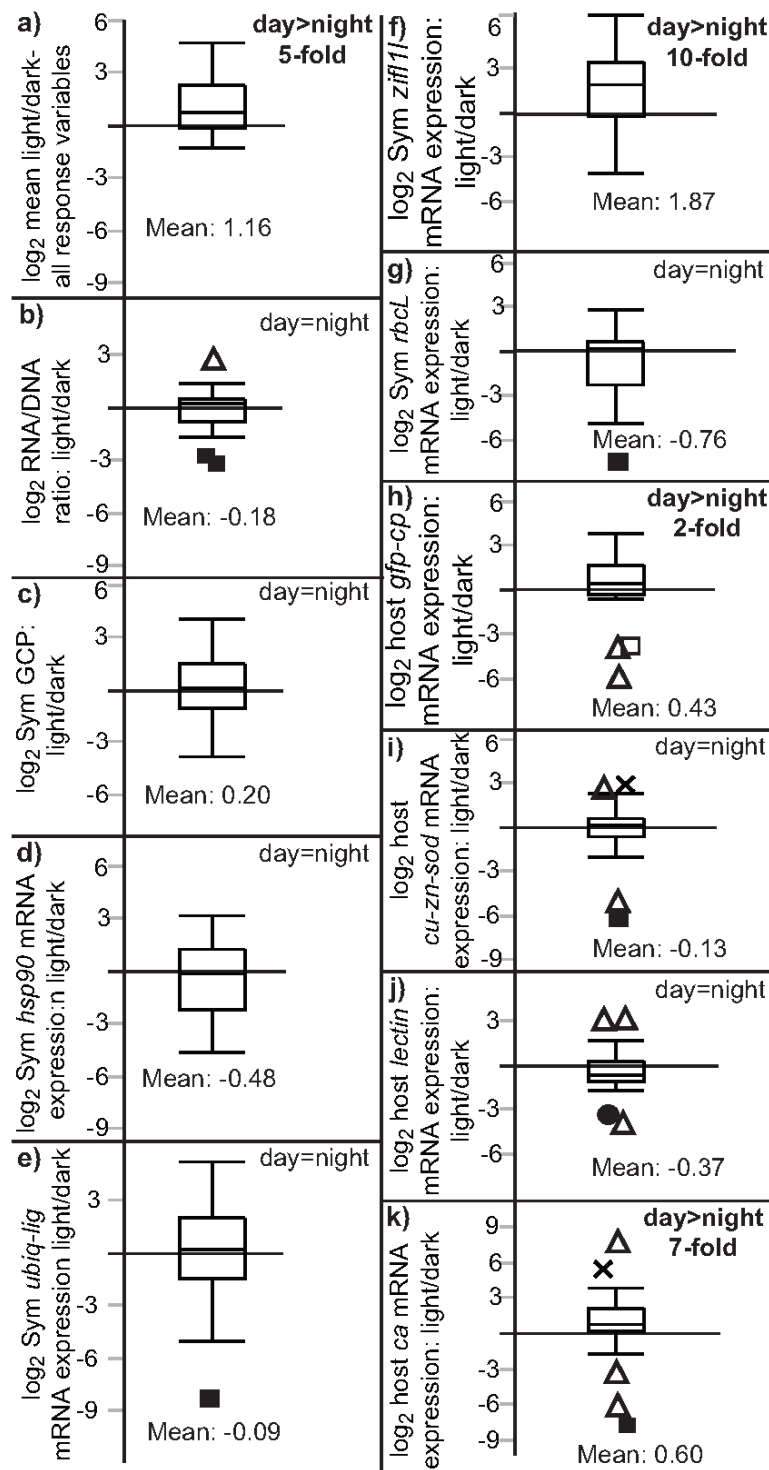


Figure 4. Mean light/dark ratios for the 26 coral colonies sampled at both midday and night. Ratio data were log₂-transformed, and signed-rank (non-parametric) tests were used to determine whether the transformed values of the geometric mean (a) plus the 10 response variables (b–k) were significantly higher than 0 (equivalent to whether the untransformed ratios were significantly higher than 1); only the geometric mean (a), Symbiodiniaceae (Sym) *zif11* (f) mRNA expression, and host coral *gfp-cp* (h) and *ca* (k) mRNA expression fulfilled this criterion ($p < 0.02$ for all). The null hypothesis value of 0 is plotted as a solid horizontal line in each panel. The icon legend follows Figure 2.

4. Discussion

Prior works have found that anthozoan–dinoflagellate endosymbioses vary significantly in molecular composition (e.g., endosymbiont density and lipid levels) and gene expression over short- (mins–hrs [24]), medium (hrs–days [25]), and long-term (days–months [26]) timescales (including control corals not exposed to stress-inducing conditions); however, in many cases, these findings have either been made from laboratory specimens (e.g., [27]) or from opportunistic sampling regimens (e.g., [28]). Herein, we instead had the opportunity to re-sample field corals at multiple points in the light–dark cycle (for a subset of samples) to assess intra-day changes in molecular physiology, and expression levels of a subset of both host coral and endosymbiont genes varied over time (as well as across other environmental gradients). This represents the first opportunity to observe natural variation in field coral molecular physiology in New Caledonia, and several notable observations are highlighted below.

Prior to doing so, however, it must be mentioned that, until coral reef researchers make a more concerted effort to account for the endosymbiotic, multi-compartmental nature of corals in their analyses of, in particular, RNA-Seq- or real-time PCR-derived datasets, it will be difficult to compare results to those obtained using the symbiosis-tailored approaches taken herein and in our prior works that accommodate the spatio-temporally dynamic nature of the coral holobiont [21]. For this reason, we generally abstained from making comparisons to prior laboratory analyses of diel variation in coral gene expression (e.g., [29–33]). It is our hope, though, that symbiologists will more carefully consider the dynamic nature of the endosymbiosis in future works on anthozoan cell biology, particularly those seeking to quantify concentrations of molecular analytes. This will be especially critical when attempting to analyze bleached or bleaching coral samples (provided that bleaching occurs via the loss of endosymbiont cells [34], rather than simply their pigments); unless proper controls are taken, bleached corals will artifactually demonstrate global increases and decreases in host coral and endosymbiont gene expression, respectively, simply because there is a higher host/endosymbiont ratio in these samples (i.e., *not* because expression levels of particular genes were actually changing within the cells analyzed). Herein it was found that endosymbiont densities, in fact, changed over the course of the day; were the molecular data *not* normalized to accommodate differing host/endosymbiont ratios over time, the gene expression data would have been inherently biased (i.e., higher endosymbiont gene expression measured at times of higher endosymbiont densities and vice versa).

As a second diversion to discussing the predominant findings of interest, one limitation of the approach employed herein must be addressed: only 10 RV were assessed in a subset of 89 coral samples. As such, this dataset in and of itself does not signify that, for instance, salinity does not affect the physiology of *P. damicornis*; it surely does [35]. Rather, it did not influence any of the RV assessed, nor did it explain multivariate variation/similarity among samples. The lack of influence may stem not only from having profiled non-responsive analytes, but instead, from low variation in salinity across the survey reefs [12]. In other words, both a lack of environmental variation *and* the incorporation of non-sensitive analytes could explain the lack of statistically significant influence of certain EP (additionally including reef zone and endosymbiont assemblage). By analyzing data from multiple LOF GRE research missions in the near future, we will ultimately gain a greater understanding of the EP that drive variation in coral physiology on a pan-ocean (*sensu* [36]), not just intra-country, scale.

Expression levels of both Symbiodiniaceae (*hsp90* and *ubiq-lig*) and host coral (*cu-zn-sod*) stress-targeted genes were high in all samples (see normalized, transformed, and standardized values in Figure 2, Figure 3, Figure 4 and the OSDF). As has been found in prior works in laboratory (e.g., [37]) and field [38] settings, high molecular chaperone levels (e.g., *hsp40*, *hsp70*, and *hsp90*) are characteristic of all corals and dinoflagellate endosymbionts analyzed to date. Whether this signifies that all corals are under some degree of stress or, more optimistically, that they are simply stress-hardened in a manner in which

cells are “prepared” (at the mRNA level) for environmental change at all times (not just when temperatures, for instance, are dramatically elevated) remains to be determined. In certain, more recent proteomic works, this mRNA-based finding was, in fact, *not* corroborated; molecular chaperones were not generally concentrated at high levels in control or high-temperature-challenged pocilloporid corals [39]. This lack of congruency between the mRNA- and protein-level stress responses must certainly be addressed, especially by those interested in creating molecular biomarker tests for proactive coral health assessment. As for now, it appears that pocilloporid corals and their endosymbionts express high levels of stress-targeted mRNAs alongside low levels of the stress proteins encoded by these genes (global gene vs. protein $R^2 = 0$).

Despite the fact that a mix of genes were responsive to sampling time/light level in both compartments, the endosymbionts were a stronger driver of the multivariate variation among times and light levels. Whether wider temporal and temperature-driven shifts in gene expression are indicative of relatively less control of cellular homeostasis than their anthozoan hosts or, in contrast, simply represent a greater degree of transcriptomic scope or plasticity (i.e., a desirable attribute), remains to be determined. Actually, the temporal variation observed in both compartments was anticipated based on prior studies that found that dinoflagellate metabolism and lipid composition fundamentally differ between day and night as a result of photosynthesis [40]. The consequent diel alternation between autotrophy and heterotrophy was also found to affect host gene expression [41] and could explain why carbonic anhydrase levels were temporally variable given the role of the encoded protein in (1) carbon regulation within the holobiont and (2) biomineralization [42].

Three of the four endosymbiont genes were expressed at higher levels at night, the exception being *zif11* (whose expression instead peaked in the morning; discussed below). The high nocturnal expression of *rbcL* was unexpected and conflicts with prior findings using laboratory cultures [43]; in cultured endosymbionts, *rbcL* expression indeed rises over the course of the evening (as was observed herein), but in laboratory-reared sea anemones, endosymbiont *rbcL* levels *in hospite* are generally lower at night. This could signify that the molecular regulation of carbon fixation differs between anemone–dinoflagellate and coral–dinoflagellate endosymbioses. Ultimately, though, there is no correlation between *rbcL* expression and RBCL protein concentrations in cultured or *in hospite* dinoflagellates [43], and so it will be critical to measure the concentrations of key photosynthesis-associated proteins or, alternatively, profile the entire proteomes of these coral samples in the future to better understand the molecular mechanisms underlying diel hysteresis in coral photosynthesis. In addition to the transfer of photosynthetically fixed carbon products (e.g., glycerol and glucose), the synthesis, modulation, mobilization, and translocation of lipid bodies surely play roles in the temporally dynamic metabolism of these mutualistic holobionts [44].

It is also possible that diel fluctuations in reactive oxygen species (ROS) levels resulting from even normal levels of photosynthesis-driven oxygen evolution from the endosymbionts to the hosts [45] could have driven not only changes in the expression of photosynthesis-targeted genes but also the aforementioned stress markers (in both compartments); indeed, oxidative stress is a key regulator of both gene expression and protein levels in corals [46]. Although *cu-zn-sod* levels and *ubiq-lig* levels were higher at night, it is worth mentioning that corals were sampled only an hour after sunset; corals were no longer photosynthesizing, yet this nevertheless could reflect the peak period of ROS production given the inherent lag between oxygen generation and macromolecular damage. Future work should attempt to sample later in the evening to determine when stress marker levels eventually drop to their lower daytime levels.

The most temporally variable RV assessed was the endosymbiont gene *zif11*, which encodes the zinc-induced facilitator protein. Not only did expression of this gene vary between morning and night, but it contributed highly to the partitioning of biopsies by sampling time in the multivariate analyses. Furthermore, SRA found it to be the most influential gene in building a predictive model for light vs. dark behavior. Interestingly, expression of

this gene was found to be down-regulated at high temperatures in prior works [25,26]. The encoded protein is in the major facilitator superfamily and was hypothesized to function in the drought response of higher plants [47]. In single-celled organisms, though, it likely plays a more general role in proton efflux from the vacuole; given this putative role in osmoregulation, concentrations of this protein could indeed be hypothesized to change over the day given the dynamic, photosynthesis-driven osmoregulatory strategy of the anthozoan–dinoflagellate endosymbiosis, in which the osmotic pressure of both members can change over the day as osmolytes are continually synthesized/replenished and then rapidly respired. Then again, given the aforementioned lack of congruency between gene expression and protein levels in reef corals, it will be critical to first show that the temporal and temperature sensitivity of this gene is corroborated at the protein level. Regardless, the importance of the zinc-induced facilitator in reef coral homeostasis over diel cycles should be addressed in future works.

5. Conclusions

Herein, we uncovered numerous drivers of variation in coral physiology in a never-before-studied/sampled region of the Coral Triangle, with light levels significantly affecting the molecular biology of the *P. damicornis* colonies sampled. Whether the large temporal swings in the expression of genes involved in the stress response and other cellular processes are simply indicative of normal phenotypic plasticity or are, in contrast, testament to considerable deviations in cellular homeostasis that could be energetically expensive, cannot be determined at present. However, these reefs have bleached annually in all years following the late-2013 LOF GRE survey [35], potentially providing evidence for the latter hypothesis. Provided that a subset of these corals ultimately withstand ASB via acclimatization or adaptation, then this dataset could be used to build physiologically based resilience models that consider the degree to which short-term fluctuations in molecular biology reflect a coral's scope and, consequently, likelihood of persisting into the next century. If no such resilience is documented, then this dataset may only have value in serving as a record for future scientists to understand how New Caledonian corals functioned in the last bleaching-free year. Indeed, the preserved tissue biopsies may unfortunately reflect the last biological record of the coral colonies from which they were sampled, many of which have likely already perished on account of climate change.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/oceans3010002/s1>: Supplementary Materials containing supplemental methods, Tables S1–S3, Figure S1, and the online supplemental data file (xlsx).

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