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Transcriptome-wide Changes in Coral Gene Expression at Noon and Midnight Under Field Conditions

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Abstract. Reef-building corals experience high daily variation in their environment, food availability, and physiological activities such as calcification and photosynthesis by endosymbionts. On Ofu Island, American Samoa, we investigated day-night differences in gene expression under field conditions of changing pH, temperature, light, and oxygen. Using RNASeq techniques, we compared two replicate transcriptomes from a single coral colony of *Acropora hyacinthus* over six noons and five midnights. We identified 344 contigs with significant expression differences across 16,800 contigs in the transcriptome, most with small fold-changes. However, there were 21 contigs with fold-changes ranging from 10 to 141. The largest changes were in a set of transcription factors strongly associated with day-night gene regulation in other animals, including cryptochromes, thyrotroph embryonic factor, and D site-binding protein. We also found large daytime increases in a set of genes involved in glucose transport and glycogen storage. We found small expression differences in genes associated with aerobic ATP production and hypoxia response, along with slightly higher expression of most calcification genes at noon. Although >40-fold-changes in expression occur in important transcription factors, downstream gene regulation seems very stable in corals from day to night compared to other animals studied.

Introduction

Reef-building corals seem to be well suited to large daily changes in gene expression. The metabolism of coral colonies is in part driven by the photosynthesis of their symbionts, dinoflagellates of the genus *Symbiodinium.* During daylight, corals receive a substantial portion of the photosynthate produced by the endosymbionts living in their gastrodermis (32%–45% in *Pocillopora damicornis*) (Muscatine and Cernichiari, 1969), which can make up a significant portion of the coral's energy budget—in *Porites compressa* and *Montipora capitata* 65%–79% of daily metabolic energy is autotrophically derived (Grottoli *et al.*, 2006). In turn, high daytime photosynthesis powers high daytime calcification rates, and as a result corals produce more skeletal material during the day (*e.g.,* Moya *et al.*, 2006). The diurnal periodicity of photosynthesis and calcification results in a physiological rhythm within the coral holobiont that follows the day-night cycle. In addition, corals living in back-reef lagoons can experience large daily fluctuations in pH, temperature, and dissolved oxygen availability (Craig *et al.*, 2001; Hofmann *et al.*, 2011; Smith *et al.*, 2013). In tune with these rhythms, some coral genes show diurnal fluctuations in gene expression. Some are under circadian control (Hoadley *et al.*, 2011; Levy *et al.*, 2011) and others fluctuate diurnally because they are light-induced (Levy *et al.*, 2007; Hoadley *et al.*, 2011; Levy *et al.*, 2011). However, a surprising feature of studies thus far completed is that relatively few genes have diurnal fluctuations in expression: Levy *et al.* (2011) found that only 2.3% (*i.e.,* 200 of 8606) of transcripts examined had circadian expression patterns in *Acropora millepora*; to date this remains the only study to examine circadian rhythm in a coral species across thousands of genes.

Despite the low number of genes with circadian expression, Levy *et al.* (2011) found that the expression patterns imply a degree of separation of specific cellular functions. For example, three key cell cycle genes (ribonucleotide

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reductase, histone H4, and cyclin D2) had significantly higher expression at midnight, leading the authors to hypothesize that DNA replication in corals is a nocturnal activity. Barott *et al*. (2013) examined day-night differences in protein levels of cyclic adenosine monophosphate (cAMP) in *Pocillopora damicornis* in the laboratory and found significantly higher levels during the day than in the dark (0500 h), leading the authors to suggest that soluble adenylyl cyclase, which catalyzes the production of cAMP and whose activity is stimulated by bicarbonate, may have a role in regulating acid-base homeostasis during the day.

There are several hypotheses for why coral studies have found low numbers of genes whose expression is under day-night regulation. First, some species of corals have high variance in gene expression from colony to colony (*e.g.,* Barshis *et al*., 2013); this might lower statistical power to detect day-night variation for many loci. Second, the subset of genes examined in gene-chip studies may have excluded a large number of day-night variable genes. For instance, a study by Tovin *et al*. (2012) identified circadian genes in the zebrafish pineal gland by using both DNA microarray and RNASeq methods. Although they concluded that the two results were reasonably comparable, they suggest that the RNASeq approach has higher detection power due to its unbiased measure of the entire transcriptome and a higher accuracy of expression measurements. Third, most of these studies have been conducted under controlled light conditions in the laboratory. However, more than light varies on a daily basis in coral reefs, particularly in shallow back-reef environments where pH, temperature, oxygen levels, and depth rise and fall daily (Craig *et al*., 2001; Hofmann *et al*., 2011; Smith *et al*., 2013). These environmental cues could spark larger changes in gene expression than seen in laboratory studies.

The aim of our study was to identify genes with different expression levels at noon and midnight in a colony of *Acropora hyacinthus* (Dana, 1846) living in the natural setting of a back-reef of Ofu Island, American Samoa. We focused our study on a colony in a back-reef because many coral species inhabit these environments, which can vary greatly from day to night (*e.g.,* Craig *et al*., 2001). We multiple sampled one colony to reduce intra-individual variation and to estimate the amount of error from one RNASeq study to another. With these data, we asked (1) how many genes in the transcriptome have expression patterns that differ significantly between noon and midnight; (2) in which cellular activities are the gene products involved; and (3) do genes involved in carbohydrate metabolism, oxygen homeostasis, or calcification have expression patterns that follow the diurnal fluctuation of conditions that occurs in the reef and within the holobiont?

To examine differential gene expression patterns between noon and midnight, we sequenced six noon and five midnight coral transcriptomes from the same colony (a total of 11 time points) and included duplicate samples per time point in order to estimate sample-to-sample variability. In addition, we monitored a suite of environmental factors close to the sampled coral in order to estimate their impact on coral gene expression. Sampling multiple colonies could have allowed us to estimate variation in day/night control of gene expression from colony to colony. However, marked variation in gene expression among colonies (*e.g.,* Seneca and Palumbi, 2015; N. Rose *et al*., Stanford University, Hopkins Marine Station; unpubl.) limits detection of subtle differences from day to night. Instead we sought to increase the depth of biological replication for independent branches taken at widely spaced regions of a single large colony, and used the additional precision to more powerfully test for day/night expression differences. We used RNASeq on an Illumina platform to expand the number of genes beyond that available to previous chip-based surveys. We focused on day/night patterns under field conditions in order to estimate gene expression changes that coral colonies are likely to experience in natural habitats. As a consequence, we have not tested for classically defined circadian genes (*i.e.,* those that maintain rhythmic expression patterns in constant dark conditions). Our study is designed to maximize detection of genes with day/night differences in expression, and included in our results are some of the classic circadian genes along with those that might be induced by daily environmental changes.

We found major changes (up to 100-fold) in a small subset of contigs (21 total). Many of these are transcription factors known to be involved in daily control of gene expression in other animals. Despite these genes with major changes, only about 2.0% of contigs in the transcriptome had different expression levels at noon and midnight, and most of those had mild shifts in expression (<2-fold). Such low numbers of genes with differential expression from day to night suggest that although corals experience different physiological and environmental states across the daily cycle, transcriptional control is not the dominant mode of regulating daily changes in metabolism.

Materials and Methods

Sample collection

In August 2011, individual coral branches were collected from a single colony of *Acropora hyacinthus* living in a back-reef environment on Ofu Island, American Samoa (14.1750°S, 169.6180°W) (all necessary permits were in hand; American Samoa Government Department of Marine and Wildlife Resources Permit No. 2011/44). From 21–30 August the selected colony (about 1.75 m across at the time of sampling) was sampled at six noon and five midnight time points. For each sampling, three branches adjacent to each other (each <2 cm long) were cut from the colony and placed in separate plastic zip-closure bags. Throughout the

Table 1

Mean environmental conditions in the hour prior to sampling (sample sizes are n = 6 *at noon, and* n = 5 *at midnight)*

Parameter	Noon mean ± SD	Midnight mean ± SD
Aragonite saturation	4.6 ± 0.3	4.0 ± 0.2
pH	8.16 ± 0.04	8.08 ± 0.03
Temperature (°C)	28.2 ± 0.3	27.6 ± 0.1
DO saturation (%)	124 ± 6	93 ± 3

sampling period, random locations around the perimeter of the colony were selected so as not to sample the same area twice. On shore (~5 min after collection) the branches were placed individually into 5 ml of RNALater. Upon arrival at the field laboratory, fresh RNALater was placed in the vials and the samples were stored at 4 °C for 24 h, then transferred to −20 °C for 1 to 10 days until being transported to Hopkins Marine Station, Pacific Grove, California. Samples were then stored at −80 °C until processing.

Environmental data gathered

During sampling, environmental data were gathered from the vicinity of the colony. A temperature logger (HOBO Pendant) about 20 cm from the colony recorded temperature every 12 min. A YSI DataSonde (model 6600V2-4), which was attached to the substratum 0.5 m from the colony, recorded dissolved oxygen saturation, depth, and pH every 1–2 min. Discrete water samples were collected beginning on the fourth time point, alongside the coral samples, to obtain aragonite saturation and pH of the water within 0.5 m of the colony. Seawater was collected (nine 30-ml borosilicate glass bottles filled each time) every 6 h from 1800 22 August to 1200 24 August and from 1200 28 August to 1800 30 August—in total 18 time points. Mercuric chloride (0.03% of total volume) was added to the water samples used to measure total dissolved inorganic carbon (TDIC) and total alkalinity (TA). For each discrete water sample, TDIC was measured using a 5011 CO_2 coulometer (UIC Inc.); TA was measured by open-cell potentiometric titration; and salinity was measured with a Guildline Autosal salinometer. Using the TDIC, TA, salinity, and temperature data for each sampled time point, we calculated aragonite saturation and pH *via* the program SeaCarb ver. 2.4.3 (Lavigne and Gattuso, 2011) in R ver. 3.0 (CRAN.R-project.org). The seawater pH for the first (1200), second (0000), and third (1200) coral samples was measured by the YSI DataSonde and was used to calculate the mean pH for noon and midnight during our survey; the seawater pH of the subsequent eight coral samples was measured with the discrete water samples (see Table 1).

RNA extraction, cDNA synthesis, and transcriptome sequencing

Two biological replicates per sampled time point (six noon and five midnight) were prepared for gene expression analysis *via* RNASeq. Total RNA was extracted from each sample using TRIzol (Life Technologies). An Illumina TruSeq RNA prep kit, ver. 2, was used to separate the mRNA from the total RNA and make cDNA libraries for each sample. Briefly, the mRNA was isolated from the total RNA *via* poly-A selection. The isolated mRNA was fragmented and purified in preparation for cDNA synthesis. Random hexamer primers were used in cDNA synthesis to ensure that sequence reads represent the entire length of the mRNA transcript. The double-stranded cDNA library underwent end repair, which converted overhangs into blunt ends. A single adenine was added to the 3′ end of the blunt-end fragments. Because lanes in the flow cell were multiplexed, adapters were used to identify samples. Adapters have a single thymine that ligates to the single adenine on the fragment. The adapter-ligated fragments underwent PCR amplification of 15 cycles to ensure that transcript representation in the library was not skewed.

After quantification of DNA concentration (Qubit, Life Technologies), the samples were submitted to the University of Utah Microarray and Genomic Analysis Core Facility. At the Core Facility, the concentration and quality of the 22 cDNA libraries were determined with an Agilent Bioanalyzer DNA 1000 chip and by qPCR. The 22 samples were divided into two groups of 11 that were each pooled and multiplexed together in one lane on the flow cell. The two lanes were run on an Illumina HiSeq 2000 with 50-cycle single-end read sequencing. All reads per sample were processed after the quality check and filtering steps outlined in De Wit *et al*. (2013). The 22 samples represented 11 time points—five midnights and six noons—each with two biological replicates.

Sequence reads that passed specific quality checks (duplicate reads removed, length >25 base pairs, and quality score >20) were mapped against a reference transcriptome for *A. hyacinthus*, which is composed of 33,496 contigs (14.9 MB) (Barshis *et al.*, 2013) (see Supplementary Table 1. *Acropora hyacinthus* reference transcriptome with contig annotations, http://www.biolbull.org/content/supplemental). Reads belonging to the coral host were separated from reads belonging to *Symbiodinium* and other organisms found in the coral holobiont. The number of reads that map to each contig in the reference transcriptome provides a measure of gene expression. Prior to read count normalization, the number of reads per sample ranged from 1.1 to 1.5 million after duplicate reads were removed. Previous coral studies have validated transcriptomic data with real-time PCR and demonstrated a high correlation between expression measured by RNASeq and real-time PCR methods (*e.g.,* Meyer

et al., 2011). Due to our experimental design—two biological replicates for each of the six noon and five midnight time points—and the previous RNASeq validations done for corals, we felt confident in our transcriptomic data and decided not to validate by real-time PCR.

Read count normalization and filtering

The package DESeq ver. 1.12.0 (Anders and Huber, 2010) was used in R ver. 3.0.1 (CRAN) to normalize the raw read counts (see Supplementary Table 2. Raw read count data of the 33,496 contigs in the *Acropora hyacinthus* reference transcriptome, http://www.biolbull.org/content/supplemental). The normalization procedure was as follows: (1) for each contig the geometric mean across all samples was calculated; (2) for each sample every contig's raw read value was divided by the geometric mean for that contig; (3) for each sample the median of all the ratios of raw read number over geometric mean per contig was used as a size factor; and (4) all raw read values for a sample were divided by the sample's size factor to produce the normalized read value. After the read counts were normalized, contigs with mean read depth less than 5 were removed. Of the 33,496 contigs in the reference transcriptome for *A. hyacinthus*, 16,800 contigs had a mean read depth across all samples greater than 5 and were used in subsequence analyses (see Supplementary Table 3. Normalized read count data for the 16,800 contigs in the filtered dataset, http://www.biolbull.org/content/supplemental).

Gene expression analysis by two-way ANOVA

The 11 time points, each with two biological replicates, were analyzed to identify differences in coral expression between noon and midnight. Differentially expressed contigs were identified with a two-way ANOVA in R ver. 3.0.1. The two factors were time of day, either noon or midnight, and sample. A two-way ANOVA was done on the 16,800 contigs that passed the filtering step. A multiple test correction using the Benjamini and Hochberg (1995) method was applied. All differentially expressed contigs that are significant for time of day have $P < 0.05$ after false discovery rate (FDR) correction (Benjamini-Hochberg method).

Functional enrichment and pathway analysis

Functional enrichment analyses were used to identify the cellular activities that were enriched by contigs with significantly higher expression at noon or midnight, compared to all cellular activities represented in the reference dataset using the Database for Annotation, Visualization, and Integrated Discovery ver. 6.7 (DAVID) bioinformatics resource (Huang *et al.*, 2009a, b). In DAVID, a hypergeometric test was used to identify gene ontology (GO) terms that were significantly ($P < 0.05$ after Benjamini correction) over-represented by contigs with higher expression at noon or midnight. First, a reference list of the contigs present in the filtered dataset with UniProt accession matches was assembled, resulting in 12,595 contigs. Of the 344 contigs with significant differences in expression according to time of day (FDR corrected $P < 0.05$), 239 had higher expression at noon and 105 had higher expression at midnight. Only contigs that were annotated with UniProt accessions were used in the analyses, resulting in 183 for noon and 76 for midnight. Two DAVID analyses were conducted, one comparing the list of 183 noon contigs and a second comparing the list of 76 midnight contigs to the reference list of 12,595 contigs. Default parameters were used with a Benjamini correction at 5%.

Post hoc of the DAVID analyses, we identified the components of the fatty acid degradation, the citric acid (TCA) cycle, glycolysis, and oxidative phosphorylation pathways that were present in the filtered dataset. We then determined how many of those components were represented in our data by contigs with at least a 20% increase in expression at noon or midnight relative to the other time point (*i.e.,* fold-changes of 1.2 or greater at either noon or midnight). The R package Pathview ver. 1.8.0 (Luo and Brouwer, 2013) was used to map the Kegg Orthology terms present in the filtered dataset onto the four Kegg pathways (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2013), and we identified which terms were associated with contigs with at least a 20% increase in expression at noon or midnight.

Analysis of a response to diurnal oxygen fluctuations at the gene expression level

From previous observations, we know that the back-reef environment in which the sampled colony lives has diurnal fluctuations in dissolved [O_2] (P. Craig, U.S. National Park Service; pers. comm.). During the sampling period we recorded diurnal fluctuations in dissolved oxygen saturation, with higher values during the day than at night. To examine if the colony was responding to these changes in oxygen levels, we examined the expression of genes that are annotated with either or both of the GO terms "oxygen homeostasis" or "response to hypoxia," or contained the term "hypoxia-inducible" in the gene name. There were 38 contigs in the filtered dataset of 16,800 contigs that meet our criteria and whose expression we examined. A two-way ANOVA of the 38 contigs was used to identify contigs that were significant for time of day ($P < 0.05$ after FDR correction, Benjamini-Hochberg method); we also report the FDR corrected P value from the transcriptome-wide ANOVA.

Calcification gene expression analysis

The reference transcriptome of *A. hyacinthus* (Barshis *et al.*, 2013) was searched for genes previously identified as

involved in coral biomineralization. Within the filtered dataset of 16,800 contigs, we identified 70 calcification contigs: five galaxin contigs, nine Ca^{2+} ATPase contigs (seven of these contigs map to two *Acropora digitifera* genes), 16 carbonic anhydrase contigs (eight of these map to four *A. digitifera* genes), two biomineralization contigs identified in the *A. digitifera* genome (both contigs map to one *A. digitifera* gene) (Shinzato *et al.*, 2011) that were not already in our list, and 38 skeletal organic matrix protein contigs (not including galaxins) identified in *Acropora millepora* (31 of these contigs map to 10 *A. digitifera* genes) (Ramos-Silva *et al.*, 2013).

The expression of the 70 calcification contigs was examined to determine if there were significant differences between noon and midnight. A two-way ANOVA of the 70 contigs was used to identify those that were significant for time of day. A multiple test correction using the Benjamini and Hochberg (1995) method was applied. All significant differentially expressed contigs have $P < 0.05$ after FDR correction; we also report the FDR corrected P value from the transcriptome-wide ANOVA.

Results

Environmental conditions at time of sampling

There were measurable changes in aragonite saturation, pH, temperature, and dissolved oxygen saturation (DO) from day to night during the period of our sampling (Table 1). For example, aragonite saturation declined to 4.0 (SD 0.2) at midnight from 4.6 (SD 0.3) at noon (max range, 3.75–4.97 across all 18 discrete water samples). Likewise, pH varied by 0.17 units (max range, 8.04–8.21), and temperature varied by 1 °C (max range, 27.4–28.4 °C). DO saturation was higher at noon than at midnight and ranged from 51.5% to 142.8% during the sampling period. Diurnal shifts in these conditions are typically larger for this particular back-reef (Craig *et al.*, 2001; Oliver and Palumbi, 2011; Koweek *et al.*, 2015; Ruiz-Jones *et al.*, unpubl.) and were most likely dampened by a continuous 2–3-ft swell that delivered more than the usual wave-driven flow across the back-reef during our sampling. As a result, environmental change in our sample is probably more similar to that experienced regularly by fore-reef corals.

Transcriptome-wide differences in expression between noon and midnight

Our dataset includes expression levels for 16,800 contigs with mean read depth greater than 5. Biological replicates taken at the same time had low variation. The coefficient of variation of the normalized counts across contigs for these replicates averaged 0.20. Replicates from date to date were slightly more variable, with an average coefficient of variation across contigs of 0.25.

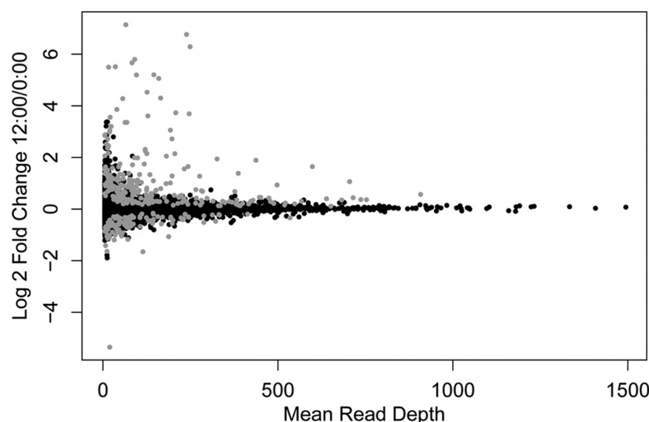

Figure 1. Log base 2-fold change and mean read depth of the 16,800 contigs analyzed. The 344 contigs identified by the 2-way ANOVA to have significant differences in expression between noon and midnight (FDR correct $P < 0.05$) are in gray.

We did a principal component analysis on the normalized counts for the 16,800 contigs to visualize the extent of variation between the 22 samples and found that the samples were distinctly separated by time of day (see Appendix Fig. 1). For the day-night comparison, a two-way ANOVA found that 344 contigs had significantly different expression levels at noon *versus* midnight (Fig. 1; contigs with $P < 0.05$ after Benjamini and Hochberg (1995) false discovery rate (FDR) correction are in gray). Of these contigs, 239 had higher expression at noon, and the remaining 105 had higher expression at midnight. Of the 344 significant contigs, 259 are annotated with UniProt matches, 216 of which are unique (see Supplementary Table 4. Metadata for the 239 contigs with significantly higher expression at noon and Table 5. Metadata for the 105 contigs with significantly higher expression at midnight, http://www.biolbull.org/content/supplemental).

The expression patterns of these 344 contigs showed highly consistent changes from day to night across the six noon and five midnight time points and between replicates; they revealed the presence of three major clusters (Fig. 2). The first cluster (labeled Cluster I in Fig. 2) represents 20 contigs with very large increases in noontime expression (mean fold change, 39.0; range, 10.3–141.1). In Clusters II and III, fold-changes were much lower. Cluster II contains 105 contigs with higher expression at midnight (mean fold change 1.9 (range, 1.1–40.7)), and Cluster III consists of 219 contigs with higher expression at noon (mean, 2.1; range, 1.1–9.2).

These patterns had low variance among replicate branches of this single colony, allowing us to detect a larger number of differentially expressed genes. For example, a majority of our highly significant genes (248 out of 341) differed in expression by less than 2-fold from noon to midnight. However, because we heavily sampled only a

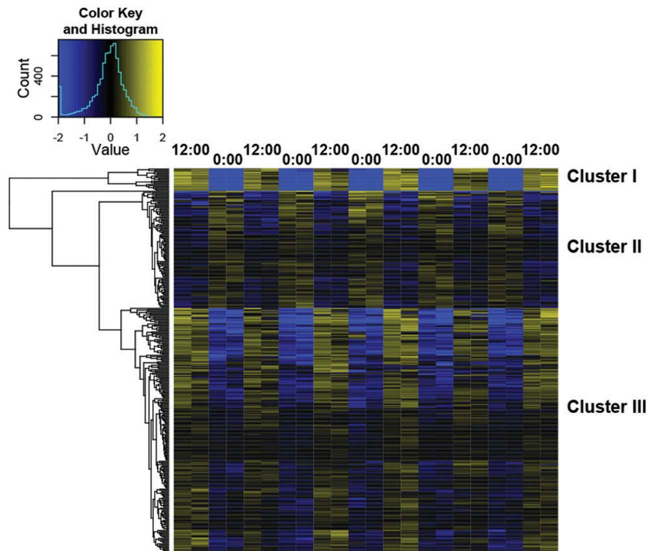

Figure 2. The 344 significant contigs (value = Log$_2$(normalized count/mean normalized count for contig)). Cluster I includes 20 contigs with very large increases in expression at noon (mean fold change 39.0; range 10.3–141.1). Cluster II includes 105 contigs with higher expression at midnight (mean 1.9; range 1.1–40.7). Cluster III consists of 219 noon contigs with a moderate fold change (mean 2.1; range 1.1–9.2). Samples are in columns and contigs are in rows. Biological replicates are paired.

single colony, these data may represent some gene expression patterns unique to this single coral colony (Coral AH09 in the highly variable pool; see Oliver and Palumbi, 2011; Barshis *et al.*, 2013, for site description).

Strong differences for 21 contigs

Transcription factors. Five of the 21 highly changing contigs (fold change 10) are not annotated, but the other 16 have homologies to proteins that suggest something about their function. Among the genes in Cluster I are three transcription factors: cryptochrome-1 (CRY1), thyrotroph embryonic factor (TEF), and a Hairy related transcription factor (Fig. 3A). We found four contigs of cryptochrome-1 in Cluster I (FDR corrected $P < 0.05$), with an average fold change of 36.7 (range, 13.3–50.8). These four contigs map (two each) to two *Acropora digitifera* genes. TEF, a PAR-domain basic leucine zipper (PAR bZip) transcription factor, increased 78.4-fold at noon in *A. hyacinthus*. CRY1 and TEF are known to be involved in circadian rhythm (Gavriouchkina *et al.*, 2010; Chaves *et al.*, 2011). The cryptochrome-1 and -2 genes are known circadian transcriptional regulators in corals (Levy *et al.*, 2007; Hoadley *et al.*, 2011) and other animals (Cashmore, 2003). The contig with the highest fold change at midnight was a D site-binding protein (DBP) (fold change, 40.7) (Fig. 3A), a second PAR bZip transcription factor with a daily expression pattern opposite to TEF (see Supplementary Table 6. Metadata for the

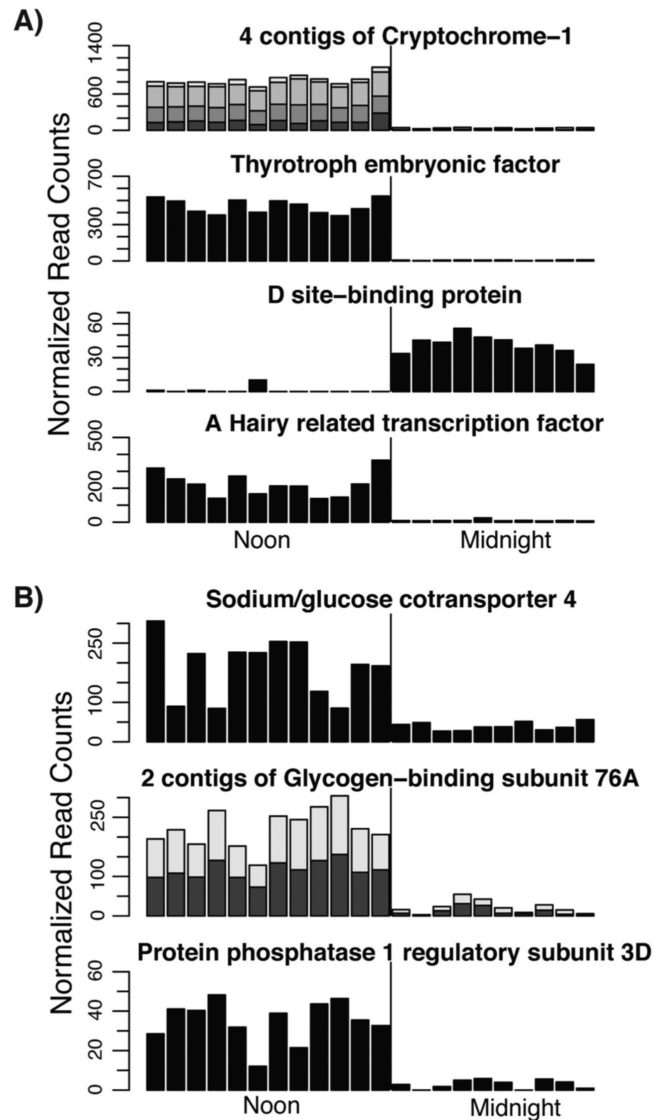

Figure 3. Expression levels for contigs with large fold-changes. (A) Four transcription factors: four contigs of cryptochrome-1, thyrotroph embryonic factor, D site-binding protein, and a Hairy related transcription factor. (B) Three genes involved in glucose metabolism: sodium/glucose cotransporter 4, two contigs of glycogen-binding subunit 76A, and protein phosphatase 1 regulatory subunit 3D. All had significant differences in expression between noon and midnight (FDR corrected $P < 0.05$) (noon $n = 6$ and midnight $n = 5$, both in duplicate). Each bar represents a single sample; duplicates are paired.

21 contigs with fold changes greater than 10, http://www.biolbull.org/content/supplemental).

Glycogen and glycoside metabolism. We found 10.2 times higher daytime expression of two contigs of glycogen-binding subunit 76A and 11.8 times higher daytime expression of protein phosphatase 1 regulatory subunit 3D (Fig. 3B). Both are glycogen-targeting protein subunits that anchor protein phosphatase 1 (PP1) to glycogen-associated

substrates. Furthermore, we found three contigs of glycoside hydrolase family 63, which all align to the same *A. digitifera* gene, that increased an average of 15.4-fold during the day.

Functional analyses

Genes in Clusters II and III have low average fold-changes, and the biological relevance of these differences is unclear. However, if these changes represent functional shifts in metabolism, then genes in similar pathways should show parallel expression differences (Fraser *et al.*, 2004). We searched functional categories in three ways: (1) *via* gene ontology terms in standard enrichment analyses, (2) *via* metabolic pathway analysis using Kegg, and (3) by grouping genes together that we *a priori* hypothesized would be affected by the day/night cycle: oxygen homeostasis genes and calcification genes.

Functional enrichment analyses. The enrichment analysis of the 183 noon contigs with UniProt accessions (out of the total 239 contigs) found four significant gene oncology (GO) terms: carbonate dehydratase activity, DNA photolyase activity, hydro-lyase activity, and photoreceptor activity (Benjamini corrected $P < 0.05$) (see Supplementary Table 7. Results from the DAVID functional enrichment analysis of significant noon contigs, http://www.biolbull.org/content/supplemental). The analysis of the 76 midnight contigs with UniProt accessions (out of the total 105 contigs) found one significant GO term: extracellular region (Benjamini corrected $P < 0.05$) (see Supplementary Table 8. Results from the DAVID functional enrichment analysis of significant midnight contigs, http://www.biolbull.org/content/supplemental).

Metabolic pathway analysis. To further explore the metabolic pathways that are affected by daily changes in gene expression, we compared day-night fold-changes in expression among genes within metabolic pathways. There are 5769 contigs in the filtered dataset that are annotated with Kegg Orthology (KO) terms. We mapped contigs with KO terms and a minimum of 20% increase in expression at either noon or midnight to the Kegg database (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2013). Of the 2019 contigs with fold-changes of 1.2 or greater at noon, 692 have KO terms; of the 2126 contigs with fold-changes of 1.2 or greater at midnight, 666 have KO terms. We then identified contigs in these lists that are associated with components of the pathways for fatty acid degradation, the citric acid (TCA) cycle, glycolysis, and oxidative phosphorylation (Table 2).

For example, among the 12 components of the fatty acid degradation pathway represented by contigs in our dataset; four had a 20%–30% increase in expression at noon. Similarly, among 15 components of the TCA cycle pathway represented in our dataset; five had a 20%–100% increase in expression at noon. In this pathway, however, two contigs had a 20%–50% increase at midnight. The other metabolic pathways had about equal numbers of contigs with 20% increase in expression at noon and at midnight (*e.g.,* the glycolysis pathway and the oxidative phosphorylation pathway, Table 2) (see Supplementary Table 9. Metadata for the contigs used in the Kegg pathway analysis, http://www.biolbull.org/content/supplemental).

Table 2

Differences in the number of components of the fatty acid degradation, TCA cycle, glycolysis, and oxidative phosphorylation pathways with at least a 20% increase in expression at noon or midnight

	No. of components of the pathway represented by contigs with at least a 20% increase in expression		
Pathway	in filtered data set	at noon	at midnight
Fatty acid degradation	12	4	0
TCA cycle	15	5	2
Glycolysis	13	3	5
Oxidative phosphorylation	43	9	4

The expression of genes involved in oxygen homeostasis. During the sampling period there were diurnal fluctuations in dissolved oxygen (DO) saturation (Table 1). To examine if the coral colony was responding at the transcriptional level to changes in DO saturation between noon and midnight, we used a two-way ANOVA to analyze the expression of 38 contigs that were annotated with either one or both of the GO terms “oxygen homeostasis” and “response to hypoxia” or that contained the term “hypoxia-inducible” in the gene name. Of the 38 contigs, 12 had significant differences in expression between noon and midnight (FDR corrected $P < 0.05$) with an expression range of 0.4–1.7-fold change (1200 expression level/0000 expression level) (gray dots in Fig. 4). Eight of the 12 significant contigs had FDR corrected $P < 0.05$ in the transcriptome-wide ANOVA (see Supplementary Table 10. Metadata for the 38 oxygen related contigs, http://www.biolbull.org/content/supplemental). Four of the 12 significant contigs had higher expression at noon (fold-change range, 1.3–1.7). All of these map to the same *A. digitifera* hypoxia-inducible factor 1α gene (HIF1A), and two were found to be significant in the transcriptome-wide ANOVA (FDR corrected $P < 0.05$). In addition, six Egl nine homolog 1 contigs (four of which map to a single *A. digitifera* gene, and all of which were significant in the transcriptome-wide ANOVA) were expressed more highly at midnight (fold change 1.5 to 2.7). There is also one thrombospondin-1 contig (fold change 2.2 higher at midnight), and one heat shock protein 23 contig (fold change 1.5 higher at midnight).

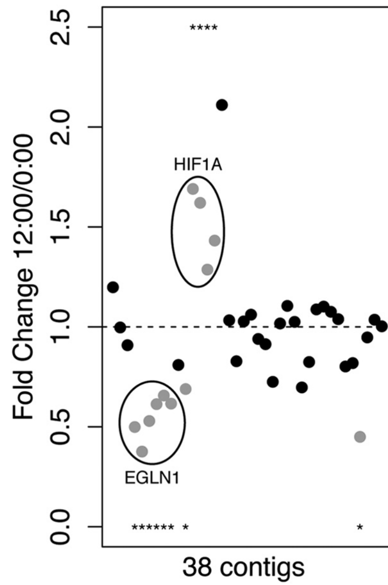

Figure 4. The fold change (noon/midnight) in expression for 38 contigs associated with one or both of the gene ontology terms "oxygen homeostasis" and "response to hypoxia," or contained the term "hypoxia-inducible" in the gene name. The 12 significant contigs (FDR corrected $P < 0.05$) are in gray. HIF1A, hypoxia-inducible factor 1α contigs. EGLN1, Egl nine homolog 1.

Expression of calcification genes at noon and midnight. We were interested *a priori* in the regulation of calcification genes from day to night, because skeletogenesis occurs more strongly during the day than at night (Cohen and McConnaughey, 2003). Our analysis of transcriptome-wide gene expression patterns at noon and midnight allowed us to investigate whether calcification genes were differentially expressed between these two time points. We focused our analysis on 70 contigs involved in calcification: five galaxin contigs, nine Ca^{2+} ATPase contigs (seven of these contigs map to two *A. digitifera* genes), 16 carbonic anhydrase contigs (eight of these map to four *A. digitifera* genes), and two biomineralization contigs identified in the *A. digitifera* genome (both contigs map to a single *A. digitifera* gene) (Shinzato *et al.*, 2011) that were not already in our list. In addition, we considered 38 skeletal organic matrix protein contigs (not including galaxins) identified in *Acropora millepora* (31 of these contigs map to 10 *A. digitifera* genes) (Ramos-Silva *et al.*, 2013). Fifty-two of these 70 contigs had higher expression at noon than at midnight (chi-square test, $P < 0.00005$; Fig. 5); 14 of the 52, with an expression fold-change range of 1.2–2.0, were significantly higher at noon (FDR corrected $P < 0.05$) (gray dots in Fig. 5). Ten of the 14 significant contigs had FDR corrected $P < 0.05$ in the transcriptome-wide ANOVA (see Supplementary Table 11. Metadata for the 70 calcification-related contigs, http://www.biolbull.org/content/supplemental). These 14 significant contigs include 11 carbonic anhydrase contigs (fold-change range, 1.2–2.0) (five of which map to three *A. digitifera* genes) and three skeletal organic matrix protein contigs (fold-change range, 1.2–2.0), one of which is galaxin. Nine of the 11 carbonic anhydrase contigs were

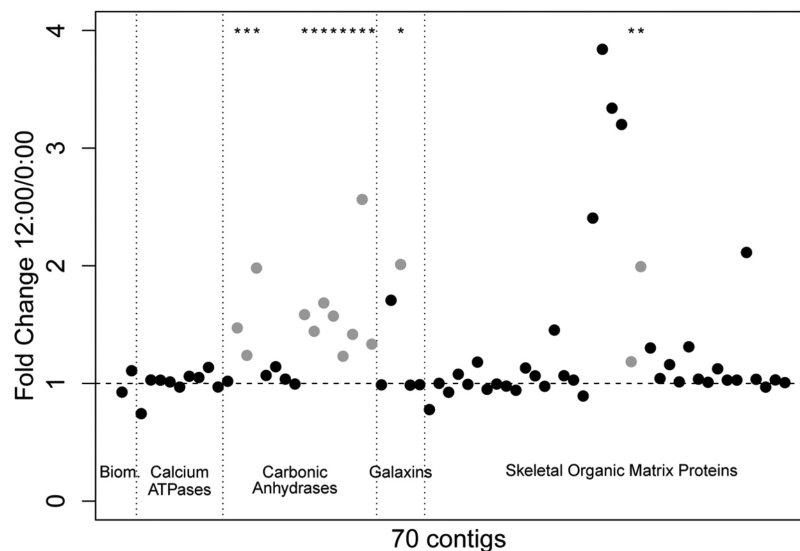

Figure 5. The fold change (noon/midnight) in expression for 70 calcification contigs. The 14 contigs with significant differences in expression between noon and midnight (FDR corrected $P < 0.05$) are in gray. These 70 contigs are separated into five groups: biomineralization (Biom.) genes identified in the *Acropora digitifera* genome (Shinzato *et al.,* 2011) ($n = 2$), Ca^{2+} ATPase contigs ($n = 9$), carbonic anhydrase contigs ($n = 16$), galaxin contigs ($n = 5$), and skeletal organic matrix protein contigs identified in *Acropora millepora* (Ramos-Silva *et al.*, 2013) ($n = 38$).

also significant in the transcriptome-wide ANOVA (FDR corrected $P < 0.05$).

Discussion

We found that 344 of 16,800 contigs in the coral transcriptome of *Acropora hyacinthus* showed highly repeatable and significant differences in expression levels between noon and midnight (FDR corrected $P < 0.05$) (Fig. 2). The largest changes are in transcription factors known to be associated with day-night gene regulation in other animals and in a set of genes involved in glucose transport and glycogen storage. In addition, based on the functional roles of genes, we found mildly increased expression of genes involved in aerobic ATP production at noon; a complex shift in genes normally involved in response to hypoxia; and slightly higher expression of most calcification genes at noon.

Large shifts in the expression of transcription factors from day to night

We found 21 contigs with changes greater than 10-fold from noon to midnight: 20 with higher expression at noon (mean fold change 39.0; range 10.3–141.1) (Cluster I in Fig. 2) and one with higher expression at midnight (fold change 40.7). Experimentally imposed temperature stress has been shown to change gene expression on average 3.6-fold (range 1.7–27.2) in *A. hyacinthus* from the same back-reef of Ofu Island, American Samoa (Barshis *et al.*, 2013). When compared to these changes, the fold-changes of the 21 contigs in Cluster I are substantial considering that no stress was imposed.

Cryptochrome-1 and cryptochrome-2 were identified as having much higher expression at noon (Fig. 3A, fold-change range 13.3–50.8, FDR corrected $P < 0.05$). Cryptochromes are blue-light photoreceptors that have circadian expression patterns in plants and animals where they are involved in entraining the molecular oscillator, which coordinates circadian rhythm through transcription/translation feedback loops, to the 24-h light-dark cycle (Chaves *et al.*, 2011). They can function as transcription factors, inhibiting expression of other components of the circadian clock (Cashmore, 2003). Cryptochromes may also be involved in phototropic growth of axial corallites in acroporid branching corals (Kaniewska *et al.*, 2009).

We also found large but opposite diurnal changes in transcript abundance of two PAR-domain basic leucine zipper (PAR bZip) transcription factors. Thyrotroph embryonic factor (TEF) had a 78.4-fold increase at noon, and D site-binding protein (DBP) had a 40.7-fold increase at midnight (FDR corrected $P < 0.05$). In zebrafish, some isoforms of TEF are directly induced by light, whereas others show circadian expression. TEF and DBP transcript levels have circadian oscillation in rats; however, in rats they both have peak expression at 1800 h, with a daily 5–10-fold increase for TEF and 200–400-fold for DBP (Fonjallaz *et al.*, 1996).

Both TEF and DBP transcription factors affect transcription of circadian clock genes that have D-box promotors (Zhang and Kay, 2010). The TEFα isoform in zebrafish regulates the expression of many light-induced genes, including cryptochromes (Gavriouchkina *et al.*, 2010). In turn, DBP activity is inhibited by the light-induced cryptochrome-1 (CRY1) (Stratmann *et al.*, 2010). As has been seen in mice (Stratmann *et al.*, 2010), we found increased expression of CRY1 at noon and increased expression of DBP at midnight, suggesting that in *A. hyacinthus* CRY1 also inhibits the expression of DBP during the day. In *A. hyacinthus*, we also found increased expression of TEF during the day. These data suggest that major elements of circadian gene regulation are found in *A. hyacinthus* (see also Levy *et al.*, 2011); yet our full-transcriptome survey could not detect other well-known circadian genes such as *Cycle*, *Clock*, and *Hlf*.

A fourth transcription factor also changed radically in our dataset: a Hairy related transcription factor increased 23.1-fold during the day. Many of the Hairy/Enhancer-of-split genes have oscillatory expression patterns that control developmental timing during embryogenesis (Nakagawa *et al.*, 2000; Kageyama *et al.*, 2007). The Notch signaling pathway controls the expression of Hairy related transcription factors (Mumm and Kopan, 2000; Nakagawa *et al.*, 2000), but none of the Notch genes in our dataset changed expression from day to night. The two contigs of Hairy/Enhancer-of-split genes that increased during the day also have high inter-sample correlation with cryptochrome-1. Either these gene families are highly co-regulated or these Hairy/Enhancer-of-split contigs may be misidentified cryptochrome genes.

The expression patterns of cryptochromes we found in *A. hyacinthus* corroborate results from previous studies (Levy *et al.*, 2007; Hoadley *et al.*, 2011; Levy *et al.*, 2011). In other scleractinian corals the expression of these genes is driven by external light conditions and is not endogenously entrained (Levy *et al.*, 2007; Hoadley *et al.*, 2011; Levy *et al.*, 2011). We have not conducted experiments with *A. hyacinthus* in constant darkness to test whether the other transcription factors in our dataset (TEF, DBP, and Hairy related) are truly circadian or controlled by the environment.

Nevertheless, strong cycling of TEF, DBP, CRY1, and Hairy suggests that many downstream genes might be regulated on a day/night basis in corals. Yet we see noon-midnight differences in only 2.0% of contigs analyzed, and most of these show very low changes. The strong diurnal changes in transcription factors may function to change transcription in only the handful of genes in Cluster I. Alternatively they may set in

motion other cellular shifts that are not driven by transcriptional change. Future investigation of the genes targeted by TEF and DBP in corals, perhaps with a search for TEF and DBP binding sites in D-box promoter regions in coral genomes, may lead to a better understanding of the significance of the temporal difference in expression of these two transcription factors and their physiological implications.

High expression of glucose- and glycogen-related genes at noon

Several sets of genes involved in glucose and glycogen metabolism were highly upregulated at noon, including glycogen-binding subunit 76A and a regulatory subunit of protein phosphatase 1; both are glycogen-targeting subunits that anchor protein phosphatase 1 (PP1) to glycogen-associated substrates. In addition, we found that the *Acropora* sodium/glucose co-transporter 4 had 4.8-fold higher expression at noon. Burriesci *et al*. (2012) found that during the day, when endosymbionts are actively photosynthesizing, glucose is the major translocated metabolite in the cnidarian-dinoflagellate symbiosis, though other metabolites, including some amino acids, fatty acids, and lipids, are also translocated (Gordon and Leggat, 2010). In *Aiptasia,* a sodium/(glucose/myo-inositol) transporter 2 shows significantly higher expression in symbiotic *versus* aposymbiotic animals, suggesting it has an important role in transporting glucose from *Symbiodinium* into host cells (Lehnert *et al.*, 2014). The overall pattern of higher expression of glucose-related genes in *A. hyacinthus* at noon likely corresponds to an increased availability of glucose during the day.

Glucose is stored by cnidarians in the form of glycogen (Gauthier, 1963; Taatjes and Rivera, 1983), and increases in glycogen binding and PP1 protein activity may play a role in increasing glycogen synthesis during the day. Through interactions with glycogen-binding subunits, PP1 dephosphorylates glycogen synthase (the rate-limiting enzyme of glycogen synthesis), which activates it; and dephosphorylates phosphorylase (the rate-limiting enzyme of glycogen breakdown), which deactivates it. Ultimately this results in increased glycogen storage (reviewed in Ceulemans and Bollen, 2004). Ten-to-twelve-fold higher expression of these genes during the day suggests a diurnal increase in glycogen storage, possibly creating an energy reserve to be used at night.

Metabolism and reactive oxygen species

Outside the major expression shifts are 323 other contigs that changed from day to night. About 80 of these changed more than 2-fold, but the rest had low diurnal changes. To explore whether these slight changes might be biologically significant, we asked whether they were clustered into specific metabolic pathways. In other systems, the evolution of expression changes results in coordinated shifts within specific pathways (Fraser *et al.*, 2004).

We hypothesized that there may be increased metabolism at noon compared to midnight due to higher availability of carbon during the day as a result of photosynthesis by endosymbionts (Muscatine and Cernichiari, 1969). We saw significantly higher expression at noon of malate dehydrogenase (MDH) (fold change, 2.5), catalase (CAT) (fold change, 3.8), and superoxide dismutase (SOD) (fold change, 1.1)—all have FDR corrected $P < 0.05$. MDH is involved in aerobic ATP production, and CAT and SOD are antioxidant enzymes involved in converting reactive oxygen species (ROS), which are produced during aerobic respiration, to water (Hochachka and Somero, 2002). In the intertidal mussel *Mytilus californianus*, the enzymatic activities of MDH and CAT are positively correlated with food availability, suggesting that as food availability increases, aerobic ATP production increases, which increases the production of ROS and the need to combat ROS. In addition, SOD activity was correlated with MDH activity, further suggesting that as aerobic metabolic rates increase, the need to combat ROS increases (Dowd *et al.*, 2013). The gene expression patterns of MDH, CAT, and SOD we observed in *A. hyacinthus* may reflect increased aerobic ATP production and ROS production at midday. Alternatively, SOD and CAT may be involved in eliminating ROS produced by the photosynthetic activity of endosymbionts (Levy, 2006).

There may be a link between CAT activity and important transcription factors such as cryptochromes. In zebrafish, catalase (zCAT) is involved in negatively regulating light-dependent circadian gene expression by regulating levels of H_2O_2. Light induces the production of H_2O_2, which in turn induces the expression of cryptochrome-1a (zCRY1a). When zCAT activity reduces H_2O_2 levels, the expression of zCRY1a is inhibited (Hirayama *et al.*, 2007). Whether this system operates in corals is not known. Prior experiments with *A. hyacinthus* (Seneca and Palumbi, 2015) show cryptochrome expression increasing 5 h after imposition of heat stress, then returning to near control levels after 15 h at control temperatures. At the same time, catalase shows no clear pattern of increased expression after the onset of stress exposure.

Aerobic ATP production

We found slight shifts from noon to midnight in the expression of some of the components of the fatty acid degradation, TCA cycle, glycolysis, and oxidative phosphorylation pathways. Although we did not see significant differences in transcript abundance for most of the components of these pathways from noon to midnight, the slight shifts in expression may indicate small day-night adjustments in their activity. We hypothesize that the slight increase in expression at noon of some of the components of the fatty acid degradation pathway (20%–30%) and the

TCA cycle (20%–100%) may be linked. A slight increase in the activity of the TCA cycle pathway during the day may be supported by the increased oxygen availability during the day at the coral diffusive boundary layer (Shashar *et al.*, 1993). These results lead us to hypothesize that the levels of aerobic ATP production are slightly higher during the day than at night. Additional evidence is the slight increase (20%–70%) in expression of nine components of the oxidative phosphorylation pathway at noon, compared to four components with a slight increase (also 20%–70%) at midnight. A complete characterization of the day-night differences in aerobic ATP production in corals will require future investigation.

Response to changes in oxygen levels

We found Egl nine homolog 1 (EGLN1) to have significantly higher expression at midnight (both transcriptome-wide ANOVA and oxygen ANOVA, FDR corrected $P < 0.05$; gray dots in Fig. 4). EGLN1 encodes the Egl nine homolog 1 protein, homologous to prolyl hydroxylase domain protein 2, an oxygen-sensing enzyme that uses O_2 as a substrate and is up-regulated under conditions of hypoxia (Epstein *et al.*, 2001; D'Angelo *et al.*, 2003). Coral reef environments experience diurnal fluctuations in dissolved oxygen saturation (Table 1), and at night the coral diffusive boundary layer very likely has even lower DO levels than the surrounding seawater (Shashar *et al.*, 1993). Several studies have addressed how cnidarians in symbiotic associations with zooxanthellae cope with high oxygen concentrations during the day (*e.g.,* Shick and Dykens, 1985; Levy, 2006), but little is known about how they cope with low oxygen levels at night. During our 10-day sampling period, DO saturation levels never dropped below 52%, but we know that DO saturation in this particular back-reef can be as low as 20% at night (P. Craig, U.S. National Park Service; pers. comm.); the swell during the sampling period cancelled out the back-reef effect, resulting in conditions that are more typical in the fore-reef (as mentioned in the Results section). The significantly higher expression levels of the EGLN1 contigs at midnight could suggest that this was a response to the lower oxygen levels at night.

Our data also show significantly higher expression of a hypoxia-inducible factor 1α (HIF1A) gene at noon (fold-change range among four contigs, 1.3–1.7) (oxygen ANOVA, FDR corrected $P < 0.05$; gray dots in Fig. 4). In well-studied mammalian systems, the physiological response to hypoxia begins with hypoxia-inducible factor 1 (HIF-1), a transcription factor that is composed of subunits HIF1A and HIF1B (reviewed in Guillemin and Krasnow, 1997). Orthologs of HIF-1 are found across metazoans (*e.g., Caenorhabditis elegans* (Epstein *et al.*, 2001), *Carassius carassius* (Rissanen *et al.*, 2006), and *Acropora millepora* (Levy *et al.*, 2011)), and the role of HIF-1 in maintaining oxygen homeostasis in response to hypoxia is believed to be conserved (Lendahl *et al.*, 2009). According to Levy *et al.* (2011), there is an ortholog of HIF1A in *A. millepora* that has an oscillating pattern of temporal gene expression.

Under normoxic conditions, prolyl hydroxylase domain protein 2 (PHD2), homologous to EGLN1, constitutively hydroxylates specific residues on HIF1A, resulting in its degradation (Salceda and Caro, 1997; Epstein *et al.*, 2001). Under hypoxic conditions, the expression of HIF1A increases and it is not degraded, because the hydroxylation reaction carried out by PHD2 requires O_2; therefore, HIF-1 assembles into its active form and accumulates in the cell (Huang *et al.*, 1998; Semenza, 1999; Epstein *et al.*, 2001). An enzyme known as factor inhibiting HIF-1 (FIH-1) can inhibit the transactivation of HIF-1 in the presence of oxygen (Semenza, 2010). In our dataset, a FIH-1 gene shows slightly higher expression at noon (fold-change range across three contigs, 1.0–1.1), but the increase is not statistically significant.

If HIF1A were playing its typical role in the coral cell, its expression would be expected to increase as oxygen levels decrease (Huang *et al.*, 1998; Semenza, 1999; Epstein *et al.*, 2001). However, we see the opposite—30%–70% higher expression of HIF1A at noon when oxygen levels are higher (Fig. 4). There are examples of HIF1A being active in normoxic conditions in mouse stem cells (Iyer *et al.*, 1998), adult mice (Huang *et al.*, 2004), and *Carassius carassius* (Rissanen *et al.*, 2006). These studies suggest that HIF1A has important physiological roles in addition to being the master regulator of the cellular hypoxic response.

Increase in the expression of calcification genes during the day

Calcification-related contigs showed an overall trend of higher expression at noon. This result parallels an observed 2.6–4.0-fold increase in daytime calcification in corals (Furla *et al.*, 2000; Moya *et al.*, 2006). In our dataset of 70 calcification contigs, 52 had higher mean expression at noon than at midnight, and 14 of these differences were significant (calcification ANOVA, FDR corrected $P < 0.05$) (Fig. 5).

Previous work has shown that a secreted form of carbonic anhydrase (STPCA) localized to the calicoblastic ectoderm in *Stylophora pistillata* increases 2-fold in expression during dark conditions (Moya *et al.*, 2008b). We found the opposite pattern: 11 carbonic anhydrase contigs had significantly higher expression at noon (1.2–2.6-fold increases) and none increased at night (calcification ANOVA, FDR corrected $P < 0.05$; gray dots in Fig. 5) (five of these contigs map to three *A. digitifera* genes). In corals, carbonic anhydrases are involved in external uptake of dissolved inorganic carbon and delivery of CO_2 to endosymbionts, and in providing HCO_3^- to the site of calcification (reviewed in Bertucci *et al.*, 2013). The 11 carbonic anhydrase

contigs with significantly higher expression at noon include five secreted or membrane-associated isoforms; four of these are homologs of STPCA. Increases in carbonic anhydrase may indicate an increased demand for CO_3^{2-} for daytime calcification. Laboratory experiments will be needed to refine our understanding of the diurnal role of carbonic anhydrases in coral physiology.

Plasma membrane calcium ATPase is involved in pH regulation in the calicoblastic space and has increased activity during the day (Al-Horani *et al.*, 2003). However, in *Stylophora pistillata,* plasma membrane calcium ATPase is transcribed at the same rate during the day and night (Moya *et al.*, 2008a). Our data confirm this pattern in *A. hyacinthus* (Fig. 5).

Galaxin is a major constituent of the skeletal organic matrix (Fukuda *et al.*, 2003), and we found two galaxin contigs with about a 2-fold increase in expression at noon (FDR corrected $P < 0.05$; gray dots in Fig. 5). Likewise, six other skeletal organic matrix proteins increased 2–4-fold during the day (Fig. 5). High variance in expression of these contigs from day to day prevents these differences from being significant in our analysis. Overall, expression in calcification genes is more variable than in other genes: across sampling dates the coefficient of variation of expression averaged 0.78 for calcification contig compared to a transcriptome-wide average of 0.25.

Number and roles of circadian/diurnal genes in other taxa

We found that 2.0% of the transcriptome of *A. hyacinthus* was differentially expressed from noon to midnight. Our data include replicate samples at each of 11 time points from a single coral colony and therefore minimize variation that might otherwise prevent day-night differences from being statistically significant. As a result, our low number of significant genes is probably not a function of low statistical power. The addition of other colonies to our analysis, at the cost of biological replication, would have resulted in an even smaller fraction of the transcriptome showing differential expression from noon to midnight. For example, Seneca and Palumbi (2015) examined gene expression patterns for different colonies of *A. hyacinthus* and found that over half of the contigs (8,509 out of 15,753) showed strong variation among individuals (FDR $P < 0.001$). In an earlier study of this species, Barshis *et al.* (2013) examined gene expression variation among 11 colonies in response to heat stress. Very few genes with expression changes less than 2-fold were significant (less than 2%) (Barshis *et al.*, 2013). By strong contrast, in our current study, biological replication within the same coral colony reduced variance among samples and produced evidence for significant gene expression change of less than 2-fold in 248 of 341 contigs. We suspect that some of the 248 contigs with mild expression differences from day to night in the sampled colony may not be differentially expressed in all conspecific colonies. However, the major shifts we observed (*e.g.,* Fig. 2. Cluster I, fold change 10) are likely to be more general for *A. hyacinthus*, and perhaps for other coral species. For example, Levy *et al.* (2011) detected similar patterns in *A. millepora* for cryptochrome genes, though other transcription factors we found to be highly variable may not have been on the DNA chip they used.

The number of diurnally fluctuating coral genes identified here and by Levy *et al.* (2011) appears to be lower than found in other species. In *Mytilus californianus,* 37.8% of 10,410 genes examined are circadian (Connor and Gracey, 2011). In mouse liver, the number of circadian genes ranges from 10.5%to 19.7% (Miller *et al.*, 2007; Hughes *et al.*, 2009). In maize, 10.0% of 13,339 genes are circadian (Khan *et al.*, 2010). A few systems show lower levels of differentiation in day/night expression. In mouse skeletal muscle, the circadian fraction is much smaller: from 0.6% to 3.4% (out of 36,182) (McCarthy *et al.*, 2007; Miller *et al.*, 2007). In *Arabidopsis,* 2.0%–6.0% of genes are circadian (Harmer *et al.*, 2000; Schaffer *et al.*, 2001).

Across these species there is some consistency in the functions of circadian genes. In mouse liver and skeletal muscle tissue, circadian genes are mostly involved in metabolism and biosynthesis (Miller *et al.*, 2007). The timing of peak expression and functional roles of mouse circadian genes suggests that the circadian clock is involved in maintaining homeostasis (McCarthy *et al.*, 2007). In flies and mammals, the circadian clock is involved in coordinating the timing of feeding with the timing of the activity of proteins that function in digestion (Panda *et al.*, 2002). In *Arabidopsis,* most circadian genes are involved in metabolism and response to light (Harmer *et al.*, 2000). And in maize, the functional role of circadian genes suggests there is circadian regulation of energy/carbohydrate metabolism and photosynthesis (Khan *et al.*, 2010). Likewise in corals, our data suggest there are diurnal shifts in glucose metabolism.

In many non-mammalian species, circadian genes have been found to have peak expression either at dawn or dusk. This is true for circadian transcripts in *M. californianus* (Connor and Gracey, 2011), maize (Khan *et al.*, 2010), and *Arabidopsis* (Michael *et al.*, 2008). Because we sampled at noon and midnight, our dataset may miss these cyclic gene patterns. Few such patterns were seen by Levy *et al.* (2011); however, many genes examined in that study, such as heat shock proteins and cell cycling proteins, peak in the late afternoon rather than exactly at noon, and so our data might underestimate the change they undergo.

Conclusion

Two major physiological activities with known day-night cycles occur within the coral holobiont: photosynthesis by endosymbiotic dinoflagellates (Muscatine and Cernichiari,

1969) and calcification of the coral skeleton (reviewed in Cohen and McConnaughey, 2003). In addition to these internal day-night shifts, corals are exposed to daily environmental variability in pH, temperature, and dissolved oxygen saturation (Craig *et al.*, 2001; Hofmann *et al.*, 2011; Smith *et al.*, 2013). Our analysis of day-night differences in genome-wide patterns of gene expression in *Acropora hyacinthus* shows a set of transcription factors with large changes. We also see milder diurnal shifts in glucose, fatty acid, and reactive oxygen metabolism, in addition to the slight up-regulation of most calcification contigs at noon. So little is known about the way coral genes are regulated and how transcription factors operate that we can build hypotheses about the functional significance of these gene expression changes only by comparison to other animals and by examining correlations between changes in gene expression. Our data suggest that the transcription factors TEF, CRY1, and DBP interact during the day-night cycle, just as they do in other animals. The mechanisms of these changes and their physiological consequences are not known.

Despite major day-night differences that occur within the holobiont and externally in the environment, we found significant differences in expression in only 2.0% of contigs analyzed, many of which have been identified as circadian genes by previous studies. This low fraction may suggest that corals, which seem to be more affected by day-night differences in their environment than other species, have low rates of day-night transcriptional regulation. Such transcriptional stability may point to alternative modes of regulating metabolism and maintaining homeostasis over the daily cycle.

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Appendix

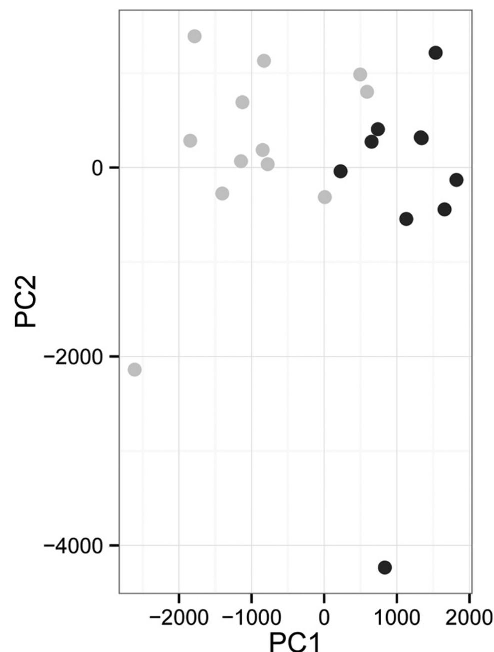

Figure A1. Principal components one and two from the principal components analysis of the normalized counts for the 16,800 contigs. Gray, noon samples. Black, midnight samples.