Title: Diurnal and nocturnal transcriptomic variation in the Caribbean staghorn coral, *Acropora cervicornis*

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Running Title: Diel transcriptomic variation in *Acropora*

Abstract

Reef-building corals experience large diel shifts in their environment, both externally due to changes in light intensity, predator activity and prey availability, and internally as a result of diel fluctuations in photosynthesis by their endosymbiotic algae, *Symbiodinium*. Diel patterns of tentacle
behavior, skeletal growth, and gene expression indicate reactions of the coral animal in response to light and through circadian regulation. Some corals, such as the Caribbean Acropora, have strong within-colony division of labor, including specialized fast-growing apical polyps, accompanied by large gene expression differences. Here we use RNA-seq to evaluate how diel changes in gene expression vary within the branching Caribbean staghorn coral, Acropora cervicornis, between branch tips and branch bases. Multifactor generalized linear model analysis indicated that 6% (3005) of transcripts were differentially expressed between branch tips and bases, while 1% (441) of transcripts were differentially expressed between day and night. The gene expression patterns of 220 transcripts were affected by both time of day and location within the colony. In particular, photoreceptors, putative circadian genes, stress response genes and metabolic genes were differentially expressed between day and night, and some of these, including Amcry1, tef and hebp2, exhibited location-specific regulation within the coral colony as well. These findings indicate that the genetic response of the coral to day and night conditions varies within the colony. Both time of day and location within the colony are factors that should be considered in future coral gene expression experiments.

Introduction

The daily rotation of the earth subjects organisms to predictable oscillations of abiotic factors, such as light, temperature and tides, and biotic factors, such as predation and prey availability. To moderate the effects of these diel changes, organisms often alter their behavior and physiology by responding directly to environmental cues (e.g. light/dark) or through circadian regulation (Reitzel et al. 2013). A significant portion an organism’s transcriptome can exhibit daily rhythms; for example, more than 20% of gene transcripts show daily fluctuations in intertidal
mussels (Connor & Gracey 2011) and up to a third of expressed genes are under circadian control in plants (Covington et al. 2008).

Hermatypic (or reef-building) corals inhabit tropical marine waters and live in symbiosis with dinoflagellate algae (Symbiodinium, aka zooxanthellae) that provide 40% to 100% of the coral’s energy requirements (Falkowski et al. 1984; Muscatine et al. 1984). Corals experience large diel changes in their physiology between day and night to deal with the shift from acquiring food from their photosynthetic endosymbionts during the day to being heterotrophic at night, when they feed on demersal zooplankton (Heidelberg et al. 2004). Daytime stresses include high UV radiation (UVR) and light exposure and the production of reactive oxygen species (ROS) by Symbiodinium during photosynthesis (Lesser 1996; 1997), while nighttime stress results from respiration by coral and Symbiodinium which can lead to hypoxic conditions and decreased pH within the coral tissues (Kühl et al. 1995).

As sessile organisms, coral behavioral changes include polyp and tentacle extension and retraction, which often display diel patterns (Levy et al. 2006). Tentacle behaviors, which may help the animal avoid predation while maximizing photosynthesis, heterotrophic feeding and gas exchange (Levy et al. 2006), appear to be light-responsive, but may also have a circadian component of regulation (Tsang et al. 1997; Levy 2003). In some corals, such as the Caribbean Acropora corals, tentacle behavior varies between different parts of the colony (E. Hemond & S. Vollmer, personal observation), suggesting complex regulation beyond uniform light-response. Tentacles of Symbiodinium-rich radial polyps throughout most of the colony are extended during the day to capture light and remain extended at night, while axial polyps at the tip of the branch, which contain few Symbiodinium, are retracted during the day and only extended at night for feeding on active zooplankton.

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Diel gene expression patterns have been observed in anthozoans, including corals, in response to both light and circadian regulation (Levy et al. 2007; Reitzel et al. 2010; Brady et al. 2011; Levy et al. 2011). Alteration of gene expression and subsequent protein production at regular intervals based on time of day is probably necessary for the coral to cope with cyclic patterns of stress (UV and ROS), facilitate digestion of molecules obtained from Symbiodinium or heterotrophy, and manage algal densities. Corals and other anthozoans possess a number of circadian genes (Vize 2009; Reitzel et al. 2010) as well as photoreceptors, such as cryptochromes (crys) and opsins, that may also interact with circadian genes (Levy et al. 2007; Shoguchi et al. 2013). It is not yet understood what coral functions may be under circadian regulation (Sorek et al. 2014); however, some stress response chaperone proteins, specifically heat shock proteins, are expressed with circadian rhythmicity (Levy et al. 2011). Coral photoreceptors are suspected to be involved in a number of light-responsive functions, such as growth (Kaniewska et al. 2009), polyp expansion or contraction (Levy 2003), and spawning (Levy et al. 2007; Brady et al. 2009).

In many corals, calcification increases in the light in a phenomenon now referred to as ‘light-enhanced calcification’ (LEC) (Goreau & Goreau 1959), which is related to light availability and Symbiodinium photosynthetic activity (Moya 2006). The Caribbean staghorn coral, Acropora cervicornis, is a good model for studying coral growth processes because of its rapid growth rate, its branching morphology, and a polyp-based division of labor (DOL) within the colony. A. cervicornis has dimorphic polyps: 1) radial polyps that contain a high concentration of Symbiodinium along the branch and are the site of gamete production, and 2) axial polyps at the branch tip that are the location of linear skeletal growth and that contain lower Symbiodinium concentrations. Although A. cervicornis branch tips contain fewer Symbiodinium, they have the highest calcification rates (Goreau & Goreau 1959), probably due to transfer of Symbiodinium-produced photosynthate.
Calcification in *A. cervicornis* exhibits a diel pattern in the quality of skeleton deposited at the axial corallite (Gladfelter 1983a) and predictable changes in the density of skeleton at different regions along the colony branch (Gladfelter 1982).

The DOL within Caribbean *Acropora* coral colonies represents substantial differences in function. For example, gamete production occurs only in mature radial polyps at least 2 cm from the branch tip (Szmant 1986), and the metabolic rate in branch tips is higher than the rest of the colony (Gladfelter *et al.* 1989). Differences in gene expression between branch bases and tips have been found to represent a substantial fraction, approximately 10%, of the transcriptome, including functions such as developmental signaling, metabolism and calcification (Hemond *et al.* 2014). In corals with DOL and variation in the density of *Symbiodinium* within the colony, such as *Acropora*, there may also be location-specific, diel changes in gene expression. While studies of Pacific *Acropora* corals have indicated gene expression differences between day and night, or light and dark, with particular interest in circadian regulation or LEC (Levy *et al.* 2007; Brady *et al.* 2011; Levy *et al.* 2011), no study has yet compared full transcriptome differences between day and night in functionally distinct parts of the colony.

To identify diel and within-colony gene expression differences, we compared transcriptome-wide gene expression patterns of branch tips and bases in the Caribbean staghorn coral, *A. cervicornis*, in its natural reef environment at mid-day versus mid-night using RNA sequencing (RNA-seq). Full transcriptome data was analyzed with a multi-factor generalized linear model (GLM) to identify transcripts whose expression differs between day and night and within the coral colony (branch tips vs. bases), as well as whose expression depends on both factors.
(significant for both factors or having a significant interaction between factors). We hypothesized that transcripts involved in LEC, phototropism or polyp behavior should show differences in expression between day and night in tips, while transcripts related to the coral's response to the symbiont's photosynthetic activity and population growth should display differences between day and night in bases, where symbiont densities are highest. Transcripts with a significant interaction effect may also be involved in these processes. Genes expressed differentially between day and night throughout the colony may be involved in stress response, circadian rhythms and other functions that may be shared among all polyps.

Materials and Methods

Sample collection & RNA extraction

*Acropora cervicornis* samples were collected from three large coral colonies located at least 10 meters apart at 5-6 meters depth in Crawl Cay, Bocas del Toro, Panama (09 15.517N 082 07.625W). Paired samples were taken from individual branches, with the base sample taken at 25-30 cm from the tip of the branch, and the tip sample being the top 1 cm of the branch, including the axial polyp. For each colony, one branch was sampled at 12:00-13:00 (~6 hrs post-sunrise) on August 2 (sunrise 6:09, sunset 18:39, moonrise 23:46, third quarter), and one branch was sampled at midnight-00:30 (~5.5 hrs past sunset) on August 3, 2010. At the time of the nighttime sampling, corals had been exposed to little, if any, moonlight; however, some exposure to dive lights was unavoidable while collecting. Temperature data was collected from a permanent data logger near Crawl Cay (Cayo Agua; data publicly available at http://biogeodb.stri.si.edu/physical_monitoring/research/bocas). Seawater temperature was 29.7-29.8°C during daytime sampling (12:00-13:00) and 29.6°C during nighttime sampling (24:00-00:30).
Coral samples were transported to the boat in covered plastic containers, then wrapped in aluminum foil, immediately preserved in liquid nitrogen, and stored at -80°C.

Illumina RNA-seq library preparation

Total RNA extraction was performed using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) following the manufacturer’s protocol, with an additional 75% ethanol wash step. Total RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and RNA Pico Chips, and only extractions with distinct 18s and 28S ribosomal RNA peaks were used (RIN values 4.9-8.0). mRNA was isolated using Dynabeads® oligo(dT) mRNA isolation beads (Life Technologies, Grand Island, NY) to exclude non-protein coding RNAs and non-eukaryotic mRNAs from the samples. cDNA was produced using random hexamer primers and SuperScript® II reverse transcriptase (Life Technologies) for first strand synthesis and DNA polymerase I (New England BioLabs, Ipswich, MA) for second strand synthesis. RNA-seq libraries were prepared by fragmenting the double stranded cDNA with dsDNA fragmentase (NEB) for 30 minutes at 37°C, followed by library preparation using NEBNext® next-generation sequencing modules. This included end-repair, A-tailing, and ligation of custom 4bp barcoded adapters. The cDNA transcripts with barcoded adapters were then size selected at 250 bp by gel extraction and amplified with 15 rounds of PCR. RNA-seq libraries were sequenced (6 samples per lane) using single-end 50 bp sequencing on the Illumina Hi Seq 2000 platform (Illumina, Inc., San Diego, CA, USA) at the FAS Center for System Biology at Harvard University.
Bioinformatics

Read quality control and barcode removal were conducted with custom Perl scripts in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). RNA-seq reads were mapped against our published de novo transcriptome for *A. cervicornis* and *A. palmata* (Libro *et al.* 2013; Hemond *et al.* 2014) using CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Reference transcriptome annotation was conducted using translated nucleotide queries against the curated UniProt/Swiss-Prot database and a threshold E-value of $10^{-5}$. Coral genes were identified by aligning the de novo reference transcriptome contigs to the genomes of congener *A. digitifera* (Shinzato *et al.* 2011) and *A. millepora* (D. Miller, unpublished). Transcripts with a significant BLAST hit (E-value < $10^{-10}$) were identified as coral and retained for the analyses. Putative non-coral transcripts were removed.

Statistical analysis of differential expression

Using the DESeq package v. 1.14.0 (Anders & Huber 2010) in R v. 3.0.2 (R Development Core Team, 2012), the RNA-seq read count data were normalized by sample library size. Non-metric multidimensional scaling (nMDS; isoMDS) and PERMANOVA (Adonis) were conducted on the normalized data using the MASS v. 7.3.29 and vegan v. 2.0.10 packages (Venables & Ripley 2002; Oksanen *et al.*) in R to examine transcriptome-wide differences in gene expression. Differentially expressed (DE) gene transcripts were then identified using a three-factor negative binomial Wald test (design = ~ colony + position * time) in DESeq2 v 1.2.10 (Anders & Huber 2010; Love *et al.* 2014) with colony position (base vs. tip), time (day vs. night) and colony (genet) as the factors. An interaction effect between position and time factors was also tested. Colony (genet) was included as a factor only to control for inter-colony variation. An adjusted p-value < 0.05 was used to evaluate significance (Benjamini & Hochberg 1995). For the colony position factor, gene expression values in
bases were assigned as the baseline for comparison, so DE transcripts are considered ‘up-regulated’ or ‘down-regulated’ in tips, relative to bases. For the time of day factor, gene expression values in night samples were assigned as the baseline, so DE transcripts are considered ‘up-regulated’ or ‘down-regulated’ during the day. The moderated log$_2$(fold change) values provided are maximum a posteriori estimations based on data-driven prior distributions calculated in DESeq2 (Love et al. 2014).

Functional genetic pathway analysis

Using UniProt accession IDs, contigs were annotated with Gene Ontology (GO) terms (www.geneontology.org). Transcripts of interest were determined based on GO terms and a review of the literature for transcripts that were DE between day and night, DE for both factors or that had a significant interaction between the two primary factors. Heat maps of gene expression data were created using log-transformed normalized count data in GENE-E (Gould). For the cryptochrome genes, an additional annotation step was performed using nucleotide BLAST (blastn) through NCBI’s reference databases to more precisely discuss their known roles as putative circadian and light-response genes in cnidarians.

Results & Discussion

For the twelve samples (six sets of paired samples) RNA-seq libraries ranged in size from 8.4 to 22.5 million reads (16.6 million ± 1.2 million, mean ± SE) (Supplementary File S1), with 4.1 to 15.8 million reads (9.2 million ± 0.9 million) mapping to coral transcripts. PERMANOVA analysis revealed significant differences in transcriptome-wide gene expression patterns between branch bases and tips (d.f. = 1, Pseudo-F = 5.9631, P = 0.001), but not between day and night (d.f. = 1, Pseudo-F = 1.9308, P = 0.072) or among colonies (d.f. = 1, Pseudo-F = 1.9992, P = 0.08).
interaction between colony position and time of day was also not significant (d.f. = 1, Pseudo-F = 1.1943, P = 0.261). The difference in gene expression profiles between tips and bases can be seen in the nMDS plot (Figure 1), in which transcription profiles cluster according to colony position along the x-axis.

Of the 47,688 transcripts in the analysis, a greater number of DE transcripts were observed between tips and bases of the *A. cervicornis* colony (3005 transcripts) than between day and night (441 transcripts) (Figure 2A). This indicates strong within-colony division of labor (DOL) between branch tips and bases in addition to moderate diel variation. The 231 transcripts that differed by colony position and time of day or had a significant interaction effect demonstrate that gene expression patterns underlying the within-colony DOL include a diel component. Approximately 55% of the DE transcripts had strong protein annotations (Figure 2B; Supplementary File S2).

While corals have previously been shown to exhibit clear diel patterns of calcification (Barnes & Crossland 1980; Gladfelter 1983a; Moya 2006; Schneider et al. 2009) and tentacle behavior (Levy 2003; Levy et al. 2006), as well as light-responsive and circadian patterns of gene expression (Levy et al. 2007; Brady et al. 2011; Levy et al. 2011; Sorek et al. 2014), our results suggest that diel shifts in gene expression between mid-day and midnight occur at less than 1% of *A. cervicornis*’s transcriptome, and thus diel variation may be relatively modest compared to that observed in some other marine organisms, like mussels, where over 20% of the transcriptome shows diel oscillations (Connor & Gracey 2011). In *A. cervicornis*, genes that differed significantly between day and night were involved in a variety of processes including regulating circadian rhythm and photoreception, mediating stress responses and immunity, as well as nitrogen, lipid.
and carbohydrate metabolism and carbohydrate transport. Selected DE transcript expression patterns are shown in figure 3.

Circadian rhythms & Photoreception

Eight transcripts representing regulatory and response components of the animal circadian clock (circadian locomoter output cycles protein kaput, \textit{clock}; neuronal PAS domain-containing protein 2, \textit{npas2}; cryptochromes, \textit{cry} & \textit{Amcry}; d site-binding protein, \textit{dbp}; and thyrotroph embryonic factor, \textit{tef}) were DE by time of day (Table 1; Figure 4). Two transcripts of \textit{Amcry1} and a transcript of \textit{tef}, had a significant interaction effect in which each exhibited a much higher (doubled) fold change up-regulation in day in tips compared to bases. In animals, the core circadian oscillation of genes occurs through positive and negative transcriptional feedback loops. Our results indicate that some circadian genes are differentially expressed between day and night; however, further studies sampling at multiple time points and observing rhythmicity of gene expression under manipulated light conditions would be required to determine endogenous circadian regulation of these genes.

We observed down-regulation of the circadian transcription factor \textit{clock} during the day, which is consistent with previous findings of down-regulation of \textit{clock} in light in \textit{A. millepora} adult colonies; although larvae were previously found to up-regulate \textit{clock} in light (Brady et al. 2011). Interestingly, \textit{npas2}, a \textit{clock} paralog that acts as a distinct circadian regulator in some mammalian tissues (Reick 2001), and which has not previously been studied in cnidarians, was up-regulated during the day, indicating that Clock and NPAS2 may function independently of one another in cnidarians. The other circadian gene down-regulated during the day, \textit{dbp}, is a circadian output PAR.
bZip transcription factor that may enhance production of enzymes involved in detoxification and metabolism important for processing food (Gachon et al. 2006). Corals would have a greater requirement for such enzymes at night when a greater abundance of demersal planktonic prey are available for consumption (Heidelberg et al. 2004).

Cryptochromes (Crys) are blue light sensitive photolyase-like proteins that act as photoreceptors to entrain the circadian clock in insects, but not in mammals, where they act in a light-independent part of the circadian oscillator feedback loop (Lin & Todo 2005). Cnidarian crys are more closely related to their mammalian counterparts, yet cnidarian cry gene expression appears to be light-induced (Levy et al. 2007; Reitzel et al. 2010) with only Amcry2 showing some circadian periodicity (Brady et al. 2011). Correlation of Amcry2 with lunar light exposure may be involved in regulation of coral spawning behavior (Levy et al. 2007). In this dataset, Amcry2 and a transcript annotating to a mammalian-type cry2, were up-regulated during the day; whereas two Amcry1 transcripts were up-regulated during the day and had a significant interaction effect due to greater fold change in tips, similar to the circadian output transcription factor tef. This within-colony variation observed in the expression of Amcry1 and tef genes might reflect an effect of Symbiodinium on coral circadian gene expression, possibly through photosynthetic activity which strongly alters the oxygen content of the polyp tissue (Kühl et al. 1995). In a previous study of symbiont-containing adult versus aposymbiotic A. millepora larvae, a higher fold change of Amcry1 between light and dark was found for larva than adults (Brady et al. 2011). It is possible that Amcry1 expression change may be affected in cells containing or located near a high concentration of Symbiodinium through the effect of redox state on the circadian clock (Stangherlin & Reddy 2013). In A. cervicornis during the day, polyps located in the branch bases having a high density of Symbiodinium are likely to experience higher levels of ROS, including hydrogen peroxide (H₂O₂).
compared to polyps in branch tips with a lower density of algal cells. H$_2$O$_2$ is able to diffuse through cell membranes (Lesser 2006), and could therefore move freely between the endoderm where Symbiodinium are located and ectoderm where cryptochrome proteins have been found (Levy et al. 2007). A link may exist between tef expression and ROS due to the potential circadian regulation of DNA repair and ROS stress response proteins by tef, which has been observed in zebrafish (Gavriouchkina et al. 2010). A possible connection between ROS stress and regulation of circadian genes in corals warrants further investigation.

An alternate hypothesis for a greater magnitude change in expression of Amcry1 in tips is a potential role outside of circadian regulation, specifically in the blue-light-activated phototropic growth observed in branching Acropora. In Acropora, the production and direction of growth of new axial corallites respond to blue light (Kaniewska et al. 2009), but the mechanism for this is not known. Crys, which are known to modulate phototropic growth in plants (Goyal et al. 2013) and may play a phototaxic role in sponges (Rivera et al. 2012), should also be considered as a potential candidate for phototropism in corals.

Genes involved in production of components of rhodopsin-like photoreceptors were found to be DE both by time of day and colony position. Rhodopsin, comprised of a transmembrane opsin apoprotein with photosensitive retinal chromophore, is a low-light photoreceptor that allows nighttime vision in animals. The production of retinal from retinol (vitamin A) is catalyzed by the enzyme Retinol dehydrogenase (Rdh). One rhodopsin-like opsin was down-regulated during the day and three transcripts of rdh were observed with differing patterns of expression. rdh3 was most highly expressed in tips at night, rdh7 was most highly expressed in tips during the day, and...
rh8 was down-regulated in tips. In addition to its role in producing retinal, Rdh can also produce retinaldehyde, which can be further oxidized to retinoic acid, an important signaling molecule used in embryonic patterning as well as circadian gene regulation (Shirai et al. 2006). The multiple gene expression patterns found for rdh genes suggest that Rdhs are involved in multiple functional pathways. Up-regulation of rdh7 in tips during the day suggests it may be involved in phototropic growth.

**Stress response & immunity**

Predictably, UV and ROS stress response genes were mostly up-regulated during the day, but interestingly the degree of up-regulation for some genes varied within the colony. The UV stress response gene deoxyribodipyrimidine photo-lyase (phr), which codes for a light-induced DNA-repair enzyme, was up-regulated both during the day and also in branch tips. Photoreactivation, the light-induced repair of UV-damaged DNA through correction of pyrimidine dimers, is the primary DNA-repair mechanism in coral larvae (Reef et al. 2009), and it appears to be important for mature colonies as well, particularly in the growing tips. Growing tips may be more vulnerable to UV-induced DNA damage due to the production of new polyps through asexual reproduction, the high rate of mitosis, and the lower concentration of Symbiodinium, which absorb and block UVR through shading and mycosporine-like amino acids (MAAs; Dunlap & Shick 1998).

Additionally, while three transcripts of GFP-like fluorescent chromoproteins (FP486 and FP506) were up-regulated in tips only, one GFP-like non-fluorescent chromoprotein (gtCP), was up-regulated both in tips and during the day. Elevated expression of GFP-like proteins in growing regions of corals, has previously been observed in Acropora and other species (Bay et al. 2009;
D'Angelo et al. 2012), and up-regulation during the day is consistent with the observed increase in transcription and production of GFP-like proteins in response to blue light (D’Angelo et al. 2008). Our results indicate that different chromoproteins may be functioning differently within the colony. Numerous roles have been suggested for GFP-like proteins, including photoprotection, stress/redox response, symbiont interactions and a potential role in growth (D’Angelo et al. 2008). Higher expression of both known and putative UV stress genes in branch tips suggests that this region of the colony must cope with elevated UV stress compared to branch bases.

Excessive oxidative stress resulting from ROS produced by Symbiodinium and coral under high light and temperature conditions is believed to be the primary cause of mass coral bleaching episodes (Weis 2008). Multiple oxidative stress response genes have been proposed to be important in maintaining the symbiotic partnership (Downs et al. 2002; DeSalvo et al. 2008; Sunagawa et al. 2009). Three of these genes showed both diel and within-colony differential expression. These include thioredoxin, glutathione S-transferase (gst), and extracellular superoxide dismutase (sod). Thioredoxin is a small redox protein found in all organisms that can participate in redox signaling or antioxidant activity by reducing other proteins by cysteine thiol-disulfide exchange. GST is an enzyme that inactivates the oxidizing activity of substrates by conjugation with the antioxidant glutathione. Both thioredoxin and gst were up-regulated during the day, particularly in branch tips. Higher ROS during the day resulting from increased symbiont photosynthesis is expected to increase oxidative pressure on the host and trigger elevated expression of oxidative stress response proteins. However, these redox proteins were also up-regulated in tips, which due to a lower concentration of photosymbionts are expected to have lower ROS stress. One explanation for up-regulation in tips may be that UVR itself can induce ROS in animal tissues (Pourzand & Tyrrell 1999). Elevated UVR exposure in branch tips, which are shallower than bases and probably
benefit less from symbiont-produced photo-protective MAAs, may account for the observed elevated oxidative stress response. Alternatively, production of antioxidants by the symbiont itself may alleviate the burden of oxidative stress in symbiont-rich regions of the coral (Rodriguez-Lanetty et al. 2006). Interestingly, although counterintuitive based on the expectation of ROS generation by algal symbionts (Lesser 1997; 2006), an inverse relationship between Symbiodinium and ROS response genes, including \(gst\) and \(sod\), has been observed in multiple studies comparing symbiotic and non-symbiotic cnidarians (Rodriguez-Lanetty et al. 2006; Ganot et al. 2011; Lehnert et al. 2014). One transcript of \(sod\) had a significant interaction effect with the lowest expression during the day in tips and highest at night in tips. SOD is an antioxidant enzyme that catalyzes the dismutation of the free radical superoxide (\(\bullet O_2^-\)) into hydrogen peroxide (\(H_2O_2\)). The pattern of expression observed for \(sod\) suggests that it has a function other than (or in addition to) coping with Symbiodinium-produced ROS, possibly related to ROS produced during metabolism of heterotrophically obtained food. As mentioned earlier, transcription of stress response genes may also be directly or indirectly linked to circadian rhythm. For example, in mammals \(gst\) transcription may be stimulated by \(tef\) and/or \(dbp\) transcription factors (Gachon et al. 2006).

Three iron homeostasis genes with putative roles in oxidative stress response, heme-binding protein 2 (\(hebp2\)), soma ferritin (\(sof\)), and mitoferrin-2, were up-regulated during the day, indicating differential use of iron between day and night. The reaction of ferrous iron (\(Fe^{2+}\)) with \(H_2O_2\) produces the highly reactive hydroxyl radical (\(\bullet OH\)); therefore, proteins that chelate iron or bind heme can act as antioxidants (Yu 1994). In this dataset, \(hebp2\) had a similar expression pattern with \(Amcry1\) and \(tef\), being up-regulated during the day with a 2-fold greater change in tips than bases. \(hebp2\) was previously observed to follow a synexpression pattern with \(\alpha\)-CA, cry1 and cry2 in
A. millepora, indicating a potential link between redox state, hebp2 and circadian expression (Levy et al. 2011).

Regulation of Symbiodinium by the coral host

The breakdown of the coral-algal symbiosis during coral bleaching under thermal and UV stress is considered one of the most serious threats to the future of coral reefs (Hoegh-Guldberg 1999). The healthy coral-algal symbiosis depends on the coral regulating the population biomass of algae through three proposed mechanisms: expulsion or degradation of excess symbionts or inhibition of symbiont growth and division (Davy et al. 2012). We identified two candidate genes that may be involved in such regulation: a nitrogen metabolism gene, glutamine synthetase (gs), and an immunity-related gene, hemagglutinin/amebocyte aggregation factor (haaf). In addition to gs, other glutamine/glutamate pathway genes (Figure 5) had diel and within-colony differences, indicating that the coral may use transcriptional regulation of this nitrogen metabolic pathway to regulate its symbiont population.

Nitrogen metabolism may play an important role in regulating Symbiodinium population growth by restricting the available nitrogen (Rees 1986). Glutamine synthetase (GS), which is required for the assimilation of nitrogen from ammonium into glutamine (Figure 5), has been suggested as a mechanism of symbiont control (Rees 1986). The pattern of gs expression, which was higher in branch bases than branch tips during the day, with a significant interaction effect, supports the hypothesis of nitrogen control in symbiont-rich base polyps during the day when Symbiodinium growth would be at its height (Wang et al. 2008). However, it should be noted that GS protein levels may also be regulated post-translationally by the level of glutamine present (Labow...
Interestingly, the highest expression of gs was in symbiont-poor tips at night, which may be related to processing nitrogen waste derived from heterotrophy. *Glutamate synthase ([NADH]) (gogat)*, which breaks down glutamine to glutamate, was elevated at night, particularly in branch bases. GOGAT produces two molecules of glutamate from glutamine and 2-oxoglutarate, without producing ammonia. Glutamate is an excitatory amino acid that may be used in signaling and to synthesize other amino acids. In a study of symbiotic and aposymbiotic *Aiptasia*, Lehnert et al. (2014) observed increased gs and gogat expression in symbiotic anemones, supporting the idea that host cnidarians alter their nitrogen metabolism in the presence of *Symbiodinium*. Genes for carbamoyl-phosphate synthase I (CPS1), which catalyzes the synthesis of carbamoyl phosphate from glutamine-derived ammonia and bicarbonate, and a putative glutamine amidotransferase (GATase), which transfers the ammonia group from glutamine to other substrates, were up-regulated during the day and had the lowest expression in bases at night. The expression patterns of these genes suggest that nitrogen cycling is elevated in bases during the day, where there is a higher concentration of symbionts, and that nitrogen availability for *Symbiodinium* may be controlled by conversion to glutamine followed by transfer of the ammonia group to carbamoyl phosphate and ultimately to urea or to other molecules.

The daytime up-regulation of the immune response gene *haaf* in bases suggests enhanced amebocyte activity to promote phagocytic clearance of dysfunctional or damaged *Symbiodinium*. Mobile amebocytes are part of the coral’s innate immune response and remove infecting pathogens or necrotic tissue via phagocytosis (Libro et al. 2013), but *haaf* and amebocytes have also been found to respond to elevated heat stress in soft corals (octocorallia) (Woo et al. 2006; Mydlarz et al. 2008). It has been suggested that amebocytes may be activated under heat stressed conditions to dispose of damaged *Symbiodinium* cells (Strychar et al. 2004). Phagocytosis is one potential
mechanism of coral bleaching, but may also be a way for corals to manage symbiont populations when subjected to stressful (e.g. high light and ROS) conditions, even when bleaching is not the ultimate outcome (Stat et al. 2006). Because no sign of disease, bleaching or wounding was observed on these corals prior to collection and there was no difference in the way the corals were collected, the location and timing of elevated expression of an amebocyte-related gene suggests a role for these cells in regulating Symbiodinium populations under high light conditions.

Light-enhanced calcification

In Acropora spp. quantity and quality of CaCO$_3$ deposited into the skeleton as well as mitotic activity differ between day and night. At night the linear branch extension rate and mitotic activity are higher and CaCO$_3$ crystals accumulate in random orientation at the growing edge of the skeleton (Barnes & Crossland 1977; Gladfelter 1983a; Gladfelter et al. 1989). During the day, a larger quantity of CaCO$_3$ is incorporated to reinforce the skeleton (Barnes & Crossland 1977; Gladfelter 1983a). Functionally, this suggests that nighttime conditions promote abiotic precipitation of CaCO$_3$, while in the daytime coral-regulated activity requiring energetic inputs, such as skeletal organic matrix (SOM), encourages directed skeleton formation. We have identified two genes with putative roles in LEC, carbonic anhydrase 2 (CA2) and mucin-4, and multiple genes that may provide the energy for LEC through metabolic functions or transport.

Two transcripts of CA2, an α-CA, were up-regulated in tips and also up-regulated during the day; however another transcript of CA2 was up-regulated in bases but did not show differential expression between day and night. Carbonic anhydrase (CA), including CA2, was previously identified as highly up-regulated in the growing tips of Acropora corals (Hemond et al. 2014) and is
known to be involved in coral skeleton growth (Goreau 1959; Tambutte et al. 2006). CA, which catalyzes the interconversion of carbon dioxide and water to bicarbonate and protons, may participate in coral chemistry by providing bicarbonate for CaCO₃ production and by regulating pH, but it may also provide CO₂ for symbiont photosynthesis (Bertucci et al. 2013). Increased expression of CA during the day in colonies of A. millepora (Levy et al. 2011) supports a possible role of CA in LEC.

A potential SOM protein, mucin-4, that was up-regulated during the day in tips is similar to a protein extracted from A. millepora SOM (Ramos-Silva et al. 2013) and may play a role in LEC. Mucins, which are the primary functional protein component of the gel-like mucus secreted by corals to protect against stresses and trap food particles, may also have been some of the first molecules that evolved to control biomineralization (Marin et al. 2000). Examples of mucin-like calcification-related proteins have previously been found in corals, galaxin (Reyes-Bermudez et al. 2009), and molluscs, mucoperlin (Marin et al. 2000).

Carbohydrate and lipid metabolism and transport

Carbon metabolic processes, particularly mitochondrial respiration, were largely up-regulated during the day, consistent with a previous study in A. millepora (Brady et al. 2011). This should correspond to the increased production of photosynthetically-fixed carbon by Symbiodinium. Elevated respiration in tips indicates a high amount of energy consumption involved with growth during the day, which may permit the increase in mitotic activity at night previously observed in A. cervicornis tips (Gladfelter 1983b).

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The elevated expression of lipid synthesis genes in branch bases (e.g. fatty acid synthase) observed in this study confirms a similar finding previously observed for Acropora corals (Hemond et al. 2014). Lipid synthesis also appears to be elevated during the day (acyl-coA desaturase), which may be a result of increased effort to store energy produced by symbiont photosynthesis. Up-regulation of lipid synthesis, storage, and catabolism genes has previously been associated with the symbiotic state (Ganot et al. 2011; Lehnert et al. 2014). However, lipid synthesis may also be increased in branch bases to support the high lipid requirements of gamete (egg) production (Arai et al. 1993) in bases, an activity suggested by elevated expression of the egg yolk protein gene vitellogenin.

Lipid metabolism genes that were up-regulated in tips have putative roles in arachidonic acid metabolism and eicosanoid production. These include delta(5) fatty acid desaturase, which was also up-regulated during the day, and phospholipase A2, which was also down-regulated during the day. Eicosanoids are thought to function as part of the coral’s innate immune system (Libro et al. 2013). Interestingly, arachidonate 5-lipoxygenase, involved in production of leukotriene eicosanoids, and seven transcripts of allene oxide synthase-lipoxygenase, which are involved in biosynthesis of allene oxide from arachidonic acid, were also up-regulated in tips. Increased phenoloxidase activity in coral growth zones observed by D’Angelo et al. (2012) indicates that multiple immune pathways may be up-regulated in growing tissues of corals. Another interpretation can be that immune response is down-regulated in mature polyps to facilitate the symbiosis with Symbiodinium (Lehnert et al. 2014). The highest expression of phospholipase A2 at night in tips, however, suggests that the protein may act as a toxin in the nematocyst complex (Nevalainen et al. 2004).
Transport of carbohydrates across organelle membranes and among polyps is critical for coral growth. *Symbiodinium*, the coral’s primary source of carbohydrates, are isolated within symbiosomes, vacuoles comprised of both coral-derived and symbiont-derived membranes (Wakefield et al. 2000). In *Acropora*, carbohydrates must also be transported from radial polyps to the growing tip of the branch (Pearse & Muscatine 1971). The three carbohydrate transport genes that were up-regulated during the day (*slc5a2, slc5a10, slc5a11*) are sodium/glucose and sodium/myo-inositol transporters, which have previously been observed to be up-regulated in symbiotic compared to aposymbiotic anemones (Lehnert et al. 2014). These are strong candidates for involvement in transfer of fixed carbon involved in LEC (Pearse & Muscatine 1971).

**Conclusion**

Though conditions within the coral animal have previously been shown to fluctuate dramatically between day and nighttime conditions, the coral response via gene expression is relatively modest. Transcriptomic differences between day and night reflect putative circadian clock functions, response to light and oxidative stress, lipid and nitrogen metabolic functions and carbohydrate transport. By exploring the effect of location within the colony (branch bases versus branch tips) on diurnal versus nocturnal gene expression, we have identified genes that show within-colony temporal differences and provide candidates for further exploration into LEC, phototaxis and the regulation of *Symbiodinium* by the host coral. These results support roles for carbonate ion regulation (*CA2*) and a mucin SOM protein in LEC, and two photoreceptor-related genes, a retinol dehydrogenase and *Amcry1*, in blue-light phototaxis. Furthermore, these results support the hypothesis that corals control *Symbiodinium* population densities by limiting nitrogen availability through uptake of ammonia by Glutamine synthetase activity, and additionally suggest removal of dysfunctional symbionts by phagocytotic amebocytes. Symbionts may also affect the
coral circadian rhythms through an effect of oxidative stress on circadian gene regulation. These findings add to a growing body of knowledge about coral growth and coral host-symbiont interactions.

Acknowledgements

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References


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Supporting Information

Supplementary File S1. Mean and standard error of RNA-seq library sizes and number of mapped reads for each treatment group of samples.

Supplementary File S2. Data set of annotated (UniProt/Swiss-Prot E-val < 10^{-5}) coral transcripts. Includes adjusted P and moderated log_{2}(fold change) values, GO terms and putative functions of genes discussed in the manuscript and/or included in Table 1. * Indicates annotation from GenBank.

Data Accessibility

The reference transcriptome sequences are available on Bioproject [accession number PRJNA222758]. Demultiplexed Illumina read data (.fastq) and normalized read count data (.csv) are available on Dryad doi:10.5061/dryad.7qq74.

Author Contributions

EMH and SVV conceived and designed the experiment and wrote the manuscript. EMH generated and analyzed the data.
### Table 1. Number of differentially expressed transcripts

For functional categories of interest. For transcripts with a significant interaction, *indicates also DE by colony position, † indicates also DE by time of day, § indicates DE for both factors.

<table>
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<tr>
<th>Function</th>
<th>Total DE</th>
<th>Colony Position only</th>
<th>Time of Day only</th>
<th>Both Factors</th>
<th>Interaction</th>
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</thead>
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<tr>
<td>Circadian/photoreception</td>
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<td>Carbohydrate transport</td>
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Figure Legends

Figure 1. nMDS plot of transcription profiles for tips (triangles) and bases (squares), day (gray) and night (black). Clustering of base and tip samples indicated by dashed lines.

Figure 2. Number of DE genes for model factors (colony position and time) and their interaction considering all coral transcripts (A) and annotated transcripts (B).

Figure 3. Heat map of selected annotated DE transcripts. Expression data is log-transformed. Significance of GLM tests indicated by bands to the left of expression data: time of day (black), colony position (light gray), interaction (dark gray). Moderated log2(fold change) for position (POS) and time of day (TIME) provided where significant (ns = not significant). Annotation ID for cryptochromes based on blastn results.

Figure 4. Possible circadian and light-responsive gene interactions and roles in diel processes in corals. Genes DE by time of day or showing an interaction between factors were up-regulated in day (left) or night (right). $\dagger$ indicates a significant interaction effect. Dashed lines indicate hypothetical relationships. Rdh enzymes (purple) may either produce retinal or retinaldehyde; retinal interacts with the opsin apoprotein (purple) to form rhodopsin, while retinaldehyde may be further reduced to retinoic acid. Amcrys and phr (gray), are in the photo-lyase family of light-responsive proteins. Cryptochrome expression may regulate the circadian clock, Clock and NPAS2 activity in a light-dependent manner. Clock and NPAS2 proteins (red) are two of the primary bHLH-PAS transcription factors that, when bound to BMAL1 as a heterodimer, direct the circadian rhythm of animals and activate expression of Crys. TEF and DBP PAR bZip transcription factors (green) activate transcription of circadian-regulated genes.

Figure 5. Glutamate and glutamine biosynthesis pathways including enzymes (boxes) with genes DE by time of day or with an interaction (black text) and present but non-DE enzymes (gray text). $\dagger$ indicates significant interaction effect. Enzymes are GLDH, Glutamate dehydrogenase; GOGAT, Glutamate synthase; GLN, Glutaminase; GS, Glutamine synthetase; GATase, Glutamine amidotransferase; and CPSI, Carbamoyl phosphate synthetase I.