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Transcriptome dynamics over a lunar month in a broadcast spawning acroporid coral

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Abstract

On one night per year, at a specific point in the lunar cycle, one of the most extraordinary reproductive events on the planet unfolds as hundreds of millions of broadcast spawning corals release their trillions of gametes into the waters of the tropical seas. Each species spawns on a specific night within the lunar cycle, typically from full moon to third quarter moon, and in a specific time window after sunset. This accuracy is essential to achieve efficient fertilization in the vastness of the oceans. In this report, we use transcriptome sequencing at noon and midnight across an entire lunar cycle to explore how acroporid corals interpret lunar signals. The data were interrogated by both time-of-day-dependent and time-of-day-independent methods to identify different types of lunar cycles. Time-of-day methods found that genes associated with biological clocks and circadian processes change their diurnal cycles over the course of a synodic lunar cycle. Some genes have large differences between day and night at some lunar phases, but little or no diurnal differences at other phases. Many clock genes display an oscillation pattern indicative of phase shifts linked to the lunar cycle. Time-independent methods found that signal transduction, protein secretion and modification, cell cycle and ion transport change over the lunar timescale and peak at various phases of the moon. Together these data provide unique insights into how the moon impinges on coral transcription cycles and how lunar light may regulate circalunar timing systems and coral biology.

Keywords: biological clocks, cnidarians, genomics, invertebrate reproduction, proteomics, transcriptomics

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Introduction

Most reef-building corals reproduce by broadcast spawning. This process is under extraordinarily tight temporal control, with the typical 30- to 60-min-long time windows of spawning of some species being predictable to within 20 min from year to year (Levitan *et al.* 2004, 2011; Vize *et al.* 2005). The month of spawning is determined by long-term local weather and temperature patterns and typically occurs in the window with the least wind and waves (Penland *et al.* 2004; Van Woesik 2010; Keith *et al.* 2016). The date of spawning is set by the lunar cycle (Harrison *et al.* 1984; Babcock *et al.* 1986; Oliver *et al.* 1988), and the actual time of spawning is set by sunset time (Levitan *et al.* 2004; Brady *et al.* 2009). These three parameters can be used to accurately predict spawn dates and times. Broadcast spawning corals are often hermaphrodites, and within the right time window, every polyp in a colony releases a bundle of eggs and sperm that slowly floats to the surface where it and other bundles break apart, gametes mix and fertilization occurs (Wallace *et al.* 1986). The scale of this event is gargantuan, with an individual colony being capable of producing hundreds of millions of eggs. How simple radial animals that lack eyes, brains

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or even a central nervous system manage such amazing temporal coordination remains largely unknown.

Many periodic biological processes are regulated by entrained biological clocks, and transcription–translation feedback loops are central elements of these systems. How, or whether, these systems intersect with the lunar cycle that sets coral spawn dates is not known. Some transcriptional changes in genes driving biological clocks at different phases of the moon have been described. In the fruit fly *Drosophila*, both nocturnal behaviour and *Clock* protein levels can shift in response to quarter moon levels of illumination at night (Bachleitner *et al*. 2007). In marine fishes, decreased transcription of *AANT*, a rate-limiting enzyme in melatonin production (Kashiwagi *et al.* 2013), has been reported on full moon evenings, as have altered *Period* and *Cryptochrome* transcription (Fukushiro *et al*. 2011). A marine polychaete also shows differences in gene expression under laboratory conditions between new moon and constant full moon for *Clock*, *Period* and *Pdp1* genes (Zantke *et al*. 2013). The most complete data on lunar responses to the moon to date are from *Acropora millepora*, a common Pacific reef-building coral. In this species, RNA-seq comparison between new and full moon identified a number of differentially expressed transcripts (Kaniewska *et al*. 2015), and a qPCR analysis of biological clock genes found preliminary evidence of differences in the phase of expression of circadian genes between new and full moon (Brady *et al.* 2016). We do not yet know, however, what happens to transcription cycles over a full lunar month.

In mammals, reproductive cycles are linked to seasonal changes through shifting melatonin and biological clock cycles (Hanon *et al.* 2008). A combination of these cycles aligns as daylight length increases, activating transcription of TSH-beta and driving ovulation. While a number of studies have examined daily cycles of transcription in corals (Brady *et al*. 2011; Bertucci *et al*. 2015; Hemond & Vollmer 2015; Kaniewska *et al*. 2015; Oren *et al*. 2015; Ruiz-Jones & Palumbi 2015), the lack of data on lunar-associated cycles blocks our ability to detect potential cycle overlaps that may act in a similar manner to drive biological processes that are regulated by the moon, such as broadcast spawning. We report here an RNA-seq analysis of transcription in the reef-building coral *Acropora gemmifera* (Brook 1892) over an entire lunar month, under stabilized environmental conditions. The results show that transcription patterns change in a number of different dynamic ways over a synodic lunar month. These shifts may act in combination with seasonal or daily solar cues to regulate coral biological responses to the moon. As transcription patterns change so profoundly over a lunar month, experiments examining both daily and more long range cycles should include moonlight cycles in their design to achieve biologically relevant results.

Methods

Samples and sampling

SCUBA was used to collect three individual *Acropora gemmifera* coral colonies from Houbihu Bay, southern Taiwan, at 21.931501 °N, 120.745207 °E and approximately 2 m depth. Following transport to the NMMBA husbandry facility, corals were maintained in outdoor aquaria with sand-filtered, temperature-controlled flow-through seawater, monitored by temperature and light loggers. This set-up was designed to mimic local solar and lunar illumination patterns but isolate corals from tidal flux and weather effects. A previous study by Boch *et al.* (2011) demonstrated that constant spectra lunar lighting timed and intensity matched to local conditions can direct normal broadcast spawn timing in *Acropora humilis*. A separate study (Mayfield *et al.* 2013) found that transplanted corals cultured at the NMMBA had similar physiological parameters to field corals over a 4-week culture period, as employed here. Transparent roof panels shielded corals from rain but allowed exposure to a normal local day: night light cycle with sunrise at 05:30 and sunset at 18:14 local time (UTC+8) on the start date, and also allowed associated twilight periods. At night, this facility has no lighting and walls block much of the moonlight. Cultured corals were therefore exposed to a broad-spectrum lamp (Mr. Aqua 15 watt, cat. no. EA-063; see Fig. S1, Supporting information for spectrum) filtered through layers of shade cloth to mimic lunar lighting. The intensity of artificial moonlight was matched to local lunar illumination using an Extech Easyview 3.3 light meter and the moonlight on/off timing also matched to the local moonrise and moonset. Sampling began after 7 days of conditioning to the tank environment, and a table of all sampling dates and associated lunar calendar data is available in Table S1 (Supporting information).

Individual coral branches were sampled from each colony on four dates, corresponding to third quarter moon (3Q), new moon (NM), first quarter moon (1Q) and full moon (FM). On each of these dates, samples were collected both at noon and at midnight. Upon completion of sampling, all remaining coral colonies were returned to the sampling site and reattached to the reef with epoxy.

RNA isolation and sequencing

Coral tissue was sampled using a chisel to snap off a single peripheral branch, or finger, from each colony. In *A. gemmifera*, such branches are approximately 1.5 cm

wide at the base and 2–3 cm long, with a single axial polyp and hundreds of radial polyps. The entire branch was ground to a slurry in 1 mL TRIzol (Ambion) per g of tissue using a mortar and pestle and then processed according to the manufacturer's protocol. Samples were stored in a −80 °C freezer and then shipped to the laboratory in Canada under CITES export permit number FTS503W0040611.

RNA was precipitated by adding 0.25 mL of isopropyl alcohol and 0.25 mL of salt solution (0.8 M sodium citrate + 1.2 M NaCl) per 1 mL of TRIzol, incubated at room temperature for 10 min, centrifuged at 10 000 ***g*** for 10 min, rinsed with 70% ethanol and redissolved in water. RNA quality was assessed by agarose gel electrophoresis, and samples with sharp ribosomal bands were selected for sequencing. Tag-based library preparation and sequencing was performed at the University of Texas using the TagSeq protocol and reagents described in Meyer *et al.* (2011). This method generates one read from the 3′ end of each RNA and so requires lower read depths than whole mRNA sequencing strategies. Both QPCR and spike in tests have shown that 6 million Tag-end reads provide more quantitative results than do 30 million whole mRNA reads (Lohman *et al.* 2016). Between 5 and 16 million 50-base single-end reads were generated using an Illumina HISEQ 2500 per library and RNA sample. After quality filtering, 74.3% of reads were retained, and read counts for each sample are available in Table S2 (Supporting information). All sequence reads are available at the NCBI SRA accession nos. listed in Table S2 (Supporting information).

Annotation

The *A. millepora* transcriptome annotations of Moya *et al.* (2012), modified as described by Dixon *et al.* (2015), were further updated for genes with differential expression patterns using BLASTX against the NCBI nonredundant *Homo sapiens* gene set, or when no matches were returned, against the entire nr database. As with all major model organisms, gene symbols matching human symbols approved by the Human Gene Nomenclature Committee (HGNC) were used whenever possible. When multiple isoforms (splice variants or different parts of the same gene) were identified, sequential numbers were applied, for example *cry1*, *cry1.1* and *cry1.2*. A table listing annotation updates used herein is available in Table S2 (Supporting information).

Data processing pipeline 1 – Bowtie2 and DESeq2

Raw sequencing reads were analysed with the FastQC quality control tool to evaluate the read sequence quality. Trimming and filtering was performed with the FASTX_-TOOLKIT. The ARRAYQUALITYMETRICS package (Kauffmann *et al.* 2009) was then used to detect outliers, and two outlying samples (colony 2, day, 3Q moon; and colony 2, night, FM) were removed. Trimmed reads were aligned to the *A. millepora* transcriptome (no high-quality annotated *A. gemmifera* transcriptome or genome is available) using Bowtie2 with the very-sensitive-local setting (Langmead & Salzberg 2012). The *A. gemmifera* reads typically had 96–100% identity to the *A. millepora* transcripts. Read counts were assembled according to isogroups – a collection of contigs of the same gene representing different splice forms or assembly variants (Dixon *et al.* 2015). Reads mapping to multiple isogroups were discarded, as were any reads where 25 of the first 35 bases failed to achieve a quality score of over 30. A table of read count numbers and alignment statistics is available in Table S2 (Supporting information).

Transcripts that had a low abundance were filtered by DESeq2 to optimize the rate of differentially expressed gene discovery. The likelihood ratio test (LRT) was used to look for circadian and circalunar differences by taking into account the daytime and nighttime expression across all four periods in the lunar month. The R code used is available at: https://github.com/Vize-Lab/RNA-Seq_RScripts. Gene expression graphs were generated with GGPLOT (Wickham 2009), and heatmaps were created with the PHEATMAP package, also in R (Kolde 2012).

Data processing pipeline 2 – Salmon and generalized linear model (GLM)

The second data analysis approach was tested using pseudoalignment via Salmon (Patro *et al.* 2015) version 0.6.0 using the *A. millepora* transcriptome (Dixon *et al.* 2015) as the reference. Raw transcript counts were imported into R (R Core Team 2015) and transcript counts summed across genes which were then analysed using DESeq2 (Love *et al.* 2014, 2015). GLMs were used to investigate the impact of the lunar cycle and sampling time. A term for individual was included in each model to control for variation between the individual corals. For the lunar cycle, likelihood ratio tests were used to compare models with and without the terms of interest, which performs analysis of deviance (ANODEV) and the GLM equivalent of ANOVA (Love *et al.* 2014, 2015). Genes with a P value of <0.05 were considered to be statistically significant.

GO and KOG analyses

The GO terms were updated for all genes identified as showing differential expression using Blast2GO (Conesa

et al. 2005). GO cluster analysis was then performed, once again using Blast2GO, for each of the three different sets of genes identified by different statistical methods. The updated GO terms are available on Dryad. KOG analysis was performed by selecting the top-level KOG term from the *A. millepora* transcriptome annotations.

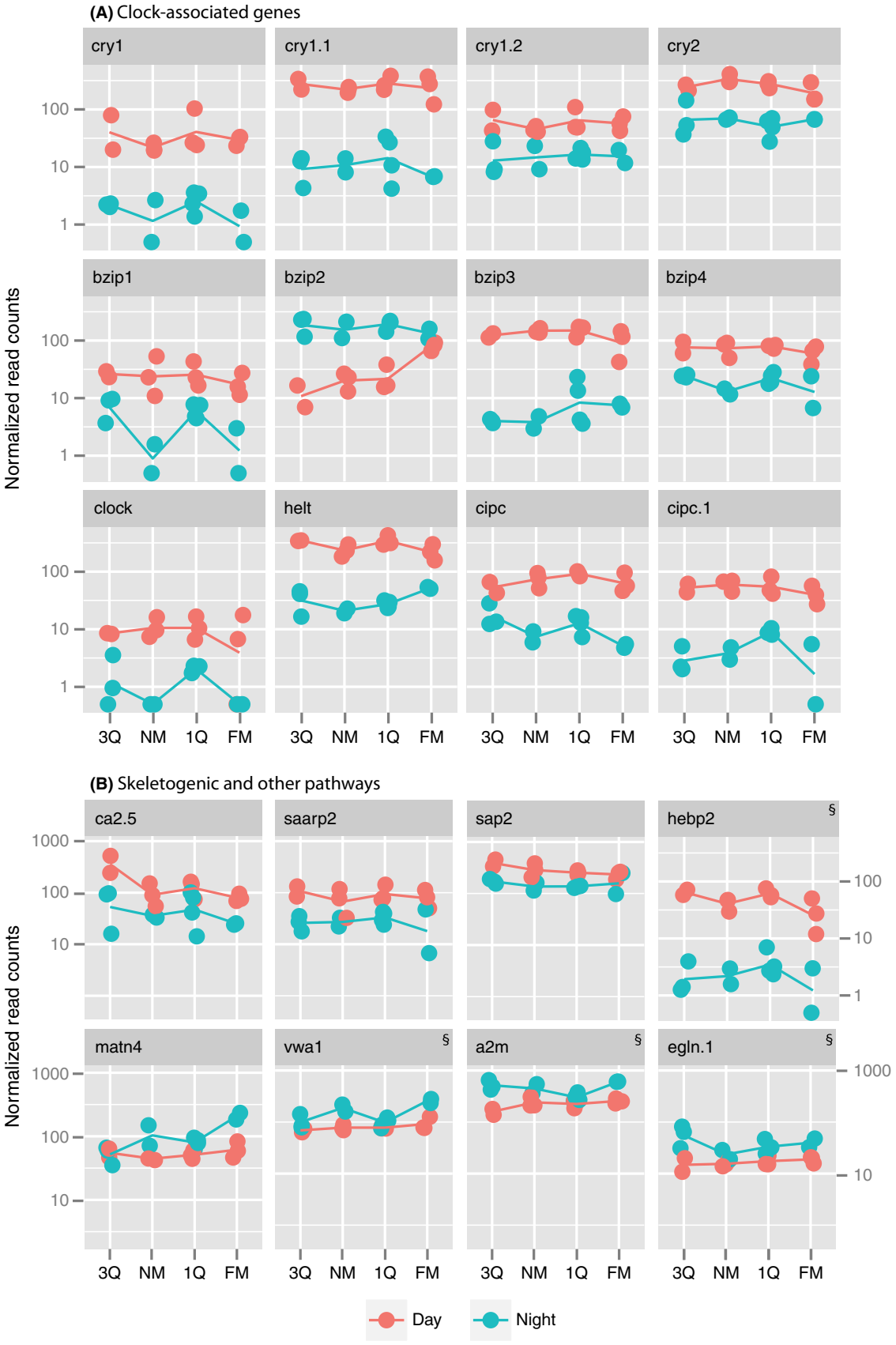
Results

Samples were collected at both noon and midnight at four lunar stages, 3Q, NM, 1Q and FM. RNA was isolated from each sample and subjected to RNA-seq via TagSeq (Meyer *et al.* 2011; Lohman *et al.* 2016), generating 5 and 16 million 50-base single-end reads per sample. Two data sets that did not pass quality control were removed (colony 2, day, 3Q moon; and colony 2, night, FM). The first method of RNA-seq data analysis used a standard bioinformatics pipeline, with read trimming and quality filtering, followed by aligning reads to the *Acropora millepora* transcriptome (Moya *et al.* 2012), modified as described by Dixon *et al.* (2015), using Bowtie2. DESeq2 was then used to normalize and contrast data sets. The initial analysis compared levels of expression that varied between noon and midnight that changed over a lunar month. This method was chosen as diurnal expression cycles have previously been shown to shift with lunar phase (Brady *et al.* 2016). The statistical test performed by DESeq2 used a likelihood ratio test (LRT) with a 10% false discovery rate (FDR) (Benjamini & Hochberg 1995). This analysis identified 55 genes with diurnal patterns that changed over the course of a lunar cycle, with P values of <0.05. A selection of the genes showing the strongest changes in differential expression between day and night over a lunar month are displayed as graphs of (log) normalized read counts versus lunar phase in Fig. 1, and the output for all 55 genes is illustrated in Fig. S2 (Supporting information) and the complete list of all genes and supporting statistics, including standard error and standard deviation, is available in Table S3 (Supporting information). GO cluster analysis indicated that genes associated with biological clocks were predominant (23 genes in total), and all 10 of the genes showing the lowest P values were associated with biological clocks (Fig. 1). These clock genes included previously characterized diurnal genes (Brady *et al.* 2011; Bertucci *et al.* 2015; Hemond & Vollmer 2015; Kaniewska *et al.* 2015; Oren *et al.* 2015; Ruiz-Jones & Palumbi 2015) such as *cryptochrome 1* and *2* (*cry1* and *cry2*) and recently described genes such as *PAR-bzips* (*bzip*), *clock-interacting circadian pacemaker* (*cipc*), and the hairy-related transcription factor *helt*. The identified *rbm38* gene has not previously been shown to function in clocks, but a related RRM gene, *rbm4/lark*, regulates circadian phase periodicity in *Drosophila* (Huang *et al.* 2014) and likely functions in the *Acropora* clock. Other highly significant differences were observed in genes driving skeletogenesis and those producing components of the extracellular matrix/skeletogenic organic matrix (ECM/SOM; Fig. 1).

These data were output as a heatmap as an alternative visualization method. In this case, expression levels were colour-mapped to indicate $\log_2$ fold changes in expression in each individual and at each time point. Selected examples are illustrated in Fig. 3 and the full 55 genes in Fig. S3 (Supporting information). In addition to the trends visible in the results illustrated in Fig. 1, the heatmap highlights differences between individual coral colonies at each time point, each of which is plotted in separate columns.

The above-described method was very effective at detecting changes in diurnal patterns over a lunar month; there are also two additional factors we also sought to address with further analyses, boosting read count numbers to detect genes expressed at lower levels, and accounting for individual effects in specific coral colonies that might bias the above methods, and which are indicated by the variation between individuals observed in Fig. 3. As greater sequencing depth was not possible, an alternative method that would assign more reads to transcripts was adopted – Salmon (Patro *et al.* 2015)-based lightweight alignment of untrimmed and unfiltered reads. Generalized linear models (GLMs) were then used to investigate the impact of the lunar cycle and the sampling time. A term for individual was included in each model to control for variation between the individual coral colonies. For the lunar cycle and sampling time, LRTs were used to compare models with and without the terms of interest, which performs analysis of deviance (ANODEV) and the GLM equivalent of ANOVA (Love *et al.* 2014, 2015).

The Salmon data set was first used to detect differences between the day and night data, with all four sampling dates added together for noon data and compared to all four combined midnight sets. Once again, genes with a P value of <0.05 were considered to be statistically significant, and the analysis detected 11 genes with a P value of <0.05, with a 10% FDR. This set included many of the genes also illustrated in Fig. 1, including those encoding *cry1* and *cry2*, *cipc*, *bzip3*, *helt*, *pepk* and the calcium-binding ECM/SOM protein *matn4*. The only novel annotated gene in the Salmon/GLM analysis, not detected in the Bowtie2/DESeq2/LRT (Figs 1 and 2), was the dynein-associated gene *roadblock* (*dynlrb*). This gene was also detected in the earlier data set (see Table S3, Supporting information), but it had a P value of 0.1 and so was excluded. GO analysis of this gene set showed very similar patterns of enrichment as



did the Bowtie2/DESeq2/LRT analysis, with the majority of genes being associated with biological clocks or rhythmic processes (Fig. 2). The complete list of genes displaying day/night differences detected via the GLM and additional data are available in Table S3 (Supporting information).

The Salmon/GLM data set, accounting for individual effects, was then analysed for genes that differed over a lunar month, without requiring any time-of-day effect. By combining the two time points (noon and midnight) for each lunar phase, this doubled the read depth available. This analysis identified a total of 273 genes that showed differential expression over the lunar cycle (Figs S4 and S5, Table S3, Supporting information), a considerably higher number than were identified with lower read counts and when individual effects were not included in the previous pipeline (55 genes; Fig. 1 and Table S3, Supporting information). KOG class analysis of these genes indicates that signal transduction is the most variable process over a lunar month, with 59 genes having this classification (Table 1). Over 20 of the genes in this group were associated with calcium-based signal transduction, including *calmodulin* (*calm3*), *calbindin* (*calb2*) and various calcium-responsive proteins such as *hippocalcin-like* (*hpcal1*) and *synaptotagmin* (*syt*). The next most high represented class was post-translational modification, protein turnover and chaperones with 24 genes. When this class is combined with intracellular trafficking and extracellular structures, as all ECM/SOM is both post-translationally modified and secreted via vesicles, this combined KOG class totals 50 genes, making it equivalent in predominance to signal transduction (Table 1). Another KOG class that was common is genes associated with the cell cycle. Histone genes, which are only expressed during S-phase and therefore excellent markers of cell division, are not in this category (they are included in chromatin modification). If the five detected histone transcripts are combined with cell cycle class genes, the two classes, cell cycle (plus histones) and ion transport, equal as the third most frequent KOG classes showing lunar cycle differences with 14 genes each. The expression profiles of representatives of these various KOG classes are shown in Fig. 4. As this figure illustrates, different processes peak at different parts of the lunar cycle, such as the cell cycle at new moon, calcium signalling at the first quarter moon and wnt signalling at the full moon. GO

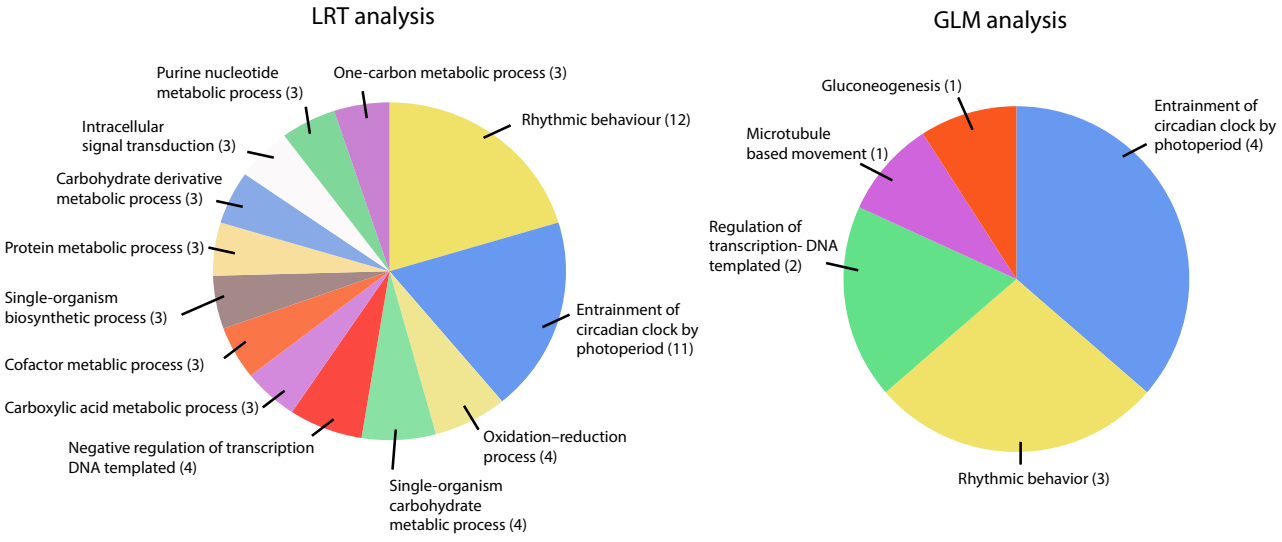


Fig. 2 GO analysis of differentially expressed genes. The GO terms for biological process enriched in differentially expressed genes identified by LRT analysis for genes with diel patterns that change with lunar phase (top) or GLM analysis for genes that show differences in diel expression over a whole lunar month (bottom).

Fig. 1 Noon and midnight gene expression comparisons that change over a lunar month. Each data point in the time series plots three biological replicates except for during the day on the 3Q moon (colony 2 was removed as outlier) and at night during the full moon, where once again colony 2 was an outlier. The *X*-axis shows the lunar cycle and *Y*-axis the normalized counts (log scale). The read count number scale on the left applies, except for panels marked with a '§' symbol, in which case the read count scale on the right applies. Daily and nightly expression levels are shown in red and blue, respectively. The SD and SE scores for these data are available in Table S3 (Supporting information).

Table 1 KOG analysis of lunar-regulated genes

KOG class	Number
Signal transduction mechanisms	59
Post-translational modification, protein turnover, chaperones	24* (50)
Intracellular trafficking, secretion and vesicular transport	17*
Inorganic ion transport and metabolism	14
Cytoskeleton	11
Amino acid transport and metabolism	11
Extracellular structures	9*
Cell cycle control, cell division, chromosome partitioning	9† (14)
Chromatin structure and dynamics	9†
Transcription	8
Translation, ribosomal structure and biogenesis	7
RNA processing and modification	7
Lipid transport and metabolism	6
Energy production and conversion	6
Defence mechanisms	6
Carbohydrate transport and metabolism	4
Replication, recombination and repair	3
Coenzyme transport and metabolism	2
Cell wall/membrane/envelope biogenesis	2
Cell motility	2

*All three of this KOG class are associated with protein secretion.
†Both of these KOG classes contain proteins involved in cell division.

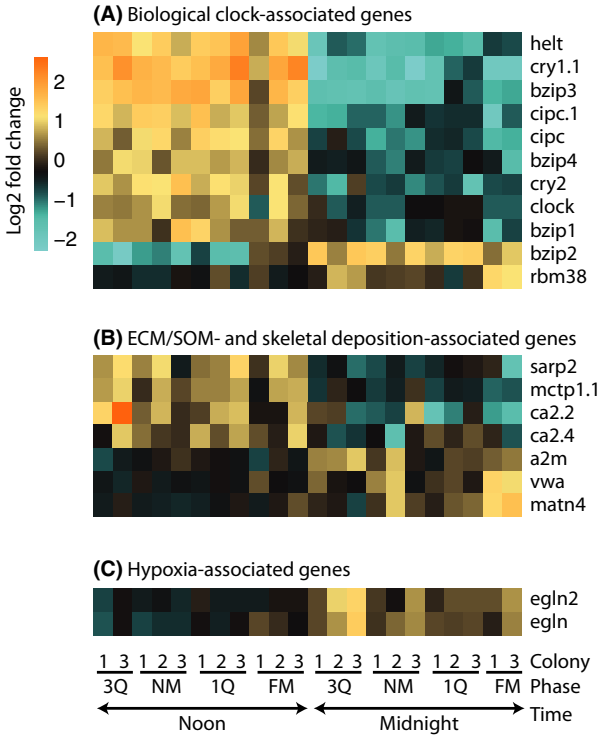


Fig. 3 Diel patterns of gene expression over a lunar month. Expression heatmaps for the three groups of genes showing diurnal expression patterns after FDR correction ($P < 0.05$). (A) Biological clock-associated genes, (B) Extracellular matrix- and skeletal deposition-associated genes and (C) hypoxia-associated genes. Each row represents one transcript (isogroup), and each column represents one time point. Expression levels for each gene are shown in $\log_2$ fold change (relative to the mean) such that orange (+2) and blue (−2) indicate high expression and low expression, respectively. Individual coral colonies, numbered 1, 2 and 3, were sampled at four different lunar phases (3Q, NM, 1Q and FM) at both noon and midnight. The data sets for colony 2 at 3Q day and FM night were found to be outliers and therefore excluded (see Methods).

analysis was also performed for this gene set and it generated very similar results, with signal transduction being by far the largest class of differentially expressed genes (Fig. S4, Supporting information).

Discussion

The results presented above detected multiple patterns of differential gene expression over a full lunar cycle. One method of analysis tested for differences in expression in genes with diurnal transcription profiles and did not account for individual effects. This approach detected 55 differentially expressed genes. A related approach looking only for time-of-day effects independent of lunar phase identified 11 genes, all but one of which (*roadblock*) were detected by the first method. The third contrast tested for changes across a lunar month independent of diurnal changes, and this method did account for individual effects. The later approach detected 273 differentially expressed genes. The two approaches that detected most lunar-associated changes, diel patterns changing over a lunar month and lunar phase-specific patterns each detected genes associated with different biological processes and so are discussed separately.

Changes in diel patterns over a lunar month

Genes showing changes in diurnal profiles over a lunar month were predominantly associated with biological clocks, but other processes, such as skeletal deposition and oxidation/reduction/hypoxia, also showed changes (Figs 1–3). Many of the genes detected have been identified as showing diel expression patterns by previous studies (Brady *et al.* 2011; Bertucci *et al.* 2015; Hemond & Vollmer 2015; Kaniewska *et al.* 2015; Oren *et al.* 2015; Ruiz-Jones & Palumbi 2015), but their expression over a complete lunar cycle has not been previously described.

Some of the changes in these diel patterns over a synodic lunar month are quite dramatic and could not be visualized by sampling at less frequent intervals. For example, genes such as *bzip1*, *clock* and *cry1* (Fig. 1) display relatively constant daytime expression levels, but midnight levels oscillate broadly, with night-time peaks at third and first quarter moons. Some genes have profiles that loosely fit this pattern but were less dramatic, for example *cipc*, which is not as obvious as the aforementioned examples. Two potential explanations for these extraordinary patterns are a 2-week periodicity (a semilunar pattern) and a month-long phase sliding of an oscillating cycle. In the first case, spring or neap tidal influences could explain a 2-week periodicity, but as our corals were isolated from tidal influences in tanks, these responses would need to be entrained and

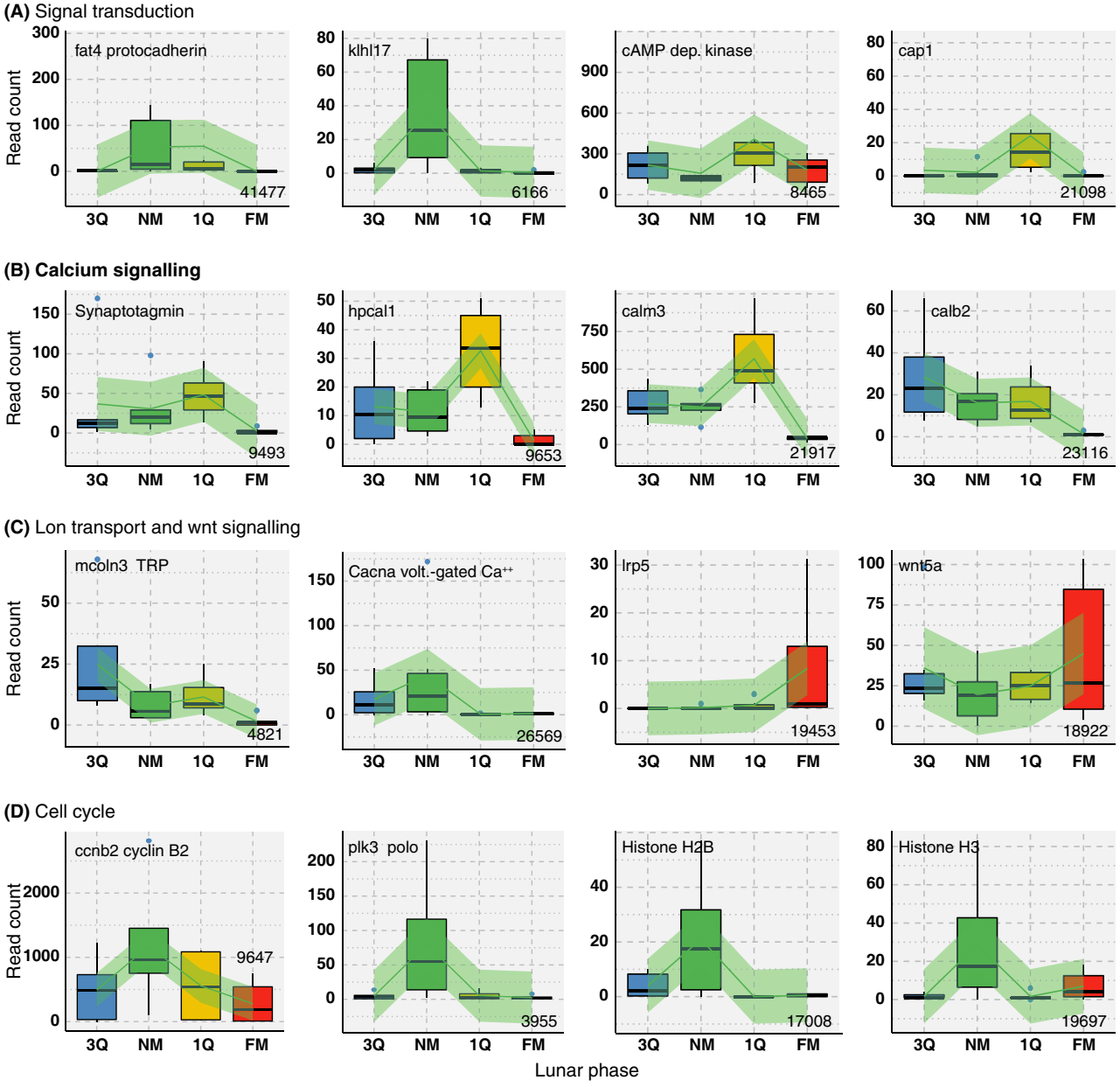


Fig. 4 Box plots for selected genes that had significant variation over the lunar cycle. The *y*-axis shows the normalized counts, and the *x*-axis is the lunar cycle. The boxes (also coloured by lunar cycle, blue for third quarter, green for new moon, yellow for first quarter and red for full moon) show the mean, 25th and 75th percentiles with the bars showing the minimum and maximum values (within 1.5 * interquartile range). Blue points are outliers (beyond 1.5 * interquartile range). A local regression (green line) with 95% confidence intervals (transparent green ribbon) was fitted for each gene to show the trends over the lunar cycle. Gene names are in the top-left corner, and the gene number (isogroup number) is in the bottom-right corner.

cycle for a full month following isolation from the entraining process to explain the results. While this is possible, the second option seems more likely. There is already evidence that in corals, the genes *cry2* and *clock* alter the phase of their daily oscillations between new moon and full moon (Brady *et al.* 2016). If the phase of the daily expression cycle changes, but the sampling time was constant, as in the present experiments, this would result in relative expression levels increasing and decreasing over the sampling window, as illustrated by a model in Fig. 5. As some data already exist to support this possibility (Brady *et al.* 2016) and there is no evidence to support entrained semilunar tidal-driven fluctuations in transcription to date, and the corals were isolated from tidal influences, we propose that these data support the sliding phase model. The strongest oscillation was observed in *bzip1* (Fig. 1). As a related gene, *tef1*, drives transcription via D-box elements in the promoters of genes such as *period* and *cryptochrome* in vertebrates (Vatine *et al.* 2009; Weger *et al.* 2011), this gene may also be the driver regulating expression of other oscillating genes in our data shown in Fig. 1. A phase shift in diurnally expressed clock genes in response to changing lunar illumination that intersected with relatively constant day/night cycles, for example in calcium signalling (Hilton *et al.* 2012) or melatonin (Kaniewska *et al.* 2015), could form the basis of an accurate lunar timing mechanism.

Another novel pattern observed in these data for a different set of genes is modulation of the level of diurnal regulation, or day:night (D:N) ratio over a lunar month. The most extreme example was *bzip2*, where the D:N ratios were 11:1 (3Q), 9:1 (NM), 8:1 (3Q) and then 1.6:1 (FM) (Fig. 1). Seasonal reproductive cycles in mammals are driven by differences in cycle phases controlled by day length (Dardente *et al.* 2010). The elevated midnight expression of *bzip2* during full moon shown in Fig. 1 could drive a similar timing system in *Acropora*. If this transcription factor acts as a repressor, downstream targets would be active at night only during 3Q, NM and 1Q lunar phases. If *bzip2* was a transcriptional activator, its targets would only be active at night during the full moon. Similar effects on targets limited to specific phases of the moon may also occur for other genes with related lunar transcription cycles, such as *helt* and *cipc*.

Genes involved in skeletal deposition or production of the ECM/SOM also show changes in diurnal patterns over the lunar month. These genes can be divided into two classes based on expression profiles. Genes involved in calcium carbonate precipitation are more active in the daytime, as has been previously described (Bertucci *et al.* 2015; Hemond & Vollmer 2015; Kaniewska *et al.* 2015; Oren *et al.* 2015; Ruiz-Jones & Palumbi 2015); examples include *carbonic anhydrase 2* (*ca2*), *secreted acid-rich protein* (*sarp*) and *secreted acidic protein 2* (*sap2*), with expression levels showing general decreases approaching the full moon (Fig. 1). In the second group, those associated with ECM/SOM production show higher levels of expression at night; examples include *matrilin 4* (*matn4*) and *von Willebrand factor A domain-containing 1* (*vwa1*). A gene with similarity to *alpha-2-macroglobulin* (*a2 m*), a secreted protease inhibitor, shows a similar pattern. This trio of genes shows minimal differences at first quarter moon, followed by larger differences at the full moon. Genes associated with hypoxia and stress, such as *egln* and *hepb2*, also show changes over the lunar cycle (Fig. 1).

Some of the genes showing the most significant differences across the lunar month are genes that have been paid little attention in previous diel-focused analyses. These include the *helt* transcription factor, the *clock-interacting circadian pacemaker cipc* and *RNA-binding protein rbm38*. At the moment, nothing is known of how these genes function in invertebrate clocks, but our data indicate that this is very worth exploration.

Changes over a lunar month, accounting for individual effects

The approach discussed above did not account for individual to individual variation, and purposely focussed on genes that have different levels of transcription between day and night. The next approach described here examined our RNA-seq data for genes that differed over a lunar month, pooling day and night scores for each sampling window, thereby doubling the read counts for each date (six sets of TagSeq data per lunar phase), and included accounting for individual effects. This approach identified 273 differentially expressed genes. Both KOG and GO term enrichment analyses were used to identify biological processes that change over the course of a lunar month (Table 1; Fig. S4, Supporting information). The predominant process changing over the course of a lunar month by both KOG and GO analysis was signal transduction, with 59 differentially expressed genes belonging to this KOG class. Of these 59 genes, 24 are associated with calcium-based cell signalling or calcium binding. There is already evidence that corals use calcium as a second messenger in light signal transduction (Hilton *et al.* 2012; Kaniewska *et al.* 2015). The data presented here indicate that the transcription of some of the genes associated with this process peaks during the first quarter moon (Fig. 4). Some genes that may be directly involved in detecting light display interesting patterns, such the recoverin family protein, *hippocalcin-like* (*hpcal*), which regulates

photoreceptor phosphorylation in a calcium-dependent manner (Klenchin *et al.* 1995) and peaks at first quarter moon; *synaptotagmin* (*syt*), also peaking at the 1Q moon; and *mucolipin* (*mcoln/TRPML*), a transient receptor potential cation channel that boosts intracellular signals to trigger action potentials, are expressed at highest levels at third quarter moon. These results indicate that proteins associated with calcium signalling change relative expression levels over a lunar cycle. Calcium signalling has also recently been linked to lunar-associated emergence timing in the midge, *Clunio marinus* (Kaiser *et al.* 2016). In this study, the *calcium/calmodulin-dependent kinase II* gene was found to have different activities in insect populations with different lunar/tidal eclosion timing. This pathway clearly functions in biological clocks in multiple phyla, but untangling its roles in solar and lunar cycles will require extensive further research. As calcium signalling is extremely dynamic, the development of methods to detect calcium levels in vivo, such as expressing genetically encoded calcium indicators (e.g. Hires *et al.* 2008), will be necessary to observe how solar irradiance and lunar irradiance impact cytoplasmic calcium flux.

Other signal transduction pathways were also differentially active over a lunar month, including the wnt pathway (also detected in the LRT analysis; Fig. 1, Table S3, Supporting information), and tyrosine kinase signalling. In the LRT analysis, one of the genes showing the strongest differential expression was *helt*, a *hairy/enhancer-of-split*-related transcription factor that acts downstream of notch signalling (Kokubo *et al.* 1999), so this pathway may also be involved in circalunar timing (Fig. 1). This pathway has been associated with other oscillating timers, including cell division in *Drosophila* (Hunter *et al.* 2016) and somite segmentation in mammals (Conlon *et al.* 1995), a process that also requires circadian genes in the frog, *Xenopus* (Curran *et al.* 2014). Typically, this pathway oscillates in a short-phase ultradian manner in mammals and amphibians (Shimojo *et al.* 2008; Bonev *et al.* 2012), but the results presented here indicate that these ultradian oscillations, if also present in corals, may change over a longer circalunar scale. The availability of small-molecule inhibitors for many signal transduction pathways will make direct testing of the importance of each of these pathways possible.

Following signal transduction, the second most abundant class of genes that differed over a lunar month were those associated with protein secretion and modification, represented by three different KOG classes (Table 1). This result is consistent with the results from the LRT analysis shown in Figs 1 and 3, where proteins associated with skeletal deposition showed changes in both diel expression profiles and a month-long trend in changes in expression level. The expanded gene set detected by the GLM analysis includes many gene products that would be required to fold, process and transport proteins associated with the skeletal organic matrix to sites of skeletal growth such as *copII, pdi* and *arf1* (Table S3, Supporting information).

One of the most fascinating classes of genes showing lunar-associated regulation is those regulating cell division. Cell division has long been known to occur at higher rates at night in mammalian cells (Halberg 1960; Johnson 2010), possibly to reduce UV induced errors in DNA replication. In mice, the activation of mitosis at night is controlled by circadian clock genes regulating *cyclin B/cdc2* (Matsuo *et al.* 2003). The results in Fig. 4 and Table S3 (Supporting information) show that multiple markers of cell division, including *cyclin B* and a *cyclin-dependent kinase* (annotated as *cdc28*), strongly peak at the new moon indicating that lunar light may regulate the same molecular mitotic switch. This is most likely exerted by biological clock genes regulating *cyclin* and *cdk* genes in a manner akin to that previously reported in mammals, and that we demonstrated to change over a lunar month in the results presented in Fig. 4. This is the first demonstration of lunar-associated cell replication patterns to our knowledge. While cell cycle genes were not detected by other recent RNA-seq studies (Brady *et al.* 2011; Bertucci *et al.* 2015; Hemond & Vollmer 2015; Kaniewska *et al.* 2015; Oren *et al.* 2015; Ruiz-Jones & Palumbi 2015), this may be due to low read counts of these genes. These genes were presumably detected in our analysis due to six samples being combined for each lunar time point, together with the greater representation offered by TagSeq (Lohman *et al.* 2016). As corals rely on the moon to coordinate reproduction and therefore must be sensitive to lunar illumination, the link between mitosis and the lunar cycle may be restricted to species with similar lunar dependencies. Some vertebrates, for example the reef fish *S. guttatus* (Fukushiro *et al.* 2011), also link reproduction to the lunar cycle, as do a wide variety of other marine phyla, and it will be interesting to determine whether cell division rates also show a lunar association in these other species.

Circadian versus circalunar systems

Interestingly, some authors have argued for an independence between circadian and circalunar timing, based on experiments in the polychaetes (Zantke *et al.* 2013) and midges (Kaiser *et al.* 2016). Our data show that multiple circadian clock regulators change transcription patterns over a lunar cycle in coral (Figs 1 and 3) and that such changes are in some cases only observable by analysing at least four lunar phases. Given the

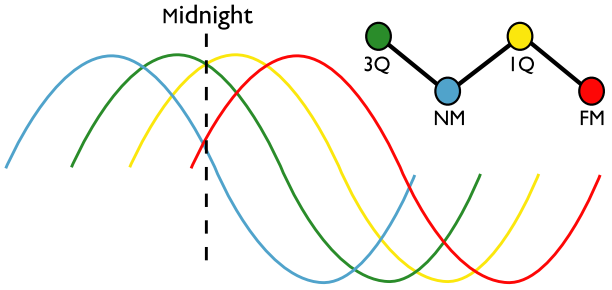


Fig. 5 Model for transcription cycle phase shifting over a lunar month. In this model, the phase cycle of a theoretical gene shifts over the lunar cycle and the point at which a specific clock time intersects the transcription cycle, in the order indicated below, cycles up and down.

importance of lunar phase to reproductive timing, more detailed studies in these other systems may find similar results. Circatidal behaviour in a marine crustacean depends on circadian clocks, as pharmacological inhibition of casein kinase I (CKI) blocks both circatidal and circadian behaviour in this isopod (Zhang *et al.* 2013) in a similar manner to that we propose for circalunar systems in corals. However, similar inhibition of CKI in *Platynereis* did not block circalunar spawning, although it is possible this is due to free run of gametogenesis cycles (Zantke *et al.* 2013). The results both in *Platynereis* (Zantke *et al.* 2013) and in the reef fish *S. guttatus* (Fukushiro *et al.* 2011) did in fact find differences in core circadian gene expression between full and new moon, consistent with the observations reported here and the model presented in Fig. 5. Direct experimentation will be required to resolve whether circadian and circalunar systems are interdependent, as our results imply. Should the circadian clock indeed be dispensable for circalunar timekeeping, the pathways identified in the lunar phase analysis in Fig. 4, namely signal transduction via calcium second messengers, would be the prime candidate as the regulating process.

Not only do our results have implications for lunar responsivity in corals, they also indicate that other studies exploring clock-associated systems should take the lunar phase into account when designing experiments. For example, an analysis of diurnal differences in coral transcription would not detect *bzip2* if sampled at full moon but would at other phases (Fig. 1). Simplified laboratory light cycles do not always recapitulate more complex light-driven processes in the real world, where long twilights and temperature shifts are intertwined with day:night differences in light intensity in regulating clock genes in *Drosophila* (Vanin *et al.* 2012). The lunar cycle may be similarly important and may explain some of the puzzling differences in phase peak timing in published diurnal studies in cnidarians (e.g. Oren *et al.* 2015).

Together these data show that lunar light regulates biological clocks and many other processes in a reef-building coral. Corals therefore do not simply cue, or link behaviour to the full moon; our data imply that the lunar cycle controls transcriptional processes in these organisms and thereby directly regulates cnidarian biology. As other organisms, including some vertebrates, also link their reproduction to long-term changes in light cycles, both seasonal day length in terrestrial mammals (e.g. Dardente *et al.* 2010) and lunar phase in marine fishes (e.g. Fukushiro *et al.* 2011; Ikegami *et al.* 2015), it will be fascinating to determine whether similar transcriptional changes occur in other organisms in response to long-term changes in solar or lunar illumination.

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Data accessibility

RNA sequence data used in this publication are available from the NCBI SRA under the accession nos. shown in Table S2 (Supporting information). R scripts can be accessed via GITHUB at https://github.com/Vize-Lab/RNA-Seq_RScripts. Ancillary data, such as read-count files, the transcriptome and its annotations, are available via Dryad at doi: 10.5061/dryad.gk1qk.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Spectrum of the lamp used to generate lunar lighting.

Fig. S2 Noon and midnight expression comparisons for all 55 genes that change over a lunar month.

Fig. S3 Diel patterns of gene expression over a lunar month.

Fig. S4 GO analysis of GLM data over a lunar month, as per text Fig. 4.

Fig. S5 Individual expression profiles for all 273 genes showing lunar phase differential expression, as per data in Table S3.

Table S1 Lunar phase and sampling details over experimental time window.

Table S2 Read counts per sample, SRA accession nos. and transcriptome annotation updates.

Table S3 Data analysis.