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Biological clock driven circadian transcription cycles in *Acropora millepora*

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Abstract. Circadian rhythms regulate many physiological, behavioral and reproductive processes. These rhythms are often controlled by light, and daily cycles of solar illumination entrain many clock regulated processes. In scleractinian corals a number of different processes and behaviors are associated with specific periods of solar illumination or non-illumination, for example, feeding and spawning. In order to explore if the genetic pathways that regulate many diurnal processes in other animals are conserved and active in stony corals we have undertaken an analysis of diurnal expression of a number of candidate circadian genes in the coral *Acropora millepora* using deep RNA sequencing (40 million reads) and quantitative PCR. Many examples of diurnal cycles of RNA abundance were identified, some of which are under the direct control of light cycles (eg *cryptochrome 1* and *timeless*) and others that are under the control of an entrained biological clock and continue to cycle in a robust manner when kept in constant darkness (eg *cycle, clock, cryptochrome 2* and *eyes absent*). Entrained cycles of circadian gene expression occur in both coral larvae that lack zooxanthellae and in adult holobiont tissue. Corals therefore exhibit entrained circadian patterns of gene expression that may participate in the regulation of diurnal biological processes. We tested the importance of entrained clocks in regulating the timing of coral spawning after sunset. Our data show that this is not an entrained process and is directly regulated by light cycles. We are currently exploring the role of biological clocks in coordinating the date of spawning with lunar cycles.

Key words: circadian rhythm, circalunar rhythm, coral spawning, broadcast spawning.

Introduction

Broadcast spawning in corals is an extraordinary display of biological timing. In the Caribbean multiple species spawn on just one major evening just once per year, and do so in a time window that is predictable to within 20 minutes from year to year (Vize et al. 2005; Levitan et al. 2011). This timing plays a critical role in broadcast spawning synchronization, and corals that spawn outside of this narrow time window have little chance of reproductive success (Levitan et al. 2004). Our research explores how this timing is achieved.

Many biological processes are controlled by entrained biological clocks. Environmental factors, such as exposure to light or consumption of food entrain cycles of transcription, translation and post translational modifications that regulate metabolism and optimize the biochemistry of the organism in different states. These cycles of molecular responses continue for long periods even when the entraining signals are no longer present. A classic example is sleep/wake cycles continuing on a similar schedule when an animal is kept in constant darkness. Such biological clocks can be very robust and could potentially provide the accuracy necessary to achieve to level of synchrony required to achieve the extraordinary accuracy of coral broadcast spawning.

We report that corals express many of the genes required to drive a circadian biological clock, that transcription of these genes cycles in a daily manner and that many of these genes continue their cycles of transcription even when kept in constant darkness. The role of entrained clocks in controlling the time at which corals spawn after sunset was then tested. Spawn timing was found to be directly controlled by sunset time, demonstrating that entrained clocks have no role in this process.

Circadian cycles are used in a multitude of circumstances to improve and predict biochemical status. For example, bat body temperature increases prior to waking and flight so that muscles work more effectively and energy metabolism is linked to the sleep/wake cycle, lowering energy production as animals approach sleep (for review see Dunlap et al. 2004). Although an entrained biological clock does not control the time of spawning after sunset, and may not be accurate enough to achieve the level of sophistication displayed by such behavior, it may be involved in other aspects of spawn timing, such as setting the date of spawning via the lunar cycle.

Material and Methods

Details of coral collection and culturing and molecular techniques are described by Brady et al. (2009, 2011). *Montastraea franksi* were collected from the Flower Garden Banks National Marine Sanctuary (Texas, USA) and *Acropora millepora* from the environs of Orpheus Island Research Station (Queensland, Australia).

Illumina deep sequencing was performed on two larval RNA samples 7 days old, one from the late day and one from late night. 20 million reads were generated from each sample by the BC Genome Centre. Sequence reads were aligned against the *Acropora* transcriptome (Meyer et al. 2010) by MegaBLAST with a 1e-10 cutoff, and a custom perl script used to score the number of matches to each transcript. The full analysis (approximately 20 MB) is available online at: ftp://ftp.xenbase.org/pub/Coral/ as are the primary sequence data.

Before determining differences in gene expression profiles between coral larvae in light and in darkness, the deep sequencing results were used to look for genes that had similar levels in both light and dark. This was for two reasons: i) to validate that the approach demonstrated similar expression levels in housekeeping genes under both conditions; and ii) to identify optimal controls for comparative analysis. Over 11,000 transcripts were found to have expression levels that differed by less than 20% between the two samples, at least one third of the transcriptome. We chose to use a value of 150% difference to indicate a transcript differentially expressed between the two samples. Unfortunately, the common control gene, cytoplasmic actin, was found to differ in transcript abundance 2.4 fold between day and night samples, so is not appropriate as a reference standard. RNA polymerase 2 (*rnap2*) was found to be expressed with ratios of 1.01 between light and dark and also to be expressed at similar levels to circadian genes and was used as a control in all experiments.

Results

Genes known to regulate biological timekeeping in other organisms were sought in corals by BLASTing mammalian protein sequences against an *Acropora millepora* transcriptome (Meyer et al. 2010). Candidates for most of the key transcriptional regulators of biological clocks were identified (Table 1): highly conserved (BLAST Expect values < e-50) transcripts for *clock*, *cycle*, *cryptochromes 1* and *2*, *eya*, *six* and *timeless*; and less conserved transcripts (BLAST Expect values > e-50) for many other components including *period*, *grin*, *vri*, *bhlbh2/3*, *slmb* etc. (Vize, 2009; Brady et al. 2011). A number of gene products that control the activity of the transcription factor network were extremely highly conserved, most especially the kinases *csnk1d/e*, *ckIIalpha/beta* and *dco*, while no convincing homologs were found for elements of the cAMP pathway, important in entraining mammalian clocks, such as *adcyap1* and *adcyap1r1*. Selected examples of genes found by sequence alignment are shown in Table 1, along with E values. The lower the E value the higher the match, with zero being the highest possible score.

Protein	*Acropora*	*Nematostella*
clock	e-55	e-62
cry1	e-136	0
cry2	e-130	0
cycle	e-60	e-103
eya	e-21	e-124
period	e-13	e-11
six	e-88	e-109
timeless	e-58	0
ckIIalpha	e-112	e-151
csnk1d	e-149	0
shaggy	e-176	e-172
adcyap1	e-4	e-12

Table 1. Coral and *Nematostella* protein similarities to known biological clock elements.

Matches to *Nematostella* proteins were also identified (Table 1). These had typically lower e-values than the *Acropora* matches, presumably due to the anemone proteins being longer.

We next undertook an analysis of whether these candidate circadian genes were expressed in a cyclical manner similar to that of their mammalian and arthropod relatives. This study was performed on developing *A. millepora* larvae to avoid confounding effects from endosymbiotic zooxanthellae. For six days post fertilization, larvae were kept in two culture tanks both exposed to a 12 hour light:dark (LD) cycle. On the seventh day one tank continued on the 12:12 LD cycle while the second was completely covered in thick black plastic to render it totally dark. Larval samples (approximately 200) were collected from both tanks at four hour intervals for 24 hours, frozen, and shipped to the laboratory for further analysis. RNA was isolated from each sample, reverse transcribed using a poly A primer, and subjected to two different types of analysis- RNA-seq and quantitative PCR (QPCR). RNA-seq was performed on polyA primed RNA on one day and one night sample. 20 million sequence reads were generated from each sample. These were matched against the

coral transcriptome by BLAST and the number of matches to each transcript counted using a perl script (Brady et al. 2011). The scores for a set of genes from Table 1 are shown in Table 2.

Gene	Day Reads	Night Reads	Fold Change
clock	681	216	3.15
cry1	5724	353	16.22
cry2	1843	309	5.96
cycle	6	10	1.67
eya	4	23	5.75
six	345	589	1.71
timeless	27	69	2.56
vrille	12	2499	208.25
RNAP2	745	750	1.01

Table 2. RNA-seq analysis of transcription levels under light and dark conditions.

The 24 hour profiles of seven of these transcripts under light:dark (LD) and dark:dark (DD) conditions were then assayed by QPCR. Each transcript was assayed in triplicate and compared to RNAP2 controls, also assayed in triplicate. The results were then plotted using the ARSER algorithm (Fig.1). ARSER is an algorithm that combines both time domain (sinusoid-based patterning matching) and frequency domain (e.g. Fischer's G-test that detects periodic gene expression profiles) analyses, and is available at http://bioinfo.cau.edu.cn/BioClock/index.php (Yang and Su 2010).

As Fig. 1 shows, many genes continue to be transcribed in a circadian rhythm even when kept in constant darkness. Genes displaying this behavior include *cry2, clock, cycle* and *eya*. Genes that responded to light, but which did not display entrained expression include *cry1* and *six*. Of these latter genes, *six* expression, normally higher at night (Table 2), is constantly elevated under DD conditions. These genes displayed similar cycles of expression both in larvae that lack zooxanthellae (Fig.1) and in adult holobionts (Brady et al. 2011).

Solar cycle regulation of coral spawning time

To test if the time of spawning after sunset is under the control of an entrained biological clock we performed a sunset shifting experiment. Work by others had shown that applying artificial early sunset times for a few days prior to spawning could shift spawning to an earlier time (Knowlton et al. 1997). However this time shift was performed over multiple evenings and with limited sample sizes. To ensure that there was no time for a biological clock to reset

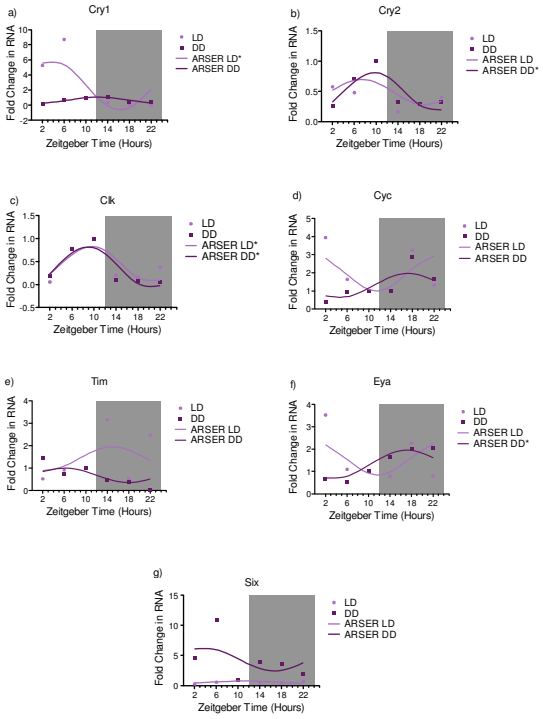

Figure 1: QPCR analysis of 24 hour transcription cycles in *A. millepora.* The control gene was RNAP2, and levels indicate the mean of triplicate measurements. A white background indicates day, and a grey background night LD were samples exposed to light and dark, DD were samples exposed to constant darkness. Asterisks represent statistically rhythmic expression.

we collected 12 samples of *Montastraea franksi* on the day of spawning. Each sample was split into three clonal fragments, and these subjected to different sunset times; normal, one hour early, and two hours early. Of the 12 samples, six spawned and the times at which this occurred are displayed in Fig. 2.

Shifting sunset time by one hour on the day of spawning resulted in a correspond shift in spawning by one hour. Shifting by two hours interfered with all six replicates ability to spawn with one exception, and this sample released only a single gamete packet. The two hour shift may not have left the coral sufficient time to move gamete bundles into the mouths of polyps ready for spawning. Further details of this experiment are available in Brady et al. (2009).

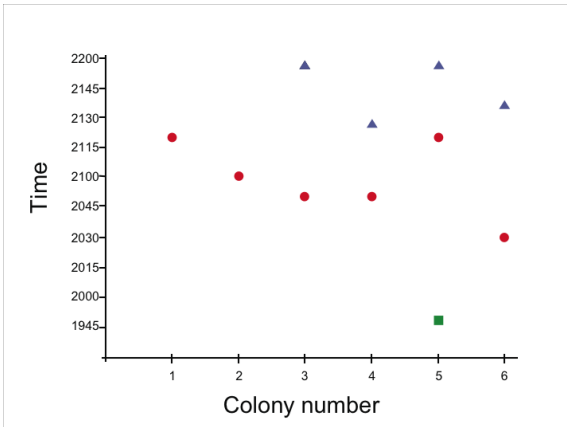

Figure 2. Spawn time after sunset is a direct response to the onset of darkness and is not controlled by a biological clock. Blue triangles represent spawning by normal sunset time fragments, red circles represent spawning by samples with a sunset time pushed forward by one hour, and the green square spawning by a sample with sunset pushed forward by two hours. From Brady et al. (2009).

Discussion

These data, and those of others (e.g. Hoadley et al. 2011; Levy et al. 2011; Reitzel et al. 2010) clearly show that corals expresses genes related to those driving biological clocks in other animals, that these genes display circadian transcription cycles, and that some of these genes are under transcriptional regulation by an entrained biological clock. The spawn timing experiments however show that this clock does not control spawning time relative to sunset. This was at first little surprising as it would seem to be a useful system to improve spawn timing consistency under different weather conditions. However, the alternative that corals employ, an hourglass mechanism that starts immediately upon darkening, does make sense especially in an evolutionary context. As corals respond directly to light, photoreceptors must detect light and alter signal transduction pathways resulting in different states under light and dark conditions. Intracellular light responses are mediated by controlling the levels of second messengers, in some species these are cyclic nucleotides, in others, cytoplasmic calcium. During speciation, a shift in spawning time- a key element in reproductive isolation- would be complex to achieve via an entrained clock with their complex feedback loops. However, in an hourglass mechanism a simple shift in activity of the enzymes that generate or destroy cyclic nucleotides, or the proteins that manage calcium levels or calcium responses, would result in a shift in spawn timing with a change in a single gene.

These observations raise the obvious question of what is the endogenous biological clock of corals doing? Does it have any role in regulating broadcast spawning behavior? Many biochemical and cellular processes shift in a cyclic manner between day and night, for example cell division rates, metabolism, energy production etc (Brady et al. 2011, Levy et al. 2011). The entrained clock very likely regulates many of these processes, though this remains to be demonstrated. In terms of reproductive behavior we are currently exploring if the biological clock participates in setting the date of spawning. This is under the control of the lunar cycle and may well be an entrained behavior (Jokiel et al. 1985). Current experiments are exploring two options, whether corals display circalunar transcription cycles or if circadian cycles intersect with lunar illumination in some other manner.

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