

CRYPTOCHROME AND CLOCK GENE EXPRESSION PATTERNS OVER DIEL AND
REPRODUCTIVE CYCLES IN *FAVIA FRAGUM*

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ABSTRACT

Natural light cycles are known to be important for synchronizing behavioral and physiological cycles over varying time periods in both plants and animals. Many corals exhibit diel cycles of polyp expansion and contraction entrained by solar light patterns and monthly cycles of spawning or planulation that correspond to nocturnal lunar light cycles. In *Drosophila melanogaster* and mammalian species rhythmic and light entrained cycles are underlined by a molecular clock involving the gene transcription factors *clock* and *cycle*. To determine if the molecular clock mechanism might also be involved in the regulation of diel and reproductive behaviors in corals, I identified cDNAs encoding the clock genes *cry1*, *cry2*, *clock*, and *cycle* in the scleractinian coral *Favia fragum*. Using qPCR I measured fluctuations in expression of these transcripts over both diel and monthly spawning cycles. Additionally, I assayed gene expression and polyp expansion/contraction behavior in experimental corals under normal light:dark (control) or constant dark treatments. Well-defined and reproducible diel variations in *cry1*, *cry2*, and *clock* expression were observed in both field-collected and the experimental colonies maintained in control light:dark conditions. Colonies in the control light:dark treatment also displayed diel rhythms in tentacle expansion and contraction. Experimental colonies in the constant dark treatment lost diel variations in *cry1*, *cry2*, and *clock* expression and displayed a diminished and more irregular pattern of tentacle expansion and contraction. I observed no pattern in *cry1*, *cry2*, *clock*, or *cycle* expression correlated with monthly spawning events suggesting these genes are not involved in the entrainment of reproductive cycles to lunar cycles in *F. fragum*.

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INTRODUCTION

Diurnal cycles of sunlight and monthly cycles of lunar light vary in a predictable and cyclic patterns in most geographic locations around the globe. Accordingly, many species have evolved mechanisms to entrain behaviors to these environmental light patterns (Lin, 2002; Lin and Todo, 2005). Scleractinian corals display behavioral and reproductive changes corresponding to both diel solar and monthly lunar light cycles. Many shallow water coral species retract tentacles during the day and extend them at night to feed (Yonge, 1940; Sweeney, 1976; Sebens and Deriemer, 1977; Lasker, 1979). Over longer periods of time, the lunar cycle provides a light cue that is thought to play a role in synchronization of reproductive events in some scleractinian coral species (Abe, 1939; Jokiel *et al.*, 1985). Specifically, many coral species will spawn or release larvae during a certain phase in the lunar cycle, typically on an evening a few nights after the full moon (Harrison *et al.*, 1984; Szmant-Froelich *et al.*, 1985). Within a given geographic region, spawning time occurs simultaneously for all corals of a particular species. This precise and simultaneous release of gametes is thought to be an adaptation for increasing the probability of successful fertilization (Baird *et al.*, 2009).

Although rhythmic coral behaviors such as diel tentacle contraction and synchronous spawning have been well characterized, little is known about the molecular signaling pathways responsible for these behaviors. In model systems such as *Drosophila melanogaster* and *Mus musculus* circadian behaviors are maintained by a well-studied core molecular “clock” composed of the transcriptional activators CLOCK and CYCLE (orthologous to BMAL1 in vertebrates) and other positive and negative regulatory components including PERIOD, TIMELESS and CRYPTOCHROME (Lin and Todo, 2005; Looby and Loudon, 2005; Hardin, 2009).

A recent metagenomic analysis has shown that these genes are present in the cnidarian species *Acropora millepora* and *Nematostella vectensis* (Vize, 2009). Correlative evidence suggests that at least part of the molecular clock mechanism may play a role in reproductive synchronization within a cnidarian coral species. Specifically, upregulation of a particular cryptochrome transcript, *cry2*, was observed during a full moon seasonal spawning event in the broadcast spawning coral *A. millepora* (Levy *et al.*, 2007). In mammalian model organisms, molecular clock components, including cryptochromes, are also thought to play fundamental roles in the timing of reproductive processes (Dolatshad *et al.*, 2009).

Recently, several of the core clock genes including, *clock*, *cycle*, *timeless* and several *cryptochrome* variants were identified within *N. vectensis* (Reitzel *et al.*, 2010). Of these genes, *clock* and two *cryptochrome* variants were shown to undergo rhythmic circadian expression similar to that observed for mammalian and *D. melanogaster* clock gene orthologs (Looby and Loudon, 2005). These findings suggest the presence of a conserved molecular clock mechanism within this group of early metazoans. Based on the established role these genes play in maintaining both short term circadian and long term reproductive rhythms within insect and mammalian species (Boden and Kennaway, 2006; Dolatshad *et al.*, 2006; Sandrelli *et al.*, 2008; Dolatshad *et al.*, 2009; Hardin, 2009), these genes may also be important in synchronizing both diel and monthly behaviors within the scleractinian coral *Favia fragum*, a small Caribbean reef coral that has a lunar cycle of reproduction (Szmant-Froelich *et al.*, 1985).

In this study, I aimed to correlate monthly and diel variations in transcript abundance of *cry1*, *cry2*, *clock*, and *cycle* in *F. fragum* to key events during the monthly reproductive and daily diel cycle. In contrast to *A. millepora* and many other broadcast spawning corals which reproduce annually (Harrison *et al.*, 1984; Baird *et al.*, 2009), *F. fragum* is a brooding coral

species that reproduces monthly throughout the year in a predictable lunar pattern (Szmant-Froelich *et al.*, 1985). Understanding the patterns of expression of these genes will help elucidate the circadian molecular clock mechanism in corals and the evolution of clock mechanisms within the metazoan lineage.

MATERIALS AND METHODS

Study Site

Colonies of *F. fragum* were collected along the western side of Isla Magueyes, Puerto Rico, during August 2009 and June 2010. Only healthy colonies visually assessed to be free of disease, with full tissue coverage and more than 20 polyps were selected for use in this study. Coral samples collected as described below were shipped to UNC Wilmington on either dry ice or in liquid nitrogen for RNA extraction or in 70 % ethanol for histology. Water temperature and light intensity at the collection site were recorded using Hobo data loggers (Onset Computer Corporation, Pocasset, Massachusetts) throughout each collection period.

Sample Collections

Diel Sampling: To investigate the diel expression of *cry1*, *cry2*, *clock*, and *cycle* in field collected *F. fragum* colonies, relative transcript abundances were measured using qPCR on samples collected over a 24 h (August 2009) and again over a of 60 h (May 2010) period. On 5 August 2009, the day of the full moon, samples were collected every 4 h over a 24 h period at 1400, 1800, 2200, 0200, 0600, 1000, and 1400 h local time. A second series of samples were

collected every 4 h over a 60 h period starting on 31 May 2010 at 1000 h local time, spanning days 19 through 21 of that lunar month.

Lunar cycle: To investigate the possible involvement of *cry1*, *cry2*, *clock* and *cycle* genes in the monthly spawning cycle of *F. fragum*, relative transcript abundances were measured using qPCR in samples collected at 3 diel time points on each of 5 days and 2 diel time points on a sixth day. Days were strategically chosen to correspond with specific reproductive events, especially spawning and onset of embryogenesis (days 15-20), in the *F. fragum* life cycle (Szmant-Froelich *et al.*, 1985). Sample dates were 29 July and 3, 5, 7, 10, and 15 August 2009, which encompass days 7 through 25 of the lunar cycle. 5 samples were collected each day every 8 h at 0600, 1400, and 2200 local time.

Collected coral colonies were cleaved in half. One half was immediately flash frozen in liquid nitrogen for future RNA extraction. The other half was prepared for histology by fixing in Zenker's formaldehyde (Szmant-Froelich *et al.*, 1985) for 5 h, decalcifying in 10% HCl for approximately 24 to 48 h, and storing the sample in 70 % ethanol for transportation back to UNC Wilmington.

Light Manipulation Experiments

To examine the light-dependence of diel patterns of gene expression, clock gene expression measured in corals maintained under a normal light:dark conditions was compared to expression measured in corals maintained in constant darkness. Polyp expansion/contraction behaviors were also monitored during the experiment. During May 2010, 60 freshly collected colonies were transferred to an outdoor, flow-through water table measuring 1.83 m long by 0.6 m wide with flow rates of 0.4 liters s⁻¹, under full natural light. Seawater for the Magueyes

seawater system is pumped from a location near the sampling site and, therefore, is similar to the water quality and temperature of field collected corals. For all laboratory coral colonies, monitoring of polyp expansion began 4 h after initial collection and was monitored thereafter every 4 h for 5 days. Colonies were visually scored as having fully expanded polyps (1), partially expanded polyps (0.5), or retracted polyps (0) as described previously (Sweeney, 1976). After a 24 h acclimation period, 30 of the corals were moved at night (in the dark) to an adjacent water table covered with a sheet of dark plastic to provide constant dark. Polyp expansion was monitored in the constant dark treatment with a low intensity red light emitting diode (LED) head lamp. After 72 h in either the normal light:dark or constant dark conditions, 5 colonies were collected and immediately flash frozen in liquid nitrogen for RNA extraction. Additional sets of colonies were collected over a 2 day period at 1000, 1400, 1800, 0200, 1000, 1400 and 1800 local time. Throughout the experiment, water temperature and light intensity in both tanks were recorded using Hobo data loggers (Onset Computer Corporation).

RNA Extraction

Frozen coral samples were ground into a powder using a mortar and pestle chilled on a bed of dry ice. Total RNA was extracted and purified from each sample using TRIzol Reagent (Invitrogen) and Pure Link (Invitrogen) clean up kits and a modified version of the manufacturer's protocols that included an additional chloroform extraction and sodium acetate precipitation step. Purified RNA samples were then analyzed using a NanoDrop 2000 spectrophotometer (ThermoScientific) and a 2100 Bioanalyzer (Agilent Technologies) to assess RNA quantity and quality. (RNA integrity numbers were on average 9.2 and always > 8.4.) Only samples with concentrations greater than 50 ng μl^{-1} were used for subsequent analyses.

Reverse Transcription PCR Amplification (rtPCR)

Reverse transcription PCR reactions were performed using 200 U μL^{-1} Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers following the manufacturer's protocols. cDNA reactions for rtPCR and also quantitative real time PCR were made from 800 ng and 10 ng of total RNA respectively. For rtPCR amplification, 1 μL of cDNA was used in each 50 μL reaction. Degenerate primers for *cry 1*, *cry 2*, *clock*, *cycle* and the housekeeping gene *ef1 α* (*elongation factor 1 α*) were designed using Primer Express software (AppliedBiosystems) or Primer Quest software (Integrated DNA Technologies) and available sequence data for *N. vectensis* and *A. millepora* (Appendix B). All rtPCR reactions were carried out with either TSG polymerase (Bio-Basics, Inc.) or high fidelity polymerase (BioRad) and then purified using QIAquick PCR purification kits (Qiagen). For rtPCR reactions using TSG polymerase, each 50 μL reaction contained 0.4 μL polymerase, 1 μL 10 mM dNTPs, 5 μL 10X buffer, 3.5 μL 25 mM MgSO_4 , 1 μL each of 50 mM forward and reverse primer, 36.1 μL ddH₂O (nuclease-free water, GrowCells) and 2 μL of cDNA. All rtPCR reactions using TSG polymerase were performed using the following protocol: 94 °C for 1:20 min followed by 30-40 cycles of 94 °C for 40 s, 50-60 °C for 40 s, 70° C for 1 min and a final extension at 72° C for 9 min. For rtPCR reactions using high fidelity polymerase, each 50 μL reaction contained 0.5 μL polymerase, 1 μL 10 mM dNTPs, 10 μL 5X buffer, 1 μL 25 mM MgCl_2 , 1 μL each of 50 mM forward and reverse primer, 34.5 μL ddH₂O (nuclease-free water, GrowCells) and 1 μL of cDNA. All rtPCR reactions using high fidelity polymerase were performed using the following protocol: 98 °C for 30 s followed by 30-40 cycles of 98 °C for 8 s, 50-65 °C for 25 s, 72° C for 1 min and a final extension at 72° C for 8 min. Purified PCR products were sequenced commercially (MacrogenUSA, Maryland). Resulting sequences were assembled using Sequencher software (Gene Codes Corporation) and

sequence identity was confirmed using BLASTx and tBLASTx searches through the NCBI server on the Gen Bank database.

Quantitative Real Time PCR Assay (qPCR)

Primers for qPCR were designed with Primer-Quest software (Integrated DNA Technologies) or PrimerExpress (Applied Biosystems) (Table 1.). qPCR reactions were performed using a Bio-Rad iQ5 real time detection system with 2X SYBR Green master mix (BioRad). Each 25 μ L reaction contained 12 μ L SYBR Green, 0.1 μ L 25 mM $MgCl_2$, 0.25 μ L each of 10 mM forward and reverse primer, 9.9 μ L ddH₂O (nuclease-free water, GrowCells) and 2 μ L of cDNA. All qPCR reactions were performed using the following thermal profile: 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 59 °C for 1 min. Samples were run on 96 well plates with optical film. Standards were constructed from pooled total RNA samples from multiple time points and diluted in a 4-fold dilutions series prior to cDNA synthesis. Standards were run in triplicate and samples run in duplicate. Immediately after the qPCR reaction, a dissociation curve between 59 and 95 °C in 0.5 °C intervals was performed. Efficiency values for each gene were calculated using the formula $E = 10^{(-1/\text{slope})}$ (Rasmussen, 2000). Average efficiency values for each gene are reported along with primer sets (Table 1). Correlation coefficients (r^2) for all genes were between 0.975 and 1.00. All data were normalized to *efl α* expression from the same sample and presented as a ratio relative to *efl α* . The gene *efl α* was used to normalize data because it showed no significant variation throughout our sampling assays. Relative expression values for each gene were calculated by dividing the above value by the highest value within each gene assay. Negative control reactions were carried out on a subset of the samples and on average contributed less than 0.1 % of the overall signal. To exclude the

possibility of amplification of cDNA from symbiotic algae (zooxanthellae) in the corals, all qPCR primers were screened by rtPCR against cDNA and genomic DNA extracted from *Symbiodinium sp* to ensure no amplification (algal samples provided by Dr. Mary Alice Coffroth, State University of New York, Buffalo).

Assessment of Reproductive Stage

To confirm that the *F. fragum* colonies sampled during the lunar sampling schedule were undergoing the expected monthly pattern of reproduction, histology was used to detect the presence of mature oocytes and spermaries in a subset of the samples analyzed by qPCR. Decalcified coral tissues were processed and stained using methods described previously (Szmant-Froelich et al., 1985). Briefly, samples were dehydrated in ethanol, cleared with xylene and embedded in Paraplast-Plus. 7µm thick sections were stained with Heidenhain's azocarmine-aniline blue. The presence of mature spermaries and oocytes within each colony was determined by examining 4 to 8 polyps per colony. The data are presented as the proportion of colonies on any given sampling day with mature gametes (n = 5-12 colonies). Mature oocytes were identified by their large size (>250 µm), distinctive indentation of the nuclei, and dark red coloration. Mature spermaries were identified by the distinctive bouquets of tails.

Statistical Analyses

Proportional qPCR data for all sampling sets were either log arcsine or log10 transformed prior to statistical analysis and analyzed using SigmaPlot 11 (Systat Software Inc). Data from the diel sampling set from 2009 and 2010 were analyzed separately using a Kruskal-Wallis one-way Analysis on Ranks with a Dunn's post-hoc test with the factor being time. Data points from the

same time for different days were not found to be statistically significantly different and were, therefore, pooled and analyzed over a single 24 h cycle. Gene expression data for the light manipulation experiments and for samples collected over the lunar cycle were analyzed using a two-way (time of day and lunar day as the two factors for the lunar set and time of day and treatment for the light manipulation set) ANOVA with a Holm-Sidak post-hoc test. Analysis of interactions within the lunar cycle was not possible because of an incomplete data set (2200 h day 25 missing). The expression assays for *cry1*, *cry2* and *clock* over the lunar cycle failed the normality test. However, a normal probability plot of each assay suggested that failure to meet normality was only attributed to one or two data points.

RESULTS

Identification of Clock Genes in *Favia fragum*

Reverse transcription PCR with degenerate primers was used to identify and sequence partial cDNAs encoding the genes *cry1*, *cry2*, *clock*, and *cycle* within the *F. fragum* transcriptome. Gene identity was initially confirmed using BLASTn and tBLASTx searches in NCBI. Phylogenetic analyses using the neighbor joining method through MEGA 4 was used to confirm gene identity and also identify cnidarian orthologs (Appendix D, Figure 5 and 6). The resulting analyses suggest *A. millepora cry1* and *N. vectensis cry2* are most similar to *F. fragum cry1*, *A. millepora cry2* and *N. vectensis cry1a* and *cry1b* are most similar to *F. fragum cry2*, and *N. vectensis clock* and *cycle* are most similar to *F. fragum clock* and *cycle*.

Diel Patterns of Gene Expression

Significant diel peaks in expression were observed for *cry1* at 1000 h for both the August 2009 and May 2010 collection periods (Figure 1). Compared to expression levels measured at dawn (0600 h), dusk (1800 h), and nighttime (2200 h and 0200 h), *cry1* relative expression at (1000 h) was always elevated ($p < 0.05$; Figure 1A). Interestingly, *cry2* showed a similar pattern of cyclic expression, but with expression peaking at dusk (1800 h) and significantly elevated compared to transcript levels measured at night time (0200 h) and dawn (0600 h; $p < 0.05$; Figure 1B). Like *cry2*, *clock* expression peaked at 1800 h (dusk) and was significantly elevated compared to levels measured at dawn (0600 h) and morning (1000 h) ($p < 0.05$; Figure 1C). Although *cycle* showed significantly increased relative expression at night (2200 h) compared to dawn (0600 h) and midday (1000 h and 1400 h) during the August 2009 period ($p < 0.05$; Figure 1D gray symbols), no significant diel variation was observed during the more extensive May 2010 period ($p = 0.129$; Figure 1D black symbols).

Light Dependence of Gene Expression

During an initial 24 h normal light:dark period, experimental coral polyp expansion and contraction occurred as expected, with nighttime polyp expansion higher than daytime expansion in both groups (Figures 2A-C). After collection of this baseline data, one group of corals remained in normal light:dark conditions while a second group was switched to a constant dark treatment. Under normal light:dark treatment, nighttime polyp expansion continued to be greater than daytime expansion levels throughout the experiment (Figure 2A). In contrast, polyps in constant dark initially showed a prolonged period of expanded and partially expanded polyps

followed by a period of mostly contracted polyps (Figure 2C). The data suggest that polyp expansion/contraction behavior was different between the two treatments (Figure 2A and C).

Relative expression of *cry1*, *cry2*, and *clock* in corals maintained in the light:dark treatment conditions showed peaks in transcript abundance that matched those times observed in the field (compare Figure 3 with Figure 1A-C). Specifically, *cry1* expression was elevated at 1000 h and *cry2* and *clock* expression were significantly elevated at 1800 h over nighttime (0200 h) and dawn (0600 h) expression levels ($p < 0.05$; Figure 3A-C). In contrast, the relative expression of *cry1*, *cry2*, and *clock* in corals maintained in constant darkness showed no pattern in expression over the sampling period ($p > 0.05$ Figure 3A-C). Peak levels in transcript abundance for *cry1* (1000 h, for both diel cycles), *cry2* (at 1800 h for the first diel cycle), and *clock* (at 1800 h for both diel cycles) observed in corals maintained in normal light:dark were significantly elevated compared to expression at those time points in corals maintained in constant dark ($p < 0.001$; Figure 3A-C). Although there was no rhythmic pattern of expression for *cry1* throughout the constant dark treatment, the level of expression was high when compared to normal nighttime (0200 h) levels ($p < 0.001$; Figure 3A). Only *cycle* showed no statistically significant differences in expression between corals maintained in normal light:dark compared to those in constant dark ($p = 0.52$; Figure 3D). However, statistical analyses did indicate that there was a small but significant increase in *cycle* expression for both light:dark and constant dark conditions at 1400 h compared to 0200 h ($p < 0.05$; Figure 3D). This increase was not repeated at 1400 h on the second day and is likely due to the large arrhythmic variation observed in *cycle* expression.

Lunar Pattern of Expression

As expected from the diel cycle data, levels of expression for *cry1* and *cry2* were elevated in corals collected at 1400 h compared to those collected at 0600 h and 2200 h. Expression levels of *clock*, but not *cycle*, were elevated in corals collected at 1400 and 2200 h over those at 0600 h. Thus, for detection of lunar cycles of gene expression, comparison among lunar days was done independently for each time of day sampled, and the most informative sampling times were those at 1400 h and 2200 h.

Although there appears to be a trend of increasing *cry1* and *cry2* transcript abundance over the sampling timeframe, no statistically significant differences in transcript abundance among lunar days were detected for *cry1* (Figure 4A). There was, however, a significantly elevated daytime expression of *cry2* on days 20 and 25 compared to transcript abundances measured on day 8 ($p < 0.01$; Figure 4B). No significant differences in expression among lunar days were detected for *clock* ($p = 0.369$; Figure 4C). Transcript abundance of *cycle* did vary temporally, with expression levels on day 25 being significantly elevated over days 8, 17 and 20 ($p < 0.05$; Figure 4D).

Histological analysis of the colonies sampled for the lunar reproductive cycle showed that the number of colonies with mature oocytes and spermaries peaked on day 15 (the full moon) and declined to almost none on day 20 of the lunar cycle, reflecting spawning and initiation of embryogenesis by day 20 (Figure 4E). Thus, the *F. fragum* samples collected in this study were undergoing the same pattern of gamete maturation, spawning, and embryogenesis over the collection time period as described previously (Szmant-Froelich, et al. 1985).

DISCUSSION

Significant diurnal fluctuations in *cry1*, *cry2*, and *clock* expression in *F. fragum* were observed within diel sampling sets. Specifically, *cry1* showed elevated expression at 1000 h and both *cry2* and *clock* showed elevated expression at 1800 h. In contrast, *cycle* expression was highly variable with no repeatable pattern of expression. These findings are consistent with observations of *cryptochrome* expression in *A. millepora* (Levy *et al.*, 2007) and largely consistent with observations of *cryptochrome*, *clock*, and *cycle* in *N. vectensis* (Reitzel *et al.*, 2010). Both *cry1* and *cry2* (similar to *F. fragum cry1* and *cry2*) showed significantly elevated expression in *A. millepora* in response to light (Levy *et al.*, 2007). With *N. vectensis cry1a* and *cry1b* (similar to *F. fragum cry2*) and *clock* showed elevated expression in response to light, with *cry1a* and *cry1b* peaking in expression during the middle of the subjective day and *clock* peaking in expression just before the onset of subjective night (Reitzel *et al.*, 2010). Also similar to our observations in *F. fragum*, *cycle* expression showed no light-dependent variation in *N. vectensis* (Reitzel *et al.*, 2010). The rhythmic and repeatable expression of *clock* but not *cycle* in both *F. fragum* and *N. vectensis* are similar to observations in *D. melanogaster* (Hardin, 2009). Although *clock* and *cycle* are the essential positive transcriptional regulators of the molecular clock in *D. melanogaster* and *M. musculus*, diel expression of these genes differs between these groups (Looby and Loudon, 2005; Hardin, 2009). In particular, *clock* but not *cycle* shows diel variations in expression in *D. melanogaster*, whereas *bmal1* (the mammalian ortholog of *cycle*) but not *clock* shows diel variation in *M. musculus* (Looby and Loudon, 2005). The cyclical expression of the *cryptochrome* and *clock* genes within *F. fragum* suggests a molecular and mechanistically conserved animal circadian clock most similar to that found in *D. melanogaster*.

Although the similarities in our findings in *F. fragum* with those in *N. vectensis* suggest largely similar mechanisms of circadian clock function between these two cnidarians, I did observe differences in *cryptochrome* gene expression. Specifically, I observed significantly elevated diurnal expression of both *cry1* and *cry2* in *F. fragum*, whereas expression of *N. vectensis cry2* (similar to *F. fragum cry1*) remained relatively stable over the subjective day and night (Reitzel *et al.*, 2010). Possible differences in *cryptochrome* gene expression may reflect differences in the life history strategies of *F. fragum* and *N. vectensis*. *N. vectensis* does not have symbiotic algae nor does it secrete a carbonate skeleton. Both feeding and calcification have diel cycles in many scleractinian corals and could require different or additional molecular mechanisms to coordinate those behaviors. Interestingly, peaks in *cry1* and *clock* expression are offset, whereas peaks in *cry2* and *clock* expression are coincident in *F. fragum*. In both *D. melanogaster* and *M. musculus*, *cryptochrome* gene expression is activated by CLOCK:CYCLE/BMAL1 and mediates the negative regulatory loop of the circadian clock (Looby and Loudon, 2005; Ko and Takahashi, 2006). Diurnal and nocturnal animals show differences in the relative timing of *cry* and *clock* gene expression (Kume *et al.*, 1999; Caldelas *et al.*, 2003). Differences in *cry1* and *cry2* gene expression relative to *clock* gene expression in *F. fragum* may reflect its diurnal calcification and symbiosis (Kawaguti, 1948); and nocturnal feeding; (Abe, 1939; Yonge, 1940) behaviors.

Experiments examining the expression of *F. fragum* clock genes in light:dark and constant dark conditions revealed that the diel peaks in expression of *cry1*, *cry2*, and *clock* are dependent on light and not observed under conditions of constant dark. These results are consistent with observations of the loss of diurnal peaks in expression of *cry1* and *cry2* in *A. millepora* (Levy *et al.*, 2007) and *cry1a* and *cry1b* (similar to *F. fragum cry2*) in *N. vectensis* in

the absence of light. Interestingly, in both *D. Melanogaster* and many vertebrates, rhythmic expression of clock genes continues under constant dark conditions (Zheng *et al.*, 2001; Chong *et al.*, 2003; Ko and Takahashi, 2006; Hardin, 2009). I additionally examined diel tentacle expansion and contraction, a potential behavioral output of the molecular circadian clock, in light:dark and constant dark conditions. I observed significant loss of rhythmicity in tentacle contraction and expansion in *F. fragum* in the absence of a diel light cycle. Under conditions of constant dark, *F. fragum* maintained the normally daytime phenotype of retracted tentacles, but the nighttime expansion pattern was less pronounced. The loss of behavioral rhythm in *F. fragum* is in stark contrast to observations in *D. melanogaster* and many vertebrates, which both display robust behavioral rhythms (level of activity and sleep cycles) even in the absence of light (Honma *et al.*, 1998; Thresher *et al.*, 1998; Zhu and Green, 2001; Chong *et al.*, 2003; Hardin, 2009). However, knockout *period* strains of both *D. melanogaster* and mice displayed diel clock gene expression under natural light cycles and arrhythmic clock gene expression under constant dark conditions (Hardin *et al.*, 1992a, b; Zheng *et al.*, 2001). I was unable to isolate the clock gene *period* within the *F. fragum* transcriptome. Analyses of the fully sequenced *N. vectensis* genome also failed to identify *period* and have led to the suggestion that this gene may have been lost within the cnidarians (Reitzel *et al.*, 2010) or evolved after the split between cnidarians and bilaterian metazoans. Because PERIOD is an essential negative regulator of the molecular clock in *D. melanogaster* and *M. musculus* (Looby and Loudon, 2005; Ko and Takahashi, 2006; Hardin, 2009), its absence in cnidarians suggests differences in the negative regulatory loop of the circadian clock between cnidarians and bilaterians.

Parallel histological analyses of gamete maturity and number and quantification of the relative *cry1*, *cry2*, *clock*, and *cycle* expression in *F. fragum* during a three-week period centered

on the full moon showed that, although spawning occurred as expected, just after the full moon, it was not associated with a relative peak in expression of the genes *cry1*, *cry2*, *clock* or *cycle*. I did, however, observe a gradual and significant increase in expression of *cry2* and *cycle* 10 days after the full moon. These results contrast with observations of increased *cry2* expression in *A. millepora* on the night of the full moon relative to the new moon night (Levy *et al.*, 2007).

In summary, I saw no evidence to support a correlation between peaks in expression of several clock genes and timing of spawning by *F. fragum*. However, the clear diel fluctuations observed in *cry1*, *cry2* and *clock* gene expression suggest a molecular clock mechanism exists within the scleractinian taxa. The rhythmic expression in *clock* and not *cycle* suggests a mechanism more similar to that found within *D. melanogaster* than *M. musculus*. An arrhythmic pattern of expression during constant dark conditions differs from what has been observed in both *D. melanogaster* and vertebrates, suggesting an ancestral mechanism in *F. fragum* of circadian regulation that does not involve the *period* gene. Future research on the cnidarian molecular clock should focus on the protein interactions within this system and how they produce the novel clock gene expression profiles observed in this work.

Table 1. qPCR Primers

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Avg. Efficiency (%)	Size (bp)
<i>ef1a</i>	ATCAGGTGATGCTGCCATTGTCTC	GCCAAACAGCTACAGTCTGCTTCAT	101.5	124
<i>cry1</i>	CAGGAAATCCTTTCAGGACTGGCA	ATGCTGGTAACTGGATGTGGCTCT	97.1	143
<i>cry2</i>	TTGCGGATAGAGGGTTGGATTCT	CCACAGCCAGTTGCCTACATTCAA	101.8	158
<i>clock</i>	CGACTACTGTGGTCCTGAAGATGTC	CGCAACCAAACCCAAGATTGT	100.1	125
<i>cycle</i>	TGTAATGCCATGTCACGCAAGCTG	AGCCATCAGCTGCCTCAAGAATCA	98.8	169

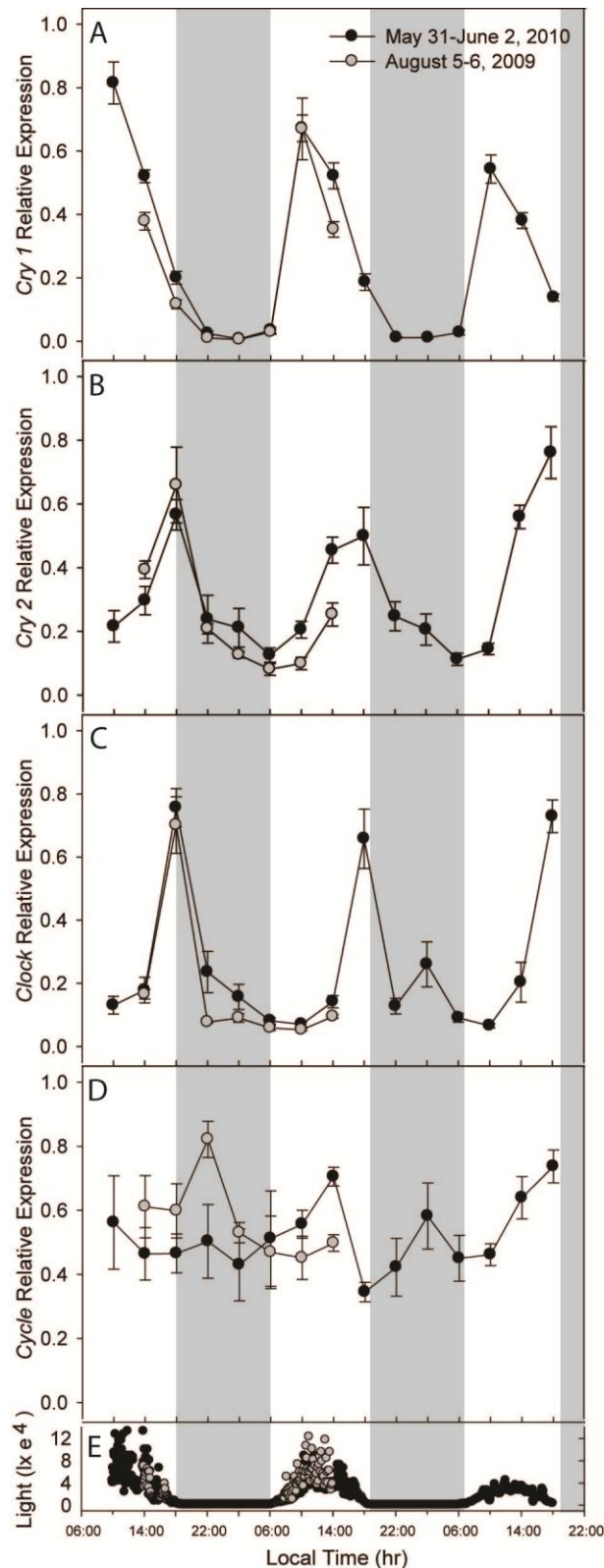


Figure 1. Relative Expression of *F. fragum* Clock Genes Over the Diel Cycle: *F. fragum* samples were collected between August 5 and 6, 2009 (gray circles) and May 31 and June 2, 2010 (black circles). Horizontal gray bars indicate nighttime. Relative expression of *cry1* (A), *cry2* (B), *clock* (C), and *cycle* (D) normalized to total RNA and *efl α* expression are shown as the mean $\pm$ s. e. m. (n = 5). Natural light levels (in lx) were measured every 5 min at a depth of 0.5 m (E). Dunn's method post-hoc analysis of May 2010 diel rhythms indicate the following significant ($p < 0.05$) differences among times: *cry1* expression at 1000 and 1400 h are significantly elevated compared to expression at 0200, 2200, and 0600 h and expression at 1000 is also significantly elevated compared to expression at 1800 h; *cry2* expression at 1800 and 1400 h are significantly elevated compared to expression at 0200, 0600, and 1000 h, and expression at 1800 h is also significantly elevated compared to expression at 2200 h; *cycle* expression showed no significant differences among sampling times ($p = 0.364$); *clock* expression at 1800 h is significantly elevated compared to expression at 0200, 0600, 1000, 1400, and 2200 h expression. Statistical analyses for the diel data collected August 2009 is provided in (Table. 3).

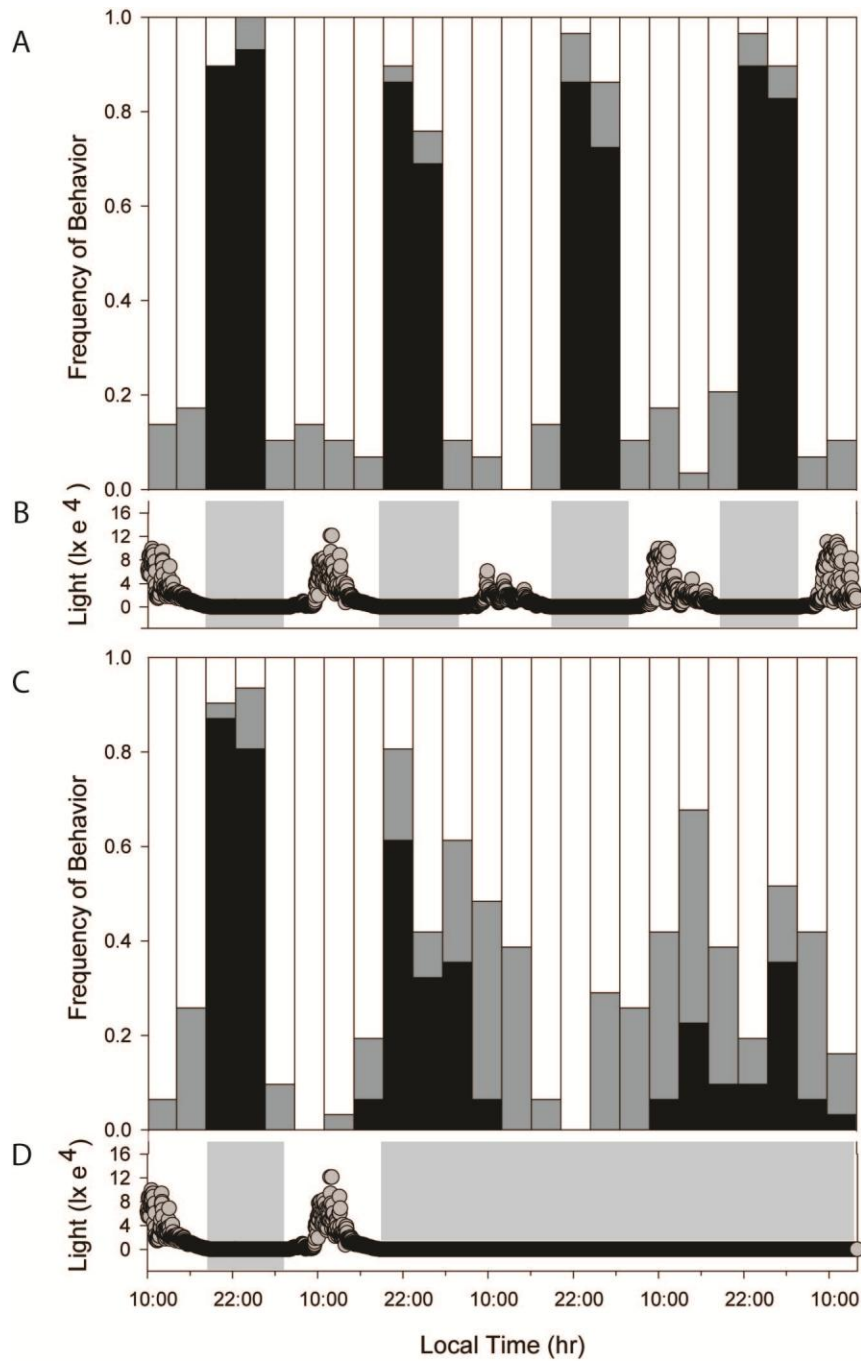


Figure 2. Diel Polyp Expansion and Contraction in *F. fragum* in Light:Dark and Constant Dark Conditions: The fraction of colonies with contracted (white), partially expanded (gray), or fully expanded (black) in light:dark (A; n = 29) and constant dark (C, n = 31) conditions. Light levels (lx) were measured every 5 min for light:dark (B) and constant dark conditions (D). Horizontal gray bars indicate dark periods.

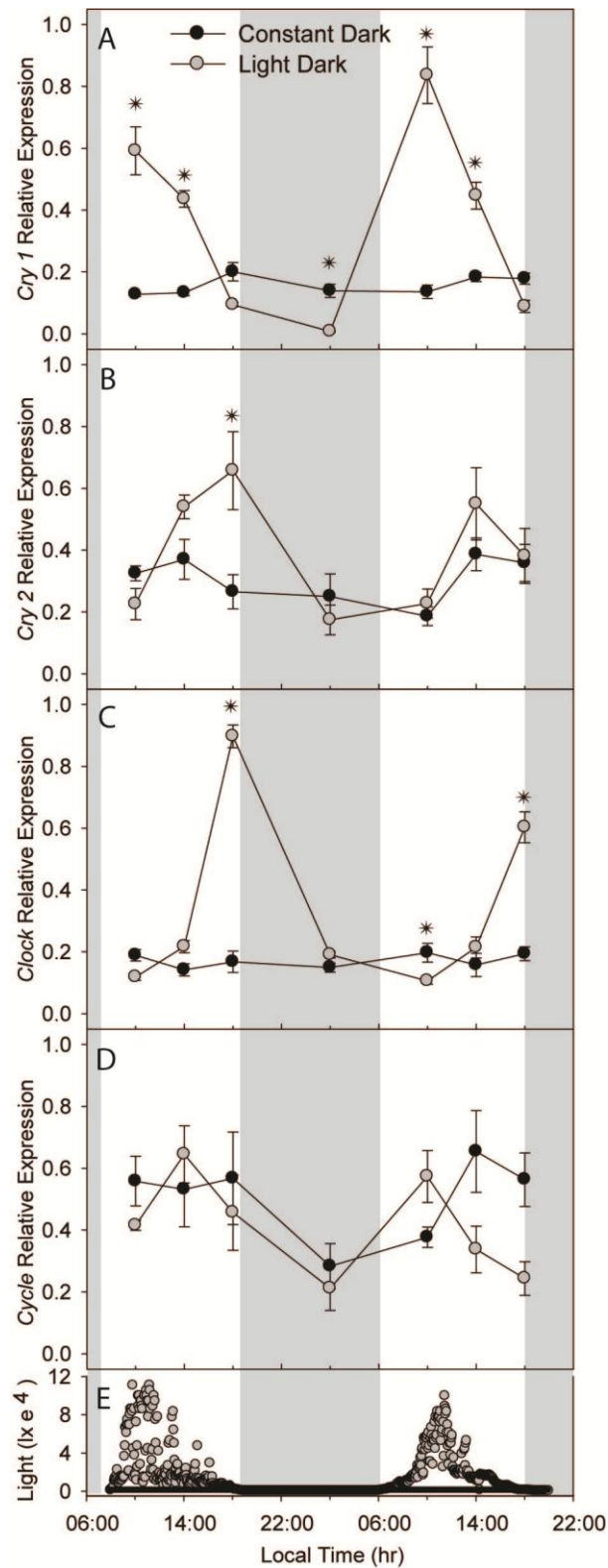


Figure 3. Relative Expression of *F. fragum* Clock Genes in Light:Dark and Constant Dark Conditions: Relative expression of *cry1* (A), *cry2* (B), *clock* (C), and *cycle* (D) normalized to total RNA and *eflα* expression are shown as the mean $\pm$ s. e. m. ($n = 4$) are shown for light:dark (white circles) and constant dark (black circles) conditions. Light levels (lx) were measured every 5 min for light:dark (E, white circles) and constant dark conditions (E, black circles). * indicates statistically significant ($p < 0.05$) differences in gene expression between light:dark and constant dark conditions.

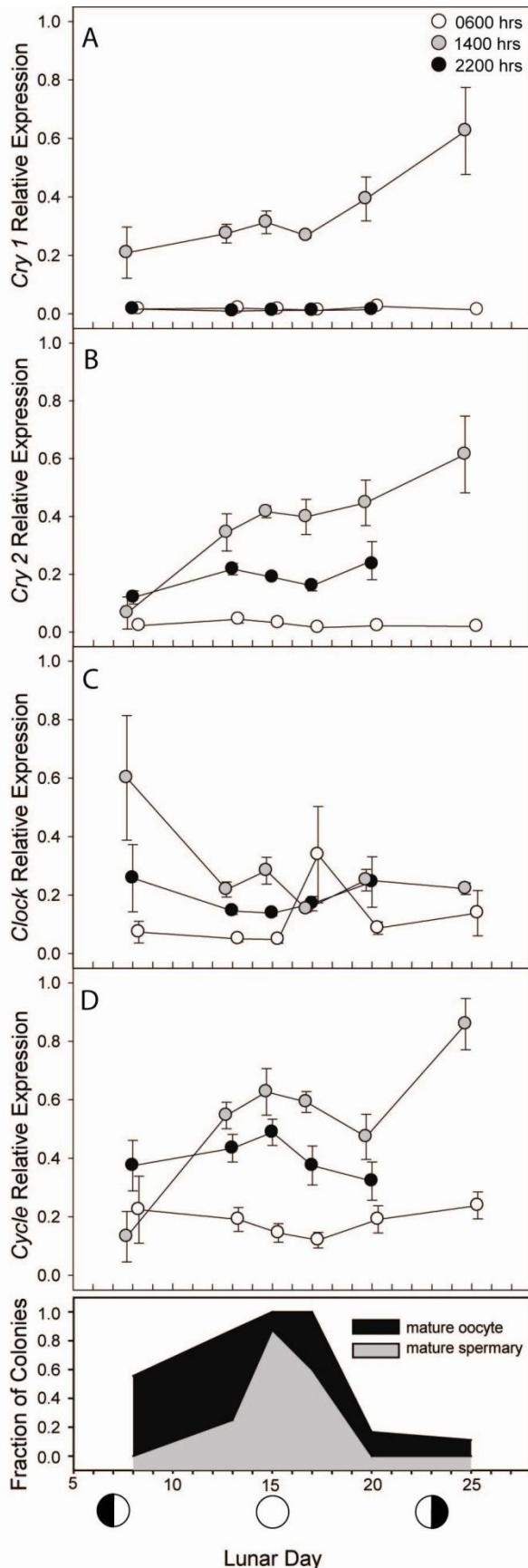


Figure 4. Relative Expression of *F. fragum* Clock Genes Over the Lunar Cycle: *F. fragum* samples were collected between July 7 and August 25, 2009 at 0600 h (white circles), 1400 h (gray circles) and 2200 h (black circles). Relative expression of *cry1* (A), *cry2* (B), *clock* (C), and *cycle* (D) normalized to total RNA and *ef1a* expression are shown as the mean $\pm$ s. e. m. (n= 4 to 5). The fraction of *F. fragum* colonies determined to contain mature oocytes (black) and spermaries (gray) is shown over the same time period (E). Two-way ANOVA indicates the following significant ($p < 0.05$) differences among times and days: *cry1* expression at 1400 h is significantly elevated compared to expression at 0600 and 2200 hrs with no significant differences in expression among lunar days; *cry2* expression at 1400 h is significantly elevated compared to expression at 0600 and 2200 h with expression on lunar days 20 and 25 significantly elevated compared to expression on day 8; *clock* expression at 1400 h and 2200 h is significantly elevated compared to expression at 0600 h with no significant differences in expression among lunar days; *cycle* expression at 1400 h significantly elevated compared to expression at 0600 h and 2200 h with expression on lunar day 25 significantly elevated compared to expression on lunar days 8, 17 and 20.

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APPENDIX A: *F. fragum* Nucleotide Sequences

clock

GAGAAACGGAGAAGGGAACGTTTCAATATACTTGTGCAAGAGCTAGCGGGGATTGT
GTCACCTAAAGAAGGTCGAAAGTTGGAGAAAACAGCTGTGCTAGAACTTGCTATAA
GCTATTTACGAAATCATCAGTCACAACAACACAAGTCTCTCCCAAGGGGGAGTAGA
GACGTCGATGCCTTGAAGTGGCAGCCTTCGTTCACTACAGACCCTGAATTTAACTTG
ATTTTAACAGATGCACTAGATTCTTTTACAATTGTGATTGAAAATGACGGGACCATA
TTATATGCCTCGCAAAGTGTTTTGCCAATCTTGGGCCATCTTTGCGATGACCTAGTTG
GCACGAGTTTGTTCAGCCACCTGAGTGAAGACGAAGCTTTGAGAGTCTGGTCGCAGT
TGGCCGTTATTGTCCCAAAAGGCGATAAAACCGGAGTAGCCTCCAACAGTAATCCTT
TCACTTTTCGCATGAAGTGTGGAAGTTTTTGTTCACGAAGCTCCTACCAAATGATTCA
TTGTAATGCTGCTGTTATTCGTCCATCCATTGACGAGAAAACCATGAACTGCTTTGTT
CTGATAGGAAAGGTCACGCACCCGCAACCGAACAAAATAATAACGAGCTCGGATAG
TACTGAAAAGGAATTTTCTTATCGCCTAACCATGGACTGGAAATATATTTATATCGA
TCACAGGGCGCCTTCNTATTATTGGATTTCTGCCTTTTGAGGTGCTGGGCACTTCAAT
TTACGACTACTGTGGTCCTGAAGATGTCTTCAATATTTCCCGGTACCACAATTTTTTA
ATTTGTGTCGGCAAGGTAACCACTTGTTACTACCGACATCTAACGAAAGGACAATCT
TGGGTTTGTTGCGAAGTTCTGGCTACGTGTCCTACAATCAGTGGAACTC

cycle

GGATCCCAATGTGTAATGCCATGTACGCAAGCTGGATAAACTGACTGTTTTGAGAA
TGGCTGTGCAGCATATGAAGACTTTAAAAGGAGCACTTGACCCATTCATGGAAACA
AACTACAAACCAGCTTTCTTGTCAGATGAGGATTTGAAGAACCTGATTCTTGAGGCA
GCTGATGGCTTTTTATTTGTTGTTGGTTGTGACAGGGGGTGTCTTCTCTATGCATCAG
ATTCCATTCAGAGTTACCTGCAACAGGCTCCTTCTGACCTTGTGGGGCATAGCTTCCT
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AGAATCAACCAACTCCAACAAGATTGTGCTCTGGGGCCAGGAGATCATTCTTTTGCC
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CTTACATTGTCGTTCACTGCACAGGTTATTTGAAATCATGGCCGCCATCAGGCTGTT
ATTTGATGATGATGACGAAGACAGTGAATCGAGAAATCTCAGCTGCCTCGTAGCGG
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GCAAAAGAGTTTCATTTACGCCACAGTATAGATGGAAAATTCATTTTTGTGCGATCAG
CGAACAACGGCTATAACGGGTTATCTTCCCCAGGAATTAATCGGAAGTTCAGCCTAC
GAATTTTTTTTACCAAGACGATTTGAATTCTATAGCCTTGTCGCATCGCCAGTCACTCC
TCGGAGAGACAGTAGTGACA

cry1

CTGGCACGACATGCAGTTGGGTGCTTCCTTACAAGAGGAGACCTTTGGATAAGCTGG
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GCTGGTAACTGGATGTGGCTCTCATAACAGTGCATTTTTTCAGCAGTTCTTTAACTGCC
TGTGTCCTGTGGGGTTTTGGAAGGAACTGGATCCCAATGGCGATTATGTCAAAAAAT
ATTTGCCAGTCCTGAAAGGATTTCTTGCAAAATATATCCATGCTCCCTGGACAGCCC
CAGAGAGTGTACAGAAAGCAGCAAGGTGTATCATTGGACAAGATTACCCACGCCT
ATTGTAGATCACAACAAAGTCAGTCGAACTAATCTGGAAAAGTTGAGAAACATTTTT
AAGGCACTCCTCTGTTACAAAGATTCATTTACTGCATCTTCTGAGAAAAAGGATAAT
GTACCAGATCGGCAGGAAAAGTTGAAACAGCAATTGTTCTCTTGGAAAGACAAAGA
AAATGATCTGTCTACTTAAAAAGAACTTTGGGTTCAAATACACTTCACTGGCCTTTA
TCAGTACAAGTCTACCAAACAACAATACTGTTATTAATTAATATTTACATAAATC
TAGTGAAGATCACGTCTCATTATAAGTGATATTAATGGTATCCAGCTAATGCTCAAT
GTTTCAAATTACTCACTATTTTAACTTATGTCCAAATGATAGATCAGCGAACCTCG
GAAAAGTGTAGAGAATCCTGCAGATCA

cry2

TTGGATTGATGCAATMATGCGACAGTTGCGGATAGAGGGTTGGATTCCTCACGTAG
CACGTCAGGCTGTTGGATGTTTCTTGACCAGGGGATGCCTTTGGGTCAACTGGGAAG
AGGGATACAAGGTTTTTTGACGAGCTTCAGTTAGATGCAGAGTGGAGTTTGAATGTAG
GCAACTGGCTGTGGCTGTCAGGAAGCACTTTTGTGAAAGAGCATGTTCSST

efla

AAAGCTGAGNNTGAAGGCAAGACTACCAAGGNNGCNGCAAAGGCCCAAAAGAAAA
AGTGAAATTCTGTGCACAATTTAAATTCAAAGAGACTCCGCTTACAAGTCAATCATG
CTGTTGTAGTAGAATGGCTTCTTTGCTATTTGTTCCATCGCAAGTCTGTCAGGAGGCG
GAGACAGCCGTGCTGTTACCTTAATTTCTGAACGATAAGTGTACTTCGTAGAAATAA
AATTAAAAAA

APPENDIX B: Degenerate Primer Sets and Alignment Sequences

Table 2. Degenerate Primer Sets and Alignment Sequences. Degenerate primers were based on alignments using *A. millepora* and *N. vectensis* sequences using Sequencher software. Primers were selected using PrimerExpress software.

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Expected length (bp)
<i>efla</i>	TGATTGTCCTCMACCATCCAGGTGA	TCCACYTCCTTAATRACRCCAACAGC	210
<i>cry1</i>	ATGGKGGWGAGACAGAAGC	CAAAYACCTTCATTCCWKCYTCC	350
<i>cry2</i>	TGGATWGATGCAATMATGCGACAG	GTGGGAAGGYKTCTTAKTTCKGG	260
<i>clock</i>	CCDTTGGARGTKCTRGAAC	AATCGRAATWTGAAYGARAAGAARCG	800
<i>cycle</i>	TGTAATGCCATGTCACGCAAGCTG	GTGTCCTCCYTTGCATYGGAATCTGTA	800

APPENDIX C: August 2009 Diel Statistical Analyses

Table 3. August 2009 Diel Statistical Analyses. Groups showing statistically significant ($p < 0.05$) differences in gene expression as determined using a Kruskal-Wallis one-way analysis on ranks with a Dunn's method post-hoc.

Gene	Local Time (24h)	Group
<i>cry1</i>	0200	C
	0600	BC
	1000	A
	1400	AB
	1800	ABC
	2200	C
<i>cry2</i>	0200	BC
	0600	C
	1000	C
	1400	AB
	1800	A
	2200	ABC
<i>clock</i>	0200	ABC
	0600	BC
	1000	C
	1400	AB
	1800	A
	2200	ABC
<i>cycle</i>	0200	B
	0600	B
	1000	B
	1400	B
	1800	AB
	2200	A

APPENDIX D: Phylogenetic Analysis of Clock and Cryptochrome Genes

A translated sequence for *F. fragum clock* (gi| to be determined) was queried under BLASTp in NCBI for the top 500 sequence hits. Similarly, the translated sequence for *F. fragum cry1* (gi| to be determined) was also queried using BLASTp in NCBI for the top 250 sequence hits. Both *clock* and *cry1* results were then reduced to a total of 54 and 37 sequences respectively to reflect a diverse range of taxa and genes identified from the BLASTp query. For the *clock* alignment, translated sequences for *A. millepora* (gi|222781555, gi|222807278) and *F. fragum cycle* (gi| to be determined) were also included. *F. fragum cry2* (gi| to be determined) was added to the *cryptochrome* alignment. Both sets were aligned using clustalx 2.0.11 software. Phylogenetic analyses were then performed using the Neighbor-Joining method with pairwise deletions in MEGA 4. Sequences were analyzed with all four models, p-distance, Poisson correction, Dayhoff and JTT (1000 bootstrap replicates).

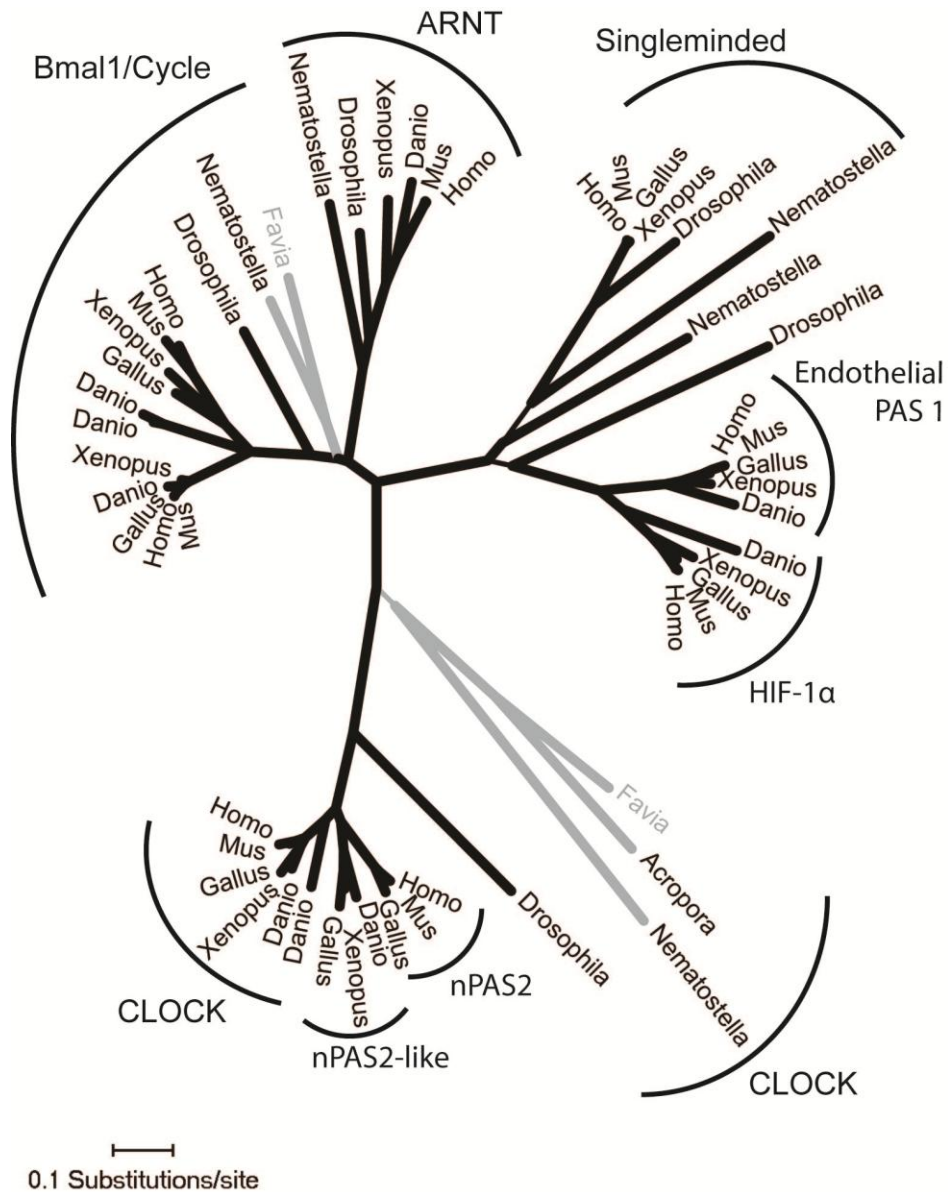


Figure 5. Phylogenetic Relationship of the *F. fragum* Genes *clock* and *cycle*: Tree depicts a radial consensus of 1000 bootstrap replicates using the Neighbor-Joining method with pairwise deletions and Poisson control. Analyses with 1000 bootstrap replicates under Dayhoff or JTT models were also run and resulted in similar phylogenetic relationships (data not shown). Thin lines represent nodes where less than 70% of the 1000 bootstrap replicates show support for the topology depicted above. Gray lines represent cnidarian lineages.

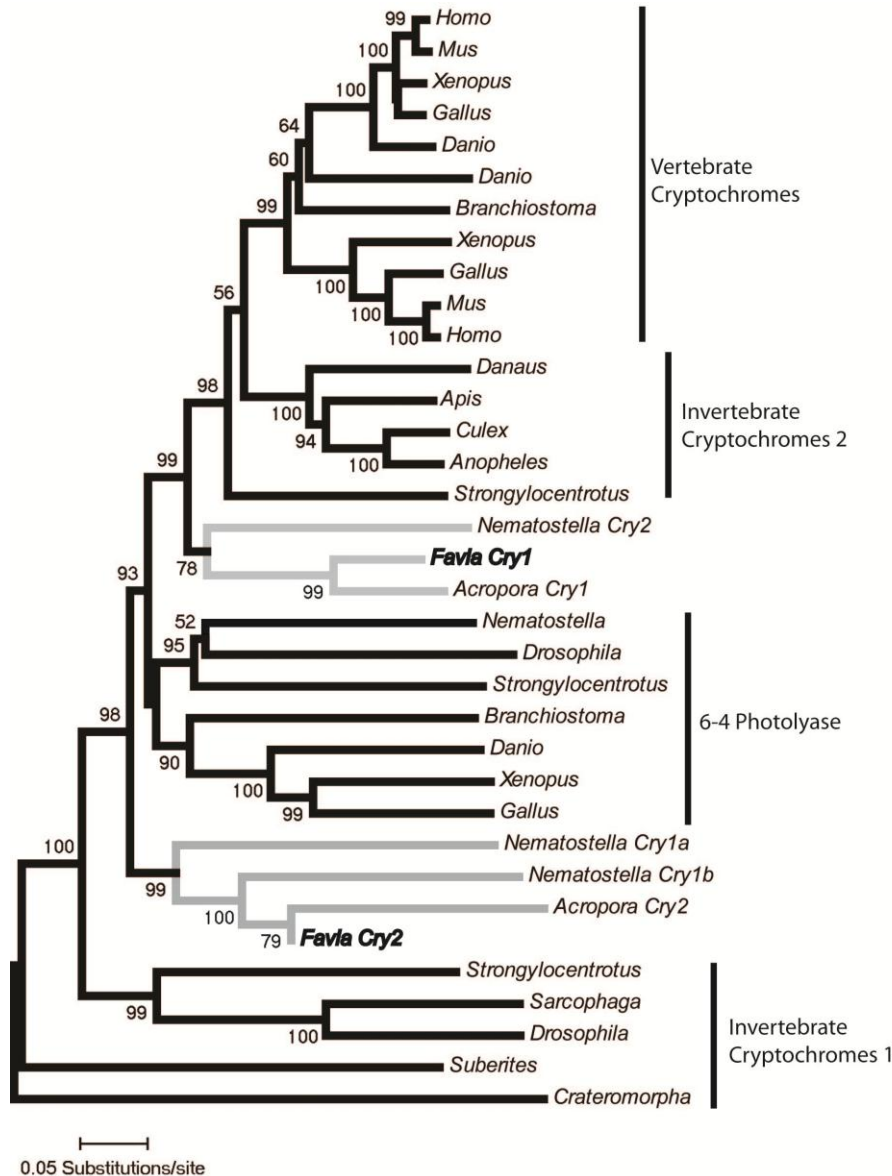


Figure 6. Phylogenetic Relationship of *F. fragum* Genes *cry1* and *cry2*: Tree depicts a consensus of 1000 bootstrap replicates using the Neighbor-Joining method with pairwise deletions and Poisson control. Analyses with 1000 bootstrap replicates under Dayhoff or JTT models were also run and resulted in similar phylogenetic relationships (data not shown). Gray lines represent cnidarian lineages.

Table 4. GI Numbers for Sequences Used in Phylogenetic Analyses

Gene	Species	NCBI Protein GI
Vertebrate cryptochromes	<i>Homo sapiens</i>	4758072
	<i>Mus musculus</i>	6681031
	<i>Xenopus tropicalis</i>	291290511
	<i>Gallus gallus</i>	45383636
	<i>Danio rerio</i>	55251266
	<i>Danio rerio</i>	66910245
	<i>Branchiostoma floridae</i>	260829102
	<i>Xenopus laevis</i>	147901075
	<i>Gallus gallus</i>	45383642
	<i>Mus musculus</i>	27312016
	<i>Homo sapiens</i>	3327130
Invertebrate cry2	<i>Danaus plexippus</i>	77166866
	<i>Culex quinquefasciatus</i>	170059953
	<i>Anopheles gambiae</i>	78191297
	<i>Apis mellifera</i>	136255185
	<i>Strongylocentrotus purpuratus</i>	115738187
	<i>Nematostella vectensis</i>	156353900
	<i>Acropora millepora</i>	145881071
	<i>Favia fragum</i>	To be determined
6-4 Photolyase	<i>Strongylocentrotus purpuratus</i>	72012168
	<i>Drosophila melanogaster</i>	3986298
	<i>Branchiostoma floridae</i>	260800305
	<i>Nematostella vectensis</i>	156383457
	<i>Danio rerio</i>	27882257
	<i>Xenopus tropicalis</i>	194332801
	<i>Gallus gallus</i>	110626125
	<i>Favia fragum</i>	To be determined
	<i>Acropora millepora</i>	145881069
	<i>Nematostella vectensis</i>	156378195
	<i>Nematostella vectensis</i>	156383455
Invertebrate cry1	<i>Strongylocentrotus purpuratus</i>	72115950
	<i>Drosophila melanogaster</i>	17137248
	<i>Sarcophaga bullata</i>	210136298
	<i>Suberites domuncula</i>	293321543
	<i>Crateromorpha meyeri</i>	293321545
Cycle/bmal1	<i>Homo sapiens</i>	2094735
	<i>Mus musculus</i>	34098759
	<i>Gallus gallus</i>	47825375
	<i>Xenopus tropicalis</i>	291290509

	<i>Danio rerio</i>	30231256
	<i>Danio rerio</i>	28279879
	<i>Danio rerio</i>	94733888
	<i>Danio rerio</i>	169158685
	<i>Xenopus tropicalis</i>	156717516
	<i>Gallus gallus</i>	45383840
	<i>Mus musculus</i>	187466535
	<i>Homo sapiens</i>	17979654
	<i>Drosophila melanogaster</i>	24667005
	<i>Nematostella vectensis</i>	156359347
	<i>Favia fragum</i>	To be determined
ARNT	<i>Nematostella vectensis</i>	156373864
	<i>Drosophila melanogaster</i>	13124680
	<i>Xenopus laevis</i>	147903135
	<i>Danio rerio</i>	113674860
	<i>Mus musculus</i>	74220117
	<i>Homo sapiens</i>	30795242
clock	<i>Favia fragum</i>	To be determined
	<i>Acropora millepora</i>	222781555
	<i>Nematostella vectensis</i>	156402728
	<i>Drosophila melanogaster</i>	3219726
	<i>Danio rerio</i>	55962414
	<i>Danio rerio</i>	190338076
	<i>Xenopus tropicalis</i>	6466210
	<i>Gallus gallus</i>	4903294
	<i>Mus musculus</i>	148705948
	<i>Homo sapiens</i>	6478192
nPAS	<i>Homo sapiens</i>	118572678
	<i>Mus musculus</i>	80479005
	<i>Gallus gallus</i>	71896369
nPAS2-like	<i>Gallus gallus</i>	118089716
	<i>Xenopus tropicalis</i>	301610093
	<i>Danio rerio</i>	30231248
singleminded	<i>Homo sapiens</i>	194239684
	<i>Mus musculus</i>	1213283
	<i>Xenopus tropicalis</i>	301624571
	<i>Gallus gallus</i>	118083884
	<i>Drosophila melanogaster</i>	3769480
	<i>Nematostella vectensis</i>	156397887
	<i>Nematostella vectensis</i>	156392022
	<i>Drosophila melanogaster</i>	24651293
Endothelial PAS1	<i>Homo sapiens</i>	40254439
	<i>Mus musculus</i>	2895756

	<i>Gallus gallus</i>	46048879
	<i>Xenopus tropicalis</i>	148227427
	<i>Danio rerio</i>	292619504
<i>HIF1α</i>	<i>Danio rerio</i>	125833414
	<i>Xenopus tropicalis</i>	102621855
	<i>Gallus gallus</i>	45383550
	<i>Mus musculus</i>	148704554
	<i>Homo sapiens</i>	3790535