Title: Circadian clocks in the Cnidaria: Environmental entrainment, molecular regulation, and organismal outputs[1]

Authors: Adam M. Reitzel,[*,^,%], Ann M. Tarrant[+,%], and Oren Levy[#]

* Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223

[+] Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

[#] The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel

% These two authors made equal contributions

^ Corresponding author:

9201 University City Blvd.
Woodward Hall 381A
Charlotte, NC 28223
Phone: 704-687-5018
Fax: 704-687-1488
Email: areitze2@uncc.edu

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ABSTRACT

The circadian clock is a molecular network that translates predictable environmental signals, such as light levels, into organismal responses, including behavior and physiology. Regular oscillations of the molecular components of the clock enable individuals to anticipate regularly fluctuating environmental conditions. Cnidarians play important roles in benthic and pelagic marine environments, and also occupy a key evolutionary position as the likely sister group to the bilaterians. Together, these attributes make members of this phylum attractive as models for testing hypotheses on role for circadian clocks in regulating behavior, physiology, and reproduction as well as those regarding the deep evolutionary conservation of circadian regulatory pathways in animal evolution. Here, we review and synthesize the field of cnidarian circadian biology by discussing the diverse effects of daily light cycles on cnidarians, summarizing the molecular evidence for the conservation of a bilaterian-like circadian clock in anthozoan cnidarians, and presenting new empirical data supporting the presence of a conserved feed-forward loop in the starlet sea anemone, *Nematostella vectensis*. Furthermore, we discuss critical gaps in our current knowledge about the cnidarian clock, including the functions directly regulated by the clock and the precise molecular interactions that drive the oscillating gene-expression patterns. We conclude that the field of cnidarian circadian biology is moving rapidly toward linking molecular mechanisms with physiology and behavior.

Introduction

In many habitats, light is a predictable signal that provides information about the environment on daily, lunar, and seasonal time-scales. The need to anticipate and prepare for periodic changes in the environment is strong, evidenced by the nearly universal presence of molecular timekeeping mechanisms in both unicellular and multicellular organisms. Circadian rhythms in behavior and physiology are driven by daily cycles in expression of, interactions between, and degradation of, the underlying molecular components. The genes forming the core timing mechanism are not shared among distantly related organisms, e.g., bacteria (Xu et al. 2003), plants (Pruneda-Paz and Kay 2010), fungi (Salichos and Rokas 2009), and animals (Harmer et al. 2001; Panda et al. 2002), which suggests that circadian regulation has evolved independently within these lineages (Rosbash 2009).

Three main hypotheses have been put forward regarding the driving forces that led to the evolution of circadian clocks. The first hypothesis is that clocks arose primarily to minimize UV damage to DNA by ensuring that replication occurred in the dark. Evidence comes from the presence of blue light-sensitive cryptochromes in plants (Somers et al. 1998) and many animals, including insects (Zhu et al. 2008) and cnidarians (Levy et al. 2007; Reitzel et al. 2010). Light-sensitive cryptochromes provide input to the central clock and are thought to have evolved from photolyases, which use blue light to repair UV-induced DNA damage. A second hypothesis is that clocks arose in the context of the requirements for redox homeostatic mechanisms, which are linked to the Great Oxidation Event (GOE) that occurred approximately 2.5 billion years ago (Edgar et al. 2012). A third hypothesis is that the real driving force for the evolution of clocks followed the symbiotic fusion of a prokaryote with an archaebacterium that gave rise to the first eukaryotic organism (DeCoursey 2003). This symbiosis required metabolic synchronization and

coordination of the cell cycles of both partners. Optimization of this interaction may have driven the evolution of an internal pacemaker.

In animals, understanding of circadian mechanisms has progressed primarily through studies of a few animal groups, particularly mammals and insects. Recently, studies of additional animal models, such as non-drosophilid insects, have revealed a more complete picture of the diversity and complexity of circadian pathways in animals (Rubin et al. 2006; Yuan et al. 2007; Zhu et al. 2008). Advances in sequencing technology have fueled an explosion of available genomic and transcriptomic databases, enabling studies of the evolution of circadian genes and their expression patterns in diverse animal models, including cnidarians (Levy et al. 2007; Reitzel et al. 2010; Hoadley et al. 2011). These molecular studies have led to hypotheses regarding circadian regulation in cnidarians and to initial functional studies. In this paper, we review the state of knowledge regarding circadian signaling in cnidarians, with a focus on sea anemones and corals, in which most studies of cnidarian circadian regulation have been conducted. We consider entrainment of the clock by light cues, molecular regulatory pathways, and the physiological and behavioral outputs of the clock. In addition to reviewing published studies, we provide new data regarding possible components of a feed-forward loop and hypotheses regarding regulation of the circadian clock of the starlet sea anemone, *Nematostella vectensis*.

Why Cnidarians?

Cnidarians, the "stinging-celled animals" that include hydras, jellyfish, corals, and anemones, are intriguing models for circadian research for several reasons. First, the lineages leading to bilaterians and cnidarians diverged early in metazoan evolution, prior to the

65 divergence of protostomes and deuterostomes. The presence of shared regulatory mechanisms
66 between cnidarians and bilaterians should provide insight into the early origins of circadian
67 regulation in animals. By studying early-diverging animals, such as cnidarians, fundamental
68 questions can be addressed regarding the evolution of photosensing, entrainment of circadian
69 clocks, and transduction of light signals to the circadian clock. Second, cnidarians are an
70 ecologically important group, and light regulates the distribution, behavior, and physiology of
71 many cnidarian species (as discussed in the following section). Understanding how cnidarians
72 anticipate, detect, and respond to light and other environmental cues will lead to a more complete
73 understanding of their physiology and ecology.

74 In addition, many reef-building corals and other cnidarians live in symbiotic relationships
75 with photosynthetic dinoflagellates in the genus *Symbiodinium*. Photosynthesis, growth, and
76 bioluminescence can all exhibit circadian periodicity, both in free-living dinoflagellates
77 (reviewed by Hastings 2007) and in those living within cnidarians or other animal hosts (Sorek
78 and Levy 2012). Many aspects of the physiology of dinoflagellates and their cnidarian hosts are
79 deeply integrated. To give two examples, corals' calcification rates vary on a daily cycle along
80 with changes in the carbonate chemistry associated with photosynthesis by the symbionts
81 (reviewed by Tambutté et al. 2011), and activities of antioxidant enzymes in scleractinian corals
82 are correlated with rates of photosynthesis in the symbionts (Levy et al. 2006). It is not currently
83 known whether the hosts and/or the symbionts use circadian mechanisms to anticipate some of
84 these daily changes. Further, it is not known whether the two timekeeping pathways (i.e., the
85 host and symbiont clocks) are entirely separate or interact with one another in any way.
86

87 **Organismal Responses of Cnidarians to Light**

Several aspects of cnidarian biology vary on daily cycles, including vertical migration, larval phototaxis, settlement behavior, expansion and retraction of the body column, and feeding behaviors, including extension of the tentacles (reviewed in Taddei-Ferretti and Musio 2000; Hendricks et al. 2012). Some of these behaviors are directly cued by light or other external signals. For example, simultaneous diel vertical migration in jellyfish has been modeled to result from individual responses to light intensity (Dupont et al. 2009). Similarly, daily cycles in corals' extension of their tentacles disappear under constant light conditions in most species and are most likely a direct response to light (Sweeney 1976; Hoadley et al. 2011). On the other hand, other rhythmic behaviors have been shown to persist in the absence of an external light cue. Recent studies of locomotor activity in the sea anemone, *Nematostella vectensis*, have shown that when animals are maintained on a 24-hour photoperiod (12 hours light: 12 hours dark), activity increased approximately two-fold during the subjective night (Hendricks et al. 2012). Animals exposed to constant light or constant darkness maintained rhythmic cycles in behavior for a period of several (3-8) days, supporting the presence of a free-running clock.

In many cnidarian species, gametogenesis and spawning are cued by seasonal, lunar, and daily changes in light intensity and spectral quality. Considerable effort has been devoted to documenting the temporal patterns of spawning by scleractinian coral species and into identifying the proximal cues used to synchronize the release of gametes or larvae; however, the role of an endogenous clock in regulating reproductive timing in cnidarians has not been demonstrated.

On a daily time-scale, manipulations of the light environment to simulate a change in the time of sunset can alter the timing of spawning (Brady et al. 2009). Following this observation, it has been proposed that the release of gametes or larvae by scleractinian corals is a direct

response to light that is unlikely to be regulated by a circadian clock (Brady et al. 2009; Hilton et al. 2012). An alternative possibility is that manipulations of the light environment provide an immediate stimulus that overrides the endogenous clock, a phenomenon known as “masking” (Aschoff 1960). For example, light typically increases activity in diurnal mammals and suppresses it in nocturnal mammals (Aschoff and Vongoetz 1988; Redlin et al. 2005). The possible role of masking following experimental manipulations of the coral light environment has not yet been evaluated. Under natural conditions, masking has the adaptive value of confining animals to their appropriate temporal niche and may complement the circadian clock by fine-tuning activity patterns in response to environmental stimuli (Redlin et al. 2005; Smarr et al. 2013). Thus, masking may be an important mechanism in the natural response of corals to moonlight.

On monthly scales, nocturnal illumination from moonlight is thought to provide a cue to synchronize late stages of gamete maturation and the night of release in corals (Baird et al. 2009). It has been demonstrated that mimicking different lunar phases over a period of days to weeks can shift the timing of spawning or planulation (Jokiel et al. 1985; Hunter 1988), and that corals can detect low levels of blue light similar to the light produced by a full moon in shallow clear water (Gorbunov and Falkowski 2002). Although the molecular mechanisms mediating this circa-annual and circa-lunar synchronization of reproduction by reef-building corals remain elusive, cryptochromes may be involved in this process (Levy et al. 2007; Hoadley et al. 2011) and may link the circadian clockwork with reproductive synchrony over longer time scales.

Light-Sensing Mechanisms in Cnidarians

Most animals contain specialized visual structures that range greatly in complexity and organization[2]. Some cnidarians, including box jellyfishes, such as *Tripedalia cystophora*, have complex visual structures, including camera-type eyes (Nilsson et al. 2005). In contrast, anthozoans (the class of cnidarians that includes anemones and corals) and many hydrozoans (the class that includes *Hydra*) do not have image-forming visual structures, pigmented eyespots, or other specialized light-sensing organs, yet these animals are able to detect and respond to light as an environmental signal. Notably, although anthozoans are sessile as adults, they produce free-swimming larvae that exhibit phototaxis and use light as a cue to guide settlement behavior (Mundy and Babcock 1998). Coral larvae respond to a range of wavelengths of light (Mason and Cohen 2012) and preferentially settle on red substrates (Mason et al. 2011). Together, these observations imply that at least some anthozoan larvae are able to obtain information regarding the intensity, direction, and wavelength of light.

Because many anthozoans contain algal symbionts, light may be initially detected by algal photosynthetic pigments and indirectly used to cue cnidarian physiology and behavior. For example, positive phototaxis by the sea anemone *Anthopleura elegantissima* only occurs in organisms containing algal symbionts (Pearse 1974). However, it is also clear that cnidarians can directly detect and respond to light. As in bilaterians, light detection in cnidarians is most likely mediated through at least two classes of photosensitive molecules: opsins and cryptochromes

Opsins are a family of transmembrane proteins that form complexes with light-sensitive chromophores, usually 11-cis-retinal. These complexes, called rhodopsins, function as G-

[2] This was the subject of the symposium : *Integrating Genomics with Comparative Vision Research of the Invertebrates* presented at the Annual Meeting of the Society of Integrative and Comparative Biology, 3-7 January 2013, at San Francisco, California. Integrative and Comparative Biology 2013. Vol: pages-pages.

protein-coupled receptors (Shichida and Matsuyama 2009). While the role of rhodopsins in animal photoreception is ancient and widespread, the types of opsins used and the architecture of photoreceptive cells and structures vary among animal groups. Most of the opsins present in cnidarians are more closely related to the ciliary opsins (c-opsins) found in vertebrates than to the rhabdomeric opsins (r-opsins) found in insects (Suga et al. 2008). Some opsins, identified in the anthozoans *Nematostella vectensis* (Plachetzki et al. 2007; Suga et al. 2008) and *Acropora millepora* (Anctil et al. 2007) are more divergent and appear to be specific to cnidarians. In the hydrozoan jellyfish, *Cladonema radiatum*, some opsins show specific expression within the eye and are hypothesized to act for photoreception (Suga et al. 2008). In addition, functional studies have shown that cnidarian opsins can activate specific classes of G-proteins in response to light (Koyanagi et al. 2008; Mason et al. 2012). Hilton et al. (2012) observed that using pharmacological compounds that raise cytoplasmic calcium levels in corals resulted in proteomic changes similar to those observed when corals were exposed to light. They inferred that cytoplasmic calcium probably acts as a secondary messenger for coral photoreceptors, such as rhodopsins and melanopsins.

Mason et al. (2012) recently suggested that phototaxis in coral larvae may be mediated through opsins. They found that in *Acropora palmata*, acropsin2 is expressed within solitary epithelial cells that are concentrated at the aboral end of the larvae; this polar expression pattern may allow the larvae to detect the intensity, quality, and direction of light. In contrast, Anctil et al. (2007) showed that expression of four opsins in *Acropora millepora* was not polar in larvae, but rather was scattered throughout the endoderm. Because anthozoans contain numerous opsins that form at least three distinctive clades, phylogenetic analysis is needed to determine the evolutionary relationship between the opsins identified in these two coral species. Evaluating the

177 specific expression patterns and functions of opsins in cnidarians and their phylogenetic
178 relationships is necessary to elucidate the functional diversity of opsins in anthozoan cnidarians.
179 Studies across diverse animal groups show that while many opsins serve as ocular
180 photoreceptors, others are expressed extraocularly and can serve other functions, such as
181 entrainment of circadian rhythms by vertebrate melanopsins (reviewed by Hankins et al. 2008).
182 The role of opsins, if any, in entrainment of cnidarian circadian pathways has not been tested.
183 Cryptochromes are a part of a large family of conserved proteins present throughout the
184 biological kingdom that includes light-activated DNA-repair enzymes called photolyases
185 (Chaves et al. 2011). Within this family, different groups of cryptochromes have independently
186 lost their enzymatic activity and evolved as central players in light-sensing and in circadian
187 regulation both in animals and plants. The animal cryptochromes that are involved in circadian
188 signaling fall into two evolutionary clades with distinct properties and functions, Type I and
189 Type II (Zhu et al. 2005; Yuan et al. 2007). Both cryptochrome clades are present in anthozoans
190 (Levy et al. 2007; Reitzel et al. 2010; Hoadley et al. 2011). For historical reasons, nomenclature
191 within individual taxa does not always correspond directly to these cladal designations (Table 1
192 shows nomenclature of the Type I and Type II cryptochromes identified in anthozoans). Type I
193 cryptochromes, first characterized in *Drosophila* but present in most animals except vertebrates,
194 contain a flavin cofactor that is reduced upon exposure to blue light, thus their designation as
195 blue light sensitive proteins (Chaves et al. 2011). *Nematostella vectensis* and *Acropora spp.* each
196 contain at least two Type I cryptochromes, which have resulted from a duplication within the
197 cnidarian lineage (Reitzel et al. 2010; Shoguchi et al. 2013). In *Acropora digitifera*, these genes
198 are ordered sequentially and in the same direction on the chromosome, suggesting that they
199 resulted from a recent tandem duplication (Shoguchi et al. 2013). Type II cryptochromes, first

200 characterized in mammals, but present in most animals except drosophilid insects, are not
201 typically light sensitive and act to repress signaling by CLOCK and CYCLE (discussed in more
202 detail in the following sections). One Type II cryptochrome gene has been identified in *N.*
203 *vectensis* and in several coral species (Table 1, Levy et al. 2007; Reitzel et al. 2010; Hoadley et
204 al. 2011; Shoguchi et al. 2013). The photosensitivity of cnidarian cryptochromes and their
205 possible activity as transcriptional regulators have not yet been investigated.
206
207 **Molecular Mechanisms of the Circadian Clock**
208 In most cases, circadian clocks consist of regulatory loops composed of a small set of
209 genes, mostly transcription factors, with oscillating expression on intervals of 24 hours. From
210 extensive studies in mammals (Ko and Takahashi 2006) and diverse insects (Williams and
211 Sehgal 2001; Rubin et al. 2006; Yuan et al. 2007), it is clear that many of the core clock genes
212 and their interactions are conserved in these two disparate animal groups, suggesting that this
213 molecular clock dates back to at least the ancestor of deuterostomes and protostomes (Dunlap
214 1999). Until recently, the components of the circadian clock of cnidarians had not been studied
215 for assessment of whether the molecular players in the bilaterian clock are more ancient.
216 Furthermore, it was unknown whether any of these genes would exhibit an oscillating expression
217 pattern consistent with a role in mediating the observed effects of diel light cycles on cnidarian
218 behavior, physiology, and reproduction. In the past few years, our understanding of molecular
219 components of the circadian clocks in one class of cnidarians, the Anthozoa, has greatly
220 progressed, showing both conserved and novel elements of the circadian clock when compared
221 with bilaterians and even among different anthozoan species (Levy et al. 2007; Reitzel et al.
222 2010; Brady et al. 2011; Hoadley et al. 2011). Here, we review these data as well as present new

data for one anthozoan, the starlet sea anemone *Nematostella vectensis*, to highlight the relative conservation of the cnidarian clock by deconstructing the three portions of the transcription-translation feedback loops common to bilaterian clocks: positive elements, feedback loops, and feed-forward loops (Figure 1).

Positive elements

The basic helix–loop-helix Per-ARNT-Sim (bHLH-PAS) transcription factors *Clock* and *Cycle* are the critical core components, called positive elements, of circadian clocks in bilaterian animals. These two genes appear to be nearly universal members of bilaterian circadian clocks. Regulation of both mammalian and insect clocks is based on regulation of expression and function of either *Clock* or *Cycle* (also called *Bmal1*/*Mop3* in mammals). They are termed positive elements because they directly stimulate the transcription of clock-controlled genes (CCGs) and keep the oscillations of the clock from damping or "winding down" (Dunlap 1999). In a species-dependent manner, the expression of one of these two transcription factors oscillates in neuronal tissue (*Bmal1* in mammalian suprachiasmatic nucleus [SCN], and *Clock* in insect dorsal ganglion and antennae) with a 24-hour periodicity, whereas the other gene shows little to no oscillation. CLOCK and CYCLE proteins form a heterodimer that translocates to the nucleus and regulates downstream expression of CCGs through specific sequence motifs called E-Box motifs (Hardin 2006).

Work with the sea anemone *N. vectensis* and the corals *Favia fragum* and *A. millepora* has shown that all three species contain *Clock* and *Cycle*; peak *Clock* expression occurs during subjective day, and *Cycle* transcript expression from *N. vectensis* and *F. fragum* remains constant over a day (Reitzel et al. 2010; Brady et al. 2011; Hoadley et al. 2011). These data support the

hypothesis that the cnidarian-bilaterian ancestor possessed these two bHLH-PAS transcription factors and that the ancestral expression pattern most likely was similar to the patterns observed in modern anthozoans and insects. Reitzel et al. (2010) and Hoadley et al. (2011) have shown that the rhythmic expression of *Clock* is lost when individuals are cultured in all-dark conditions. Brady et al. (2011) found that *Clock* continued to oscillate in all-dark conditions in *A. millepora* larvae, but they only maintained the larvae in darkness for the 24-hour period of sampling with no acclimation period. Thus, the ability of the cnidarian clock to maintain a free-running rhythm is still under investigation. In contrast to these anthozoans, recent sequencing of the *Hydra magnipapillata* genome has revealed that this hydrozoan has lost both *Clock* and *Cycle* (Chapman et al. 2010); however, this species displays photoperiodic behavior in response to light cycles (Taddei-Ferretti and Musio 2000).

Reitzel *et a*l. (2010) showed that heterodimerization of CLOCK and CYCLE was conserved in *N. vectensis*, suggesting that conservation of the positive loop extends to protein-protein interactions. The Levy lab has recently documented similar heterodimerization by CLOCK and CYCLE in the coral *Stylophora pistillata* (Shemesh et al., in preparation). Through informatics searches of promoters for genes with potential roles in circadian-clock regulation (discussed below), Reitzel *et al*. (2010) only observed E-Box motifs upstream of genes that show light-dependent cycling in transcription, consistent with a role for this protein heterodimer in the circadian clock of this cnidarian. Available data collectively suggest that the positive loop of bilaterians is likely conserved in cnidarians.

<u>Feedback loop</u>

The feedback, or negative loop, is composed of proteins that inhibit the CLOCK:CYCLE heterodimer via direct interactions of proteins, and thus downregulate their own expression. The composition of the feedback loop varies among bilaterians. In mammals, the feedback loop is composed principally of *period* and Type I cryptochromes. The PERIOD and CRYPTOCHROME proteins form dimers (Tei et al. 1997; Sancar 2004), and the cryptochromes repress signaling of the CLOCK:CYCLE heterodimer. In insects, the feedback loop is composed of different combinations of PERIOD, TIMELESS, and/or cryptochromes, depending on the species (Bae et al. 1998; Yuan et al. 2007). It has recently become understood that the molecular composition of the feedback loop in *Drosophila* is atypical for insects, likely due to the loss of Type II cryptochromes (Reppert 2007; Yuan et al. 2007). In *Drosophila*, a Type I cryptochrome exerts indirect repression of CLOCK:CYCLE function by degrading TIMELESS in a light-dependent manner and thus influences PER localization and repression of CLOCK:CYCLE. In other insects (e.g., monarch butterfly Zhu et al. 2005; Zhu et al. 2008), Type II cryptochromes act as the principal component of the feedback loop, as in mammals. Collectively, available data suggest that cryptochromes and *Period* are the principal shared elements of the feedback loops from both vertebrates and insects. Both in mammals and in non-drosophilid insects, only cryptochromes interact directly with the CLOCK:CYCLE heterodimer to inhibit its transcriptional activity (Griffin et al. 1999; Cashmore 2003; Yuan et al. 2007; Zhu et al. 2008).

Based on searches of available genomes, cnidarians lack *Period* genes as well as *Timeless* (Reitzel et al. 2010; Shoguchi et al. 2013). However, anthozoan cnidarians have both Type I and Type II cryptochromes. In contrast, the hydrozoan *H. magnipapillata* has lost both classes of cryptochromes. As described previously, Type I cryptochromes are typically sensitive to blue light. In both corals (Levy et al. 2007, Hoadley et al. 2011, Brady et al. 2011) and *N. vectensis*

(Reitzel et al. 2010), expression of Type I cryptochrome(s) increases during subjective day. Experiments with *N. vectensis* show that up-regulation of *Cry1b* transcripts requires blue or full-spectrum light (Reitzel et al. 2010). Type II cryptochrome is strongly up-regulated during subjective day in corals (Levy et al. 2007, Hoadley et al. 2011, Brady et al. 2011) but does not show strong cycling in *N. vectensis* (Reitzel et al. 2010), suggesting a difference in the regulatory pathways between the two groups. Interestingly, the peak in expression of Type II cryptochrome consistently occurs earlier than expression of Type I cryptochrome both in *A. millepora* and *F. fragum* (Levy et al. 2007, Hoadley et al. 2011, Brady et al. 2011). Two studies have shown that diel variation in cryptochrome does not persist under constant darkness (Reitzel et al. 2010, Hoadley et al. 2011). Brady et al. (2011) found that when *A. millepora* larvae were placed in constant darkness, daily fluctuation in Type I cryptochrome expression ceased immediately, but fluctuation in Type II cryptochrome expression persisted for at least 24 hours.

Feed-forward loop

Activity of the feedback loop results in degradation of the positive elements and is balanced by a feed-forward loop composed of transcription factors regulate transcription of either *Clock* or *Cycle* (Looby and Loudon 2005). The feed-forward loop is composed of bZIP genes in the PAR family in insects and mammals (Cyran et al. 2003; Gachon 2007) and the nuclear receptors REV-ERB (NR1D) and ROR (NR1F) in mammals (Guillaumond et al. 2005). In *Drosophila*, the PAR-bZIP proteins VRILLE and PDP1 regulate transcription of *Clock* through competitive binding to specific DNA motifs termed V/P-Box motifs (5’ – ATTAYRTAAY – 3’), where they suppress and activate transcription, respectively. In vertebrates, evolutionary related PAR-bZIPs (e.g., hepatic leukemia factor [HLF], nuclear factor

- interleukin 3 [NF-IL3]) similarly regulate transcription of downstream genes in the circadian clock through conserved sequences referred to as D-Box binding sites (Vatine et al. 2009).

There has been very little research directed toward characterizing a feed-forward loop in any cnidarian. Comparative genomic analysis of the nuclear receptors has clearly shown that cnidarians, as well as other early-diverging phyla, do not contain members of the nuclear receptor 1 (NR1) family, including homologs of REV-ERB and ROR (Reitzel and Tarrant 2009; Reitzel et al. 2011). On the other hand, phylogenetic analyses of the bZIP superfamily of transcription factors identified cnidarian genes that group in the PAR-bZIP family (Amoutzias et al. 2007). In a study of transcriptome changes associated with diel treatments of the coral *A. millepora*, Brady et al. (2011) identified one PAR-bZIP that showed elevated expression during subjective night. These previous data suggest that PAR-bZIPs may have a role in the cnidarian circadian clock.

To further investigate the potential role for PAR-bZIPs in the cnidarian circadian clock, we used phylogenetic methods, quantitative real time PCR (qPCR), and promoter analysis to look for evidence of the feed-forward loop in *N. vectensis*. We used PAR-bZIPs from human (HLF [NP_002117], D-site binding protein [D-site, NP_001343], and NF-IL3 [NP_005375]) and *Drosophila* (PDP1 [NP_729301] and VRILLE [NP_477191]) as query sequences to BLAST the *N. vectensis* genome. Based on these searches, we identified three genes that were reciprocal matches to bilaterian PAR-bZIPs. Similar searches of the *Acropora digitifera* genome (Shinzato et al. 2011) also recovered three PAR-bZIP genes. Phylogenetic analyses with representative genes from bilaterians confirmed that these anemone genes group with strong support (Figure 2A) to the exclusion of the nearest outgroup bZIP family, C/EBP (Amoutzias et al. 2007). PAR-bZIPs from *N. vectensis* and *A. digitifera* grouped together with high support, but did not group

with bilaterian genes, suggesting an independent radiation of this subfamily in anthozoan cnidarians. To address whether these *N. vectensis* genes are expressed in a rhythmic manner under an oscillating daily light cycle, like bilaterian genes, we utilized qPCR to measure transcription of each gene in animals exposed to light:dark (12 h : 12 h) or to constant darkness (see Reitzel et al. 2010 for experimental details). Two of the three NvPAR-bZIP genes (A and C) showed strong oscillating expression under light:dark conditions, while one showed no significant changes in expression (Figure 2B-D). The rhythmic gene expression was not present in animals that were cultured in constant darkness. The timing of peak expression for the each of the oscillating PAR-bZIPs differed. *NvPAR-bZIPA* showed highest expression at the beginning of subjective day (ZT = 3), while *NvPAR-bZIPC* showed highest expression during subjective night (ZT = 19). The expression of these two PAR-bZIPs is consistent with a role in regulation of *NvClock* transcription because they bookend the transcription of *NvClock*, which is expressed during subjective day (see above). *N. vectensis* PAR-bZIPs show high conservation in amino-acid sequence for the region of this family of transcription factors involved in DNA binding (Figure 2E). Assuming that a similar DNA-binding domain would result in similar DNA-binding sites, we looked at the promoter region of *NvClock* for the signature V/P-box motifs recognized by PAR-bZIPs. Through these searches, we identified four candidate V/P-Box sites within 2 kb of the start site for *NvClock* promoter (-1311: ATTACATGAT, -1177: ATTACATGGC, -733: ATTAAATAAC, -196: GTTATATAA), suggesting a conserved role for these transcription factors in regulation of the anemone's clock.

Looking forward

Connecting Molecular Mechanisms with Organismal Processes

The circadian clock in bilaterian animals coordinates numerous gene networks, cellular pathways, and physiological processes (Doherty and Kay 2010) through clock-controlled genes (CCGs). As we review above, cnidarians exhibit diverse organismal-level processes, including behavior, reproduction, and physiology, which co-vary with 24-hour light cycles. One clear area of future research is to integrate what researchers have recently learned about the molecular cogs of the cnidarian circadian clock with the observed oscillations in organismal processes. Initially, these connections could be made using a combination of transcriptome-level studies to measure oscillations of gene expression, similar to what has been reported for candidate clock genes, and experimental measurements of organismal responses. Quantitative measurements of transcriptome-wide variation in gene expression are a direct experimental method of identifying potential CCGs. To date, two studies have taken this approach to measure differential gene expression for the coral *A. millepora* over a daily cycle (Brady et al. 2011; Levy et al. 2011). Levy et al. (2011) exposed *A. millepora* to either oscillating or constant dark conditions and used microarrays to identify approximately 200 genes differentially regulated in relation to a 24-hour period, including genes with known or suspected roles in metabolism, response to oxidative stress, and molecular chaperones (e.g., heat-shock proteins). Similarly, Brady et al. (2011) sampled *A. millepora* during different times of the day and conducted Illumina-based transcriptional profiling to identify differentially expressed genes. However, because this coral is symbiotic, the oscillations in gene expression may reflect not only potential genes regulated by the host's circadian clock, but also interactions with the symbionts. While these interactions are certainly of interest, it is also important to study the clock in species lacking algal symbionts in an effort to identify genes directly regulated by the cnidarian circadian machinery. To this end, species like *N. vectensis* are useful models. Not only does *N. vectensis* lack algal symbionts, but

383 also the genome has been sequenced, enabling analysis of binding motifs in the promoters of
384 differentially expressed genes. The combined analysis of differential transcriptional profiles
385 with motif representation in promoters will identify likely CCGs to better characterize what
386 processes the circadian clock may regulate and how these relate to previous studies of
387 organismal-level responses to diel light environments.

388 In cnidarians, current data suggest that light-entrained behavior and gene expression both
389 lose rhythmicity within a few days when individuals are removed from a light:dark environment.
390 For *N. vectensis*, Reitzel et al. (2010) has shown that 30 days of constant darkness are sufficient
391 for loss of cyclic gene expression for genes inferred to constitute the circadian clock. Data from
392 different anthozoans have shown loss of the rhythmicity of some clock genes with 24 hours (*A.*
393 *millepora*) (Brady et al. 2011) or 72 hours (*F. fragum*) (Hoadley et al. 2011) of constant
394 darkness. The loss of cyclic gene expression correlates with organismal-level characteristics. For
395 example, colonies of *F. fragum* show partial loss of daily rhythms in polyp extension 24 hours
396 after removal of the light cue and near complete loss after 48 hours. By some definitions, a true
397 circadian clock must maintain regular rhythmic output (e.g., behavior, physiology, gene
398 expression) upon removal of the entraining cue. Vertebrate and insect circadian clocks have been
399 well-characterized for the ability to maintain cyclic outputs for extended periods of time under
400 constant conditions. In vertebrates, particularly mammals, the signaling is maintained by the
401 suprachiasmatic nucleus (SCN), and in *Drosophila*, signaling is maintained through the ventral
402 group of lateral neurons (Emery et al. 2000). Together, these data suggest that loss of rhythmic
403 gene expression and behavior may be characteristic of the cnidarian clock, in opposition to the
404 classical description of the bilaterian clock, which is capable of maintaining rhythmicity even
405 after several days in constant darkness. These apparent differences between cnidarians and

bilaterians could be a product of measuring gene expression via whole-animal homogenates, thus missing cycling of circadian genes in a small number of neuronal cells. In addition, by measuring behavior and gene expression in groups of animals as opposed to individuals, persistent cycles may be obscured by gradual asynchrony among individuals. Future research at both the molecular and organismal level will help clarify these potential differences between cnidarian and bilaterian circadian clocks.

Establishing Links in the Cnidarian Circadian Clock

Transcriptional oscillations in genes comprising the circadian clock are hallmarks of animal circadian clocks. Mechanistically, these oscillations are driven by protein-protein and protein-DNA interactions (arrows in Figure 1). Previous research in anthozoan cnidarians (reviewed above) has provided strong correlative evidence that the molecular components of the circadian clock date back to the cnidarian-bilaterian ancestor. However, in the absence of data on protein-protein and protein-DNA interactions, the cnidarian clockwork remains to be functionally tested to address hypotheses about the conservation of the gene network. Currently, the only protein-level interaction studied has been the conserved dimerization between the positive elements CLOCK and CYCLE in the sea anemone *N. vectensis* (Reitzel et al. 2010). Future research is needed to test for other potential conserved and novel protein-protein interactions. In the feedback loop, cnidarians lack TIMELESS and PERIOD, which are important proteins for the repression of the CLOCK:CYCLE dimer. However, as indicated above, cnidarians have both Type I and II cryptochromes, both of which play roles in the feedback loop of bilaterians. Although additional proteins could be involved, a parsimonious hypothesis is that cryptochromes, particularly Type II, are centrally involved in suppression.

This mechanism could be tested using luciferase reporter assays in heterologous expression systems with co-incubations of *Clock*, *Cycle,* and the cryptochromes. A similar approach could be used to assess the ability of the cnidarian PAR-bZIPs to drive transcriptional activation and suppression of *Clock* via V/P-box motifs. These approaches have been instrumental methods for characterizing the clockwork of bilaterian circadian clocks and are likely to reveal the mechanistic links between the identified clock genes.

Ultimately there is a need to follow up work in heterologous systems with *in vivo* studies conducted within cnidarians. With the generation of specific antibodies, it will be possible to conduct co-immunoprecipitation studies to examine protein-protein interactions in cnidarian tissues and chromatin immunoprecipitation studies to directly identify CCGs. While morpholinos have been developed as a robust technology for knocking down gene expression during early development, techniques for generating cnidarian knockout strains or for knocking down expression in adults would be extremely beneficial in directly demonstrating the necessity of individual genes for circadian regulation.

Finally, we should be prepared for surprises by identifying novel mechanisms in the cnidarian clock. Research in mammalian systems continues to identify additional molecular mechanisms that drive the circadian clock, including chromatin structure (Koike et al. 2012) and RNA-binding proteins (Morf et al. 2012). Cnidarians have undergone millions of years of independent evolution since diverging from the animal stem and have surely evolved novel molecular mechanisms that drive the circadian clock. Indeed, one cnidarian (*Hydra magnipapillata*) has lost principal genes (*Clock*, *Cycle*, cryptochromes) that are central components of the cnidarian-bilaterian clock, yet displays photoperiodism at the organismal level. Thus, while much of the current work with cnidarians has been motivated by

452 characterizing the similarities with bilaterian clocks, future studies will doubtless uncover
453 molecular novelties that drive the organismal-level responses to diel light cycles.
454

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FIGURE LEGENDS

Figure 1. Diagrams of the gene networks composing the circadian clock of two model bilaterians (human and *Drosophila*) and the hypothesized network for the cnidarian *Nematostella vectensis*. The circadian clock for bilaterians is composed of three loops: the positive elements, the feedback loop, and the feed-forward loop. *Clock* and *Cycle* proteins dimerize and act as positive elements by upregulating transcription of target genes, including members of the other regulatory loops. Some of the genes composing the feedback loop (*period* and Type II cryptochromes in human; *period* and *timeless* in *Drosophila*) and the feed-forward loop (PAR-bZIPs and nuclear receptors *ROR* and *Rev-erb* in human; PAR-bZIPs in *Drosophila*) differ between animal lineages. One or more members of the feedback loop bind to, and suppress, the CLOCK:CYCLE dimer, leading to their own repression. Members of the feed-forward loop are direct transcriptional activators and repressors of either *Clock* or *Cycle*. Presently, molecular research in cnidarians via gene expression and promoter searches has provided correlative evidence that these loops may be conserved, suggesting that the topology of the circadian gene network predates the cnidarian-bilaterian ancestor. However, mechanistic studies to characterize protein-protein and protein-DNA interactions are needed to test for the hypothesized connections in the cnidarian circadian clock (see section “Looking Forward” for discussion).

Figure 2. Identification of PAR-bZIP transcription factors in the cnidarian, *Nematostella vectensis*, and their expression under diel (12 h light : 12 h dark) lighting conditions. (A) Maximum-likelihood tree showing the relationship of three identified *N. vectensis* PAR-bZIPs

(A, B, C) with coral (*Acropora digitifera*) and bilaterian genes in the same subfamily. Phylogenetic analyses were conducted with RAxML 2.6 (Stamatakis 2006), using protein models determined by AIC criteria with ProtTest 2.4 (Abascal et al. 2005). Trees were visualized with FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/). All *N. vectensis* genes form a monophyletic grouping with bilaterian PAR-bZIPs to the exclusion of the bZIP sister family, C/EBP. *N. vectensis* genes did not group with any specific bilaterian sequences within the PAR family but did group with genes identified in the coral *A. digitifera*. Nodes above labels indicate percent of 1000 bootstrap replicates (ML), in which values below 40 were omitted. Accession values in parentheses are from the Joint Genome Institute (JGI) databases for *N. vectensis, Lottia gigantea,* and *Capitella teleta;* the Okinawa Institute of Science and Technology (OIST) for *A. digitifera*, and NCBI for all other species. (B – D) Temporal gene expression of *NvPAR-bZIPA-C* from 12:12 light:dark treatment and constant dark, showing light-dependent expression. Animal experiments, RNA isolation and quality, and synthesis of cDNA were performed using previously described methods (Reitzel and Tarrant 2009; Reitzel et al. 2010). For each *N. vectensis* PAR-bZIP, we produced a plasmid standard from an amplified portion of each transcript cloned into pGEM-T Easy (Promega). The qPCR primers were designed and data generated on a MyiQ instrument, as previously described (Reitzel and Tarrant 2009, see Supplemental Table 1). (B) *NvPAR-bZIPA* was significantly upregulated in subjective day in only the light:dark treatment, with no cycling of transcription when animals where cultured in all dark. (C) *NvPAR-bZIPB* had no differences in expression over time in either experimental treatment. (D) *NvPAR-bZIPC* was upregulated in subjective night, only in the light:dark treatment, similar to *NvPAR-bZIPA*. (E) Alignment of a portion of bZIP domain for PAR-bZIPs in the phylogenetic tree in panel A. Bar indicates amino acids that contact DNA at V/P sequence

motifs. *N. vectensis* genes show high conservation in this region, as well as the bZIP domain in general, suggesting that similar binding sites may be recognized by anemone PAR-bZIPs.

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