

Title: Circadian clocks in the Cnidaria: Environmental entrainment, molecular regulation, and organismal outputs<sup>1</sup>

Authors: Adam M. Reitzel,<sup>\*,^,%</sup>, Ann M. Tarrant<sup>+,%</sup>, and Oren Levy<sup>#</sup>

\* 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

Running title: Cnidarian circadian clock

Key words: circadian clock, coral, cryptochrome, *Nematostella*, photoperiod

---

<sup>1</sup> From the symposium: *Keeping Time During Animal Evolution: Conservation and Innovation of the Circadian Clock* presented at the Annual Meeting of the Society of Integrative and Comparative Biology, 3-7 January 2013, at San Francisco, California.

1 **ABSTRACT**

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

19 **Introduction**

20 In many habitats, light is a predictable signal that provides information about the  
21 environment on daily, lunar, and seasonal time-scales. The need to anticipate and prepare for  
22 periodic changes in the environment is strong, evidenced by the nearly universal presence of  
23 molecular timekeeping mechanisms in both unicellular and multicellular organisms. Circadian  
24 rhythms in behavior and physiology are driven by daily cycles in expression of, interactions  
25 between, and degradation of, the underlying molecular components. The genes forming the core  
26 timing mechanism are not shared among distantly related organisms, e.g., bacteria (Xu et al.  
27 2003), plants (Pruneda-Paz and Kay 2010), fungi (Salichos and Rokas 2009), and animals  
28 (Harmer et al. 2001; Panda et al. 2002), which suggests that circadian regulation has evolved  
29 independently within these lineages (Rosbash 2009).

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

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

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

60

## 61 **Why Cnidarians?**

62 Cnidarians, the “stinging-celled animals” that include hydras, jellyfish, corals, and  
63 anemones, are intriguing models for circadian research for several reasons. First, the lineages  
64 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**

88           Several aspects of cnidarian biology vary on daily cycles, including vertical migration,  
89 larval phototaxis, settlement behavior, expansion and retraction of the body column, and feeding  
90 behaviors, including extension of the tentacles (reviewed in Taddei-Ferretti and Musio 2000;  
91 Hendricks et al. 2012). Some of these behaviors are directly cued by light or other external  
92 signals. For example, simultaneous diel vertical migration in jellyfish has been modeled to result  
93 from individual responses to light intensity (Dupont et al. 2009). Similarly, daily cycles in  
94 corals' extension of their tentacles disappear under constant light conditions in most species and  
95 are most likely a direct response to light (Sweeney 1976; Hoadley et al. 2011). On the other  
96 hand, other rhythmic behaviors have been shown to persist in the absence of an external light  
97 cue. Recent studies of locomotor activity in the sea anemone, *Nematostella vectensis*, have  
98 shown that when animals are maintained on a 24-hour photoperiod (12 hours light: 12 hours  
99 dark), activity increased approximately two-fold during the subjective night (Hendricks et al.  
100 2012). Animals exposed to constant light or constant darkness maintained rhythmic cycles in  
101 behavior for a period of several (3-8) days, supporting the presence of a free-running clock.

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

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

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

122         On monthly scales, nocturnal illumination from moonlight is thought to provide a cue to  
123 synchronize late stages of gamete maturation and the night of release in corals (Baird et al.  
124 2009). It has been demonstrated that mimicking different lunar phases over a period of days to  
125 weeks can shift the timing of spawning or planulation (Jokiel et al. 1985; Hunter 1988), and that  
126 corals can detect low levels of blue light similar to the light produced by a full moon in shallow  
127 clear water (Gorbunov and Falkowski 2002). Although the molecular mechanisms mediating this  
128 circa-annual and circa-lunar synchronization of reproduction by reef-building corals remain  
129 elusive, cryptochromes may be involved in this process (Levy et al. 2007; Hoadley et al. 2011)  
130 and may link the circadian clockwork with reproductive synchrony over longer time scales.

131

## 132 **Light-Sensing Mechanisms in Cnidarians**

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

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

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

---

<sup>2</sup> 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.

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

169 Mason et al. (2012) recently suggested that phototaxis in coral larvae may be mediated  
170 through opsins. They found that in *Acropora palmata*, *acropsin2* is expressed within solitary  
171 epithelial cells that are concentrated at the aboral end of the larvae; this polar expression pattern  
172 may allow the larvae to detect the intensity, quality, and direction of light. In contrast, Anctil et  
173 al. (2007) showed that expression of four opsins in *Acropora millepora* was not polar in larvae,  
174 but rather was scattered throughout the endoderm. Because anthozoans contain numerous opsins  
175 that form at least three distinctive clades, phylogenetic analysis is needed to determine the  
176 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

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

227

### 228 Positive elements

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

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

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

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

266

267 Feedback loop

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

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

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

303

#### 304 Feed-forward loop

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

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

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

326         To further investigate the potential role for PAR-bZIPs in the cnidarian circadian clock,  
327 we used phylogenetic methods, quantitative real time PCR (qPCR), and promoter analysis to  
328 look for evidence of the feed-forward loop in *N. vectensis*. We used PAR-bZIPs from human  
329 (HLF [NP\_002117], D-site binding protein [D-site, NP\_001343], and NF-IL3 [NP\_005375]) and  
330 *Drosophila* (PDP1 [NP\_729301] and VRILLE [NP\_477191]) as query sequences to BLAST the  
331 *N. vectensis* genome. Based on these searches, we identified three genes that were reciprocal  
332 matches to bilaterian PAR-bZIPs. Similar searches of the *Acropora digitifera* genome (Shinzato  
333 et al. 2011) also recovered three PAR-bZIP genes. Phylogenetic analyses with representative  
334 genes from bilaterians confirmed that these anemone genes group with strong support (Figure  
335 2A) to the exclusion of the nearest outgroup bZIP family, C/EBP (Amoutzias et al. 2007). PAR-  
336 bZIPs from *N. vectensis* and *A. digitifera* grouped together with high support, but did not group

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

357

## 358 **Looking forward**

359 *Connecting Molecular Mechanisms with Organismal Processes*

360           The circadian clock in bilaterian animals coordinates numerous gene networks, cellular  
361 pathways, and physiological processes (Doherty and Kay 2010) through clock-controlled genes  
362 (CCGs). As we review above, cnidarians exhibit diverse organismal-level processes, including  
363 behavior, reproduction, and physiology, which co-vary with 24-hour light cycles. One clear area  
364 of future research is to integrate what researchers have recently learned about the molecular cogs  
365 of the cnidarian circadian clock with the observed oscillations in organismal processes. Initially,  
366 these connections could be made using a combination of transcriptome-level studies to measure  
367 oscillations of gene expression, similar to what has been reported for candidate clock genes, and  
368 experimental measurements of organismal responses. Quantitative measurements of  
369 transcriptome-wide variation in gene expression are a direct experimental method of identifying  
370 potential CCGs. To date, two studies have taken this approach to measure differential gene  
371 expression for the coral *A. millepora* over a daily cycle (Brady et al. 2011; Levy et al. 2011).  
372 Levy et al. (2011) exposed *A. millepora* to either oscillating or constant dark conditions and  
373 used microarrays to identify approximately 200 genes differentially regulated in relation to a 24-  
374 hour period, including genes with known or suspected roles in metabolism, response to oxidative  
375 stress, and molecular chaperones (e.g., heat-shock proteins). Similarly, Brady et al. (2011)  
376 sampled *A. millepora* during different times of the day and conducted Illumina-based  
377 transcriptional profiling to identify differentially expressed genes. However, because this coral is  
378 symbiotic, the oscillations in gene expression may reflect not only potential genes regulated by  
379 the host's circadian clock, but also interactions with the symbionts. While these interactions are  
380 certainly of interest, it is also important to study the clock in species lacking algal symbionts in  
381 an effort to identify genes directly regulated by the cnidarian circadian machinery. To this end,  
382 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

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

412

### 413 *Establishing Links in the Cnidarian Circadian Clock*

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

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

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

443         Finally, we should be prepared for surprises by identifying novel mechanisms in the  
444 cnidarian clock. Research in mammalian systems continues to identify additional molecular  
445 mechanisms that drive the circadian clock, including chromatin structure (Koike et al. 2012) and  
446 RNA-binding proteins (Morf et al. 2012). Cnidarians have undergone millions of years of  
447 independent evolution since diverging from the animal stem and have surely evolved novel  
448 molecular mechanisms that drive the circadian clock. Indeed, one cnidarian (*Hydra*  
449 *magnipapillata*) has lost principal genes (*Clock*, *Cycle*, cryptochromes) that are central  
450 components of the cnidarian-bilaterian clock, yet displays photoperiodism at the organismal  
451 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

455 **Funding**

456 This work was supported by the United States – Israel Binational Science Foundation award  
457 2011187 (AMT and OL), the National Institutes of Health / National Institute of Child Health  
458 and Human Development award HD062178 (AMR), and generous funding from the University  
459 of North Carolina at Charlotte (AMR). This paper resulted from the symposium “Keeping Time  
460 During Animal Evolution: Conservation and Innovation of the Circadian Clock” presented at the  
461 2013 Annual Meeting of the Society of Integrative and Comparative Biology. The symposium  
462 was supported by the Society of Integrative and Comparative Biology and award 1239607 from  
463 the Integrative Organismal Systems Program at the National Science Foundation.

464

465 FIGURE LEGENDS

466

467 Figure 1. Diagrams of the gene networks composing the circadian clock of two model bilaterians  
468 (human and *Drosophila*) and the hypothesized network for the cnidarian *Nematostella vectensis*.

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

483

484 Figure 2. Identification of PAR-bZIP transcription factors in the cnidarian, *Nematostella*  
485 *vectensis*, and their expression under diel (12 h light : 12 h dark) lighting conditions. (A)

486 Maximum-likelihood tree showing the relationship of three identified *N. vectensis* PAR-bZIPs

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

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

512

513 **References**

514

515 Abascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein  
516 evolution. *Bioinformatics* 21:2104-2105.

517 Amoutzias GD, Veron AS, Weiner J, Robinson-Rechavi M, Bornberg-Bauer E, Oliver SG,  
518 Robertson DL. 2007. One billion years of bZIP transcription factor evolution:

519 Conservation and change in dimerization and DNA-binding site specificity. *Mol Biol*  
520 *Evol* 24:827-835.

521 Anctil M, Hayward DC, Miller DJ, Ball EE. 2007. Sequence and expression of four coral G  
522 protein-coupled receptors distinct from all classifiable members of the rhodopsin family.  
523 *Gene* 392:14-21.

524 Aschoff J, Vongoezt C. 1988. Masking of circadian activity rhythms in hamsters by darkness. *J*  
525 *Comp Physiol A Sens Neural Behav Physiol* 162:559-62.

526 Bae K, Lee C, Sidote D, Chuang K-y, Edery I. 1998. Circadian regulation of a *Drosophila*  
527 homolog of the mammalian clock gene: PER and TIM function as positive regulators.  
528 *Mol Cell Biol* 18:6142-6151.

529 Baird AH, Guest JR, Willis BL. 2009. Systematic and biogeographical patterns in the  
530 reproductive biology of scleractinian corals. *Annu Rev Ecol Evol Syst* 40:551-71.

531 Brady AK, Hilton JD, Vize PD. 2009. Coral spawn timing is a direct response to solar light  
532 cycles and is not an entrained circadian response. *Coral Reefs* 28:677-680.

533 Brady AK, Snyder KA, Vize PD. 2011. Circadian cycles of gene expression in the coral,  
534 *Acropora millepora*. *PLoS ONE* 6:e25072.

535 Cashmore AR. 2003. Cryptochromes: enabling plants and animals to determine circadian time.  
536 *Cell* 114:537-543.

537 Chapman JA, Kirkness EF, Simakov O, Hampson SE, Mitros T, Weinmaier T, Rattei T,  
538 Balasubramanian PG, Borman J, Busam D, Disbennett K, Pfannkoch C, Sumin N, Sutton  
539 GG, Viswanathan LD, Walenz B, Goodstein DM, Hellsten U, Kawashima T, Prochnik  
540 SE, Putnam NH, Shu S, Blumberg B, Dana CE, Gee L, Kibler DF, Law L, Lindgens D,  
541 Martinez DE, Peng J, Wigge PA, Bertulat B, Guder C, Nakamura Y, Ozbek S, Watanabe  
542 H, Khalturin K, Hemmrich G, Franke A, Augustin R, Fraune S, Hayakawa E, Hayakawa  
543 S, Hirose M, Hwang JS, Ikeo K, Nishimiya-Fujisawa C, Ogura A, Takahashi T,  
544 Steinmetz PRH, Zhang X, Aufschnaiter R, Eder M-K, Gorny A-K, Salvenmoser W,  
545 Heimberg AM, Wheeler BM, Peterson KJ, Bottger A, Tischler P, Wolf A, Gojobori T,  
546 Remington KA, Strausberg RL, Venter JC, Technau U, Hobmayer B, Bosch TCG,  
547 Holstein TW, Fujisawa T, Bode HR, David CN, Rokhsar DS, Steele RE. 2010. The  
548 dynamic genome of Hydra. *Nature* 464:592-596.

549 Chaves I, Pokorny R, Byrdin M, Hoang N, Ritz T, Brettel K, Essen L-O, van der Horst GTJ,  
550 Batschauer A, Ahmad M. 2011. The cryptochromes: blue light photoreceptors in plants  
551 and animals. *Annu Rev Plant Bio* 62:335-364.

552 Cyran SA, Buchsbaum AM, Reddy KL, Lin M-C, Glossop NRJ, Hardin PE, Young MW, Storti  
553 RV, Blau J. 2003. *vriille*, *pdp1*, and *dClock* form a second feedback loop in the  
554 *Drosophila* circadian clock. *Cell* 112:329-341.

555 DeCoursey PJ. 2003. The behavioral ecology and evolution of biological timing systems. In:  
556 Dunlap JC, Loros JJ, and DeCoursey PJ, editors. *Chronobiology: Biological*  
557 *Timekeeping*. Sunderland, Massachusetts: Sinauer Associates. p. 58-60.

558 Doherty CJ, Kay SA. 2010. Circadian control of global gene expression patterns. *Annu Rev*  
559 *Genet* 44:419-444.

560 Dunlap JC. 1999. Molecular bases for circadian clocks. *Cell* 96:271-290.

561 Dupont N, Klevjer TA, Kaartvedt S, Aksnes DL. 2009. Diel vertical migration of the deep-water  
562 jellyfish *Periphylla periphylla* simulated as individual responses to absolute light  
563 intensity. *Limnol Oceanogr* 54:1765.

564 Edgar RS, Green EW, Zhao Y, van Ooijen G, Olmedo M, Qin X, Xu Y, Pan M, Valekunja UK,  
565 Feeney KA, Maywood ES, Hastings MH, Baliga NS, Meroow M, Millar AJ, Johnson CH,  
566 Kyriacou CP, O'Neill JS, Reddy AB. 2012. Peroxiredoxins are conserved markers of  
567 circadian rhythms. *Nature* 485:459-64.

568 Emery P, Stanewsky R, Helfrich-Förster C, Emery-Le M, Hall JC, Rosbash M. 2000. *Drosophila*  
569 CRY is a deep brain circadian photoreceptor. *Neuron* 26:493-504.

570 Gachon F. 2007. Physiological function of PAR-bZip circadian clock controlled transcription  
571 factors. *Ann Med* 39:562-571.

572 Gorbunov MY, Falkowski PG. 2002. Photoreceptors in the cnidarian hosts allow symbiotic  
573 corals to sense blue moonlight. *Limnol Oceanogr* 47:309-315.

574 Griffin EA, Staknis A, Weitz CJ. 1999. Light-independent role of CRY1 and CRY2 in the  
575 mammalian circadian clock. *Science* 286:768-771.

576 Guillaumond F, Dardente H, Giguere V, Cermakian N. 2005. Differential control of *Bmal1*  
577 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms* 20:391-  
578 403.

579 Hankins MW, Peirson SN, Foster RG. 2008. Melanopsin: an exciting photopigment. *Trends*  
580 *Neurosci* 31:27-36.

581 Hardin PE. 2006. Essential and expendable features of the circadian timekeeping mechanism.  
582 *Curr Opin Neurobiol* 16:686-692.

583 Harmer SL, Panda S, Kay SA. 2001. Molecular bases of circadian rhythms. *Annu Rev Cell Dev*  
584 *Biol* 17:215-253.

585 Hastings JW. 2007. The *Gonyaulax* clock at 50: Translational control of circadian expression.  
586 *Cold Spring Harb Symp Quant Bio* 72:141-144.

587 Hendricks WD, Byrum CA, Meyer-Bernstein EL. 2012. Characterization of circadian behavior  
588 in the starlet sea anemone, *Nematostella vectensis*. *PLoS ONE* 7:e46843.

589 Hilton JD, Brady AK, Spaho SA, Vize PD. 2012. Photoreception and signal transduction in  
590 corals: proteomic and behavioral evidence for cytoplasmic calcium as a mediator of light  
591 responsivity. *Biol Bull* 223:291-9.

592 Hoadley KD, Szmant AM, Pyott SJ. 2011. Circadian clock gene expression in the coral *Favia*  
593 *fragum* over diel and lunar reproductive cycles. *PLoS ONE* 6:e19755.

594 Hunter CL. 1988. Environmental cues controlling spawning in two Hawaiian corals, *Montipora*  
595 *verrucose* and *M. dilatata*. In *Proceedings of the 6th International Coral Reef*  
596 *Symposium*. Townsville, Australia.

597 Jokiel PL, Ito RY, Liu PM. 1985. Night irradiance and synchronization of lunar release of  
598 planula larvae in the reef coral *Pocillopora damicornis*. *Mar Biol* 88:167-174.

599 Ko CH, Takahashi JS. 2006. Molecular components of the mammalian circadian clock. *Hum*  
600 *Mol Genet* 15:R271-277.

601 Koike N, Yoo S-H, Huang H-C, Kumar V, Lee C, Kim T-K, Takahashi JS. 2012. Transcriptional  
602 architecture and chromatin landscape of the core circadian clock in mammals. *Science*  
603 338:349-354.

604 Koyanagi M, Takano K, Tsukamoto H, Ohtsu K, Tokunaga F, Terakita A. 2008. Jellyfish vision  
605 starts with cAMP signaling mediated by opsin-Gs cascade. Proc Natl Acad Sci USA  
606 105:15576-15580.

607 Levy O, Achituv Y, Yacobi YZ, Stambler N, Dubinsky Z. 2006. The impact of spectral  
608 composition and light periodicity on the activity of two antioxidant enzymes (SOD and  
609 CAT) in the coral *Favia favaus*. J Exp Mar Biol Ecol 328:35-46.

610 Levy O, Appelbaum L, Leggat W, Gothlif Y, Hayward DC, Miller DJ, Hoegh-Guldberg O.  
611 2007. Light-responsive cryptochromes from a simple multicellular animal, the coral  
612 *Acropora millepora*. Science 318:467-470.

613 Levy O, Kaniewska P, Alon S, Eisenberg E, Karako-Lampert S, Bay LK, Reef R, Rodriguez-  
614 Lanetty M, Miller DJ, Hoegh-Guldberg O. 2011. Complex diel cycles of gene expression  
615 in coral-algal symbiosis. Science 331:175.

616 Looby P, Loudon ASI. 2005. Gene duplication and complex circadian clocks in mammals.  
617 Trends Genet 21:46-53.

618 Mason B, Beard M, Miller MW. 2011. Coral larvae settle at a higher frequency on red surfaces.  
619 Coral Reefs 30:667-676.

620 Mason B, Schmale M, Gibbs P, Miller MW, Wang Q, Levay K, Shestopalov V, Slepak VZ.  
621 2012. Evidence for multiple phototransduction pathways in a reef-building coral. PLoS  
622 ONE 7:e50371.

623 Mason BM, Cohen JH. 2012. Long-wavelength photosensitivity in coral planula larvae. Biol  
624 Bull 222:88-92.

625 Morf J, Rey G, Schneider K, Stratmann M, Fujita J, Naef F, Schibler U. 2012. Cold-inducible  
626 RNA-binding protein modulates circadian gene expression posttranscriptionally. *Science*  
627 338:379-383.

628 Mundy CN, Babcock RC. 1998. Role of light intensity and spectral quality in coral settlement:  
629 Implications for depth-dependent settlement? *J Exp Mar Biol Ecol* 223:235-255.

630 Nilsson D-E, Gislén L, Coates MM, Skogh C, Garm A. 2005. Advanced optics in a jellyfish eye.  
631 *Nature* 435:201-205.

632 Panda S, Hogenesch JB, Kay SA. 2002. Circadian rhythms from flies to human. *Nature* 417:329-  
633 335.

634 Pearse VB. 1974. Modification of sea anemone behavior by symbiotic zooxanthellae: phototaxis.  
635 *Biol Bull* 147:630-640.

636 Plachetzki DC, Degnan BM, Oakley TH. 2007. The origins of novel protein interactions during  
637 animal opsin evolution. *PLoS ONE* 2:e1054.

638 Pruneda-Paz JL, Kay SA. 2010. An expanding universe of circadian networks in higher plants.  
639 *Trends Plant Sci* 15:259-265.

640 Redlin U, Hattar S, Mrosovsky N. 2005. The circadian Clock mutant mouse: impaired masking  
641 response to light. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 191:51-59.

642 Reitzel AM, Behrendt L, Tarrant AM. 2010. Light entrained rhythmic gene expression in the sea  
643 anemone *Nematostella vectensis*: the evolution of the animal circadian clock. *PLoS ONE*  
644 5:e12805.

645 Reitzel AM, Pang K, Ryan JF, Mullikin J, Martindale MQ, Baxevanis A, Tarrant AM. 2011.  
646 Nuclear receptors from the ctenophore *Mnemiopsis leidyi* lack a zinc-finger DNA-

647 binding domain: lineage-specific loss or ancestral condition in the emergence of the  
648 nuclear receptor superfamily? *EvoDevo* 2:3.

649 Reitzel AM, Tarrant AM. 2009. Nuclear receptor complement of the cnidarian *Nematostella*  
650 *vectensis*: phylogenetic relationships and developmental expression patterns. *BMC Evol*  
651 *Biol* 9:230.

652 Reppert SM. 2007. The ancestral circadian clock of monarch butterflies: role in time-  
653 compensated sun compass orientation. *Cold Spring Harb Symp Quant Biol* 72:113-8.

654 Rosbash M. 2009. The implications of multiple circadian clock origins. *PLoS Biol* 7:e1000062.

655 Rubin EB, Shemesh Y, Cohen M, Elgavish S, Robertson HM, Bloch G. 2006. Molecular and  
656 phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis*  
657 *mellifera*) and shed new light on the molecular evolution of the circadian clock. *Genome*  
658 *Res* 16:1352-1365.

659 Salichos L, Rokas A. 2009. The diversity and evolution of circadian clock proteins in Fungi.  
660 *Mycologia*:09-073.

661 Sancar A. 2004. Regulation of the mammalian circadian clock by cryptochrome. *J Biol Chem*  
662 279:34079-34082.

663 Shichida Y, Matsuyama T. 2009. Evolution of opsins and phototransduction. *Phil Trans R Soc B*  
664 364:2881-2895.

665 Shinzato C, Shoguchi E, Kawashima T, Hamada M, Hisata K, Tanaka M, Fujie M, Fujiwara M,  
666 Koyanagi R, Ikuta T, Fujiyama A, Miller DJ, Satoh N. 2011. Using the *Acropora*  
667 *digitifera* genome to understand coral responses to environmental change. *Nature*  
668 476:320-323.

669 Shoguchi E, Tanaka M, Shinzato C, Kawashima T, Satoh N. 2013. A genome-wide survey of  
670 photoreceptor and circadian genes in the coral, *Acropora digitifera*. *Gene* 515:426-431.

671 Smarr B, Schwartz M, Wotus C, de la Iglesia H. 2013. Who you callin' diurnal? Redefining  
672 temporal niche. *Integr Comp Biol* (this issue):xx-xx.

673 Somers DE, Devlin PF, Kay SA. 1998. Phytochromes and cryptochromes in the entrainment of  
674 the *Arabidopsis* circadian clock. *Science* 282:1488-90.

675 Sorek M, Levy O. 2012. Influence of the quantity and quality of light on photosynthetic  
676 periodicity in coral endosymbiotic algae. *PLoS ONE* 7:e43264.

677 Stamatakis A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with  
678 thousands of taxa and mixed models. *Bioinformatics* 22:2688 - 90.

679 Suga H, Schmid V, Gehring WJ. 2008. Evolution and functional diversity of jellyfish opsins.  
680 *Curr Biol* 18:51-55.

681 Sweeney BM. 1976. Circadian rhythms in corals, particularly Fungiidae. *Biol Bull* 151:236-246.

682 Taddei-Ferretti C, Musio C. 2000. Photobehaviour of *Hydra* (Cnidaria, Hydrozoa) and correlated  
683 mechanisms: a case of extraocular photosensitivity. *J Photochem Photobiol B: Biol*  
684 55:88-101.

685 Tambutté S, Holcomb M, Ferrier-Pagès C, Reynaud S, Tambutté É, Zoccola D, Allemand D.  
686 2011. Coral biomineralization: From the gene to the environment. *J Exp Mar Biol Ecol*  
687 408:58-78.

688 Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, Sakaki Y. 1997. Circadian  
689 oscillation of a mammalian homologue of the *Drosophila* period gene. *Nature* 389:512-  
690 516.

691 Vatine G, Vallone D, Appelbaum L, Mracek P, Ben-Moshe Z, Lahiri K, Gothilf Y, Foulkes NS.  
692 2009. Light directs zebrafish *period2* expression via conserved D and E boxes. PLoS Biol  
693 7:e1000223.

694 Williams JA, Sehgal A. 2001. Molecular components of the circadian system in *Drosophila*.  
695 Annu Rev Physiol 63:729-755.

696 Xu Y, Mori T, Johnson CH. 2003. Cyanobacterial circadian clockwork: roles of KaiA, KaiB and  
697 the kaiBC promoter in regulating KaiC. EMBO J 22:2117-2126.

698 Yuan Q, Metterville D, Briscoe AD, Reppert SM. 2007. Insect cryptochromes: gene duplication  
699 and loss define diverse ways to construct insect circadian clocks. Mol Biol Evol 24:948-  
700 955.

701 Zhu H, Sauman I, Yuan Q, Casselman A, Emery-Le M, Emery P, Reppert SM. 2008.  
702 Cryptochromes define a novel circadian clock mechanism in monarch butterflies that  
703 may underlie sun compass navigation. PLoS Biol 6:e4.

704 Zhu H, Yuan Q, Froy O, Casselman A, Reppert SM. 2005. The two CRYs of the butterfly. Curr  
705 Biol 15:R953-R954.

706

707