Current Understanding of the Circadian Clock Within Cnidaria

31

Kenneth D. Hoadley, Peter D. Vize, and Sonja J. Pyott

Abstract

Molecularly-based timing systems drive many periodic biological processes in both animals and plants. In cnidarians these periodic processes include daily cycles in metabolism, growth, and tentacle and body wall movements and monthly or yearly reproductive activity. In this chapter we review the current understanding of biological clocks in the cnidaria, with an emphasis on the molecular underpinnings of these processes. The genes that form this molecular clock and drive biological rhythms in well-characterized genetic systems such as *Drosophila* and mouse are highly conserved in cnidarians and, like these model systems, display diel cycles in transcription levels. In addition to describing the clock genes, we also review potential entraining systems and discuss the broader implications of biological clocks in cnidarian biology.

Keywords

Circadian rhythms • Biological clocks • Reproductive timing • Non-visual photodetection • Light perception

31.1 Overview

Entrainment of physiological rhythms to environmental cues is ubiquitous among living organisms and allows coordination of biology and behavior with daily environmental changes. This coordination improves survival and reproductive fitness, and, thus, it is not surprising that an endogenous "clock" has evolved to maintain rhythmicity over a circadian (24 h) period. From the identification of the first circadian gene (Konopka and Benzer 1971), the past 40 years have produced thousands of studies focusing on the molecular basis of the circadian clock. Across species, from bacteria, to fungi, to plants and animals, this molecular circadian clock involves transcription and translation feedback loops with a self-sustained period of about 24 h (reviewed in Dunlap 1999). Investigation in the model genetic species, mouse and fly, has identified a core set of genes that form the central oscillator in animals (reviewed in Panda et al. 2002). Input pathways to this central oscillator reset the circadian clock to environmental cues, such as sunlight, and output pathways direct changes in biology and behavior, such as sleep-wake cycles. Coordination of behaviors, like reproduction and migration, to seasonal environmental changes, such as photoperiod (day length) also utilizes components of the circadian clock (reviewed in Hut and Beersma 2011, Ikegami and Yoshimura 2012, and Coomans et al. 2014). This core set of genes appears conserved among animals including, as revealed by very recent work (reviewed here), cnidarians. These findings are evidence of the very early evolutionary origin of the molecular circadian clock within the animal lineage (Young and Kay 2001).

The existence of a molecular circadian clock within cnidarians is not unexpected. Many cnidarian species display

K.D. Hoadley
School of Marine Science and Policy, University of Delaware, 700 Pilottown Rd., Lewes, DE 19958, USA
e-mail: khoadley@udel.edu

P.D. Vize
Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada
e-mail: pvize@ucalgary.ca

S.J. Pyott (✉)
Department of Otorhinolaryngology, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands
e-mail: s.pyott@umcg.nl

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behavioral and reproductive changes that are synchronized to diel solar or lunar light cycles. Tentacle retraction during the day to avoid predation and expansion at night to feed is a well-known behavior observed within many shallow water scleractinian coral species (Yonge 1940; Sweeney 1976; Sebens and DeRiemer 1977) and rhythmic body wall contractions in response to light have been described in Hydra (Passano and McCullough 1962). In addition, rates of calcification, photosynthesis, and respiration also display diel rhythmicity and are thought to utilize the daily light cycle as a principle entrainment cue (Lasker 1979; Moya et al. 2006; Sorek and Levy 2012). Importantly, these behaviors maintain their rhythmicity even under conditions of constant dark, an important feature suggesting regulation by an intrinsically generated molecular circadian clock. Behavioral patterns over longer time periods, such as gamete release during annual mass spawning events or monthly coordinated larval release in brooding corals species, may also be entrained responses, utilizing environmental cues to synchronize reproductive efforts (Harrison et al. 1984; Szmant-Froelich et al. 1985; Baird et al. 2009). Although synchronous coral spawning is well-characterized, little is known about the molecular signaling pathways responsible for such precise timing and entrainment of reproductive effort has not been demonstrated. As with tentacle retraction, an important environmental entrainment cue is thought to be light, both solar and lunar. Even small changes in low intensity light can induce behavioral responses, such as tentacle retraction or larval release in scleractinian corals, indicating sensitivity to small changes in light such as those experienced throughout the lunar cycle (Jokiel et al. 1985; Gorbunov and Falkowski 2002).

This chapter discusses what is currently known about the molecular mechanisms governing environmentally regulated behavior in cnidarians. The chapter begins with a brief description of the molecular mechanisms elucidated from model mammalian and insect species to build a framework for understanding the cnidarian molecular circadian clock. In describing the cnidarian circadian clock, emphasis is placed on outlining the molecules that form the circadian clock, describing what is known about the input pathways that set the clock, and then discussing possible output pathways that direct entrained behaviors. The chapter concludes with a discussion of the work that still needs to be done to understand more fully the molecular basis of the circadian clock in cnidarians and how it regulates behavior.

31.2 The Molecular Circadian Clock as Revealed from Model Metazoan Species

The first investigations of molecular circadian clocks in cnidarians were naturally guided by knowledge gained from well-studied model systems, and, therefore, it is useful to review briefly the molecular basis of circadian clocks as elucidated in the genetically tractable organisms mouse (*Mus musculus*) and fly (*Drosophila melanogaster*). A number of excellent reviews much more extensively outline the current molecular understanding of clocks in these species (Ko and Takahashi 2006; Hardin 2011; Ozkaya and Rosato 2012) and are summarized below. Importantly, early work unraveled the molecular mechanisms within the central oscillators, which are contained within a specific set of neurons known as the superchiasmatic nucleus (SCN) in mammals (reviewed in Hastings et al. 2014) or the lateral and the dorsal neurons within the fly brain (reviewed in Nitabach and Taghert 2008). Circadian clocks are now known to be composed of a hierarchy of oscillators that function not just centrally but also peripherally at the cellular, tissue, and systems level (reviewed in Mohawk et al. 2012). The molecular mechanisms regulating the synchrony and coherence of these various oscillators as part of a larger circadian "system" is the focus of intense investigation and may prove informative to understanding how circadian clocks operate in cnidarians (as discussed later in this chapter). Finally, the molecular understanding of circadian clocks has expanded to a variety of other organisms, including a diversity of animals, plants, and even unicellular prokaryotes and eukaryotes (reviewed in Crane and Young 2014). Whereas a similar molecular mechanism appears conserved among animals, now reinforced by our knowledge of these mechanisms in cnidarians, circadian clocks in plants (*Arabidopsis thaliana*, see Nakamichi 2011), fungi (*Neurospora crassa*, see Baker et al. 2012), and bacteria (*Synechococcus elongates*, see Mackey et al. 2011) utilize different cores sets of molecules, suggesting the independent evolution of circadian clocks among these different lineages (reviewed in Young and Kay 2001 and Bell-Pedersen et al. 2005).

31.2.1 The Molecular Circadian Clock: The Central Players

The period of oscillation of the molecular clock (approximately 24 h) is set by two interacting transcription/translation feedback loops. This periodicity is set autonomously and maintained even in the absence of external cues. At their simplest examination, these feedback loops involve two activators, CLOCK and BMAL1 in mouse and CLOCK and CYCLE in fly, and two repressors, PER and CRY in mouse and PER and TIM in fly. Once transcribed, CLOCK and BMAL1/CYCLE form a heterodimer that translocates to the nucleus and acts as a transcriptional activator by binding to specific DNA elements, especially E-box regulatory elements within the promoter region of additional clock genes or genes under clock-regulated control (Hardin 2004). As expected for transcriptional activators, binding increases

transcription rates. In particular, CLOCK/BMAL1 (mouse) or CLK/CYC (fly) initiate the transcription of the *Per* and *Cry* genes (mouse) or *per* and *tim* genes (fly). Following transcription and heterodimerazation, PER/CRY or PER/TIM translocate to the nucleus and suppress CLOCK/BMAL1 or CLK/CYC activity, resulting in reduced transcription of *Per* and *Cry* or *per* and *tim* as well as other clock-controlled genes. The degradation of PER and CRY or PER and TIM are necessary to terminate suppression. Thus, the stability and rates of degradation of these repressors determine the period of the central oscillator. CLOCK/BMAL1 and CLK/CYC also initiate transcription of a second transcription/translation feedback loop (reviewed in Hardin and Panda 2013). In brief, in flies, this feedback loop is controlled by the transcription factor VRILLE, which represses *Clk* activation. In mammals, this feedback loop is controlled by the proteins ROR (α, β, and γ) and REV-ERB (α and β), which bind to regulatory elements and serve to regulate *Bmal1* transcription (Yang 2010). This second transcription/translation feedback loop serves to improve the robustness and fidelity of the circadian clock.

These feedback loops form an endogenous rhythm that enables genes to be turned on and off at a regular cycle, thereby regulating behavioral and/or physiological outputs regardless of the presence or absence of environmental signals. Both the positive and negative feedback loops undergo significant post-translational modification (Gallego and Virshup 2007), including ubiquitination and phosphorylation which help set the approximate 24 h cycle of the molecular circadian clock. In addition to these core genes, mutants have identified various accessory genes that serve to enhance the robustness of periodicity.

31.2.2 Input Pathways: Setting the Clock

Because the period of the molecular clock is only approximately 24 h and because shifts in environmental cues can occur (such as changing day length in different seasons), mechanisms must exist to synchronize circadian rhythms with important environmental cues. (The best example of a mismatch in biological and environmental time occurs during jet lag.) These cues are called "zeitgebers" or time givers and can include light, temperature, food availability, day length, as well as social parameters. The strongest entrainment cue appears to be light, and especially the daily light cycle, and, because it is also easy to manipulate experimentally, it is the most well-studied entrainment cue. It has long been appreciated that light pulses reset circadian activity (Pittendrigh and Minis 1964): light pulses in the early night (or dusk) delay circadian activity whereas light pulses in the late night (or dawn) advance circadian activity. Light induces these shifts in circadian activity by molecularly resetting the oscillation of the transcription/translation feedback loops.

In mammals the role of opsin, melanopsin, and cryptochrome based systems in the entrainment of biological clocks and light responses have been extensively studied. Mice lacking either opsin-based light detection (via either retinal degeneration that causes loss of the opsin-containing rod and cone photoreceptors or by targeted mutagenesis of the transducin and the cyclic GMP-gated channel activated by transducing) or melanopsin still show pupillary light responses and can still be entrained to light (reviewed in Lucas et al. 2012). However, in the absence of both opsin- and melanopsin-based photoreception pupillary light responses and photoentrainment is eliminated (Hattar et al. 2003). These findings suggest that cryptochromes do not have a light-detecting role in the retina as had been suggested previously in mice lacking *Cry1* and *Cry 2* (Vitaterna et al. 1999; Van Gelder et al. 2003). Thus, at least in mice, the central pacemaker clock is synchronized by opsin- and melanopsin-based photoreception, and cryptochromes function as transcription factors in the clocks they entrain. This central clock, in turn, regulates peripheral clocks whose synchronization is driven by the SCN and not light itself.

In the fly, environmental light syncs the molecular clock in part via CRY (reviewed in Ashmore and Sehgal 2003). CRY within the nucleus competes with PER to bind with TIM, forming a heterodimer. Formation of the CRY/TIM heterodimer leads to proteosomal degradation of the TIM protein, and CRY itself is degraded in a light-dependent manner. In addition, the disassociation of the PER/TIM heterodimer exposes PER to phosphorylation and subsequent degradation. The reduction of the PER/TIM heterodimer releases the CLOCK/CYCLE heterodimer from inhibition, which, in turn, increases the transcription of gene products with E-box motifs within their promoter regions. In this way, CRY resets the molecular clock. Importantly, the original *cryb* mutant flies do retain some light-driven entrainment (Stanewsky et al. 1998), indicating the existence of redundant circadian photoreceptors. Furthermore, CRY activity is also dependent on the redox state of the cell (Lin et al. 2001). Thus, in fly, CRY acts as both a sensor of multiple cellular parameters and also a transcription factor within the core circadian clock.

31.2.3 Output Pathways: Timing Biological Processes

The molecular mechanisms linking the circadian clock to circadian rhythmicity are very poorly understood in any species. The most readily observed circadian outputs include, in fly, eclosion (the emergence of the adult fly from the pupa case) and locomotor activity, and, in mammals, sleep-wake

and fasting-feeding cycles. However, as is being increasingly appreciated, the circadian clock regulates a range of metabolic processes, including but not limited to glucose and lipid metabolism, body temperature, and endocrine hormone secretion. Complicating research is the fact that metabolic processes both receive input and also return input to central as well as peripheral clocks (Rutter et al. 2002; Bass and Takahashi 2010). For example, feeding behavior is regulated by the circadian clock and yet food can also act as a zeitgeber (reviewed in Challet 2013). Not surprisingly then, many mutants of core components of the circadian clock also exhibit metabolic disorders (reviewed in Marcheva et al. 2013 and Richards and Gumz 2013). In general, output pathways require neuroendocrine circuits to link central clocks with central and also peripheral targets that direct activity. For example, in the mouse, the SCN targets (among other structures) the hypothalamus (reviewed in Li et al. 2012). Via the pituitary gland, the hypothalamus serves as an important link between the nervous system and the endocrine system and regulates a number of circadian behaviors. Also via the hypothalamus (and other intermediate structures), the SCN triggers the increased release of the signaling molecule cAMP from cells of the pineal gland during the dark. Increased cAMP increases the nighttime production of the hormone melatonin (reviewed in Pevet and Challet 2011). Melatonin receptors are expressed throughout the body. Temporal patterns of melatonin levels encode not only day-night but also photoperiod; thus, melatonin functions in both the regulation of circadian and also seasonal behaviors (see Coomans et al. 2014). Output pathways from the central clocks have been reviewed in animals in greater detail elsewhere (Hardin 2000; Bass and Takahashi 2010).

31.3 Investigating the Cnidarian Molecular Circadian Clock

When applying principles derived from work on mammalian and insect systems to the cnidarian molecular clock, major physiological differences between these taxa become immediately apparent. For mammals and insects alike, the central oscillator is located within specialized cells of the central nervous system and provides master control of peripheral oscillators. In contrast, most cnidarian nervous systems revolve around a nerve-net-like system with many small ganglia but no clear centralization (Marlow et al. 2009). Thus, the location of oscillators and the presence of central or peripheral oscillators in cnidarians is unclear. Furthermore, in mammals and insects, complex light entrainment pathways may be necessary since the core molecular clock exists within the central nervous system and is unlikely to be directly exposed to environmental light. Cnidarians however are largely translucent organisms and for those species living within the photic zone, solar irradiance likely penetrates through most of their living tissues. This is especially true of scleractinian coral species where highly reflective calcium carbonate skeletons greatly increase the light field for symbiotic algal cells living within the host (Enriquez et al. 2005).

31.3.1 Molecular Basis of the Cnidarian Circadian Clock: The Players So Far

The molecular clock machinery in cnidarians has been elucidated from genomic (Shoguchi et al. 2013) and transcriptomic (Vize 2009; Brady et al. 2011) analyses aimed to identify clock genes and also investigation of diel (and lunar) differences in expression of smaller sets of presumed clock genes (Levy et al. 2007; Reitzel et al. 2010; Hoadley et al. 2011). Genetic information is presently limited to anthozoan coral species.

Utilizing the framework established in fly and mouse, *Clock* and *Cycle* (sometimes referred to as *Bmal/Cycle* in the literature), the positive elements of the molecular clock feedback loop, have been identified in the genomes of *Acropora digitifera* (Shoguchi et al. 2013) and *Nematostella vectensis* (Reitzel et al. 2010) and transcript expression has been verified in *Acropora millepora* (Levy et al. 2007, 2011; Vize 2009; Brady et al. 2011), *Favia fragum* (Hoadley et al. 2011), and *Nematostella vectensis* (Reitzel et al. 2010, 2013; Peres et al. 2014). In genomic analyses, *A. digitifera* expresses three clock and one clock-like gene compared to one clock and one clock-like gene identified in *N. vectensis* (Shoguchi et al. 2013). In both species, one *Bmal/Cycle* gene has been identified. Comparison of expression profiles of these genes among studies is complicated by experimental differences in sampling frequency, light regimes, and life stages of the species utilized. Nonetheless, molecular clock expression profiles for *A. millepora* (Levy et al. 2007, 2011; Brady et al. 2011), *F. fragum* (Hoadley et al. 2011) and *N. vectensis* (Reitzel et al. 2010, 2013; Peres et al. 2014) appear to share many of the same features both among cnidarians and in comparison to model (fly and mouse) systems. Under a 12:12 h light:dark (LD) cycle, expression profiles for *Clock* and *Cycle* are similar for *N. vectensis* and *F. fragum.* Moreover, only *Clock* shows cyclical expression, with peak gene expression occurring near the LD transition (Reitzel et al. 2010; Hoadley et al. 2011). For *A. millepora*, both *Clock* and *Cycle* appear to peak in expression a few hours after the LD transition, with the lowest expression values occurring during the subjective day (Brady et al. 2011). However, because the *A. millepora Clock* and *Cycle* genes were only profiled within a 22-h window within this study, a cyclic diel pattern of expression is difficult to identify.

The negative elements of the molecular clock feedback loop have also been examined. The *Cry* genes have been

most extensively examined. Three *Cry* gene variants have been identified within the cnidarian genomes: *Cry1*, *Cry2*, and *Cry-DASH*. In addition, *N. vectensis* contained more than twice as many *Cry* variants (seven) as compared to the three identified in *A. digitifera* and *A. millepora* (Shoguchi et al. 2013). It is unclear if this suggests a gene loss or gene duplication between the scleractinian and actinarian taxa and/or if all seven of the *N. vectensis Cry* genes are important to the molecular clock mechanism. Peak *Cry* expression within the two scleractinian coral species, *A. millepora* and *F. fragum*, occur during the daytime, with *Cry1* expression peaking earlier in the day than *Cry2* (Levy et al. 2007; Hoadley et al. 2011). The *Cry2* ortholog in *N. vectensis, Cry1b*, also peaks during mid to late daytime hours. However, in *N. vectensis*, peak expression of *Cry2* (which is orthologous to *Cry1* in *F. fragum* and *A. millepora*) occurs just a few hours prior to the dark:light transition (Reitzel et al. 2010). The offset in peak expression for this *Cry* variant can potentially be explained by differences in the life history and physiology of the three species. Whereas *F. fragum* and *A. millepora* are calcifying scleractinian corals harboring symbiotic dinoflagellate species, the anemone *N. vectensis* is aposymbiotic and does not secrete a calcium carbonate skeleton. The presence of photosynthetic alga, which presumably express their own light-entrained molecular clock, within the cells of *F. fragum* and *A millepora*, may influence expression patterns within the host (Sorek et al. 2013). Regulation of cnidarian circadian clocks by symbiotic algae will be discussed more later on.

An additional negative feedback gene in model organism clocks is the transcription factor VRILLE (Cyran et al. 2003). In other model systems VRILLE binds to the promoter of the CLOCK gene and inhibits its expression (Hardin 2005). The *A. millepora Vrille* gene displays a very strong diel cycle of transcription and shows 200-fold higher levels of expression at night versus day (Brady et al. 2011) and is an excellent candidate as a repressor of the *Clock* mediated positive elements of the coral network.

Considering these negative regulatory elements, a very surprising and yet consistent finding has been the absence of the *Per* genes within the cnidarian genomes (Hoadley et al. 2011; Shoguchi et al. 2013; Reitzel et al. 2013). Cnidarian genomes contain PAS family members but the overall level of conservation is modest and none of the three family members analyzed in *A. millepora* larvae showed any differential expression over diel cycles (Brady and Vize, unpublished findings). Another feature is that a single *Timeless* gene is present within all three cnidarian species, and may be orthologous to *Timeless2/Timeout*, although the N-terminal Timeless domain alone clusters with *Timeless* and not *Timeless2* (Oldach and Vize, unpublished findings). The *Drosophila timeless2/timeout* gene plays two roles, one in chromosome stability and one in circadian timing (Benna et al. 2010) while *timeless* is a dedicated clock gene (Hunter-Ensor et al. 1996). Although it is unclear whether TIM is involved in the negative feedback loop of the cnidarian molecular clock, its transcription does follow a 24 h cycle suggesting that it may be under clock regulation (Reitzel et al. 2010; Brady et al. 2011). It is possible that *Timeless2/Timeout* also acts in the negative feedback loop within the cnidarian molecular clock or, as in fly, binds with CRY in a light sensitive fashion to serve as a repressor of transcription. However, physical protein interactions would need to be studied in order to confirm this interaction.

Another striking contrast between adult cnidarians and fly and mammal clock gene expression patterns is the loss of circadian rhythmicity in conditions of constant dark in some studies, suggesting that there may be no (or at least reduced) intrinsic maintenance of rhythmicity. Intrinsic rhythmicity in gene expression may be masked by the sampling techniques utilized when sampling from adult colonies: most expression studies have ground up relatively large portions of coral tissue. Thus, the resulting mRNA samples represent the combined expression profiles for multiple tissue layers. Because larvae are small and relatively undifferentiated in terms of tissue types, an intrinsic rhythm may be easier to observe in larval samples. In support of this hypothesis, differences in expression profiles are observed between larval and adult coral colonies (Brady et al. 2011; Peres et al. 2014) and rhythmic expression under constant dark conditions has been documented over 24 h for *A. millepora* planulae (Brady et al. 2011). *N. vectensis* larvae also displayed dark:dark rhythmic expression of the molecular clock genes *Clock*, *Timeout*, *Cry1a*, *Cry1b* and *Cry2* but lost this effect after 96 h of constant darkness (Peres et al. 2014). Interestingly, mouse and fly *Per* mutants display disrupted free running rhythms (Hardin et al. 1992; Zheng et al. 2001). Perhaps the absence of *Per* in the cnidarian genome contributes to the degraded free running rhythms observed in adult cnidarians, suggesting that function of the core clock mechanism to maintain intrinsic transcriptional rhythmicity may differ between cnidarians and other animal (mammalian and insect) groups.

In addition to the core elements of the circadian molecular clock, a number of other clock genes have been identified in cnidarians. Based on transcriptomic analysis in *A. millepora*, matches with strong confidence levels were found to 22 key insect and mammalian circadian clock genes have been identified (Vize 2009). Both positive and negative elements of the feedback system were identified, along with key phosphatases and ubiquitinases important to posttranslational modifications of the core clock proteins. As discussed above, in fly and mouse these mechanisms are important regulators of the approximate 24 h periodicity of the clock. A comparison of clock genes across the three available cnidarian genomes, *A. millepora, N. vectensis*, and *A. digitifera* revealed differences in gene copy numbers and duplications

for several of the core molecular clock components (Shoguchi et al. 2013). This research proposes various hypotheses on the evolutionary origin of clocks within these groups.

31.3.2 Input Pathways: Light as an Entrainment Cue in Cnidarians

Cnidarians respond to a wide range of different light wavelengths and intensities (Levy et al. 2003; Mason and Cohen 2012). A variety of potentially light-sensitive molecules have been identified in cnidarians and presumably the co-expression of multiple photosensitive molecules enables light detection over a broad range of wavelengths. The likely candidates currently include cryptochromes, which are discussed in more detail below. In addition, genome-wide searches have identified opsins in *N. vectensis* (Suga et al. 2008) and *A. digitifera* (Shoguchi et al. 2013), melanopsin in *A. millepora* (Vize 2009), as well as rhodopsin-like G protein-coupled receptors in *A. millepora* (Anctil et al. 2007; Mason et al. 2012).

Cryptochromes were introduced earlier as part of the core circadian clock, and, based on their known role as blue light flavoprotein photoreceptors (photosensors) in plants (Chaves et al. 2011), have been the most investigated potential molecular photosensors in cnidarians. Unlike the vitamin-A derived chromophore used by opsins and melanopsins, cryptochromes use FAD as their chromophore. When light strikes the FAD and reduces it to FADH, a conformational shift in the cryptochrome occurs in plants and changes its transcriptional activity (reviewed in Christie et al. 2015). A potential role for cryptochromes as cnidarian photosensors has been proposed (e.g. Levy et al. 2007), but, importantly, no data demonstrating intrinsic photosensitivity of cnidarian cryptochromes has yet been published. Although the transcription of *Cry1* and *Cry2* appears to depend on light in *A. millepora* and *F. fragum* (Levy et al. 2007; Brady et al. 2011; Hoadley et al. 2011), this observation indicates only that these genes are a part of the light-mediated response and not necessarily the photosensor per se.

In cnidarians cryptochromes display diel cycles in transcription (Levy et al. 2007; Reitzel et al. 2010; Brady et al. 2011; Hoadley et al. 2011), with high levels of expression in the daytime and low levels of expression at night. In *A. millepora* planulae, the cycle of transcription for *Cry2* can continue in complete darkness, indicating that expression of this gene is, in fact, driven by the circadian clock and not directly by light (Reitzel et al. 2010; Brady et al. 2011). Thus, cryptochromes in *A. millepora* (analogous to those of mammals) may serve as components of the core circadian clock rather than as photosensors. Similar results have been obtained in the anemone, *N. vectensis* (Reitzel et al. 2010), and possibly also in the coral *F. fragum* (Hoadley et al. 2011). Results vary somewhat between studies, and these differences may arise from differences in tissue- or cell-specific expression and/or as the result of expression profiles that vary between central versus peripheral clocks. In other systems, cryptochromes are targeted for proteosomal degradation after shifting to their light-activated configuration (Ashmore and Sehgal 2003). Thus, if cnidarian cryptochromes are intrinsically photosensitive, light-triggered degradation may be essential to their diel changes in expression. On the other hand, or additionally, redox state can regulate the transcriptional activity of the CLOCK and NPAS proteins (Rutter et al. 2002) and presumably a similar form of regulation may operate for cryptochromes independently of any intrinsic light sensitivity (see Lin et al. 2001).

Much less is known about the physical structures that house the photosensors important for the entrainment of cnidarian circadian clocks. In other animals, both visual and non-visual light detection systems convey information about environmental light to the central circadian clock. Visual systems include specialized light sensing organs known collectively as eyes, though variations in structure and function can vary widely. Eyes with a single lens are known as camera eyes, single simple eyes with no lens are known as ocelli, and eyes with multiple lenses are known as compound eyes. Within cnidarians the most highly developed eyes are found in cubozoan medusae, which have both camera eyes capable of detecting specific features for hunting and navigation (Kozmik et al. 2008). Cubozoan planulae also have pigment spot ocelli. These are individual specialized cells that contain a pigmented pit that allows for directional light detection. These cells also contain a cilium with a standard 9+2 axoneme, the activity of which is thought to be directly controlled by light striking the photoreceptor containing villi within the pigment pit (Nordstrom et al. 2003). This arrangement allows for directional motility in response to light without any relay via a nervous system.

Detailed studies on cnidarian light-induced signal transduction have only been performed in detail in cubozoans. In the camera type eyes of these animals ciliary-type opsins and a cyclic nucleotide pathway is utilized, followed by a transient receptor potential channel triggered response that initiates an action potential in camera eyes (Koyanagi et al. 2008). Cubomedusae also contain less well-developed ocelli. The opsins and transduction pathway in the ocelli have been shown to lack ciliary opsins (Ekstrom et al. 2008), and they likely use rhabdomeric-type opsins and calcium-mediated transduction pathways. In coral planulae treatment with compounds that alter cytoplasmic calcium levels induce changes matching those triggered by light, providing at least indirect evidence that light triggers a calcium-mediated response (Hilton et al. 2012). A separate series of experiments identified opsins in *A. palmata planulae* that localized to the aboral end of the larvae and were capable of in vitro

GDP-GTP exchange in response to light (Mason et al. 2012). These results are all consistent with the pigment pit ocelli of cubozoans and both adult and larval scleractinians using a classical rhabdomeric opsin and G protein- and calcium-mediated transduction cascade. If and how these visual systems convey information to the cnidarian molecular clock has yet to be elucidated. Moreover, the contribution of non-visual systems, using cryptochromes and/or melanospin, to the entrainment of the cnidarian molecular clock has yet to be functionally investigated.

31.3.3 Output Pathways: Molecular Mechanisms That Regulate Circadian and Seasonal Physiology in Cnidarians

As introduced earlier, very little is known about the molecular mechanisms that link the circadian clock to circadian rhythmicity, even in model genetic systems. Nonetheless, recent research has begun to investigate output pathways of circadian clocks in cnidarians using a variety of strategies. At the genetic level, E-box motifs, specific DNA elements found within the promoter region of many clock-regulated genes, have been identified within the promoter region of several cnidarian genes, including those known to be core clock components (Reitzel et al. 2013). At the transcriptional level, previous research (discussed above) has uncovered additional genes with cyclical transcriptional profiles. These types of investigations should, without a priori assumptions, identify genes that are under clock control and, therefore, may comprise the output pathways that regulate circadian behaviors.

More targeted approaches to examine output pathways in cnidarians have also been utilized. Specifically, melatonin, the circadian and output signaling molecule important in vertebrates (see discussion above), has been examined using immunoreactivity, histology, and HPLC (Mechawar and Anctil 1997; Roopin and Levy 2012a, b; Peres et al. 2014). Although there are conflicting findings on the diel expression patterns of melatonin, there is agreement on the likely involvement of melatonin in reproductive maturation. In the most recent of these investigations, Peres and others found *e*xpression that peaked at the end of light period in *N.* vectensis adults maintained under diel conditions (2014), expression patterns that would be expected from vertebrate systems. This study also provided data indicating that exogenously applied melatonin may restore circadian oscillation of clock transcripts that have lost transcriptional rhythmicity after being transferred to conditions of constant darkness. This research suggests that melatonin may serve as an intermediate connecting input and output pathways. Such dual roles for clock signaling molecules have been observed in fly and mouse as well (as discussed above) and may be an inherent feature of circadian clock regulation. Much work remains to be done to determine the mechanism by which melatonin functions to regulate circadian and seasonal behaviors in cnidarians. Importantly, previous work in *N. vectensis* (Roopin and Levy 2012b) used in-situ hybridization to localize transcripts of two representative melatonin receptor orthologs (identified previously by Anctil 2009) and found abundant expression of these putative melatonin receptors in both reproductive tissues and also highly neuralized areas. These findings begin to uncover the circuitry of the melatonin signaling pathway in (at least select) cnidarians and suggest interesting hypotheses on the role of melatonin signaling in anthozoans.

Transcript differences in clock genes have also been investigated during development as well as during reproductive cycles, offering insight into the genetic mechanisms regulating these behaviors. For *N. vectensis*, diel periodicity in molecular clock expression profiles is absent in larvae at 48 h, with rhythmic expression only emerging later (Peres et al. 2014). When sampled at 1 week of age, *A. millepora* larvae displayed similar diel periodicity (Brady et al. 2011) as do 1–2 week old *N. vectensis* larvae (Peres et al. 2014). Importantly, the spatial expression of *Cry1a*, *Cry1b*, *Clock* and *Cycle* changes considerably within the initial 168 h of embryonic development, with diffuse expression first observed within the blastula stage, localizing mostly within the endoderm by the planulae stage (Peres et al. 2014). As pointed out by the authors of that study, expression of clock genes during the early stages of development has been well-documented within fly and mouse (Curran et al. 2008; Dekens and Whitmore 2008), where there expression may be important to the proper timing and formation of organs and tissues. In cnidarians, developmental and spatial differences in localization suggest additional roles for these genes directing the initial stages of development.

The earliest investigations of clock genes in cnidarians actually investigated the role of these genes in regulating seasonal reproductive events (Levy et al. 2007). Increases in *A. millepora Cry 2* were observed during a full moon (Levy et al. 2007) and these authors have suggested, therefore, that cryptochromes may play a role in reproductive synchronization. However, peaks in cryptochrome expression for the brooding coral *F. fragum* did not correlate with either larval or gamete release (Hoadley et al. 2011), both of which are known to follow the lunar light cycle (Szmant-Froelich et al. 1985) and very different nightly profiles of these genes have been described by others (Brady et al. 2011). Thus, the role of clock genes and cryptochromes in coordinating reproductive cycles is unclear. However, all work on cnidarian molecular clock rhythms thus far has been mostly focused on expression levels of core clock genes. Although informative, this assay requires peaks in expression to be synchronized with reproductive outputs in order to conclude a positive correlation.

Capturing such a correlation requires precise timing between sampling regimes and the biological/behavioral output of interest and interactions at the protein and post-translational rather than transcriptional levels may be informative. Moreover, events downstream of the core clock genes may be involved in the timing of reproductive cycles and, therefore, not captured by assays that examine expression patterns of the core clock genes over reproductive cycles.

Finally, recognizing that metabolic processes are not only a product of circadian clocks but also provide important entrainment cues in other animals (Rutter et al. 2002; Bass and Takahashi 2010), the importance of translocation of metabolites and signaling molecules between the host coral and its endosymbiont (Whitehead and Douglas 2003; Yellowlees et al. 2008) need to be considered when discussing output and input pathways in cnidarains. Adult scleractinian coral colonies may contain up to four million *Symbiodinium* cells per cm^2 and are, therefore, under significant influence from their symbiotic algae via photosynthesis and translocation of glucose rich photosynthates (Fitt et al. 2000). Additionally, in contrast to aposymbiotic corals such as *N. vectensis*, major changes in both oxygen saturation and carbonate ion concentrations occur within the host as a direct result of daily photosynthesis by their symbionts (Sorek and Levy 2012; Sorek et al. 2014). Several behavioral and physiological outputs of the host/symbiont (holobiont), including respiration, photosynthesis, calcification, tentacle retraction and feeding are known to exhibit 24 h peridocitiy (Moya et al. 2006; Sorek and Levy 2012). Additionally, endosymbiotic algae, such as *Symbiodinium*, have molecular clock mechanisms of their own (Levy et al. 2003; Sorek and Levy 2012; Sorek et al. 2013). The degree to which there is cross talk between host and symbiont clock mechanisms is also largely unknown and certainly worthy of further investigation.

31.4 The Road Ahead

The last few years have seen a transformative increase in our understanding of the molecular basis of circadian clocks in cnidarians, largely through the use of genomic and transcriptomic approaches. As is typical in science, this increased understanding presents many new questions. Some of the most exciting questions get at fundamental differences in the circadian clock between cnidarians and other animals. For example, in which tissues are clock genes located and expressed and to what extent are their central versus peripheral clocks in cnidarian species? Is the periodicity of this clock intrinsic or does it require entrainment cues? What, then, are the most salient entrainment cues (zeitgebers) in cnidarians and what are the receptive molecules detecting these cues? What are the anatomical circuits that direct input to and output from the cnidarian circadian clock and how do these pathways converge? Finally, what role do symbionts play in entraining the clock?

Answering these questions will require the development and application of a variety of new tools. Firstly, the utility of genomic approaches will be greatly improved as the number and accessibility of full cnidarian genomes increases. Currently only four cnidarian genomes are available, and three of these four are sessile cnidarians within the Anthozoan class. A jellyfish genome would be particularly useful and would allow investigation of the mechanisms regulating other circadian behaviors, like diel vertical migrations. Secondly, moving the clock mechanism from gene to transcript to protein will require a better understanding of the direct physical interactions among the identified cnidarian molecular clock proteins. Presently, only the core molecular clock proteins CLOCK and CYCLE have been examined and confirmed by coimmunoprecipitation to form a heterodimer in *N. vectensis* (Reitzel et al. 2010). More systemic application of biochemical techniques will be required to validate the protein interactions predicted in cnidarians based on knowledge obtained from model systems. These techniques also promise to identify the physical location of the cnidarian circadian clock. Interestingly, recent investigation of developing *N. vectensis* embryos and planulae suggest that the major site of expression of clock genes is the endoderm, rather than the nervous system or specialized sensory cells (Peres et al. 2014). Finally, the development of techniques to eliminate gene expression via genetic modification or protein interference will allow testing of the necessity and sufficiency of predicted clock proteins to the circadian molecular clock in cnidarians.

Decoding the cnidarian circadian clock is an important piece of understanding the evolutionary origin of fundamental mechanisms that coordinate physiological rhythms to environmental cues and improve survival and reproductive fitness. In turn, the cnidarian circadian clock is essential to understanding the basic metabolic processes among of these animals and may prove useful to their future conservation.

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