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Biological clocks and rhythms in intertidal crustaceans

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1. ABSTRACT

Animals with habitats within the intertidal zone are exposed to environmental cycles that include the ebb and flow of tidal waters, changes in tidal levels associated with the lunar month, the light-dark cycle and the alternation of seasons. This intricate temporal environment results in the selection of biological timing systems with endogenous clocks that can oscillate with this wide range of periodicities. Whereas great progress has been made in our understanding of the molecular and neural bases of circadian rhythms, that is, endogenous rhythms synchronized to the solar day, there is little understanding on how circatidal rhythms, namely endogenous rhythms synchronized to tides, are generated. Intertidal crustaceans have been a pivotal group for the demonstration of the endogenous nature of circatidal rhythms and their mechanisms of entrainment. We review here some of the classic work using intertidal crustaceans to unmask basic properties of circatidal systems, as well as work from our laboratory that aims to identify putative chemical signals that could be involved in the circatidal systems of decapod crustaceans.

2. THE TEMPORAL ENVIRONMENT OF THE INTERTIDAL ZONE

Living systems have evolved under the influence of environmental cycles that result from the movement of the earth and moon and from their relative position to each other and the sun. The alternations of day and night, and of seasons, affect most living species, including humans. Intertidal organisms, on the other hand, are not only subjected to the solar day and earth year but their habitat is under the cyclic ebb and flow of tidal waters that results from the gravitational pull of the moon and the sun. The 24.8-h lunar day results from the combined rotation of our planet and the moon's orbit around it. The gravitational pull of the moon induces high tides on the point immediately below it, as well as in a point diametrically opposed to the moon, leading to 12.4-h tides. Because the moon's orbit is not usually on the equatorial plane, the 12.4-h tides can be highly asymmetrical, leading to so-called diurnal tides, namely tides that show one predominant high tide and one predominant low tide every 24.8 h. The degree of semidiurnality (12.4 h) or 'diurnality' (24.8 h) of the tidal cycle depends on how far

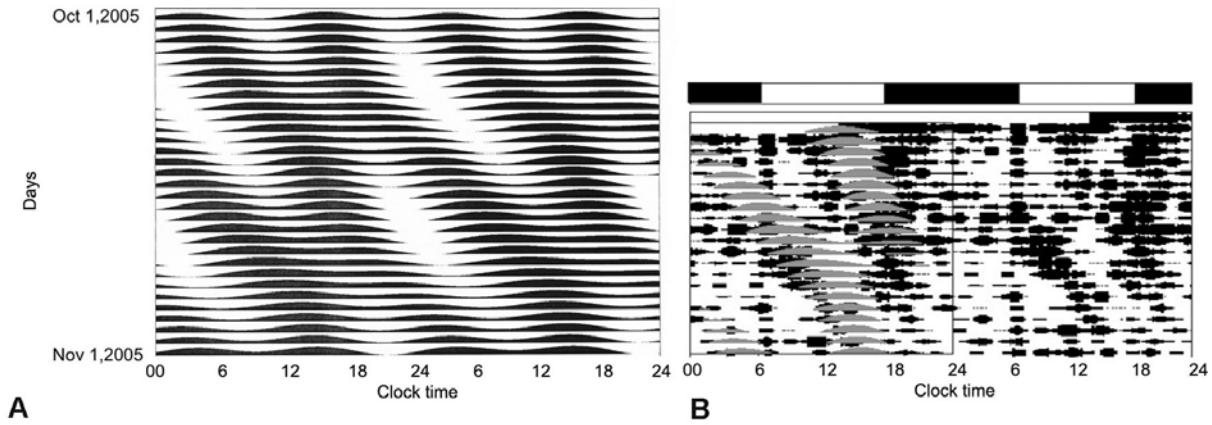

Figure 1. Circatidal locomotor activity rhythm in the crab *Hemigrapsus nudus*. A: Double-plotted tide levels on successive days (vertically stacked) at a beach in Seattle, WA, where *H. nudus* crabs were collected. B: Locomotor activity rhythm in a single crab collected from the site, displayed as a double-plotted actogram. Spontaneous locomotor activity recorded with a time-lapsed camera system is expressed as 60-min moving averages of arbitrary locomotor activity units per minute. Laboratory conditions were 10ºC, a constant water level of 10 cm and an LD cycle (horizontal black and white bars on top) of 11 h of light (approximately 300 lux) and 13 h of dark (dim red light of 0.5 to 1 lux) matching the LD cycle at the site of collection. The gray bands on the left 24-h block represent the tides at the site of collection of each of the species until November 3, 2005.

from the equator —where tides are typically more semidiurnal— the coast is, as well as other geographical factors. Of note, the term diurnality in the context of tides is used to refer to tides that have a total semidiurnal inequality, that is one maximally high tide and one maximally low tide per 24.8-h period. It does not refer to the pattern of activity of organisms that are active during the light phase of the light-dark (LD) cycle. We will use the term semidiurnal inequality to avoid confusion.

The relative position of the moon changes across the lunar month between phases in which it is aligned with the earth-sun axis and phases in which it is perpendicular to it. The sun also exerts gravitational pull on the earth waters. When the moon and sun are aligned on the same axis, the additive effect of their gravitational pulls leads to *spring tides*, namely high tides that are maximally high and low tides that are maximally low. Thus, gravitational pull in relation to the lunar day and lunar month generates temporal tidal patterns of remarkable complexity. An example of the tidal levels in the coast of Seattle, WA, is shown in Figure 1A.

3. BIOLOGICAL TIMING SYSTEMS IN INTERTIDAL ANIMALS

After a quick glance at Figure 1A it may be hard to conceive a biological timing system that could predict these cyclic changes. Nevertheless, if the 24-h LD cycle has exerted strong selective pressure for the existence of circadian clocks, one would imagine that an environmental cycle that forces animals to switch between water and land environments should have been equally powerful in selecting for biological clocks that anticipate these changes. Indeed, the existence of circatidal rhythms, or biological oscillations with periods close to the tidal period and synchronized (*entrained*) by tides, has been described several decades ago (reviewed in (1-3)). Figure 1B shows an example of a male purple shore crab (*Hemigrapsus nudus*) caught in the coasts of Seattle and housed under a 11:13 LD cycle under constant tidal conditions. Despite the fact that this animal had no cues about the ebb and flow of water present in its home environment during captivity, it could clearly predict the time at which the high (daytime) tide would occur, as it is indicated by its increased locomotor activity.

The presence of rhythms in the absence of cyclic environmental cues is taken as proof of a biological clock, an endogenous biological mechanism that can oscillate autonomously and sustain specific physiological and behavioral rhythmic outputs. Crustaceans have represented a pivotal group for proving the existence of circatidal clocks. In several species including decapods and isopods, both the endogenous (*free-running*) nature of these clocks and their ability to be entrained by environmental cycles have been established. Interestingly, locomotor activity rhythms have been described in crustaceans that are active swimmers during the high tides, such as the crab *Carcinus maenas* (4) and the isopod *Eurydicce pulchra* (3), as well as in animals that are active foraging and mating in the land environment offered by low tides, such as the fiddler crab *Uca pugnax (*1). In some cases, as in *C. maenas*, this circatidal rhythmicity is modulated by the LD cycle through a circadian clock, leading to a semidiurnal (~12.4-h) circatidal rhythm with a higher locomotor activity peak during the night (3, 4). Thus, biological timing systems in intertidal species can generate endogenous rhythms that are as complex as their temporal environment. This has been clearly shown by a study in fiddler crabs of the species *U. princeps* (5). Specimens collected from habitats with different tidal patterns along the Pacific coast of Mexico had locomotor activity patterns that strikingly mirrored the tidal patterns of their home environment, even when these

patterns range dramatically in their tidal semidiurnality and semidiurnal inequality.

The endogenous nature of biological rhythms in intertidal organisms is not limited to those in association with diurnal and semidiurnal tides. Rhythms in synchrony with lunar-monthly spring tides (approximately every 28 days) or with lunar-bimonthly spring tides (approximately every 14 days) have been shown in several species from annelids to insects and crustaceans (reviewed in (1)). The classic work by Neuman on the midge *Clunio marinus*, an insect whose larvae live in sea water, showed that the rhythm of larval emergence is synchronized to the spring tides, which optimizes the survival probability of the larvae. His work not only showed the endogenous nature of this rhythm but also the ability of simulated full-moon light to synchronize this rhythm. Other studies have reported endogenous rhythms of locomotor activity, egg maintenance and larval release in crustaceans, which typically optimize the release of larvae into the sea waters ((6) and see review in (2)). Molting in the crab *C. maenas* shows an endogenous circatidal rhythm (with a semidiurnal period), which is in turn modulated with a lunar-bimonthly periodicity that is apparently endogenous as well (7). This dual regulation leads to molting during maximally high tides instead of during low tides when dehydration could represent a major challenge.

Taken together, the presence of diverse endogenous biological rhythms synchronized to semidiurnal and unequal semidiurnal tides, as well as to lunar-month associated tides, clearly shows that the complexity of the temporal environment of the intertidal zone is no rival to the intricacy that biological timing systems can achieve.

4. ENTRAINMENT OF CIRCATIDAL RHYTHMS

A key property of 'circa-rhythms' is their ability to be synchronized to environmental cycles (8). Whereas the LD cycle is clearly the most pervasive signal for the entrainment of circadian clocks, entrainment of circatidal rhythms may rely on different and multiple cycling environmental cues that result from the ebb and flow of water. Among these, the most apparent are periodic inundation and changes in salinity, hydrostatic pressure, water turbulence, temperature and food availability. Simulated cycles of these variables have been successful in the entrainment of circatidal rhythms in different crustaceans. *C. maenas* appears to use several cyclic cues to entrain its locomotor activity rhythms, as cycles in temperature (9), hydrostatic pressure (reviewed in (2)) and salinity (10) are all successful in entraining circatidal rhythms. Similarly, cycles of water turbulence (11) and hydrostatic pressure (12) can entrain circatidal rhythms in *E. pulchra*. The locomotor activity rhythm of the horseshoe "crab" *Limulus polyphemus* (a non-crustacean arthropod related to spiders) can be synchronized by water depth oscillations (13).

Entrainment of circadian rhythms is achieved in part through daily light-induced phase shifts of the circadian clock that drives overt rhythmicity. The effects of light are such that the direction (advance or delay) and magnitude of the shifts depend on the phase at which light stimulates the circadian clock. This circadian phase dependence of the effects of light is portrayed by the phase response curve (PRC), which depicts the phase shifts achieved by light pulses as a function of circadian phase (14, 15). Several attempts have been made to derive crustacean circatidal PRCs. Of these, the PRC of the locomotor activity rhythm to 3-h cold pulses (from 15 to 10ºC) in the crab *Hemigrapsus edwardsi* (16) and to 2-h shaking pulses in the isopod *Excirolana chiltoni* (17) show both phase dependent advances and delays. Although circatidal PRCs are typically not as precise and reproducible among species as circadian PRCs are, this may be related to the fact that locomotor activity rhythms recorded in most crustaceans show lower phase stability from cycle to cycle. Accordingly, a recent study in intertidal crickets shows a circatidal PRC to inundation pulses with well-defined delays and advances similar to those of circadian PRCs (18).

5. NEURAL SUBSTRATES OF CIRCATIDAL RHYTHMS

Biological timing systems have three basic components: a clock or pacemaker that can oscillate autonomously, input pathways through which cyclic environmental factors can entrain the pacemaker, and output pathways through which the pacemaker can sustain physiological and behavioral rhythms. Circadian pacemakers have been identified within discrete neuroanatomical sites in several animals and in some cases show a striking localization of function, as it is the case in the mammalian suprachiasmatic nucleus (SCN) (19) and the lateral neurons of *Drosophila* (20). However, there has been limited success in identifying the neuroanatomical sites of pacemakers that generate circadian and, in particular, circatidal rhythmicity in crustaceans.

Decapod crustaceans have a distributed nervous system characterized by several ganglia and primary neuroendocrine sites (21-23). The supraesophageal ganglion (the brain), located dorsal to the anterior digestive tract, and the optic ganglia and the X-organ-sinus gland (XO-SG) complex, located within the eyestalk optic peduncle (Figure 2A), have received most of the attention as putative sites for biological clocks. The XO-SG complex is the primary neuroendocrine site in crustaceans and together with the optic ganglia they receive afferent input from the brain. Removal of eyestalks in the crab *C. maenas* led to arrhythmic and high locomotor activity, pointing to the eyestalk as a putative site of a circatidal clock (24). This idea was further supported by the fact that eyestalk extracts taken from crabs during the low activity phase of their circatidal activity rhythm and injected into eyestalkless crabs reduced their locomotor activity. This result suggested that the XO-SG complex was the site of the clock, and a hormone released by the complex was a key chemical signal driving the locomotor activity rhythm. However, the abolition of rhythmicity by a neural lesion does not necessarily pinpoint the location of the clock and

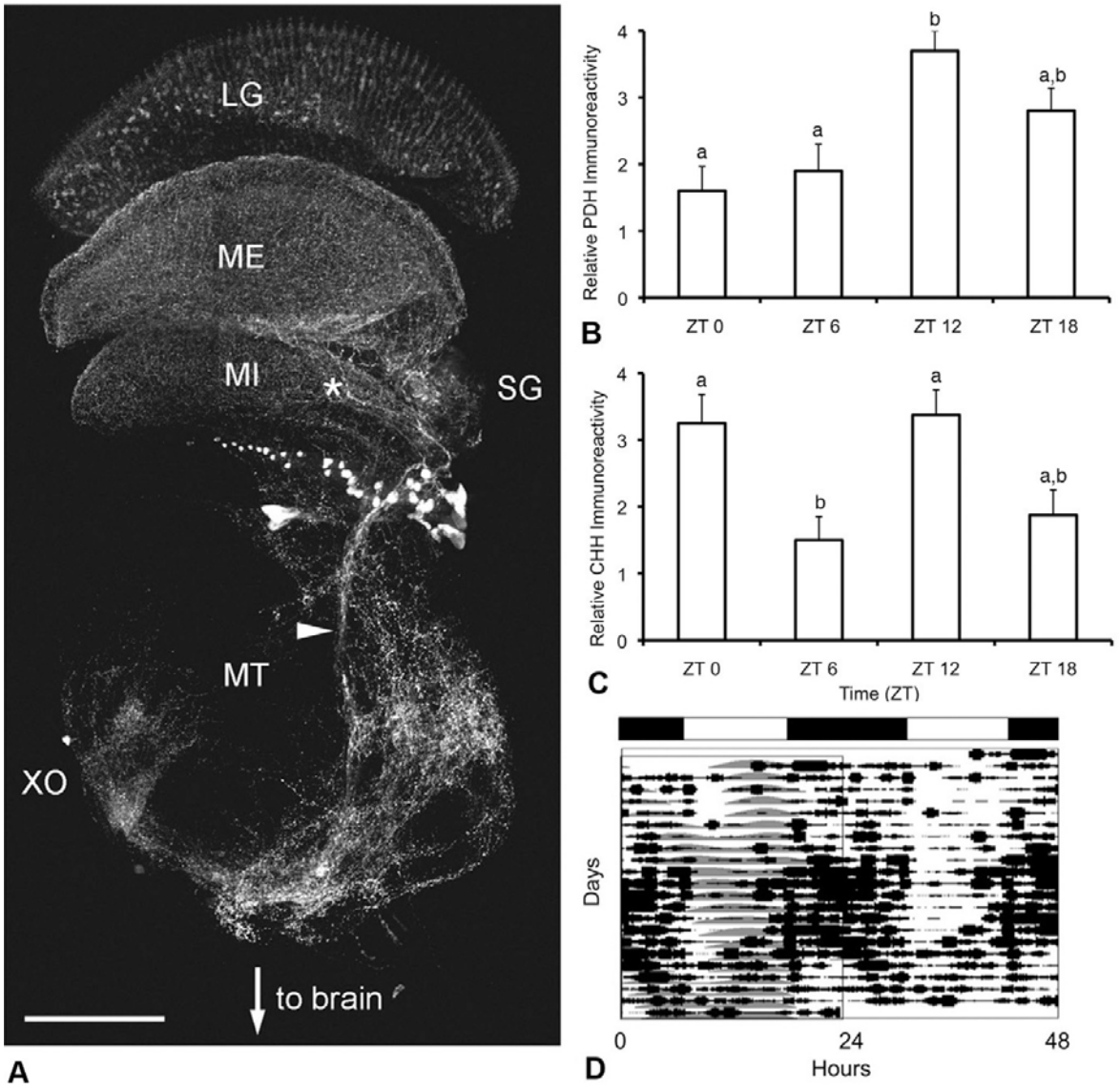

Figure 2. Putative chemical signals mediating biological rhythms in the crab *Cancer productus*. A: Beta-PDH-like immunoreactivity within the eyestalk showing cell bodies in the lamina ganglionaris (LG), the region between the medulla interna (MI) and medulla terminalis (MT), and the X-organ (XO) as well as neuropils throughout the eyestalk. The sinus gland (SG), a major neuroendocrine site in crustaceans, was also labeled. No beta-PDH-positive axons were observed to emanate from the XO, which typically provides the majority of the axonal innervation to the SG. The beta-PDH-positive axon bundle that innervated the SG appeared to originate from the MI-MT cells and projected proximally (arrowhead) to give rise to neuropils in the MT before exiting the eyestalk via the optic nerve. This axon bundle also projected distally (asterisk) and provided innervation to the MI and medulla externa (ME). Note that the antibody used labels two isoforms of beta-PDH, namely beta-PDH I and beta-PDH II. B: Beta-PDH immunoreactivity in the SG shows as daily oscillation with a peak towards dusk (1-way ANOVA, $p < 0.003$). C: CCH immunoreactivity shows a daily bimodal oscillation with peaks at dawn and dusk (1-way ANOVA, $p < 0.009$). D: Locomotor activity in a single *C. productus* crab recorded with a time-lapsed camera system. A bout of activity with a period longer than 24 h as well as peaks at dawn and dusk are observed. Image in A is modified from (47). Units in B and C represent arbitrary units from 1-4, where 4 is maximum immunoreactivity to an experimenter masked to the time of dissection of each animal; bars labeled with different letters indicate statistically significant differences (Tukey contrasts, alpha = 0.05). Lab conditions, analysis and symbols in D are as in Figure 1B.

could merely be the consequence of removing part of an output pathway that links the clock to locomotor activity centers. Indeed, later studies showed that ablating the optic tract while leaving the XO-SG complex intact led to arrhythmic behavior similar to that of eyestalkless crabs (25), suggesting that a central clock in the brain could regulate locomotor activity rhythms through the regulation of the neuroendocrine machinery of the eyestalk. Partial

ablation of the eyestalk in the estuarine crab *Sesarma haematocheir* suggested that the medulla terminalis may contain a circatidal pacemaker that regulates hatching of embryos and release of larvae in females (26), although in this case the presence of extra-eyestalk oscillators was not discarded as well.

Most physiological studies searching for the location of clocks in crustaceans have been done in the crayfish *Procambarus clarkii*. Although not an intertidal animal, the findings in this species may serve as a framework for the locations of circatidal clocks in other crustaceans. *P. clarkii* exhibits a circadian rhythm of light responsiveness. The anatomical and functional complexity of the eyestalk neural system, and its direct access to photic information, suggested that the eyestalk could be the site of a 'complete' biological timing system, with an oscillator and both entrainment and output pathways regulating retinal sensitivity. Aréchiga and Rodríguez-Sosa (27) demonstrated that a pacemaker modulating light sensitivity of retinal photoreceptors is located within the eyestalk in the crayfish *P. clarkii*, by isolating the retina and lamina ganglionaris (Figure 2A) and recording a circadian electrical response to short light pulses.

The brain of *P. clarkii* also exhibits a circadian rhythm of spontaneous electrical activity and a circadian response of electrical activity to light (28). These authors proposed that a central clock in the brain may regulate rhythmic outputs through input via the optic tract to the XO-SG complex, which in turn would release hormones that induce the dispersion of retinal pigments (see below). The presence of a clock and photic entrainment pathways within the brain was recently confirmed by a study that showed the persistence of locomotor activity circadian rhythms in two species of crayfish (*P. clarkii* and *Cherax destructor*) lacking extra-brain photoreceptors (29). These authors demonstrated that after ablation of both compound eye photoreceptors within the eyestalk and caudal photoreceptors within the terminal abdominal ganglion, the remaining brain photoreceptors were sufficient to entrain circadian rhythms of locomotor activity.

Taken together, these results indicate that the crayfish biological timing system, and likely that of intertidal crustaceans, is a multioscillatory system in which individual oscillators located in different CNS areas can drive independent outputs and be independently entrained. Whether and how this multioscillatory system serves as the foundation for the generation of circatidal rhythmicity remains unknown but, as we review below, it is conceivable that circadian and circatidal clocks share common molecular and neural pathways.

6. CHEMICAL SIGNALS UNDERLYING CIRCATIDAL RHYTHMICITY

Several neurochemical signals, particularly neuropeptides, have been identified in the crustacean CNS, and their putative physiological role has been characterized to a great extent (see review by Christie in this issue). While a few neuropeptides have been implicated in the expression of circadian rhythms in crayfish and shrimps, virtually nothing is known about the role of neuropeptides in circatidal systems. The basic roles of neural signals in any biological timing system can be (a) relaying environmental entraining signals to the clock, (b) being a constitutive component of an neuronal-network oscillator and/or (c) representing output signals from the clock that sustain rhythmic outputs. Because there is little information on signals that are essential to sustain circatidal rhythms, here we review neural signals that have been demonstrated to be important in the regulation of circadian rhythms in intertidal and non-intertidal crustacean species, as this may represent the basis for the future identification of signals underlying circatidal rhythmicity.

6.1. Pigment-Dispersing Hormone (PDH)

The pigment-dispersing hormones (PDHs) of crustaceans and the pigment-dispersing factors (PDFs) present in insects form a large family of structurally related neuropeptides (30). Several neuropeptides have been identified in the *Drosophila* circadian system (31); among them, PDF is localized within the circadian pacemaker cells of *Drosophila* (32) and is necessary for normal expression of circadian locomotor rhythms (20). Interestingly, PDF is critical as a signal that mediates communication between neurons in the circadian clock neuronal network, as well as an output signal that drives specific rhythmic outputs (33-38).

The first PDH/PDF identified was the distal retinal pigment hormone or DRPH (NSGMINSILGIPRVMTEAamide) from the eyestalk of the caridean shrimp *Pandalus borealis* (39). It was able to elicit pigment movement in the eye in response to light as well as disperse pigment in dermal chromatophores (39, 40). A second pigment-dispersing peptide (NSELINSILGLPKVMNDAamide) was identified from the eyestalk of the fiddler crab *U. pugilator* (41). DRPH was later renamed as alpha-pigment-dispersing hormone (alpha-PDH) and isoform identified in *Uca* was named beta-pigment-dispersing hormone (beta-PDH). Beta-PDH is one of the neuropeptides synthesized within the eyestalk and has been implicated in the control of rhythmic physiological processes such as the induction of melanophore and retinal pigment dispersion (42, 43).

PDH immunoreactivity in decapod crustaceans reveals a similar anatomy to that of the insect PDF system (44-47). PDH immunopositive neurons and fibers are present in the brain of the crab *C. productus* (47) and two species of crayfish (29). Interestingly, PDH neuropils emerging from these cells in the crayfish arborize in close proximity to the neuropils emerging from brain photoreceptor neurons that express cryptochrome (CRY) (29), a photoreceptor protein critical for circadian photoreception in *Drosophila* (48). As indicated above, these brain photoreceptors are sufficient to synchronize circadian locomotor activity rhythms in crayfish and cells within this region show an apparently circadian gated response to light (49). Furthermore, within PDH neurons that project to the photoreceptor neuropils, PDH immunoreactivity oscillates with a daily rhythm (29).

Taken together, these results suggest that a similar circadian network to that found in *Drosophila* is present in the crayfish brain.

Within brachyurans, *C. productus* and *C. maenas*, which exhibit circatidal locomotion (Figure 2D), (45-47)), show a similar PDH neuronal network. While several crustacean species have been found to possess multiple isoforms of beta-PDH, isoform-specific roles have only been proposed in the crabs *Callinectes sapidus* (50) and *C. productus* (47). In *C. sapidus*, data suggest that beta-PDH I and II functions as a hormone and local modulator, respectively (30, 50, 51). In contrast, based in their respective presence and absence in the sinus gland of *C. productus*, beta-PDH II appears to have a hormonal function whereas beta-PDH I could act as a locally released neurotransmitter/modulator (47).

It remains to be determined whether PDH within the eyestalk optic ganglia may serve as a circadian output/network-synchronizing signal, as PDF does in *Drosophila*, or if its sole role is that of pigment dispersion. Also unknown is whether each of these roles are served by specific PDH isoforms, particularly beta-PDH I and II, which are not expressed by the same neurons in some regions of the eyestalk (47). Using semi-quantitative beta-PDH immunolabeling in the SG of *C. productus* we found that beta-PDH exhibits a daily rhythm with a peak before the time of lights off (Figure 2B). Since mass spectrometry analysis indicated that the isoform present in the SG of *C. productus* is most likely beta-PDH II (47), it remains to be determined whether the physiological role of beta-PDH II is indeed pigment dispersion and whether the observed immunoreactivity rhythm is involved in sustaining pigment dispersion rhythms. Interestingly, an injection of PDH induced phase shifts in the circadian rhythm of electroretinogram (ERG) response in the crayfish *P. clarkii* (52), suggesting that beta-PDH in the eyestalk may not only be an endocrine mediator of rhythms of pigment dispersion but it could also serve as an endogenous entraining signal to biological clocks.

6.2. Red pigment-concentrating hormone (RPCH)

Like the identification of PDH (39), red pigment-concentrating hormone (RPCH) was first identified in the caridean shrimp *P. borealis* (53). There currently is only one known RPCH isoform and a related peptide, adipokinetic hormone (AKH), has been identified in insects, and both are involved in pigment concentration with additional functions in the perspective species (30). In the crayfish *Cherax quadricarinatus*, the mRNA levels of RPCH oscillate in a circadian fashion in the eyestalk (54). The levels of RPCH undergo a seasonal variation in the eyestalk of the crayfish *P. clarkii* (55), and it also enhances the response of ERG in a dose-dependent manner (56).

6.3. Crustacean Hyperglycemic Hormone (CHH)

Crustacean hyperglycemic hormone (CHH) is a neuropeptide that also shows a daily rhythm of secretion and circulating levels in crayfish. Its known physiological actions include the regulation of hemolymph glucose levels (for review see (57)) and the development of circadian retinal sensitivity in crayfish (58). In the crayfish *Orconectes limosus*, the levels of CHH and hemolymph glucose increase after the onset of darkness (59). In the crayfish *Astacus leptodactylus*, the glucose concentration in the hemolymph exhibits a 24-h rhythm (60) and the authors hypothesized that increased CHH release from the SG at dawn and dusk is responsible for a rhythm in blood glucose.

CHH immunoreactivity has been localized in several *Cancer* species (61, 62). Known isoforms of CHH and those predicted through post-transcriptional modifications have been identified for brachyuran species; amongst them *C. productus* is the only species with two distinct XO-SG isoforms (63). Levels of CHH immunolabeling in the SG of *C. productus* also varied in a daily bimodal pattern (Figure 2C) and mirrored a locomotor activity rhythm (Figure 2D), presumably reflecting higher glucose mobilization to sustain higher metabolism. Whether this crepuscular locomotor activity and the increased CHH immunoreactivity represent the outputs of circadian and/or circatidal clocks in this intertidal species remains to be determined. Until now, a circatidal rhythm in glucose levels has only been described in *C. maenas* (64). Although mass spectral analysis suggests the presence of both CHH isoforms in the XO-SG complex (46, 65), the functional significance of two CHH isoforms in *C. productus* is unknown.

6.4. Serotonin

Serotonin has been localized in several crustaceans using immunohistochemistry (66, 67) and it is important in the establishment and regulation of circadian retinal sensitivity. Both its levels and its receptor binding exhibit a daily rhythm in parts of the eyestalk of the crayfish *P. clarkii* (68, 69). Photosensitive neurons with serotonin receptors have also been localized in the abdominal ganglion of crayfish, and serotonin is able to induce a phase shift in their circadian rhythm of electrical activity (70). Finally, serotonin levels in the brain of the American lobsters show an endogenous circadian rhythm that can be entrained by light (71).

6.5. Melatonin

Melatonin has been identified in almost all organisms and studied extensively in mammals as an internal entraining signal for circadian clocks. Melatonin has also been found in crustaceans and its levels oscillate over a 24-h period in various invertebrates including crabs and several crayfish (72-76). Melatonin injection caused a shift in hemolymph lactate levels in *U. pugilator* and a delay in hyperglycemia in both intact and eyestalkless *U. pugilator*, suggesting a direct pathway not regulated through CHH (77). In addition to exhibiting a circadian rhythm in *P. clarkii* (75), melatonin has been shown to modulate locomotor activity, glucose and lactate levels in hemolymph (78), and the circadian response of ERG (79).

7. THE CIRCATIDAL CLOCKWORK

The presence of two biological timing systems, circadian and circatidal, within the same

species, opens a key, though yet unanswered, evolutionary question: *Could circatidal biological timing systems have evolved from preexisting ubiquitous circadian systems?* As noted above, circatidal rhythms can be strongly semidiurnal, with a ~12.4-h period, or show semidiurnal inequality, with a predominant period of ~24.8 h. Whereas a circa-24.8-h clock cannot be distinguished from a circa-24-h clock in terms of period, the presence of circa-12.4-h rhythms in several circatidal species has led to the suggestion that true circatidal clocks, with periods close to 12.4-h and essentially different from circadian clocks, may be present in intertidal organisms (4, 80). Alternatively, Palmer proposed that circa-12.4-h rhythms in intertidal organisms could be generated by the antiphase oscillations of two or more circalunidian clocks with periods close to 24.8-h, the period of the lunar day (1, 81, 82). Due to the similarity of periods between circadian and circalunidian clocks, this second hypothesis implies that the mechanisms underlying circadian and circatidal rhythmicity may be similar. The circalunidian hypothesis is evolutionary parsimonious in the sense that it does not call for a newly emerged circatidal mechanism but rather for the reenactment of a preexisting circadian mechanism into a circatidal role. A testable prediction of this hypothesis is that some of the molecular components underlying circatidal timekeeping should be common to circadian clock components. The presence of a circa-12-h rhythm of locomotor activity in the golden hamster —obviously not an intertidal organism that lives in the Syrian desert— that represents the output of two circadian oscillators (83) represents a proof of principle that the circalunidian hypothesis is biologically feasible.

Due to our poor knowledge of the mechanisms underlying circadian rhythmicity in intertidal animals and our total lack of understanding of the circatidal clockwork in any organism, these hypotheses remain untested. Nevertheless, the identification of the first clock mutants in *Drosophila (*84) and the subsequent cloning of the first clock gene *period* (85, 86) set the basis for our current knowledge of the molecular clockwork of circadian oscillators in both invertebrates and vertebrates. In all eukaryotes studied so far, the basic mechanism at the single cell level is a set of negative and positive feedback loops that involve transcription and translation of so-called clock genes, as well as post-translational modifications of their protein products (87-89). Interestingly, the commonalities between different circadian pacemakers in animals are not limited to the basic mechanism of positive and negative feedback loops but also involve clock gene homologs that are shared between species that range from mollusks to mammals (90). This remarkable evolutionary conservation of mechanistic and structural function has represented an advantage for the study of the function of newly identified putative clock components in non-model species and will hopefully lead to the discovery of molecular components of clocks underlying circatidal rhythms.

8. ACKNOWLEDGMENTS

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