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Till Roenneberg · Martha Merrow

## “What watch?... such much!”\* Complexity and evolution of circadian clocks

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**Abstract** This review uses three examples to summarise our knowledge about the complexity and the evolution of circadian systems. The first example describes the ecology of unicellular algae, which use their circadian system to optimise the daily exploitation of resources that are spatially separated. The second example looks at the role of clocks in tissues and cells within a complex organism, and the third speculates on how the circadian system may have evolved.

**Keywords** Circadian · *Gonyaulax* · Shift worker · Oscillator · Light input

### Algae’s dilemma

Each day, the unicellular marine alga *Gonyaulax polyedra* travels more than 200,000 times its size up and down the ocean. This corresponds to a human commuting 170 km twice a day. *Gonyaulax* cannot cover this distance by active swimming but probably does so by changing its buoyancy. By making this long journey, the cells exploit two different resources. During the day, the cells aggregate in swarms in the upper layer of the ocean where they absorb sunlight and take up CO<sub>2</sub> for photosynthesis. The upper layers are, however, poor in nutrients (nitrate, phosphate, etc.), which in the ocean derive from decay of dead organisms that have settled. Another nutrient-limiting factor is the high density of organisms at the surface that compete for the sparse resources. Dur-

ing the night, *Gonyaulax* cells sink to lower layers and take advantage of higher nutrient concentrations. At the same time, they produce bioluminescence. Both exogenous light and nitrogen are not only resources for *Gonyaulax*, but they can also shift the phase of its circadian system. The role of light as a true exogenous zeitgeber is unquestioned, but nitrate is, in this case, a self-selected zeitgeber. Its “cycling” depends only on the algae’s circadian behaviour leading to a time-of-day specific exposure.

Thus, an important task of the algae’s temporal programme is to optimise the exploitation of the spatially separated resources. But how does their circadian system deal with the following situation? Let us suppose the algae have spent most of the day at the top of the ocean, replenishing their energy and carbon pools. In the late afternoon, they begin their voyage towards the bottom of the ocean, which has been exceptionally calm over the past days, so that nutrients were not distributed. It is only at the end of the night, when they normally start to rise to the top, that the cells encounter a nutrient-rich region at a thermocline, a horizontal border between water layers of different temperatures. This situation puts the algae into a dilemma. Should they rise to the top towards energy and CO<sub>2</sub> without the nutrients or should they remain at the thermocline and take the risk of running out of energy? The answer to how *Gonyaulax* may optimise these “difficult decisions” in its temporal ecology lies in the complexity of its circadian system.

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\*Quotation from the classic film *Casablanca*, where two Germans show off their English language skills to the waiter Karl in Rick’s Café

### The *Gonyaulax* circadian system and light

Light reaches the *Gonyaulax* clock via at least two separate light receptors and light input pathways (Roenneberg and Deng 1997; Roenneberg and Hastings 1988; Roenneberg and Taylor 1994), none of which has as yet been identified. One of them strongly resets the phase in response to blue light. Exposure to light for 1–2 h given in the middle of the subjective night can advance the clock by 12 h and more (Johnson and Hastings 1989;

Roenneberg and Taylor 1994), while light stimuli are almost ineffective during the subjective day. In contrast, the other light input pathway responds to both red and blue light and leads to much smaller phase advances (around dawn) and phase delays (around dusk). The two light responsive systems have a certain logic in view of the circadian behaviour of these unicells. When *Gonyaulax* receives light at greater depths during the subjective night (i.e. when the clock “says” it’s night), the cells are obviously late in their upward migration. Because the longer wavelengths of light are progressively filtered out with increasing water depth, the light input pathway that advances the clock to dawn is selective for short wavelengths.

Another bioluminescent unicellular alga, *Pyrocystis lunula*, uses a different strategy to optimise its resource necessities. *Pyrocystis* does not travel up to the surface like *Gonyaulax*. Its circadian system is relatively insensitive to long wavelength light but very sensitive to short wavelengths (Colepiccolo et al. 1993). While the cells themselves do not travel, their organelles do. During the night, its chloroplasts aggregate around the nucleus, but during the day they spread throughout the cell forming a maximum surface area to exploit light for photosynthesis (Töpperwien and Hardeland 1980). The bioluminescent particles (scintillons) travel also, but 180° out of phase with the chloroplasts, being spread out during the night and retracted towards the nucleus during the day. In *Gonyaulax*, scintillons contain the enzyme luciferase, its substrate luciferin, a luciferin-binding protein (LBP) (Mittag and Hastings 1996; Morse et al. 1989), and they are recycled by production at dusk and degradation at dawn. So, different organisms clearly have developed different circadian strategies to cope with their temporal environment.

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### The *Gonyaulax* circadian system and “food”

The fact that nitrate can reset the circadian system of *Gonyaulax* had long been overlooked because it does so only when the cells have been nitrate starved (Roenneberg and Rehman 1996). When they are cultured in supplemented sea-water medium, as is usually the case in the laboratory, nitrate has no effect. However, cells in nature rarely live in conditions where they find high nitrate concentrations throughout the circadian cycle, especially not during the day. As all environmental signals that can entrain a circadian system, single exposures of starved cells to nitrate produce a phase response curve (PRC). The nitrate PRC in *Gonyaulax* is an all-delay type with the largest effects at the crack of subjective dawn. When starved cells encounter nitrate at the end of the night, their circadian system is delayed for several hours. This capacity to delay declines rapidly throughout the day. The interplay between responses of the *Gonyaulax* circadian system to light and nitrate provide a basis for the “decisions” mentioned above. When cells encounter nitrate during the first half of the night, the course of their

circadian clock remains unchanged and they travel up before dawn. When they, however, find nutrients at the end of the night, their endogenous time course can be delayed, keeping the cells at lower depths. The more nitrate they take up, the smaller becomes its effect on the progression of the clock, so that light can speed up the temporal programme which “tells” the cells to rise towards the energy source. In most cases, the effect of a zeitgeber signal is investigated per se and not in combination with other environmental signals, and, thus, we know very little about the interplay between different clock inputs. From the *Gonyaulax* system, we do know that the effect of nitrate on the clock strongly depends on light conditions and that, conversely, the circadian effects of light depend on the cell’s nitrate pools (M. Mellow, J. Rehman, and T. Roenneberg, unpublished results). Experiments over the recent years have also shown that a strong interplay exists also between the circadian clock and its input pathways (Fleissner and Fleissner 1992; Roenneberg and Mellow 2000).

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### Feedbacks over feedbacks

Phase response curves (PRCs) of circadian clocks clearly show that the strength of a signal in resetting the phase of the circadian system strongly depends on when the stimulus is given during the circadian cycle. This has been interpreted as reflecting the ups and downs of the so-called “state variables”. If light, for example, degrades a component of the system’s “core oscillator”, it can only have a strong effect when there is something to be degraded. A good example of this scenario is the light-dependent degradation of the TIMELESS protein in *Drosophila* (Hunter-Ensor et al. 1996). However, pharmacological studies in *Gonyaulax* indicate that the time-of-day specific responses of a zeitgeber signal may additionally be due to a modification of the respective input pathway by the circadian system itself (Roenneberg and Taylor 1994). It appears to be a rule that zeitgeber signals have the greatest impact on resetting the circadian phase during the times when they normally are absent in nature, i.e. at those times when the signal’s presence has to readjust a clock that runs out of synchrony with the environment. Light has little effect during the subjective day in all circadian systems and, for example, in *Gonyaulax*, nitrate has little effect during the subjective night when it is normally encountered.

Thus, the characteristics of PRCs can derive from the cycling availability of an affected state variable, from modulation of the input pathway via feedback from the clock, or from both. The blue-sensitive light input pathway in *Gonyaulax* is active only during the subjective night and is, thus, under clock control (Deng and Roenneberg 1997; Roenneberg and Deng 1997; Roenneberg and Taylor 1994). The other light input pathway responds both to red and blue light, possibly sharing pigments with the photosynthesis machinery. Pharmacological inhibition of photosynthesis affects the *Gonyaulax*

circadian system (Johnson and Hastings 1989; Roenneberg 1994), and photosynthesis is itself an output of the clock (Prezelin and Sweeney 1977; Sweeney 1986). Thus, it represents a metabolic feedback loop. The same is true for nitrate, which affects the *Gonyaulax* clock through the known nitrogen assimilation pathway (Roenneberg and Rehman 1996). Both nitrate uptake (M. Merrow and T. Roenneberg, unpublished results) and the activity and protein concentration of the first enzyme in this pathway, nitrate reductase, are under circadian control (Ramalho et al. 1995). Interestingly, the circadian control of this input pathway is restricted to the first steps of signal transduction: the activities of the two following enzymes, nitrite reductase and glutamine synthetase, are not circadian (Eisensamer 2000), and yet the nitrate signal for the clock apparently has to proceed via glutamine synthetase, because inhibition of this enzyme blocks phase shifting by nitrate (Roenneberg and Rehman 1996). It is unlikely that the “core oscillator” of the *Gonyaulax* clock resides in the first two steps of nitrogen assimilation; these results, therefore, show that input modulation and the oscillation of the state variables can be clearly distinct functions that affect the strength of the zeitgeber signal which is represented by the PRC.

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### The two circadian oscillators in *Gonyaulax*

To add to this complexity, *Gonyaulax* regulates its temporal programme with the help of at least two circadian oscillators (Roenneberg and Morse 1993) which control different aspects of metabolism and behaviour, and which respond differently to zeitgeber. When the cells aggregate in swarms at the surface during the (subjective) day, their preference for light intensities changes over the course of the circadian cycle (Eisensamer 2000). While they move away from the light (given in these experiments from the side) during the day with a maximum avoidance at midday, they move towards a light source during the subjective night. This nocturnal, photophile behaviour is so strong that the cells even expose themselves to damaging light intensities. The self-selection behaviour in light gradients is one basis for the formation of dense swarms (all cells choose the same light intensity); another is “bio-convection” (Platt 1961; Roenneberg and Hastings 1993), a phenomenon where moving particles form a dynamic localised structure that resembles a magnetic field and stabilises the daytime *Gonyaulax* swarm. These aspects of *Gonyaulax* behaviour are controlled by one circadian oscillator while bioluminescence (Roenneberg and Morse 1993) and vertical migration (Eisensamer 2000) are controlled by another. The first oscillator responds to both red and blue light while the second responds only to blue light (Morse et al. 1994). The latter can be reset by nitrate while the first is relatively insensitive to this nutrient (Roenneberg and Rehman 1996). It is almost as if the daytime activities at the ocean’s surface and the night-time activities at greater depths are controlled by separate circadian

clocks, each optimising different aspects of *Gonyaulax* ecology.

The enormous complexity within the *Gonyaulax* circadian system may be explained by the fact that all aspects of the temporal programme must be implemented within a single cell. This does not necessarily predict that the cellular clocks in higher organisms are similarly complex. Like many other functions in higher animals and plants, the cellular clock systems could be subject to specialisation. There are, however, indications that the molecular circadian system within cells of higher organisms may be more complex than a single simple (Aronson et al. 1994; Hardin et al. 1990) or interconnected (Glossop et al. 1999; Lee et al. 2000; Shearman et al. 2000) molecular feedback loop. *Neurospora crassa*, for example, retains circadian qualities, such as entrainment, in mutant strains that lack components of the putative molecular “core oscillator” (Merrow et al. 1999, 2001).

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### The jet-lagged liver

Jet-lag is due to a circadian clock that has been flown across several time zones – this is common knowledge. When chronobiologists tell someone what they do for a living, they are often asked how to adjust as quickly as possible to a new time zone or shift work, when to sleep, eat, and whether alcohol helps or hinders. We still know little about what exactly makes us feel so bad during those first days after travelling, for example, from the United States to Europe, which is the more difficult direction for re-entrainment. Is jet-lag merely caused by our entire internal timing system being out of synchrony with the local clock or different parts of our body being internally desynchronised? For many years, the suprachiasmatic nucleus (SCN) was the only circadian clock we knew of in mammals. Then experiments showed that circadian rhythmicity can be recorded in rats with their SCNs removed (Honma et al. 1987), followed by indications that the eyes of mammals may have their own clock (Remé et al. 1991), and, finally, circadian rhythmicity was shown to exist even in mammalian cell lines in culture (Balsalobre et al. 1998). The development of “circadian” reporter genes (Plautz et al. 1997) drastically expanded our knowledge about the many circadian clocks that tick throughout our body in practically every tissue and cell (Yamazaki et al. 2000), and questions about how the different clocks in our body readjust to a new time became answerable.

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### Flying rats across the Atlantic

Transmeridian flights are simulated in the laboratory by simply advancing or delaying the light:dark cycle by 6 h. These experiments had often been done by recording the shifts of locomotor activity, but, in a recent experiment the effect of zeitgeber shifts was also recorded for tissue clocks. This project produced surprising results



(Yamazaki et al. 2000). Following a delay (corresponding to a west-bound flight), the central pacemaker in the SCN shifted within one cycle. Muscle and lung took longer, comparable with the known behavioural adjustment. The liver, however, had not fully re-entrained after 6 days. In the opposite direction (the more difficult advance), the SCN again shifted within the first cycle, but the clocks in muscle and lung tissue became arrhythmic for 1 day and took the expected 6 days to fully adjust. In contrast, the liver rhythms were not disrupted during the 1st day but were fully adjusted after six cycles. In summary, the pacemaker has the least difficulties in adjusting, lung and muscle behave similarly to the re-entrainment of behaviour, while the liver does not follow the rules. It has more difficulties in adjusting to the easier, west-bound “flight” but its rhythm is not disrupted after an advance.

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### Food for liver – food for thought

It has been long known that food as well as light:dark cycles entrain behaviour (e.g. Reid and Finger 1955; Richter 1922). Recent experiments have shown that scheduled feeding can shift the phase of the liver clock by as much as 5 h/day (Damiola et al. 2000; Stokkan et al. 2001), while the circadian clock in the SCN, judged by the activity rhythm, ignores food as zeitgeber (Honma et al. 1983). Thus, the SCN and the liver clock respond specifically to different zeitgebers. The former is strongly shifted by light while the latter responds very slowly. The liver is strongly shifted by food, while the SCN ignores it. The signal transduction pathways of the phase shifting effects of food on the liver are not yet well understood. It has been shown that glucocorticoids are potent endogenous zeitgebers for the liver and other tissue clocks (Balsalobre et al. 2000), but this hormone is unlikely to mediate food entrainment in the liver. Although dexamethasone does shift the liver clock, it has no effect at the time when food restriction causes a rise in corticosterone (Stokkan et al. 2001). In addition, experiments with mutant mice defective in the liver-specific glucocorticoid receptors indicate that glucocorticoid-signalling counteracts rather than facilitates food entrainment (Damiola et al. 2000).

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### The shift worker's liver

These new insights open a host of questions. The liver is the largest gland in the body and affects almost all physiological functions. Among other tasks, the liver provides mechanisms of immunity. Hepatocytes serve general metabolism by controlling synthesis and utilisation of carbohydrates, lipids and proteins, and, in addition, serve a major function in digesting food.

The example of the circadian system of the unicell *Gonyaulax* has shown the strong interactions between light and “food” as zeitgebers, in addition to feedbacks

between the clock and metabolism within a single cell. Surely, a similar or even higher complexity can be expected of the temporal programmes in mammals. The necessity for *Gonyaulax* to house two circadian clocks within its single cell has been explained by the relative independence of food and light. While dawn and dusk are extremely predictable, the optimal food source may be available in the morning at certain times of the year and in the afternoon at others. The fact that the liver clock can be entrained independently from that in the SCN indicates that such a multi-oscillator system also controls the temporal programme of higher animals.

But, how does the temporal programme within the liver or within single hepatocytes cope with the difficulties of changing food availability or with readjustment to new time zones or shift schedules? On one hand, the liver controls the body's energy demand, which strongly depends on motor activity. On the other hand, it optimises the digestion of food. Activity and food uptake are normally in synchrony, but in the experiments described above food was provided to nocturnal rats during the day (Damiola et al. 2000; Stokkan et al. 2001). Although they anticipate the scheduled meal times with an activity bout (Richter 1922), they continue to show an SCN-controlled activity at night, though somewhat reduced. It will be interesting to investigate how the different liver functions respond in this drastically altered temporal structure. Is the reduced nocturnal activity due to internal desynchronisation, where the liver is shifted to the day and no longer provides sufficient energy at night? Have the hepatic immune functions also shifted to the day? Further questions concern the specific entrainability of peripheral clocks. The liver clock can be shifted by food. Is the kidney clock shifted by scheduled drinking or is the muscle clock shifted by activity? The latter is conceivable because activity is known to entrain motor activity separately from the SCN (Mrosovsky and Janik 1993).

Shift workers are in a situation similar to that described above for rats. In most cases, their SCN remains entrained to the natural light:dark cycle (probably because the illumination at work cannot compete with that outside). They eat when they normally would not, and, once off work, aside from sleep they have 8 h left to be additionally active in their normal time. The possibility of examining the responses of the individual body clocks to potential zeitgebers will be of great importance for understanding potential health impairments of shift-work and jet-lag. The results will impact on how night shifts are scheduled. Many shift work rotations follow the sequence of 2–3 days on each of the following schedules: late, night, early and off work. According to the results described above, some body clocks, like the liver, would be constantly shifted while others remain entrained to the natural cycle. This creates two sources of physiological stress: internal desynchrony and constant shifting. Experiments with flies have shown that the latter shortens life expectancy by over 20% (Aschoff et al. 1971).

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## Why have a clock in the first place?

The difficulties of shift workers suggest that modern man would be better off without a clock. The value provided by a circadian system for the survival of an individual or the fitness of a species is in fact difficult to prove. The strongest indication that clocks increase fitness came from work with cyanobacteria (Yan et al. 1998). Mutants with different endogenous periods competed for growth in different light:dark cycles. In each case, those mutants which had periods closest to the zeitgeber period outlived their competitors. A conclusion would be that a wild-type circadian system optimally tunes physiology to the 24-h day. Circadian systems, which continue to oscillate in constant conditions, are entrained by zeitgebers rather than driven by them. They establish a specific phase relationship to the zeitgeber cycle, which depends on period and amplitude of both the zeitgeber and the endogenous oscillator. Circadian systems provide the possibility of anticipation, preparing physiology before the external changes occur. An organism that is simply driven by the external changes is thought to be disadvantageous relative to one that is regulated by an endogenous clock with flexible and anticipatory qualities (Beaver et al. 2002; Daan 1981).

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## Why self-sustainment?

The self-sustained oscillation in constant conditions is the most conspicuous quality of circadian systems, but is this quality really important in view of evolution predominantly proceeding in light and temperature cycles? Under zeitgeber conditions, damped oscillators could show the same qualities as self-sustained systems. Thus, we either have not yet fully understood the multiple advantages of circadian systems, or self-sustainment is a consequence of how and why the system evolved rather than being the object of the evolutionary process (Roenneberg and Merrow 2001). We have recently taken a different view based on the assumption that parts of the circadian system have evolved prior to self-sustainment (Roenneberg and Merrow 2002). We investigated this possibility by computer modelling a network consisting of dampened oscillators with periods in the ultradian range. As a whole, this network is arrhythmic in constant conditions, but it also has difficulties to synchronise with a zeitgeber, although the single oscillators have no difficulties in synchronising individually.

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## Before circadian – BC

It is thought that the circadian system represents a phylogenetic “improvement” over purely driven systems. As our theoretical model shows, however, complex networks of metabolism which must have existed in the most primitive organisms may not be simply drivable by zeitgeber cycles. With increasing metabolic complexity,

they may have had to develop mechanisms to counteract chaotic responses of the complex network to cyclic environmental signals. If one presumes that such a mechanism is necessary for reliable synchronisation with the environment, organisms lacking this mechanism would have difficulties surviving in a periodic world. So, the mechanisms on which the circadian clock was built might be those that allowed physiology to be driven in spite of complexity.

Negative feedback is one of the most common control mechanisms within pathways at all levels; metabolism must, therefore, involve many negative feedback loops. The *Gonyaulax* circadian system shows that numerous metabolic feedbacks are involved in their daily programme. The experiments of entraining the liver clock by food indicate that metabolism also plays a role in the mammalian clock. It has recently been shown that basic cellular features, such as redox, affect the function of transcription factors which are believed to be involved in the generation of circadian rhythmicity in mammals (Merrow and Roenneberg 2001; Rutter et al. 2001). It has been proposed that the current transcriptional-translational feedbacks, although an essential part of the circadian system, may only be a subset of the entire mechanism and that one of its functions is to process zeitgeber signals on a circadian basis (Roenneberg and Merrow 1998, 1999).

One of the oscillators in the network was assigned the function to process zeitgeber signals. Because all oscillators are connected, it transduced these signals to the entire network. It is known that the clock feeds back on its own input pathways; we, therefore, investigated entrainment of the network under conditions where the input oscillator is modulated by the integral output of the entire network. This is comparable to the cell’s redox potential, which is an integral product of many metabolic functions. This input feedback creates a zeitnehmer (time taker, Roenneberg et al. 1998), an endogenous function that modulates the effects of a zeitgeber (time giver). Under these conditions, the network shows stable entrainment by the same zeitgeber that is unable to entrain the zeitnehmer-less network. The results of this theoretical modelling show that driven systems may contain special functions that enable their (metabolic) network to be synchronised to environmental cycles.

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## The gentle slope from driven to entrained

Evolution proceeds in small steps (Dawkins 1997); it is, therefore, reasonable to presume that some components of the circadian machinery have already existed before a self-sustained circadian system evolved. The example described above proposes that these may have played a role in ensuring synchronisation of a complex cellular biochemistry. Further results of the described model indicate that the small step turning a driven system into a circadian system may have been based on small changes in the multi-oscillator network. When the strength by

which individual oscillators influence each other is increased, the rhythm of the system becomes self-sustained in the circadian range and it shows all the known properties of entrainment (anticipation, transients, and specific phase angles with the zeitgeber).

Under these conditions, the network behaves like the circadian clocks investigated in so many different organisms. Yet, a core oscillator in the form of one negative feedback does not exist. The multi-oscillatory network shows great flexibility. Small changes in the connectivity, for example, can drastically change the entrained phase angle, a feature that exists in nature but can as yet not be explained on the basis of the single molecular loop. Day active migratory birds, for example, which travel long distances at night, change their activity phase at the appropriate times of the year (Gwinner 1996), and humans, which are larks both as a child and as an adult, tend to be owls during adolescence (Carskadon et al. 1998). The model also encourages experiments in organisms that are arrhythmic in constant conditions. Mutagenesis and appropriate screens could be performed to identify those components that enable reliable synchronization, and these should also play an important circadian role in organisms that have developed a daily clock.

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