

Circadian Rhythms

1. From Daily Rhythms to Biological Clocks

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Living organisms ranging from bacteria to human beings exhibit 24-h rhythms in various behaviours and physiological processes. Matching of the period of such rhythms with that of the daily environmental cycles gives an impression that they are mere passive responses to environmental changes. Here, we discuss the change in our perception about daily biological rhythms, from their identity as rhythmic phenomena occurring as passive response to environmental changes, to the biological clocks. This is the first article in a series of articles on circadian rhythms which will cover discussion on the genetic and molecular basis, circadian organization and its adaptive significance.

We all experience robust cycles of light and darkness, occurring as a consequence of continuous rotation of the earth about its axis and we call such a twenty-four hour cycle, 'a day'. Almost all living beings are exposed to such daily environmental cycles and they too exhibit daily rhythms in various biological processes. For example, most of us are awake during the daytime and asleep during night; leaves of many plants are open during the day and are closed at night (*Box 1*). Daily rhythms of such behaviours thus appear to be simple responses of living beings to cyclic presence/absence of environmental stimuli. However, studies on daily rhythms in a range of living organisms over the last 250 years, have made it amply clear that they are not as simple as they appear.

Daily Rhythms: Are they Simple Responses to Cyclic Environmental Stimuli?

The French astronomer Jacques d'Ortous de Mairan (1729) could be credited with one of the earliest attempts to test whether daily

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Box 1. Plant Leaf Movement Rhythms.

Although daily rhythms of leaf movement are quite conspicuous, they can be more informative in the form of graphical representations. For example, during leaf movement rhythms, leaves alternate between open and closed states (*Figure A*). If we arbitrarily assign a value of 0 to the closed state and 1 to the open state of leaves, a graph of values representing leaf state on *y*-axis and time on *x*-axis helps visualise such time-dependent changes in the state of leaves (open/close) and enhance the perception of leaf movement rhythms. It is common practice to plot such graphs for each day (24 h time scale) and to stack graphs of consecutive days one below the other such that the uppermost graph represents the first day and the lowermost one represents the last day of observations (*Figure B*). Each graph is plotted with 24 h scale and therefore, if opening and closing of leaves occurs at the same time every day (i.e., period of rhythm is 24 h), then the pattern would look exactly vertical (*Figure B*).

If the period of the rhythm is less than 24 h, leaf opening and closing time would occur earlier than the previous day and the open leaf state would appear drifting towards the left (*Figure C*). On the other hand, if the period is longer than 24 h, leaf opening and closing would occur later than the previous day, leading to a pattern of open leaf state slanting towards the right (*Figure D*).

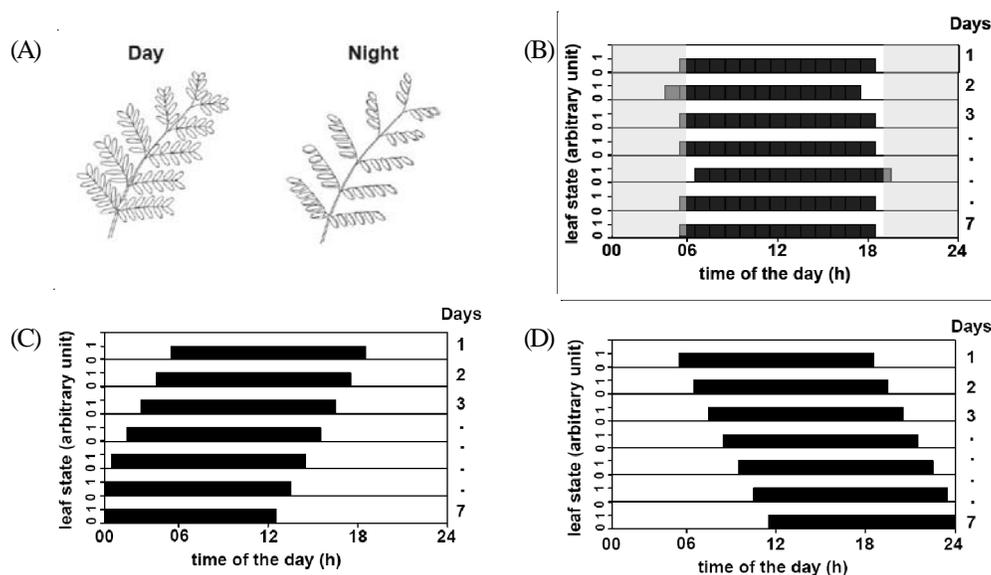


Figure (A). Open and closed states of leaves. **(B)** Graphical representation of leaf movement rhythms under laboratory light/dark (LD) cycles. The *y*-axis represents leaf state (open (0)/ closed (1)) and *x*-axis represents the time of the day. Black portions in graph indicate open leaf state and empty spaces indicate closed state. Period of the rhythm is 24 h. **(C and D)** Leaf movement rhythm under constant light (LL) when period of the rhythm is less than 24 h **(C)** and more than 24 h **(D)**.



Daily rhythms persist under constant environmental conditions which indicate that they are endogenous.

rhythms are really simple responses of living beings to cyclic environmental stimuli, through his observations of the daily rhythms of leaf movements in the common touch-me-not plant *Mimosa*. To test whether the daytime opening and night-time closing of *Mimosa* leaves were responses of plant to daily cycles of light and dark, he placed the plant in a dark cupboard only to find that although plants were deprived of external light/dark cycles, its leaves continued to remain open when it was day outside and closed when it was night. Although de Mairan's experiment showed that plants did not require light/dark cycles to continue their leaf movement rhythm, it left the possibility open that persistence of the rhythm could have been the result of response of plants to environmental variables other than light such as temperature and humidity, which also oscillate with a 24 h period. In 1758, Duhamel showed the persistence of daily rhythms of leaf movement in a cave where apart from constant darkness, temperature and probably humidity also remained constant.

Although such experiments ruled out the possibility of daily rhythms being a response to cyclic changes in light and temperature, many other known/unknown environmental variables associated with the earth's rotation were considered as possible driving forces of daily rhythms for quite a long time. In the early twentieth century, many studies tested the role of environmental factors such as gravitational field which oscillates with a 24 h period by systematically nullifying their effects [1]. However, evidence came from a different line of experiments during the 1920s to indicate that the cause of daily rhythms is not external and that they are in fact generated from within the organism. Erwin Bünning¹, a German botanist of the early twentieth century, observed that plants kept under constant light were also able to maintain rhythmic leaf opening and closing, however, with a period clearly different from 24 h (*Box 1, Figures C and D*). This led him to argue that if these rhythms were in fact driven by environmental factors correlated with the Earth's rotation, they could not possibly assume non-24 h periods. He went on to

¹ See *Resonance*, Vol.1, No.7, 1996.



propose that these rhythms are generated from within the plant. Eventually, several studies established the persistence of rhythms with periods deviant from 24 h in many behaviours such as activity/rest in mammals, and adult emergence in insects, under constant environmental conditions (i.e., under conditions of constant light, temperature, and humidity). Rhythms persisting under constant conditions with near 24 h period are referred to as *free-running rhythms* and their period as the *free-running period*. Free-running rhythms thus became acceptable evidence for endogenous generation of daily rhythms.

One caveat with the demonstration of the endogenous nature of daily rhythms is that it does not indicate whether rhythms are an inherent property of living beings, since, in principle, rhythmic pattern of behaviours could be a result of learning to be rhythmic under rhythmic environments during previous stages of that organism's life. However, experiments showing persistence of rhythms with a near 24 h period in organisms kept under constant environmental conditions for several generations clearly demonstrated that prior exposure to rhythmic environments is not essential for the initiation of rhythms. These observations thus ruled out the possibility of rhythms being learned phenomena and consequently, daily rhythms were accepted as an inborn/innate property of living systems.

Only a subset of behavioural and physiological processes is innate and endogenously generated [2, 3]. Innate and endogenously generated daily rhythms are termed as '*circadian rhythms*' to distinguish them from those which occur as mere passive responses of organisms to cyclic environments, after the Latin word *circadian* meaning 'about a day' (*circa* – about, *diem* or *dies* – day).

So how do these non-24 h rhythms manage to attain 24 h periods under cyclic environments? The proposed mechanism is *entrainment*, basically the synchronization of one oscillation to the other, in this case synchronization of the endogenously generated biological rhythm to 24 h environmental cycles [4–6]. The process

Endogenous near 24 h rhythms are an innate/inborn property of the living beings.



of entrainment is characterised by (1) matching of period of the endogenous rhythm with that of environmental cycles, and (2) attainment of specific time difference (phase-relationship) between the phase of the endogenous rhythm and that of environmental cycles. Evidence from several studies showed that endogenous rhythms entrain to cycles of light and temperature and thereby schedule occurrence of biological processes to specific times of the day. Cycles of light, temperature and of other environmental factors, which serve as synchronizing agents to schedule cyclic biological events at specific time of the environmental cycles were named as *zeitgebers* after the German words *zeit* (time) and *geber* (giver).

From Endogenous Rhythms to Biological Clocks

The historical account of studies on daily rhythms suggests that the early studies were primarily aimed at understanding the causes and properties of these rhythms without any reference to their possible role as biological timekeepers as we recognize them today. However, some remarkable discoveries during the 1950s changed the way circadian rhythms were being viewed. Interestingly, the scientists behind the discoveries which triggered such change in the course of research on circadian rhythms were in fact trying to decipher how animals navigate while foraging or during long distance migrations, and were not studying daily rhythms *per se*.

A variety of animals use the Sun's position relative to terrestrial landmarks as a compass to navigate their path. Although, the Sun serves as a reliable reference point, we know that its position relative to terrestrial landmarks changes with time of the day (at an average rate of 15° per hour). Therefore, use of the Sun as reference would require appropriate corrections depending on the time of the day at which directional information is being used.

Gustav Kramer, a well-known German zoologist and ornithologist of his time, in an elegant experiment (*Box 2*) to study the navigational ability of birds, demonstrated that birds (starlings), use the Sun as reference for navigation, and are also able to find

Box 2. Gustav Kramer's Experimental Set-up to Study Bird Navigation

The set-up consisted of a bird kept in a circular enclosure with 12 feeders placed equidistantly along the circumference of the enclosure (*Figure A*). One artificial light source was fixed at a specific position along the circumference in the enclosure which mimicked the Sun. Individual birds were trained to obtain a food reward in feeders located at a definite angle relative to the position of the artificial Sun. During the test sessions, all the feeders were kept empty and the feeder preferred by the bird indicated the direction it had chosen in anticipation of food.

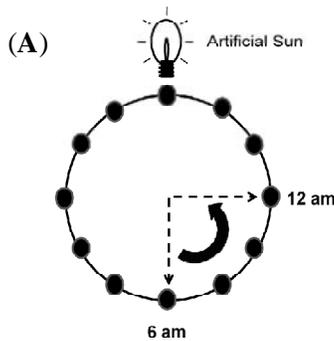
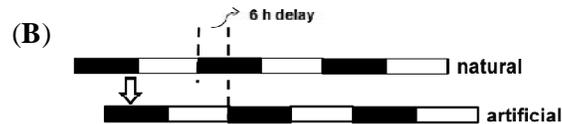
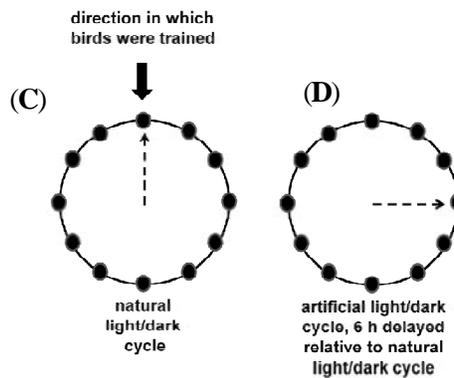


Figure (A). Change in the direction relative to the artificial Sun, preferred by birds in anticipation of food, in test sessions at different times of the day. The big circle represents circumference of the enclosure and solid circles on circumference are feeders. Dashed arrows indicate direction chosen by the birds in anticipation of food in test sessions.



Hoffman's clock shift experiment. (B) Shifting of birds' endogenous clocks by entraining them to artificial light/dark (LD) cycle, 6 h delayed relative to natural LD cycle. (C) Direction chosen by the birds when tested under natural LD cycles. (D) Direction chosen by the birds when tested under artificial LD cycles. Dashed arrows indicate direction chosen by the birds in anticipation of food in test sessions.



the direction correctly despite the continuous changing position of the Sun during the course of the day. In the initial set of experiments, birds were exposed to natural sunlight and hence also to daily changes in the position of Sun. Birds trained to obtain food from a specific feeder position relative to the Sun, at a specific time of the day, continued to show their preference for the same feeder position even at other times of the day, indicating the presence of mechanisms compensating for change in the



position of the Sun and therefore the passage of time. Further experiments using a fixed artificial Sun revealed systematic change in the direction birds chose in anticipation of food with the progression of time of the day, which provided even more compelling evidence for the time compensation mechanisms (*Box 2, Figure A*). Around the same time Karl von Frisch, an Austrian ethologist who received the Nobel Prize in Physiology or Medicine in 1973, along with Nikolaas Tinbergen and Konrad Lorenz, independently found the existence of such time-compensated Sun compass in honey bees [1,7].

The observations that birds are able to compensate for the passage of time or the change in Sun's position, clearly suggest the presence of mechanisms by which birds are able to judge local time. Two processes were proposed as possible mechanisms of time compensation. One of the propositions was that birds probably learn to associate diurnal change in local environment with the direction of food, whereas the other possibility considered endogenous daily rhythms as potential time measuring systems. Interestingly, in further experiments, when birds were deprived of diurnal environmental changes, including the Sun's movement, they were able to anticipate food in the right direction when tested with an artificially simulated Sun, strengthening the view that endogenous rhythms serve to measure time.

The ability of endogenous diurnal rhythms to synchronize with light/dark cycles offered a great opportunity to test their role as mechanisms underlying estimation of passage of time, by examining the effect of shifting of endogenous rhythms in the direction chosen by the bird in anticipation of food. In a brilliantly conducted experiment, birds were first trained to anticipate food in a particular direction under natural light/dark cycles and then the phase of their endogenous rhythms were shifted by synchronizing them to artificial light/dark cycles where the day began and ended with a 6 h delay relative to natural light/dark cycles (*Box 2, Figure B*). When birds were tested for the direction they chose, in anticipation of food under artificial light/dark cycles (which was 6 h phase-delayed relative to natural light/dark



cycles), they showed 90° change in the preferred direction as expected from 90° shift in the phase of their endogenous rhythm (6 h = 90° phase shift) (*Box 2, Figures C and D*), clearly suggesting involvement of endogenous diurnal rhythms in the estimation of the passage of time, in the time-compensated Sun compass. Similar experiments in other bird species, fish, and many arthropods also showed that the direction chosen by animals in anticipation of rewards could be altered predictably by shifting the phase of their endogenous rhythms, which further confirmed the role of endogenous diurnal rhythms, or circadian rhythms as timekeepers [7]. These studies suggesting the role of circadian clocks as systems measuring passage of time inspired many to evaluate their properties as true biological timekeepers.

Upon hearing Gustav Kramer's exciting account of a probable role of endogenous diurnal rhythms in time measurement by birds during navigation, Colin S Pittendrigh², one of the pioneers in the field of chronobiology, wondered if the endogenous rhythms of similar period exhibited by plants and insects also serve the same purpose of measuring time [7]. This thought inspired Pittendrigh to further evaluate the possible role of endogenous diurnal rhythms as true biological timekeepers, a question which he eventually pursued throughout his life. Pittendrigh noticed that if endogenous rhythms were to serve as timekeepers using their 24 h period as template, the period of the rhythm should be refractory to external environmental conditions to estimate local time correctly. Temperature, an important environmental variable was seen as a factor which could potentially affect time measurement. Pittendrigh therefore set out to test his hypothesis that if environmental temperature was to serve as a reliable biological timekeeper, it should not affect the period of endogenous diurnal rhythm generating mechanisms. Since poikilotherms (organisms whose internal body temperature changes are easily affected by environment) would serve as good model systems to test such a hypothesis, Pittendrigh went on to examine the period of adult emergence rhythm exhibited by fruit flies *Drosophila pseudoobscura* (*Box 3, Figure A*). Adult emergence

² See *Resonance*, Vol.11, No.5, 2006.



Box 3. Adult Emergence Rhythm in Fruit Flies

Circadian rhythms have been studied extensively in many species of *Drosophila*, the favourite model organism. Like other holometabolus insects, *Drosophila* too, has a lifecycle consisting of four stages, namely, egg, larva, pupa, and adult (fly). Single larva hatches out from individual egg, which feeds on the available food source and develops into a pupa. Finally an adult form, a fly, emerges from a pupal case after completion of development. The event of emergence of a fly from pupal case is known as eclosion (emergence). Under laboratory LD 12:12 h cycle, *Drosophila* exhibits robust 24 h rhythm of emergence. As each individual emerge as adult (fly) only once in its lifecycle, emergence rhythm is not observable on individual flies, but only on the asynchronous population of developing individuals, where the frequency of emergence events varies rhythmically. In every cycle, emergence primarily occurs during the daytime. Highest frequency of emergence events occurs close to dawn and then decreases gradually leading to the end emergence by dusk (Figure A). Adult emergence rhythm is thus exhibited by a population of insects and not by individuals.

Incidence of emergence primarily during the light phase suggests that eclosion is under the control of environmental cues/stimuli such as light. Persistence of the rhythm under constant darkness, however, suggests that emergence rhythm is not driven directly by environmental cycles but by circadian clocks. Evidence from several studies indicates that each developing individual alternates between phases of its circadian oscillation which either allow emergence or does not, each spanning approximately 12 h. Rhythmic emergence thus occurs as a result of synchrony among circadian oscillations of individuals in a population and is thus a reflection of population of circadian oscillators in synchrony. It is easy to perceive emergence rhythm by plotting frequency of emergence against time. Figure A shows rhythmic emergence in fruit fly *Drosophila melanogaster* under laboratory LD cycles, and Figure B shows its persistence under constant darkness at constant low and high ambient temperatures. Approximately 24 h rhythm visible from inter-peak intervals (Figure B) at different temperatures suggests that the period of emergence rhythm is temperature compensated.

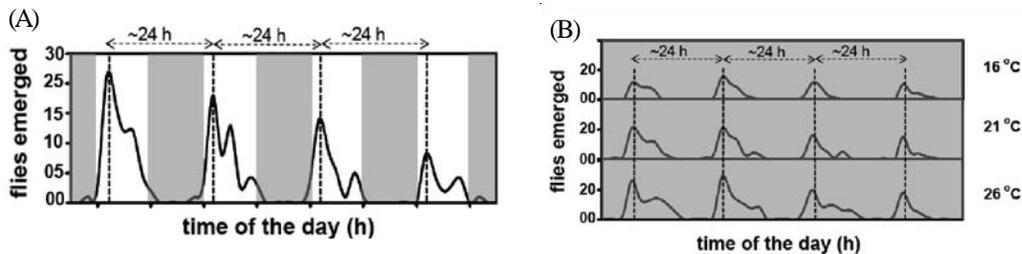


Figure (A). Adult emergence rhythm under laboratory LD cycles. **(B)** Emergence rhythm under constant dark condition at three constant ambient temperatures: 16, 21 and 26 °C (adapted from [8]). Dashed vertical lines indicate timings of emergence peak.

rhythm studies carried out under constant darkness and at two different constant temperatures of 16 and 26 °C, showed that the period of the rhythm as calculated by measuring inter-peak time



intervals was found to be only marginally longer at 16 °C than at 26 °C, suggesting that the period of endogenous diurnal rhythm of adult emergence was largely unaffected due to temperature changes within physiological limits (*Box 3, Figure B*). This property of the clock period to remain unaltered with cyclic changes in temperature is commonly known as temperature compensation [8].

Thus, evidence suggesting the role of endogenous daily rhythms as the time measuring component of time-compensated Sun compass, revealed, for the first time, the possible functional significance of endogenous daily rhythms in measuring the passage of time. Subsequent demonstration of temperature compensation of the circadian period and functioning of circadian rhythms akin to physical oscillators in organisms ranging from protists to mammals further strengthened the notion of circadian rhythms as timekeeping systems. Together, these developments changed the identity of circadian rhythms from mere endogenous rhythms of near-24 h period to biological time-measuring systems: the biological clocks.

Mechanisms of Entrainment

Study of daily rhythms under constant conditions revealed their endogenous nature. Subsequent studies showed that daily rhythms are endogenous oscillations entrained to environmental cycles, and during the 1950s and 1960s, the focus of circadian rhythm researchers moved towards understanding how cycles of light and dark entrain circadian rhythms. Entrainment of circadian rhythms by light/dark cycles has been explained with the help of two models, which primarily differ in the nature of response of endogenous rhythms to light, although both models try to explain matching of endogenous period (τ) with that of light/dark cycles (T). The model proposed by Jürgen Aschoff, one of the three founders of the field of circadian biology proposed that continuous effect of light on endogenous rhythm alters its period and matches it with that of environmental cycles. Since this model proposed alteration of an important clock parameter, the τ , it is

Daily rhythms are endogenous near-24 h rhythms synchronized to environmental cycles.



known as the parametric model of entrainment. The second model put forth by another founding figure, Colin S Pittendrigh proposed that τ of the rhythm is unchanged; however, entrainment occurs by daily instantaneous phase-resetting of circadian rhythms. For example, if τ is shorter than T then light would delay, the phase of the rhythm by an amount equal to $\tau - T$. The model can be summarised as $\Delta\phi = \tau - T$, where $\Delta\phi$ is the daily phase-shift necessary for entrainment to correct for the difference between τ and T . Although the two models propose entirely different ways of achieving entrainment, it is becoming increasingly clear that in real life a combination of both may be at work [4–6].

Conclusion

The study of daily rhythms over the past 250 years has shown that these rhythms are not passive responses of the living organisms to cyclic environmental changes; but are endogenously generated and entrained by cyclic environment. The ability of these rhythms to synchronize to environmental cycles serves to function as timekeepers and hence the mechanisms underlying these rhythms are metaphorically called as circadian clocks.

Suggested Reading:

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