

# Zeitgebers (*time cues*) for biological clocks

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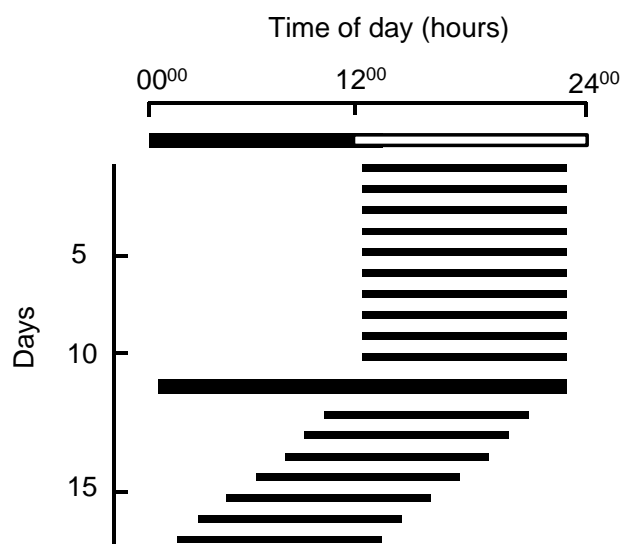
The spatial and temporal aspects of the geophysical environment act as prominent selection forces for the evolution of life on this planet. The spatial features of the environment open up a choice of spatial niches and the temporal aspects on the other hand provide opportunities for adopting different temporal niches. Hence, both the spatial and the temporal properties of the environment together enhance the possibility for living organisms to exploit a given ecological niche at a given time of the day. The temporal selection pressures of the geophysical environment are composed of a number of abiotic factors such as light/dark cycles, temperature cycles, humidity cycles, and a range of biotic factors such as inter-individual interactions, interactions with preys, predators and parasites. Although the study of temporal organization in living organisms is relatively a recent phenomenon in biology, we now have access to a fair amount of knowledge about it in a number of organisms ranging from cyanobacteria to humans. In this review, we shall focus mainly on three core questions related to timekeeping in living organisms: How are circadian clocks made to oscillate at desired frequencies?; What are the geophysical cycles that fine-tune circadian clocks?; Why are circadian clocks circadian?

**Keywords:** Entrainment, free-run, free-running period, phase-response curve, zeitgebers.

Life on earth has evolved around two key variables, space and time. The spatial features of each organism's biotic and abiotic environments are to a certain extent a familiar concept to all of us, but the temporal features remain more abstract and often go unnoticed at least by an unfamiliar observer<sup>1</sup>. It is only now becoming progressively clear that adaptation to the temporal structures of the geophysical environment is at least as important as adaptation to spatial factors, more so for organisms living in periodically fluctuating environments<sup>2</sup>.

The earth, spinning on its axis approximately once every 24 h, exposes living organisms to highly predictable daily rhythms of light and temperature. In addition, planetary movements also give rise to tidal, lunar and annual cycles, and to match these cycles living organisms have evolved tidal<sup>3,4</sup>, daily<sup>5-7</sup>, lunar<sup>8</sup>, and annual<sup>9</sup> rhythms. The most ubiquitous and hence the most extensively studied among these biological rhythms are those which manifest themselves

in daily patterns of physiology and behaviour<sup>1</sup>. Although some of these rhythmic processes may be mimics of environmental cycles, many more are expressions of endogenous mechanisms, believed to be an outcome of millions of years of interaction between the biological and the geophysical world<sup>7,10,11</sup>. When organisms are isolated from the influence of periodic environmental factors – by maintaining them under constant laboratory conditions with light, temperature, humidity and sound constant – a large majority of them display biological rhythms with a near 24 h free-running period ( $\tau$ ) (hence circadian rhythms; *circa* = approximately, *dies* = day) (Figure 1). After several decades of extensive research at various structural levels of biological organization, we are now in a position to recognize and appreciate the importance of the time keeping machinery for living organisms. Until recently it was believed that circadian rhythms would provide adaptive advantage only to organisms with complex cellular organization and therefore may not be displayed by prokaryotes, at



**Figure 1.** Schematics of activity/rest cycle of a diurnal animal in presence (top) and in absence (bottom) of light/dark (LD) cycles. The abscissa represents time of day, and the ordinate represents number of days. Activity/rest data is arranged one below another chronologically to facilitate easier visualization of activity patterns. Presence of dark bars represents activity and its absence represents rest. The activity/rest rhythm of the animal entrained to 24 h LD cycles, i.e. it matches the periodicity of the LD cycles. When the animal is introduced into constant darkness, its activity/rest cycles are set free from the influence of the zeitgeber, and they start free-running with a near-24 h (circadian) periodicity.

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least by those that multiply faster than<sup>12</sup> 24 h. However, this is no longer true: circadian rhythms of photosynthesis and nitrogen fixation have now been reported in a few species of cyanobacteria<sup>12</sup>.

Circadian rhythms are unique among biological oscillations in more than one respect, (i) they are temperature and nutrition compensated, i.e. they maintain a nearly constant period at different temperatures and nutrition levels within physiological limits, and (ii) they entrain to periodic environmental factors<sup>1,11</sup>. Under entrained state, circadian clocks adjust their phase and/or period to synchronize their 'internal time' with the phase and period of the geophysical world, and thus acquire a stable phase relationship with the environment<sup>13-15</sup>. Thus, entrainment of circadian clocks by appropriate zeitgebers appears to have served as the proximal and ultimate mechanism for their evolution. Although it is not yet clear why and how organisms evolved circadian clocks in an exactly 24 h environment, there is little doubt about their advantages, specially to organisms living in periodic environments. In this review, we shall discuss about the journey of circadian clocks from their free-running to entrained states, focusing mainly on the proximal factors and mechanisms that might have acted as selection pressures for their evolution.

### Entrainment of circadian clocks

Biological clocks will be of no use if they cannot help their owners in timing their physiology and behaviour to match the biotic and abiotic cycles of the environment. Therefore, in order to fully appreciate the functions of circadian clocks one needs to understand how living organisms keep time in the ever fluctuating environments. Circadian clocks can synchronize with zeitgebers merely by passively responding to them. Whereas, in the entrained state circadian clocks make adjustments so as to match the period of the environmental cycles and thereby adopt a stable phase relationship ( $\gamma$ , defined as the time interval between a well-defined phase of a biological rhythm and a phase of the light-dark (LD) cycle when the biological rhythm is synchronized by the periodic time cue) with the LD cycle<sup>2,10</sup>. Therefore, the necessary and sufficient conditions for such entrainment are (i) the clock mechanisms should be oscillatory, and (ii) they should be sensitive to zeitgebers in a periodic manner. There is little doubt that circadian clocks are oscillatory in nature and in a number of organisms they have been demonstrated to reset their phase and/or period in a periodic manner, thus acquiring a characteristic, and reproducible  $\gamma$ <sup>15</sup>.

### Potential zeitgebers for circadian clocks

The earth's rotation around the sun creates a number of time cues in the natural environment and living organisms have evolved mechanisms to use them to keep time. Evi-

dence from studies of the last couple of decades indicates that circadian clocks possibly use several time cues simultaneously in order to occupy temporal niches in their natural environment<sup>2,16</sup>. Further, there is a general belief among rhythm researchers that circadian clocks, as they exist now, might have evolved as an adaptation, primarily to daily cycles of the environment<sup>11</sup>. Several cyclic factors such as LD cycles, temperature cycles, and humidity cycles may have initially acted as selection pressure, while the rhythmic activities of prey, predators, parasites, intra- and inter-species competition, and availability of food may have fine-tuned them subsequently.

### Light/dark cycles

The LD cycles act as an important time cue for the circadian clocks of a wide variety of organisms ranging from cyanobacteria to mammals<sup>1</sup>. The daily LD cycles are believed to be the primary force for the emergence and evolutionary maintenance of circadian clocks, serving both as proximal and ultimate cause<sup>10</sup>. Pittendrigh<sup>7</sup> believed that organisms might have restricted some of their metabolic processes to night to avoid adverse effects of light. It was also thought that a temporal programme, whereby light-sensitive processes are timed to avoid sunlight-induced damage, would be advantageous and would therefore be selected<sup>17</sup>. Indeed, several organisms are known to have shifted various UV-sensitive cellular processes such as DNA replication to night, presumably to avoid exposure to deleterious UV radiations<sup>17</sup>. For example, the unicellular alga *Chlamydomonas reinhardtii* was found to be most sensitive to UV radiations during the end of the day, and at the beginning of the night, and sensitivity was highest at the time of nuclear division<sup>17</sup>. Alternatively the emergence of circadian clocks has been attributed to an increasing level of free oxygen in the environment<sup>18</sup>; according to this hypothesis, increased level of free oxygen is believed to have forced organisms to minimize the deleterious effects of the diurnal photo oxidative exposures by developing circadian rhythmicity of metabolic activities. Therefore, it is possible that early eukaryotes might have rescheduled their metabolic activities to protect themselves against deleterious effects of light and free oxygen.

### How do LD cycles time biological clocks?

The issue of entrainment has been central to circadian rhythm research since its conception, and most of our understanding about it has come from mainly the entrainment of circadian clocks by LD cycles. It is now known that in mammals, light perceived by specialized photoreceptors in the eye<sup>19,20</sup> regulates clock genes<sup>21,22</sup> and immediate early genes<sup>23,24</sup>. However, the molecular mechanisms facilitating entrainment of a circadian clock still remain a mystery<sup>25</sup>. Entrainment of circadian clocks by LD cycles is

believed to be due to daily discrete phase shifts equal in magnitude to the difference between the periodicity of LD cycles and the  $\tau$  of the circadian rhythms<sup>13,15</sup>. Thus entrainment depends on two key properties of circadian clocks, the phase response curve (PRC) and the  $\tau$ , and it is assumed that they remain unchanged during the process of entrainment (non-parametric model). According to this model of entrainment, the stability of circadian clocks under entrained conditions depends upon the  $\tau$  and the PRC<sup>15,26,27</sup>. Empirical evidence includes some key features of entrainment in *Drosophila*<sup>28</sup> and mice<sup>13</sup>, the maximal and minimal period lengths of LD cycles to which circadian clocks entrain (limits of entrainment)<sup>13,29,30</sup>, and regions of bistability when skeleton photoperiods (two brief light pulses per cycle to mimic 'dawn' and 'dusk') were used for entrainment<sup>13,14,29,30</sup>. Further, the functional relationship between  $\mathbf{y}$  and  $\tau$ , the occasional lack of entrainment, and the dependence of minimum tolerable night on  $\tau$  of the circadian clocks in the nocturnal field mouse *Mus booduga*<sup>14,15,31</sup>, suggest that entrainment of circadian clocks occurs via changes in phase. However, the non-parametric model of entrainment fails to explain the complete compression of activity in LD cycles with longer photoperiods<sup>32</sup>, the relationship between  $\mathbf{y}$  and  $\tau$  for entrainment in complete LD cycles<sup>33</sup>, and the results from some of the recent experiments using natural LD cycles<sup>34</sup>. Thus it appears that entrainment mechanisms of circadian clocks are probably more complex than the tenets implicit in the non-parametric model.

The  $\tau$  of circadian clocks varies in response to different environmental conditions, often reflecting residual effects of prior environmental conditions experienced, typically referred to as 'after-effects'<sup>35</sup>. Mice assayed in DD after being exposed to LD cycles of either 20 h or a 28 h period length, continue to exhibit rhythmic locomotor activity with  $\tau$  close to the periodicity of the experienced LD cycles for about 100 days<sup>13</sup>. Such after-effects may have some functional significance, as they may help organisms to maintain a stable  $\mathbf{y}$ , even when the environmental LD cycle is perturbed, especially during cloudy days and due to behavioural fluctuations<sup>13,15,26</sup>. These results suggest that the  $\tau$  of circadian clocks can change, thus violating the basic assumption of the non-parametric model of entrainment. Aschoff<sup>36</sup> suggested that circadian clocks could also entrain to periodic LD cycles by changing  $\tau$ , by lengthening or shortening of  $\tau$  in a periodic manner. Thus, just as the PRC is a prerequisite for entrainment in the non-parametric model, period response curve ( $\tau$ RC), i.e. changes in period as a function of phase of perturbations, is essential for entrainment in the parametric model<sup>15,26,27,37</sup>.

In a recent paper, Beersma and coworkers<sup>26</sup> have discussed an elegant way of integrating the two models of entrainment, by analysing the stability of circadian clocks during entrainment. They used a model circadian clock characterized by its instantaneous phase and instantaneous velocity, and assumed that it responds to light by

changing both phase and period, and then evaluated its performance by analysing the stability of such circadian clocks during entrainment. The results of the simulations provided great insights into the entrainment mechanisms of circadian clocks. When phase shifts were assumed to occur without changes in period, the stability of the model circadian clock increased with increasing amplitude of PRC<sup>26</sup>, whereas when entrainment was assumed to occur due to changes in period, the stability of the model circadian clock increased with increasing amplitude of  $\tau$ RC<sup>26</sup>. When entrainment was assumed to occur both by phase and period changes, the stability of the model circadian clock was maximal when the amplitude of the PRC was about six times greater than those of the  $\tau$ RC, which closely corresponds to the empirically observed ratio for diurnal mammals. The results of simulations further demonstrated that the stability of the model circadian clock was high when phase advances occur along with shortening of  $\tau$ , and phase delays occur along with lengthening of  $\tau$ <sup>26,27</sup>. Based on the results of simulations it was predicted that nocturnal burrowers would benefit less from period responses compared to the diurnal burrowers<sup>26</sup>. Comparison of the stability of circadian clocks in diurnal burrowers, non-burrowers and nocturnal burrowers, revealed that the stability was maximal in diurnal burrowers at relatively high values of amplitude of the PRC and the  $\tau$ RC<sup>27</sup>. Optimal stability for the nocturnal burrowers was obtained at much smaller amplitude of  $\tau$ RC. Therefore, it appears that circadian clocks enhance their stability in ever fluctuating environments by a number of mechanisms, which include both phase and period responses to light<sup>15</sup>.

### Temperature cycles

Although circadian clocks are temperature compensated, daily temperature cycles do entrain circadian rhythms of plants and poikilothermic animals, and help them keep time of the day, even in the absence of LD cycles<sup>10</sup>. Thus, although the period of circadian clocks is temperature compensated, their phases are not. Besides LD cycles, temperature cycles are undoubtedly the most important zeitgeber for circadian clocks. Temperature step-ups and step-downs as well as temperature pulses evoke phase advances and phase delays in a phase-dependent manner, and temperature pulses entrain circadian rhythms in a manner similar to the LD cycles<sup>16</sup>. In *Drosophila*, the high temperature pulse (HTP) and low temperature pulse (LTP) PRCs were roughly mirror images of each other, although the magnitude of phase shifts differed<sup>38</sup>. The phase shifts evoked by HTP and LTP pulses corresponded closely to the sum of the phase shifts caused by the temperature steps. Further, under natural conditions, temperature cycles interact directly with the LD cycles. Similarly, temperature pulses initiated circadian rhythmicity

of eclosion in the leafcutter bee, *Megachile rotundata*, which otherwise was arrhythmic in a 24 h LD cycle<sup>10</sup>. Temperature cycles synchronized eclosion of the Queensland fruit fly, *Dacus tryoni*, which was otherwise refractory to LD cycles, especially during the pupal stage<sup>38</sup>. Under the presence of both temperature and LD cycles the locomotor activity rhythm of the heliothermic lizard *Uta stansburiana* was more strongly dependent upon the temperature cycles and not so much on the LD cycles<sup>10</sup>.

Studies on the effect of temperature pulses and cycles on heterothermic animals are very few. Evidence from a number of mammalian and bird species indicates that temperature is only a weak zeitgeber for homeotherms<sup>10,16</sup>. For example, some animals such as the little pocket mouse, *Perognathus longimembris*, and a few heterothermic bats, *Molossus ater* showed entrainment to temperature cycles whereas the rhythms of homeothermic species *Phyllostomus discolor* were altogether refractory to temperature cycles<sup>10,16</sup>.

### Other abiotic cycles

The relative humidity of the natural environment also acts as time cues for the circadian clocks of several insect species<sup>38</sup>. For example in the field, the flight activity patterns of mosquitoes match the periodicity of the daily cycles of humidity more closely than those of light or temperature<sup>39</sup>. Gated eclosion of several species of insects, timed appropriately with respect to the humidity cycles of the natural environment, can prove to be of adaptive value. For example in *Drosophila* the timing of adult eclosion is believed to have evolved to coincide with the time of the day when the relative humidity levels are the highest, which is close to dawn<sup>38</sup>.

In certain mammals, sound cues such as levels of noise and quietness also act as time cues for their biological timing system. For example in humans, gong signals<sup>40</sup> promoted entrainment when coupled with LD cycles. A 6 h intermittent buzz, 25 decibels above ambient noise level entrained circadian rhythms of birds<sup>41</sup>.

The electrostatic fields also act as time cue for the wheel-running activity rhythm of mice<sup>42</sup>. Further, humans<sup>43</sup> and other animals<sup>44</sup> can not only detect magnetic field, but circadian rhythms of humans entrain to periodically experienced electromagnetic fields<sup>40</sup>. In addition, atmospheric pressure cycles have also been reported to entrain the body temperature rhythm of mice<sup>45</sup>.

### Food availability cycles

In the natural environment, optimal foraging occurs in space as well as in time. Some food sources such as nectar, pollen, insects and rodents can be preyed upon only at certain time of the day. Consequently, it is not at all surprising that many animals display the ability to 'anticipate' periodic

meals, referred to as meal anticipatory (meal-AA) rhythm<sup>46</sup>. Even in herbivores such as rabbits, foraging at the appropriate time of the day may be adaptive in terms of avoiding predators. In addition, cycles of eating and fasting may also be expected to provide an important temporal constraint on an animal's behaviour, especially under natural conditions where food may not be available at all times of the day<sup>16</sup>. For example, honeybees synchronize their foraging activity to the daily cycling of nectar and pollen production of plants<sup>47,48</sup>. Rhythmic feeding has been reported in several studies on honeybees<sup>49,50</sup>. Honeybees can also be trained to arrive at feeding sites if food is offered only for a limited time each day<sup>51,52</sup>. Such feeding schedules were effective in entraining the activity rhythms of honeybee colonies, only when they followed periodicities<sup>49</sup> between 22 h and 25 h. Under constant laboratory conditions, restricted feeding entrained locomotor activity rhythm of a number of mammals<sup>16,46,53</sup>. Periodic availability of food also modulated the phase of activity rhythm of animals maintained in LD cycles<sup>42,54</sup>. Further, a wide range of animals such as rabbits, weasels, and squirrel monkeys develop meal-AA rhythm a couple of hours before the meal is presented, under the restricted feeding schedules<sup>46</sup>. Within a limited range of imposed restricted feeding periodicities, the  $\gamma$  of the meal-AA rhythm shows relationship with the period of the restricted feeding rhythm, in a manner similar to those seen for light-entrained rhythm and the period of the LD cycle<sup>54,55</sup>. Meal-AA also displays gradual resynchronization following a shift in the daily feeding timing<sup>46</sup>.

### Social cycles

There are a number of anecdotal reports, incidental observations<sup>56</sup> and empirical studies on the influence of social interactions on circadian clocks. Under isolation, social zeitgebers entrained circadian clocks of humans<sup>57</sup>, and domestic sparrows<sup>58,59</sup>. In natural and semi-natural conditions, social time cues entrained circadian rhythms of bats<sup>60</sup>, and of a few other mammals<sup>56</sup>. In rodent females, the circadian clocks besides regulating other functions also control oestrus cycle and seasonal reproductive cycles<sup>61</sup>. Therefore, a role of social cues on circadian clocks would require evidence that social interactions influence these reproductive cycles. The exposure to pheromones from male mouse entrained the oestrus cycles of female mice<sup>10</sup>. Female rats and hamsters when housed together showed a non-random degree of synchrony among their estrous cycles<sup>62</sup>, similar to the remarkable synchrony seen in menstrual cycles of human females<sup>63</sup>.

Mammals experience a variety of time cues both prenatally and postnatally, which help them to know the time of the day and season in the external world. In rat pups, the pineal *N*-acetyltransferase rhythms and the activity rhythms<sup>64</sup> were entrained by the time cues from mothers,

both pre- and post-natally<sup>65</sup>, while such entrainment in Syrian hamsters occurred *in utero*<sup>66,67</sup>. Maternal time cues, even mere presence and absence of mother act as an effective zeitgeber, postnatally, for their pup's circadian clocks<sup>68,69</sup>.

Social interactions act as time cues even in some species of insects. For example, honeybee colonies consist of multiple forager groups, each with a high degree of fidelity to its own food source which varies during the day, e.g. each flower opens at its own specific time, however the colony as a whole maintains perfect synchrony<sup>70</sup>. Nectar forager bees, when transferred to a foreign colony, were found to visit food source at the foraging times of their parental and host colony, suggesting a temporal influence through social interactions<sup>71</sup>. Under isolation, the activity of isolated bees drifted from that of the colony, and the  $t$  of the activity rhythm of the individuals was either longer or shorter than the  $t$  of their respective colonies, suggesting that under colony conditions these bees must be mutually synchronized<sup>72</sup>. Similarly when two groups of bees were allowed physical contact, their metabolic rhythms became synchronized<sup>73</sup>, a phenomenon also seen in humans<sup>40,74</sup>, beavers<sup>75</sup>, bats<sup>60</sup>, vertebrates<sup>69,75-78</sup>, and recently in *Drosophila*, an organism that was considered to be the least social at least amongst insects<sup>79</sup>.

#### Other biotic cycles

Animals can influence each other's circadian clocks not just by maintaining a common phase, but also by competitive temporal isolation. Such exclusions have been reported to occur both intra- and inter-specifically in rats<sup>10</sup>. Competition between sympatric species could also act as proximal and ultimate force for the evolution of circadian clocks. Two sympatric species of kangaroo rats (*Dipodomys*) have been reported to fight aggressively for favoured feeding sites at a favoured time<sup>80</sup>. The locomotor activity and the oviposition rhythms of three species of *Drosophila* parasitoids were out of phase, and the competitively inferior parasitoid species started activity earlier than the superior competitor. The  $t$  of the inferior competitor *Asobara tabida* (21.3 h) was shorter than that of the superior competitor *Leptopilina boulardi* (24.3 h)<sup>81</sup>. Such temporal segregation, at least partially, was reported to be mediated through and determined by the  $t$  of the circadian clocks, which also varied between species and was correlated with the timing of activity<sup>81</sup>. Thus it appears that the temporal segregation of competing species could be endogenously controlled, and this could in turn allow coexistence of sympatric species under natural conditions.

Temporal niche sharing has also been described as a useful mechanism for reducing inter-specific competition. On a long time scale, evolutionary forces may have caused divergence of activity patterns among species that now allows efficient sharing of temporally distributed resources. Al-

though evolution of temporal partitioning to share spatial niches would involve natural selection over a long period of time, but an alternate mechanism could be immediate behavioural responses rather than selection. Although it is difficult to tease apart these two possibilities, field observations have shown that several ant species occupy distinct temporal niches in the natural LD environment<sup>82</sup>, most probably for temporal partitioning of resources. Some species such as *Myrmecia*, *Rhytidoponera*, *Docryon* and *Iridomyrmex humilis* start their activity during mid-day and remain active throughout the light phase of LD cycle, whereas other species like *Colobostruma* and *Camponotus*, make their appearance in a regularly staggered succession during the night<sup>82</sup>.

#### Temporal patterning of internal metabolic processes

The temporal co-ordination of internal metabolic processes can also act as an internal zeitgeber for circadian clocks. Incompatible processes that may require different physicochemical conditions for their successful functioning appear to be separated efficiently in time and space<sup>16</sup>. For example, in heterocystous cyanobacteria, incompatible processes such as oxygen-producing photosynthesis, and oxygen-labile nitrogen fixation occur by spatial separation of the site of nitrogen fixation from that of photosynthesis<sup>11</sup>. Non-heterocystous species, on the other hand, show endogenously generated daily rhythms in these two functions, with peaks occurring at different times<sup>11</sup>. Although the use of circadian clocks in segregation of various metabolic processes may clearly appear advantageous, it is argued that this may not be the only driving force behind the emergence of circadian clocks. It is possible that the circadian rhythm of photosynthesis has evolved first, as a response to LD cycles and only then the circadian rhythm of nitrogen fixation may have become advantageous.

#### Behavioural feedback

Besides the external time cues, several clock-controlled processes such as behavioural manipulations involving locomotor activity, arousal, or correlated states themselves can also reset the phase and the period of circadian clocks<sup>83,84</sup>. Such changes influence both the free running and the entrained states of circadian clocks. The possibility that even the clock-controlled processes might have significant and non-negligible feedback on circadian clocks has been considered by several researchers<sup>83</sup>. It is believed that the coupling between circadian clocks and overt rhythms is bi-directional<sup>83</sup>. Phase shifts and entrainment of circadian clocks by various non-photoc stimuli such as scheduled voluntary exercise<sup>85-87</sup>, forced treadmill running<sup>88-90</sup>, arousal due to saline injections and triazolam injections<sup>91,92</sup>, daily schedules of cage changes and social

interactions<sup>83</sup>, restricted foraging<sup>93</sup>, triazolam-induced wheel running<sup>91</sup>, and novelty-induced wheel running<sup>83</sup>, are now well-documented in the literature, specially in nocturnal mammals. It is also known that non-photic stimuli induce large phase advances in nocturnal mammals during the subjective day and evoke less or no phase shifts during the subjective night<sup>83,90,91,94,95</sup>. In the European ground squirrel (*Spermophilus citellus*), wheel running activity entrained circadian rhythms in a manner similar to those seen in nocturnal animals<sup>34</sup>. On the other hand, scheduled wheel running did not entrain the locomotor activity rhythm of the crepuscular animal *Octodon degus*, instead, it exerted weak phase control<sup>96</sup>, suggesting that behavioural feedbacks can be a weak zeitgeber for some animals. In humans, the effect of intense physical activity on circadian clocks has been well documented<sup>97</sup>. Brief exercise, especially during mid-subjective night phase delayed the circadian rhythms of body temperature and hormone levels<sup>98,99</sup>. Long duration exercise phase shifted human circadian clocks in a manner qualitatively similar to that seen in nocturnal rodents<sup>97</sup>.

In nocturnal mammals, some of the strongest non-photic effects involve manipulations that induced initiation of locomotor activity, at times when the animal would normally be resting, i.e. the subjective day<sup>83,100,101</sup>. When mice were provided with 'novel running wheels' during the subjective day, they usually become active, started digging and scratching their body, started scent marking, and pushing grains<sup>83</sup>. These actions serve to establish a home territory for the animals and therefore might have social implications. Previous studies on hamsters showed that access to novel running wheels for 3 h phase shifted the circadian clocks and the magnitude of the phase shift was a function of the total amount of novelty-induced running per unit time<sup>83,102</sup>. Similarly in *tau* mutant hamsters phase shifts and period changes were obtained following a single exposure to novel running wheel<sup>83</sup>. In rats, entrainment due to periodic access to novel running wheels produces sizeable changes in post-entrainment  $\tau$ <sup>103</sup>. Under constant dim light, mean  $\tau$  of Syrian hamsters housed in spring-suspended cages without running wheel was shorter than those housed in cages with running wheel<sup>83</sup>. In constant darkness (DD), slightly shorter values of  $\tau$  have been reported for hamsters with access to running wheels compared to the hamsters housed in a simulated burrow system<sup>104</sup>. A single 3 h access to novel running wheels was found to lengthen the  $\tau$  of the locomotor activity rhythm in hamsters<sup>83,105</sup>. Shortening of  $\tau$  due to running wheel access has also been reported in blinded rats<sup>103,106,107</sup> and mice<sup>108</sup>. Such differences in responses to novel running wheel access have also been reported in rats<sup>109</sup>, mice<sup>108</sup> and hamsters<sup>104</sup>. These intra- and inter-species differences in the response to non-photic cues lead to the notion that non-photic zeitgebers are weak in nature<sup>83</sup>. In addition to phase shifts and period changes, behavioural activity is also known to accelerate re-entrainment of circadian clocks to phase

shifted LD cycles<sup>110</sup>. Evidence such as effects of activity on the  $\tau$  of the free-running locomotor activity rhythm in rats<sup>108,109</sup>, and acceleration of rate of re-entrainment to phase shifted LD cycles in hamsters<sup>102,110</sup>, suggests that behavioural feedbacks influence the phase of circadian clocks and therefore can act as potential zeitgebers.

### *Melatonin as an internal zeitgeber*

The pineal hormone melatonin has been hypothesized to be an internal zeitgeber for the circadian clocks of vertebrates<sup>111</sup>. In mammals, melatonin is synthesized and secreted by the pineal gland in a rhythmic manner, and is regulated both by the circadian clocks and the LD cycles<sup>84,112,113</sup>. Melatonin level begins to rise in the evening, and remains high throughout the night<sup>84,111</sup>, hence the level of melatonin varies periodically in mammals. Besides this, in mammals the phase shifts evoked by external melatonin administrations vary periodically<sup>84,114,115</sup>.

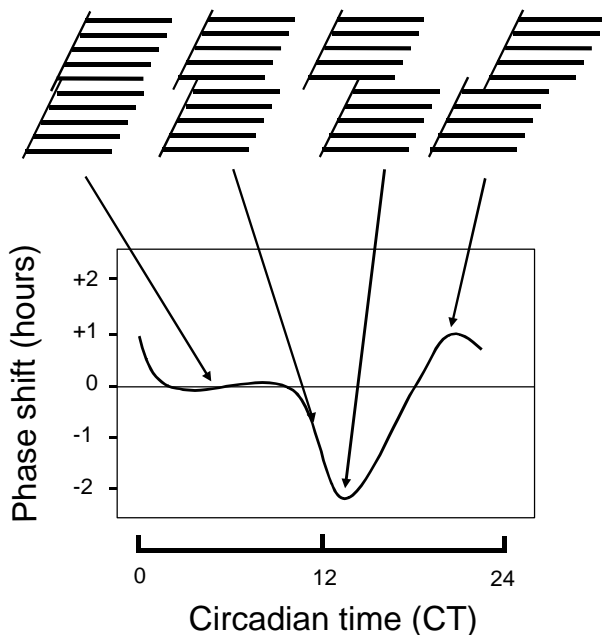
A number of mammalian species entrained to daily administration of melatonin<sup>84,111,116</sup>. Timely administration of melatonin to rodents accelerated re-entrainment to phase shifted LD cycle<sup>84,117-119</sup>, altered metabolic and electrical activities of the suprachiasmatic nucleus (SCN)<sup>112</sup>, and phase shifted the firing of the SCN neurons *in vitro*<sup>120,121</sup>. These studies suggest that the SCN, the circadian clocks of mammals, are sensitive to melatonin. However, in adult hamsters, a single dose of melatonin had no effect on the free-running activity rhythm when administered without handling the animals<sup>95,122</sup>. The SCN of mammals regulates a number of circadian rhythms such as wheel-running, feeding, drinking, plasma corticosterone and growth hormone levels, heart rate, learning, sleep-wake cycle, pineal serotonin and *N*-acetyltransferase rhythms<sup>122,123</sup>. Studies involving autoradiography using <sup>125</sup>I-melatonin suggested existence of melatonin-binding sites in the rat SCN<sup>124</sup>. Selective activation of neurotransmitters or neurohormones in the SCN induced phase shifts in circadian rhythms<sup>100</sup>. Melatonin is also ubiquitous with respect to permeation of all cells, in all tissues in all organs and its synthesis and periodic release is directly controlled by SCN<sup>125</sup>. These findings suggest a direct action of melatonin on the circadian pacemaker of mammals<sup>112</sup>, and a possible role of melatonin as an internal zeitgeber.

### **Why circadian clocks are preferred over exactly 24 h clocks**

An interesting and still unanswered question, which haunts rhythm researchers is how approximately 24 h timing systems could evolve in an exactly 24 h environment? It is obvious that the forces of natural selection might act on the time of expression of circadian clocks rather than its other parameters, and therefore the precision (stability) of maintenance of temporal niches in the natural environ-

ment may be crucial for the survival of any organism. To analyse this aspect, we shall undertake a comparative study of circadian (near-24 h) and exactly 24 h clocks by evaluating their stability under a 24 h environment.

Although at first sight it might appear that natural selection has failed to produce exactly 24 h clocks, a close analysis would make it clear that it is not true, because in a 24 h environment a near-24 h clock would provide greater stability to the biological rhythm compared to an exactly 24 h clock<sup>13-15,26,27,126</sup>. Many night-active animals spend most of the daylight hours in light-excluding shelters, emerging at dusk and returning to their shelters at dawn. Depending on the species, time of the year and geographical location, animals sample light specifically during dawn, dusk or both, and adopt a reproducible  $\mathbf{y}$ . The  $\mathbf{y}$  reflects interaction of the  $\mathbf{t}$  and the periodicity of the external LD cycle through the light-induced PRC<sup>13,14,28</sup> (Figure 2). The  $\mathbf{y}$  and  $\mathbf{t}$  often follow a sigmoidal relationship<sup>13,14,28</sup>. As a consequence of this, small fluctuations in  $\mathbf{t}$ , when  $\mathbf{t}$  is close to 24 h, would cause large fluctuation in  $\mathbf{y}$ , whereas fluctuations in  $\mathbf{t}$  of comparable magnitude, when  $\mathbf{t}$  is farther from 24 h (either  $<$  or  $>$  24 h), would cause only a slight change in  $\mathbf{y}$  (Figure 3). Such fluctuation in  $\mathbf{y}$  could also arise due to fluctuations in the zeitgebers. Thus the  $\mathbf{y}$  of biological clocks with  $\mathbf{t}$  far removed from 24 h will be more stable in the face of fluctuations in  $\mathbf{t}$ , than those with  $\mathbf{t}$  closer to 24 h. Therefore, organisms with  $\mathbf{t}$  exceeding by far from 24 h would be

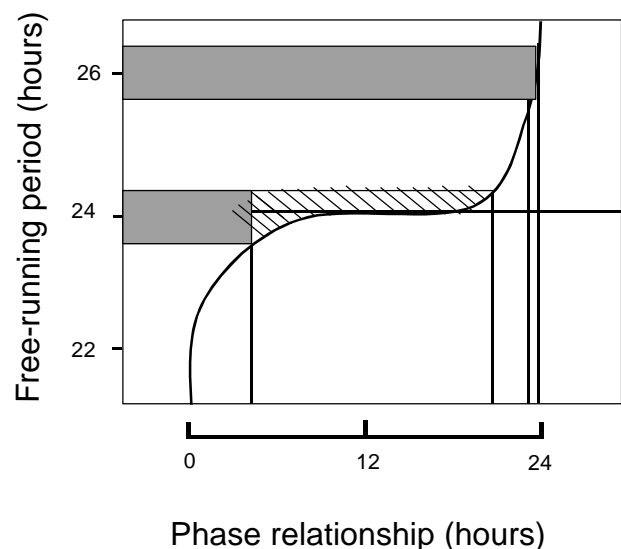


**Figure 2.** The activity/rest rhythm of animals phase shift by different magnitudes when exposed to light pulses at various circadian time (CT). The phase response curve (PRC) evoked by light pulses, show regions of phase advances and phase delays. Activity/rest cycles undergo phase delays and phase advances when animals are exposed to light pulse during early and late subjective night, respectively. Light pulses presented during subjective day cause minimal phase shifts.

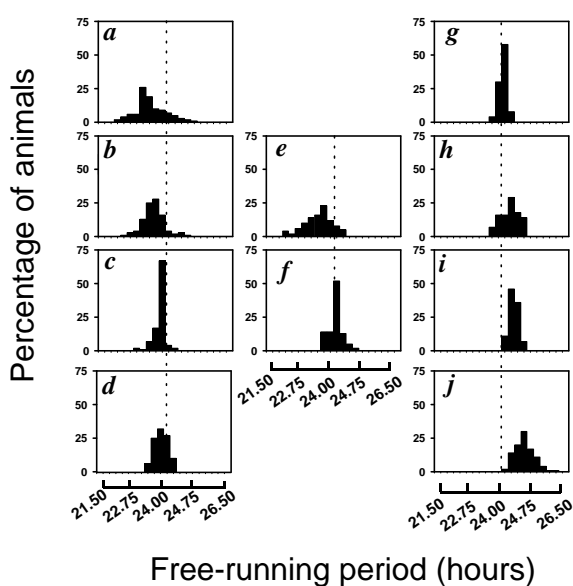
preferred by natural selection over those with  $\mathbf{t}$  of exactly 24 h. Empirical data from several species of rodents suggest that the periodicity of locomotor activity rhythm is close to but rarely equal to 24 h<sup>13,16,127</sup> (Figure 4). However, there is a non-negligible fraction of individuals of several species, which display exactly 24 h period in constant conditions (Figure 4). How do then these individuals deal with large fluctuations in  $\mathbf{y}$ ? We can arrive at least at an approximate answer to this question if we simultaneously consider the stability in  $\mathbf{y}$  and  $\mathbf{t}$ . The  $\mathbf{y}$  of rhythms driven by circadian clocks with  $\mathbf{t}$  far removed from 24 h are more stable, their  $\mathbf{t}$ s are not. The precision of clocks with exactly 24 h period was usually higher than those far removed from 24 h<sup>97,126,127</sup>. Hence the imprecision in  $\mathbf{y}$  for individuals with  $\mathbf{t}$  values exactly 24 h appears to be compensated by greater precision in  $\mathbf{t}$ . Thus it appears that the very circadian nature of  $\mathbf{t}$  has functional significance in the conservation of  $\mathbf{y}$ , which is likely to be the prime target of natural selection<sup>2</sup>.

### Conclusions

It is important to appreciate a subtle but important distinction that circadian clocks' free-run because they have evolved as entrained clocks in the periodic environments, and not that they entrain because they free-run. One of the most important functions of circadian clocks is to en-



**Figure 3.** Dependence of phase relationship ( $\mathbf{y}$ ) on the free-running period ( $\mathbf{t}$ ) of circadian rhythms in DD. The relationship shows interaction of  $\mathbf{y}$  and  $\mathbf{t}$ , mediated through the light-induced phase response curve (PRC) (Figure 2). The  $\mathbf{y}$  and  $\mathbf{t}$  often follow a sigmoidal relationship, as a consequence, small fluctuations in  $\mathbf{t}$ , when  $\mathbf{t}$  is close to 24 h, would cause large fluctuation in  $\mathbf{y}$ , whereas fluctuations in  $\mathbf{t}$  of comparable magnitude, when  $\mathbf{t}$  is farther from 24 h (either  $<$  or  $>$  24 h), would cause only a slight change in  $\mathbf{y}$ . Thus the  $\mathbf{y}$  of biological clocks with  $\mathbf{t}$  far removed from 24 h will be more stable in the face of fluctuations in  $\mathbf{t}$ , than those with  $\mathbf{t}$  closer to 24 h.



**Figure 4.** The frequency distributions of free-running periods ( $t$ ) of ten mammalian species: *a*, *Peromyscus maniculatus*; *b*, *Mus musculus*; *c*, *Glaucomys volans*; *d*, *Mus platythrix*; *e*, *Mus booduga*; *f*, *Peromyscus leucopus*; *g*, *Mesocricetus auratus*; *h*, *Rattus exulans*; *i*, *Saimiri sciureus*, and *j*, *Homo sapiens*. All experiments except those with humans were conducted under similar environmental conditions. Each species displayed a characteristic distribution of  $t$ , with the mean  $t$  displaced from 24 h. The vertical dotted line at  $t$  equal to 24 h is drawn to provide a reference for the frequency distribution of different species of mammals.

sure that various behavioural and metabolic processes are appropriately timed with respect to the daily events in the environment, and thereby to help organisms adopt a characteristic temporal niche. Those temporal niches would then complement the spatial niches occupied by them. It is believed that circadian clocks have evolved under the influence of temporal factors of geophysical environments as forces of natural selection. Several such forces in the natural environment impose constraints on organisms to schedule their metabolic processes at appropriate time of the day. It is also believed that organisms have evolved under the simultaneous action of several such zeitgebers, getting influenced by them differentially. Given that organisms are sensitive to several time cues of the geophysical environment to different degrees, one can construct an apparent hierarchy of zeitgebers for any given organism.

After several decades of extensive research we still do not know exactly why circadian clocks have evolved, how they work, or how they interact with various zeitgebers in the geophysical environment. What we now know is that organisms from a wide range of taxa display circadian rhythms at various levels of complexity, regulated by molecular mechanisms in the pacemaker cells, involving clock genes, and that they use special mechanisms to sense LD cycles of the environment. In recent years emphasis of the field has shifted from describing circadian rhythms and demonstrating its inherent control, to assessing zeti-

gebers of the geophysical environment using advanced experimental protocols incorporating several times cues, acting simultaneously. The mechanisms and pathways via which photic and non-photoc zeitgebers act on the circadian clocks of organisms from different ecological environments are also being extensively explored.

Although at this juncture many questions about circadian clocks, and their interaction with zeitgebers, cannot be answered to complete satisfaction, we know enough about them, and their various manifestations, at least to justify the conclusion that they represent a powerful and versatile physiological mechanism, important for the overall functioning of the organism. They help organisms to measure (i) lapse of time and (ii) exact time in the local environment by maintaining stability in their period and phase. The purpose of this review was to highlight the central role of the proximal forces of natural selection for the evolution and maintenance of circadian timing systems, an issue that we think will remain alive for several decades to come.

We would like to point out that a review of zeitgebers and their interactions with circadian clocks involves covering voluminous amounts of literature. In the interest of keeping the list of references to a reasonable length we have not cited some of the primary papers, rather we have mentioned recent reviews where the issue has been discussed at length. Thus, omissions of papers from the list of references should not be taken to imply our intention to underrate the importance of those studies.

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ACKNOWLEDGEMENTS. We acknowledge financial support from the Department of Science and Technology, New Delhi and Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore. We thank Amitabh Joshi for carefully reading the manuscript and suggesting improvements.