

Entrainment Concepts Revisited

Till Roenneberg,^{*,1} Roelof Hut,[†] Serge Daan,[†] and Martha Merrow[†]

**Institute for Medical Psychology, University of Munich, Germany, †Centre for Behaviour and Neurosciences, University of Groningen, Haren, The Netherlands*

Abstract The traditional approaches to predict entrainment of circadian clocks by light are based on 2 concepts that were never successfully unified: the non-parametric approach assumes that entrainment occurs via discrete daily phase shifts while the parametric approach assumes that entrainment involves changes of the clock's velocity. Here the authors suggest a new approach to predict and model entrainment. Unlike the traditional approaches, it does not assume a priori the mechanism of how the internal and external cycle lengths are matched (via phase shifts, velocity changes, or even other mechanisms). It is based on a circadian integrated response characteristic (CIRC) that describes how the circadian system integrates light signals at different circadian phases, without specifying exactly when and how fast its progression is affected. Light around subjective dawn compresses the internal cycle; light around subjective dusk expands it. While the phase response curve (PRC) describes the results of experiments using light stimuli (of specified duration and intensity), the CIRC reflects how the system integrates any given light profile, be it single pulses or any form of light-dark cycle (from skeleton photoperiods to natural light profiles). CIRCs are characterized by their shape (determining the extent of their dead zone) and their asymmetry (the ratio of its compressing and expanding portions). They are dimensionless (time/time), and their maximum is by definition 1. To make predictions about entrainment, the CIRC is multiplied with the light intensity/sensitivity at any given time point. Unlike the PRC and the velocity response curve, the CIRC can be assessed on the basis of entrained steady states, by modeling experimental results. The CIRC approach makes several predictions that can be tested experimentally.

Key words circadian, entrainment, *Neurospora crassa*

Nothing in circadian biology makes sense except in the light of entrainment¹; thus, the principles of entrainment should be to chronobiologists what grammar is to linguists. The question of how circadian clocks entrain to zeitgebers, the light-dark cycle in particular, has been a focus of clock research from its beginning. The field's 2 pioneers, Jürgen Aschoff and Colin Pittendrigh, were close friends, but they

had different views on how to approach entrainment (Daan, 2000). Aschoff emphasized the sustained (tonic) effects of light, which potentially change how fast internal (clock) time moves in relation to external (zeitgeber) time, while Pittendrigh focused on shifts of internal phase due to light-dark transitions.

Both the velocity and the phase shift approaches are based on so-called phase-only models; their only

1. To whom all correspondence should be addressed: Till Roenneberg, Institute for Medical Psychology, University of Munich, Goethestr. 31, D-80336 Munich, Germany; e-mail: roenneberg@lmu.de.

readout is phase, be it a phase shift due to a single intervention, a change in phase when the velocity of the oscillator changes, or the phase relationship between the internal cycle and that of the zeitgeber. Phase-only models are simplifications of biological oscillators, missing the mechanistic details, but they are so far the most useful models generating quantitative predictions.

Entrainment has also been modeled by more elaborate mathematical differential equations (by Wever, Pavlidis, and many others; for review, see Roenneberg et al., 2008). Differential equations provide analogs to biological entrainment, but the elements in these models are usually not intuitively clear and are less reliable in generating quantitative predictions.

Finally, entrainment has been modeled with chemical transition equations (for review, see Leloup and Goldbeter, 2008), which try to mimic natural processes. These approaches usually rely on an enormous number of equations, generating a large parameter space with many degrees of freedom. Another complication with these models is that the mechanisms generating the circadian oscillation are still insufficiently known.

Here we propose a new approach, which stays within the realm of phase-only models that have proven to be the most effective. It focuses exclusively on entrained situations for 3 reasons: 1) They are the conditions under which the circadian system evolved, 2) entrainment by a regularly repeating zeitgeber differs from responses to single perturbations applied in constant darkness (DD), and 3) entrainment protocols offer much richer data sources than do phase response curves (PRCs).

The new approach avoids assumptions on the dynamics of phase progression during illumination and aims to explain entrainment under all conditions. It provides a rich spectrum of experimental predictions that will lead to an understanding of how entrainment is implemented at all levels of the circadian system, from molecules to organism. Before describing the new approach, we first highlight the main difficulties faced by the traditional phase-only models.

PROBLEMS OF THE EXISTING THEORIES

Entrainment by instantaneous shifts in response to transitions is nonparametric since it does not change parameters of the underlying oscillator. Entrainment

by velocity changes necessarily affects 1 or more oscillator parameters when exposed to light and is thus parametric. Since velocity changes obviously also change the phase, parametric and nonparametric entrainment differ only by the nature of the effective stimulus (transition or continuous). The nonparametric theory is widely accepted, although no unequivocal evidence exists for phase responses to LD transitions (see Comas et al., 2008; Nelson and Takahashi, 1999). In contrast, velocity (period) changes in response to constant light are well documented and widespread (Aschoff, 1979). Both approaches use the effects of light compared with DD. The PRC is derived from single light pulses, and the velocity response curve (VRC) is estimated from the PRC (Daan, 1977).

The PRC approach makes important assumptions that cannot be directly measured since we can observe only the steady-state consequences of an intervention 1 or even more days after it has been presented (e.g., due to transients; Pittendrigh et al., 1958). We do not know whether the steady state was due to a transient change in velocity (parametric) or to an acute change in phase (nonparametric). Considering our more recent understanding of how complex the circadian system is at all levels (Baggs et al., 2009; Roenneberg and Merrow, 2003) and in view of the poor temporal resolution of circadian experiments, it is conceivable that the system needs much of its cycle to manifest a response.

Pittendrigh performed a series of experiments to investigate what happens during the 1st cycle after an intervention by a light pulse (Pittendrigh, 1967, 1981). He first constructed a PRC for light pulses given in DD and then—for selected phases of the 1st, single-pulse PRC—predicted the PRC generated by a series of 2nd pulses. If the 1st phase shift was instantaneous, the 2nd PRC should be predictably shifted by the magnitude of the 1st pulse. Similar experiments had been performed earlier by Chandrashekar, and the predictions are true in most of the *Drosophila* work (Chandrashekar, 1980; Pittendrigh, 1981), especially in *Drosophila pseudoobscura* characterized by strong (type-0) light resetting since the 1st pulse resets the system to nearly the same phase.

In nocturnal rodents, which show weak (type-1) resetting on the behavioral level, the predictive power of the PRC approach breaks down when the 2 pulses are close to each other (Comas et al., 2007; Pittendrigh and Daan, 1976b, figs. 3 and 13, respectively). Phase shifts with 2 pulses given 1 h apart

cannot be explained by complete instantaneous resetting, although such an explanation could not be rejected when the pulses were 2 h apart (Best et al., 1999). Note that if the pulses are repeated indefinitely, the clock should stop: a 15' light pulse presented once per hour will somewhere hit a phase where, according to the PRC, it delays the system by 1 h. The next pulse should then again hit the same phase, and delay again, and so on. The prediction of instantaneous phase resetting that potentially stops the clock is also not upheld in other species since rhythms continue in high-frequency LD cycles, for example, in fish (Eriksson and Veen, 1980), cockroaches (Roberts, 1982), and several species of birds and mammals (Aschoff, 1999).

Entrainment has to correct for differences in cycle lengths—external and internal—and thereby produces a specific phase relationship (ψ). Internal cycle length becomes apparent in constant conditions as free-running period (in constant darkness, as τ_{DD}). The nonparametric approach predicts the phase of entrainment by calculating the phase of the PRC at which the light transition has to occur in order to produce the daily phase shifts ($\Delta\phi$), necessary to correct for the difference between τ_{DD} and the period of the zeitgeber (T): $\Delta\phi = \tau_{DD} - T$ (Pittendrigh, 1981). This prediction works well for different T. It works only to a limited extent for skeleton photoperiods (2 single pulses per cycle), since the subjective night is compressed in rodents into much shorter intervals than predicted (Pittendrigh and Daan, 1976b, figs. 13, 15). The most significant difficulty for the nonparametric approach is that it yields no quantitative predictions for long photoperiods without making additional assumptions on the action of light between lights-on and lights-off (Pittendrigh and Daan, 1976b).

But which transition should be considered as effective: onset, offset, or both? At least one study in mice has shown that the offset does not determine phase, at the least if the velocity changes during a long light pulse are considered (Comas et al., 2006). Step PRCs have been used to explore the individual contributions of the transitions (Albers, 1986; Aschoff, 1994; Comas et al., 2008; Kramm, 1974; Subbaraj and Chandrashekar, 1981), but their results show that entrainment cannot be predicted alone by a nonparametric approach: step-up and step-down light signals produce all delay and all advance PRCs, respectively (Comas et al., 2008). In contrast to light pulses, steps from dark to light induce the largest phase delays around subjective dawn, and steps

from light to dark give the largest phase advance around subjective dusk. After using step-up ramp-down and ramp-up step-down signals to avoid masking, Comas et al. (2008) concluded that the clock does not phase-shift by nonparametric responses.

Extrapolations from PRCs to entrainment face the fundamental problem that the shape and the amplitude of the PRC (see fig. 9 in Pittendrigh, 1981) as well as the underlying τ (Aschoff, 1979) are bound to change with zeitgeber conditions (Evans et al., 2004; Pittendrigh et al., 1984). Wever (1989) already stated that “all parameters (e.g., phase, amplitude, wave shape) of response curves reflect the state of the original rhythm. These parameters are different for rhythms under constant conditions and those under a 24-hr zeitgeber. Only the responses of synchronized rhythms are relevant to all applied situations. It is therefore preferable to generate the response curves directly under zeitgeber conditions.” In a recent study using *Neurospora*, we constructed a circadian surface by systematically varying the 3 pillars of entrainment: τ , T, and photoperiod (Rémi et al., 2010), showing that the phase of entrainment cannot be predicted for all the tested conditions by computing PRC and τ_{DD} , especially in long photoperiods (for *Neurospora* PRCs, see Dharmananda, 1980; Johnson, 1990; Lakin-Thomas et al., 1991).

Explaining entrainment nonparametrically becomes especially problematic in species that are not exposed to dawn and dusk, such as the European ground squirrel (Hut et al., 1999). This problem was resolved by modeling entrainment with a combination of a PRC and a VRC that exploits the light intensities changing over the course of the day (Beersma et al., 1999). The parametric approach was first advocated by Swade (1969), but again, formal assumptions had to be made. Since light pulses can cause delays larger than their own duration, they theoretically produce negative velocity. This was solved by assuming that responses are reduced during prolonged light exposure and restored by prolonged darkness (Comas et al., 2008, 2006, 2007; Daan, 1977; Daan and Pittendrigh, 1976). VRC-derived models can predict steady-state entrainment reasonably well under different photoperiods and different zeitgeber cycle lengths (Comas et al., 2006, 2007; Daan, 1977; Taylor et al., 2010). However, VRCs are also based on experiments using single light pulses given in DD, and they therefore inherently make the same assumptions as discussed above for the PRC approach.

A NEW APPROACH TO UNDERSTANDING AND PREDICTING ENTRAINMENT

Chronobiologists have intuitively extrapolated over the shortcomings of the traditional paradigms to make them applicable to different conditions and species. The new approach described here formalizes these intuitive extrapolations. It is also a phase-only model and as such a simplification of biological oscillators, not going into the mechanistic details. Yet phase-only models are so far the most useful in generating quantitative predictions. The new approach makes no assumptions about local velocity changes or instantaneous phase shifts, by considering only the integrated outcome over a cycle. Its predictions are not based on responses in DD but exclusively on more abundant and accessible entrainment data. It uses the integrated effect of light at different internal times (formalized by a circadian integrated response characteristic [CIRC]) as the parameter that adjusts the internal cycle length to that of the zeitgeber. Its use of only a few variables simplifies the search for biological correlates (intercellular and intracellular) that contribute mechanistically to circadian entrainment, an understanding that is still lacking after several decades of molecular and cellular research into circadian clock mechanisms.

The Circadian Integrated Response Characteristic

PRCs have shown that light produces advances around dawn and delays around dusk (Hastings and Sweeney, 1958; Pittendrigh, 1981), and VRCs assume that light accelerates the clock's velocity around dawn and decelerates it around dusk (Daan and Pittendrigh, 1976; Swade, 1969). The CIRC (Fig. 1A) combines these insights by assuming that light around subjective dawn compresses the internal cycle and light around subjective dusk expands it. It integrates the responses over the entire light signal, be it a single pulse, skeleton photoperiods, or any other form of daily light profile (rectangular, ramped, twilight, or noisy light environments). The CIRC is dimensionless since it represents the system's capacity to compress or expand the internal cycle length (unit: time) integrated over the time of light exposure. Its maximum is by definition 1. To make quantitative predictions about entrainment, the CIRC has to be calibrated for zeitgeber strength (intensity/sensitivity); that is, a calibration factor c_{ZG} has to be derived

experimentally (see "Assessing the CIRC by Experimental Protocols" below).

Predicting entrainment by the CIRC is possible not only in LD cycles but also in dim:bright light cycles (see Suppl. Fig. S1 in supplementary online materials; this approach will also enable the establishment of CIRC for temperature entrainment, which we have not considered here). Since the CIRC is a product of experimental observations (like the PRC and the VRC), any circadian modulation of sensitivity should already be part of a derived CIRC. Thus, as with the PRC, the CIRC currently provides no possibility to separate the parts of its dynamics that result from oscillator function or from a circadianly modulated light input-pathway.

The forms of the CIRC shown in Figures 1 through 4 are chosen only for demonstration purposes to illustrate the rules of entrainment laid out by this new approach (for different forms of the CIRC, see Fig. 5). Unlike the PRC, the CIRC offers a template to predict entrainment under all conditions in conjunction with a given light environment. Figure 1B-D demonstrates how a single CIRC can be used to construct different PRCs. With the help of computer modeling, this process can also be reversed, so that the CIRC's form for a given condition and a given species can be constructed from systematic entrainment results (an example of applying such a reversed process is described elsewhere; Roenneberg et al., 2010).

Under a typical laboratory light profile (Fig. 2B), the integrative effect of the CIRC is as shown in Figure 2C (filled areas). The effect of light on the system can be estimated by adding/subtracting the integration of the profile to/from the current internal cycle length, $\tau_E(\tau_E - T = \int \text{CIRC} \times c_{ZG} \times \text{intensity}$; integrating the CIRC from lights-on to lights-off). Note that τ_E may be different from τ_{DD} (this concept will be discussed in detail elsewhere; Roenneberg et al., 2010). Figures 2D and 2E show an example of how a more realistic twilight exposure would be integrated by the system, and Figures 2F and 2G illustrate this process for a noisy light profile as it is to be expected under natural conditions, predicting that the resulting compression/expansion profile can be quite similar for very different light environments.

The simplest way to explain the predictions of the CIRC for entrainment are when $T = \tau$ and when the CIRC is symmetrical in its compression (C) and expansion (E) portions (as indicated in Fig. 2A). For the zeitgeber profiles shown in Figures 2B and 2D, internal midday (InT12) would coincide with external

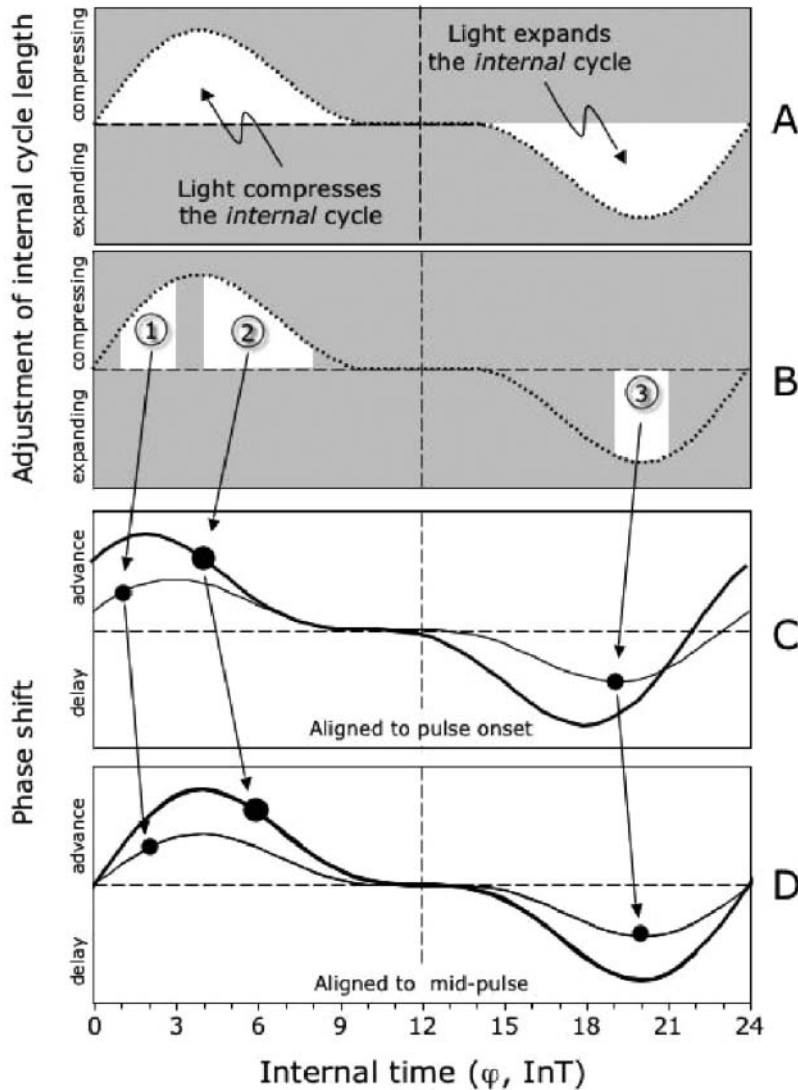


Figure 1. (A) A circadian integrated response characteristic (CIRC) predicts how a clock integrates the light signal and by how much light exposure will compress or expand the internal cycle length at different internal times. (B, D) How a single CIRC can be translated into many different PRCs. The light pulses shown in (B) represent examples in the context of single-pulse experiments (1: a 2-h light pulse starting at InT 1; 2: a 4-h light pulse starting at InT 4; 3: a 2-h light pulse starting at InT 19). The resulting changes in internal cycle length are calculated by integrating the area under the curve and are represented as phase shifts in (C) and (D), aligned to pulse onset and to midpulse, respectively (2 h: small dot; 4 h: large dot). The CIRC is dimensionless since it represents the system's capacity to compress or expand the internal cycle length (unit: time) integrated over the time of light exposure (for details, see text).

midday (ExT12) because the integrals under C and E cancel each other. InT12 would slightly lead ExT12, hiding more of its compression region in the dark less of the morning light (and thereby resulting in an earlier chronotype) when the system's CIRC is exposed to the light profile shown in Figure 2F due to more light being present in the morning than in the evening.

Phase of Entrainment

The CIRC concept provides a simple, intuitive way to predict the phase of entrainment (e.g., chronotype in different individuals and for different light environments). The entraining process must mold the internal cycle into the external cycle, so that $\tau_{LD} = T$. If the internal and the external cycle already are equal in length (Fig. 3A), the integral under the CIRC must add up to zero (compression or expansion must cancel each other out). The CIRC predicts that if 1) $\tau = T$ and if 2) the CIRC's compression and expansion regions are symmetrical, then a) internal midday (triangle) and external noon (vertical line) coincide (as in Fig. 3A and 3D) and b) the phase of entrainment will be independent of zeitgeber strength and photoperiod (because these changes affect both portions equally, always balancing compression with expansion; compare Fig. 3A and 3D).

If the dead zone of the example given here (i.e., $\tau = T$ and a symmetrical CIRC) is zero, 2 alternative solutions fulfill the criterion of producing no net compression or expansion (see top panel in Suppl. Fig. S2): 1) when the light is centered around internal midday (InT12), and 2) when it is centered around internal midnight (InT0). However, the latter example does not lead to stable entrainment because the responses will drive the internal phase away from the light pulse if τ is not exactly equal to T . If light hits the internal phase too early (i.e., exposing more of the expansion portion), the next light signal will hit an even earlier phase, and so on. If

light hits the clock slightly too late, it will do so even later in the next cycle. Not so if the light signal settles around InT12. If light hits too early, it will fall on a later internal phase the next cycle, and if it hits too late, it will come at an earlier internal phase the next cycle. Since most circadian systems are made up of populations of oscillators, this principle will lead to a broadening of the phase distribution when light falls

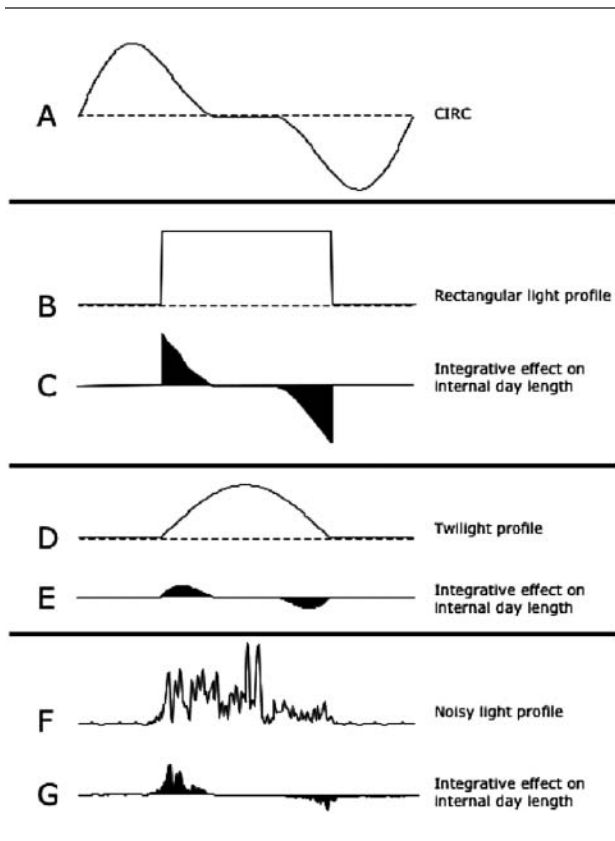


Figure 2. The effect of light on the circadian clock can be separated into 3 levels: 1) the circadian response characteristics (CIRC; same y-axis as in Fig. 1), 2) the physical qualities of the zeitgeber, and 3) the qualities of this signal after processing (adaptation, response reduction, etc.). (A) A hypothetical CIRC. (B) Physical light signal. (C) Net effect on internal cycle length. (D-G) Examples of a twilight profile and a noisy (natural) light profile. Ordinates are as described for Figure 1.

at around $\text{InT}0/24$ and will narrow the distribution when falling at around $\text{InT}12$ (Suppl. Fig. S2).

For $\tau > T$ (as it is in most humans), the integrated response must lead to a compression of the internal cycle (Fig. 3B). Internal phase must therefore move to a later external phase (white arrow to the right in Fig. 3B) to expose more of the CIRC's compressing portion to the light and hide more of its expanding portion in the dark. The hypothetical individual represented in Figure 3B is a later chronotype compared with that in Figure 3A. In this case, different zeitgeber strengths have an influence on chronotype because they will exert an unbalanced effect on the compression and the expansion portions (although the CIRC is dimensionless and normalized to a maximum of 1, its amplitude was adjusted according to zeitgeber strength for demonstration purposes). If the individual represented in Figure 3B is exposed to a stronger zeitgeber (Fig. 3E), the compressing

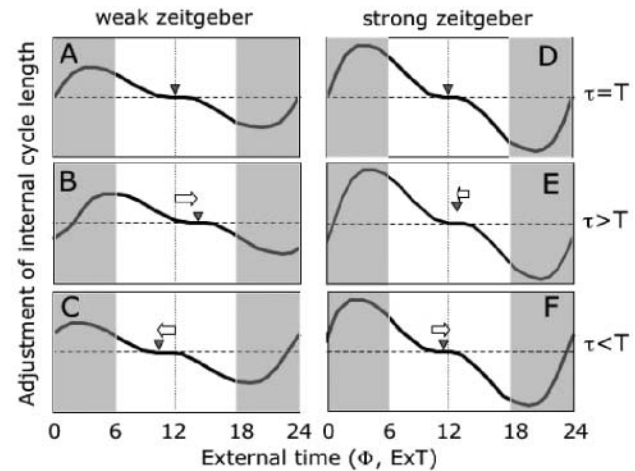


Figure 3. Entrainment is accomplished by differentially hiding and exposing different parts of the circadian integrated response characteristic (CIRC) in light and darkness (shaded areas). Phase of entrainment (chronotype) in individuals with different internal cycle lengths is predicted by the CIRC (an arbitrary shape of a CIRC is chosen for demonstration purposes). Graphs are aligned to external time (vertical stippled lines represent noon of the zeitgeber: ExT 12). Internal time is indicated by the triangles (representing $\text{InT} 12$). The left panels show examples for weak and the right panels for strong zeitgebers. The different relationships between τ and T are shown to the right. The white arrows show the direction in which the CIRC has "moved" in order to achieve stable entrainment. Those in the left panels indicate the movements compared with (A), and those in the right panels (E and F) show the movements compared with the weaker zeitgeber condition on the left (B and C, respectively). Note that although the CIRC is dimensionless and normalized to a maximum of 1, its amplitude was adjusted according to zeitgeber strength for demonstration purposes.

portion of the profile increases more than its expanding portion, making this individual an earlier chronotype compared with the weaker zeitgeber situation (white arrow to the left in Fig. 3E).

For $\tau < T$, the integrated response must lead to a compression of the internal cycle (Fig. 3C), producing an earlier chronotype compared with the first 2 examples (white arrow to the left in Fig. 3C). In this case, an increase in zeitgeber strength has the opposite effect as for the individual with $\tau > T$ (Fig. 3B and 3E), generating a slightly later chronotype as compared with a weak zeitgeber situation (compare Fig. 3C and 3F).

Limits of Entrainment

As for other oscillators, the circadian clock has a range of entrainment within which it can stably entrain. The CIRC allows estimating the limits of this

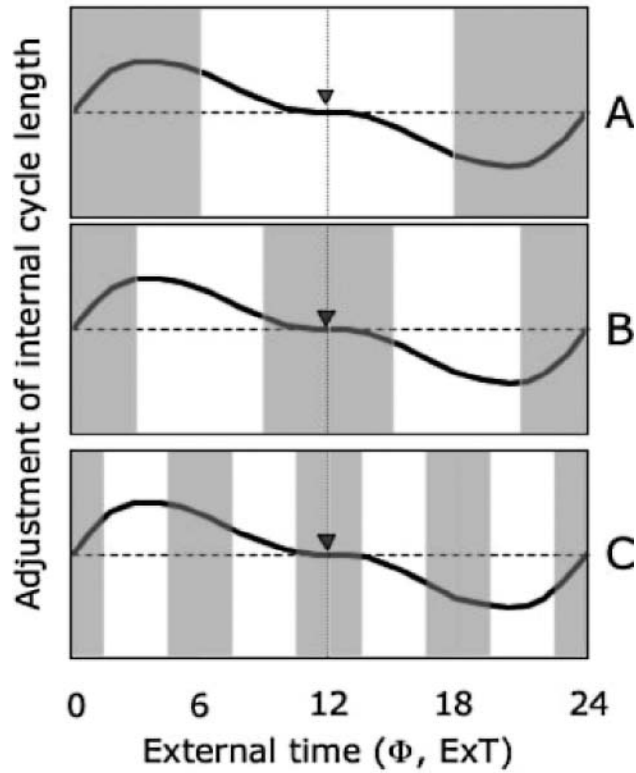


Figure 4. Frequency demultiplication is traditionally explained by oscillator theory, but it can also be described as entrainment to a structured light profile. (A) LD 12:12; (B) LD 6:6; (C) LD 3:3. The time axis is as described in Figure 3. Which of the dark episodes (in B and C) will represent ExT 0 depends on prior history of the system, as shown extensively in *Drosophila* by Pittendrigh (1981). See text for details.

range once its variables (s and a) and the calibration factor c_{ZG} have been established, as described below. Theoretically, the lower limit is reached when its entire compression portion is exposed to light while the entire expansion portion is hidden in darkness (Fig. 4B), and the upper limit is reached in the inverse condition (Fig. 4E). If $c_{ZG} = 1$, $\tau_{DD} = 22$, with an integral under the entire compression portion of 4, and that under the entire expansion portion of 3, the range of entrainment is defined by $T = 18$ and $T = 25$. These can only be crude estimations due to the fact that the dynamics of the CIRC as well as τ_E can change with T-cycle length (Roenneberg et al., 2010).

Frequency Demultiplication

Outside of the range of entrainment, the clock will not entrain stably and will therefore run through the zeitgeber cycle (possibly showing relative

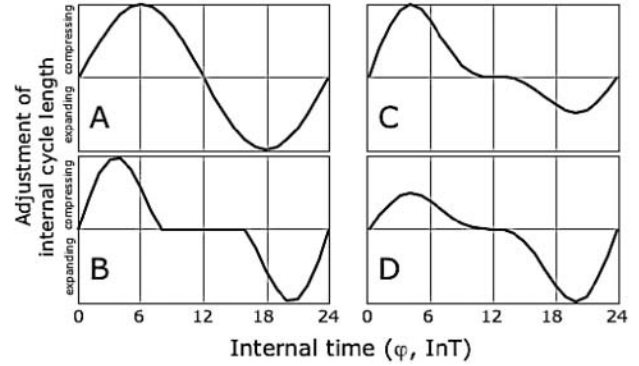


Figure 5. The form of the circadian integrated response characteristic (CIRC) can vary in shape and asymmetry. Its shape can range from pure sinusoidal (A) to showing smaller (C and D) or larger (B) dead zones. Its asymmetry determines the ratio of the integrals under its compressing and expanding portions (C and D).

coordination). When the zeitgeber lengths, however, approach approximately half of the internal cycle length, the system can be stably entrained again to 2 zeitgeber cycles at a time, a process called frequency demultiplication. This feature of the clock can also be easily explained by the CIRC. To make things simple, the example shown in Figure 4 is again based on a symmetrical CIRC and on the presumption that $\tau = T$, so that the sum of integrals covered by light must always add up to zero to ensure stable entrainment. Because many different symmetrical light-dark profiles can produce a balanced integral, frequency demultiplication simply reflects the fact that systematic light-dark profiles meet the demand that the sum of integrals is equal to $\tau - T$ (although the example shown refers to $\tau = T$, demultiplication will also occur for $\tau \neq T$).

Assessing the CIRC by Experimental Protocols

So far, we have explored the CIRC by using a hypothetical example with symmetrical compression and expansion portions and a moderate dead zone. As predicted by the diverse forms of PRCs produced in many different species (Johnson, 1990), the form of the CIRC will also be different between species and individuals. Figure 5 demonstrates how the CIRC's form can be changed by only 2 variables. The extent of the dead zone can vary from zero (making the CIRC more sinusoidal; Fig. 5A) to extending over a large part of the internal cycle (Fig. 5B). This feature of the CIRC can be modeled by a single shape factor. The symmetry of the compression and expansion portions can be changed by an asymmetry factor, as

shown in Figure 5C and 5D. These 2 variables are essentially the 2 degrees of freedom that can be used to model CIRC based on phases of entrainment generated experimentally, as will be described in detail (Roenneberg et al., 2010) in the context of using the CIRC to model the results of the circadian surface generated for *Neurospora* (Rémi et al., 2010).

The advantage of CIRC is that they can be assessed under entrainment proper, thus avoiding extrapolation from interventions in constant conditions to entrainment. Figure 6 proposes a protocol to establish a CIRC's shape and asymmetry for a given organism and individual. Unlike for PRCs, the control condition for the CIRC is entrainment by an anchor pulse of, for example, 3 h. The interval between successive anchor pulses (T or cycle length of the zeitgeber) should be only slightly shorter than τ_{DD} for reasons described below. The entrained system will settle at a phase at which the integral under the curve exposed to the light will compress the internal cycle length so that $\tau = T$ (Fig. 6A). To construct the CIRC, the clock is then probed with additional short probing pulses (for example, 1 h; Fig. 6B-E).

The readout of these experiments is phase of entrainment (ψ , the phase angle difference between oscillator and zeitgeber). If a given probing pulse increases ψ compared with the control situation (e.g., activity onset moved to the left), it must have fallen into the compressing portion of the CIRC (Fig. 6B and E), so that the sum of the 2 pulse integrals compresses the internal cycle too much (beyond $\tau - T$). In response, the clock has changed its phase relationship to the zeitgeber, so that part of the light exposure moves to the dead zone. If a probing pulse has no effect, it can only have fallen in the dead zone (Fig. 6C). If a given probing pulse delays ψ compared with the control situation, the probing pulse must have fallen into the expanding portion (Fig. 6D). The different movements of the phase of entrainment, therefore, are directly proportional to the form of the CIRC. Note that we assume that the form of the CIRC, unlike that of the PRC (Elliott, 1976), is insensitive to photoperiod and can thus be used as an internal reference to measure external changes of day length. This protocol can also be used to test whether the form of the CIRC remains the same for all combinations of the 2 pulses. If so, certain combinations should not permit stable entrainment because the net integral cannot compensate for the $\tau - T$ difference.

The reason this protocol should be performed with a zeitgeber period just slightly shorter than τ_{DD}

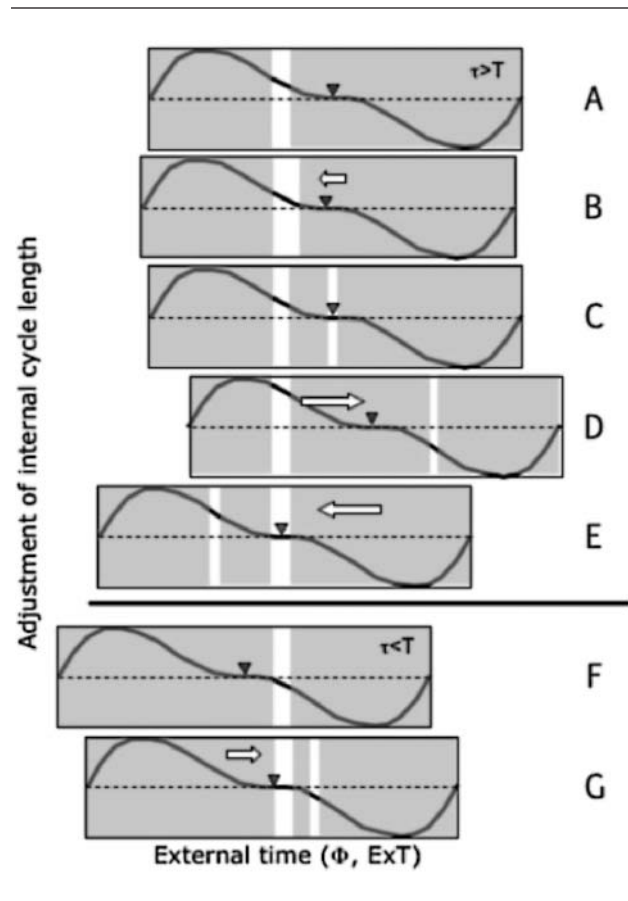


Figure 6. A protocol for probing the form of the circadian integrated response characteristic (CIRC) under continuous entrainment. Time axis as described in Figure 3. See text for details.

is that one can also investigate possible interactions between the pulses that may have nothing to do with the CIRC's form, such as response saturation-restoration (Comas et al., 2007). To distinguish between the CIRC's form and potential interaction effects, a 2nd series of experiments should be performed with a zeitgeber cycle that is slightly longer than τ_{DD} . Under these conditions, the system will settle at the other end of the dead zone (the phase angle difference between the 2 control conditions already gives an indication of the extent of the dead zone). The rest of the cycle is then, again, probed with 1-h probing pulses. Although the temporal relationships between anchor and probing pulses will be the same as in the 1st series of experiments (with $T < \tau_{DD}$), their relationship to internal phase will be different. It is essential for this protocol that the 2 chosen T -lengths are as close as possible since our modeling results indicate that the CIRC's form changes as a function of T (Roenneberg et al., 2010).

Once the form of the CIRC has been established, one can assess the factor (c_{ZG}) that translates the impact of the zeitgeber on the CIRC into actual values of compression/expansion in hours. If $\tau_{DD} = 24.5$ h, for example, the anchor pulse in the protocol described above could be given with $T = 24$ h to establish the situation shown in Figure 6A. Under these conditions, one can calculate the integral under the CIRC from lights-on to lights-off. If the result were, for example, 0.5, then $c_{ZG} = 1$ (since the τ_{DD} has to be compressed by 0.5 h; larger integral values produce a smaller c_{ZG} factor and vice versa). Repeating this protocol with different intensities gives rise to the function $c_{ZG} = f(\text{fluence})$.

CONCLUSION

Evolution has shaped circadian clocks in a cyclic world; temporal constancy of environmental qualities must have been an extremely rare exception. It is therefore the mechanism of entrainment that has evolved and not sustained rhythmicity in constant conditions. The latter is theoretically not even essential for a functional entrained circadian system. If free-running rhythms can be recorded under special constant conditions (not all allow the expression of a free run), this must reflect how the system evolved to function optimally under entrainment rather than being the object of the evolutionary process (Roenneberg and Mellow, 2002).

The CIRC approach avoids the assumptive leaps from constant conditions to entrainment (while still revisiting many aspects of the traditional paradigms) and—unlike the traditional approaches—allows studying entrainment under exactly those conditions that lead to synchronization of the clock with its environment. The CIRC resolves many of the difficulties inherent in the PRC and VRC approaches. For a given T-cycle length, a single CIRC can be used to predict entrainment for all light signals, from single pulses (see examples in Fig. 1) to noisy light profiles (Fig. 2). It also makes entrainment independent of experiencing actual sunrise and sunset, as in the case of the day-active European ground squirrel (Hut et al., 1999). Even a self-selected exposure to a natural light profile (see example in Fig. 2F and the resulting light response characteristics in Fig. 2G) would always keep the clock entrained to the 24-h day at the appropriate phase, maintaining a narrow distribution of chronotypes within the population.

The phase of entrainment of individuals with different internal cycle lengths and different light exposure can be easily predicted by the CIRC (Fig. 3). Entrainment of the human clock also depends on the natural light profile (Roenneberg et al., 2007). People living in urban regions largely create their own light environment (by rarely being outdoors and by using artificial light sources); the extremes in the human chronotype distribution are therefore almost 12 h out of phase (Roenneberg and Mellow, 2007) despite a relatively narrow proposed distribution of internal cycle length in humans (Czeisler et al., 1999; Wright et al., 2001).

The functional significance of this ubiquitous biological timing system is to internally represent a *zeitraum* (German for time-space distinguishing a temporal structure from the flow of time) to allow organisms to orient within its structure (light, dark, warm, cold, food, etc.), to specialize in temporal niches, and to anticipate its regular changes. To do so optimally, clocks must be able to change their phase relationship to the zeitgeber. The reason why evolution has provided clocks with the flexibility to entrain to very different zeitgeber lengths (different ratios of T/τ) although T changes by only 20 s every million years (Pannella, 1972) may simply reflect the fact that the entrainment process uses flexibility in τ to vary phase of entrainment. But does the form of the CIRC change with the T/τ ratio? Our modeling results (Roenneberg et al., 2010) indicate that it does. They also show that there is an aspect to τ that has so far been seen only as τ aftereffects in many circadian systems, from unicells (Roenneberg, 1995) to rodents (Pittendrigh and Daan, 1976a) and humans (Scheer et al., 2007): after release to constant conditions, τ depends systematically on the prior length of T , indicating that τ changes with the qualities of the zeitgeber. Such aftereffects have also been observed after single brief light pulses, resulting in τ RCs (Beersma et al., 1999; Pittendrigh and Daan, 1976a). In the process of modeling the form of *Neurospora's* CIRC based on the circadian surface (Rémi et al., 2010), we found that τ (which we call τ_E to denote the intrinsic period of the system under entrainment conditions) is necessary as a variable for explaining the observed phase angles of entrainment, despite the fact that τ_{LD} is always equal to T when the system is stably entrained (Roenneberg et al., 2010).

Applying the CIRC concept in modeling should make it possible to derive the form of the CIRC from

existing data on entrained systems, for example, by computing on a day-to-day basis the interaction between activity recordings and concurrently recorded light profiles (including natural effects of cloud cover and behavioral effects of self-selection), or by modeling the form of the CIRC based on phases of entrainment under a wide range of different entrainment protocols that produce a circadian surface (Rémi et al., 2010). This latter modeling approach has shown to be successful and has produced a host of predictions (Roenneberg et al., 2010). Entrainment to very different zeitgeber conditions can be modeled by changing 3 parameters: the CIRC's shape and asymmetry (Fig. 5) and τ_E in addition to calibrating the responses by the impact of zeitgeber strength (intensity and/or sensitivity). With large experimental data sets, the new approach allows us to estimate all 3 and thus provides a solid framework for future molecular analysis of the pathways of entrainment.

As with all new concepts and approaches, many questions are still open, and aspects of the CIRC will surely have to be modified as a result of experimentation in different model systems. For example, how can the CIRC help to explain that the period of a free-running rhythm depends on the intensity of constant light (Aschoff, 1979)? Similar to the PRC concept, one would predict that this behavior depends on the asymmetry of the CIRC (Fig. 5C and 5D). In this introduction of the new approach, we have concentrated on the zeitgeber light, but CIRC's should also exist for other zeitgebers. How stable is the form of a CIRC under different conditions, and does it change with photoperiod and season? Based on our modeling results (Roenneberg et al., 2010), we hypothesize that the CIRC remains constant in different photoperiods and can therefore be used as a reference to read out time of year. In that, the CIRC could be used similarly to the circadian rhythm of photoperiodic photosensitivity, which forms the basis of the external coincidence model proposed by Bünning (1936).

ACKNOWLEDGMENTS

Our work is supported by EUCLOCK (FP6 IP), the DFG (Deutsche Forschungsgemeinschaft), the NWO (the Dutch Science Foundation), the Hersenstichting Nederland, and the Rosalind Franklin Fellowships of the University of Groningen.

NOTES

Supplementary online material for this article is available on the journal's website: <http://jbr.sagepub.com/supplemental>.

1. We have derived this statement from Dobzhansky's famous quote, "Nothing in biology makes sense except in the light of evolution," which was first published in *American Biology Teacher*, 1973, volume 35, pages 125-129.

REFERENCES

- Albers HE (1986) Response of hamster circadian system to transitions between light and darkness. *Am J Physiol* 250:R708-R711.
- Aschoff J (1979) Circadian rhythms: influences of internal and external factors on the period measured under constant conditions. *Z Tierpsychol* 49:225-249.
- Aschoff J, ed. (1994) *On the Aging of Circadian Systems*. Sapporo (Japan): Hokkaido University Press.
- Aschoff J (1999) Masking and parametric effects of high-frequency light-dark cycles. *Japanese J Physiol* 49:11-18.
- Baggs JE, Price TS, DiTacchio L, Panda S, FitzGerald GA, and Hogenesch JB (2009) Network features of the mammalian circadian clock. *PLoS Biol* 7:e52.
- Beersma DG, Daan S, and Hut RA (1999) Accuracy of circadian entrainment under fluctuating light conditions: contributions of phase and period responses. *J Biol Rhythms* 14:320-329.
- Best JD, Maywood ES, Smith KL, and Hastings MH (1999) Rapid resetting of the mammalian circadian clock. *J Neurosci* 19:828-835.
- Bünning E (1936) Die endogene Tagesperiodik als Grundlage der photoperiodischen Reaktion. *Ber Dtsch Bot Ges* 54:590-608.
- Chandrasekaran MK (1980) Apparent absence of a separate beta-oscillator in phasing the circadian rhythm of eclosion in *Drosophila pseudoobscura*. *Basic Life Sci* 16:417-424.
- Comas M, Beersma DG, Hut RA, and Daan S (2008) Circadian phase resetting in response to light-dark and dark-light transitions. *J Biol Rhythms* 23:425-434.
- Comas M, Beersma DG, Spoelstra K, and Daan S (2006) Phase and period responses of the circadian system of mice (*Mus musculus*) to light stimuli of different duration. *J Biol Rhythms* 21:362-372.
- Comas M, Beersma DG, Spoelstra K, and Daan S (2007) Circadian response reduction in light and response restoration in darkness: a "skeleton" light pulse PRC study in mice (*Mus musculus*). *J Biol Rhythms* 22:432-444.
- Czeisler CA, Duffy JF, Shanahan TL, Brown EN, Mitchell JF, Rimmer DW, Ronda JM, Silva EJ, Allan JS, Emens JS, et al. (1999) Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science* 284:2177-2181.
- Daan S (1977) Tonic and phase effects of light in the entrainment of circadian rhythms. *Ann N Y Acad Sci* 290:51-59.

- Daan S (2000) The Colin S. Pittendrigh Lecture. Colin Pittendrigh, Jürgen Aschoff, and the natural entrainment of circadian systems. *J Biol Rhythms* 15:195-207.
- Daan S and Pittendrigh CS (1976) A functional analysis of circadian pacemakers in nocturnal rodents. III. Heavy water and constant light: homeostasis of frequency? *J Comp Physiol A* 106:267-290.
- Dharmananda S (1980) Studies of the circadian clock of *Neurospora crassa*: light-induced phase shifting. PhD thesis, University of California, Santa Cruz.
- Elliott JA (1976) Circadian rhythms and photoperiodic time measurement in mammals. *Fed Proc* 35:2339-2346.
- Eriksson L-O and Veen TV (1980) Circadian rhythms in the brown bullhead, *Ictalurus nebulosus* (Teleostei): evidence for an endogenous rhythm in feeding, locomotor, and reaction time behaviour. *Canad J Zool* 58:1899-1907.
- Evans JA, Elliott JA, and Gorman MR (2004) Photoperiod differentially modulates photic and nonphotic phase response curves of hamsters. *Am J Physiol Regul Integr Comp Physiol* 286:R539-R546.
- Hastings JW and Sweeney BM (1958) A persistent diurnal rhythm of luminescence in *Gonyaulax polyedra*. *Biol Bull* 115:440-458.
- Hut RA, van Oort BE, and Daan S (1999) Natural entrainment without dawn and dusk: the case of the European ground squirrel (*Spermophilus citellus*). *J Biol Rhythms* 14:290-299.
- Johnson CH (1990) *PRC-Atlas*. Nashville (TN): Vanderbilt University.
- Kramm KR (1974) Phase control of circadian activity rhythms in ground squirrels. *Naturwissenschaften* 61:34.
- Lakin-Thomas PL, Brody S, and Coté GG (1991) Amplitude model for the effects of mutations and temperature on period and phase resetting of the *Neurospora* circadian oscillator. *J Biol Rhythms* 6:281-298.
- Leloup JC and Goldbeter A (2008) Modeling the circadian clock: from molecular mechanism to physiological disorders. *Bioessays* 30:590-600.
- Nelson DE and Takahashi JS (1999) Integration and saturation within the circadian photic entrainment pathway of hamsters. *Am J Physiol* 277:R1351-R1361.
- Pannella G (1972) Paleontological evidence on the Earth's rotational history since early precambrian. *Astrophys Space Sci* 16:212-237.
- Pittendrigh CS (1967) Circadian systems: I. The driving oscillation and its assay in *Drosophila pseudoobscura*. *Proc Natl Acad Sci U S A* 58:1762-1767.
- Pittendrigh CS, ed. (1981) *Circadian Systems: Entrainment*. New York: Plenum.
- Pittendrigh CS, Bruce V, and Kaus P (1958) On the significance of transients in daily rhythms. *Proc Natl Acad Sci U S A* 44:965-973.
- Pittendrigh CS and Daan S (1976a) A functional analysis of circadian pacemakers in nocturnal rodents: I. The stability and lability of circadian frequency. *J Comp Physiol A* 106:223-252.
- Pittendrigh CS and Daan S (1976b) A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J Comp Physiol A* 106:291-331.
- Pittendrigh CS, Elliott J, and Takamura T (1984) The circadian component in photoperiodic induction. In *Photoperiodic Regulation of Insect and Molluscan Hormones*, Porter R and Collins JM, eds, pp 26-47. London: Pitman.
- Rémi J, Merrow M, and Roenneberg T (2010) A circadian surface of entrainment: varying T , τ and photoperiod in *Neurospora crassa*. *J Biol Rhythms*. Forthcoming.
- Roberts S (1982) Circadian periodicity in cockroaches altered by high frequency light-dark cycles. *J Comp Physiol A* 146:255-259.
- Roenneberg T, ed. (1995) *The Effects of Light on the Gonyaulax Circadian System*. Chichester (UK): John Wiley & Sons.
- Roenneberg T, Chua EJ, Bernardo R, and Mendoza E (2008) Modelling biological rhythms. *Curr Biol* 18:826-835.
- Roenneberg T, Kumar CJ, and Merrow M (2007) The human circadian clock entrains to sun time. *Curr Biol* 17:R44-R45.
- Roenneberg T and Merrow M (2002) Life before the clock: modeling circadian evolution. *J Biol Rhythms* 17:495-505.
- Roenneberg T and Merrow M (2003) The network of time: understanding the molecular circadian system. *Curr Biol* 13:R198-R207.
- Roenneberg T and Merrow M (2007) Entrainment of the human circadian clock. *Cold Spring Harb Symp Quant Biol* 72:293-299.
- Roenneberg T, Rémi J, and Merrow M (2010) Modeling a circadian surface. *J Biol Rhythms*. Forthcoming.
- Scheer FA, Wright KP Jr., Kronauer RE, and Czeisler CA (2007) Plasticity of the intrinsic period of the human circadian timing system. *PLoS ONE* 2:e721.
- Subbaraj R and Chandrashekar MK (1981) Mirror imaging phase response curves obtained for the circadian rhythm of a bat with single steps of light and darkness. *J Interdiscipl Cycle Res* 12:305-312.
- Swade RH (1969) Circadian rhythms in fluctuating light cycles: toward a new model of entrainment. *J Theor Biol* 24:227-239.
- Taylor SR, Webb AB, Smith KS, Petzold LR, and Doyle FJ (2010) Velocity response curves support the role of continuous entrainment in circadian clocks. *J Biol Rhythms* 25:138-149.
- Wever RA (1989) Light effects on human circadian rhythms: a review of recent Andechs experiments. *J Biol Rhythms* 4:161-185.
- Wright KPJ, Hughes RJ, Kronauer RE, Dijk D-J, and Czeisler CA (2001) Intrinsic near-24-h pacemaker period determines limits of circadian entrainment to a weak synchronizer in humans. *Proc Natl Acad Sci U S A* 98:14027-14032.