CHRONOBIOLOGY INTERNATIONAL
Vol. 20, No. 5, pp. 741–774, 2003

REVIEW

Entrainment of Circadian Programs

Carl Hirschie Johnson,[1,*] Jeffrey A. Elliott,[2] and Russell Foster[3]

[1]Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee, USA
[2]Department of Psychiatry, University of California, San Diego, La Jolla, California, USA
[3]Department of Integrative & Molecular Neuroscience, Imperial College, London, Charing Cross Hospital, London, UK

ABSTRACT

Of the three defining properties of circadian rhythmicity—persisting free-running rhythm, temperature compensation, and entrainment—the last is often poorly understood by many chronobiologists. This paper gives an overview of entrainment. Where have we been? Where are we now? Whence should we be going? Particular emphasis is given to a discussion of the Discrete vs. Continuous models for entrainment. We provide an integrated mechanism for entrainment from a limit-cycle perspective.

Key Words: Entrainment; Continuous; Discrete; Parametric; Non-parametric; Limit cycles; Temperature compensation; Phase response curve; Aftereffects; Transients; Zeitgeber; Phase angle.

"Mechanism is not the biologist's only business. He is, or should be, concerned also with questions of both function and history."

Colin Pittendrigh (1966)

This article is about entrainment of circadian oscillators. It does not purport to be a comprehensive view, and we apologize in advance to many of you who have published

*Correspondence: Dr. Carl Johnson, Department of Biological Sciences, Box 1634-B, Vanderbilt University, Nashville, TN 37235, USA; Fax: 615-343-0336; E-mail: carl.h.johnson@vanderbilt.edu.

DOI: 10.1081/CBI-120024211
0742-0528 (Print); 1525-6073 (Online)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

important studies on this subject that we did not have space to include. This article is our attempt to describe *some* of the aspects of entrainment that we think are central and to point toward questions that we think future investigations concerning entrainment need to explain. A primary aim of this review is to highlight how powerful models of entrainment can be in helping researchers design approaches to look for and understand the molecular mechanisms of the clock. Throughout this review we have defined "Wanted" topics where we feel critical questions in circadian biology will benefit from a consideration of both theoretical and "wet-lab" approaches. Except for very general comments, this article will not describe photoreceptors, phototransduction pathways, or the molecular underpinnings of entrainment. These topics are admirably covered in a recent Special Topics Issue of the *Journal for Biological Rhythms* (volume 18, issue 3, June 2003).

Of the "big three" fundamental properties of circadian rhythmicity—persisting free-running rhythm, temperature compensation, and entrainment (Pittendrigh, 1960)—the last is often poorly understood by many chronobiologists. Unlike temperature compensation, about which we know practically nothing, there is a lot of information about entrainment. Nevertheless, many practicing chronobiologists appear not to understand some basic principles of entrainment. We all know something about photoreceptors and some of the transduction pathways, but many of us become uneasy at the first mention of a "phase angle." This is unfortunate, because the presumed Key Function of circadian clocks is to tell time, or to put it another way, to provide an internal estimate of external environmental time. In that context, entrainment is the most important fundamental property to understand. For example, entrainment theory may provide an explanation for the sleep/wake patterns of a human family exhibiting "familial advanced sleep phase syndrome" (FASPS) in which a mutation in the *hPer2* gene results in an early phasing of sleep (Toh et al., 2001). In this family, affected individuals go to sleep at about 19:30h and wake up about 03:00–4:00h. Although the basis of this example of FASPS is not yet clear, it might be due to a faster than average clock due to the mutation in *hPer2*.

Entrainment literally means "to get aboard a train" (originally from the French *entrainer*, "to carry along"). Therefore, entrainment means to be carried along by a "train" of controlling stimuli. In the specific context of this review, entrainment means that an internal biological clock with a free-running period (FRP) which is *not* exactly 24h is carried along so that its period conforms to the exactly 24h period of the environment. "Entrainment" is *not* the same as "synchronization," which implies that the waveform of the driving rhythm (the environment) coincides with the waveform of the driven rhythm (the internal clock). The biological clock is not necessarily *synchronized* to the environmental cycle when it is *entrained*. The consequences of entrainment are that the period of the biological rhythm becomes equal on average to that of the entraining stimuli and that a stable phase relationship is established between the entraining and entrained oscillations. An environmental stimulus that can act to entrain circadian clocks is called a "zeitgeber," from the German for "time giver" (Zeitgeber).

There are many papers in the chronobiological literature that purport to show entrainment but that are incomplete. For example, it is *not* enough to show a 24h rhythm in a light/dark cycle as a demonstration of entrainment. To establish that a zeitgeber cycle has indeed entrained the rhythm, it is necessary to show that (1) the period of the rhythm equals the period of the zeitgeber cycle with a stable, unique phase angle, and (2) that after "releasing" the organism from a zeitgeber cycle, the FRP resumes with a phase determined by the zeitgeber cycle. In some cases, a rhythm can appear to have been

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

entrained by periodic stimuli, but after removing the stimuli the free-running rhythm starts up from a phase that is not predictable from the exposure to the periodic stimuli. In such a case, the stimuli may have forced expression of the overt behavior without actually entraining the central pacemaker. This phenomenon is called "masking," and it can be a serious artifact of investigations that study entrainment. Masking is a complete topic in itself, and is admirably reviewed elsewhere (e.g., Mrosovsky, 1999).

One of the best ways to demonstrate entrainment is to test zeitgeber cycles with periods (T) that are not 24h in duration. These non-24-h cycles are called "T-cycles." When an organism appears to entrain to several different T-cycles with stable phase angles that are different and specific for each value of T, this is an excellent demonstration of entrainment (Aschoff, 1965b). On the other hand, if the observable rhythm shows a similar phase angle on different T-cycles, it is likely that the rhythm is due to masking rather than entrainment of an endogenous oscillator. The concept underlying this test will be explained further in Fig. 4. A recent paper used the T-cycle approach to demonstrate the existence of an unexpected oscillator in a strain of *Neurospora* that had previously been considered to be totally clock-null (Merrow et al., 1999). An up-to-date paper by Daan and Aschoff discusses the experimental criteria for entrainment in more detail (Daan and Aschoff, 2001).

OUR PERSPECTIVES

One of our perspectives is that the overriding function of circadian rhythms is to provide an internal estimate of the external local time, i.e., to allow the organism to program its activities to occur at an appropriate phase relationship, or *phase angle*, to the daily environmental cycle. In this paper, we will refer to this function—the conservation of phase angle—as the *Key Function* of circadian clocks. Another important function exhibited by some organisms is to accurately measure the ongoing lapse of time throughout the daily cycle, which is particularly relevant in the case of clock involvement in sun-compass orientation. In many organisms, a third function of the circadian clock is to provide a "measuring stick" to estimate the day- and/or night-length so that seasonal phenomena that depend upon estimating the time-of-year can be regulated appropriately. Circadian biologists often study organisms under free-running conditions, so we are apt to forget that the function of the circadian clock is not to provide a precise timekeeper that persists in *constant* conditions. All organisms (with the possible exception of organisms dwelling in caves or deep in the ocean) live in a rhythmic environment, so we must always consider the function of a circadian system in the real environment.

Our second perspective is to interpret circadian phenomena in the context of limit-cycle oscillator theory. There is considerable evidence that supports the conviction that circadian oscillators are limit-cycle oscillators (Jewett et al., 1991; Johnson and Kondo, 1992; Pavlidis, 1981; Peterson, 1980; 1981; Taylor et al., 1982; Winfree, 1970; 1971). As a result, our discussion of entrainment will often refer to limit-cycle explanations of the phenomena we describe below, especially in the context of molecular explanations. In particular, limit-cycle depictions of phase resetting can prove to be valuable in both the design and interpretation of experiments. We expect that an ultimate understanding of the phenomenology *and* molecular bases of entrainment will require limit cycle modeling of the responses of identified molecules to entraining stimuli. Based on a limit cycle model

proposed by Eric Peterson (Peterson, 1980), we provide the beginnings of such a model that attempts to integrate the discrete and continuous models of entrainment (see below, especially Fig. 7). Circadian limit-cycle oscillators are likely to be multidimensional, but in this paper we will consider only *two*-dimensional limit cycle models for simplicity. Even a two-dimensional limit cycle is helpful to illustrate the relevant concepts.

Phase Angle Is Influenced by the FRP and Temperature Compensation

Entrainment per se is not the only one of the "big three" properties of circadian rhythms that influences phase angle; the other salient characteristics of circadian clocks are also crucial. The first fundamental property—that circadian clocks have an endogenous period (FRP) that is not 24h is the basis for the name *circa*dian—from the Latin for "about" (*circa*) and "day" (*dies*). Why do circadian clocks have FRPs that are close to 24h? In general, entrainment between two oscillators will be optimal if their inherent frequencies are nearly the same. In the case of circadian oscillators, the driving oscillation is the 24h cycle resulting from the earth's rotation. Therefore, to entrain stably to the 24h cycle, the driven circadian oscillator needs to have an intrinsic frequency that is consonant with the earth's daily whirl.

Usually, circadian FRPs are exquisitely precise from day to day. This should not be taken to mean, however, that the FRP is rigid and invariable. Indeed, in most organisms there appears to be a range of allowed values of the FRP within which the FRP can be modulated, particularly in response to environmental conditions. For example, the LD cycle to which a circadian clock is exposed can influence the FRP, as can be gauged by measuring the FRP after releasing the organism from an LD cycle into constant conditions. Light/dark cycles of different photoperiods or different periods ($=$"T") can have profound "aftereffects" on the subsequent FRP in constant conditions (Aschoff 1960; 1981; Pittendrigh, 1960; Pittendrigh and Daan, 1976a). Other conditions that can cause aftereffects include the ambient temperature, light intensity (in LL), developmental stage, and prior history (Aschoff, 1960; 1981; Barrett and Page, 1989; Eskin, 1971). Aftereffects usually decay in time after transfer of the organism to constant conditions.

***Wanted**: a cellular/molecular explanation of aftereffects. Because the FRP is central toward calculating phase angle (see below), aftereffects are likely to be very important in the entrainment of circadian clocks under natural conditions, especially in the context of seasonal tracking. Nevertheless, there is zero information on how aftereffects might be explained at the molecular and/or cellular levels.*

Therefore, circadian FRPs are precise and close to 24h. Why are they not closer to 24h? In other words, why are circadian clocks *circa*-24h? A probable reason why most circadian systems have FRPs that are significantly different from 24h (i.e., usually between 23 and 25h) is discussed later, in the section entitled, "Why are circadian clocks 'circa' 24h?".

The second fundamental property—temperature compensation—is also crucial for conservation of phase angle (Pittendrigh, 1993). Circadian biologists are fond of saying that the biological clock is temperature compensated because if it were not, then the "clock" would only be good as a thermometer, not as a timekeeper. What does this statement *really* mean, however, given that temperature pulses/cycles can entrain the

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

circadian clock? In other words, what is the function of temperature compensation of the *period* if the *phase* of the clock can be modified by temperature transitions? Returning to our Key Function, the answer probably lies in buffering the biochemistry of the clock against running faster or slower when the temperature changes so that an appropriate phase angle to the solar day is conserved in the presence of day-to-day temperature variations.

For example, if the period of a circadian clock were not temperature compensated, consider what would happen to its entrainment on two successive days, a warm day followed by a cool day. The *phase* of the temperature cycle is the same on both days (therefore, there might be no difference in the entraining input of the temperature cycle), but the average level of temperature is different. If the clock's period were not compensated, it would run fast on the first day (warm day), whereas it would run slower on the second day (cool day). Because the value of FRP is critical for determining the phase angle under entrainment (see below), shortening of the FRP on day 1 vs. day 2 would advance the *phase* of the circadian pacemaker on day 1 vs. day 2, merely because the average temperature was higher. Therefore, our first Key Function—conservation of phase angle—would be violated if the period of the oscillator were not temperature compensated.

Wanted*: a molecular explanation for temperature compensation. Temperature compensation is the property that put biological clocks "on the map" with the characterization by Colin Pittendrigh (Pittendrigh, 1954). Nevertheless, there is no successful molecular explanation for temperature compensation. The lack of an explanation for the mechanism(s) of temperature compensation is a partial reason why some early researchers of daily rhythms believed that circadian phenomena had to be exogenously driven.*

RHYTHMIC ENVIRONMENTAL STIMULI ENTRAIN CIRCADIAN CLOCKS

Entrainment is the "big three" property that is most essential to our Key Function: a clock providing an internal estimate of external local time. To estimate local time, the internal timekeeper must be entrained, or synchronized, to the 24h environmental cycle. The consequences of entrainment are that the period of the biological rhythm becomes equal on average to that of the entraining stimuli with a stable phase relationship (or phase angle) between the entraining and entrained oscillations. Entrainment can occur by modulation of the *period* and/or *phase* of the biological rhythm so that its period conforms to the period of the environment (Aschoff 1960; 1981; Daan and Aschoff, 2001; Pittendrigh, 1981b).

In nature, multiple environmental factors oscillate over the daily cycle, including light and darkness, temperature, humidity, food availability, and social cues. Some of these factors can function as zeitgebers. The most consistent environmental time cue is the 24h cycle of light and darkness, and almost all circadian rhythms can be entrained to LD cycles. This article will focus upon LD signals because they are likely to be very significant environmental determinants of phase angle and because most information about entrainment derives from studies using LD cycles. The principles described here for entrainment by LD stimuli can be applied to any potential zeitgeber. Multiple zeitgebers

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

exist in nature, however, and a selection of non-photic zeitgebers will be discussed briefly in the final section of this paper.

Light influences phase, but it is also important to remember that illumination affects both the period and amplitude of circadian rhythms as well. For example, high-amplitude rhythms of most photosynthetic organisms are optimally expressed in LL, while LL tends to repress the amplitude of circadian rhythms in many other organisms. Jurgen Aschoff collected much data that demonstrated that the FRP in LL is a function of the intensity of illumination (Aschoff, 1960; 1981). Additionally, he observed that increasing the light intensity tends to produce on average a net acceleration of the rhythms of day-active vertebrates, while it slows those of night-active vertebrates. This intriguing correlation has come to be known as *Aschoff's Rule*. The effects of LL on the angular velocity of circadian rhythms may derive from light accelerating the clock at some phases and slowing it at other phases, as described by so-called "Velocity Response Curves" (Daan and Pittendrigh, 1976).

TWO DIFFERENT MODELS HAVE BEEN PROPOSED TO EXPLAIN CIRCADIAN ENTRAINMENT

As mentioned above, entrainment to a LD zeitgeber means that the period of the biological rhythm becomes equal to that of the LD cycle with a stable phase relationship between the LD cycle and the biological rhythm. But which aspect of an LD cycle in nature is responsible for entraining the biological rhythm? Is it the dawn and dusk transitions, the increase and decrease of light intensity during the daytime, the changes in spectral quality (i.e., color) of light, the continuous presence of light during the daytime, or some other factor? (Roenneberg and Foster, 1997).

Two major classes of models have been proposed to explain the mechanism(s) by which circadian clocks are entrained to environmental cycles: the discrete (also called non-parametric or phasic) and the continuous (also called parametric or tonic) (Aschoff, 1960; Bruce, 1960; Daan, 2000; 1977; Daan and Aschoff, 2001; Pittendrigh, 1966; 1981b; Pittendrigh and Minis, 1964; Pittendrigh and Daan, 1976b; Swade, 1969). The *continuous* entrainment model has been based on the observation by Aschoff that the FRP is dependent upon light intensity, and suggests that light has a continuous action on the clock to entrain it to the LD cycle. One mechanism that has been suggested is that the acceleration and deceleration of FRP (i.e., angular velocity) by daily changes in light intensity could allow the circadian pacemaker to continuously adjust its cycle length to that of the environment. Entrainment achieved by modulation of FRP has sometimes been called "parametric entrainment," but this term does not conform to the formal mathematical definition of parametric entrainment. For this reason, and also because there are other mechanisms by which light could continuously entrain circadian oscillators (see below), we will follow others in using the more general term, continuous entrainment. Aschoff was a strong advocate of continuous entrainment, and its potential involvement in circadian entrainment will be addressed later (Aschoff, 1960; 1999; Daan and Aschoff, 2001).

The *discrete* model was championed by Pittendrigh. It has been the most successful model to date in predicting the entrainment of some organisms, most notably *Drosophila* and nocturnal rodents, and will therefore be described in detail (Pittendrigh, 1966; 1981b; Pittendrigh and Minis, 1964; Pittendrigh and Daan, 1976b). The basic premise of the

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

model is that an entrained circadian pacemaker is in equilibrium with an LD cycle consisting of repetitive light pulses (the zeitgeber). That equilibrium is achieved when each light pulse falls at a phase so as to elicit a phase shift that is equal to the difference between the FRP and the period of the entraining cycle (T). In nature, the zeitgeber is the dawn and dusk transitions, which can be mimicked in the laboratory by brief light pulses. Because the effective action of light is considered to be due essentially to discrete time cues, e.g., in nature at dawn and/or dusk, this mechanism of entrainment has been called the discrete or nonparametric model. The earliest incarnations of the Discrete model assumed that the FRP was a constant, but later reincarnations allowed FRP to vary (Pittendrigh and Daan, 1976b). The elegant simplicity of this model lies in its excellent predictive properties based on only two pieces of information: the FRP, and the map of phase-dependent resetting called the phase response curve (PRC) (Aschoff, 1965a; DeCoursey, 1960; Johnson, 1999; Pittendrigh, 1960; Pittendrigh and Minis, 1964).

PHASE RESPONSE CURVES MAP THE PHASE-DEPENDENT RESPONSES OF CIRCADIAN CLOCKS TO ZEITGEBERS

According to the discrete model, the circadian oscillator must respond differently to light at different phases of its cycle to entrain to the daily light/dark cycle. Phase response curves are useful descriptions of this phase-dependent response. A PRC is a plot of phase shifts of a circadian rhythm as a function of the circadian phase that a stimulus, or zeitgeber, is given (Aschoff, 1965a; Johnson, 1999; Pittendrigh and Minis, 1964). Light pulses presented in the subjective day (CT 0–12) have little or no effect on the onset of activity (phase of activity rhythm) on subsequent days. This is a characteristic feature of PRCs: light has less phase-resetting efficacy during the organism's subjective day than during its subjective night. This subjective-day portion of the PRC during which little phase-shifting can be elicited is often referred to as the dead zone (Fig. 1A).

By contrast, light presented during the organism's subjective night will usually phase-shift the free-running rhythm. During the first half of the subjective night, light pulses phase delay the activity rhythm (i.e., subsequent activity starts later, plotted as $-\Delta\phi$). During the second half of the subjective night, light pulses will phase advance the activity rhythm (i.e., activity starts earlier, plotted as $+\Delta\phi$). A PRC is constructed by plotting the phase shifts (advance and delays) against the circadian time of light-pulse administration (Fig. 1A). It is important to note that the precise waveform of the PRC can vary greatly depending upon the strength of the zeitgeber stimulus (intensity and/or duration), the amount of time an organism has been in constant conditions (DD or LL), and previous photoperiodic history (Pittendrigh, 1981b, Pittendrigh et al., 1984).

In many multicellular organisms, there is often transient behavior in the first few cycles after a perturbation by a light or temperature stimulus [Pittendrigh, 1960; Pittendrigh and Bruce, 1959; Pittendrigh et al., 1958; (intriguingly, transients are rare in unicellular organisms)]. Because the underlying pacemaker—as judged by the resetting of the PRC—appears to reset rapidly after these perturbations, these transient cycles are thought to reflect disequilibrium (e.g., altered phase angle) between the overt rhythm and the pacemaker in response to a phase shift (Pittendrigh, 1981a,b). Such transients are usually more common after stimuli that provoke phase advances. The phase shifts used in constructing a steady-state PRC should be calculated after the transients have subsided and

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

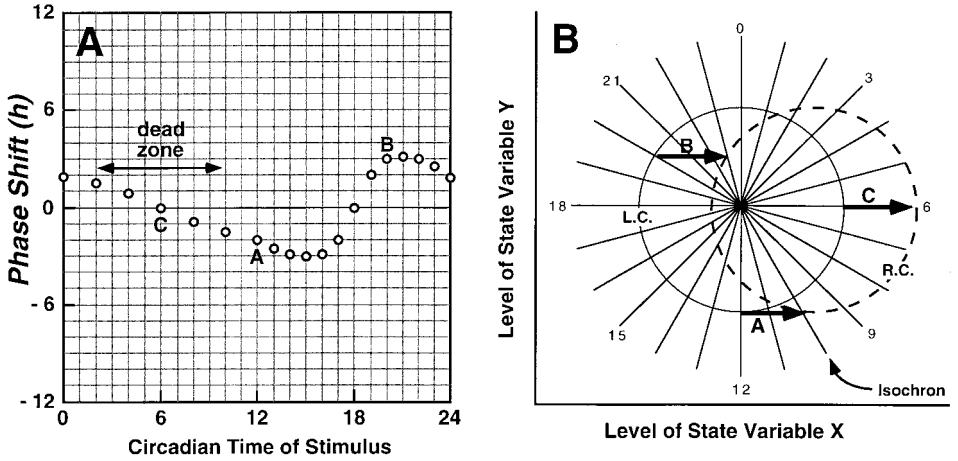


Figure 1. A limit-cycle interpretation of PRCs. Panel A: A representative Type 1 phase response curve, plotted as circadian time of the stimulus (abscissa) vs. phase shift in hour (ordinate). Panel B: Limit-cycle interpretation of the PRC depicted in panel A. Stimuli (illustrated as the bold arrows) are postulated to increase the value of state variable *X*. These stimuli move the state variables that define the oscillator from the limit cycle (L.C.) to a point on the resetting curve (R.C.). After the stimulus ends, the state variables oscillate back to the limit cycle from the resetting curve in phase with oscillators that are also on the same isochron. If the oscillator has been moved to a different isochron by a stimulus (as in the examples A and B) a steady-state phase shift is obtained. The phase shifts resulting from the example stimuli shown are a 2h delay (A) or a 3h advance (B). On the other hand, if the stimulus moves the oscillator to another portion of the phase plane but *on the same isochron* (e.g., example C), no phase shift results.

a stable FRP is established. For simplicity, transients will not be considered further herein. From now on, we will assume that the observed rhythm phase shifts as rapidly as does the central oscillator.

Wanted*: a physiological/molecular explanation of transients. Pittendrigh provided extensive formal evidence for the postulate that transients were a result of hierarchical multioscillator organization (Pittendrigh, 1960; 1981a,b; Pittendrigh and Bruce, 1959). However, physiological/molecular evidence for this interpretation of transients is lacking. Transients are crucially important as they relate to human jet lag, shift work, alertness, and performance. With new evidence that circadian oscillations of gene expression occur in peripheral tissue and of differential responses of that expression to shifted zeitgeber cycles (Abe et al., 2002; Yamazaki et al., 2000), new investigations into the physiological and molecular bases of transients are needed (for an excellent recent example, see Reddy et al., 2002).*

There is considerable evidence for multioscillator organization of circadian systems, both from behavorial studies and physiological studies of, e.g., the suprachiasmatic nuclei (Shirakawa et al., 2001). The multioscillator structure of circadian pacemakers certainly has a significant impact upon entrainment properties as elegantly argued by Pittendrigh

and Daan in formulating the Evening–Morning model of the circadian pacemaker (Pittendrigh and Daan, 1976c). An in-depth discussion of the impact of this multioscillator pacemaker structure on entrainment is beyond the scope of this review, which will operate from the simpler assumption that circadian systems are driven by a single oscillator.

In the natural environment, dawn and dusk are characterized by gradual changes in light intensity. Most laboratory studies of entrainment have used light stimuli with abrupt lights-on and lights-off transitions. In general, these step changes for lights-on and lights-off appear to mimic entrainment under natural conditions. A recent study of entrainment in hamsters, however, showed that the range of *T*-cycles to which a hamster can entrain is larger if the dawn and dusk transitions are gradual, as they are in nature (Boulos et al., 2002). This effect of gradual lights-on and lights-off transitions suggests the participation of continuous entrainment in nature (see below).

PRCs CAN BE MODELED IN TERMS OF LIMIT CYCLES

Limit-cycle oscillators are those whose state variables oscillate in phase space around a stable trajectory (the "limit cycle") like horses trotting around a racetrack. If the oscillator is perturbed such that the state variables are "pushed off" the limit cycle (= the "track"), they will return to the limit cycle. In the case of such a perturbation, the state variables will return in phase with a specific point on the limit cycle, and the set of points in phase space that specify values for the state variables which will return to the limit cycle in phase are called isochrons. A less precise, but more intuitive definition of an isochron is those points in phase space that are at the same time (iso = same; chronos = time).

In the application of limit-cycle modeling to biological oscillators, the fundamental concept is that some core clock components cycle. Consequently, the rhythmic process can be described by a system of differential equations in which the oscillating components are "state variables." The equations also include state parameters that establish the amplitude and period of the oscillations (Johnson, 1999; Lakin-Thomas, 1995; Peterson, 1980; Winfree, 1970; Johnson et al., 2003). In a constant environment, the parameters can be constant while the levels of the state variables oscillate periodically. External stimuli (e.g., zeitgebers) may reset a limit-cycle oscillator by changing the parameters (parametric excitation), or additional terms in the differential equations may allow direct changes of the state variables. Thus, state variables could change rapidly in response to excitation by zeitgebers.

The limit-cycle interpretation of phase resetting that is presented here assumes that zeitgebers change the value(s) of one or more state variables (X and/or Y), so that the oscillator is perturbed from the limit cycle to some other position in phase space. If this change moves the state variables from one isochron to a different isochron, a steady-state phase shift will be observed (Johnson, 1999; Lakin-Thomas, 1995; Peterson, 1980; Winfree, 1970; Johnson et al., 2003). Why? A phase shift occurs because as the state variables move back to the limit cycle, they reach it at a different phase than they would have if no perturbation had occurred. In Fig. 1B, light is postulated to increase the value of variable X (Y is insensitive to brief light stimuli). A light pulse given at CT 12 moves the state variables from the CT 12 isochron to the CT 10 isochron ("A" on Fig. 1B). The state variables spiral back to the limit cycle in phase with an oscillator at CT 10, evoking a 2h phase delay. A light pulse at CT 20 moves variable X the same amount, but this time the

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

oscillator is reset to the isochron of CT 23, eliciting a phase advance of 3h ("B" in Fig. 1B). When this analysis is applied to many phases around the limit cycle, a "resetting contour" can be derived that shows the positions of the state variables immediately after the resetting stimulus (dashed circle in Fig. 1B).

It might be assumed that stimuli presented at phases in the dead zone would not modify the state variables, since no phase shift results from stimuli administered in the dead zone. While this can be true for some specific models, it is not a necessity of a limit-cycle model. The other, equally plausible, alternative is that stimuli presented during the dead zone induce changes of the state variables, but these altered values move the variables approximately along the original isochron ("C" in Fig. 1B). Therefore, no steady-state phase shift results. Consequently, state variables of the oscillator are not necessarily insensitive to the stimulus during the dead zone—in fact, the stimulus could induce large changes of the state variables, but these changes do not move the oscillator to a different isochron. This insight gleaned from limit-cycle modeling has important implications for identifying molecular correlates of state variables. In particular, we should not make as a criterion for a state variable of the clockwork that its responsiveness to phase-resetting stimuli must correlate directly with the magnitude of phase-shifting at every phase—especially at phases in the dead zone (Johnson, 1999).

PRC AMPLITUDE: TYPE 1 AND TYPE 0 PRCs

As illustrated in Fig. 2A and C, there are two "types" of PRCs—Type 1 and Type 0. Type 1 PRCs display relatively small phase shifts (e.g., usually less than 6h phase shifts) and have a continuous transition between delays and advances, whereas Type 0 PRCs show large phase shifts. If the phase shifts of a Type 0 PRC are plotted as advances and delays, a discontinuity (the "breakpoint") often appears at the transition between delay and advance phase shifts. The breakpoint discontinuity of Type 0 PRCs is in some cases merely a plotting convention of arbitrarily assigning phase shifts as delays vs. advances. The basis for the names Type 1 and Type 0, and the breakpoint convention is discussed elsewhere (Johnson, 1999; Winfree, 1980).

Whether Type 1 or Type 0 resetting is exhibited often depends upon the strength of the stimulus. For example, it has been shown in the flies *Drosophila* and *Sarcophaga*, and in the mosquito *Culex* that increasing the light dose of the stimulus converts Type 1 into Type 0 resetting (Peterson, 1980; Pittendrigh, 1960; Saunders, 1978; Winfree, 1970). Similar results have been observed for temperature pulses of different step sizes (e.g., Gooch et al., 1994). A limit-cycle interpretation of Type 1 vs. Type 0 resetting is illustrated in Fig. 2B. Type 1 resetting is expected if the resetting contour is not moved beyond the central point from which the isochrons radiate. This central point is called the "singularity." In other words, the resetting contour encloses the singularity in Type 1 resetting. On the other hand, Type 0 resetting occurs if the stimulus is strong enough to move the variables beyond the singularity (e.g., stimulus #2, resulting in resetting contour #2). In this case, the resetting contour does not enclose the singularity. Visualizing phase shifting from the limit-cycle perspective is valuable in that it illustrates how a stimulus that changes the value of a state variable by an equivalent amount at every phase could cause a wide variety of observable phase-shifting behaviors: delay vs. advances, breakpoints, and an apparently discontinuous switch between Type 1 and Type 0 resetting. Visualization of the

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

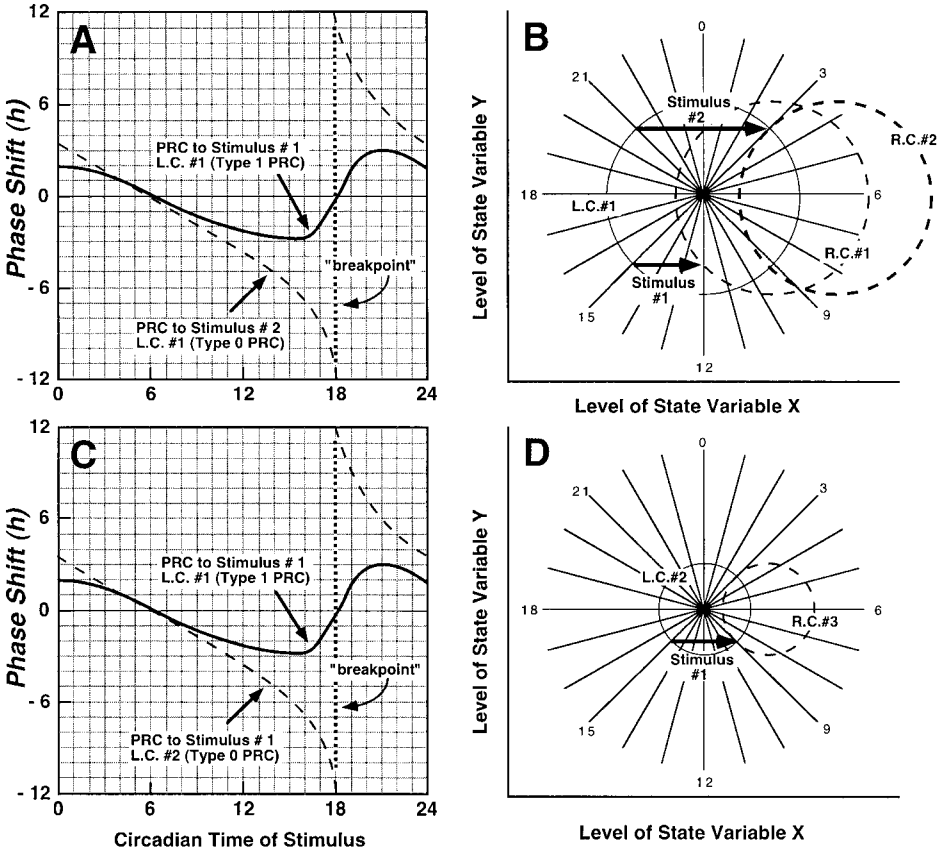


Figure 2. Type 1 and Type 0 PRCs; limit-cycle interpretations. Panel A: Type 1 (lower amplitude) and Type 0 (higher amplitude) PRCs generated by stimuli of different strengths, as illustrated in Panel B. When the phase shifts of Type 0 PRCs are plotted as advances and delays (−12h delays, +12h advances), there is often a "breakpoint" at the transition between delays and advances. Panel B: Limit-cycle interpretation of the PRC Types illustrated in Panel A. Given the same limit cycle, a weaker stimulus (Stimulus #1) that does not displace the resetting curve beyond the singular region (R.C. #1) results in Type 1 resetting. On the other hand, a slightly stronger stimulus (Stimulus #2) that moves the resetting curve beyond the singular region (R.C. #2) will result in Type 0 resetting. Panel C: Another way to effect a transition between Type 1 and Type 0 resetting is by changing the diameter of the limit cycle. Panel C replots the data from panel A for the limit cycle #1 subjected to the weaker stimulus (Stimulus #1), and compares it with a Type 0 PRC obtained with the same stimulus acting upon a smaller diameter limit cycle. Panel D: Limit-cycle interpretation of the Type 0 PRC illustrated in Panel C. A smaller-diameter limit cycle (L.C. #2) subjected to a weak stimulus (Stimulus #1) will nevertheless exhibit Type 0 resetting if the diameter of the limit cycle is small enough to allow the resetting curve to move beyond the singular region (R.C. #3).

PRC alone might lead to the fallacious reasoning that delay phase shifts must be mechanistically different from advance phase shifts, e.g., that advance phase shifts result from pacemaker components being changed in one direction, while delay phase shifts change components in the opposite direction. Limit-cycle models suggest that mechanistic differences between advances and delays are not a necessary conclusion, as is the case for some other kinds of oscillators (Johnson, 1999). Moreover, limit-cycle models interpret that the transition from Type 1 to Type 0 resetting can follow simply and directly from an increase in the magnitude of the stimulus so that it shifts the resetting contour beyond the singularity (Johnson, 1999; Lakin-Thomas, 1995).

In addition to increasing stimulus strength, another way in which the transition from Type 1 to Type 0 resetting could occur is by reducing the amplitude of the oscillator (Johnson, 1999; Lakin-Thomas, 1995). An amplitude reduction may be visualized in a limit cycle model by a smaller diameter limit cycle (i.e., the peak-to-trough oscillation of the state variables is smaller, therefore the limit cycle is smaller). A stimulus that causes an equal change of state variables might lead to Type 0 resetting for a limit cycle that has a small diameter while the same stimulus could elicit only Type 1 resetting for a larger diameter limit cycle. Why? Because a stimulus that displaces the state variables by an amount sufficient to move a small-diameter limit-cycle oscillator beyond the singularity (therefore, Type 0 resetting) could fail to move the variables in a large-diameter limit-cycle beyond the singular region (therefore, Type 1 resetting), as illustrated in Fig. 2.

Therefore, a prediction that can be drawn from the limit-cycle interpretation of phase-shifting is that oscillator systems which differ only in the amplitude of the oscillating state variables (and therefore the diameter of the limit cycles) may show very different PRCs to a stimulus that elicits equal changes in the level of the state variable(s). The tim^{UL} mutant of *Drosophila melanogaster* may provide an example of this prediction (Rothenfluh et al., 2000). In this mutant, the amplitude of the oscillation of two presumptive state variables (the levels of *per* and *tim* mRNAs) is significantly less than in wild-type flies. This result is consistent with the interpretation that the diameter of the limit-cycle in tim^{UL} flies is smaller than for wild-type flies. The PRCs for wild-type and tim^{UL} flies to 10 min light pulses show the predicted result: the wild-type PRC is Type 1 (no phase shifts greater than 4h), while the tim^{UL} PRC is clearly Type 0 (10h advances, −6h delays) (Rothenfluh et al., 2000). [In *Drosophila*, light *reduces* the level of TIM protein (Williams and Sehgal, 2001), whereas light increases the level of *frq* mRNA in *Neurospora* (Crosthwaite et al., 1995), but the concept described here applies to both.] The tim^{UL} case is an excellent example of classical phase-shifting experiments and state-of-the-art molecular approaches converging upon explanations based on modeling insights.

***Wanted**: molecular explanations for phase shifting in the context of limit-cycle models. Molecules whose abundance and/or modification status (e.g., phosphorylation) are thought to act as molecular correlates of state variables should be studied as a function of circadian time and before/after phase-shifting stimuli. These data should be plotted as limit cycles for freeruns and as resetting contours for responsiveness to perturbation. Some work exists along these lines in which limit-cycle explanations are mentioned (e.g., for Neurospora: Ruoff et al., 2001), but there remains an extreme reluctance to publish data on molecular oscillations in terms of limit-cycle maps. We must overcome this reluctance.*

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

A final note: circadian oscillators (indeed, all limit-cycle oscillators) are nonlinear oscillators. This means that their responses to incremental increases in stimulus strength will not necessarily be linear. Frequently fluence (for light) and/or dose (for drugs) responses to resetting stimuli have been observed to be discontinuous for circadian clocks, especially when in the range of strength that is close to the Type 1-to-Type 0 transition (Dharmananda, 1981; Johnson and Hastings, 1989; Johnson and Kondo, 1992; Taylor et al., 1982). A discontinuous fluence response curve may mean that some process "downstream" from the photo-pigment's absorption of light is converting the initially continuous photochemical response into a discontinuous biological response. For circadian rhythms, limit-cycle organization is likely to be responsible for converting the initially monotonic response into a discontinuous response as the light pulse moves the pacemaker past the singular region. Because of their nonlinear basis, PRCs ought not be "added" or "subtracted," or otherwise manipulated from the viewpoint of "linear thinking."

ENTRAINMENT BEHAVIOR OF MANY ORGANISMS CAN BE PREDICTED FROM THE PRC AND FRP BY THE "DISCRETE ENTRAINMENT MODEL"

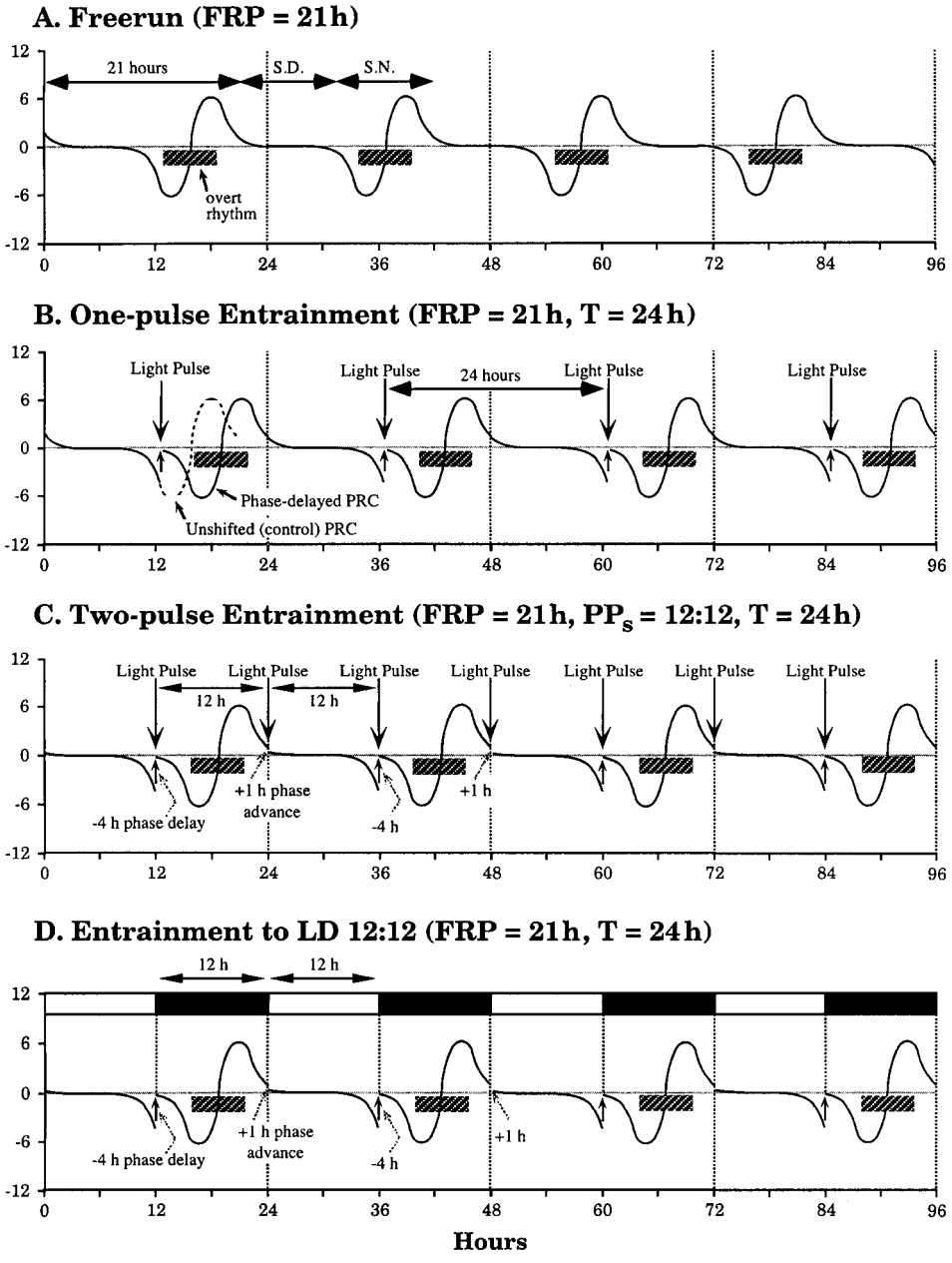
A PRC is a map of the phase-dependent responsiveness of a circadian pacemaker. As mentioned above, this map resets rapidly in response to light stimuli (within one cycle), whereas overt rhythms often require many *transient* cycles to attain a steady-state phase shift. The discrete entrainment model uses the light PRC as a tool to predict entrainment behavior. In the discussion of the discrete model that follows, the predictions of entrainment will be limited to steady-state entrainment. That is, transients will be ignored and the steady-state PRC will be used to make predictions of entrainment behavior. It is important to keep in mind two key assumptions of the model: (1) that the FRP measured in constant conditions accurately reflects the circadian period functioning under entrained conditions and (2) that the stimuli used in entrainment and to derive the PRC are effectively the same. Because the shape and/or amplitude of the PRC is often a function of the strength/duration/type of stimulus, the *same* stimulus used for modeling entrainment must be used to derive the PRC used for modeling.

For one light pulse per cycle, the discrete entrainment model proposes that the circadian pacemaker is entrained solely by light pulses which fall at that phase of the pacemaker specified by the PRC such that a phase shift is evoked which is equal in magnitude to the difference between the FRP and the period of the entraining cycle (i.e., T) (Pittendrigh, 1981b; Pittendrigh and Minis, 1964). In other words,

$$\text{Phase shift} = \text{FRP} - T$$

For example, if the FRP is 23h, then the pacemaker must experience a net delay phase shift of 1h (−1h) in order to entrain to a 24h LD cycle. For a FRP of 21h, the phase shift in steady-state must be a delay of 3h, which will be accomplished by the light pulse striking the PRC in the early subjective night (Fig. 3B). Conversely, for a FRP of 27h, the steady-state phase shift must be an advance of 3h, so that the light pulse will strike the PRC in the late subjective night. Because the light pulse must strike a different phase of the pacemaker (as gauged by the PRC) for different FRPs to achieve steady-state entrainment to

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016



MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

a LD cycle of 24h, the phase angle (or phase relationship) between the entraining light pulse and a given pacemaker must be different for different FRPs, as shown in Fig. 4A and B.

These predictions have been upheld by studies in which organisms whose FRP has been changed by mutation are entrained to LD cycles. For example, the *tau* mutation of hamsters changes FRP from a wild-type value of 24h to a mutant value of 20h (in homozygotes). As predicted, homozygous *tau* hamsters entrain to LD cycles with a significantly earlier phase angle than do wild-type hamsters (Ralph and Menaker, 1988). Mutant *Drosophila melanogaster* flies that are entrained to LD exhibit phase relationships as predicted by the theory depicted in Fig. 4—evening activity peaks occur earlier in short-period flies and later in long-period flies than in wild-type flies (Hamblen-Coyle et al., 1992). Even lowly pond scum (cyanobacteria) display these same entrainment characteristics (Ouyang et al., 1998), clearly indicating that these patterns of entrainment span phylogenetic divisions. Because a stable phase relationship of clock to zeitgeber cycle depends on a FRP that is consistent from cycle to cycle, our Key Function of circadian timekeepers predicts that the FRP must be relatively stable. In general, that prediction is upheld, but as mentioned above, there are a number of factors that can modulate the FRP within a narrow range and thereby affect the phase angle of clock to zeitgeber. To some extent, the PRC acts as an internal compensator for small day-to-day variability in FRP—for example, on a day in which the FRP is a little longer or shorter than usual, the zeitgeber will strike the PRC at a phase that will elicit a slightly larger or smaller phase shift, thereby counterbalancing the effect of the period change (Pittendrigh and Daan, 1976b).

Figure 3. Depiction of circadian entrainment using PRC diagrams. The impact of phase-resetting light pulses on the time course of circadian PRCs and overt activity rhythms is shown. The examples shown in this figure are for a circadian oscillator of a night-active organism which free-runs in DD with an FRP = 21h. Abscissa, hours; ordinates, phase shifts of the PRCs in hours (advances = +values, delays = −values). Panel A: Free-running of PRC and overt rhythm with an FRP = 21h. Notice that the PRC and rhythm recur earlier each day as compared with the abscissa. "S.D." = subjective day; "S.N." = subjective night. Panel B: "One-pulse" entrainment. A discrete light pulse is administered to the same organism (FRP = 21h) once every 24h. In the first cycle, a control "unshifted PRC" is shown to emphasize the phase shift in the experimental PRC. After entrainment has stabilized, the light pulse will occur at a circadian phase such that the steady-state phase shift will be a 3h delay in every cycle. In the case shown in this figure, the light pulse strikes the PRC at approximately CT 13. Notice that under entrainment, the phase of the PRC and rhythm is the same each day relative to the abscissa. Panel C: "Two-pulse" entrainment by a "skeleton" photoperiod of LD 12 : 12 (= PP_s 12 : 12). A discrete light pulse is administered once every 12h to the same organism as shown in Panels A and B. After entrainment has stabilized, the light pulses will occur at circadian phases such that the *net* steady-state phase shift will be a 3h delay in every cycle. In the case shown in this figure, the net phase shift is achieved by a 4h delay (by one light pulse striking the PRC in the early subjective night) combined with a 1h advance (by the other light pulse striking the PRC at the beginning of the subjective day) in each cycle. Again, notice that under entrainment by this skeleton photoperiod, the phase of the PRC and rhythm is the same each day relative to the abscissa. Panel D: Entrainment of the same organism to a complete photoperiod (LD 12 : 12) as predicted by the discrete model of entrainment. In this case, the dawn transition is postulated to cause a discrete 1h phase advance, while the dusk transition is thought to elicit a discrete 4h delay. Again, the net phase shift is a 3h delay, which enables an oscillator with an FRP = 21 to entrain to a $T = 24$h. The bars across the top of the panel illustrate the light/dark cycle: white bars are the 12h of light, black bars are the 12h of darkness.

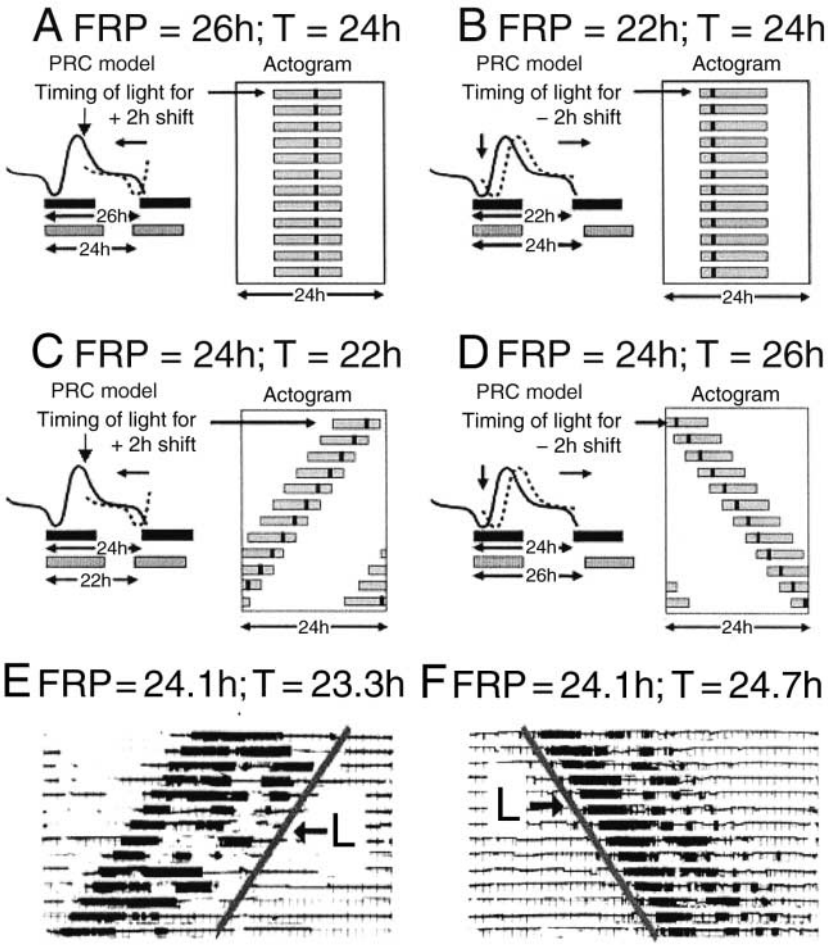


Figure 4. The phase relationship (i.e., phase angle) of a circadian rhythm under entrainment to a particular light/dark cycle can be predicted from the PRC, *T*, and FRP. This figure illustrates how the phase shift needed for stable entrainment (as predicted by the equation: phase shift = FRP− *T*) varies as a function of FRP (panels A and B) or *T* (panels C–F). In each panel A–D, the left side depicts a PRC explanation for entrainment. The solid-line PRC with the filled bars underneath represents the timing of overt activity (for a night-active organism) under free-running conditions. Under entrainment, the action of light at the indicated phase angle is represented by the dotted-line PRC, which shows the phase shift, and by the shaded bars representing activity. The right side of each panel is a simulated 12 day actogram with activity (shaded bars) and the timing of entraining light pulses (short solid bars) presented cyclically with period (*T*). Panel A: Entrainment of a circadian rhythm with FRP = 26h to a light pulse cycle with period *T* = 24h. Stable entrainment requires that the light pulse occur in the late subjective night where it elicits a phase advance of +2h. Panel B: For stable entrainment of a rhythm with FRP = 22h to a light pulse cycle of *T* = 24h, the light pulse will fall in the early subjective night to elicit a delay phase shift of −2h. Panels C and D: The same interdependency of phase angle on both FRP and *T* holds for entrainment to light pulse cycles with *T* either shorter (C) or longer (D) than 24h. Panels E and F: Real data, showing nocturnal wheel running activity (black bars) entrained to *T* = 23.3h and *T* = 24.7h. The gray lines connect midpoints of 1h light pulses (data from Elliott, 1974).

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

In addition to changing the FRP under entrainment to a fixed light cycle, the phase angle between driving light cycle and driven circadian clock can be easily modulated in the laboratory by simply changing the period of the light cycle, *T* (Aschoff, 1981; Elliott 1976; 1981; Pittendrigh and Daan, 1976b). These exotic light cycles, which have no corollary in the natural environment, are called *T*-cycles. Thus, a 24h FRP that is entrained by a 22h light/dark cycle must phase shift by advancing 2h in each cycle. Again, the light pulse will be expected to strike the late subjective night region of the PRC in order to achieve a 2h advance shift (Fig. 4C). For a given FRP and PRC, the phase angle between the light/dark cycle and oscillator will vary as a function of *T*, as shown in Fig. 4C–F. As mentioned previously, the variance of phase angle with varying *T* was used to unveil the existence of an unexpected oscillator in mutant *Neurospora* strains (Merrow et al., 1999).

STABLE ENTRAINMENT OCCURS WITHIN LIMITS DEFINED BY THE PRC AND FRP

Since entrainment is determined by a circadian pacemaker's PRC and FRP, the range (limits) of zeitgeber *T*-cycles that permit stable entrainment is also largely determined by these two factors. Because aftereffects can modulate the FRP and PRC, the prior entrainment history can affect whether a circadian pacemaker will be able to entrain to a given *T*-cycle. Phase response curves with large phase shifts can permit synchronization to *T*-cycles of a broader range as compared with low amplitude PRCs. Stable steady-state entrainment is achieved when the zeitgeber strikes a phase on the PRC (1) where the phase shift equals $(\mathrm{FRP} - T)$ and (2) where the slope of the PRC lies between 0 and -2 (Pittendrigh, 1981b; 1993). When the zeitgeber cycle fails to meet these two conditions, either *frequency demultiplication* or *relative coordination* occurs (Aschoff, 1965a; Pittendrigh, 1981b; Pittendrigh and Daan, 1976a,b). Frequency demultiplication occurs when the zeitgeber repetitively falls at the same phase of the PRC, but in every *second* cycle (or third, etc.). In this case, therefore, the period of the biological clock and of the zeitgeber cycle are not equal. The other case—relative coordination—occurs when the phase shift evoked by the zeitgeber is not large enough to equal $(\mathrm{FRP} - T)$. Under these conditions, the rhythm looks somewhat like a free-running rhythm, but with a scalloped pattern that is due to the phase-shifting effects of the repetitive stimuli as the pacemaker attempts—but fails—to stably entrain to the zeitgeber cycle. As mentioned above, light cycles with gradual lights-on and lights-off transitions may increase the limits of entrainment (Boulos et al., 2002).

WHY ARE CIRCADIAN CLOCKS "CIRCA" 24h?

Why are not circadian periods exactly or nearly exactly 24h? *Drosophila* and *Sarcophaga* pupae have FRPs that are nearly equal to 24h, so cellular biochemistry is capable of precise, nearly 24h periodicity. Two explanations have been suggested as possible answers to this question: (1) to stabilize the phase angle under one-pulse entrainment and/or (2) to allow the phase angle to track dawn (or dusk) throughout the seasons of the year.

The first explanation is related to the difference between oscillator systems that strive for synchrony among the component oscillations as opposed to systems that are designed

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

for entrainment. Circadian clocks appear to fall into the latter class, implying that they are not designed to achieve synchrony with the environmental cycle—rather, they entrain to it with a stable phase angle (Hanson, 1978; Strogatz, 1994). The coupling of circadian oscillators to environmental cycles is described by PRCs that often have a significant dead zone with a region of several hours in the mid-subjective day where a light pulse elicits no reproducible phase shift response or in which the amount of phase resetting is very small and the slope of the PRC is slight. For entrainment described by a PRC with a dead zone, stability of phase angle is maximized for 1-pulse entrainment by a FRP that is close to, but significantly different from, 24h. Why? If the FRP and the T are equal or nearly equal, the phase shift per cycle required to satisfy FRP $-$ T is zero (or very small). This phase shift can be obtained by the zeitgeber striking at any of many phases throughout the dead zone, assuming a certain amount of day-to-day variability in the FRP (Pittendrigh and Daan, 1976b). Thus, phase instability (i.e., many possible phase angles) is allowed (Fig. 5), which violates our Key Function.

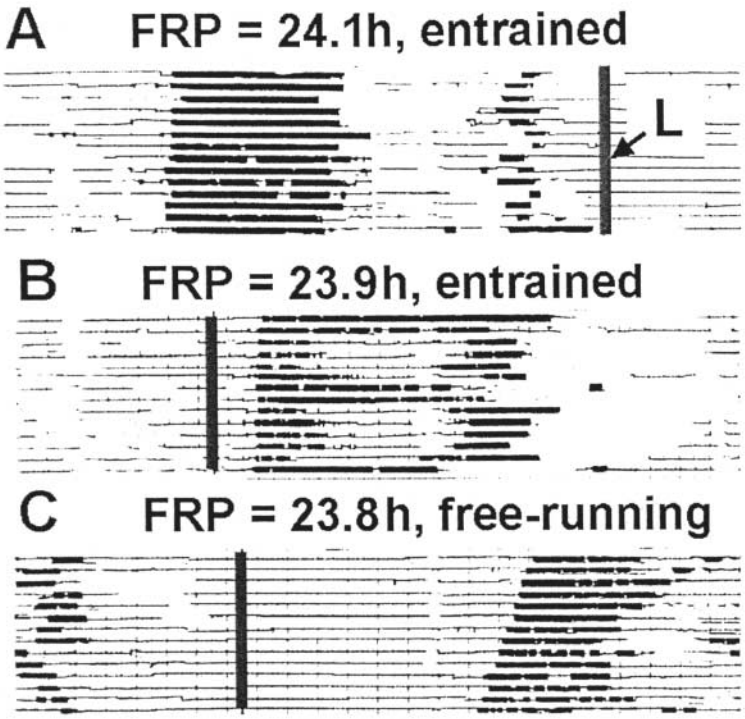


Figure 5. Phase angle of entrainment under 1-pulse entrainment may vary widely. In hamsters the FRP_{DD} is close to 24h and the PRC has a broad "dead zone." Consequently, stable entrainment to a short photoperiod at $T = 24$h may occur either with light falling in the phase advance (panel A, calculated phase advance shift $=$ FRP $- T = +0.1$h) or phase delay (panel B, calculated phase delay shift $=$ FRP $- T = -0.2$h) or the activity rhythm may "free-run" for weeks at a time as the light pulse scans the "dead zone" of the PRC (modified from Elliott, 1974).

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

On the other hand, if FRP and *T* are not equal, there is only one stable phase in the circadian cycle where both 1) the phase shift will equal [FRP − *T*] and 2) where the slope of the PRC will be between 0 and −2. (Note that Type 1 PRCs have another point at which the phase shift is zero—namely, at the transition between delay and advance shifts—but this point does not allow stable entrainment because the slope of the PRC at this point is positive.) Consequently, for unequal FRP and *T*, there will be a unique phase angle obtained under steady-state entrainment (Fig. 4). Therefore, one current hypothesis for why circadian pacemakers have a non-24h FRP is that a circa-24h FRP will always establish a specific phase relationship of clock to a 24h environment, fulfilling the Key Function of circadian systems (Pittendrigh and Daan, 1976b).

The second (and not mutually exclusive) explanation for non-24h FRPs is that circa-24h FRPs may aid the adaptation to the changes in the environment that occur over the course of a year. Annual changes in the daylength are an important challenge for the circadian pacemaker in fulfilling its Key Function of maintaining an ecologically appropriate phase angle. Environmental cycles are only constant over the year at the equator; everywhere else, daily environmental cycles vary significantly with season. This challenge to adaptive phasing has somehow been overcome in the evolution of circadian clocks; vertebrate activity patterns accurately track seasonal changes in photoperiod (Daan and Aschoff, 1975; DeCoursey, 1960). Pittendrigh and Daan found that by using appropriate combinations of FRP values and PRC shape, the discrete entrainment model could mimic the observed tracking of rhythms to seasonally changing photoperiods. They therefore concluded that circadian oscillators could be a "clock for all seasons" (Pittendrigh and Daan, 1976b). In particular, a long FRP (e.g., 25h) coupled with a PRC that has a predominately advancing shape (i.e., a large advance-to-delay ratio) allows an entrainment pattern that causes a daily phase advance and will track dawn over a wide range of photoperiods—a day-active strategy. Conversely, a short FRP (e.g., 23h) in combination with a predominately delaying PRC (small advance-to-delay ratio) will cause a daily phase delay and track dusk. The latter combination would be appropriate for a night-active organism that needs to initiate its activity at dusk throughout the year. To appreciate the satisfying simplicity of these predictions, once again remember that the seasonal tracking is predictable from merely the appropriate FRP value and PRC shape using the discrete entrainment model. Moreover, these modulations of FRP could provide a potential explanation for the adaptive basis of Aschoff's Rule (Pittendrigh, 1993).

Is it really true that organisms do it this way? When the data for all the organisms whose PRCs had been published as of 1990 are compared, the correlation between day- and night-activity patterns and FRP/PRC combinations is not convincing (Johnson, 1999). This comparison is perhaps misleading, however; it is based on a large number of studies from many different laboratories whose protocols vary widely. Our intuition suggests that the Pittendrigh and Daan hypothesis may prove to be an excellent model in the case of nocturnal animals, whose daily exposure to light in nature is behaviorally minimized, but that for organisms exposed to the complete photoperiod each day, other factors will need to be taken into account, as suggested in the section on continuous entrainment (see below).

Wanted*: more comparisons to test the Pittendrigh/Daan model for seasonal tracking in a variety of organisms. Both day active (PP_c exposed) and night active organisms under*

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

controlled, equivalent laboratory conditions should be tested. These experiments should include laboratory simulations of seasonal change in natural photoperiod and complementary field studies to evaluate how closely predictions from laboratory models match behavior in nature.

CIRCADIAN PACEMAKERS CAN ENTRAIN TO "SKELETON PHOTOPERIODS"

The 1-pulse version of the discrete entrainment model described above leads to many insights into circadian entrainment. With the exception of some nocturnal organisms, however, single brief light pulses every day do not replicate the daily light information perceived by most organisms. A surprising discovery of the discrete model has been that it can be applied to rather accurate modeling of entrainment to complete photoperiods (i.e., an LD 12 : 12 cycle) in some organisms by merely assuming that the relevant parts of the photoperiod for entrainment are the discrete light-on and light-off transitions. The light-on and light-off transitions could apparently be mimicked with a light/dark cycle of, e.g., 15-min light pulses every 12h to provide a "skeleton" of the photoperiod (Pittendrigh, 1966; 1981b; Pittendrigh and Minis, 1964; Pittendrigh and Daan, 1976b). That skeleton photoperiods consisting of light pulses could mimic complete photoperiods was counterintuitive because a light pulse includes both light-on and light-off transitions, and so it was unexpected that a pulse at dawn would mimic the single light-on transition of a real dawn, and that a pulse at dusk would mimic the single light-off transition of a real dusk. Nevertheless, skeleton photoperiods have been found to mimic complete photoperiods reasonably well in some organisms (Pittendrigh, 1981b; Pittendrigh and Daan, 1976b).

Skeleton photoperiods provide a zeitgeber of two pulses per circadian cycle, a "2-pulse entrainment." From the discrete model of entrainment, entrainment to skeleton photoperiods is determined by the net effect of the phase-shifting by both pulses:

$$\text{First phase shift} + \text{second phase shift} = \text{FRP} - T$$

Figure 3C shows a PRC subjected to a skeleton photoperiod of 12 : 12. In Fig. 3D, entrainment to a complete photoperiod as predicted by the discrete model is depicted for comparison. In the case of the *Drosophila* pupal eclosion rhythm, entrainment to skeleton photoperiods is similar to that for complete photoperiods within the photoperiod range of 1h to 13–14h. For skeleton photoperiods longer than 13–14h, the eclosion rhythm undergoes a phase-angle "jump" (Pittendrigh, 1966; Pittendrigh and Minis, 1964). This means that fruit-fly pupae will entrain in steady state to skeleton photoperiods such that the subjective day portion of the PRC coincides with the shorter of the two dark intervals. Furthermore, the rhythm of *Drosophila* pupae can entrain stably to either dark interval over a narrow range of skeleton photoperiods—this phenomenon is called "bistability," and it is well described elsewhere (Daan and Aschoff, 2001; Pittendrigh, 1966; 1981b). The discrete model predicts well the experimental responses of *Drosophila* pupae to skeleton photoperiods, including those that allow bistability, and these observations have historically been a major support of the discrete model.

An interesting insight provided by simulations of 2-pulse entrainment is that the phase angle of a circadian pacemaker is much more stable under 2-pulse than under 1-pulse entrainment. Assuming some imprecision in the day-to-day FRP, the phase angle is

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

optimally stabilized under 2-pulse entrainment to a skeleton photoperiod of sufficient duration to elicit both advance and delay phase shifts in each cycle, as shown in Fig. 3C. In so doing, the phase shifts act as "checks and balances" that prevent the zeitgeber striking near the dead zone region of the PRC where phase-angle instability is most likely (Pittendrigh and Daan, 1976b).

***Wanted**: measurements of levels of molecules putatively acting as state variables in response to complete vs. skeleton photoperiods to determine if the molecular oscillator and behavioral outputs exhibit similar responses.*

CIRCADIAN OSCILLATORS THAT ARE EXPOSED TO COMPLETE PHOTOPERIODS MUST ALSO EXPERIENCE CONTINUOUS ENTRAINMENT

As mentioned above, the continuous entrainment model was originally based on the observation that the FRP is dependent upon light intensity, and suggests that light can have a continuous action on the clock to modulate its entrainment to LD cycles. Even in organisms whose entrainment behavior apparently conforms to that expected from the discrete entrainment model for short photoperiods, one unarguable impact of the continuous action of light is to prevent the "phase-angle jump" observed in skeleton photoperiods of long durations (Daan, 2000; Pittendrigh, 1981b). In addition, for some short-duration photoperiods, skeleton photoperiods do not mimic well the entrainment to natural photoperiods, as has been shown in some studies of Syrian hamsters (Pittendrigh and Daan, 1976b). A dramatic example for which the discrete model is inadequate is that of day-active ground squirrels that entrain to LD cycles without ever seeing dawn or dusk. These animals stay in their burrows until several hours after sunrise and return to the burrows well before sunset—therefore, they never see the transitions upon which the discrete model depends. And yet, they entrain stably in natural conditions (Daan, 2000). How do they do it? The discrete model does not provide an answer.

Even in the cases where skeleton photoperiods appear to mimic complete photoperiods well (the flies *Drosophila* and *Sarcophaga*, and some nocturnal rodents), there have been concerns that those experiments are not adequate. In particular, Johnsson and Karlsson (1972) have pointed out that tests of skeleton photoperiods in which T is equal to, and FRP is close to, 24h (as is true for all the aforementioned organisms), the daily net phase shift is very small, and that this situation is not a definitive condition in which to test whether skeleton photoperiods mimic complete photoperiods. They suggest doing skeleton photoperiod experiments in which T is significantly different from FRP. Consequently, the discrete model might not be as well supported by the data as we have come to believe, even in flies and nocturnal rodents.

Moreover, the organisms on which complete vs. skeleton photoperiod entrainment has been most rigorously tested have been nocturnal rodents and fly pupae, which may behaviorally limit their light exposure in nature to skeleton photoperiods (DeCoursey, 1986). Therefore, their entrainment properties and sensitivity to light may be optimized

to those conditions in a way that is not representative of organisms that are exposed to a complete photoperiod every day. It seems intuitively obvious to us that the entrainment of organisms that are exposed to complete photoperiods (e.g., plants and day-active animals) are likely be a composite of continuous and discrete mechanisms. Why then has so little attention been paid to continuous entrainment by chronobiologists? One probable reason that the continuous model of entrainment has not achieved the same level of acceptance is that it is difficult to model quantitatively, whereas it is easy to make quantitative predictions with the discrete model using simple pencil and paper calculations, and the quality of its predictions range from adequate to excellent, depending on the organism.

One major distinction between the discrete and continuous models is that in the original versions of the discrete model, FRP is generally taken to be a constant and the adjustment of FRP to *T* occurs solely by phase shifts elicited by discrete transitions (i.e., darkness to light at dawn) that occur in the environment. The continuous model allows the FRP to modulate continuously throughout the day and/or allows other effects (see later) to occur that cannot be strictly inferred from the PRC. With respect to FRP modulation, the acceleration and deceleration of FRP (angular velocity) by daily changes in light intensity could allow the circadian pacemaker to continuously adjust its cycle length to that of the environment. It has been proposed that the magnitude of this effect can be predicted from the shape of the PRC as a velocity response curve that transforms the observed discrete effects of light on phase into a continuous effect of light on period. One experiment supporting this interpretation has been the entrainment of some organisms to sine-curve changes of light intensity in LL (Swade, 1969).

A related example comes from studies of humans. For many years, it was thought that the FRP of humans was significantly longer than 24h (25–26h in young subjects). However, these estimates of human FRP were derived from studies in which the subjects were allowed to turn on a light during the subjective daytime. A recent re-evaluation of human FRP suggests that this daytime light (even when dim) has a significant impact upon period. When the data are corrected for this light exposure, the calculated endogenous FRP_{DD} for humans is very close to 24h (24.18h; Czeisler, 1999). This re-evaluation leads to substantially different conclusions about the entrainment of the human circadian oscillator in which continuous entrainment plays a larger role.

As mentioned above, a corollary to the consequences of period changes on entrainment is their potential contribution to allowing the pacemaker's adjustment of phase angle throughout the annual cycle. Because the FRP is subject to aftereffects depending on photoperiod, it will change during the year. If it is true that alterations in FRP can account for annual tracking of the pacemaker to the annual cycle, then this FRP modulation may be a seasonal adaptation as well and could help to explain Aschoff's observations of the responses of day-active and night-active vertebrates to LL that have become known as "Aschoffs Rule" (Pittendrigh, 1993; Pittendrigh and Daan, 1976b).

Wanted: *more entrainment studies of organisms that are exposed to the complete photoperiod. These studies may wish to measure PRCs to long pulses (10–12h) and/or to "step-up" and "step-down" transitions, as suggested by Aschoff (1999) and Daan and Aschoff (2001). Step-up and step-down PRCs may be a more appropriate model for continuous entrainment. Changes in FRP caused by the exposure to light during the day (Aschoff's Rule) must also be taken into account.*

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

LIMIT-CYCLE MODELING CAN PROVIDE AN INTEGRATED ENTRAINMENT MECHANISM

Past discussions of discrete vs. continuous entrainment mechanisms have sometimes indicated that one or the other mechanism is operative in entraining circadian clocks. Rather, it is likely that most organisms utilize both mechanisms to some degree, or rather, that light might have fundamentally equivalent effects in all organisms, but differences in (e.g.) photobiological sensitivity could make it appear that the discrete entrainment adequately models the entrainment of some organisms, but not of others. Based on a limit-cycle model proposed by Eric Peterson (1980), we will describe another perspective on entrainment that provides a concept for integrating discrete vs. continuous entrainment concepts.

In LL, some insects express no discernable rhythm, but transfer to DD induces a rhythm that appears to start at approximately CT 12 (Fig. 6A). The most straightforward explanation for this phenomenon had been that the circadian pacemakers in these organisms were "stopped" by light at CT 12 (Pittendrigh, 1966). Peterson (1980), however, saw these data with new eyes and noticed that the phase of the rhythm that resumed in DD was seen to vary with the duration of the LL exposure (Fig. 6A). This variation with LL exposure had a period of about 24h. This phase effect was so slight that it might have been ignored except that Peterson noticed the same rhythmic pattern in the phases of the flight-activity rhythm of mosquitos and in the eclosion rhythm of fly pupae.

Eric Peterson used the limit-cycle concept to reinterpret these data by proposing that the state variables of the pacemaker oscillate around a different limit cycle in DD than they do in LL (Peterson, 1980). The LL limit cycle is displaced from the DD limit cycle and centered on its CT 12 isochron, as shown in Fig. 6B. After exposures to LL of greater than 12h, the state variables oscillate on the LL limit cycle, moving between the isochrons of CT 11 and CT 13 of the DD limit cycle. Therefore, upon transfer from LL to DD, the state variables find themselves on isochrons of the DD limit cycle somewhere between CT 11 and CT 13. They then return to phases on the DD limit cycle corresponding to a range between CT 11 and CT 13. The exact phase of return to the DD limit cycle depends upon the phase of the state variables on the LL limit cycle at the time of LL to DD transfer. Because the state variables rhythmically fluctuate on the LL limit cycle, the final phase of the rhythm in DD will show a slight rhythmicity, as seen in Fig. 6A. This model provides an elegant alternative to the original interpretation that LL "stops" the clock; the clock may still be running in LL, but on a different limit cycle (Peterson, 1980; Pittendrigh, 1981b). Another limit-cycle reinterpretation of "LL stops the clock" has been reported for *Neurospora* (Ruoff et al., 2001).

***Wanted**: molecular tests of Peterson's model. For example, in organisms that display the behavior shown in Fig, 6A, do putative state variables oscillate in LL? (The light intensity in LL may be important, as noted by Peterson.) If they oscillate, do they oscillate around values that are typically CT 12 values for the putative state variables? (Or more precisely, the isochrons of CT 12.)*

Peterson's limit-cycle model has important implications beyond its interest in explaining the subtle oscillation of insect rhythms about the lights-off signal. One potential insight involves the aforementioned issue of discrete vs. continuous entrainment. Peterson's model suggests that light has a single action on circadian pacemakers, namely

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

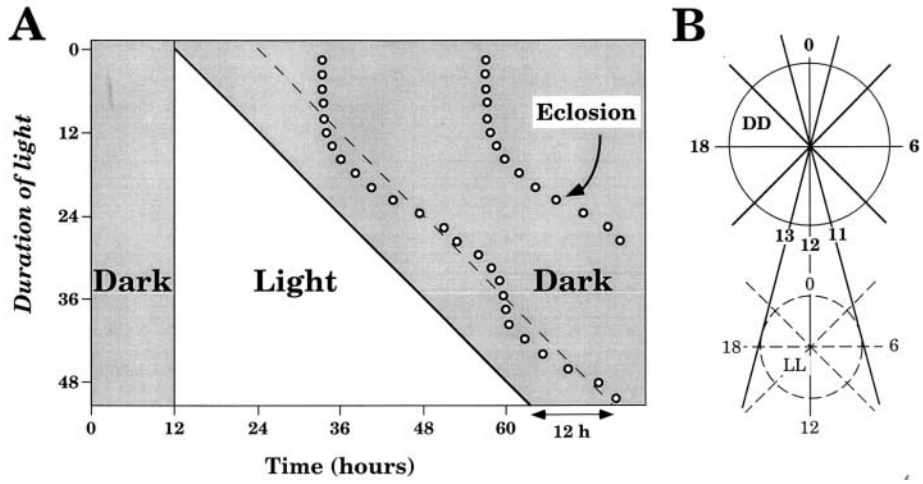


Figure 6. Limit-cycle interpretation of transitions from light to darkness—a reinterpretation of the idea that constant light "stops" the clock at CT 12. Panel A: Depiction of an experiment using long light pulses of various durations to affect the phase of circadian pacemakers in a population, based on the data of Peterson and Saunders (1980) reporting the eclosion behavior of *Sarconhaga* pupae. To a population of pupae free-running in DD, light pulses of 0 to 50h) are presented beginning at the same phase. Horizontally, each group is a population that has been exposed to a different duration of light from each other horizontal group. The circles are the median phase of eclosion for each daily peak. The effect of light pulses that are 12h or shorter is that the phase of the eclosion rhythm appears to be set by lights-on. On the other hand, for light exposures that are longer than 12h, lights-off appears to reset the eclosion rhythms to an average phase of CT 12. The "average phase of CT 12" has been displaced to 12h after the end of the light exposures and appears as the diagonal dashed line for easy comparison with the eclosion data. However, the rhythmic behavior does not coincide *exactly* with what would be expected if the underlying pacemaker starts from precisely CT 12 at the light-to-dark transition. In particular, there is a rhythmic "scalloping" of the eclosion onsets around the average phase of extrapolated CT 12. (Panel modified from Peterson and Saunders, 1980.) Panel B: Limit-cycle interpretation of the "scalloping" illustrated in Panel A. The state variables of the pacemaker are postulated to oscillate on two different limit cycles (LL and DD limit cycles). The LL limit cycle is centered on the isochron of CT 12 of the DD limit cycle. In this hypothetical example, as the state variables oscillate around the 24h of phase on the LL limit cycle i.e., the pacemaker *is not* stopped at CT 12), they oscillate only between CT 11 and 13 on the DD limit cycle. Therefore, when the organism is exposed to light, the state variables are attracted away from the DD limit cycle toward the LL limit cycle. In LL, the state variables oscillate around the LL limit cycle. When the organism is transferred back to DD, the state variables return to the DD limit cycle from a narrow range of isochrones (e.g., CT 11–13) with an average phase of CT 12. Consequently, the pacemaker is reset by the light-to-dark transition to an *average* phase of CT 12 that actually shows a small but significant oscillation of phase.

to move the state variables to a different region of phase space, from the DD limit cycle to the LL limit cycle. An interpretation of entrainment based upon Peterson's data (Fig. 6B) can (i) enable discrete and continuous modes of entrainment behavior to co-exist while (ii) explaining how complete photoperiods can prevent the phase-angle jump. The basic premise is that the primary action of light on circadian oscillators is to define two distinct

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

limit cycles, the DD and LL limit cycles. Transitions between light and dark initiate movements of the state variables between these limit cycles, and the kinetics of these movements are crucial.

Figure 7 illustrates the basic model. The action of light is first to increase the level of variable *X*, thus moving the oscillator toward the right, as also shown in Figs. 1 and 2. With longer exposures to light, however, the value of variable *Y* is also affected, so that the oscillator moves downward toward the LL limit cycle. If the DD and LL limit cycles are well separated, as is apparently the case for mosquitos, a complete photoperiod will elicit a larger amplitude oscillation of the state variables than would occur in either DD or LL, as shown in Fig. 7A. (The amplitude of the oscillation is defined by the approximate diameter of the limit cycles as in Fig. 2B and D.) On the other hand, entrainment to skeleton photoperiods would be expected to look different (Fig. 7B). In the case of skeleton photoperiods, the brief light pulses move the oscillator slightly to the right (as in

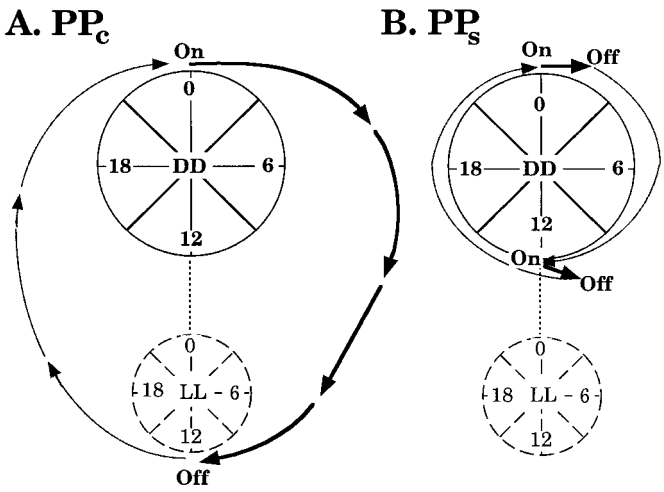


Figure 7. Predicted behavior of circadian oscillators under entrainment to complete and skeleton photoperiods of LD 12 : 12 (simulation based on Eric Peterson's data from the mosquito, *Culex*). DD limit cycles are shown at the top of each panel, with LL limit cycles at the bottom of each panel. The direction of motion of state variables across the phase planes are indicated by arrowed lines—solid black lines in the case of trajectories in light, gray lines in the case of trajectories in darkness. Panel A: Entrainment to complete photoperiods of LD 12 : 12. Under stable entrainment to LD, the state variables at lights-on ("On") move from the DD limit cycle near CT 0, initially to the right, then they arc toward the LL limit cycle. If the light were to remain on continuously, the state variables would oscillate around the LL limit cycle, but at lights-off ("Off"), the state variables are attracted back to the DD limit cycle. (Panel modified from Peterson, 1980.) Panel B: Entrainment to a skeleton photoperiod of LD 12 : 12 (composed of brief light pulses). At lights-on ("On" near CT 0), the state variables move away from the DD limit cycle. When the brief light pulse is turned off ("Off" near the isochron of CT 1.5), the state variables return to the DD limit cycle until the next light pulse begins ("On" near CT 12), at which time the state variables move again toward the right. When the second light pulse is turned off ("Off" near the isochron of CT 11), the state variables again spiral back toward the DD limit cycle.

Fig. 1B). Therefore, the amplitude of the state variables' oscillation under entrainment could be much smaller and remain close to the DD limit cycle. Therefore, even though the entrainment of a circadian rhythm to skeleton photoperiods might appear to be essentially the same as that to complete photoperiods, the state variables of the pacemaker could be oscillating in significantly different areas of phase space.

One result of the effects shown in Fig. 7 would be to explain the phase-angle jump phenomenon observed with skeleton photoperiods (Pittendrigh, 1966; Pittendrigh and Minis, 1964). With skeleton photoperiods, the state variables always remain close to the DD limit cycle and after each lights-off, the state variables are at different isochrons (Fig. 7B). This allows the oscillator to interpret the smaller dark interval as subjective day regardless of the prior phase of the oscillator. Therefore, either light pulse could become the sunrise or sunset pulse once steady-state entrainment is achieved. For example, in a skeleton photoperiod of 16 : 8, the state variables are free to select the 8h interval as subjective day (it might take many cycles to attain steady-state entrainment). On the other hand, with complete photoperiods, the state variables are displaced far off the DD limit cycle during the day (Fig. 7A). Therefore, at lights-off, the variables are constrained to return to the DD limit cycle along a restricted range of paths (for sufficiently long photoperiods, the return path is approximately in phase with the isochron of CT 12). Therefore, the oscillator can interpret the photoperiod in only one phase relationship. For example, in a complete photoperiod of LD 16 : 8, the state variables cannot select the 8h interval as subjective day because the 16h light exposure forces the variables to remain in the region of the CT 12 isochron of the DD limit cycle while oscillating around the LL limit cycle. Consequently, lights-off can only be interpreted as approximately CT 12 in a complete photoperiod. The limit-cycle interpretation of entrainment to LD thereby provides an explanation for why circadian oscillators do not undergo a phase-angle jump in complete photoperiods (Peterson, 1980; Pittendrigh, 1981b).

***Wanted**: molecular tests of the entrainment model depicted in Fig. 7. Such a test could include the measurement of putative state variables under both complete photoperiods and skeleton photoperiods to ascertain the relative magnitude of phase shifting between these two entrainment protocols. Optimally, the method of monitoring molecular state variables would be a continuous method (such as using luminescence reporters) so that brief transitions of state variable levels are recorded. Most molecular measurements (RPAs, RT-PCR, northerns, immunoblots, etc.) are "snapshots" of molecular levels that can lose much important information about key transitory states.*

If entrainment of various organisms can be explained by this single solution, why might the entrainment properties of different organisms appear to be so different? For example, the pacemakers of *Drosophila* pupae and *Neurospora* hyphae are exquisitely sensitive to light; a 1-min light pulse of moderate intensity elicits very large phase shifts and their outputs become arhythmic in very dim-intensity LL. At the other extreme, *Chlamydomonas*, other plants, many birds, and diurnal mammals (including humans?) require long, high-intensity light pulses to achieve relatively small phase shifts. How can such differences be explained by a single solution? One possible answer is that the clock systems of different organisms may have very different sensitivities of their phototransduction pathways (in particular, in the kinetics of the light-induced displacement of state

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

variables between the DD and LL limit cycles). Such differences could be based on differential photosensitivity and/or phototransduction of various clock systems. In a very photosensitive system, light and dark transitions might very rapidly move the state variables between the DD and LL limit cycles. In this case, the dim light of dawn and dusk would be sufficient to entrain the circadian rhythm, and the behavior would appear to match the predictions of the discrete entrainment model. In such a high-sensitivity system, the only remaining function supplied by a complete photoperiod would be to prevent the phase-angle jump, as explained in the preceding paragraph.

By simply assuming a lower photosensitivity, however, the entrainment behavior might superficially appear to be quite different, even though the fundamental characteristics are similar. In this case, the state variables might commute between the DD and LL limit cycles slowly. The consequence of these kinetics is that much stronger stimuli (in duration and/or intensity) might be required to elicit significant phase resetting. For stimuli of below-threshold strength (even light pulses of several hours duration and of moderate intensity), the state variables will not complete the journey between the DD and LL limit cycles, a situation that was modeled by Peterson for the case of mosquitos (Peterson, 1980). This change in the system's photosensitivity without any change in the fundamental response of the pacemaker to light and dark could alter the overall behavior into one that is not predictable from the discrete entrainment model. There could be various reasons for slower kinetics of circadian phototransduction; it is known, for example, that the sensitivity of circadian systems to light-induced phase resetting can be influenced by many factors, including mutation, developmental stage, and the nature of the background illumination (i.e., DD vs. dim LL vs. bright LL) (Johnson, 1990; 1999; Johnson and Hastings, 1989; Nelson and Takahashi, 1991; Page and Barrett, 1989; Rothenfluh et al., 2000). Organisms that are routinely exposed to complete photoperiods probably do not need a highly photosensitive circadian system, and therefore it is reasonable to propose that their entrainment mechanism need not have developed extreme sensitivity during the course of evolution. In fact, a highly photosensitive system could be a detriment in an environment with fluctuating light intensity that could create noise in the zeitgeber signal.

Wanted*: correlative measurements of the impact of zeitgebers on the magnitude of phase shifting and the levels of putative state variables. Optimally, such comparisons would include organisms whose phase resetting is very sensitive and organisms whose phase resetting is less sensitive. Fluence/dose response curves are necessary. The effect of these stimuli on the level of molecules that are putative state variables should be measured and compared with the amplitude of their oscillation under free-running conditions from a limit-cycle perspective.*

Peterson's limit cycle model for entrainment that is described here does not take into account changes in FRP. As discussed above, it is very likely that diurnal exposure to light changes the speed at which the internal clock runs, and that this effect will have a significant impact on the entrainment of organisms exposed to complete photoperiods (Aschoff, 1981). If the hypothesis of distinct DD and LL limit cycles is correct, the observation of differing FRPs in DD vs. LL might mean that FRP can differ when the state variables oscillate in different regions of the phase plane. A different possibility is that the effect of light on FRP is an effect on state parameters that are not depicted on

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

the simple *X–Y* graphs of Figs. 1, 2, 6, and 7, but are an integral part of the mathematics underlying limit-cycle oscillators. By the latter interpretation, light has an impact both upon the level of state variables and upon the values of state parameters. In either case, assessing FRP will be essential for any calculations of entrainment based on the continuous model. Moreover, if FRP is a function of light intensity, then the instantaneous FRP will be different at different phases of any LD cycle (Daan and Pittendrigh, 1976).

Past discussions of entrainment mechanisms have sometimes indicated that either discrete or continuous mechanisms are operative in entraining circadian clocks. This either/or approach is partly a result of the battling egos of circadian pioneers such as Pittendrigh and Aschoff. We have described here a different, more integrated perspective that attempts to reconcile these differences. In so doing, we have also presented our perspective that entrainment and molecular studies can be usefully interpreted from the viewpoint of limit cycles. Our field has recently made astonishing progress in the identification of molecular candidates that may act as correlates for mathematical state variables and parameters. Along the way, limit-cycle concepts have served to establish criteria for deciding whether a candidate molecule is a state variable. We expect this trend to expand such that limit-cycle modeling will provide insights into entrainment, temperature compensation, and other circadian properties.

ENTRAINMENT IN NATURE

Our ultimate goal is to understand entrainment in nature, not in the laboratory. Entrainment in nature will necessarily be a composite of many zeitgeber inputs, each of which has entraining capacity and to which the organism has differing sensitivities/thresholds. As detailed above, these factors will influence entrainment by modulating phase and period; modulation of phase can occur by changing PRC shape and/or by modulating input sensitivity, while modulation of period can occur by changing FRP, especially via aftereffects. These factors include the following:

Photic Signals

Light/dark cycles were the most discussed zeitgeber in this paper. The strength of photic signaling can be modulated by changes of (i) intensity, including diurnal modulation of intensity (as in twilight transitions); (ii) duration (in nature, by seasonally varying photoperiods); and (iii) wavelength, including diurnal modulation of wavelength (as at twilight transitions, when there is a relative enrichment of blue light over the dome of the sky, and red-light at the horizon; Roenneberg and Foster, 1997). We need to remember that light intensity is not constant during the day in nature due to weather conditions (e.g., cloud cover), twilight, shading (e.g., by trees), et al., and our models need to explain how the circadian system sees the signal without being misled by the noise. Indeed, multiple photopigments seem to mediate the effects of light on entrainment, suggesting that the sensory task of twilight detection is much more complex than has been assumed (Foster and Helfrich-Forster, 2001).

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Non-photic Signals

Non-photic signals were barely discussed in this paper, but the same principles that apply to entrainment by light/dark cycles will apply to any signal that can reset circadian clocks in a phase-dependent manner. An incomplete list of non-photic signals includes (i) temperature cycles; (ii) feedback of muscular activity or arousal state upon the central pacemaker; and (iii) feedback of feeding time and/or food availability upon the central pacemaker. The developmental stage of the organism can also influence the responsiveness of the organism to zeitgebers.

In most cases, it is not clear how significant these non-photic signals are as compared with LD cycles in nature. Classic studies using conflicting phasing of LD vs. temperature cycles showed that the LD cycle predominately determines the entrained phase angle in insects (Pittendrigh, 1960; Pittendrigh and Minis, 1971) but more recent experiments using *Neurospora* have questioned the generality of that conclusion (Liu et al., 1998). The topic of temperature cycles as entraining agents has been comprehensively reviewed elsewhere by authors who argue that the efficacy of temperature as an entraining agent in nature—even in homeotherms—has been under-appreciated (Rensing and Ruoff, 2002).

Wanted*: a comprehensive examination of relative zeitgeber strengths and interactions. Entrainment in nature will necessarily be a composite of differing zeitgebers. Examinations of the relative strengths of these zeitgebers are needed. Zeitgebers to be tested should include cycles of light (varying both irradiance and spectral composition), temperature, food availability, locomotor activity, arousal/sleep, and social cues. To be meaningful, the amplitude (and/or magnitude) of the zeitgebers must correspond to values that are typically encountered in the natural environment for the individual species.*

A final understanding of entrainment in nature will take into account the relative contribution of all the potential zeitgebers toward determining the phase angle of entrainment. Complex? Certainly. Exciting? Definitely. Although we know much about entrainment, there is still much to discover. And those discoveries will be keys to our understanding of the function and adaptive value of circadian clocks—a biochemical timekeeper whose function is to provide an internal estimate of external time.

ACKNOWLEDGMENTS

We thank Drs. Richard Kronauer and Ken-ichi Honma for their contributions to a prior manuscript for a textbook chapter from which some of the material for this paper was adapted. We are grateful to Drs. Terry Page and Shin Yamazaki for their comments on the manuscript and to Dr. Marty Zatz for his suggestion of the "Wanted" sections to identify unexplored territory. We dedicate this paper to the memory of Colin Pittendrigh and Jurgen Aschoff, whose "battling egos" did so much to explore the terrain of circadiana, and to the memory of Arthur Winfree, who provided the first persuasive experimental evidence for the limit cycle organization of circadian oscillators.

REFERENCES

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., Block, G. D. (2002). Circadian rhythms in isolated brain regions. *J. Neurosci.* 22:350–356.

Aschoff, J. (1960). Exogenous and endogenous components in circadian rhythms. In: *Cold Spring Harbor Symposia on Quantitative Biology. Volume 25. Biological Clocks.* Cold Spring Harbor, NY: Cold Spring Harbor Press, pp. 11–28.

Aschoff, J. (1965a). Response curves in circadian periodicity. In: Aschoff, J., ed. *Circadian Clocks.* Amsterdam: North-Holland, pp. 95–111.

Aschoff, J. (1965b). The phase-angle difference in circadian periodicity. In: Aschoff, J., ed. *Circadian Clocks.* Amsterdam: North-Holland, pp. 262–276.

Aschoff, J. (1981). Freerunning and entrained circadian rhythms. In: Aschoff, J., ed. *Handbook of Behavioral Neurobiology. Volume 4. Biological Rhythms.* New York: Plenum Press, Chap. 6, pp. 81–93.

Aschoff, J. (1999). Masking and parametric effects of high-frequency light–dark cycles. *Japanese J. Physiol.* 49:11–18.

Barrett, R. K., Page, T. L. (1989). Effects of light on circadian pacemaker development. I. The freerunning period. *J. Comp. Physiol.* 165:41–49.

Boulos, Z., Macchi, M. M., Terman, M. (2002). Twilights widen the range of photic entrainment in hamsters. *J. Biol. Rhythms* 17:353–363.

Bruce, V. G. (1960). Environmental entrainment of circadian rhythms. In: *Cold Spring Harbor Symposia on Quantitative Biology. Volume 25. Biological Clocks.* Cold Spring Harbor, NY: Cold Spring Harbor Press, pp. 29–48.

Crosthwaite, S. K., Loros, J. J., Dunlap, J. C. (1995). Light-induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript. *Cell* 81:1003–1012.

Czeisler, C. A., Duffy, J. F., Shanahan, T. L., Brown, E. N., Mitchell, J. F., Rimmer, D. W., Ronda, J. M., Silva, E. J., Allan, J. S., Emens, J. S., Dijk, D.-J., Kronauer, R. E. (1999). Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science* 284:2177–2181.

Daan, S. (1977). Tonic and phasic effects of light in the entrainment of circadian rhythms. *Ann. N.Y. Acad. Sci.* 290:51–59.

Daan, S. (2000). Colin Pittendrigh, Jurgen Aschoff, and the natural entrainment of circadian systems. *J. Biol. Rhythms* 15:195–207.

Daan, S., Aschoff, J. (1975). Circadian rhythms of locomotor activity in captive birds and mammals: their variations with season and latitude. *Oecologia* 18:269–316.

Daan, S., Pittendrigh, C. S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. III. Heavy water and constant light: homeostasis of frequency? *J. Comp. Physiol.* 106:267–290.

Daan, S., Aschoff, J. (2001). The entrainment of circadian systems. In: Takahashi, J. S., Turek, F. W., Moore, R. Y., ed. *Handbook of Behavioral Neurobiology. Volume 12. Circadian Clocks.* New York: Kluwer/Plenum, Chap. 1, pp. 7–43.

DeCoursey, P. J. (1960). Phase control of activity in a rodent. In: *Cold Spring Harbor Symposia on Quantitative Biology, Volume. 25. Biological Clocks.* Cold Spring Harbor, NY: Cold Spring Harbor Press, pp. 49–55.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

DeCoursey, P. J. (1986). Light-sampling behavior in photoentrainment of a rodent circadian rhythm. *J. Comp. Physiol.* 159:161–169.

Dharmananda, S. (1981). Studies of the Circadian Clock of *Neurospora crassa*: Light-Induced Phase Shifting. Ph.D. thesis, University of California at Santa Cruz, p. 284.

Elliott, J. A. (1974). Photoperiodic Regulation of Testis Function in the Golden Hamster: Relation to the Circadian System. Ph.D. thesis, University of Texas, Austin.

Elliott, J. A. (1976). Circadian rhythms and photoperiodic time measurement in mammals. *Fed. Proc.* 35:2339–2346.

Elliott, J. A. (1981). Circadian rhythms, entrainment and photoperiodism in the Syrian hamster. In: Follet, B. K., Follet, D. E., eds. *Biological Clocks in Seasonal Reproductive Cycles.* Bristol: Wright, pp. 203–217.

Eskin, A. (1971). Some properties of the system controlling the circadian activity rhythm of sparrows. In: Menaker, M., ed. *Biochronometry.* Washington, DC: National Academy of Sciences, pp. 55–80.

Foster, R. G., Helfrich-Forster, C. (2001). The regulation of circadian clocks by light in fruit-flies and mice. *Philos. Trans. R. Soc. London B Biol. Sci.* 356: 1779–1789.

Gooch, V. D., Wehseler, R. A., Gross, C. G. (1994). Temperature effects on the resetting of the phase of the neurospora circadian rhythm. *J. Biol. Rhythms* 9: 83–94.

Hamblen-Coyle, M. J., Wheeler, D. A., Rutila, J. E., Rosbash, M., Hall, J. C. (1992). Behavior of period-altered circadian rhythm mutants of *Drosophila* in light : dark cycles (Diptera: Drosophilidae). *J. Insect Behav.* 5:417–446.

Hanson, F. E. (1978). Comparative studies of firefly pacemakers. *Fed. Proc.* 37: 2158–2164.

Jewett, M. E., Kronauer, R. E., Czeisler, C. A. (1991). Light-induced suppression of endogenous circadian amplitude in humans. *Nature* 350:59–62.

Johnson, C. H. (1990). *An Atlas of Phase Response Curves for Circadian and Circatidal Rhythms.* Department of Biology, Vanderbilt University, 715 pages.

Johnson, C. H. (1999). Forty years of PRCs—what have we learned? *Chronobiol. Int.* 16:711–743.

Johnson, C. H., Hastings, J. W. (1989). Circadian phototransduction: phase resetting and frequency of the circadian clock of *Gonyaulax* cells in red light. *J. Biol. Rhythms* 4:417–437.

Johnson, C. H., Kondo, T. (1992). Light pulses induce "singular" behavior and shorten the period of the circadian phototaxis rhythm in the CW15 strain of *Chlamydomonas. J. Biol. Rhythms* 7:313–327.

Johnsson, C. H., Elliot, J. A., Foster, R. G., Honma, K.-I., Kronauer, R. (2003). Fundamental properties of circadian rhythms. In: Dunlap, J. C., Loros, L. L., DeCoursey, P. J., eds., *Chronobiology: Biological Timekeeping*. Sunderland, MA: Sinauer, pp. 66–105.

Johnsson, A., Karlsson, H. G. (1972). The *Drosophila* eclosion rhythm, the transformation method, and the fixed point theorem. Department of Electrical Measurements, Lund Institute of Technology, Report No. 2, pp. 51.

Lakin-Thomas, P. L. (1995). A beginner's guide to limit cycles, their uses and abuses. *Biol. Rhythm Res.* 26:216–232.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Liu, Y., Merrow, M., Loros, J. J., Dunlap, J. C. (1998). How temperature changes reset a circadian oscillator. *Science* 281:825–829.

Merrow, M., Brunner, M., Roenneberg, T. (1999). Assignment of circadian function for the *Neurospora* clock gene frequency. *Nature* 399:584–586.

Mrosovsky, N. (1999). Masking: history, definitions, and measurement. *Chronobiol. Int.* 16:415–429.

Nelson, D. E., Takahashi, J. S. (1991). Sensitivity and integration in a visual pathway for circadian entrainment in the hamster (*Mesocricetus auratus*). *J. Physiol.* 439:115–145.

Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S., Johnson, C. H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl. Acad. Sci. USA* 95:8660–8664.

Page, T. L., Barrett, R. K. (1989). Effects of light on circadian pacemaker development. II. Responses to light. *J. Comp. Physiol.* 165:51–59.

Pavlidis, T. (1981). Mathematical models. In: Aschoff, J., ed. *Handbook of Behavioral Neurobiology. Volume 4. Biological Rhythms.* New York: Plenum Press, Chap. 4, pp. 41–54.

Peterson, E. L. (1980). A limit cycle interpretation of a mosquito circadian oscilllator. *J. Theor. Biol.* 84:281–310.

Peterson, E. L. (1981). Dynamic response of a circadian pacemaker. II. Recovery from light pulse perturbations. *Biol. Cybern.* 40:181–194.

Peterson, E. L., Saunders, D. S. (1980). The circadian eclosion rhythm in *Sarcophaga argyrostoma*: a limit cycle representation of the pacemaker. *J. Theor. Biol.* 86:265–277.

Pittendrigh, C. S. (1954). On temperature independence in the clock system controlling emergence time in *Drosophila. Proc. Natl. Acad. Sci. USA* 40:1018–1029.

Pittendrigh, C. S. (1960). Circadian rhythms and the circadian organization of living systems. In: *Cold Spring Harbor Symposia on Quantitative Biology. Volume 25. Biological Clocks.* pp. 159–184.

Pittendrigh, C. S. (1966). The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. Z. Pflanzenphysiol. 54:275–307.

Pittendrigh, C. S. (1967). Circadian systems, I. The driving oscillation and its assay in *Drosophila pseudoobscura. Proc. Natl. Acad. Sci. USA* 58:1762–1767.

Pittendrigh, C. S. (1981a). Circadian systems: general perspective. In: Aschoff, J., ed. *Handbook of Behavioral Neurobiology. Volume 4. Biological Rhythms.* New York: Plenum Press, Chap. 5, pp. 57–80.

Pittendrigh, C. S. (1981b). Circadian systems: entrainment. In: Aschoff, J., ed. *Handbook of Behavioral Neurobiology. Volume 4. Biological Rhythms.* New York: Plenum Press, Chap. 7, pp. 95–124.

Pittendrigh, C. S. (1993). Temporal organization: reflections of a Darwinian clock-watcher. *Annu. Rev. Physiol.* 55:17–54.

Pittendrigh, C. S., Bruce, V. G. (1959). Daily rhythms as coupled oscillator systems and their relation to thermoperiodism and photoperiodism. In: Withrow, R. B., ed. *Photoperiodism and Related Phenomena in Plants and Animals.* Washington, DC: A.A.A.S., pp. 475–505.

Pittendrigh, C. S., Minis, D. H. (1964). The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *Amer. Nat.* 98:261–294.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

Pittendrigh, C. S., Minis, D. H. (1971). The photoperiodic time measurement in *Pectinophora gossypiella* and its relation to the circadian system in that species. In: *Biochronometry.* Washington: National Academy of Sciences, pp. 212–250.

Pittendrigh, C. S., Daan, S. (1976a). A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. *J. Comp. Physiol.* 106:223–252.

Pittendrigh, C. S., Daan, S. (1976b). A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J. Comp. Physiol.* 106:291–331.

Pittendrigh, C. S., Daan, S. (1976c). A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. *J. Comp. Physiol.* 106:333–355.

Pittendrigh, C., Bruce, V., Kaus, P. (1958). On the significance of transients in daily rhythms. *Proc. Natl. Acad. Sci. USA* 44:965–973.

Pittendrigh, C. S., Elliott, J. A., Takamura, T. (1984). The circadian component in photoperiodic induction. *CIBA F. Symp.* 104:26–47.

Ralph, M. R., Menaker, M. (1988). A mutation of the circadian system in golden hamsters. *Science* 241:1225–1227.

Reddy, A. B., Field, M. D., Maywood, E. S., Hastings, M. H. (2002). Differential resynchronization of circadian clock gene expression within the suprachiasmatic nuclei of mice subjected to experimental jet lag. *J. Neurosci.* 22:7326–7330.

Rensing, L., Ruoff, P. (2002). Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular basis. *Chronobiol. Intern.* 19:807–864.

Roenneberg, T., Foster, R. G. (1997). Twilight times: light and the circadian system. *Photochem. Photobiol.* 66:549–561.

Rothenfluh, A., Young, M. W., Saez, L. A. (2000). TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron.* 26:505–514.

Ruoff, P., Vinsjevik, M., Monnerjahn, C., Rensing, L. (2001). The Goodwin model: simulating the effect of light pulses on the circadian sporulation rhythm of *Neurospora crassa. J. Theor. Biol.* 209:29–42.

Saunders, D. S. (1978). An experimental and theoretical analysis of photoperiodic induction in the flesh-fly, *Sarchophaga argyrostoma. J. Comp. Physiol.* 124:75–95.

Shirakawa, T., Honma, S., Honma, K. (2001). Multiple oscillators in the suprachiasmatic nucleus. *Chronobiol. Int.* 18:371–387.

Strogatz, S. H. (1994). *Nonlinear Dynamics and Chaos.* Reading, MA: Addison-Wesley, p. 498.

Swade, R. H. (1969). Circadian rhythms in fluctuating light cycles: toward a new model of entrainment. *J. Theoret. Biol.* 24:227–239.

Taylor, W. R., Krasnow, R., Dunlap, J. C., Broda, H., Hastings, J. W. (1982). Critical pulses of anisomycin drive the circadian oscillator in *Gonyaulax* towards its singularity. *J. Comp. Physiol.* 148:11–25.

Toh, L. L., Jones, C. R., He, Y., Eide, E. J., Hinz, W. A., Virshup, D. M., Ptacek, L. J., Fu, Y.-H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* 291:1040–1043.

Williams, J. A., Sehgal, A. (2001). Molecular components of the circadian system in *Drosophila*. Annu. Rev. Physiol. 63:729–755.

Winfree, A. T. (1970). Integrated view of resetting a circadian clock. *J. Theor. Biol.* 28:327–374.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Winfree, A. T. (1971). Corkscrews and singularities in fruitflies: resetting behavior of the circadian eclosion rhythm. In: Menaker, M., ed. *Biochronometry.* Washington, DC: National Academy of Sciences, pp. 81–109.

Winfree, A. T. (1980). The geometry of biological time. *Biomathematics.* Vol. 8. New York: Springer Verlag, PP. 530.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G. D., Sakaki, Y., Menaker, M., Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science* 288:682–685.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016