Phase Response Curves: What Can They Tell Us about Circadian Clocks?

Carl Hirschie Johnson

Department of Biology, Vanderbilt University Nashville, TN 37235, U.S.A.

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Introduction

Few chronobiologists have attempted to review the topic of Phase Response Curves (PRCs)—the reviews by Aschoff (1) and Pittendrigh (55) are the notable examples. Probably the task has seemed too daunting to undertake. Compiling the PRC Atlas (31) forced me to study all the published PRCs. This project led to some generalizations that I believe are worthwhile to summarize herein. This paper does not attempt to comprehensively review all PRCs—the Atlas is itself the most comprehensive review possible—but to discuss generalizations based on the PRC Atlas. The topics to be addressed are (A) PRCs as a reflection of entrainment mechanisms (resetting by light, dark, and temperature stimuli) and ecological strategies thereof; (B) phototransduction pathways; (C) PRCs as probes for the mechanism of circadian pacemakers; (D) PRCs as phase markers for the oscillator; and (E) PRCs as gauges of the amplitude of circadian oscillators. This paper shows figures, most of which come from the PRC Atlas, and refers to the PRC numbers of the Atlas format (e.g., A/Gp-2, C/Nc-10).

I. What are PRCs and PTCs?

A PRC is a plot of phase-shifts as a function of circadian phase of a stimulus. Stimuli include light pulses, temperature pulses, or pulses of drugs or chemicals. As shown in Figs. 1A, 1D, and 1G, representative PRCs of circadian oscillators for light pulses exhibit delay phase shifts in the early subjective night and advance phase shifts in the late subjective night, with little phase shifting occurring during the subjective day (hence, the subjective day portion of the PRC is often referred to as the "dead zone"). As will be discussed later, some of these topological features of light PRCs are crucial in determining the ability of circadian pacemakers to entrain to the daily light/dark cycle.

As illustrated in Fig. 1, there are two so-called "types" of PRCs—Type 1 and Type 0 (72). Type 1 displays relatively small phase-shifts (e.g., usually less than 6-hour phase-shifts) and has a continuous transition between delays and

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advances (Fig. 1A), whereas Type 0 PRCs show large phase-shifts (Fig. 1D). If the phase shifts of a Type 0 PRC are plotted as advances and delays, a discontinuity (the "breakpoint"—see below and in Fig. 2) often appears at the transition between delay and advance phase-shifts. The terms Type "0" and "1" refer to the average slope of the curve when plotted as "new phase" *vs.* "old phase"—a so-called "phase-transition curve" or PTC (whereas a PRC plots "phase-shift" *vs.* "old phase"). As shown in Fig. 1B, Type 1 resetting can be visualized as a PTC with an average slope of 1 (45° angle), whereas Fig. 1E depicts a Type 0 PTC, which has an average slope of 0 (0° angle).

Whether Type 1 or Type 0 resetting is exhibited often depends upon the strength of the stimulus. For example, increasing the light dose (*Culex*: 7.5-min *vs.* 120-min light pulses, F/Cq-1-2) or drug dosage (*Gonyaulax*: anisomycin 0.1 *vs.* 0.3 μM, F/Gp-19-20) converts Type 1 into Type 0 resetting. Other factors, however, can also cause the Type 1 to Type 0 conversion: e.g., genetic mutation (*Drosophila melanogaster*: F/Dm-1-2) and background light quality and/or intensity (*Gonyaulax*: A/Gp-4-5). These "other factors" probably affect the sensitivity of the clock to stimuli, so that they affect the perceived stimulus strength.

The "limit-cycle" interpretation of Type 1 *vs.* Type 0 resetting is also compared in Figures 1C and 1F. Phase-shifting stimuli are posited to change the state variables from the "limit cycle" (the circle of Figs. 1C, 1F, 1I) to another area of the phase plane, labelled the "resetting contour" (the heavy dashed line of Figs. 1C, 1F, 1I). If this change moves the state variables to another isochron on the phase plane, a steady-state phase shift will be observed. Type 1 resetting results if the resetting contour is not moved beyond the singularity (resetting contour on "near side" of singularity), whereas Type 0 resetting occurs if the stimulus is strong enough to move the variables beyond the singular region (resetting contour on "far side" of singularity). Figs. 1G, 1H, 1I also illustrate an interesting case of "critical stimulus" resetting in *Gonyaulax* (A/Gp-5). Light pulses given early in the subjective night provoke Type 1 resetting, while light pulses given later yield Type 0 resetting, yielding a highly asymmetrical PRC and resetting contour.

It might be assumed that stimuli presented during the dead zone (e.g., ct3-10) do not modify the state variables. 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 do not move the variables to a different isochron (see Fig. 1F). Therefore, no phase-shift results. Consequently, state variables of the pacemaker 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 pacemaker to a different isochron.

The breakpoint discontinuity of PRCs is in some cases merely a plotting convention of arbitrarily assigning phase-shifts in one half-cycle (12 hours) as delays and the other half-cycle as advances. To avoid these arbitrary distinctions, sometimes Type 0 PRCs are plotted monotonically—that is, all phase-

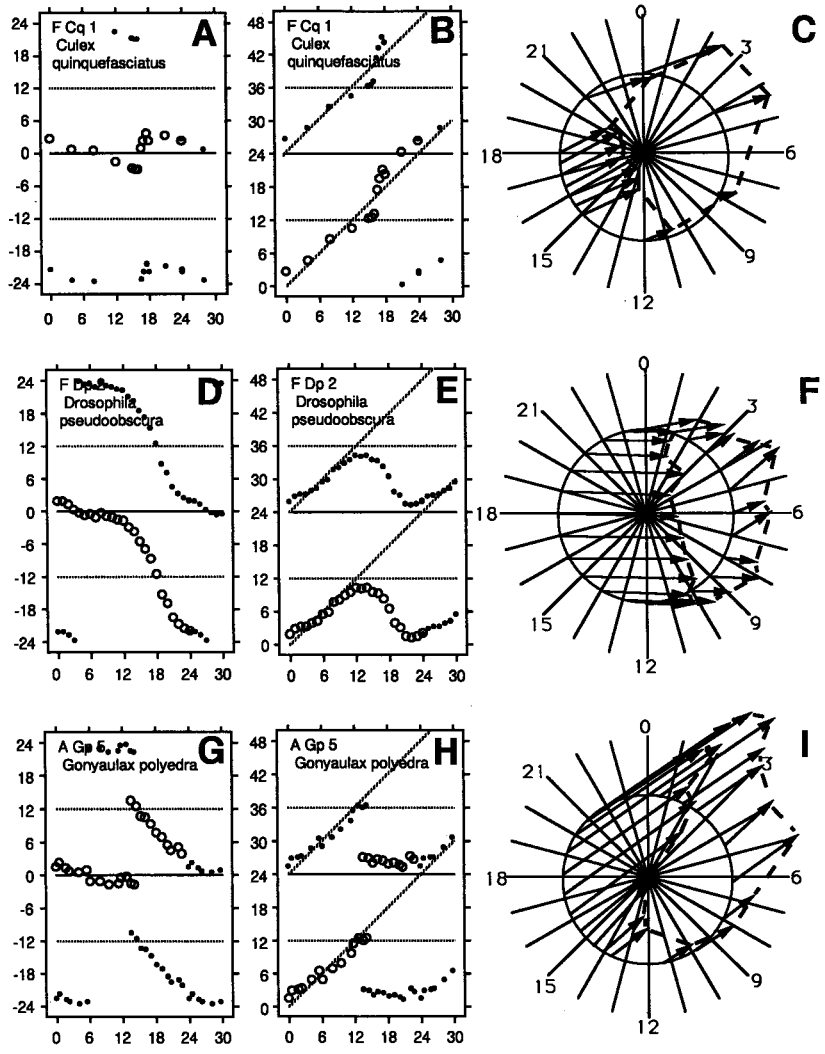

Fig. 1 Representative PRCs, PTCs, and limit cycle diagrams for Type 1 *vs.* Type 0 resetting. Figs. 1A, B, and C depict Type 1 resetting from *Culex* (F/Cq-1). Figs. 1D, E, and F depict Type 0 resetting from *Drosophila* (F/Dp-2). Figs. 1G, H, and I depict "critical stimulus" resetting from Gonyaulax (A/Gp-5). Figs. 1A, 1D, and 1G are PRCs, plotted as phase-shift (ordinate; advances positive, delays negative) *vs.* circadian time of stimulus onset (abscissa). Figs. 1B, 1E, and 1H are PTCs, plotted as "new" phase (ordinate; new phase = phase of clock after the phase-shift) *vs.* circadian time of stimulus onset (abscissa). Figs. 1C, 1F, and 1I are limit cycle diagrams, where the limit cycle is the circle with isochrons radiating from the central "singularity" point. The heavy dashed line is the resetting contour, i.e., the points on the phase plane to which state variables are changed by resetting stimuli. The resetting of state variables is depicted by the arrows from points on the limit cycle to points on the resetting contour.

shifts are plotted as delays from 0 to 24 hours, as shown in Fig. 1D. When plotting those Type 0 PRCs which happen to be asymmetric (e.g., Fig. 1G), the breakpoint is not an arbitrary convention, because the PRC has a discontinuity no matter how the PRC is plotted.

The advantage of plotting phase-shifts monotonically (or as a PTC) is that such plots do not mislead the reader into assuming that advance *vs.* delay resetting are mechanistically different, e.g., that advance phase-shifts result from a pacemaker's state variable being changed in one direction, while delay phase-shifts change the variable in an opposite direction. In fact, limit-cycle models usually suggest no mechanistic difference between advances and delays in Type 0 resetting. Moreover, the limit-cycle models usually interpret the transition from Type 1 to Type 0 resetting to be merely dependent upon whether the magnitude of the stimulus is sufficient to shift the resetting contour beyond the "singularity" (72).

In retrospect, I regret that I did not also plot phase-resetting behavior as PTCs in the Atlas, for two reasons. First, PTC_s avoid the misleading advance *vs.* delay distinction discussed above. Second, PTCs may encode other information about the pacemaker. For example, Coté (8) recently replotted a PRC published by Tamponnet and Edmunds (69) into a PTC format and may have discovered a heretofore unknown phenomenon: Type 2 resetting! Winfree (72) claims that Type 2 resetting implies three or more state variables must be intrinsic to the pacemaker's mechanism. This startling insight was obtained by merely replotting the data as a PTC. Therefore, PTCs can give us additional information. (Please note that the computer version of the Atlas allows one to switch quickly between PRC and PTC formats.)

II. How can a PRC be measured?

In principle, PRCs can be determined by a number of different protocols, as described by Aschoff (1). Four of the most commonly used protocols are described below (see ref. 1 for a discussion of other protocols):

(1) The stimulus (pulse) is applied while the oscillator is freerunning (e.g., a light pulse to an organism freerunning in DD). In this case, the individual organism serves as its own control, and accurate assignment of the circadian time of the stimulus depends upon knowledge of the circadian time of ϕr in a freerun. Usually the circadian time of ϕr is assessed by its phase in a LD 12:12 cycle, but it is important to make sure that no "masking" of ϕr occurs in LD. The possibility of masking can be evaluated by releasing the organism from LD to a freerun and confirming that ϕr in the freerun extrapolates back to ϕr in LD.

(2) The stimulus (pulse) is applied in a freerun shortly after release from entraining conditions (e.g., a light pulse to an organism in DD within a few cycles after release from LD 12:12). This is a good method when a population of organisms is being tested; it requires a few control organisms (cultures) which

do not receive a stimulus with which to compare the treated ones. This is probably the best method for estimating entrainment behavior, as the PRC shape soon after release from entrainment should be more reflective of its shape during entrainment than will its shape after a long exposure to free-running conditions.

(3) The stimulus is a "step" from one continuous condition to another (e.g., DD to LL).

(4) The PRC can be estimated from the phase angle assumed by the rhythm to different T-cycles of the stimulus (e.g., T-cycles of light pulses). An example of this method is that of Eskin (13), who compared the PRCs derived by method #1 above (see H/Pd-1) and method #4 and found them to be equivalent. Method #4 cannot give a complete PRC because the phase angle will not be stable around the breakpoint region of the PRC during entrainment to T-cycles.

III. How should a PRC be plotted ?

For compilation of PRCs into the Atlas, a standardized format was chosen so that different PRCs could be easily compared. The comments below refer to the format used in the PRC Atlas.

(1) *General information*: The abscissa is the circadian time (ct) of the stimulus, from ct0 to ct24. Circadian time 0 to ct24 is the duration of the endogenous period (τ, "tau"). Because LD 12:12 is taken to be standard entrainment conditions, circadian time 0 is defined as the beginning of the subjective daytime (therefore, subjective dawn or "lights-on"), and ct12 as the beginning of the subjective night (therefore subjective dusk). The ordinate is the magnitude of the phase shift in circadian hours (see below). Advances are plotted above the abscissa as positive values, while delays are plotted below as negative values. For Type 0 resetting, all the phase-shifts may be plotted monotonically, as in Fig. 1D.

(2) *Circadian time*: Because circadian pacemakers have different endogenous frequencies, PRCs among different organisms cannot be directly compared unless their time scales are standardized to "circadian time." The first aspect of circadian time is that the scales for both the circadian time of stimulus (abscissa) and the magnitude of phase shift (ordinate) are expressed in "circadian hours." PRCs are scaled in circadian hours so that both the horizontal and vertical axes of PRCs from different organisms may be directly compared. A circadian hour is equal to 1/24 of the endogenous period, τ (therefore, a circadian hour = tau/24 hours). To convert "real" hours to "circadian" hours, the number of real hours (e.g., of the phase shift) is multiplied by 24/tau.

(3) *Definition of circadian time zero*: The second aspect of circadian time is that PRCs must be plotted along the abscissa relative to some defined time, e.g., circadian time zero (ct0). In general, the definition of ct0 has been the least standardized variable of PRCs in the literature and yet, it is crucial for being able to compare the phase-shifting responses among organisms.

The standard definition of ct0 is as the phase in the freerun which extrapolates back to the last "dawn" (i.e., "lights-on" of the last-seen LD 12:12 cycle prior to release into constant conditions—DD or LL). In many cases, however, alternative definitions are necessary. PRCs which are measured in LL often use the beginning of LL as the extrapolated "dawn" rather than "lights-on" of the final light cycle. In addition, many PRCs have been measured from organisms which have been in constant conditions for a long time so that it is inaccurate or inconvenient to extrapolate to the final lights-on signal. For these PRCs, circadian time is usually defined from the phase reference point (ϕr). First, the phase angle in LD 12:12 of the phase reference point (ϕr) of the rhythm is measured. Then, this ϕr is assumed to define the same phase of the oscillator in freerunning conditions, and ct0 becomes a certain number of circadian hours before or after ϕr in the freerun. For example, activity onset (ϕr) of nocturnal rodents occurs at dusk in LD 12:12, and is consequently defined as occurring at ct12. Therefore, ct0 becomes that time which is twelve circadian hours before or after ϕr in a freerun. As discussed above (method #1 of PRC measurement), determining ϕr in LD can be complicated by the problem of masking. Consequently, in all cases, the circadian time of ϕr in LD should be determined by releasing the organism into freerunning conditions and extrapolating ϕr back to its phase angle in the last cycle of LD.

(4) *Estimation of the magnitude and direction of the phase-shift*: There are two major problems to be taken into account when estimating the phase-shift: (1) frequently the period (tau) changes after a stimulus ("aftereffects"), and (2) often —especially in the case of advance phase-shifts—there can be transient cycles of little or no phase resetting before the steady-state phase-shift is established. The best way to avoid both of these problems is to extrapolate ϕr for many cycles *before* and *after* the stimulus, preferably by a least-squares linear regression. Obvious transient cycles should be excluded from this regression. Then the phase-shift is calculated by the difference *on the day of the stimulus* between the extrapolated ϕr before and after the stimulus. In the case of method #2 of PRC measurement, the control ϕr's are extrapolated back to the day of the stimulus and used to compare with the extrapolations from experimental organisms.

For Type 1 resetting, it is usually easy to determine whether the phase-shift should be plotted as an advance or delay on a PRC, but when the large phase-shifts of Type 0 resetting are encountered, it is often difficult to unambiguously assign the direction of the phase-shift. One approach is merely to plot the PRC monotonically—from a limit cycle perspective, the distinction between advances and delays in Type 0 resetting is arbitrary anyway (see Fig. 1D).

Another approach to operationally distinguish between advances and delays while using a PRC-type presentation is to perform dose response experiments, thereby generating dose response curves (DRCs). DRCs assay the response at a given circadian phase of the clock to varying intensities/concentrations of the stimulus. Reducing the stimulus intensity will switch Type 0 resetting to Type

1 resetting, at which point the distinction between advances and delays becomes more obvious.

(5) *Definition of stimulus phase*: No matter what type of stimulus is considered, be it a light, temperature, or chemical pulse, the *onset* of the stimulus pulse has been plotted as the "stimulus phase" on the abscissa of the PRCs in the Atlas. In their original papers, many authors have plotted PRCs using other conventions for stimulus time—often the midpoint was used, and sometimes even the end of the pulse. There is no a *priori* reasoning which favors any of these criteria as the "stimulus phase." All are arbitrary. Aschoff urged in 1965 (1) that the stimulus midpoint be used as stimulus time. His argument —that PRCs plotted that way "line up" better—makes sense for many light PRCs. When one considers all the types of stimuli and PRC shapes, however, such a plotting convention can cause problems. In particular, I will argue later that for many chemical/drug stimuli, the *effective* duration of the pulse is unknown, since the time of recovery from a drug does not always coincide with the time of wash-out. If the effective duration is unknown, the midpoint is unknown.

The beginning of the pulse was therefore chosen as the standard marker for "stimulus phase." The phase-shifting response of an oscillator is likely to be a characteristic of the phase when the stimulus begins, that is, of the *first unperturbed phase* to be presented with a stimulus. If one uses the midpoint or end of the stimulus as the marker, then one is choosing a phase which has already been perturbed. And using the onset as the marker for stimulus phase circumvents the complications of recovery time.

IV. What are PRCs good for ?

(A) *PRCs as gauges of entrainment mechanism*

PRCs for light and temperature stimuli have been most valuable in understanding how circadian pacemakers are entrained to the daily cycle by environmental cues of light and temperature (55). Briefly, phase-resetting compensates for the fact that the freerunning period ϕ of circadian oscillators is not equal to 24 hours—therefore, entraining stimuli (e.g., light) reset the clock so as to equalize the period of the entrained oscillator (the circadian clock) to the period of the entraining oscillator (the daily rotation of the earth). This basic principle is summarized by the following equation for a circadian oscillator under steady-state entrainment:

$$\tau - \mathrm{T} = \Delta\phi$$

Light pulse resetting

Light is usually the most important Zeitgeber for entraining circadian oscillators. Therefore, PRCs for light stimuli have special interest and indeed, have

been studied most extensively. Light-pulse PRCs usually have similar characteristics: delay phase-shifts in the early subjective night, advance phase-shifts in the late subjective night, and little phase-shifting during the subjective daytime. This generalization holds true whether or not the overt rhythm peaks in the day, night, or at twilight (55). Therefore, the PRCs of nocturnal *vs.* diurnal organisms are similarly phased to the light/dark cycle, even though their rhythms are not.

The magnitude of phase-shifting exhibited by the clock is a gauge to the "limits of entrainment." Obviously, PRCs with large phase-shifts can permit synchronization to light-dark T-cycles of a broader range as compared with low amplitude PRCs. The magnitude of phase-shifting by light is dependent upon the intensity and duration of the stimulus (among other factors). As the intensity and/or duration is increased, light PRCs of limit-cycle pacemakers go through two transitions. As mentioned above, the phase-shifting first changes from Type 1 to Type 0 resetting (Figs. 1A, 1D), so the magnitude of phase-shifting increases, but the circadian time of the transition between delay and advance shifts remains fixed. There are several good examples of this type of PRC transition (Type 1 to Type 0) with increasing stimulus strength: *Chlamydomonas* (A/Cr-1-4), *Euglena* (A/Eg-3,4,7), *Kalanchoe* (D/Kb-1-11), *Leucophaea* (F/Lm-6-10), *Culex* (F/Cq-1-2), *Nauphoeta* (F/Nc-1-2), *Sarcophaga* (F/Sa-1-3), *Drosophila* (52), and *Rattus* (H/Re-1-3).

As the duration (and possibly intensity) of the light pulse is increased further, the second transition occurs: the circadian time of the break point begins to shift to earlier times (Fig. 2). This second transition has been interpreted as the clock "stopping" at ct12 until the light pulse is terminated, but other data suggests that the clock continues to oscillate during the light pulse on another limit cycle which is near an isochron of ct12 of the limit cycle in DD (51). Consequently, the clock always returns to the original limit cycle at ct12 at the end of the light pulse. Fewer examples of the second type of PRC transition (i.e., shifting breakpoint) exist for light PRCs—the best are *Drosophila* (52), and *Sarcophaga* (F/Sa-1-11, Fig. 2).

In addition to the changes of the PRC shape mentioned in the preceding paragraph, Page has recently discovered developmental plasticity of τ and PRC shape (49, 50). Page has found that raising cockroach larvae in various illumination regimes (LD, LL, DD and T-cycles) transforms the light PRCs of the mature cockroaches—i.e., the resulting PRCs become mostly-advance, mostly-delay, or symmetric PRCs, depending upon the developmental conditions (F/Lm-1-5, Fig. 3). This unexpected result demonstrates that PRCs are not developmentally immutable.

Stable entrainment does not necessarily require a PRC which has essentially the same symmetrical advance *vs.* delay topology as do the PRCs depicted in Figs. 1A and 1D. In fact, in order for entrainment to occur, a circadian oscillator's PRC need only have (1) a region of negative slope which is less than -2, and (2) a point on the PRC where the phase shift equals τ-T. In particular,

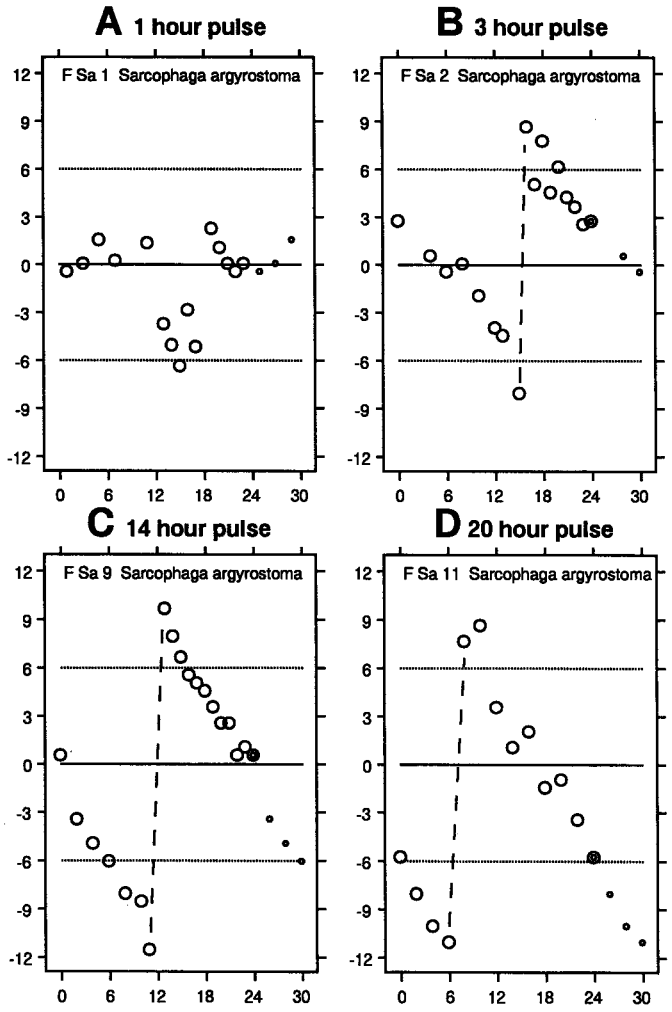

Fig. 2 Shifting breakpoint of PRCs to light pulses of various durations in *Sarcophaga* (F/Sa-1-11). The transition from delays to advances (="breakpoint") is indicated by the dashed lines. Durations of light pulses are 1 hour (2A), 3 hours (2B), 14 hours (2C), and 20 hours (2D).

it is not necessary to have a PRC with both delays *and* advances. If the freerunning period is longer than 24 hours, a PRC which only exhibits advance resetting will allow stable entrainment (this would be an example of a highly asymmetric PRC). A specific example is *Gonyaulax* cells under red light illumination (Fig. 1G, A/Gp-5): the period is 25 hours and the PRC for blue or white light pulses is essentially all advances (up to 12 hours advance). In this case, *Gonyaulax* will entrain to a light/dark cycle (or white/red light cycle) with the

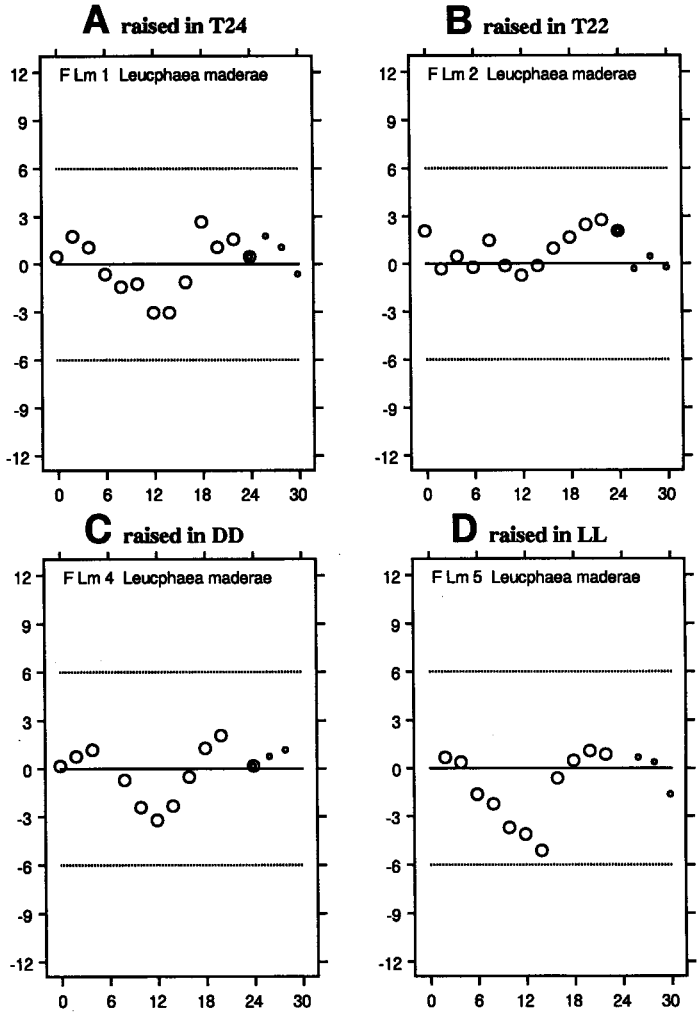

Fig. 3 Change in PRC shape depending upon illumination conditions during nymph development in *Leucophaea* (F/Lm-1-5). PRCs were measured from adults which were raised in light/dark cycles of T24 (3A) or T22 (3B), or in constant darkness (3C) or in constant light (3D).

onset of the light pulse (dawn) occurring at that circadian time which results in a one hour phase advance (33). Therefore, highly asymmetric PRCs can allow stable entrainment.

Dark pulse resetting

The apparently opposite stimulus of light pulses is to give dark pulses to organisms in LL (Fig. 4). The most simplistic model would predict that dark

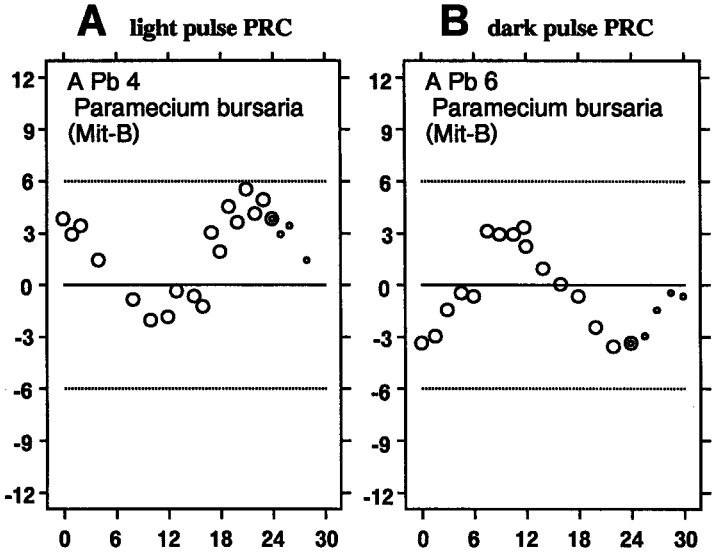

Fig. 4 Light vs. Dark pulse PRCs in *Paramecium bursaria* (A/Pb-4, 6). Fig. 4A is the PRC for 4-hour light pulses, Fig. 4B is the PRC for 6-hour dark pulses.

pulse PRCs will be the mirror-image of light pulse PRCs, which is nearly true in *Paramecium* (Fig. 4). Although this is an approximately valid description of some dark pulse resetting, dark pulse PRCs are sometimes not the exact mirror image of light pulse PRCs. One rationale for explaining why the two types of PRCs may not be mirror images is that the dark pulse stimulus in most PRCs is often longer than the corresponding light pulse stimulus. The PRC Atlas includes dark pulse PRCs for *Acetabularia*, *Euglena*, *Gonyaulax*, *Paramecium*, *Lemna*, chicken pineals, hamsters, sparrows, and bats (*Taphozous*).

Reebs and Mrosovsky (60) discovered an interesting "artifact" in hamsters with regard to dark pulses and pulses of some drugs. They noticed that dark pulses and some antidepressant drugs (e.g., the benzodiazepine triazolam) stimulate wheel-running activity in hamsters. They subsequently tested whether the stimulation of locomotor activity would alone mimic the phase-shifting action of dark pulses. It did! Furthermore, van Reeth and Turek (71) found that stimulation of activity was also the means by which phase-shifting by triazolam was accomplished. Therefore, in hamsters, dark pulses appear to reset by feedback of the overt rhythm back onto the pacemaker. It will be interesting to find other examples of feedback of overt rhythms onto pacemakers.

Temperature pulse resetting

Temperature pulse PRCs have been measured in a variety of organisms; those included in the Atlas test the clock response in *Euglena*, *Gonyaulax*, *Oedogonium*, *Neurospora*, *Bryophyllum*, *Kalanchoe*, *Lemna*, *Phaseolus*,

Hemideina, *Leucophaea*, *Uca*, *Perognathus*, and hamsters (in this final case, pulses of hypothermia). Although temperature can undoubtedly function as a zeitgeber, it apparently plays a supporting role to the light/dark cycle. In entrainment studies of conflicting light and temperature cycles, the light/dark cycle predominates in *Euglena* (5), *Drosophila*, cockroaches (52), and *Pectinophora* (57). Comparisons of the amplitudes of the light *vs.* temperature PRCs were not done in these studies. It would be interesting to repeat these types of experiments using light and temperature stimuli which elicit PRCs of equivalent amplitude, and then determine whether light still predominates.

Ecological strategies of PRC shape

The case of light-pulse PRCs which are asymmetric is especially interesting from an ecological perspective. These are PRCs which exhibit both delays and advances, but in which the area under either the advance or the delay portion of the PRC predominates (referred to below as the ratio of advance area to delay area, or A/D). Fig. 5 illustrates PRCs with various A/D shapes. Pittendrigh and Daan (53, 55, 56) have pointed out that an appropriate combination of tau values and asymmetric PRC shapes can give stable entrainment of pacemakers to various photoperiods such that ϕref. of the oscillator will always occur at a given phase angle to either dawn or dusk of the various light/dark cycles. This phase angle will be independent of the length of the photoperiod, so that it is compensated for seasonal changes in the photoperiod. For example, a tau of less than 24 hours in combination with a PRC which has relatively more delay area than advance area (=small A/D) will allow ct12 of the pacemaker's cycle to coincide with dusk on light/dark cycles which have a variety of ecologically

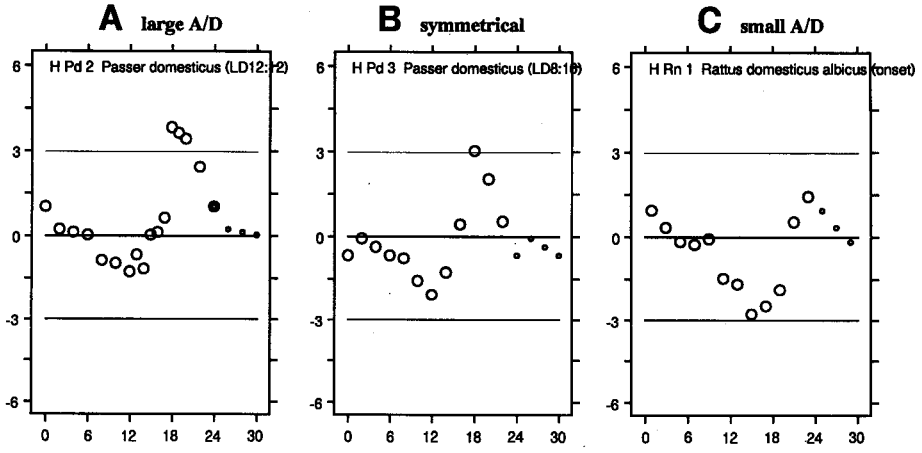

Fig. 5 PRCs exemplifying different A/D shapes. 5A has a large advance: delay ratio (*Passer*, H/Pd-2). 5B shows a PRC which is approximately symmetrical (*Passer*, H/Pd-3), while 5C depicts a PRC of small A/D from *Rattus* (H/Rn-1).

relevant daylengths (e.g., photoperiods from 6 to 18 hours). This is a strategy which may be adaptive for a nocturnal animal (e.g., a mouse). The converse example—long tau and large advance/delay PRC—yields an oscillator whose ct0 phase will coincide with dawn irrespective of the photoperiod's duration —hence, an optimal strategy for an organism which is active in the daytime (53, 55, 56).

Pittendrigh's and Daan's idea assumes that dusk is more important than dawn for night-active organisms, and that dawn is more important for day-active organisms. Obviously, this is a simplification of the ecology of ψ conservation. The crucial point is whether the organism's ecology demands adjustment of the clock to dawn *versus* dusk, not whether the organism is active in day or night. For example, one might imagine a nocturnal rodent which becomes active at an indeterminate phase sometime in the middle of the night, but must return to its burrow before dawn to escape predation. For this hypothetical rodent, a large A/D PRC and long τ might be the optimal clock system. Conversely, when a constant phase-angle to dusk is adaptive, a small A/D and short τ would be a good clock design (a common strategy for nocturnal rodents).

Another question about organisms exhibiting multiple rhythms controlled by a (presumably) single pacemaker is: which rhythm's phase is most important to conserve? A *Gonyaulax* cell has rhythms of both photosynthesis peaking in the day and bioluminescence peaking at night. Which ψ strategy the cell picks may depend upon which rhythm's phase angle is more important. As its pacemaker has a long τ and large A/D PRC, *Gonyaulax* seems to conserve its phase-relationship to dawn (33).

A different, but related, issue is whether the organism is exposed to the complete photoperiod during the day under natural conditions. (This "daylight-exposure" criterion is not the same as "day-activity"—for example, nocturnal predators such as cats may "see" the complete photoperiod during the day, but hunt at night.) Whether or not an organism is *exposed* to a more-or-less complete photoperiod (PPc) is important from the perspective of entrainment. We might suppose that clocks which are exposed to a complete photoperiod (PPc) might depend upon "continuous (parametric) entrainment" instead of "discrete (non-parametric) entrainment" (55). Moreover, clocks exposed to the full photoperiod could afford to be less light-sensitive than clocks which see only brief light pulses at dawn and/or dusk. These differences in photoperiod exposure might be correlated with differences in PRC shape (A/D), τ, and sensitivity.

To determine if PRC shape, τ, and sensitivity are correlated with PPc-exposure and/or activity patterns, information from the light PRCs in the Atlas has been distilled into Tables 1, 2, and 3. The first conclusion which is obvious from Table 1 is that it is impossible to draw any conclusion about the relative *sensitivities* between "PPc-exposed" and "non-PPc-exposed" clocks. Whether one defines "sensitivity" on the basis of (1) threshold intensity, (2) threshold duration, and/or (3) amplitude of PRC, few PRCs can be directly compared.

Table 1 Representative light PRCs

Organism (Background Ligth)	Stimulus[1]	PP_c exposure/ activity[2]	A/D	τ(h)	PRC Type (0or1)	Atlas PRC #
**Chlamydomonas* (DD)	15min W	yes	>1	25	1	A/Cr-1
**Chlamydomonas* (LL)	6h W $25Wm^{-2}$	yes	<1	25	0	A/Cr-4
**Euglena* (DD)	4h W 100ft. c.	yes	<1	23.7	1	A/Eg-4
**Euglena* (LD 3:3≈LL?)	9h W 7500lux	yes	<1	30.2	0	A/Eg-7
**Gonyaulax* (DD)	3h W	yes	>1	25.2	1	A/Gp-1 A/Gp-2
**Gonyaulax* (White LL)	4h W or B $175\mu Em^{-2}S^{-1}$	yes	>1	22.8	1	A/Gp-4
**Gonyaulax* (Red LL)	4h W or B $175\mu Em^{-2}S^{-1}$	yes	>1	25.4	0	A/Gp-5
**Paramecium bursaria* (DD)	4h W 5,000lux	yes	≈1	25.0	0	A/Pb-3
**Paramecium bursaria* (LL)	4h W 5,000lux	yes	>1	25.9	1	A/Pb-4
**Paramecium multinucleatum* (DD)	2h W 1,000lux	yes	≈1	24.1	0	A/Pm-1
**Neurospora* (bd) (DD)	5-45min. W 500-14,000lux	yes	>1	21-22	0	C/Nc-1, C/Nc-11
**Pilobolus* (Red LL)	0.5ms W	yes	≈1	26.5	0	C/Ps-1
**Albizzia* (DD)	2h B $3\times10^{14}q/cm^2s$	yes	≈1	23.5	1	D/Aj-1
**Bryophyllum* (DD)	4h W $0.6J/m^2s$	yes	<1	24.4	0	D/Bf-1
**Coleus* (LL)	4h W 1,300ft. c.(?)	yes	<1	23	1	D/Cbxf-1
**Kalanchoe* (DD)	2h R $1,600erg/cm^2s$	yes	>1	≈23	0	D/Kb-1
**Lemna* (DD)	15min. W $11Wm^{-2}$	yes	>1	25.5	0	D/Lg-1
**Lemna* (LL)	6h W $37.5Wm^{-2}$	yes	<1	25.5	1	D/Lg-2
**Phaseolus* (LL)	3h W 15,000lux	yes	≈1	27.5	1	D/Pc-1
**Phaseolus* (DD)	3h W 3,500lux	yes	≈1	24.5(?)	0	D/Pc-5
**Samanea* (DD)	5min. R $2.2J/m^2$	yes	≈1	22.2	0	D/Ss-1
**Samanea* (DD)	2h B $3\times10^{14}q/cm^2s$	yes	≈1	22.2	1	D/Ss-2
**Aplysia* (DD)	6h W 1,200lux	yes/DA	≈1	23-24	1	E/Ac-1
Aplysia (DD)	2h W 1,100lux	yes/DA	≈1	26.7	1	E/Ac-3
Anopheles (DD)	1h 70lux	?/CREP	≈1	≈23	1	F/Ag-1
**Antheraea* (DD)	30min. W	yes/DA	<1	22	1	F/Ap-1
Culex (DD)	7.5min. W 8,000lux	?/CREP	>1	24.3	1	F/Cq-1
Culex (DD)	2h W 8,000lux	?/CREP	>1	24.3	0	F/Cq-2
**D. auraria* (DD)	various	no (?)/?	≈1	≈2	1&0	D/Da-1 to D/Da-10
**D. melanogaster* (DD)	40min. W 300ft. c.	no (?)/?	<1	24.4	1	F/Dm-1
D. pseudoobscura (DD) (various conditions, various strains)	15min. W	no (?)/?	≈1	24	0	F/Dp-1 to F/Dp-15
**Hemideina*	12h W 100lux	no/NA	<1	24.6	0	F/Ht-2
**Leucophaea*	6-8h W various	no/NA	<1	23.7	1	F/Lm-1 F/Lm-6 F/Lm-7

Table 1(continued)

Organism (Background Ligth)	Stimulus[1]	PP_c exposure/ activity[2]	A/D	τ(h)	PRC Type (0or1)	Atlas PRC #
**Nauphoeta* (DD)	3h W 240μW/cm^2	no/NA	≈1	23.8	1	F/Nc-1
**Nauphoeta* (DD)	12h W 240μW/cm^2	no/NA	≈1	23.8	0	F/Nc-2
Pectinophora:						
*eclosion (DD)	15min. W 200lux	yes	≈1	22.4	1	F/Pg-2
*oviposition (DD)	15min. W 200lux	yes/DA	≈1	22.6	1	F/Pg-3
*egg hatch (DD)	8h W	yes	≈1	24	0	F/Pg-4
**Sarcophaga* (DD)	1h W 240μW/cm^2	yes/?	<1	23.8	1	F/Sa-1
**Sarcophaga* (DD)	3h–20h W 60–100lux	yes/?	≈1	23.8	0	F/Sa-2 to F/Sa-11
**Limulus* (DD)	2h W	yes/NA	≈1	23.9	1	G/Lp-1
**Procambarus* (DD)	15min. W 200lux	yes/NA	>1	23.5	0	G/Pb-1
**Ammospermophilus* (DD)	15min. W 100lux	yes/DA	<1	24.2	1	H/A1-1
Ammospermophilis (LL)	6h W 500lux	yes/DA	>1(?)	≈24.5	1	H/A1-3
**Gallus* (DD)	4h W 1,325lux	yes/DA	<1	≈24	1	H/Gd-1
**Glaucomys* (DD)	10&15min. W 0.5ft. c.	no/NA	<1	≈23.8	1	H/Gv-1 H/Gv-2
**Homo*	5h W (3×) ≈10,000lux	yes/DA	≈1(?)	24.2	0	H/Hs-1
**Mesocricetus auratus* (DD)	various 50–100lux	no/NA	>1	24.1	1	H/Ma-1 to H/Ma-21
**Mesocricetus brandti* (dim LL)	1h W 150lux	no/NA	≈1	24	0	H/Mb-1
**Mus musculus* (DD)	15min. W 100lux	no/NA	<1	23.4	1	H/Mm-1
**Mus norvegicus* (LL)	1h W 800lux	no/NA	>1	24.6	1	H/Mn-1
**Passer* (DD)	6h W 200–300lux	yes/DA	>1	24.9	0	H/Pd-1
Passer (DD)	4h W 800lux	yes/DA	>1	24	1	H/Pd-2
**Peromyscus leucopus* (DD)	15min. W 100lux	no/NA	<1	24	1	H/Pl-1
**Peromyscus maniculatus* (DD)	15min. W 100lux	no/NA	<1	22.9	1	H/Pm-1
**Rattus*	1h W 150lux	no/NA	<1	24.1	1	H/R-6
**Rattus* (dim LL)	4h W 520lux	no/NA	<1	24.3	1	H/Re-1 H/Re-2 H/Re-3
**Rattus* (DD) (norvegicus albicus)	30min. W 300lux	no/NA	<1	24.4	1	H/Rn-1 H/Rn-2
**Saimiri* (DD)	1h W 600lux	yes/DA	>1	24.3	1	H/Ss-1 H/Ss-2
**Sceloporus* (DD)	6h W 60lux	yes/DA	≈1(?)	23.6	1	H/So-1
**Taphozous* (LL)	15min. W 1,000lux	no/NA	>1	≈25	1	H/Tm-1

[1]Abbreviations: see abbreviations/definitions.

[2]=PP_c exposure/activity: "yes" or "no" refers to whether the organism is exposed to a complete photoperiod (="yes") or not (="no"); DA, NA, or CREP refers to the activity patterns of *animals*

—DA=day active, NA=night active, CREP=crepuscular activity.

*=PRCs used for the summary in Table 3.

Table 2 Activity patterns and PRC shape/τ in animals (summary of Table 1)

Active Period	A/D			τ		
	>1	≈1	<1	>24	≈24	<24
day	3	4	3	5	1	4
night	4	3	9	5	4	7
CREP	1	1	0	1	0	1

Table 3 PP_c-exposure and PRC shape/τ in all organisms (summary of Table 1)

Exposed to Complete Photoperiod?	A/D			τ			PRC Type	
	>1	≈1	<1	>24	≈24	<24	0	1
yes	12	17	10	19	4	16	17	22
no	3	5	10	6	6	6	5	13

This table summarizes the PRC data from Table 1 which has been marked with an asterisk.

Many different light intensities and durations ("stimulus" column) have been used, and few attempts to establish threshold sensitivities have been done. Although many of the studies on "PPc-exposed" organisms used long duration and/or high intensity light pulses—suggesting low sensitivity—some of the most sensitive clocks are "PPc-exposed" (e.g., *Neurospora*, *Samanea*).

Furthermore, the PRC can be "history-dependent," as shown for hamsters' and sparrows' response to prior photoperiod and T-cycles (e.g., H/Ma-6-13; H/Pd-2-4), and for cockroaches after larval growth in various T-cycles (Fig. 3, F/Lm-1-5). Because these studies have used widely varying conditions of light pulse duration and/or intensity, it is presently impossible to compare the sensitivity to light of oscillators in "PPc-exposed" and "non-PPc-exposed" organisms.

Tables 2 and 3 summarize the data of Table 1 from the perspectives of activity patterns and PPc-exposure. Table 2 shows the correlations between PRC shape, τ, and activity patterns in animals. Pittendrigh's prediction that night-active animals might prefer A/D < 1 and τ <24 is weakly supported by the data. The prediction that day-active animals might prefer A/D >1 and τ >24 is not supported by the data—no significant correlations between PRC shape and τ are obvious for day-active animals. The data for crepuscular animals are too scanty to make any conclusions.

Table 3 summarizes PRC shape and τ data for all representative organisms on the basis of PPc-exposure. PPc-exposed organisms exhibit A/D shapes of all kinds, with slightly more symmetrical PRCs. PPc-exposed organisms seem to avoid τ values close to 24 hours. Non-PPc-exposed organisms favor A/D <1 and Type 1 PRCs. When the data of Table 3 is reanalysed on the basis of plants *vs.* animals or whether the PRC was measured in background illumination (DD *vs.* LL), no new correlations emerge. (Note, however, that Aschoff's extensive analysis of τ *vs.* intensity of LL does indicate significant differences for this

response between "day-*active*" and "night-*active*" organisms—see ref. 2. Unfortunately, PRC shape is not known for most of those organisms.) The data of Table 3 demonstrate that some correlations between PRC shape and photoperiod-exposure may exist.

The data discussed above may prompt some revision of our concepts of entrainment mechanism. Our current entrainment models successfully explain the discrete entrainment mechanism of non-PPc-exposed clocks. It is in these cases that PRCs for brief light signals have helped us to understand entrainment. But organisms which are exposed to a complete photoperiod are in a quite different situation, which is not modelled well by PRCs to brief stimuli. In particular, τ may be changed by the light during a complete photoperiod (9, 55), the ψ-jump does not occur (56), and the clock can afford to be much less sensitive to light.

Pittendrigh (55) has discussed potential mechanisms of "continuous" entrainment in PPc-exposed organisms, but this mechanism(s) remains a fertile field to be tilled by future investigations, both by modelling and direct experimentation. In particular, limit-cycle modelling of continuous entrainment will undoubtedly lead to new insights (51). Additionally, some experimental questions which could be addressed in this context include:

(1) Compare threshold sensitivity (intensity and/or duration, but especially intensity) between PPc-exposed and non-PPc-exposed organisms (so far, only the hamster's threshold sensitivity has been carefully measured—see ref. 67).

(2) Test intensity/duration reciprocity for PPc-exposed *vs.* non-PPc-exposed organisms. So far, reciprocity has only been tested in hamsters (67) and *Chlamydomonas* (42).

(3) Measure PRC for long light pulses (8–16 hours) as the best gauge for predicting entrainment properties of PPc-exposed organisms.

(4) Test which aspect of a LD cycle is used as a zeitgeber in PPc- *vs.* non-PPc-exposed organisms. For example, how does the clock respond to the gradual changes of light intensity at twilight? In plants using phytochrome as clock photoreceptor, it may be the changing R:FR at dawn and dusk which is the most important zeitgeber. Also, can one predict phase-shifting by light *pulses* from the phase-shifts elicited by *steps*-LL to DD, or DD to LL?

(5) How does behavior modify the exposure of the organism to light and thereby modulate entrainment? DeCoursey (10) has shown that behavior can be a crucial component of entrainment in nocturnal rodents. Contrasting the behavioral components of entrainment in PPc-exposed *vs.* non-PPc-exposed organisms might be enlightening.

Obviously, many interesting questions about entrainment remain to be addressed.

(B) *Phototransduction pathways*

Photopigments involved in light pulse resetting

Apparently, the circadian clock became linked early in its evolution with (or had as a component) a photosensitive process which allowed the entrainment of the clock to the light/dark cycle of the sun. It might be supposed that this linkage could lead to valuable clues about the conservation or diversity of the oscillator's biochemical mechanism; if the circadian pacemaker originated once during evolution and its mechanism was subsequently conserved, then one scenario would predict that the pigment(s) involved in the photosensitive process might also be conserved.

Unfortunately, this is clearly not true. The action spectra described below show that the clock photopigments in various organisms are quite different amongst each other. This means either (a) that a conserved clock mechanism has switched its photopigment a number of times during evolution (more proximal steps in the phototransduction pathway might still be conserved), or (b) if the connection between clock mechanism and photopigment has been conserved during evolution, then the clock must have originated independently many times, and therefore the biochemical mechanisms of the clocks in various organisms could be quite different.

A first step towards characterizing the phototransduction pathway is to identify the clock's photopigment. In practice, identifying a photopigment requires measuring some specific characteristic of the photopigment and comparing it with the characteristics of known photopigments. Usually this entails action spectroscopy.

Action spectroscopy is dosimetry with light. To measure an action spectrum, one measures the photoresponse (in this case, the phase shift) at different wavelengths. For each wavelength, a range of fluences are used ("fluence" is number of photons per unit area; "fluence rate" is fluence per unit time, and is equivalent to the less precise term "intensity"). In the ideal case, where "univariance" holds, the slope of the fluence response curve (plotted as phase shift *vs.* log fluence) is the same for each wavelength, and the sensitivity at each wavelength is evaluated as the fluence necessary to achieve a given arbitrary photoresponse. On the other hand, if the shape or linearity of the fluence response is different at various wavelengths, it can mean that a screening pigment is interfering with the spectral response.

It is beyond the scope of this paper (see ref. 19, for a review of action spectroscopy) to go into greater detail about general action spectroscopy. But a specific issue about action spectra for resetting of limit cycle oscillators needs to be addressed. For Type 1 resetting, the resetting curve does not cross the singularity (Fig. 1C), and so the fluence response should match that of an ideal univariant case. This is in fact the result obtained by Takahashi *et al.* (67) for phase resetting of the hamster clock.

For Type 0 resetting, however, the expected response can be quite different-

even with an ideal univariant photoreceptor. At the phase(s) where light pulses yield maximal phase resetting, increasing the pulse fluence can cause the clock to be reset to regions close to or beyond the singularity. This can cause a discontinuity in the fluence response curve. This type of response to varying fluence has been observed in *Gonyaulax* (33), *Neurospora* (Fig. 32 in ref. 11), and *Chlamydomonas* (35). These discontinuous fluence response curves are reminiscent of the dose response of the *Gonyaulax* clock to anisomycin, which is another case of singular behavior (70).

A discontinuous fluence response curve means that some process "downstream" from the photopigment's absorption of light is converting the initially continuous photochemical response into a discontinuous biological response. In the case of clock photoreceptors, it is the limit-cycle organization of the circadian oscillator which is responsible for converting the initially monotonic response into a discontinuous response as the light pulse moves the pacemaker past the singular region.

How then should the spectral sensitivity of clock photoreceptors be measured? The following three tactics should be valid procedures to construct accurate action spectra for clock photopigments (see ref. 35 for more discussion and experimental examples). The first tactic is to use fluences and/or durations which elicit only Type 1 resetting (for a recent example, see ref. 67). In this case, the critical response is defined as an arbitrary "percent response" along a continuous fluence response curve. If Type 0 resetting is required by the experiment, the second tactic is to measure the action spectrum at a circadian phase which is not close to the PRC's "breakpoint." If this is done, the likelihood that the pacemaker will be moved through the singularity is reduced, and therefore the fluence response will probably be continuous. As in the first tactic, the critical response will be selected by the experimenter as an arbitrary "percent response?" Finally, in the case of Type 0 resetting, one can plot on the ordinate of the action spectrum the fluence at which singular behavior is elicited (i.e., arhythmicity or a discontinuity in the fluence response curve). This third tactic depends upon the pacemaker itself setting a critical threshold from which the action spectrum is derived in lieu of the experimenter selecting a "percent response" level from a continuous function (35).

Photobiology of clock photoreceptors is summarized in Tables 4 and 5. Most of these studies were performed before the complications of Type 0 resetting were fully appreciated. For example, several of the action spectra listed in Table 4 use only a single light fluence for each wavelength (an "equal-intensity" action spectrum) instead of the range of fluences required for proper action spectroscopy. This means that neither univariance nor the continuity of the fluence response was tested. Therefore, the conclusions of these studies may be compromised by artifacts. This criticism is true for the studies of *Coleus, Kalanchoe,* and Bryophyllum (possibly also *Paramecium*). In the case of *Gonyaulax*, only two fluences were checked, so this action spectrum may also have been compromised by this problem.

Table 4 Published action spectra for light resetting of circadian clocks

Organism	Background illumination	Peak(s) of action spectrum (nm)	presumed photopigment	PRC Atlas #	Reference
Bulla (not a complete action spectrum)	DD	500	rhodopsin	—	20
Chlamydomonas	DD	520,660	?	A/Cr-1	42
Chlamydomonas	LL	460-480,660-680	chlorophyll	A/Cr-2-4	34
Coleus (not a complete action spectrum)	green LL	blue=delays red=advances	?	D/Cbxf-1-2	23
Drosophila	DD	400-480 (delays and advances)	rhodopsin(?)	F/Dp-1-10	18
Drosophila	DD	450-460	rhodopsin	F/Dp-1-10	38
Gonyaulax	DD	475,650	?	A/Gp-1-2	26
Hipposideros	DD	400-480 430(delays) 520(advances)	rhodopsin(?)	—	36
Kalanchoe	DD	366,600-680	?	D/Kb-1-11	65
Mesocricetus	DD	500	rhodopsin	H/Ma-19	67
Neurospora (LL suppression of rhythm)	LL	460-470	flavin/cyt., b(?)	C/Nc-1	63
Neurospora	DD	465-470	flavin/cyt., b(?)	C/Nc-11	11
Paramecium	DD	350,430,600-700	?	A/Pb-1-5	12
Pectinophora	DD	400-480	rhodopsin(?)	F/Pg-1-3	6

Light-induced resetting in *Chlamydomonas* exhibits a remarkable feature—the action spectra for cells in DD *vs.* LL are different! The clocks of cells in LL respond to red and blue light and photosynthetic inhibitors prevent light-induced phase-resetting (34). Thus, components of photosynthesis appear to mediate clock resetting of cells in LL. On the other hand, green and red light resets the clock in DD, and photosynthetic inhibitors are ineffectual (42). The identity of this photoreceptor is unknown. The sensitivity of the two photosystems are also very different: cells in DD respond to 1/2000 of the fluence needed to reset cells in LL.

While the identification of most photopigments require action spectra to be measured throughout the visible range of wavelengths, one photopigment is exceptional: phytochrome, a common photopigment in plants, is characterized by red absorption (about 660 nm) and whose photoresponse is reversed by subsequent illumination with far-red light (about 730 nm). (Phytochrome can also exhibit the so-called "high irradiance response," or HIR, which is not reversed by far-red light, but is in fact potentiated by simultaneous irradiance with red and far-red light.) For simple phytochrome response (not HIR), far-red reversibility is usually considered to be a sufficient diagnostic and a complete

Table 5 Phytochrome as clock photopigment (light resetting partially or completely reversed by far-red light)

Organism	Background illumination	PRC Atlas #	Reference
Bryophyllum	DD	D/Bf-1	24
Lemna	DD	D/Lg-1-2	27
Phaseolus	LL (?)	D/Pc-1-5	7
Samanea	DD	D/Ss-1-2	66

action spectrum is often not done. Examples of circadian clock resetting in which phytochrome is implied are shown in Table 5.

In both *Gonyaulax* (A/Gp-8) and *Paramecium* (A/Pb-1,2), brief pulses of ultraviolet light can cause significant phase-shifting. The cells respond differently to ultraviolet light than to visible light: (1) the magnitude of the phase-shifting is not very phase dependent, and (2) the shifts are all advances (*Gonyaulax*) or all delays (*Paramecium*). Phase resetting by ultraviolet light is probably not relevant to entrainment in a natural setting, but it may imply something about the biochemical mechanism of circadian oscillators.

So far, the identification of some of these clock photopigments is not conclusive. Nevertheless, it is clear that no single photopigment is used by all circadian pacemakers. Some green plants use a phytochrome, while some animals use a rhodopsin. More studies of this type are warranted. (See ref. 47 for a more thorough, but outdated, discussion of clock photoreceptors.)

It may also be worthwhile to test the intensity *vs.* duration reciprocity of clock photoreceptors. Just as "HIR" photoreceptors show quite different reciprocity characteristics than that of phytochrome acting in its classic red/far-red mode, clock photoreceptors may likewise exhibit features not found in other photoreceptive systems. For example, Takahashi *et al.* (67) found an unusually long reciprocity (for rhodopsin) in the phase-shifting response of nocturnal hamsters. Logically, it "makes sense" for a clock photopigment to integrate light signals over long durations, but quantitative reciprocity studies have only rarely been done in the clock field. Reciprocity should also be tested in PPc-exposed organisms, which might ignore reciprocity—perhaps these organisms pay attention to the duration of the light pulse rather than to the total number of photons absorbed.

Characterization of phototransduction pathways: Chemical/drug PRCs have been used to trace the pathway of phase-shifting information from some type of receptor (e.g., photo-receptor) to the clockwork. This approach has been championed by Eskin (14). For example, Eskin has shown that the clock of the *Aplysia* eye responds to increases of cyclic GMP in much the same way as it does to light (E/Ac-1,14), suggesting that—as in light transduction within the vertebrate eye—cyclic GMP mediates the effect of light transduction within this clock's photoreceptor (16). This cGMP - induced resetting may be mediated by

new protein synthesis (59). Moreover, Eskin has discovered that the neurotransmitter serotonin causes phase-shifting within this eye (E/Ac-11), and this effect seems to be mediated by increases of the intracellular concentration of cyclic AMP because—in addition to other evidence—the adenylate cyclase activator forskolin elicits a PRC which is the same as that for serotonin (E/Ac-13; ref. 15).

Johnson and Nakashima (32) used a similar approach to study light-induced phase resetting in *Neurospora*. We found that inhibition of protein synthesis prevented light-induced phase-shifts in a dose-dependent manner. As a control, we showed that phase-shifting by light was not inhibited by the drug in mutants whose protein synthetic mechanism was resistant to the drug.

These studies exemplify some of the ways in which PRCs for chemical/drug stimuli can be used to study the transduction of phase-shifting information (either by light or other stimuli). In the case of blocking treatments which do not themselves cause phase-resetting, the interpretation of the results is relatively straightforward—if the blocking treatment inhibits the phase-shift by the tested stimulus, then the process affected by the blocking treatment may be involved in the transduction/transmission of the tested stimulus. The approach of using chemicals/drugs to block phase-shifting by another stimulus can, however, be difficult to interpret if the blocking treatment also causes phase resetting. These complications are discussed elsewhere (32).

Spectral Influences on Rhythm Expression and τ: In some plants, frequent phytochrome stimulation appears to be necessary for the *persistence* of circadian rhythmicity. This has been observed in *Albizzia* (64), *Lemna* (39, 40), and in transgenic tobacco (37). In two other plants, red *vs*. blue light has been found to differentially affect τ: in *Coleus*, red LL shortens τ, while blue LL lengthens τ (23); in Gonyaulax, the phenomenon is reversed: red LL lengthens τ, while blue LL shortens τ (62). These results have been interpreted to suggest that two photopigments are coupled to the circadian pacemakers in these organisms.

(C) *PRCs as probes for the clock mechanism: chemical/drug resetting*

Chemical stimuli have also been extensively tested for phase-resetting action. Early studies (e.g., ref. 25) suggested that the circadian clock was relatively resistant to drugs and chemicals, but now many pharmacological treatments have been discovered which reset the clock.

In general, the motivation for studying the phase response of circadian pacemakers to light or temperature stimuli has been to understand how the entrainment of the pacemaker to the solar day is accomplished. The motive for studying the pacemaker's response to chemicals and drugs is different. The hope is to unveil the biochemical mechanism of the pacemaker by assessing its pharmacological sensitivity. The impact of chemicals upon the pacemaker has been assayed by their effect upon both period and phase.

What can chemical/drug PRCs tell us about the pacemaker? PRCs for pulses of chemicals/drugs are usually interpreted to mean that the presumed

biochemical target(s) affected by the chemical is either a state variable or a state parameter of the pacemaker. In the discussion that follows, I will discuss chemical-induced clock resetting in the context of changes of a state variable. In a very simple biochemical oscillator composed only of a single state variable, chemicals which increase *vs.* decrease its level should evoke PRCs which are 180° apart and should have a predictable phase relationship to the phase of the oscillation of the state variable (see ref. 61 for an example).

The situation is considerably more complicated for an oscillator which is composed of more than one state variable, which will probably be found to be true for circadian oscillators. For multi-dimensional oscillators, the PRCs for perturbation of state variables cannot be predicted by the oscillation of any single state variable. Can PRCs still then be used to test whether biochemical entities are potential state variables? Yes—but accurate prediction of PRC shape depends upon modelling of all or most of the specific state variables and parameters in the oscillator and the interactions between these components. In the absence of such a specific model, the only unassailable prediction that can be made is that perturbation of the level of a state variable should provoke phase resetting. The shape or phase angle of the resulting PRC is not diagnostic in the absence of a specific multicomponent model. (See ref. 22 for an attempt to use chemical/drug PRCs to distinguish hands from state variables/parameters and to build a model of the pacemaker's mechanism.)

If this is true, is there any value for measuring complete PRCs for drugs/chemicals? Does measuring phase responsiveness at phases throughout the circadian cycle tell us anything more than data from a single phase point? I think the answer is yes, for several reasons. First, observing phase-shifts at various phases reassures us that the result is not an artifact. Second, knowing the phase responsiveness throughout the cycle will be useful for later modelling of the pacemaker or for designing future experiments. Finally, responsiveness must be measured at many phases in order to detect discontinuities or to distinguish Type 1 from Type 0 resetting; this information will undoubtably be crucial when the time comes to model the pacemaker's biochemistry.

Furthermore, note that the state variables of a limit cycle oscillator may be changed by phase-resetting stimuli, even if the stimuli are presented at phases of the "dead zone." As discussed previously in this article, the pacemaker is not necessarily insensitive to resetting stimuli presented during the dead zone; the state variables can be changed at these phases, but this change does not move the pacemaker to a different isochron. This phenomenon is relevant to methods of testing whether a specific biochemical substance is a state variable: phase-resetting stimuli presented during dead zone phases may modify state variables, even though no phase shift is elicited.

Trends in chemical/drug PRCs: Do different organisms show similar responses to pharmacological treatments? If so, would it suggest that the pacemaker's biochemical mechanism has been conserved during evolution? Not necessarily. For example, the fact that deuterium oxide lengthens the

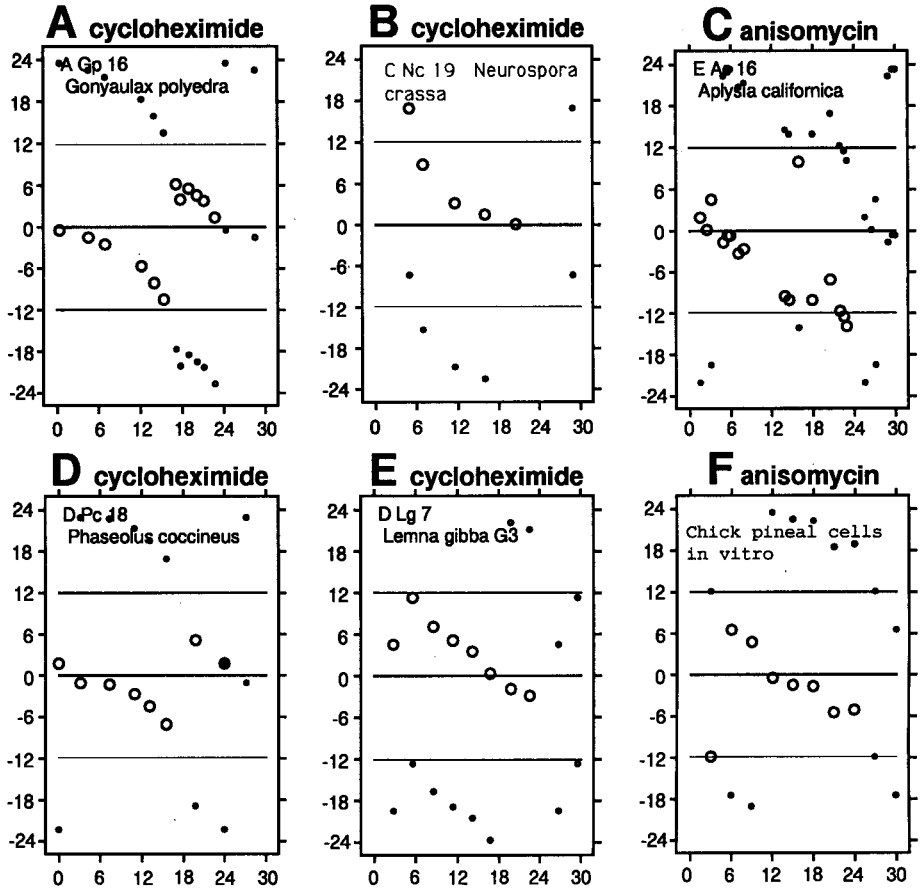

Fig. 6 PRCs for protein synthesis inhibitors. Cyclohexide phase-shifts the clocks of *Gonyaulax* (6A), *Neurospora* (6B), *Phaseolus* (6C), and *Lemna* (6E). Also shown are PRCs to anisomycin in *Aplysia* (6C) and chick pineal cells *in vitro* (6F; ref. 68).

circadian period of so many dissimilar organisms has been invoked to suggest that the clock's biochemistry has been conserved. Unfortunately, however, the action of deuterium oxide is so non-specific and its slowing effects upon biological processes is so universal that this data has not been helpful in unraveling the clock mechanism.

Other chemicals/drugs might be more useful. The class of drugs whose phase-shifting action has been best characterized in a variety of organisms is that of protein synthesis inhibitors on 80S ribosomes: cycloheximide, puromycin, anisomycin, and streptimidone. These drugs reset the clocks in a wide range of organisms: the algae Acetabularia (A/Am-2,3), and *Gonyaulax* (A/Gp-13-24, also see Fig. 10), the fungus *Neurospora* (C/Nc-19, 20), the angiosperms *Phaseolus* (D/

Pc-18,19) and *Lemna* (D/Lg-7), the eye of the mollusk *Aplysia* (E/Ac-15-18), chick pineal cells (Fig. 6F; see ref. 68), and hamsters (ref. 29). Fig. 6 illustrates that most of these PRCs show strong Type 0 resetting. Amino acid analogs also have potent resetting effects in Lemna (D/Lg-10-17; ref. 41). Cycloheximide is also known to lengthen the period of *Euglena* (17) and *Chlamydomonas* (21), but PRCs have not been reported for these organisms. Finally, it is interesting to note that inhibitors of protein synthesis on 70S ribosomes (e.g., chloramphenicol) have little or no effect on circadian rhythms in eukaryotes. Therefore, protein synthesis on mitochondrial or chloroplast ribosomes seems unnecessary for circadian precession in eukaryotic cells.

Another class of chemical/drug stimuli which have been applied to several types of organisms is that of inhibitors of metabolism. Again, the clocks of many organisms are reset by metabolic inhibitors: *Euglena* (nitrogen; A/Eg-9), *Neurospora* (azide, cyanide, antimycin A; C/Nc-26-28), *Lemna* (azide, cyanide; D/Lg-8,9), *Phaseolus* (cyanide, azide; D/Pc-20,21), *Aplysia* eyes (cyanide, dinitrophenol; E/Ac-7,8), and *Drosophila* (nitrogen; F/Dp-11). Some of these PRCs are shown in Fig. 7. In general, the responses to metabolic inhibitors are more variable amongst different species than the responses to protein synthesis inhibitors.

Drugs which affect cyclic AMP (cAMP) have also been applied to a variety of organisms: *Euglena* (theophylline; A/Eg-12), *Phaseolus* (theophylline; ref. 44), *Trifolium* (cAMP, theophylline; D/Tr-1,3), *Aplysia* (forskolin; E/Ac-13), rats (theophylline; H/R-1), and rat SCN *in vitro* (cAMP analog; ref. 58). Four of these PRCs are illustrated in Fig. 8.

Some other chemicals/drugs have been tested in various organisms, e.g., drugs which affect (1) intracellular Ca^{++} or calmodulin (A23187, chloropromazine, trifluoperazine, verapamil, EGTA, and theophylline—see Fig. 9), (2) membrane potential (K^+, Li^+, strophanthidin, electrical stimulation), or (3) other membrane properties and ion fluxes (fusaric acid, the anaesthetic quinidine, and the ionophores valinomycin, CCCP, CCmP, and A23187) but of these stimuli, no single agent has been applied to enough different organisms to allow meaningful comparison.

Interpretive problems with chemical/drug resetting: Of course, there are many caveats for the interpretation of chemical/drug PRCs. Most obvious is that one must be cautious about assigning the site of action of the chemical/drug. Side effects of pharmacological treatments abound, and are notorious for misleading researchers' conclusions. Four kinds of controls have been used as evidence for specificity of drug action. The first control is to measure the concentration dependence of the drug's effect upon the presumed site of action (e.g., protein synthesis) and compare it with the concentration dependence of the drug's phase-resetting efficacy. If the two do not correlate closely, then the drug's impact on phase is likely to be *via* a different site of action. The second way to assess the possibility of side-effects is to test mutants whose presumed site of action is resistant to the drug. If phase-shifting is concomitantly reduced

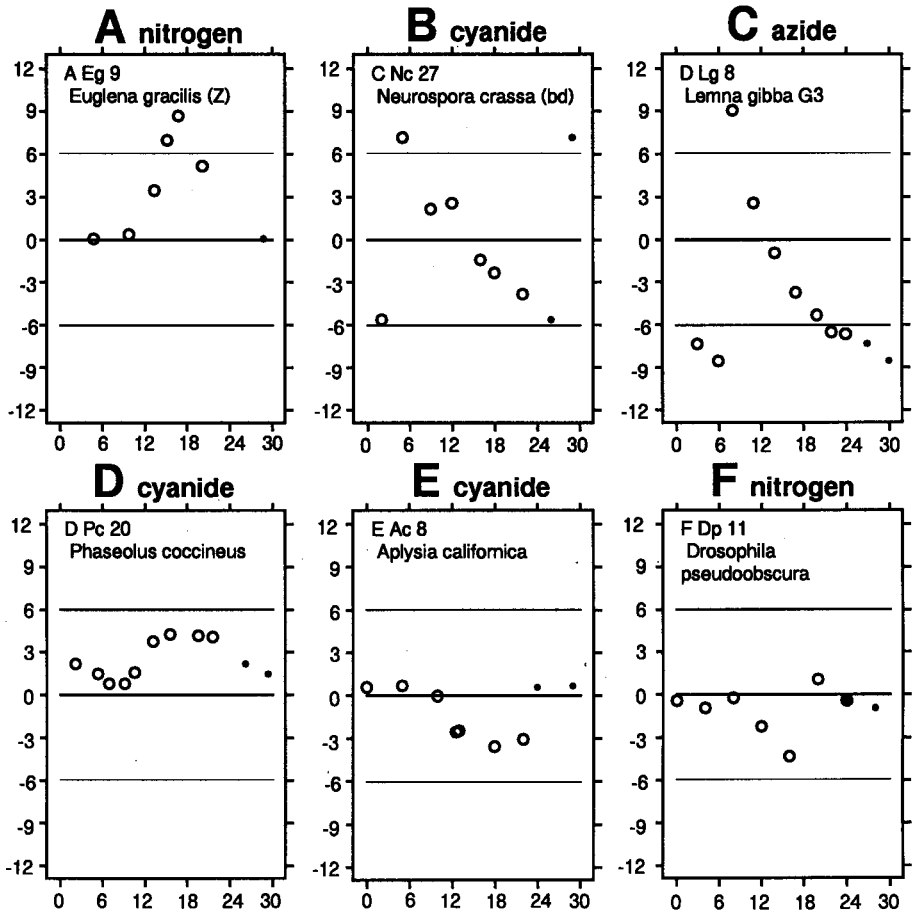

Fig. 7 PRCs for metabolic inhibitors. Cyanide phase-shifts the clocks of *Neurospora* (7B), *Phaseolus* (7D), and *Aplysia* (7E). Nitrogen resets the clocks of *Euglena* (7A) and *Drosophila* (7F), while azide resets *Lemna* (7C).

in these mutants, one may be more confident that side-effects are not responsible for phase-resetting action (46). The third method is to check whether derivatives of a drug which are inactive at the presumed biochemical target are nonetheless able to phase-shift the clock, as tested by Jacklet (30) for derivatives of the protein synthesis inhibitor anisomycin. Finally, a fourth control is to test various drugs which inhibit the same overall biochemical process, but by different mechanisms. A prime example of this control is the testing of various drugs which inhibit protein synthesis at different sites: cycloheximide, anisomycin, puromycin, streptimidone *et al.* (e.g., A/Gp-13-24, E/Ac-15-18, see Fig. 6).

Another problem with pulse application of chemicals/drugs is that the organ-

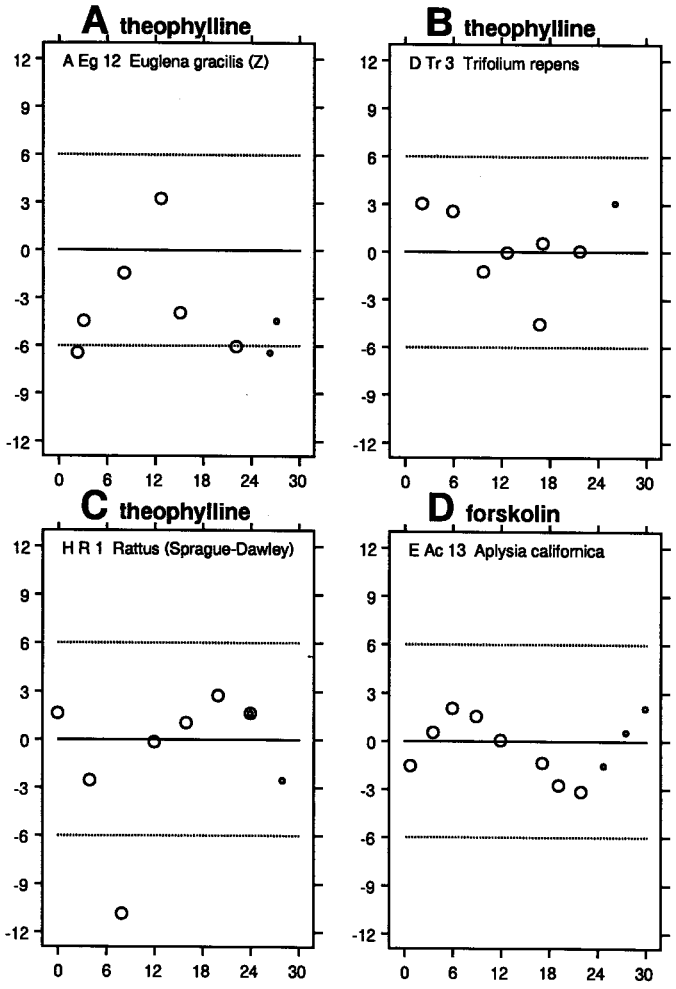

Fig. 8 PRCs for drugs which affect cyclic nucleotide metabolism. Theophylline resets the clocks of *Euglena* (8A), *Trifolium* (8B), and rats (8C). Forskolin phase-shifts the clock of *Aplysia* (8D).

ism may not recover quickly from the inhibition or stimulation (whereas, the recovery from light pulses is usually considered to be rapid). The times at which (a) a drug/chemical has begun to significantly affect a targeted biochemical process and (b) the targeted process has significantly recovered from the effects of the drug/chemical may be quite different from the times of drug/chemical (a) addition and (b) washout. These are important considerations in comparing PRCs for different drugs/chemicals whose penetration/recovery kinetics may differ, or even in comparing PRCs for the same drug/chemical in different organisms whose permeability characteristics may differ. Although

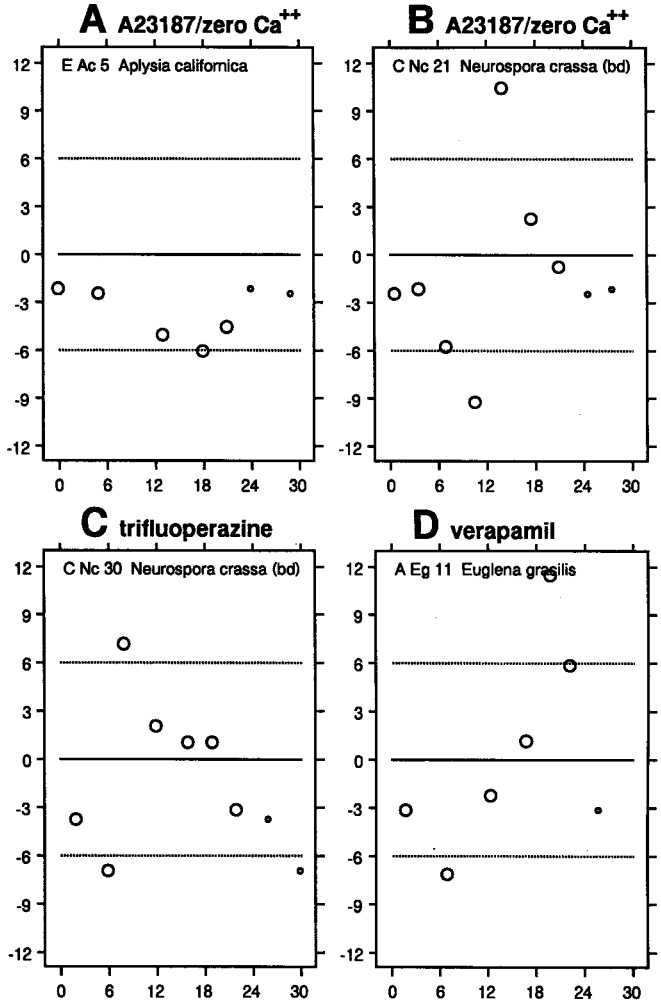

Fig. 9 PRCs for treatments which affect intracellular Ca^{++} and/or calmodulin. Resetting by the calcium ionophore A23187 is shown in 9A (*Aplysia*) and 9B (*Neurospora*). The calmodulin inhibitor trifluoperazine phase-shifts the rhythm of *Neurospora* (9C). Finally, the calcium-channel blocker verapamil resets the clock of *Euglena* (9D).

this problem may seem obvious, it has been scarcely discussed in the literature and seems to be little appreciated.

The best solution would be to measure the penetration/recovery times for each drug/chemical and for each organism. One of the very few examples where such measurements have been made is that of the inhibition of protein synthesis by anisomycin in *Gonyaulax*. In this cell, protein synthesis is significantly inhibited by anisomycin within 5 minutes after the cells are exposed to the drug. But the recovery of protein synthesis after the drug is removed can

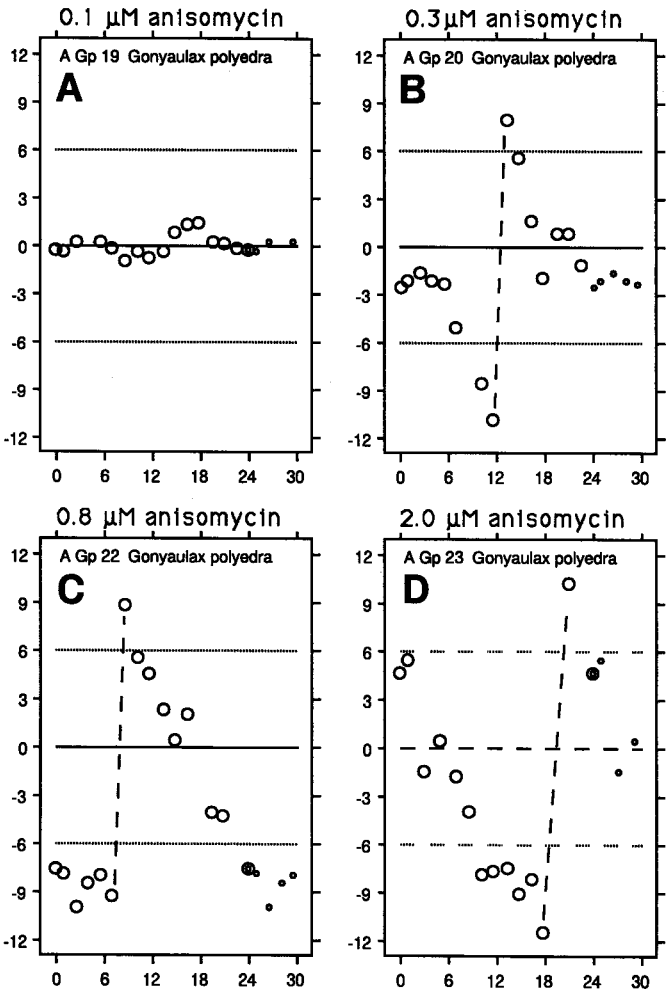

Fig. 10 Shifting breakpoint of anisomycin PRCs in *Gonyaulax* (A/Gp-19-23). The cells were given one-hour pulses of the protein synthesis inhibitor at various concentrations: 0.1 μM (10A), 0.3 μM (10B), 0.8 μM (10C) and 2.0 μM (10D).

require much more time, depending upon the concentration used. We have found that the recovery of *Gonyaulax* cells from anisomycin can require hours (perhaps even days) and that this recovery is a function of the concentration of the inhibitor which was administered to the cells: the higher the concentration, the longer the effective duration of the pulse (48). This is significant because increasing the duration of phase-shifting stimuli usually results in an increase of the magnitude of phase resetting and, for prolonged stimuli, often shifts the circadian time of the PRC's breakpoint.

This effect may help to explain the shifting breakpoints of PRCs to various

doses of anisomycin in *Gonyaulax* (70). As shown in Fig. 10, one hour pulses of anisomycin at increasing concentrations to *Gonyaulax* cells yields a Type 1 to Type 0 transition (Fig. 10A to 10B), then a progressive shift of the breakpoint to the left (A/Gp-19-23), or as displacement of the PRC downwards, depending upon your perspective (70). In Fig. 10D, the breakpoint is seen to have come through almost a full cycle. Presumably, the state variables are being pushed farther and farther out on the phase plane as the concentration is progressively increased. The effects of anisomycin persist after washout and the duration of that persistence is determined by the dose originally presented; as the dose of the anisomycin pulse is increased, the amount of time required for full recovery increases. Thus, a dose response curve for anisomycin in *Gonyaulax* is simultaneously a duration response curve. Consequently, PRCs for various doses of a drug/chemical can exhibit analogous results to that of PRCs for various durations of light stimuli, which exhibit shifting breakpoints if the duration of the light pulse is long enough, as mentioned above for the series of *Sarcophaga* PRCs (Fig. 2).

Furthermore, the ambient temperature might also affect recovery kinetics and may account for the shift in the PRC to cycloheximide at 20 C *vs.* 25 C that has been observed in *Acetabularia* (A/Am-2,3; ref. 4). Thus, one can never be completely certain *when* a chemical/drug pulse ends unless the recovery of the biochemical process which is affected is directly measured. It is for this reason that I favor the use of stimulus *onset* as the standard stimulus marker for PRCs. (In truth, the effective onset may also be uncertain since the kinetics of drug penetration can vary. In general, however, recovery usually depends upon more factors than penetration and is therefore, likely to be more variable.)

(D) *PRCs as Phase Markers for the Oscillator*

PRCs have been used frequently to probe phase position of the pacemaker underlying rhythmic behavior. This technique has led to several insights into essential features of circadian organization in multicellular organisms. One example is that many multicellular organisms display "transient" cycles after a light pulse given in the late subjective night (advance phase-shift), whereas light pulses given in the early subjective night (delay phase-shifts) usually provoke few if any transients (54). This observation prompted the hypothesis of multiple, hierarchically coupled oscillators within multicellular organisms: overt rhythms are directly controlled by "slave" (B) oscillators which are synchronized to a "master" (A) circadian oscillator. This master oscillator is, in turn, entrained to the solar cycle by the resetting mechanism reflected by the PRC (54).

This hypothesis relies upon evidence from a special kind of PRC experiment, the elegant 2-pulse PRC, which shows that the pacemaker resets rapidly after a light pulse. These results are interpreted to mean that the transient cycles observed after an advance phase-shift are a reflection of the resynchronization

of the intermediary slave (B) oscillator, not of the master (A) oscillator (54). At the present time, transient cycles after phase resetting by light have not been documented for unicellular organisms. This may mean that rhythms in single cells are controlled directly by light-sensitive pacemakers without any intermediary slave oscillators. Two-pulse light PRC experiments have been performed in *Drosophila* (54, 55), hamsters (H/Ma-12-13), and sparrows (3).

In addition, Hobohm *et al.* (28) have used the two-pulse PRC paradigm to answer a different question: is the oscillator of *Gonyaulax* precessing under some conditions in which the overt rhythm is not expressed? The answer was "yes," as indicated by two-pulse PRCs using pulses of the protein synthesis inhibitor anisomycin as stimuli.

In addition to using chemical/drug PRCs to identify biochemical processes which act as state variables/parameters in the clockwork, they have also been used for mapping phases of the circadian pacemaker. This information can then be used to determine whether mutations or pharmacological treatments which affect tau exert their effects throughout the cycle or during only a fraction of the pacemaker's time course. The first use of a PRC for this purpose was the study of Daan and Pittendrigh which used the light PRC to map the tau-lengthening effect of deuterium oxide through the circadian cycle of the mouse (see H/Mm-1,2). Another example has been to determine whether the PRC has been deformed by exposure to various photoperiods or T-cycles (H/Ma-6-11, H/Pd-2-4). More recently, Nakashima (45) has used light, temperature, and chemical/drug PRCs to map the phases affected by tau-mutations in *Neurospora*, and Kondo (41) has used amino acid analogs as resetting stimuli to map pacemaker phases in the duckweed *Lemna*. The use of PRCs to these various stimuli which have breakpoints spread throughout the pacemaker's cycle has allowed Nakashima and Kondo to do a much finer mapping of the circadian phases effected by the mutations than a single PRC would have permitted.

Mapping the pacemaker with chemical/drug PRCs can be fraught with problems, however, for the same reason that various concentrations of anisomycin shift the phase of the breakpoint in *Gonyaulax* (Fig. 10). Imagine for a moment using anisomycin to map the pacemaker in *Gonyaulax* under two different conditions (e.g., strain, mutation, temperature, background illumination). If the cells' sensitivity to anisomycin (or recovery from anisomycin) is altered by the differing conditions, then the cells can respond differently to the same concentration of anisomycin. This means that the clocks of cells under two different conditions might be perfectly in phase, but the phase of the PRCs elicited by Type 0 resetting by anisomycin could be quite different. This is a potential pitfall of using chemical/drug PRCs to map any pacemaker. Of course, recovery from some drugs may be much faster than as in the case of anisomycin in *Gonyaulax*. Nevertheless, it is obvious in retrospect that many chemicals/drugs will have effects long after the drug's washout. Therefore this is a trap which must be avoided whenever a chemical/drug is used to map the

pacemaker.

If this is true, is there any value for measuring complete PRCs for drugs/chemicals? Does measuring phase responsiveness at phases throughout the circadian cycle tell us anything more than data from a single phase point? I think the answer is yes, for several reasons. First, observing phase-shifts at various phases reassures us that the result is not an artifact. Second, knowing the phase responsiveness throughout the cycle will be useful for later modelling of the pacemaker or for designing future experiments. Finally, responsiveness must be measured at many phases in order to detect discontinuities or to distinguish Type 1 from Type 0 resetting; this information will undoubtably be crucial when the time comes to model the pacemaker's biochemistry.

Can this problem be circumvented? Yes. First, low concentrations of a chemical/drug which elicit only Type 1 resetting should yield PRCs which are an accurate reflection of the pacemaker's phase. If the larger magnitude of Type 0 resetting is helpful towards precise assignment of PRC phase, then the concentration used must be just above threshold for Type 0 resetting—before any shifting of breakpoint occurs. This Type 1 to Type 0 transition must be independently measured for each condition which is considered. The ultimate conclusion is that phase resetting to many concentrations must be measured to ensure that a chemical/drug PRC can be used to map the pacemaker under Type 0 resetting conditions. These controls have been done by Nakashima in various publications and also by Kondo (41).

(E) *PRCs as Gauges of Oscillator Amplitude*

Nakashima's light PRCs at different ambient temperatures (C/Nc-11-15) provide an example of how reinterpretating PRC data in terms of limit cycles can be interesting. The data, depicted in Figs. 11A, B, C, shows that the amplitude of the PRC to light decreases as the ambient temperature is increased. This might simply mean that the phototransduction mechanism becomes less efficient at higher temperatures. On the other hand, if one assumes that the light pulses move the state variables the same distance at all temperatures, a more interesting model emerges. Lakin-Thomas *et al.* (43) hypothesized that these data indicate that the amplitude (=diameter) of the limit cycle may increase as the temperature is raised. Thus, the same stimulus strength can provoke Type 1 resetting at high temperature (large diameter limit cycle) or Type 0 resetting at lower temperature (smaller diameter limit cycle) (shown in Fig. 11D). After proposing this interesting idea of amplitude resetting, Lakin-Thomas *et al.* (43) expand their model to suggest that changes in the amplitude of limit cycles may also explain period changes of mutants, where the circumference of the limit cycle is proportional to τ.

But I think that the idea of amplitude changes is even more interesting if one hypothesizes that τ does *not* change as the amplitude of the limit cycle changes. Indeed, such an assumption allows a limit cycle model of temperature compensa-

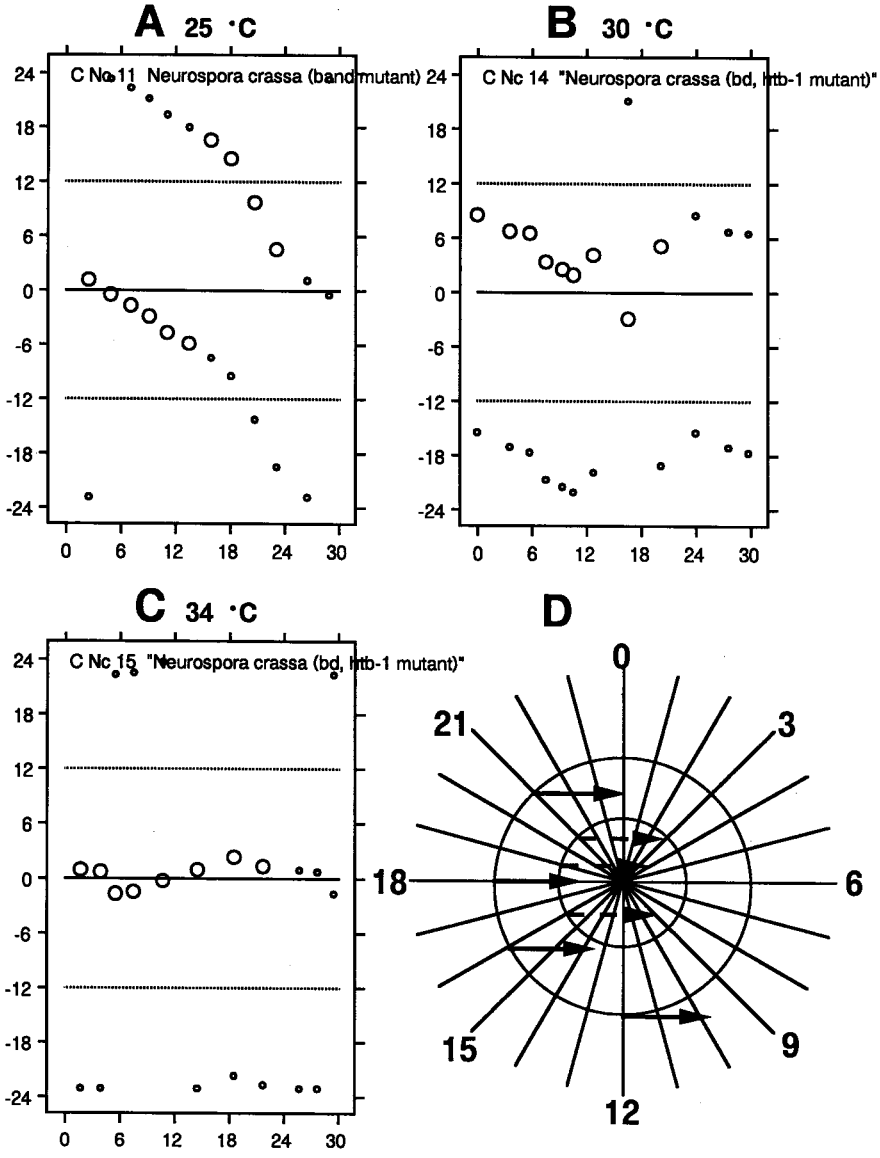

Fig. 11 Reduction of PRC amplitude at increasing ambient temperature—a limit-cycle model. PRCs to 5 min light pulses in *Neurospora* at 25°C (11A), 30°C (11B), 34°C (11C). 11D depicts a limit-cycle model where the larger diameter limit-cycle is at a higher temperature than the smaller limit cycle. Light stimuli provoke changes of the state variables, depicted by the horizontal arrows. Even though the changes of state variables are equivalent at both temperatures, the stimuli move the state variables beyond the singularity at the lower temperature (therefore, Type 0 resetting), whereas only Type 1 resetting is elicited at the higher temperature (larger limit cycle).

tion of τ. If the amplitude of the limit cycle is larger at higher temperatures, then the state parameters can be specified so that the angular velocity (τ) is the same at different limit-cycle amplitudes. If the angular velocity is conserved at different temperatures, then the linear velocity along the circumference must be faster at higher temperature (=larger circumference). In that case, the biochemical reactions which modulate the state variables can be temperature-dependent, as indicated by the larger circumference at higher temperature. By the very nature of the cycle inherent in a limit cycle, temperature-dependent reactions (linear velocity increases with temperature) can become temperature-independent rhythms (angular velocity=τ does not change with temperature) if the amplitude of the limit cycle increases as the temperature increases.

In addition to Nakashima's light PRCs at different temperatures, we have observed a similar temperature-dependency using pulses of protein-synthesis inhibitors as stimuli (4). Unfortunately, temperature dependency of light-induced phase-resetting has not been universally observed. Pittendrigh did not find the light PRCs of *Drosophila* to be temperature dependent (F/Dp-2,7,9,10). However, the *Drosophila* PRCs were measured with a saturating light stimulus, which could be so large that it would not detect significant changes in the amplitude of the limit cycle. In *Chlamydomonas*, I have measured PRCs with sub-saturating light fluence at 18°C and 25°C, and have yet not detected any significant differences (unpublished observations). At present, this intriguing hypothesis needs more testing.

Summary

In this paper, I have discussed what PRCs are, how they can be measured, and my opinion as to how they should be plotted. I have also mentioned why plotting phase resetting data in a PTC format is also advantageous in some circumstances. Finally, I have described research topics in which phase-resetting data has provided crucial insights: entrainment, phototransduction, pacemaker mechanism, phase markers of the pacemaker, and gauges of oscillator amplitude. Clearly, PRCs/PTCs have enlightened us, and will continue to do so.

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Some Abbreviations/Definitions

A/D = ratio of area of advance phase shifts to area of delay phase shifts in a PRC
B = Blue light
ft. c. = foot candles
FR = far-red light
pacemaker = oscillator = clock = master (A) oscillator
ψ = phase relationship of one cyclic phenomenon to another phase reference point
(ϕr) = a phase of the rhythm used as a reference point
PP_c = "complete" photoperiod
PP_s = "skeleton" photoperiod
PRC = phase response curve = a plot of circadian time of stimulus ("old phase") versus phase shift
PTC = phase transition curve = a plot of circadian time of stimulus ("old phase") versus "new phase" (i.e., phase of rhythm after phase shift has occurred)
rhythm = overt rhythm = "hand"
R = red light
W = white light