

CIRCADIAN SYSTEMS, I. THE DRIVING OSCILLATION
AND ITS ASSAY IN *DROSOPHILA PSEUDOOBSCURA**

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This series reports studies on circadian systems in insects and mammals. In constant darkness and temperature an organism may display one or more circadian rhythms in functions such as locomotion, drug sensitivity, or the specific activity of individual enzymes. The period, τ , of such rhythms is about 24 hours; it is usually precise and always temperature-compensated. In discussing these phenomena we describe the whole cell or organism as a *circadian system* whose output manifests (a directly observable) *circadian rhythm*, which must, *a priori*, be the result of a periodicity in its physiological control systems. We restrict the term *circadian oscillation* to this periodic driving element in the system's controls. Clearly the total system comprises, in addition to the driving oscillation, a plethora of physiological subsystems driven by this pacemaker. These driven elements may or may not be themselves periodic; in either case they are not oscillations ultimately responsible for the total system's periodicity. The driving oscillation entrains to (locks onto) a restricted class of environmental cycles, principally that of light and, to a lesser extent, temperature. It is thus responsible for phasing the entire circadian system to the daily cycle of change in the environment.

Our interest is in the following general questions: (1) What is the nature of the driving oscillation? (2) What is the mechanism of its entrainment by environmental cycles? (3) How is it coupled to the peripheral subsystems which it drives? (4) What functions does the oscillation serve in the economy of the total system (cell or organism)? (5) Are the circadian oscillations in diverse organisms alike and historically related, or are their similarities the product of evolutionary convergence?

This paper develops an operational distinction between the system's overt rhythmicity and its driving oscillation. Although one is necessarily restricted to the total system's output as an immediate assay, the properties of its driving oscillation are nevertheless open to analysis. The phase and period of the system, *when it is in free-running steady state*, are determined by the phase and period of its driving oscillation. *Steady-state* phase-shifts ($\Delta\phi$) of the free-running system thus measure *steady-state* phase-shifts of the driver. Our principal aim is to demonstrate the utility of $\Delta\phi$ of the system as an assay of the driving oscillation's state at each of its phase points: the magnitude and the sign of $\Delta\phi$ caused by a standard signal (e.g., a short light-pulse) are characteristically different for each phase-point in the system's (hence driving oscillation's) free-running cycle. The measurement of $\Delta\phi$ as a function of time during the system's free run thus measures the state of the driving oscillation as a function of time: the periodic change of state in the driver is reflected in the periodicity of $\Delta\phi$ which the system's phase-response curve¹⁻³ describes.

Experiments presented here demonstrate the utility of $\Delta\phi$ as a function of time: (1) to measure the period and phase of the driving oscillation; (2) to show that the

oscillation in *Drosophila* does not measurably change (damp) in three cycles of a free run; (3) that the phase relationship between the driving oscillation and a reference point for the system's phase is open to change; and (4) to provide new confirmation of our empirical model of the oscillation's entrainment by light-pulses.¹⁻³

The stock of *Drosophila pseudoobscura* which we use (PU 301) was collected originally in the Sierra Nevada in 1947. It has been the basis of all our published experiments on the *Drosophila* eclosion rhythm. It is maintained at 20°C in vials of Muller's *Drosophila* medium. The rhythm of pupal eclosion is assayed by fraction collector devices which trap freshly emerged adults every hour in separate vials.

We use the now-standard designations for light cycles: LL = constant light; DD = constant dark; LD 12:12 = a cycle of 12 hours light and 12 hours dark. Cycle 1, cycle 2, etc., are the first, second, etc., cycles of the free-running system in constant darkness and temperature. We also use the terminology of the oscillation analogy^{4, 5} for circadian phenomena. T is the period of any environmental cycle that entrains the rhythm. τ is the period of the free-running (unentrained) rhythm (and hence its driving oscillation). Individual phase (ϕ) points in the cycle are designated as circadian times (ct) on a scale that spans 24 hours of circadian time (= 360°); ct 12 is the phase at which the oscillation begins the "subjective night" (ct 12-24) after seeing 12 hours light in LD 12:12; ct 00 thus marks the beginning of the half-cycle we designate "subjective day." The phase relations (ψ) of the components within the system, and of each of them to the environment, require definition of phase-reference (ϕ_r) points. ϕ_r for environmental light cycles is the beginning of the light-pulse in each cycle; ϕ_r for the rhythm discussed here is the median of the distribution of pupal eclosion activity. ϕ_r for the driving oscillation is taken as the most reliably assayed point in the phase-response curve which is ct 18.5 in *D. pseudoobscura*.

In discussing phase-relations we distinguish: (1) $\psi_{R, L}$ the phase-angle (time) difference between the reference points of rhythm and light cycle; (2) $\psi_{R, O}$ the phase-angle difference between the reference points for rhythm and oscillation; and (3) $\psi_{O, L}$ the phase-angle difference between oscillation and light cycle (Fig. 2).

We use $\Delta\phi$ for phase-shifts, $+\Delta\phi$ for phase advances, and $-\Delta\phi$ for phase delays. In this paper we abandon the unfortunate convention of plotting $-\Delta\phi$ values above the zero line in phase-response curves.

Oscillation Assay in Drosophila pseudoobscura.—The phase-response curve in Figure 1, redrawn from our 1964 data, describes the steady-state $\Delta\phi$ imposed on the free-running system (and hence driving oscillation) by single light-pulses (15 min, 100 ft-c, white fluorescent) falling at different phases (ct times) in the oscillation's free-running cycle. This standard curve distinguishes advances ($+\Delta\phi$) and delays ($-\Delta\phi$). For comparing assays of the oscillation following experimental manipulation it is convenient to use a mathematically equivalent form in which the advances are displaced 360° (24 hr circadian time) on the ordinate and treated as phase delays [$-\Delta\phi = \tau - (+\Delta\phi)$] yielding a monotonic curve.

Figure 2 uses this monotonic form to show the $\Delta\phi$'s caused by single light-pulses (15 min, 100 ft-c) applied to each of 72 populations of pupae allowed to free-run in DD following entrainment by LD 12:12. The single pulse for each population

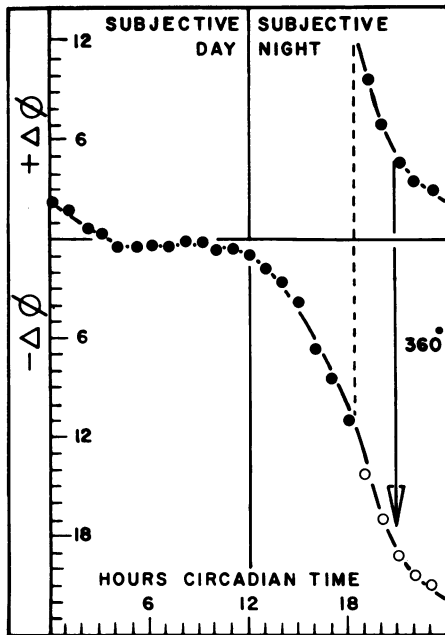


FIG. 1.—The phase-response curve for the driving oscillation in *Drosophila pseudo-obscura*. Redrawn from Pittendrigh and Minis (1964) and modified to show the derivation of its monotonic form used later in this paper. See text.

falls one hour later than the pulse for the preceding population; collectively the 72 populations assay the $\Delta\phi$ caused by a pulse through each of 72 hours following entry into a DD free run. The standard response curve, in monotonic form, is immediately evident through three full cycles showing: (1) that the oscillation does not measurably damp in at least three cycles; (2) that the period of the oscillation can be unequivocally assayed as the interval between its ϕ_r points (ct 18.5); and (3) that its phase relation to the preceding light cycle ($\psi_{o, L}$) can be assayed with equal certainty; the oscillation begins at ct 12 when the last 12-hour light went out.

Phase and period of the *free-running* oscillation can, of course, be more easily assayed via the rhythm's phase-reference $\phi_r (R)$ assuming that in the free run, $\psi_{r, o}$ is a constant (Fig. 2). There is, however, reason to believe, as we show below, that $\psi_{r, o}$ is open to change: (1) its genetic control is open to variation within the species; and (2) it does not remain fixed when the oscillation (and hence the system) is entrained by cycles with T far from τ . Under these circumstances the phase of the phase-response curve itself is the only sure, if laborious, assay of the oscillation's phase.

We have developed elsewhere¹⁻³ an empirical model for the oscillation's entrainment by light-pulses. We assume that when a light cycle (with period T) entrains the oscillation (period τ), the pulse in each cycle causes a discrete, effectively instantaneous phase-shift given by $\tau - T = \Delta\phi$. This model has been tested and upheld in various ways all involving comparison of predicted and observed values of $\psi_{o, L}$, the steady-state phase difference between oscillation and the driving light cycle. Published tests actually use $\psi_{r, L}$ as a measure of $\psi_{o, L}$: what was observed was the phase difference between the eclosion *rhythm* and the light cycle; and the justifying assumption was that $\psi_{o, R}$ remained constant. In general, observation matches prediction surprisingly well but there are significant discrepancies when T

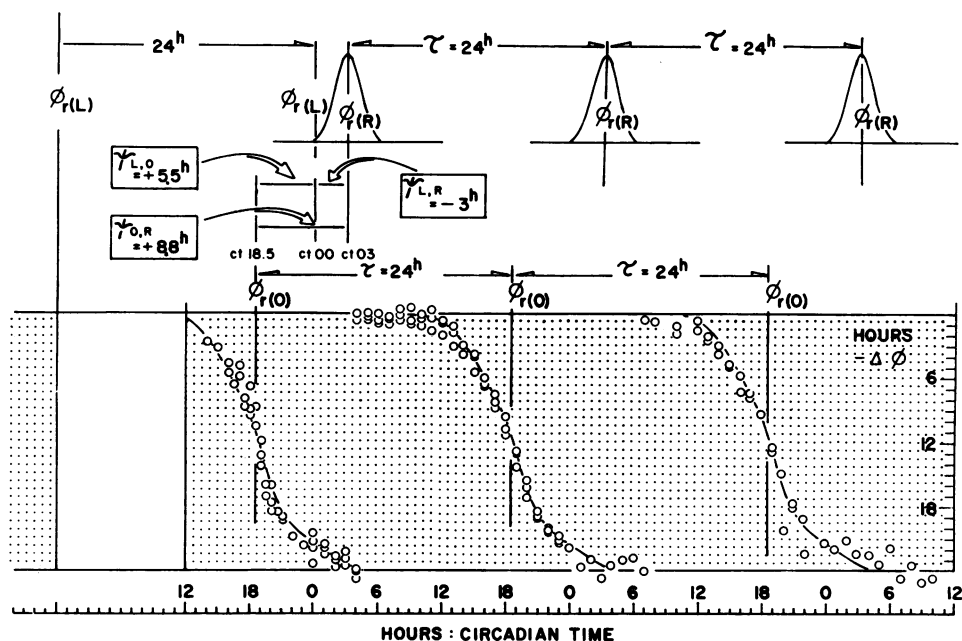


FIG. 2.—The phase-response curve for three full cycles (72 hr) of the oscillation's free run in DD following LD 12:12. The oscillation begins at ct 12 when the lights go out. Each point plots the phase-shift as $-\Delta\phi$ caused by a single 15-min light-pulse (100 ft-c, white fluorescent) at the times indicated on the abscissa.

gets far from τ , e.g., T_{21} and T_{27} . In T_{27} (one 15-min pulse/27 hr) the observed $\psi_{R,L}$ phase leads prediction by about two hours and in T_{21} its phase lags prediction by almost three hours. Figure 3 (*upper panel*) is a graphic prediction from the model of the *oscillation's* phase relative to the light-pulse ($\psi_{O,L}$) in T_{21} and T_{27} ; and the middle panel, using the monotonic form of the response curve, compares that prediction with observation during the first cycle of a free run in DD. The assay of phase of the phase-response curve is an assay of *oscillation* phase without any assumption about the constancy of $\psi_{O,R}$. The agreement of observation with prediction about $\psi_{O,L}$ based on observing $\phi_r(O)$ is very close (~ 1 -hr discrepancy), and is clearly closer (~ 2 - to 3-hr discrepancy) than the approximation to prediction of observations of $\psi_{R,L}$ based on the assumption that $\psi_{O,R}$ is constant (Fig. 3, *lower panel*). We conclude that $\psi_{O,R}$ is *not* constant when the system is driven at frequencies far from 1τ ; and we note that $\phi_r(R)$ phase leads the prediction based on $\psi_{O,R}$ constancy when $T > \tau$ and phase lags it when $T < \tau$. That is what we would expect were the driven process controlling eclosion time itself a periodic system driven by the light-sensitive driver.⁶

Genetic Change in $\psi_{O,R}$ Created by Artificial Selection.—The important distinction between the phase of an observed rhythm and the phase of its driving oscillation is made fully clear by different circadian systems created by artificial selection. In both *Drosophila melanogaster* and *pseudoobscura* we have systematically bred from parents that emerged either very *early* or very *late* in the daily distribution of emergence activity. We discuss *Drosophila pseudoobscura* here. Figure 4 shows the increasing $\Delta\psi$ between the “early” and “late” strains through 50 generations

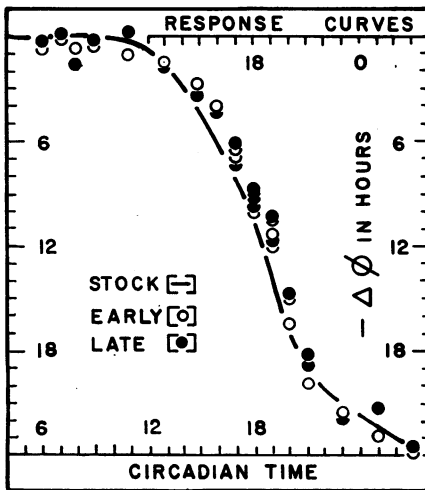
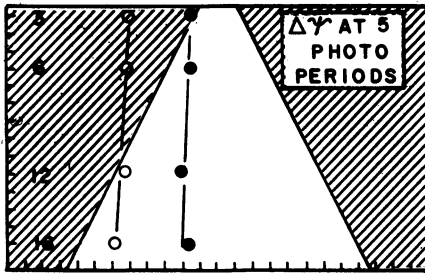
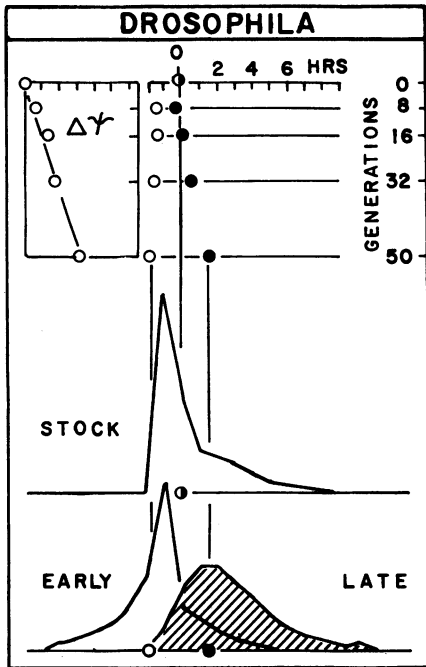


FIG. 3

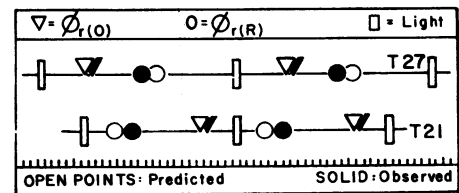
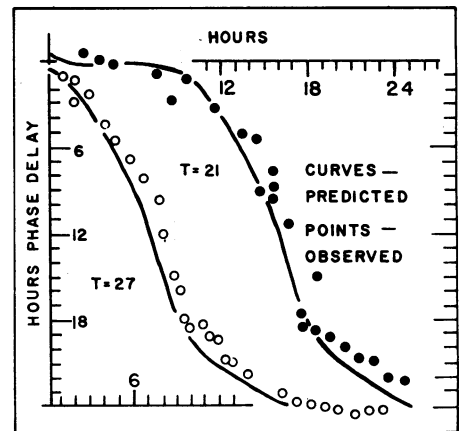
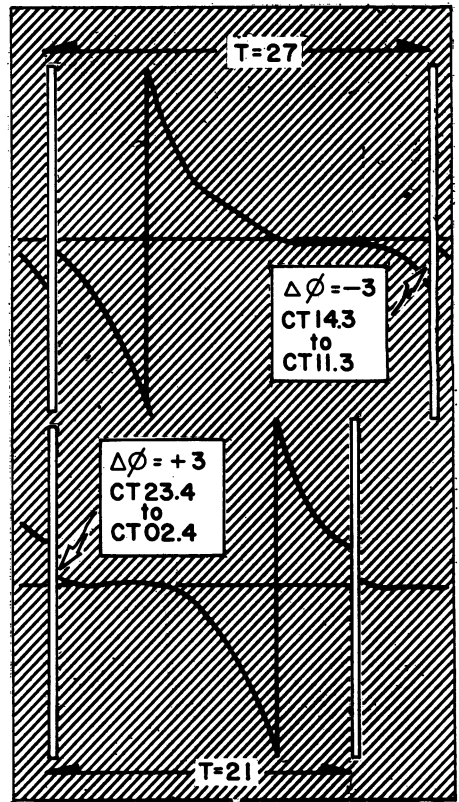


FIG. 4



FIG. 3.—*Upper panel*: Predicted steady-states of oscillation entrained to light cycles consisting of 1 light-pulse every 27 and 21 hr. *Middle panel*: Predictions from model (given as monotonic curves) of the phase of the oscillation's response curve during the 1st cycle of its free run following T_{21} and T_{27} . Points are observed $\Delta\phi$ values. The abscissa is given in hours since the final light pulse of the entraining cycle. *Lower panel*: Predicted and observed values of $\psi_{L,R}$ and $\psi_{E,O}$ for cycles of T_{21} and T_{27} .

FIG. 4.—Effect of selection for $\Delta\psi_{L,R}$ in the circadian system of *Drosophila pseudoobscura*. See text.

of selection, by which time $\Delta\psi$ was four hours. Selection was effected and its progress monitored in cultures maintained in LD 12:12; the middle panel in Figure 4 shows, however, that as ψ changes with photoperiod, the $\Delta\psi$ between the strains remains constant. $\Delta\psi$ observed in LD 12:12 is therefore not due to strain differences in the direct effect of the daily light on the emergence peak. The $\Delta\psi$ surely represents a change in the circadian system as such and could be the result of any one or more of three causes: (1) selection changed τ of the driving oscillation; or (2) it changed the shape of the response curve; or (3) it modified the coupling of the oscillation to the eclosion mechanism it controls. Analysis which we present in detail later shows that τ may indeed be slightly different in "early" and "late" but the observed $\Delta\tau$ in no way explains the observed $\Delta\psi$: τ in "early" exceeds τ in "late" by some minutes at most, and of itself this will cause a $\Delta\psi$ of opposite sign to that observed. Figure 4 (*bottom panel*) shows that selection has had, at most, trivial effects—undetectable in these data—on the driving oscillation of the system; the *shape* of the phase response curve *and its phase* relative to the last seen light-pulse are not measurably different in the "early," "late," and unselected parent stock.

Summary.— $\Delta\phi$ as a function of time measures the state of a circadian system's driving oscillation as a function of time. An arbitrary phase-reference point for the rhythmicity of the system assumes a phase relationship ($\psi_{O,R}$) to the driving oscillation that is generally but not necessarily constant. Assay of oscillation phase is thus most reliably effected by assay of the phase of its phase-response curve.

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⁴ Pittendrigh, C. S., and V. G. Bruce, in *Rhythmic and Synthetic Processes in Growth*, ed. D. Rudnik (Princeton: Princeton Univ. Press, 1957), p. 75.

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⁶ Pittendrigh, C. S., V. G. Bruce, and P. Kaus, these PROCEEDINGS, **44**, 965 (1958).