

## Pulses of Darkness Shift the Phase of a Circadian Rhythm in an Insectivorous Bat\*

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**Summary.** The circadian rhythm of a tropical insectivorous bat, *Taphozous melanopogon*, free-runs in dim light and responds to dark breaks of a few hours' duration with 'advances' and 'delays' as a function of the phase experiencing the "black out". Similarly phase shifts also follow perturbations by light breaks. The time course and the wave form of the phase response curves obtained from experiments using pulsed light and pulsed darkness are mirror images of each other.

### Introduction

In the absence of precise information about the mechanism of the "clock", the next best source of information about the temporal properties of a circadian rhythm is the so-called "phase response curve" (PRC). The 'PRC' graphically describes the qualitative and quantitative variations in the sensitivity of an endogenous rhythm over a 24-h period to experimentally administered perturbations emanating from the external world. Whereas the PRC yields continuous cue to the phases of the rhythm at any time of day or night the 'overt rhythms' which are the markers of time might not yield such cue, especially when they are discontinuous events such as animal locomotion and eclosion in insects (Bünning, 1973).

PRCs for light have been worked out for a number of organisms (Aschoff, 1965). Phase shifts can also be caused by other factors such as temperature, poisons, drugs and other chemicals (Bünning and Moser,

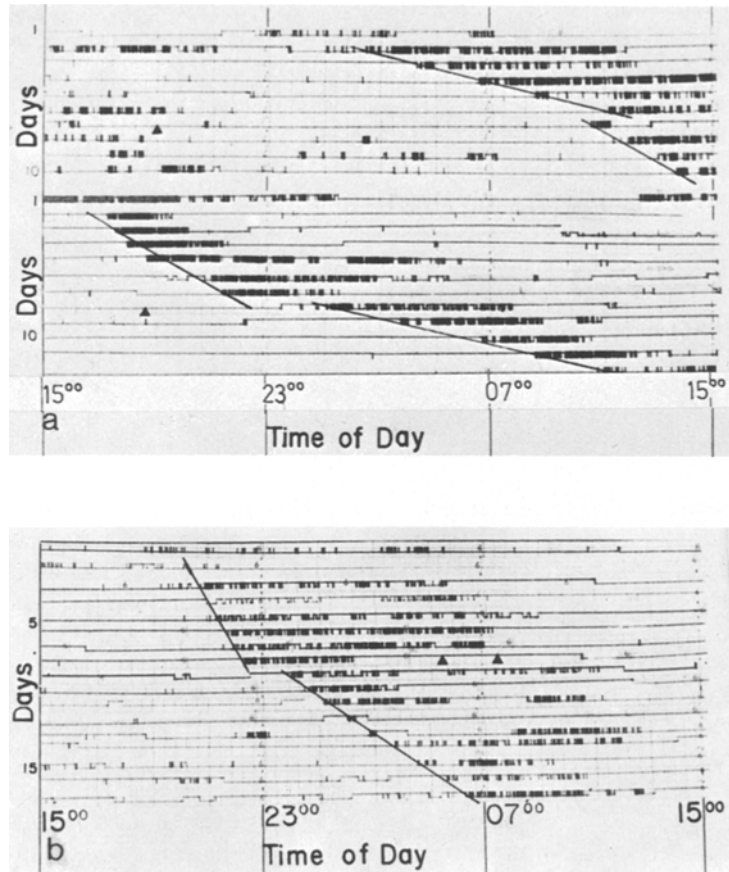
1972; Chandrashekar, 1974). In nature the entrainment of circadian rhythms arises through a process of appropriate phase shifting by the ubiquitous, 'Zeitgeber'—the L/D cycles accompanying sunrise-sunset. In LL or DD these rhythms 'free run' and systematically deviate from the strict 24-h periodicity of their geophysical environment. Thus it is implicit in the phenomenon of entrainment that D is as essential as L. Even so the literature is replete with results explaining only the role of light (to the near exclusion of even mention to D) in entrainment of rhythms (Takimoto and Hamner, 1965). The role of D in entrainment as well as in photoperiodic induction has been better studied and understood in plants. The circadian rhythm in the CO<sub>2</sub> output in the excised leaves of the plant *Bryophyllum fedtschenkoi* has been thus shown to respond to pulsed darkness with phase shifts at specific phases of the cycle (Wilkins, 1960). The *Bryophyllum* CO<sub>2</sub> rhythm is the only well-documented instance of D-shifting the phase of a rhythm. We report here the phase shifting action of pulses of D lasting 2 and 4 h when administered to the circadian rhythm in the flight activity of a crevice-dwelling tropical, insectivorous bat, *Taphozous melanopogon*. The circadian rhythm in this bat free runs in LL of intensities of 0.3 lx to 40 lx and exhibits changes in period length ( $\tau$ ) in consonance with Aschoff's rule (Hoffmann, 1965) in that the  $\tau$  increases with increasing intensities of LL. Complete PRCs to pulsed darkness have been obtained and compared with a PRC evoked with 15 min white fluorescent light pulses of ca. 1000 lx intensity.

### Materials and Methods

The activity data are based on the analyses of the locomotory activity patterns of bats as displayed by them while in confinement within tilting cages in the laboratory. The dimensions of the activity

\* Dedicated to Professor Colin S. Pittendrigh on the occasion of his 60th birthday

**Abbreviations:** L=light, D=darkness, L/D=light/darkness cycles, LL=continuous light, DD=continuous darkness, PRC=phase response curve,  $\tau$ =period length



**Fig. 1 a and b.** Aktograms showing free running locomotor rhythms in constant dim illumination of 5 lx. Consecutive 24-h activity/rest bands obtained from Esterline Angus event recorders are mounted serially one below the other. Phase shifts of activity rhythms induced by 15 min light pulses of 1000 lx intensity (a) and dark pulses of 2 h durations (b) are shown. **a** Advance phase shift induced by light pulse ( $\blacktriangle$ ) at 110°. A delay phase shift induced by light pulse ( $\blacktriangle$ ) at 300°. **b** Dark pulse ( $\blacktriangle$  ---  $\blacktriangle$ ) administered on 8th day from 120-150° induced delay phase shift in the onset of activity. The advance phase shift induced by the light pulse and the delay phase shifts induced by the light and dark pulses display no transients

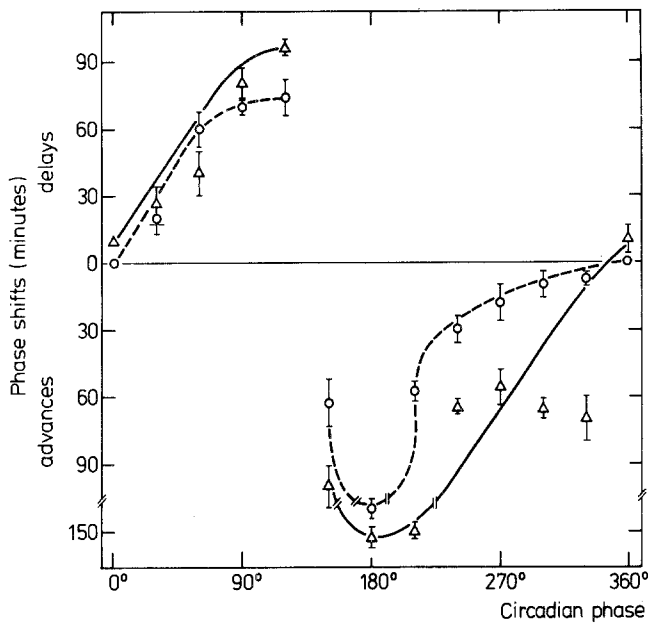
recording cages (34 × 17 × 20 cm) did not permit actual flight. Yet slight ambulatory movements of the bats during time of 'activity' were sufficient to tilt the cages poised on knife-edges. The tilts activated writing styli that registered vertical deflections on a slow moving kymograph drum. Esterline Angus event recorders were used in more recent experiments for purposes of recording. The activity patterns were recorded in LL of 5 lx in light-proof cabins at 28 °C ± 1°. An incandescent bulb provided the dim LL. The wire meshing of the cages and the postures assumed by the resting bats accounted for variations in light-intensity of the order of ca. 1 to 10 lx during the 'freeruns'. The dark pulse perturbations that lasted 2 h and 4 h were achieved by removing the bats into dark wooden boxes with a light-tight lid for the duration of the pulses. The 'control' bats were transferred to wooden boxes, illuminated from within with 5 lx intensity. The handling of the bats during transfer effects *no* phase shifts. The transition factor from LL to absolute darkness and vice-versa is presumed to be in the range of  $\alpha$ . The 15 min light pulses represented a transitory increase in the light intensity from the ambient, free running 5 lx to 1000 lx for the duration of the pulse, and back to 5 lx at its termination. The transition factor, thus, is 200-fold. Food and water were available *ad libitum*. In some instances the bats had to be forcibly fed with cockroaches. Such feeding was then done at different hours of the day. The data presented here derive from activity recordings from 751 bat-days.

In the field these bats show a remarkably precise rhythmicity in the onset of flight activity when they emerge from the Keela Kuyil Kudi caves close to the University Campus. Emergence of the first *Taphozous melanopogon* occurs between 18<sup>25</sup>-18<sup>41</sup> throughout the year (Subbaraj and Chandrashekar, 1977).

Control free running rhythms showed  $\tau$ s in the range of 25 h at an ambient LL of 5 lx. Desired phases for the L- and D-pulse perturbations were computed by taking the actual or anticipated onset of flight (ambulatory) activity as 0°. Actual phase shifts were then obtained in minutes which denoted the difference in time between the *projected onset* of activity for a bat read out from the slope of the onset of activity from its aktogram before perturbation and *actual onset* of activity after the perturbation. The altered time course set in abruptly in the very first 'onset' following perturbation without the customary 'transients' (Fig. 1). Each phase shift experiment lasted between 8 and 10 days after treatment. Untreated animals were allowed to free run in LL for at least a week.

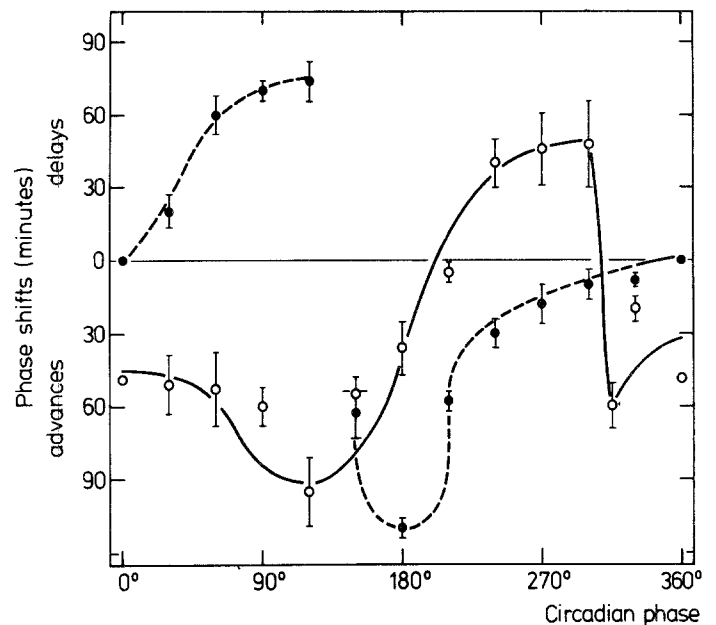
## Results

The circadian time (CT) terminology of earlier authors (Pittendrigh and Minis, 1964) is abandoned here since the  $\tau$  of the flight activity of *Taphozous melanopogon* is significantly higher than 25 h in LL. The phases are expressed in degrees between 0° to 360°. 0° denotes the onset of flight activity in this bat and hence the onset of the subjective night half-cycle. When relatively long perturbations are involved as phase shifting agents at least the 'onset', 'midpoint' and the 'end' of the perturbation come in for consid-



**Fig. 2.** PRCs for D-pulses of 2 h ( $\circ$ ) and 4 h ( $\triangle$ ) durations. Symbols denote delayed (above the horizontal line) and advanced (below the horizontal line) occurrence of the onset of activity on days following D-pulse treatment. Phase shift data include measurements obtained from day 2 onwards following perturbations for periods varying from 8 to 10 days in each case. Horizontal line dividing the delays from advances indicates the projected phase position of the rhythm of the bat prior to perturbation with D-pulses of both 2 h and 4 h durations. Abscissa: Phase of the circadian rhythm in LL of 5 lx. The  $\tau$  of approximately 25 h = 360°. 0° signifies onset of activity and the beginning of the subjective night. Ordinate: Phase shift data plotted against circadian phase of 'onset' of the D-pulses

eration as reference phases. Here we plot the phase shift data against the phase of the onset of the perturbation. It is evident from Figure 2 that D-pulses experienced by these nocturnal mammals during their subjective night (active) half-cycle lead to 'delay' phase shifts, i.e., delayed occurrence of the onset of activity on ensuing days. D-pulses experienced during the subjective day half-cycle lead to 'advances'. Both 2-h and 4-h D-pulses evoke qualitatively similar phase shifts the differences being only in magnitude. 4-h D-pulses effect more pronounced phase shifts, than 2-h pulses especially in the region of advancing responses. Figure 3 compares and contrasts a PRC for 15 min white L-pulses of intensity of ca. 1000 lx with the PRC for 2-h D-pulses. Phase advances are effected by L-pulses falling in the region of late subjective day and entire subjective night. Phase delays are effected by L-pulses falling in the early subjective day. The curves are not strictly comparable since the perturbations are not of the same dimensions with regard to duration, intensity and/or energy. The comparison must, therefore, be limited to drawing general



**Fig. 3.** Comparison of the 2 h D-pulse PRC ( $\bullet$ ) with a L-pulse (15 min 1000 lx) PRC ( $\circ$ ). The phase shift data for both curves plotted against circadian phase of the 'onset' of the L- and D-pulses. Other details as for Figure 2

inferences. L-pulses cause 'advances' in the region of the circadian cycle where D-pulses cause 'delays'. L-pulses cause largely 'delay' phase shifts during the subjective day half and D-pulses cause 'advances' in this region. Light-pulses and D-pulses cause delay phase shifts of comparable magnitude 180° apart. The 2 PRCs also 'switch' from delay responses to advance responses 180° apart relative to each other.

The 'mirror-imaging' pattern is complete and perfect for the action of L and D-pulses during the subjective night region of the circadian rhythm.

## Discussion

The time course and waveform of PRCs must reflect the ecological adaptive features a circadian physiological organization would confer on the organism. Thus, the qualitatively and quantitatively varying responses of the several phases must be explainable in terms of the action of the Zeitgeber in the special context of entrainment of circadian rhythms. Yet Pittendrigh's (1966) analysis of the functional and kinetic relationship of the PRC for the *Drosophila* rhythm to the process of its entrainment remains the only exercise of its kind. Pittendrigh (1966) explained the process of entrainment and even photoperiodic induction in terms of the phase shifts repeatedly effected by short duration (15 min, 1000 lx) light pulses and skeletal photoperiodic regimens.

Extending Pittendrigh's methodology of analysis of the PRC in the context of rhythm entrainment to the *Taphozous* case, a certain discrepancy in the region of the phase jump of the LP-PRC becomes apparent. The phases just preceding and coinciding with onset of "flight" activity display 'advance' responses. Delay responses would appear to be more appropriate for a bat erring into light ahead of time. Interestingly enough, however, the actual onset of endogenous activity coincides with the so-called "arousal" which occurs around 300° a region still showing 'delay' responses. In other words, a light perturbation experienced by a bat at this phase would still 'delay' activity. After complete arousal in nature these bats are known to emit (audible) social vocalization and await sunset to fly out (Subbaraj and Chandrashekar, 1977). Thus onset of flight activity is determined on the basis of light sampling and direct triggering. Any gross misjudgement of the bat flying out much too early would result in an LP-perturbation which then 'advances' the onset of activity on the ensuing day. Such day to day shifting of the rhythm apparently entrains and synchronizes the activity/rest pattern of the bats. No region of the PRCs evoked either by L-pulses or by D-pulses is characterised by a 'dead zone' indicating virtual unresponsiveness to light. To this extent the resultant curve is unusual and different from all the PRCs obtained on nocturnal animals (DeCoursey, 1961; Pittendrigh and Daan, 1976).

Mirror-imaging of the responses of circadian rhythms to opposing signs in the perturbations have been reported for the other organisms. The phase of the CO<sub>2</sub> rhythm in the leaves of *Bryophyllum fedtschenkoi* is reset by L-pulses at the troughs (between the crests) and the phase in LL is reset by D-pulses at the crests (Wilkins, 1960). Singular transitions of DD/LL (on) and LL/DD (off) can induce in the plant *Pharabitis nil* rhythms with time course 180° apart (Takimoto and Hamner, 1965). Similar responses occur in the eclosion rhythm of *Drosophila pseudoobscura* (Engelmann, 1966) and in the petal movement rhythm in the flowers of *Kalanchoe blossfeldiana* (Engelmann and Honegger, 1967). These rhythms, designated 'on' and 'off' rhythm for the eclosion rhythms of *Drosophila pseudoobscura* have been successfully 'simulated' by appropriately high and low intensities of light (Chandrashekar and Loher, 1969). Absolute darkness appeared to displace phases to the opposite side of the trajectory of the circadian oscillation relative to the side to which L displaces phases. Such antipodal displacement is apparently the cause of the 'mirror imaging' we report between L-pulse and D-pulse PRCs and may well be a necessary precondition for the very process of entrainment. Thus L and D

have opposite but mutually complementary influences on the circadian rhythm. In this context it is of interest that 'warm' and 'cold' steps act much as L and D-steps in affecting the time course of circadian rhythms (Zimmermann et al., 1968). Similarly 'high' and 'low' temperature pulses evoke diametrically opposed responses in the *Drosophila pseudoobscura* rhythm and the PRCs obtained with them are perfect mirror images. The kinetics of the action of L-pulses and D-pulses are also very different. In general L-pulses, at least in animal systems, exert marked phase shifts even if offered very briefly. The shortest L-pulses to shift the *Drosophila* rhythm lasted only  $1/2000$  s (Pittendrigh and Minis, 1964). D-pulses must, as a rule, last 2 h or longer. There are indications that for our *Taphozous* circadian rhythm both L-pulses and D-pulses, at least in range of magnitudes and energies employed here, evoke the type 'O' (strong) PRCs in that the rhythm switches from 'delays' to 'advances' without inertia (Winfree, 1970). The phase shifts accompanying both L and D perturbations to the circadian rhythm in our bat are unusual in that they are achieved without any transients (Fig. 1) regardless of the sign of the phase shift and strength of perturbation.

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