The *Drosophila* circadian clock*

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Abstract. The circadian rhythm in the process of eclosion of the fruitfly *Drosophila* is the best investigated with regards to properties such as entrainment, freerun and phase shifts. The system has been the basis of an important coupled oscillator model, several hypotheses, landmark papers and a monograph. The PRC for this rhythm has been extensively used in experiments designed to test the kinetics of the basic clock. The singularity point, signifying a stimulus that can 'stop' the clock, was also predicted and discovered in this rhythm. Fittingly the first clock mutant was also discovered in *Drosophila*.

Keywords. Circadian rhythm; *Drosophila*; phase response curves.

1. Introduction

The time course in the eclosion process of *Drosophila* represents perhaps the most intensively studied and best understood circadian rhythm (Saunders 1976). The system has stimulated the publication of several landmark papers (Pittendrigh and Minis 1964; Pittendrigh 1966; Engelmann 1966; Winfree 1970), postulation of several hypotheses (Pittendrigh and Bruce 1957; Chandrashekaran 1967b) and the writing of a most stimulating monograph (Winfree 1980). Work on this rhythm has also helped to analyse the formal properties of circadian rhythms, their response features and kinetics of responses. Interestingly one of the diagnostic features, the temperature compensation of circadian clocks, was first elucidated in 1954 for the *Drosophila* rhythm (Pittendrigh 1954). One of the earliest phase response curves (PRCs) was also worked out for this system (Pittendrigh 1960). The PRC of the *Drosophila* rhythm has also been used as a tool to analyse the process of entrainment (Pittendrigh and Minis 1964) and to understand the time constants involved during phase shifts ($\Delta\phi$) of the basic oscillation by light flashes (Chandrashekaran 1967a). Perhaps fittingly the first 'clock' mutant—a single locus—was also isolated for the *Drosophila* circadian rhythm (Konopka and Benzer 1971). This paper is a brief review of the author's own contributions to the gradual unravelling of the formal properties of the *Drosophila* circadian clock.
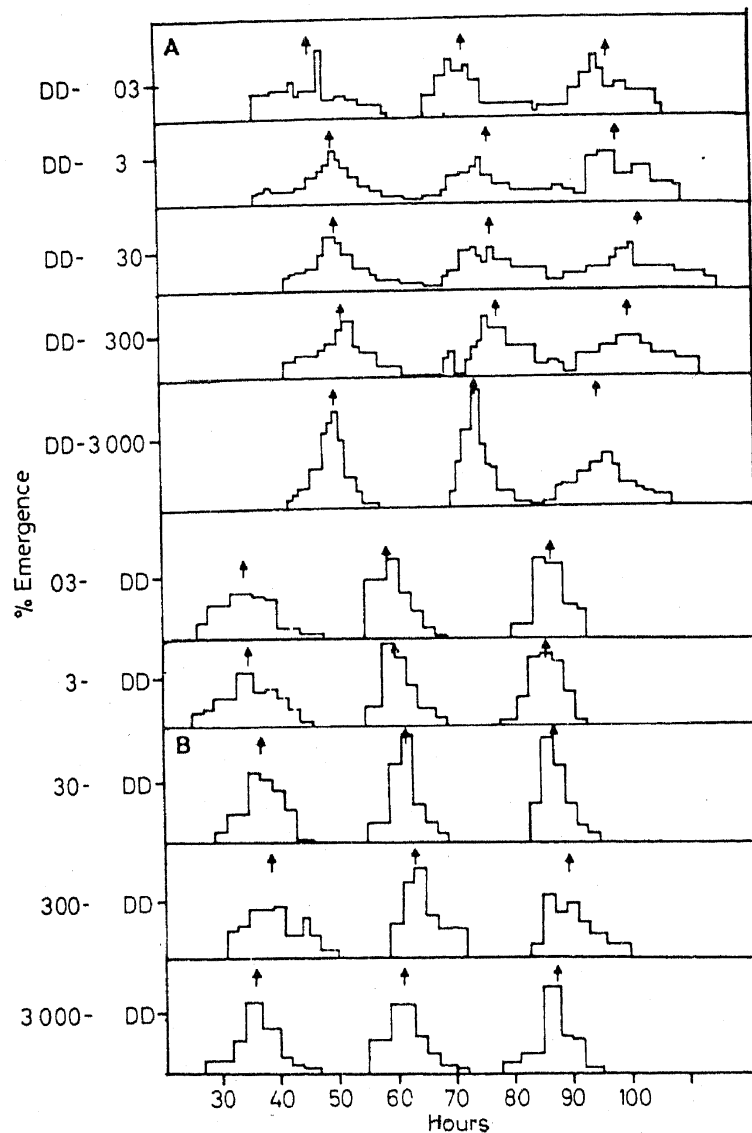
2. The eclosion rhythm

In nature, much as other insects do, the fruitflies eclose during the early hours of the morning and the last of the flies for the day to eclose would have done so by noon. The entire eclosion of a group of flies lasts over *ca* 8 hr. Thus the 'eclosion gate' is one third

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*Dedicated to Prof. Dr (multi) h c Erwin Bünning, teacher and exemplar to the author, on the occasion of his eightieth birthday.

the circadian span. If the flies are raised under light dark (LD) conditions in the laboratory eclosion peaks some 3 hr after the light comes on. Earlier it was believed that this eclosion peak appeared in response to the light-on stimulus but it became apparent that the flies take their cue from the last light-off information. In other words emergence (eclosion) of flies would start 12 hr after the L/D transition and peak (median value) 15 hr after L/D. Flies raised in continuous light (LL) or constant darkness (DD) for one or more generations do not show any rhythmicity. Adult flies eclose at all hours of the day and night. Nearly all the *Drosophila* experiments referred to in this short review were carried out at 20°C. At this temperature it takes the flies about 20–22 days to

Figure 1. 'On' and 'off' rhythms in the eclosion of pharate adult *Drosophila pseudoobscura* flies. The several populations were raised either in DD (upper panel) and then transferred to LL of intensities in lux indicated or were raised in LL of varying intensities (lower panel) and then transferred to DD. Time of transfer was on day 20 of the cultures arbitrarily and designated hour 0. First eclosion peak not shown since synchronization was generally poor in all cases. The 'on' rhythms illustrated in the upper panel and the 'off' rhythms illustrated in the lower panel show time courses *ca* 180° apart relative to each other.

progress from the egg stage, through the instars to the pupal stage and eclosion. These conditions refer to *Drosophila pseudoobscura* PU301 captured by C S Pittendrigh and Th Dobhzhansky and used by Pittendrigh and his colleagues (Pittendrigh and Minis 1964; Pittendrigh and Bruce 1957; Pittendrigh 1954, 1960; Engelmann 1966; Chandrashekaran 1967a, b; Winfree 1970).

In potentially arrhythmic pupal populations of *Drosophila pseudoobscura* raised in LL or DD it is possible to induce rhythms by a DD/LL transfer ('on' rhythms) or LL/DD transfer ('off' rhythms). The so called 'on' or 'off' rhythms are illustrated in figure 1. The two rhythms are approximately 12 hr apart (180° displacement) relative to each other. This has prompted Honegger (1967) to talk of *two* oscillators, one set in motion at sunrise and the other set in motion by sunset, and interpret that LD entrained rhythms resulted from an interaction of these 'on' and 'off' rhythms. It turned out later (Winfree 1970) that the 'off' rhythm was for all practical purposes very much like LD 12:12 entrained rhythms, so much so that LD entrainment before allowing the circadian rhythm to freerun in DD (with a period τ of 24·3 hr) was dispensed with. It was sufficient to raise the pupae in LL and transfer the populations a day or two before the first flies eclosed. Another difference, was that the 'on' rhythm waned after 4–5 days. Light intensities of just 0·0001 lux could attenuate the rhythms. Interestingly it was found that the 'on' and 'off' rhythms could be 'simulated' with appropriately higher/lower or lower/higher intensity transfer of pupae. Figure 2 describes the time course of simulated 'on' and 'off' rhythms. On the right side the magnitude of intensity differences between I_1 (rearing light intensity) and I_2 (transfer light intensity) are shown. These rhythms seemingly contradict a dictum of Pittendrigh (1966) that the *Drosophila* circadian rhythm is held
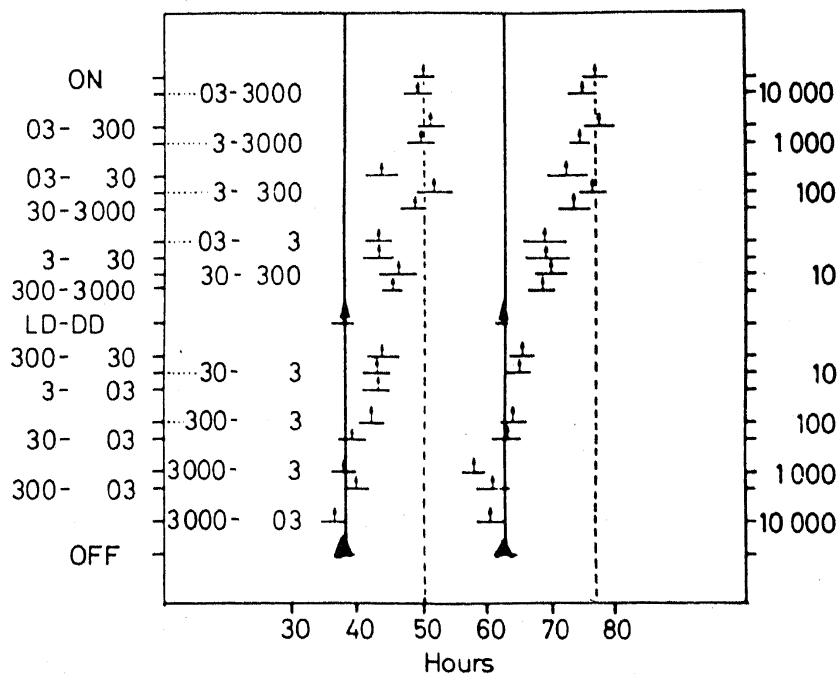

Figure 2. Illustrates the time course by means of indicating 'median' values of 'simulated on' and 'simulated off' rhythms. Appropriately high and low intensities whose actual value is given on the left and the factor of simulation is given on the right. All data above LD/DD line are simulated 'on' rhythms and those below simulated 'off' rhythms. Real 'on' and 'off' rhythm median values are given in the uppermost and lowermost lines respectively.

'fixed' at CT 12 phase by *L* extending beyond the customary 12 hr and will be released into further motion only by the restoration of *D*. Thus peaks would follow on a pattern of $n \times \tau + 15$ hr in all pupal populations when light was maintained beyond 12 hr. On the other hand the simulated 'on' and 'off' peaks may describe the behaviour of a slave oscillator(s) or may be themselves 'transients'.

3. The *Drosophila* PRC

The circadian rhythm in the eclosion rhythm of *Drosophila* is sensitive to light perturbations of 0·5 msec administered against a background of darkness. The responses of the rhythm assume the form of displacement of the peaks along the time axis. The peaks either advance or delay relative to unperturbed controls. The direction of $\Delta \phi$ s and their magnitude are direct functions of the phase being perturbed. Figure 3 describes the standard PRC for the *Drosophila* rhythm first worked out in all its details by Pittendrigh and Minis (1964). CT 0 hr represents sunrise, CT 12 hr sunset, CT 0–12 hr subjective day and CT 12–24 hr subjective night. The rhythm is refractory to light stimuli given during the subjective day but responds with increasingly dilating delays during the first half of the night. At midnight the system switches the quality of its responses from massive delays to massive advances. The magnitude of advances
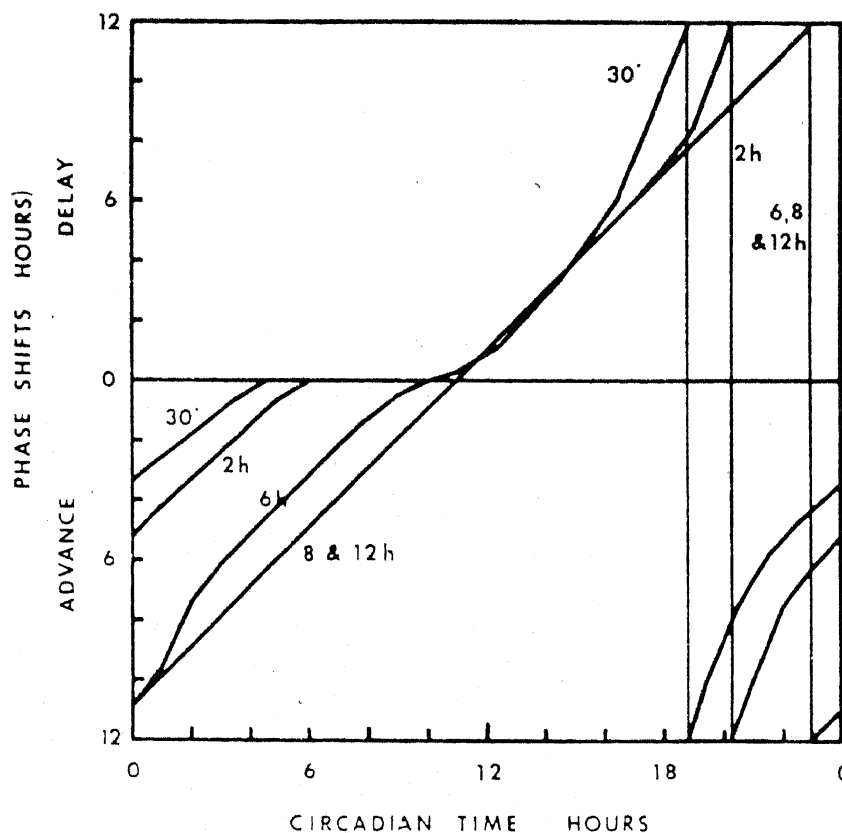
Figure 3. PRCs for 30 min, 2 hr, 6 hr, 8 hr and 12 hr light pulses of 1000 lux plotted against 'onset' of perturbation. The 30 min light pulse PRC is similar in all details to the standard 15′1000 lux PRC of Pittendrigh and Minis (1964). $\Delta \phi$ s are given in hr. Values above 0 line (control) indicate delay $\Delta \phi$ s and below the line advance $\Delta \phi$ s.

diminish in the course of the second half of the subjective night. This is one of the best worked out PRCs and was somewhat picturesquely described to represent the 'time course and waveform' of the basic oscillation gating eclosion.

4. Transients and the coupled-oscillator model

The $\Delta\phi$s that follow light perturbations do not express themselves in the same cycle or in the one after that. It takes the rhythm 3–4 cycles until the altered steady state with the stable $\Delta\phi$ is achieved. The 'creeping' fashion in which the $\Delta\phi$s express themselves has been the subject of much discussion and interpretation. One interpretation is given by a coupled-oscillator model proposed by Pittendrigh and Bruce (1957). This model postulated an A oscillator which was the pacemaker, light sensitive, temperature compensated and suffered *instantaneous* $\Delta\phi$s and a B oscillator which was the slave, light insensitive but sensitive to temperature. A influenced B but B had no feedback influence on A. The transients represent the efforts of a B oscillator trying to regain original phase angle (Ψ) coupling properties and phases with an instantaneously phase shifted A oscillator. Even though the model appears in retrospect fanciful and the transients can be explained even in terms of a single oscillator (Chandrashekaran 1980) at the time it was postulated it appeared to be an elegant way to explain the phenomenon of transients. Furthermore one of the main postulates of the model was stated without ambiguity and lent itself to direct experimentation. The main postulate was: the instantaneous resettability of the basic light sensitive A oscillator.

Soon after the coupled oscillator model was postulated Bünning and Zimmer (1962) gave a different interpretation to transients. They concluded from their studies on the petal movement rhythm of the crassulacean plant *Kalanchoe blossfeldiana* that the transient oscillation of petal movement following light signals reflects the behaviour of the underlying oscillator. They found the several phases of the transients to respond to a second light signal in a manner similar to the movement phases of the original rhythm.

This author designed critical experiments with *Drosophila* to critically test the two views of transients. In planning the experiments on graph paper *i.e.* gedanken experimental phase, the classical phase response curve (Pittendrigh and Minis 1964) was assumed to really characterize 'the waveform and time course of the basic oscillation'. The rationale was to administer light pulse 1 at a given phase and then follow up with light pulse 2 soon after to check if $\Delta\phi$ had already occurred. $\Delta\phi$s would be large and to scale of the PRC on the assumptions made by the coupled oscillator model, but small and nearly undetectable according to the assumption of Bünning and Zimmer (1962).

Figure 4 illustrates the results of an experiment where pulse 1 (15′1000 lux) was given at 15·5 CT and pulse 2 at 22 CT. Both pulses given individually to two different populations would have induced 5 hr delay $\Delta\phi$ and 5 hr advance $\Delta\phi$ respectively. According to the Bünning–Zimmer interpretation pulses 1 and 2 should have mutually counter-acted each other's influence and no $\Delta\phi$s should have shown up. The results indicate a larger than PRC-postulated delay $\Delta\phi$ which indicates a *summative* effect. This would happen if light pulse 1 had indeed shifted the basic oscillation *instantaneously* by an amount postulated by the PRC.

Figure 5 illustrates results of an experiment where it was assumed that light pulse 1 indeed shifts phase instantaneously and light pulse 2 was given to effect then an advance
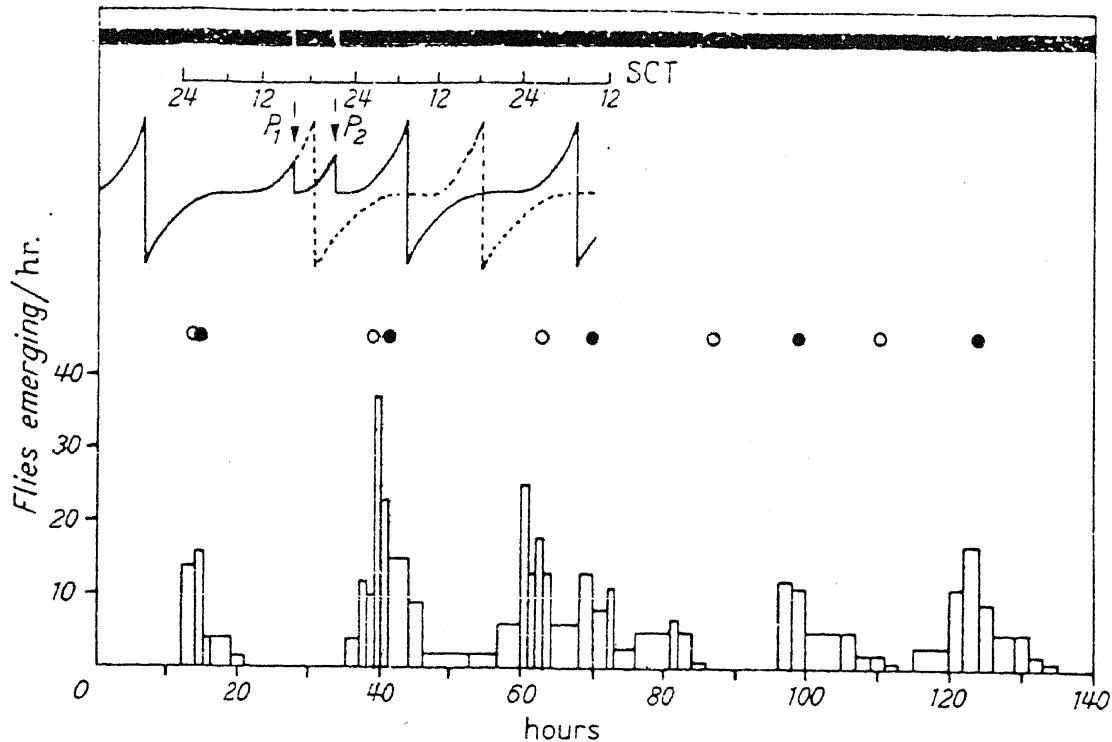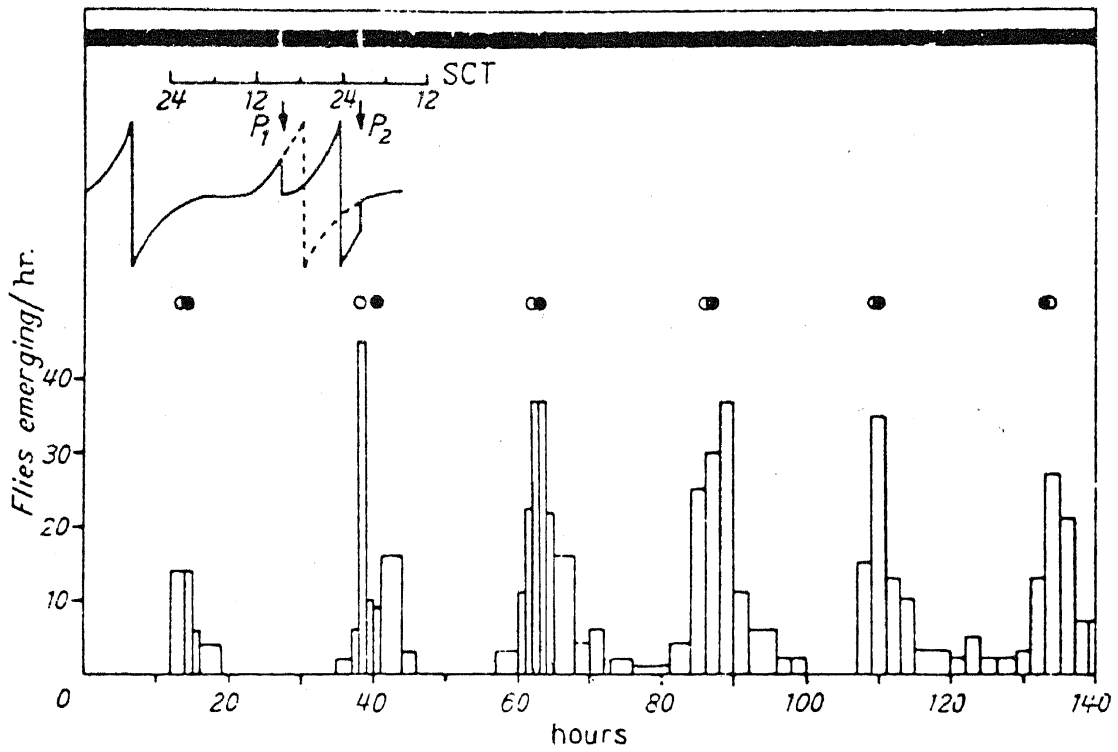
Figure 4. The effect of two light pulses (P_1 and P_2) of 15 min duration each and 300 lux intensity. P_1 was given at 15·5 CT and P_2 at 22 CT. The curve above the raw data of eclosion depicts schematically the instantaneous $\Delta\phi$ s effected by pulses. The dotted line indicates the time course of unperturbed controls. Open circles are calculated medians of peaks and solid circles indicate positions of medians of experimental populations.

$\Delta\phi$ of 5 hr *i.e.* at phase 22 CT + 5 hr = 3 CT. Now pulse 2 was seen to counteract the effects of pulse 1 with the result eclosion peaks of experimental and control pupal populations show the same time course. Figure 6 contains data of a two pulse experiment whose rationale was the same as in the first two pulse experiment except that the second pulse was administered in the second cycle. The results are unequivocal. It was concluded that the assumption of the coupled oscillator model that the basic oscillator gating eclosion in *Drosophila* is phase shifted instantaneously by light pulses holds at least for the *Drosophila* clock. But it must be mentioned in this context that no concrete proof has been forthcoming for the so-called *B*-oscillator (Chandrashekaran 1980). It is now generally assumed that most organisms, especially animals, may possess several rhythms generally coupled to each other in a hierarchical fashion (Moore-Ede *et al* 1982).

5. Dawn and dusk effects

In the course of experiments with *Drosophila* certain data tended to indicate that the first half of the subjective night of the system may shown qualitatively different responses of light on and light off transitions than the second half. In other words the first half seemed to respond only to the light 'off' *component* of a light pulse, the second half seemed to respond only to the light 'on' *component* of a light pulse. This is the gist of the

Figure 5. The effect on the rhythm of two pulses P_1 and P_2 of 15 min duration and 10000 lux intensity. P_1 at 15·5 CT, P_2 at 02·5 CT. Other details as in figure 4.
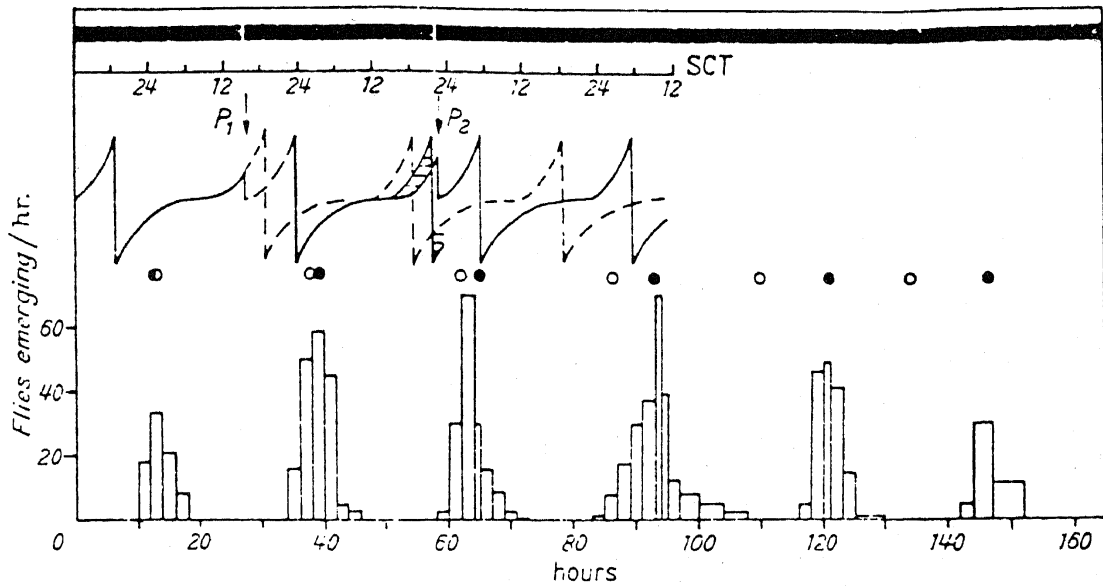
Figure 6. The effect of two light pulses of 15 min and 3000 lux. P_1 was given at 15·5 CT in the first cycle and P_2 at 22 CT of the second cycles. In practice P_1 falls 27·5 hr after LD/DD and P_2 58 hr after LD/DD. Other details as in figure 4.
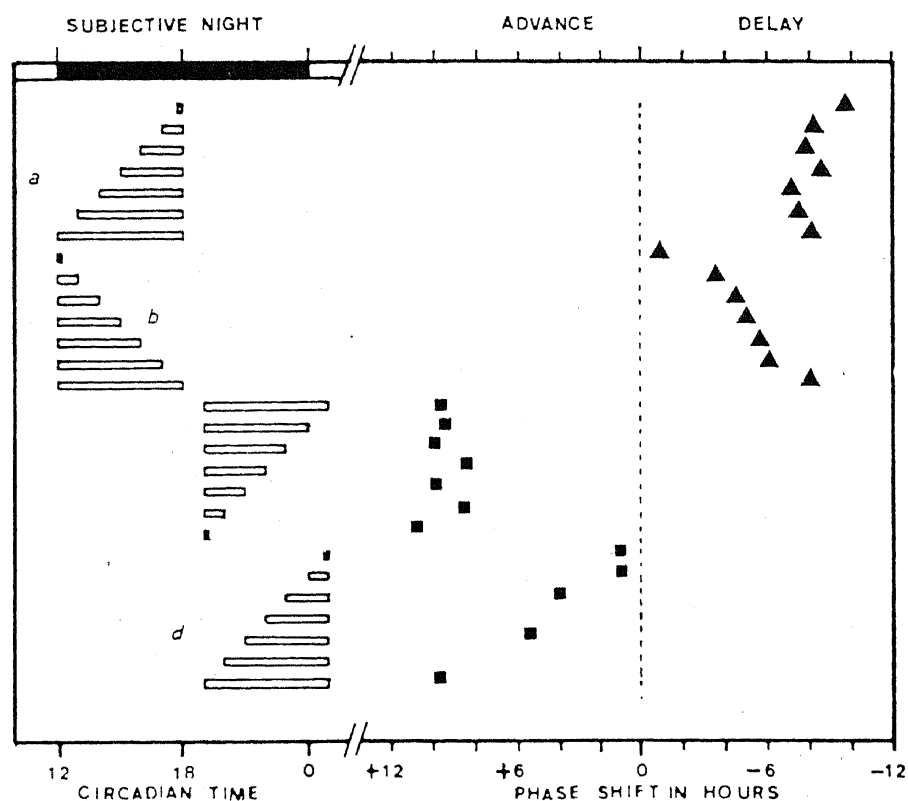
Figure 7. Shifting the phase of the *Drosophila pseudoobscura* eclosion rhythm with light pulses of 1000 lux and varying duration given in the first and second halves of the subjective night. The light pulses are represented by the unfilled bars and are arranged in 4 batches on the 'circadian time scale' of Pittendrigh and Minis (1964). In batch 'a' the different populations experienced the 'on' transition of pulses at different hours but experienced the 'off' transition at the same phase (18 CT). In batch 'b' the populations experienced the 'on' transition at the same circadian hour (12 CT) but the 'off' transition occurred for each population at a different hour. The pulses of batches 'a' and 'b' scan the first half of the subjective night. Batches 'c' and 'd' scan the hours of the second half of the subjective night. In batch 'c' the 'on' transitions of all the pulses were in alignment (at 19 CT) with the 'off' transition occurring at a different hour for each population. In batch 'd' on the other hand the 'on' transitions were systematically staggered and the 'off' transitions of pulses aligned. The filled triangles represent averaged median values of eclosion peaks of experimental populations 4–5 days after light treatment, which responded with delay phase shifts. The filled squares represent averaged median values of peaks 4–5 days after light treatment showing advancing phase shifts. Apparently the 'off' transitions of light pulses determine direction and degree of phase shifts during the first half of the night and the 'on' transitions determine phase shifts during the second half of the night.

dawn/dusk effect model postulated by Chandrashekaran (1967b). The light 'off' information simulates a dusk or sunset and the light 'on' information acts like dawn or sunrise. Figure 7 illustrates results obtained in a later series of experiments (Chandrashekaran *et al* 1973). The design was to compare the results of $\Delta\phi$ s evoked by light pulses given during the first half of the night such that they were of varying durations started at differing phases but went off at the same (18 CT) phase. If 'off' is indeed the discrete component recognized by the system then all $\Delta\phi$ s must be of equal magnitude regardless of the duration of pulses evoking them. This is the case. The opposite design was used for the second half of the subjective night. Pulses of varying duration started at the same phase (19 CT) but ended at varying phases. Since 'on' is the

discrete component implicated all $\Delta\phi$ s evoked by the pulses must be of comparable magnitude. This again is the case. Reciprocal experiments were also performed and the results further fortified these findings.

6. How to stop the *Drosophila* clock

Pavlidis (1967) predicted that the *Drosophila* clock must have a point of singularity on theoretical considerations. Drawn in the form of a phase plane limit cycle diagram the singular status will be achieved by a pulse of critical strength S^* given at a critical time T^*. Winfree (1970) discovered the values for these two parameters. T^* was 6·8 hr after an LL/DD transfer (18·8 CT) and S^* was 100 erg/cm^2/sec light of 460 nm given over 50 sec. If this treatment is indeed given to pupal populations, eclosion becomes arrhythmic. Total arrhythmicity according to the formula: number of flies outside the gate ÷ number of flies inside the 'gate' × 100 would be 200.

7. The *Drosophila* clock in contemporary research

Konopka and Benzer (1971) isolated a clock mutant for *Drosophila*. The clock could even be surgically transplanted (Handler and Konopka 1979). Engelmann and Mack (1978) showed that the PRC for the locomotor activity rhythm of *Drosophila pseudoobscura* looked very different from the PRC for the eclosion process. This is indicative of two different oscillators controlling the different rhythms. Work presently in progress in the laboratory of Engelmann in Tübingen (personal communication) indicates that the oscillatory pacemaker governing the locomotory activity in *Drosophila* is *not* situated in the optic lobes. The optic lobes are provenly the sites of the pacemakers in circadian rhythms of locomotion in the cockroach and crickets (Saunders 1976). It is to be hoped that the real nature of the elusive circadian clock in *Drosophila* might soon be unravelled using the modern techniques of gene cloning and recombinant DNA.

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