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Phase-response curves and the circadian clock in *Drosophila pseudoobscura*

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Abstract

The effects of light and temperature on the circadian rhythm of eclosion in *Drosophila* have been the subject of early investigations in chronobiology Much before the clock gene was discovered in *Drosophila melanogaster*, Pittendrigh and Bruce (1957) proposed an explicit coupled-oscillator model to account for *transients* The model postulated that (i) the basic (A) oscillator was phase-shifted *instantaneously* by brief light pulses, and that (ii) the *transients* do not represent the time course and wave form of the A oscillator Chandrashekaran performed two pulse experiments and reported that his results proferred unequivocal support to both the predictions of the coupled-oscillator model Chandrashekaran also proposed a 'dawn' and 'dusk' model for the phase-shifting effects of brief light pulses He was later able to empirically demonstrate that brief light pulses indeed shifted phase with the 'off' transition ('dusk' effect) during the first half of the subjective night and with the 'on' transition ('dawn' effect) during the second half of the subjective night Further underlining the inherent differences between the two halves of the subjective night, Chandrashekaran could demonstrate that early and late subjective night phases of the *Drosophila pseudoobscura* circadian rhythm require different energies of blue light to evoke comparable magnitudes of delay and advance phase shifts In another series of experiments he demonstrated that there are reciprocal relations between the irradiance and duration of blue light pulses which could in combination severely attenuate the amplitude of the *Drosophila pseudoobscura* circadian rhythm Chandrashekaran also reported the most complete family of PRC for HTP and LTP of 3-, 6- and 12-h pulses and demonstrated that HTP and LTP PRC were mirror images and that 6- and 12-h LTP yielded PRC of the 'strong' type (type 0) and that all durations of HTP yielded PRC of the 'weak' type (type 1)

Keywords: Phase-response curve, entrainment, phase shifts, light, temperature, circadian clocks

1. Introduction

Considering the importance of phase-response curves (PRC) in circadian rhythm studies, very few reviews of the subject are available, the works of Aschoff[1], Pittendrigh[2] and Johnson[3] being the exception A PRC is a plot of phase shifts as a function of circadian phase of a stimulus. Stimuli that can evoke phase shifts may be light, temperature, drugs or chemicals administered for brief periods, very often just once in a cycle PRC are central in processes of entrainment and are reliable phase-markers of circadian rhythms. The overt circadian phenomena monitored (eclosion, locomotor activity, sleep movements of leaves of plants, body temperature, etc) have been likened to the *hands* of the clock PRC is the closest physiological approximation (*clockworks*) to the abstraction often designated as the *oscillator(s)*.

Clock genes have been identified (in some instances even cloned) in *Drosophila*, *Neurospora crassa*, *Arabidopsis* and recently in a mouse[4]. In this paper, I review some of my own work carried out during 1965–1975 on PRC and the circadian clock underlying the eclosion of

adult fruitflies of *Drosophila pseudoobscura*. Landmark works of other authors who had published earlier to me and some very recent work only are cited.

The effect of light and temperature on the circadian rhythm of eclosion in *Drosophila* has been the subject of the earliest investigations in chronobiology[5,6] and of many subsequent studies, some of which are still landmarks[7-10]. The fascinating, if still enigmatic, phenomenon of 'temperature independence' of the period length of circadian rhythms was also established for the first time for the *Drosophila* rhythm[7].

Much before Konopka and Benzer[11] isolated three behavioural mutants of *Drosophila melanogaster* (called *period*) which drastically altered the period length of the circadian clock 'gating' both pupal eclosion and adult locomotor activity, Pittendrigh and Bruce[12] had proposed an explicit formal model to account for the response features of the circadian rhythm of eclosion in *Drosophila pseudoobscura* to light and temperature perturbations. The principal merit of this coupled-oscillator model is that it 'explained' the phenomenon of transients rather picturesquely, even though other models[13] could also explain transients implicating a single oscillator. Another attraction of the Pittendrigh–Bruce model is that it made two concrete predictions which could be experimentally tested. The model predicted that (i) the basic (A) oscillator will be phase-shifted by light pulses *instantaneously* by degrees that could be read off the standard PRC, and that (ii) the *transients* do not represent the time course of the basic oscillator. Chandrashekaran[14,15] studied these issues in *Drosophila pseudoobscura* (with PU 301 strain from Pittendrigh's laboratory) and empirically confirmed that (i) the basic oscillator was indeed *instantaneously* phase-shifted by as much as depicted in PRC, and that (ii) the *transients* did not reflect the time course nor wave form of the basic oscillator.

Another series of experiments on *Drosophila pseudoobscura* addressed itself to the relationship between the intensity of light pulses and the extent of phase shifts of the circadian rhythms in eclosion[16]. Chandrashekaran[15] claimed that brief light pulses given during the subjective night conveyed *dawn* and *dusk* information depending upon the time of the night they illumine. Results of later specific and exhaustive experiments revealed[17] that light pulses indeed shift phase with the 'off' transition during the first half of the subjective night (*dusk* effect) and shift phase with the 'on' transition during the second half of the subjective night (*dawn* effect). Further accentuating the differences between the two halves of the subjective night (for which very few models made any provision[10]), it was demonstrated that tenfold higher energies of light had to be administered during the first half of the subjective night, relative to the second half, to evoke comparable magnitudes of delay and advance phase shifts, respectively[18]. A family of high-temperature pulse (HTP) and low-temperature pulse (LTP) PRC was constructed for the rhythm in continuous darkness (DD) for pulse durations of 3, 6, and 12 h[19]. In a major publication of the period, we have reported[20] the reciprocal relations between energy (S*) of light pulses and critical phase of perturbation (T*) of a rhythm attenuating singular stimulus.

2. Methods

All experiments summarized in this review were performed on *Drosophila pseudoobscura* PU 301 strain kindly provided to W. Engelmann by C. S. Pittendrigh. Cultures for earlier experi-

ments were raised in usual manner in culture bottles of approximately 200 cc volume at 20 ± 0 2°C in 12 12 light/dark (LD) cycles or in DD from the egg stage onwards

2 1 *Light treatments*

Light intensities of 10, 3,000 and 10,000 lux were obtained with the aid of projectors fitted with cinemoid grey filters For lower intensities of 0 1, 0 3, 1 0, 2 0, 3 0 and 4 0 lux, neutral density gelatin filters (Kodak Wratten), number 3 and 4 of 0 1 and 0 01% transmittance, respectively, were employed Light intensities were variously measured with light meters (Gossen and Weston) and a United Detector Technology optometer.

Low irradiances of monochromatic blue light (0 01 to 100 μW cm^2) were obtained with the aid of projectors fitted with Philips 15 V 100 W or Osram 12 V 100 W bulbs and Schott & Mainz interference filters transmitting 442 nm (± 17 nm) For higher ranges of irradiance (50 to 15,000 μW cm^2) special projectors fitted with Xenon high irradiance bulbs (XBO lamps 2500 V and 1600 W Zeutschel Systematic M 10 DC operated) were used Projector bulbs were automatically aircooled when lights were turned on In experiments with high irradiance light neutral density heat-absorption glass filters were used with interference filters for monochromatic light For the amplitude attenuation experiments[20], experimental pupal populations consisted of 1200–1500 pupae These were held in glass petridishes of 9-cm diameter during light treatment. Hollow cardboard cylinders were used to conduct high irradiance light from source to petridishes precluding stray light. Since the pupae formed just one layer in all cases, overlapping and casting shadows should have been very low Light pulses lasting a second or fraction thereof were obtained with a photographic shutter fixed to the distal end of the cardboard cylinder The irradiances were measured with a multiflex galvanometer (40 A UDT) in μW cm^2 The degree of arrhythmicity is represented by the value of R after Winfree[10] The densest consecutive 8-h eclosion is first determined which would roughly include 95% of the flies in normally rhythmic cultures R is 100 times the ratio of the number of eclosing flies *outside* this densest 8 h to the number of eclosing flies *inside* it For ideal statistical arrhythmicity (R)—the same number of flies each hour day after day—would approach $100 \times 16/8 = 200$ which never happened in our experiments nor probably in nature.

2 2 *Temperature treatment*

Temperature pulses were given to 1200–1500 pupae in glass petri dishes in a room maintained at 10°C for LTP and at 30°C for HTP On removal to the experimental room, the pupae were immediately transferred to glass petri dishes prewarmed or precooled to avoid undue time lag The pulses (10 and 30°C) did not seem to have any direct detrimental effect on the flies The hourly rate of eclosion in this series of experiments was measured for periods of 7–8 days after temperature treatment on the 20 channels of an Esterline Angus event recorder using a light beam and photocell device designed by W Engelmann to count eclosing flies

2 3. *Measuring eclosion*

In earlier experiments, the emergence rate was determined manually every two hours by tapping and collecting the flies in detergent water for the tedious duration of the experiment In

later experiments, eclosion was measured with photoelectric automated arrangement linked to a digital voltmeter and teleprinter[17], and in still later experiments, with the help of a more direct method[18]

3. Results

3.1 *'On' and 'off' rhythms*

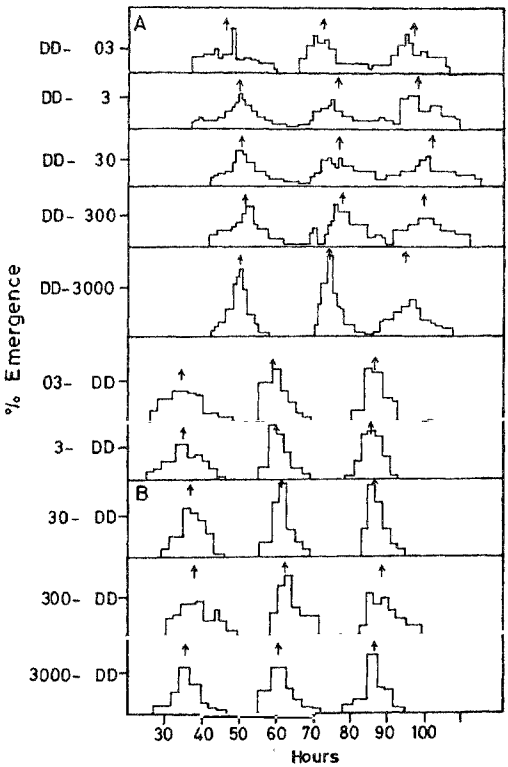
Laboratory populations of flies and pupae raised in continuous light (LL) or DD are arrhythmic But a singular transfer of the potentially arrhythmic pupal populations from LL to DD initiates the so-called 'off' rhythm in eclosion This rhythm is circadian and has a period of 24.3 h in DD The medians of eclosion peaks occur at 15, 39, 63, 87, 111, 135 and 183 h, etc after LL/DD transfer and eclosion lasts until there are pupae A singular, non-recurring transfer of potentially arrhythmic pupal populations from DD to LL initiates the so-called 'on' rhythms Figure 1 illustrates the time courses of 'on' and 'off' rhythms All transfers were made at an arbitrary '0' hour which coincided with day 20 of the cultures in 20°C The first flies emerged in the course of day 20 The first eclosion peak in all cases is not shown since synchronization was uniformly poor It may be noticed that the 'off' rhythms are stable and better synchronized than 'on' rhythms In fact, the 'on' rhythms wane even in light of very low intensities It may be noted that the 'on' and 'off' rhythms have very different time courses[16]

3.2 *The Drosophila PRC*

The circadian eclosion rhythm of *Drosophila* measured in DD is sensitive to light perturbations of 0.5 ms. The responses of the rhythm appear as displacements of eclosion peaks along the time axis and are designated 'phase shifts' ($\Delta\varphi$) The magnitude and directions (advances or delays) are both functions of phases perturbed Figure 2 illustrates the raw data and the methodology of evoking systematic $\Delta\varphi$ based on which PRC are constructed[21]. Figure 3 presents the standard PRC for 15 min, 1000 lux fluorescent light perturbations (first constructed by Pittendrigh and Minis[8]) Circadian time (CT) 0–12 h is called the subjective day and CT 12–24 h the subjective night The rhythm is refractory to light stimuli for the best part of the subjective day, but responds with increasingly *dilatory* delays during the first half of the subjective night At midnight, the system switches over from massive delays to massive advances, the latter progressively *diminishing* in magnitude as the night wears away. The *Drosophila pseudoobscura* PRC is constructed from steady-state phase shift $\Delta\varphi$ data which preclude transients The PRC describes 'the time course and wave form' of the basic oscillation[8] The PRC can and has been used as a predictive tool for single- and double-pulse light perturbations (see section 3.3)

3.3 *Transients and the coupled-oscillator model*

The $\Delta\varphi$ that follow light pulses administered to the rhythm in DD do not express themselves in full measure in the same, or even adjacent, cycle It takes the rhythms 3–4 cycles until the altered steady state with stable phase-angle difference (ψ) is achieved The cycles intervening the original and altered steady states have been called *transients* Transients in the context of circadian rhythms were first described by Pittendrigh and Bruce[12] One important interpretation of



were raised in either of different intensities arbitrarily designated after Chandrashekaran and Loher[16])

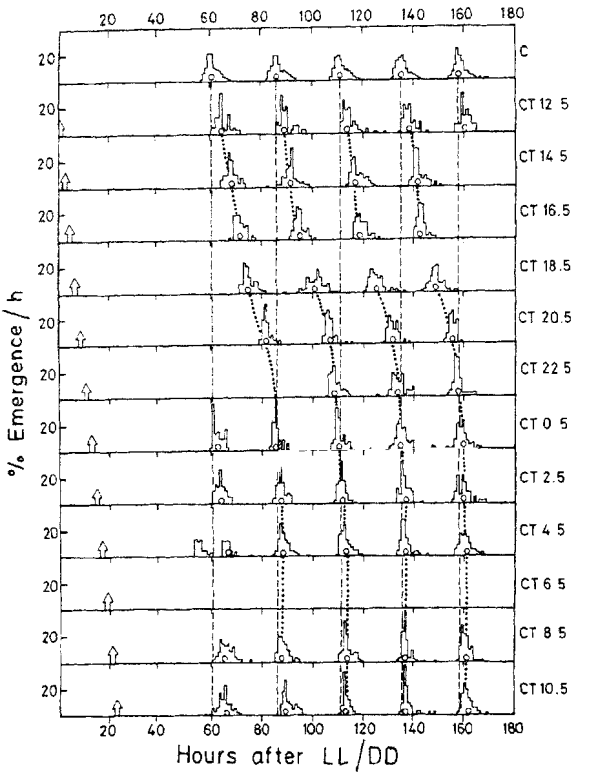


FIG 2 Circadian rhythm of eclosion in populations of *Drosophila pseudoobscura* which were transferred from LL to DD at hour 0 The upper-most row shows the pattern of eclosion of flies in a control group The other groups received 10 s of 100 μW of blue light of 442 nm ± 17 nm (after Hamm *et al* [22])

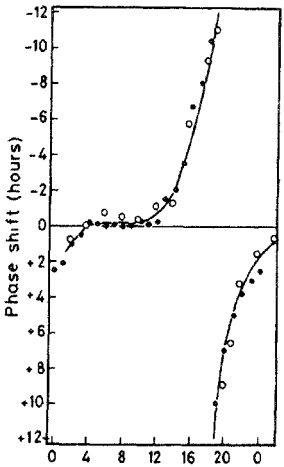


FIG 3 [illegible] from ex[illegible] Hamm *et al*[2])

the phenomenon of transients was provided by a coupled-oscillator model of Pittendrigh and Bruce[12] The model envisaged a coupled-oscillator arrangement with one of the oscillators—the A oscillator being light-sensitive and master pacemaker A oscillator was phase-shifted *instantaneously* by light pulses and was temperature-compensated qualifying it for bio-chronometry The B oscillator, on the other hand, was insensitive to light but sensitive to temperature and *slave* to the A oscillator The B oscillator did not feedback on the A oscillator. $\Delta\varphi$ of A were of magnitude represented in the PRC, B gradually caught up with A in 3 to 4 circadian cycles, restoring the original steady-state phase angle The efforts of B to restore stable phase with A expressed itself in the form of overt *transient* The coupled oscillator model elegantly and picturesquely 'explained' the transients. Furthermore, the two main postulates of the model, that (i) the basic oscillator was reset *instantaneously* by light, and that (ii) the *transients* did not reflect the phase of the basic oscillator, were stated without ambiguity and lent themselves to direct experimental verification

Soon after the coupled oscillator model was proposed, Bunning and Zimmer[13] gave a different interpretation to transients They concluded from their studies on the petal movement rhythms of the crassulacean plant *Kalanchoe blossfeldiana* that the transient oscillation of petal

movement following light signals reflected the behaviour (phase) of the underlying oscillator They found several phases of the transients to respond to a light signal in a manner similar to the movement phases of the original (steady state) rhythm

Working in Bunning's laboratory, Chandrashekaran[14,15] designed critical experiments with *Drosophila pseudoobscura* to probe the two views on the nature of transients In planning the experiments on millimeter paper (blueprint), the classical PRC[8] was assumed to really characterize *the time course* and *wave form* of the basic oscillation The rationale was to administer light pulse 1 (LP_1) at a given phase and then follow it up with light pulse 2 (LP_2) soon after to check if complete $\Delta\varphi$ had occurred Logically, $\Delta\varphi$ would be large and to scale of the PRC by norms of predictions made by the coupled-oscillator model, but small and nearly undetectable (in the same cycle at any rate) according to the assumptions of Bunning and Zimmer[13]

Figure 4 illustrates the results of an experiment in which LP_1 (15 min, 1000 lux) was given at 15 5 CT and LP_2 at 22 0 CT Both the pulses given individually to two different populations would have induced roughly 5 h delay and 5 h advance $\Delta\varphi$, respectively According to Bunning–Zimmer interpretation, LP_1 and LP_2 should have mutually counteracted each other's influence and no $\Delta\varphi$ should have shown up But the experimental results indicate a much larger

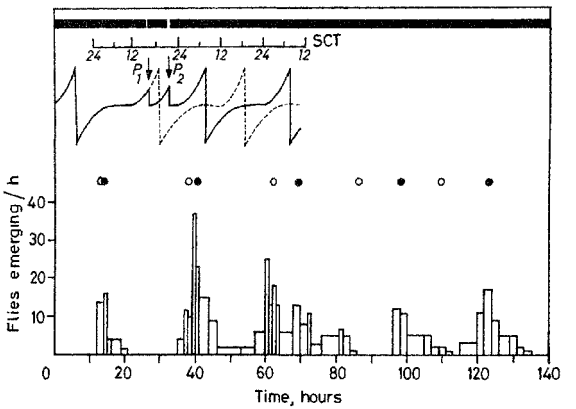


FIG 4 The effect of [illegible] was given at 15 5 h CT and P2 at [illegible] CT. The [illegible] pulses The dotted line indicates the time course of unperturbed controls [illegible] and solid circles indicate positions of medians of experimental populations (after Chandrashekaran[14])

than PRC-predicted delay $\Delta\varphi$ and imply some manner of a *summative* effect Careful calculations indicated that the large delay $\Delta\varphi$ would result if LP_1 had indeed shifted the basic oscillation *instantaneously* by an amount which can be read from the PRC.

Figure 5 illustrates the results of an experiment in which it was assumed that LP_1 indeed shifts phase *instantaneously* by a magnitude seen in PRC, and then the phase position of LP_2 calculated and administered at this phase, to effect an advance $\Delta\varphi$ of 5 h, i e at phase 22 0 CT + 5 h = 3CT The raw data and the schemata of the oscillator kinetics, being postulated as indeed happening, are given in Figs 4–6 The data illustrated in Fig 5 unequivocally demonstrate that LP_2 administered at CT 3 (of control) does evoke $\Delta\varphi$ which counteracts the effects of LP_1 such that the restored time course of the experimental and control eclosion peaks now appear to be very similar Contrast this with the results presented in Fig. 4

Figure 6 contains data of a 2-pulse experiment, whose rationale is the same as in experiment described in Fig 4, except that LP_2 in this experiment was administered in the *second* cycle or in the course of the second *transient* cycle In this figure, the schemata, reproducing events as interpreted, are given in graphic form above the histogram of raw data In all figures describing data of 2-pulse experiments, median values of controls are shown as open circles and of experimental peaks as closed circles The stipled portion in the graphic schemata (coinciding with the third peak) is the postulated, possible phase position of *transients*. LP_2 clearly does not perturb the transient phase but the phase of an *instantaneously* altered steady

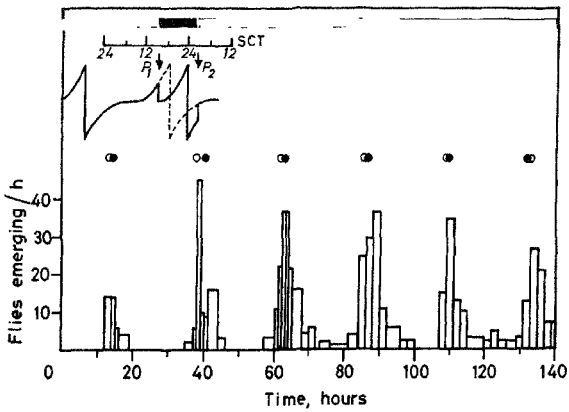


FIG 5 The effect on the rhythm of two light pulses P_1 and P_2 (15 5 h and 02 5 h CT, respectively) of 15 min duration and 1000 lux intensity Other details are as in Fig 4 (after Chandrashekaran[14])

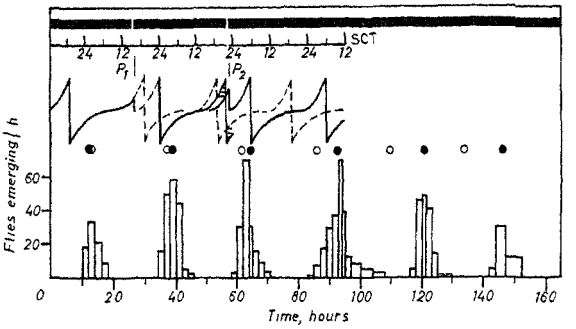


FIG 6 The effect of two light pulses of 15 min duration and 3000 lux P_1 [illegible] at 15.5 h CT, in the free-running cycle of [illegible] at 22.0 h CT of the second cycle P_2 coincides with phase 27.5 h after LL [illegible] are as in Fig. 4. (after Chandrashekaran[4])

state The results of this experiment are also unequivocal It is concluded that both the postulates of the coupled-oscillator model, (i) the basic oscillation is phase-shifted *instantaneously* by light pulses, and (ii) *transients* do not accurately reflect the time course (phases) of the basic oscillation, are valid at least for the *Drosophila* clock The last 30 years have brought to light similar findings for the circadian clocks of a fungus[22], a sparrow[23] and in our own laboratory, of a mammal[24]

3 4 *Dawn and dusk effects*

In the course of early experiments, certain data tended to indicate, that the first half of the subjective night of the circadian clock may show qualitatively different responses to the 'on' and 'off' components of even very brief light pulses, than the second half. More precisely, the first half of the subjective night seemed to respond only to the light 'off' transition of a light pulse, the second half seemed to respond selectively only to the light 'on' transition of a light pulse These possibilities seemed to hold very interesting roles for light in the context of ecology, behaviour and entrainment This is the gist of the *dawn/dusk* effect model proposed by Chandrashekaran[14] The implications are that light 'off' information simulates a *dusk* or sunset in the first half of the subjective night and the light 'on' acts like *dawn* or sunrise in the second half of the subjective night

Figure 7 illustrates the results obtained in an extended series of experiments carried out between 1969 and 1972 (Chandrashekaran *et al* [17])

1 The design in the first batch of experiments was to administer light pulses of 15 min, 1, 2, 3, 4, 5 and 6 h durations, each to a different and large population of pupae The timing of

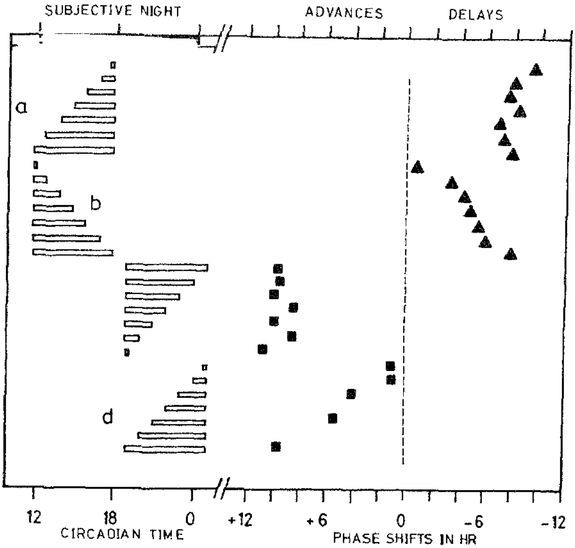


FIG 7 Shifting the p[illegible] rhythm with light pulses of [illegible] duration The light pulses are [illegible] experienced 'on' transition of the pulses at different hours and 'off' transition at 18 0 h [illegible] tions experienced 'on' transition at 12 0 CT but 'off' transition at different hours Whereas batches a and b scan [illegible] the hours of the second half of the subjective night on the [illegible] averaged median values of experimental populations on [illegible] $\Delta\varphi$) Filled squares represent averaged median values of peaks on days 4 and 5 after light treatment (advance $\Delta\varphi$) (after Chandrashekaran *et al* [17])

pulses is reproduced in the upper left panel of Fig 7 All pulses agreed in that the light 'off' in all of them coincided to occur at 18 0 CT The light 'on' component was appropriately staggered If the light 'off' component is indeed the discrete triggering event recognized by the system in shifting phase, then all delay phase shifts evoked by various light pulses must be of comparable magnitude, regardless of the duration of LP The actual responses of populations are given on the right-hand side of Fig 7 and the delays indeed appear to be of comparable magnitude

2 In the second batch of experiments, again 15 min, 1, 2, 3, 4, 5 and 6 h LP were administered, this time around all of them having the light 'off' component systematically staggered to coincide with phase points 12, 13, 14, 15, 16, 17 and 18 CT (and light 'on' coinciding in all cases with 12 CT) Here the delay phase shifts must vary as per 'off' CT phase which appears to be the case (Fig 7)

3 The opposite and reciprocal design was used in further two batches of experiments, scanning the second half of the subjective night In the third batch, pulses of varying duration (illumining as many populations of pupae) started at the same circadian phase (19 0 CT) The pulses were again of 15 min, 1, 2, 3, 4, 5 and 6 h duration Since light 'on' is the effective phase-shifting transition, all phase advances evoked must now be comparable during the second half of the subjective night, results indicate that it indeed is so

4 In a fourth batch of experiments, the light 'on' of the pulses was staggered to coincide with 19, 20, 21, 22, 23, 24 and 01 0 CT phase points The advance phase shifts must now be accordingly varied, which again is the case

3 5. *Early and late subjective night halves further differences*

Some more experiments were performed in pursuit of our quest to discover further differences in response features, energy requirements, etc We worked with blue light pulses (442 ±17 nm) of different energies Results of phase-shifting experiments performed with light perturbations are illustrated in Fig 8 18 0 and 19 0 CT, representing the end of the first half of the subjective night and the beginning of the second half, respectively, were exposed to monochromatic blue light pulses of varying energies The 18 0 CT phase was ten-fold *less* sensitive than the 19.0 CT phase point This difference is not a feature restricted to these phase points and is a defining feature inherent to the oscillator driving the rhythm

3 6 *How to stop the Drosophila clock?*

Pavlidis[25] predicted that the *Drosophila* clock must possess a 'point of singularity' on theoretical considerations Drawn in the form of a phase–plane limit-cycle diagram the singular status will be caused by light pulses of critical strength S* at critical time T*. Winfree[26] discovered the values for these two parameters, which in combination, make the eclosion of pupal populations of *Drosophila pseudoobscura* arrhythmic, with flies eclosing all hours of day and night Figure 9 presents the format of the singularity point experiments defining T*, S* and θ The T* of Winfree's experiments was 6 8 h after LL/DD transfer (18 8 CT) and S* was blue light of 460 ± 17 nm, of energy 100 ergs/cm/s lasting 50 s We undertook an extensive series of experiments in which we could repeat successfully the difficult experiments of Winfree We could severely attenuate[20] the circadian rhythm in the eclosion of *Drosophila pseudoobscura* (A. T. Winfree, W Engelmann and M K Chandrashekaran had worked on the same strain of fruitflies—PU 301) with dim blue light pulses whose product of radiant exposure was the same as the *singular simulus* of Winfree We had systematically varied in our experiments the *irradiance* (*i*) and *duration* (*t*) components within the ranges of reciprocity The range of reciprocity over which the *irradiation* and *duration* of the rhythm attenuating light pulses could be varied was surprisingly vast In terms of irradiance, it varied from very bright light of

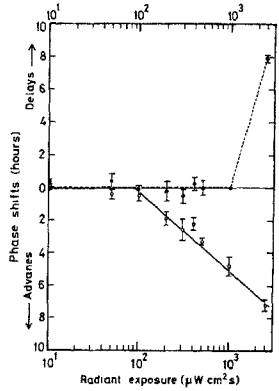


FIG. 8 The extent of phase shifts as a function of the monochromatic blue light pulses of 442 ± 17 nm. Filled circles denote delay $\Delta\varphi$ in response to light pulses given at 18 0 h CT Open circles denote advance $\Delta\varphi$ responses to light pulses given at 19 0 h CT

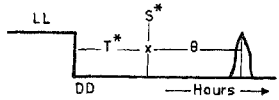


FIG 9 Format of rhythm-attenuating experiments T*, S* are explained in the text (modified after Winfree[26])

12,500 μW cm^2 to 0.01 μW cm^2 and the duration could be reciprocally varied from brief light flashes of 0 04 s to long pulses of 5,000 s Figure 10 sets forth the arrhythmicity and attenuated rhythmic trends obtained in our experiments The degree of arrhythmicity is represented by the R values of Winfree[26].

The larger open circles in Fig 11 represent populations in which the rhythms were attenuated by light pulses whose radiant exposure ($i \times t$) was 500 μW cm^2 The diameter of the open circles roughly represent degrees of arrhythmicity On the contrary, filled circles denote populations that remained often (perfectly) rhythmic in spite of exposure to pulses of radiant exposure of 500 μW cm^2, and also those populations that responded with phase shifts.

3.7. *Temperature and the Drosophila clock*

Although temperature, besides the ubiquitous LD cycles, is a strong zeitgeber in entraining circadian rhythms, very few PRC have been constructed for circadian clocks employing short-term temperature pulses Conflicting reports had been obtained by some authors on the responses of the *Drosophila pseudoobscura* rhythm to temperature perturbations Whereas Zimmerman *et al.*[9] reported persistent advance and delay phase shifts in response to LTP and HTP treatment, Winfree[27] discovered that for his strain of flies the impact of a 12-h incubation at

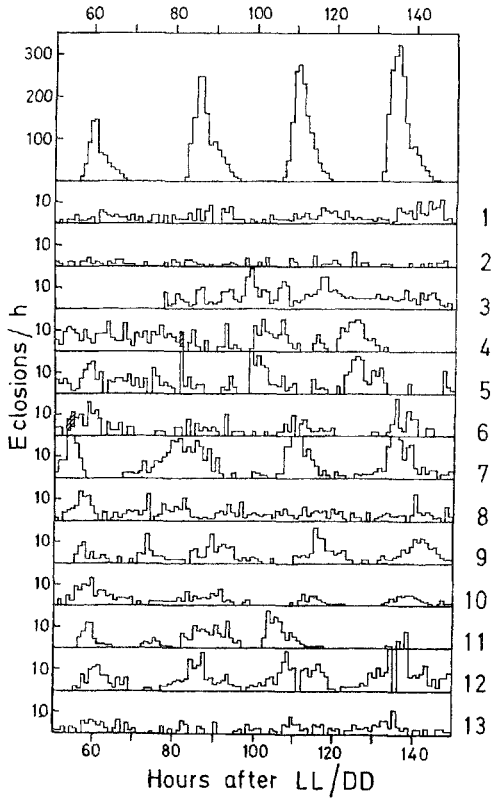


FIG 10 The actual pattern of eclosion on which Fig 11 is partly based The record shows eclosion between the 50th and 150th h after the transfer of pupae from LL to DD Uppermost histogram illustrates pooled eclosion of 11 untreated control populations The rhythmic control pattern of eclosion has an R value of 15 (after Chandrashekaran and Engelmann[20])

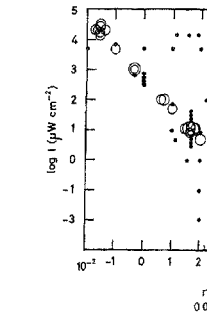


FIG 11 The relationship between irradiance (I) and duration (t) of rhythm attenuating light pulses. Larger circles represent populations in which the rhythm was attenuated and hence had R values above 50, the diameter of the circle being a direct measure of degree of arrhythmicity Filled circles indicate populations in which the rhythm was not affected (after Chandrashekaran and Engelman[20])

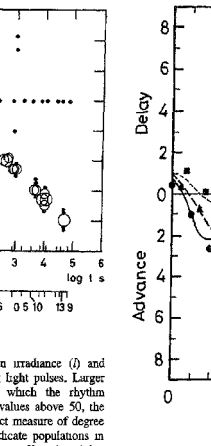


FIG 12 θ duration from LP to median of first peak of eclosion (after Chandrashekaran[16])

28°C for pupae raised at 20°C was severe at all phases. Eclosion peaks occurred 12 h + 24 h × n h after termination of the 12-h incubation regardless of the phase

We reported the most complete family of HTP and LTP of PRC for 3-, 6- and 12-h perturbations. We were able to construct 'weak' (type 1) and 'strong' (type 0) kind of PRC with temperature stimuli (Figs 12–13)[19,21]

4. Discussion

The experiments described in this brief review were performed during 1965–1975 In the intervening period, circadian rhythms in *Drosophila* have been studied intensively and two clock genes *per* (period) and *tim* (timeless) have been cloned[28] In 1997, chronobiology hit pay dirt[29] and the first circadian clock gene in mammals was identified and cloned. Although there is considerable conservation and homology between the structural features of clock genes, it is becoming increasingly clear that these genes elicit functions by completely distinct mechanisms[30] This is no matter for surprise as biological clocks underlie myriad phenomena and biochemical, physiological and behavioural processes in organisms as varied as microorganisms to humans[31] Therefore, the formal properties of circadian rhythms such as entrainment to LD cycles, freerunning under LL and DD and phase-shifting of the rhythms by light, temperature, chemical and other perturbations, and the still enigmatic phenomenon of tempera-

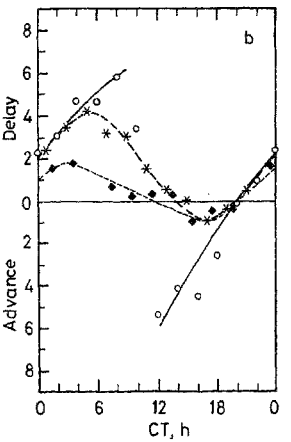


FIG. 13 Phase-response curves for LTP of 12 h (O), 6 h (*) and 3 h (◆) with values plotted against CT of the midpoint of LTP (after Chandrashekaran[9])

ture compensation of the period of circadian clocks continue to be the focus of much contemporary scientific attention[32, 33]

Engelmann[34] examined the 'on' and 'off' rhythms in the eclosion of *Drosophila pseudoobscura* and pointed out that their time courses relative to each other were displaced by 180° (as in Fig. 1) In later literature, such rhythms were likened to dawn and dusk oscillators. Pittendrigh pointed out that light staying on beyond 12 h froze the light-sensitive basic oscillator at 12 CT phase A subsequent restoration of *permissive* DD conditions released the rhythm from the 12 CT phase, the eclosion peak always following the pattern $= n \times \tau + 15$ h, i e the first peak occurring 15 h after LL/DD transfer and subsequent peaks following every 24 h (actually τ in DD is 24.3 h) The question that has never satisfactorily been answered is the nature of the 'on' and 'off' rhythms of Engelmann[34] and Chandrashekaran[14,15] and what they represent These rhythms, according to Pittendrigh's postulate, certainly do not represent the overt expressions of the pacemaker A-oscillator. The 'on' rhythms also have a different time course than 'off' rhythms, and unlike the latter, wane even in dim light as a function of time Another trend noticed in Fig 1 and in other light-intensity experiments on *Drosophila*[16] is that the kinetics of waning is in inverse proportion to the LL intensities in the range of 0.3 to 3000 lux These experiments need to be repeated and the intriguing relationship between the intensity of LL and speed of waning firmly established. It is tempting to believe that the 'on' rhythms may

represent the overt expressions of the otherwise *elusive* B-oscillator set in motion by the A-oscillator at the moment of the onset of LL

Results of experiments illustrated in Figs 4 and 5[14] unequivocally support the first tenet of the Pittendrigh–Bruce[12] coupled oscillator model, viz. the *instantaneous* resetting of the basic oscillator by light pulses to the extent predicted in the light-pulse PRC Results contained in Fig 6 further confirm the second tenet of the model that the *transients* following light perturbations do not reflect the *time course and wave form* (phases) of the basic oscillator These appear still to be the only published results of experiments that directly challenged *transients* with a second light pulse.

Instantaneous resetting of the phase has been experimentally established in *Neurospora crassa*[22] and *Passer domesticus*[23] In a recent publication, Sharma and Chandrashekaran[24] reported that the circadian rhythm in the locomotor activity of the field mouse *Mus booduga* was instantaneously reset by brief flourescent light pulses (15 min, 1000 lux), first such report for mammalian circadian rhythms. Performing two light-pulse experiments and employing a light-pulse PRC for purposes of phase scanning, they reported that the $\Delta\varphi$ was 85% complete 6 h after LP_1, and 91% after 8 h There are also indications that the *transients* following light perturbations in the mouse circadian rhythm also do not represent the time course of the basic oscillator[24] The case for possible *dawn* and *dusk* roles for light pulses (Fig 7) coinciding with the first and second halves of the subjective night of the *Drosophila* circadian clock would seem to have been made Unfortunately, these studies have not been followed up for other circadian systems A basic precondition for undertaking experiments of this nature is the construction of noise-free and fine-tuned LP-PRC of the kind constructed for *Drosophila pseudoobscura* eclosion rhythm[8]

Results of experiments illustrated in Fig. 8 indicate that the first and second halves of the subjective night of the *Drosophila* circadian system differ in their light energy requirements for comparable $\Delta\varphi$ by at least an order of magnitude During the phase of the second half of the subjective night the threshold of response to blue light of 442 ± 17 nm is ten-fold *lower* than during the course of the first half Experiments in our laboratory on the relationship between light intensity and phase resetting in a mammalian circadian system also indicate that a five-fold intensity of light is required at 15 CT (phase delays) to induce saturating $\Delta\varphi$ than is required at 20CT (phase advances)[35] Our findings that HTP and LTP of 6- and 12-h durations evoke two types of PRC-type O for LTP and type 1 for HTP in the same circadian system freerunning in DD are of obvious interest in the context of models of circadian rhythms Low temperature in general, mimics darkness and high temperature mimics light when offerred in 12 12 h cycles Interestingly, HTP and LTP PRC for *Drosophila* are *mirror images*, much as light- and dark-pulse PRC made for the circadian rhythm in the flight activity of the insectivorous tomb bat *Taphozous melanopogon*[36].

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