Early and Late Subjective Night Phases of the *Drosophila pseudoobscura* Circadian Rhythm Require Different Energies of Blue Light for Phase Shifting

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(Z. Naturforsch. **28 c,** 750–753 [1973]; received August 22, 1973)

Circadian rhythm, phase shifts, subjective night

Two phase points (18 C.T. and 19 C.T.) in the subjective night of the *Drosophila pseudoobscura* circadian rhythm of eclosion, representing the end of the first half of the night and the beginning of the second half respectively, were exposed to blue (442 nm) light pulses of varying energies. The 18 C.T. phase was less sensitive relative to the 19 C.T. phase which is nearly 10-times as sensitive. This difference is not a feature restricted to the two phase points studied since similar differences could be traced to rhythm attenuating stimuli offered slightly earlier or later than the subjective midnight. It is suggested that the differences in sensitivity to light displayed by the two halves of the night are inherent to the oscillator driving the rhythm.

An ubiquitous feature of circadian rhythms is their varying response to brief light (and temperature) pulses as a function of the phase of the rhythm[1]. The responses are reflected in a temporary lengthening or shortening of the rhythms resulting in so-called "delay" and "advance" phase shifts respectively[2]. In all organisms studied in this respect the early night phases respond to brief light perturbations in a direction (delays) opposite to that (advances) of late night phases. The switch in the direction and nature of response occurs around the subjective midnight. Several authors have studied this phenomenon of antagonistic responses of the 2 regions of the night in order to uncover differences in the dependence on the quality and energy of light[3–6].

For the circadian rhythm in the eclosion rate of *Drosophila pseudoobscura* no such overt differences could be delineated between responses of early and late night phases to different wavelengths of light[7] as well as to different intensities of white fluorescent light contained in 15-min pulses[8]. The two regions of the subjective night have been reported[9], however, to respond differently and selectively to the "on" (dawn) and "off" (dusk) transitions of brief light pulses. The problem of the responses of the early and late night phases of the *Drosophila* rhythm was re-investigated employing monochromatic (blue) light at intensity[10] levels that evoked "weak"[11] responses.

The resuslts, reported here, do reveal characteristic differences in light energy[10]-dependence within early and late night portions of the rhythm.

Material and Methods

Cultures of *Drosophila pseudoobscura* (PU 301 from C.S. Pittendrigh's laboratory) were raised in LL of 900–1100 lux at 20 ± 0.5 °C in plastic troughs 22 cm in diameter and 10 cm deep. Two circular windows each 3 cm in diameter were cut out of the walls of the trough for ventilation and covered over with fine nylon mesh. Square glass plates 25 cm × 25 cm were used as lids for the troughs. The usual cornmeal-agar-yeast-molasses medium was used in rearing the cultures. Larvae creep out of the frequently moistened medium in 17 to 18 days after oviposition and pupate on the inner wall of the trough and the glass plates. The pupae were harvested about 20 days after oviposition by a floatation method and air dried. Further details in Maier[12].

The eclosion in some of the early experiments was monitored using a photo-electric automated set-up described earlier[9]. The pattern of eclosion in other experiments was measured using a more direct method. Pupal populations were glued to glass plates 10 cm × 10 cm and placed inverted over the

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Abbreviations: LL, continuous light; DD, continuous darkness; C.T., circadian time[20] in which 0 C.T. signifies the start of day and 12 hours later, 12 C.T. denotes the onset of subjective night.

broad ends of teflon-coated glass funnels clamped upright. Eclosing flies wandered to the edge of the slippery funnels, slipped and fell through the narrow end fitted into a light (red) beam and photocell chamber. The flies, before drowning in the soap water placed below in vials, interrupted the light beam briefly and activated a relay and event recording writing pen. Further details in Maier[12].

In all the experiments the circadian rhythms in eclosion were set in motion by a transfer of the pupal population from LL to DD (red "safe" light of about 660 nm).

Monochromatic blue light was obtained with the aid of projectors fitted with Philips 15 V 150 W or Osram 12 V 100 W bulbs. The light beams projected were reflected 90° after passing through Schott interference filters transmitting 442 nm (±17 nm). Projector bulbs were aircooled automatically when the lights were on. Pupal populations, each consisting of 1000 – 1400 pupae, were held in glass petri-dishes of about 9 cm diameter during light treatment. The irradiance of light pulses were measured both with a "multiflex" galvanometer as well as an optometer (40 A, United detector Technology) in $\mu W \cdot cm^{-2}$. The radiant exposures used were obtained by varying the irradiance on the one hand and the duration of the light pulses on the other hand.

Experiments and Results

Two phase points, 18 C.T. marking the end of early night and responding with delay phase shifts, and 19 C.T. signalling the beginning of late night and responding with advance phase shifts, were chosen for light perturbations. The irradiance of light pulses administered varied between 1 and 10000 $\mu W \cdot cm^{-2}$ and durations between 1 and 1000 sec. Fig. 1 sets forth the data obtained from these experiments. It is evident that the 19 C.T. phase point exhibits an almost *10-fold* sensitivity to light pulses relative to 18 C.T. phase. Advance phase shifts of the magnitude of 25% of those induced by pulses of higher radiant exposures ($2.5 \cdot 10^3\ \mu W \cdot cm^{-2} \cdot sec$) set in even at a radiant exposure of $2 \cdot 10^2\ \mu W \cdot cm^{-2} \cdot sec$ of blue light (442 nm) whereas there are no delay phase shifts practically upto $10^3\ \mu W \cdot cm^{-2} \cdot sec$. The course and approximate magnitude of response in both cases seem, however, to be similar at and above(not shown in Fig. 1) the level of $2.5 \cdot 10^3\ \mu W \cdot cm^{-2} \cdot sec$.

Winfree[13], working with the same rhythmic system as ours, demonstrated that weak blue light stimuli of about $5 \cdot 10^2\ \mu W \cdot cm^{-2} \cdot sec$ ($10\ \mu W \cdot cm^{-2} \cdot 50\ sec$) given 6.8 hours (T) after LL/DD transfer severely deranged the emergence rhythm. This "singular stimulus" T* S* (T* = time after LL/DD; S* = radiant exposure of the pulse) was viewed by Winfree[13] "as resetting the amplitude of the oscillator to zero". This special feature of the *Drosophila* rhythm was exploited in our studies to further test the pronounced rise in sensitization to light occurring between 18 C.T. and 19 C.T. (*i. e.* between 6 and 7 hours after LL/DD). Fig. 2 illustrates the data obtained from experiments in which several combinations of T and S close to

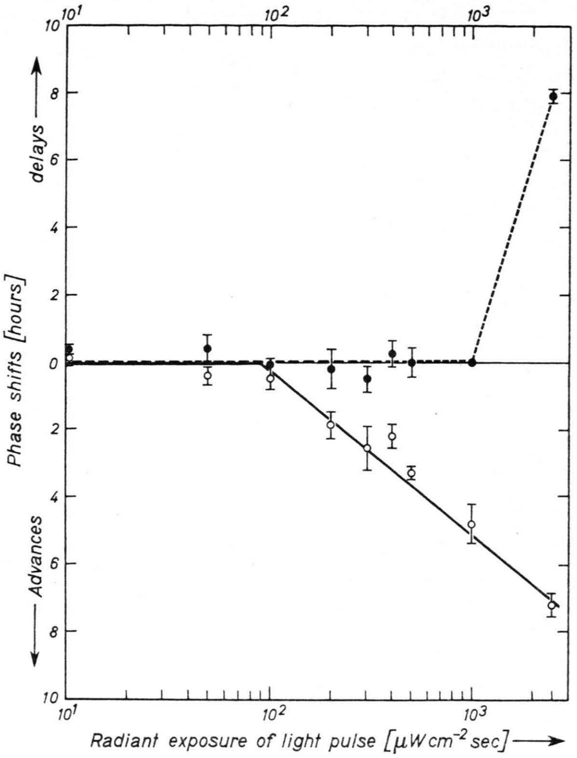

Fig. 1. The extent of phase shifts of the *Drosophila pseudoobscura* eclosion rhythm as a function of the energy of monochromatic blue light pulses of 442 nm±17. ●: Denotes 'delay' phase shifts in response to light pulses given at 18 C.T.; ○: denotes 'advance' phase shifts in response to light pulses given at 19 C.T. Ordinate: Phase shifts in hours. Abscissa: Radiant exposure of light pulses (irradiance × duration) in $\mu W \cdot cm^{-2} \cdot sec$. Horizontal line dividing advances from delays indicaets position of the peaks of unperturbed LL/DD control populations. Phase shifts in all cases were calculated from averages of the IV, V, VI and VIIth peaks of eclosion of experimental populations relative to the peaks of the controls whose medians occured about 87, 111, 135 and 159 hours after LL/DD respectively, and standard errors are indicated by vertical lines.

T* S* were tried out. It is apparent that the rhythm attenuating stimulus itself undergoes changes paralleling the changes in the energy-dependence pattern of early and late night regions. At 18 C.T. when the rhythm is less sensitive to light the radiant exposure has to be 2-fold to attenuate the rhythm in comparision to the S* at the T* defined by Winfree[13] (6.7 hours after LL/DD Fig. 2 B). At 19 C.T. when the system has already developed a higher sensitivity to light a radiant exposure of even $3 \cdot 10^2$ $\mu W \cdot cm^{-2} \cdot sec$ (Fig. 2 C) is sufficient to attenuate the rhythm.

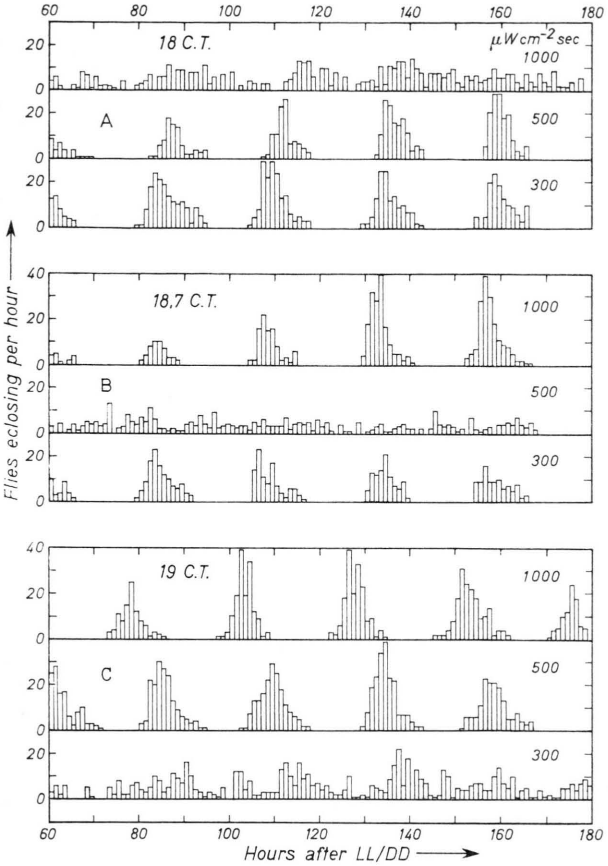

Fig. 2. A. The rhythm attenuating stimulus is 1000 $\mu W \cdot cm^{-2} \cdot sec$ at 18 C.T. Radiant exposures of 500 and 300 $\mu W \cdot cm^{-2} \cdot sec$ do not annihilate or even attenuate the rhythm at this phase. B. Various radiant exposures tested at the T* of Winfree[14]. The standard[14] S* of 500 $\mu W \cdot cm^{-2} \cdot sec$ is the most effective at this phase point of 18.7 C.T. At this later point in time 1000 $\mu W \cdot cm^{-2} \cdot sec$ has a marked phase shifting action. C. The rhythm attenuating stimulus is 300 $\mu W \cdot cm^{-2} \cdot sec$ at 19 C.T. which, as seen in Fig. 1 is far more sensitive to light pulses in general relative to 18 C.T. Radiant exposures of 1000 and 500 $\mu W \cdot cm^{-2} \cdot sec$ are stronger than the rhythm attenuating stimulus for this T and have a marked phase shifting action. Ordinate: Number of flies eclosing each hour. Abscissa: Hours after LL/DD transfer.

Discussion

The differences in the light energy relations of the delaying and advancing phases of the *Drosophila* rhythm reported here are apparently restricted to radiant exposures evoking weak responses giving rise to the "type 1" phase response curve of Winfree[14]. A similar difference between early and late night phases may be discerned in Fig. 1 of Engelmann[15] who had chosen 16 C.T. and 20 C.T. as phase points for study. Frank and Zimmerman[7], however, found no overt differences between the two regions of the subjective night in their response features to spectral values from 367 nm upto infrared of varying intensities.

Winfree[16] showed in a later report that the *Drosophila* rhythm developed a 10-fold increase in its sensitivity to light perturbations within 24 hours of transfer from LL to DD. A similar trend has been confirmed in the course of our present studies where delay and advance phases in the second cycle responded to radiant exposures one tenth of the minimal required in the first cycle. Winfree attributed this phenomenon to a recovery and regeneration of the photoreceptor/pigment complex in DD after being bleached by the preceding LL and the increase in sensitivity was not a feature of the circadian clock. The 10-fold increase in sensitivity of the late night (19 C.T.) relative to early night (18 C.T.) reported here, on the other hand, is apparently an inherent feature of the oscillation gating eclosion. The two phase points chosen by us to represent the two halves of the night are a mere 1 hour apart and the marked changes in sensitivity could hardly be attributed to processes such as regeneration of the pigment system. Furthermore a comparison of the data of Engelmann[15] and the data of the present studies reveals no comparable increase in sensitivity, for instance, between 16 C.T. and 18 C.T. perhaps owing to the fact that both points are within the early half of the night.

In our experiments dealing with the singular stimulus it is doubtful that we have "reset the amplitude of the oscillator to zero"[13] but the stimuli deranged the rhythms severely indicating that we were in each case close to the point of singularity. Winfree[13] had, in addition, used single sex (female)

X-chromosome "sex-ratio" mutants in his studies in contrast to our mixed male/female populations. This may account for the persistence of a weak rhythm such as in Fig. 2 C.

Qualitative differences between early night and late night phases have been reported for other circadian systems as well. Bünning and Moser[3], working with the leaf movement rhythm *Phaseolus*, observed that delays and advances responded to distinctly different irradiances and concluded that the nature of the primary processes in the two cases may be different. Halaban[4] found that in the leaf movement rhythm of *Coleus* blue light was effective in causing delay phase shifts whereas red light effected advance phase shifts. Recently Christianson and Sweeney[5] described the unique nature of the early part of the *Gonyaulax* rhythm: In this region a light pulse caused a *delay* when the cells were maintained in darkness but caused an *advance* when the cells were held under light intensities between 450 – 1000 lux. Truman[17] has drawn attention to some special features of the early night portion of the *Antheraea pernyi* eclosion rhythm; light coinciding with this region of the night restores the clock to te same phase each time on being switched off. Johnsson and Karlsson[18] have *theoretically* predicted a similar peculiarity in the *Drosophila pseudoobscura* circadian rhythm[19] and the standard phase response curve[20] for the organism. Occurrence of bright light starting at a particular point heralding the night, which Johnsson and Karlsson[18] call the "fixed point", would suspend the rhythm at that phase (11 C.T.) to return it to the very *same* phase (11 C.T.) on subsequent restoration of the rhythm to DD. Chandrashekaran *et al.*[9] recently suggested that early and late night phases of *Drosophila* rhythm filtered varying information from brief light pulses depending on the time of their occurrence. The "off" transition of a light pulse signified "dusk" during early night and the "on" transition adumbrated approaching "dawn". The body of evidence from earlier work cited here and our results on *Drosophila* indicate that some circadian systems show several differences between *early* and *late* night phases which may well in themselves reflect deep-seated differences in the kinetics as well as in the energy relations of the basic oscillator.

We thank Prof. E. Bünning and Dr. A. Johnsson for critically reading through the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft En42/11 and a special award under its "Schwerpunktprogramm" for "Biologie der Zeitmessung", both to Wolfgang Engelmann.

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