Temperature Sensitive Events between Photoreceptor and Circadian Clock?

Ursula Hamm, Maroli K. Chandrashekaran, and Wolfgang Engelmann

Institut für Biologie I, Tübingen

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The phase shifting action of low temperature pulses of 6 °C and 2 h duration administered to the various phases of the *Drosophila pseudoobscura* circadian rhythm and the action of light pulses given 30 min after the beginning of these low temperature pulses have been investigated. The phase response curve obtained from experiments with light pulses during low temperature cannot be explained on the basis of a straightforward and sequential phase shifting of the oscillation by the various transitions in the pulses. The response curve, after the slight phase shifting action of the temperature pulses is corrected for, resembles the standard phase response curve [4] for light pulses (at 20 °C) in its wave form but *not* in its time course. Our curve is shifted in time in a manner that indicates that the light pulses accompanying the low temperature pulses *arrived* at phase points 1.5 h later than the actual phases at which they were given. We attribute this delay to a slowing down of the information that is apparently transmitted by a process that is temperature dependent.

One of the features common to most of the known circadian rhythms (oscillatory physiological processes in organisms persisting under constant conditions with a period of about 24 hours) is their ability to respond to light pulses while being maintained in darkness. The adult flies of *Drosophila pseudoobscura* display such a circadian rhythm in eclosing out of their puparia after the termination of metamorphosis, if cultures of the flies are exposed to light/dark cycles or even if merely transferred from light to constant darkness before the beginning of eclosion. Pittendrigh and colleagues [1–4] have made a thorough analysis of the responses of the *Drosophila* rhythm to entraining photoperiods and to shorter light breaks that shift phase. The responses of the eclosion rhythm to light breaks at various points in the rhythm have been represented in the form of a phase response curve. The responses in this curve are the "advances" and "delays" in hours of the eclosion maxima evoked by brief light pulses at the various phases of an entire cycle.

The assumption regarding the action of these light pulses on the basic oscillator has been [4, 5] that they evoke *instantaneous* responses. By implication the message of the light pulses arrives straightaway at the perturbed phase of the oscillation.

We desired to put this assumption to experimental test and in doing so learn more about the physiological processes associated with light perception by the photoreceptor and the actual "arrival" of the transformed signal at the oscillator. For this purpose a series of experiments were performed to study the action of light pulses given coincidental with low temperature breaks. The resultant effects are compared with those evoked by light on the one hand and temperature on the other and some conclusions are drawn concerning possible temperature dependent processes occuring between the photoreceptor and the clock.

Requests for reprints should be sent to Dr. W. Engelmann, Institut für Biologie I, *D-7400 Tübingen*, Auf der **Morgenstelle 1.**

Material and Methods

Details of rearing the *Drosophila pseudoobscura* cultures used in this study and of the recording of the time course of eclosion are described by Maier [6]. Light pulses were administered in temperature controlled rooms equipped with monochromatic light sources. Schott interference filters (442 nm) were used to obtain the blue light, and light intensity was measured with the aid of an optometer (40 A, United Detector Technology) in $\mu W\,cm^{-2}$. For low temperature pulses the pupae were transferred to another temperature controlled room at 6 °C which was also equipped with monochromatic light sources.

Results

Derivation of phase response curves

Fig. 1 illustrates in its uppermost row a circadian rhythmicity at 20 °C in the eclosion rate of

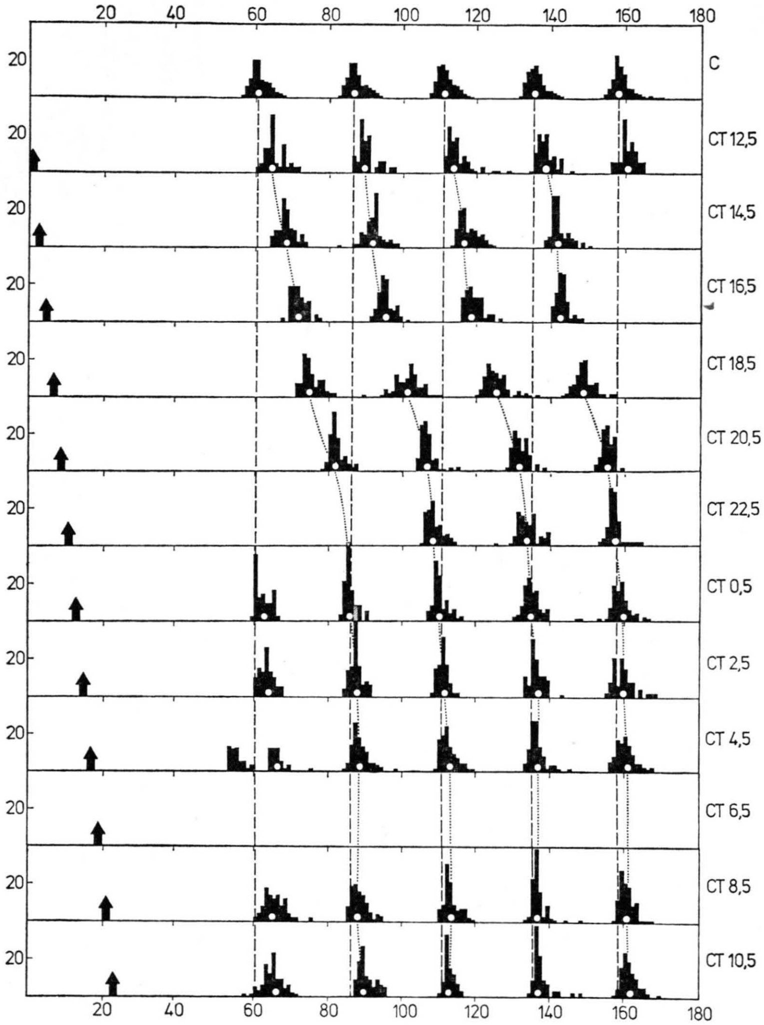

Fig. 1. Circadian rhythm of eclosion in populations of *Drosophila pseudoobscura* which were transferred from light to darkness at hour "0". The uppermost row shows eclosion of flies in a control group (C). The other groups received 10 sec-light pulses (arrows) 30 min into the 2 hour low temperature breaks at the hours indicated by the numbers alongside the groups. Eclosion within a peak have been normalized to 100%. The corresponding medians (white dots) are connected.

flies in a population which was transferred from light to darkness at hour "0" lasting for 7 days. The rest of the figure presents such rhythms in 11 populations undergoing a change in the timing of their daily eclosion peaks due to exposure to brief light pulses given during 2 hour low temperature breaks at the various hours of the cycle as indicated. It is apparent that a typical steady state pattern evolves in the eclosion medians. The medians of peaks occuring at the same hours in each cycle could be pooled and used in plotting the "phase response curve" such as the one presented in Fig. 2 which is the same as the curve formed by the medians (white dots) in Fig. 1 but tilted counterclockwise by 90°. We use the phase response curve in explaining the results of our light and temperature experiments.

Phase shifting action of light pulses during low temperature

The standard phase response curve for the *Drosophila pseudoobscura* eclosion rhythm at a constant temperature of 20 °C and for light pulses of 15 min duration and 100 ft.cd. irradiance is reproduced from the literature [4] in Fig. 2, broken line. The strongest phase shifts are effected by pulses briefly before and after the subjective midnight with the subjective day being practically light refractory. The light pulses in our experiments, which were of shorter duration (100 sec) and blue (442 nm of 100 μW cm^{-2} irradiance) would bring about identical phase responses [7]. The solid curve in Fig. 2 describes the phase shifting action of the blue light

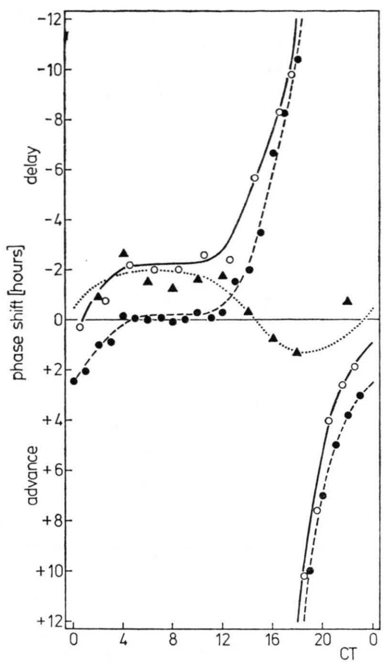

Fig. 2. Phase response curves. Broken curve and filled circles: Responses to 15 min white light 100 ftcd., from Pittendrigh and Minis 1964. Solid curve and open circles: Responses to light pulses (100 sec 100 μW cm^{-2}) during low temperature (2 hours 6 °C). Dotted curve and triangles: responses to temperature pulses (2 hours 6 °C). Standard errors of ▲ about 0.35 hours, standard errors of ○ about 0.45 hours.

pulses given during accompanying low temperature pulses (6 °C for 2 hours). The light pulses were given 30 min after the beginning of the temperature treatment. The "wave form" of this response curve is similar to that at 20 °C except for two deviant features:

1. The non-reactive subjective day part of the cycle now reacts with delays of about 2 hours.
2. The curve is shifted in its "time course" to the left by about 1 – 2 hours.

Further experiments with low temperature pulse controls clarified feature 1. and it became apparent, that the delay responses of ca. 2 hours disappear if the temperature induced effects (dotted line curve in Fig. 2) are subtracted. This leaves us with the second feature of the shift in the time course (Fig. 3). We propose the following explanation for the phenomenon:

The light signal is delayed by the low temperature by roughly 2 hours and thus reaches the oscillator that much later. The phase shift effected would be then of corresponding magnitude to the one which

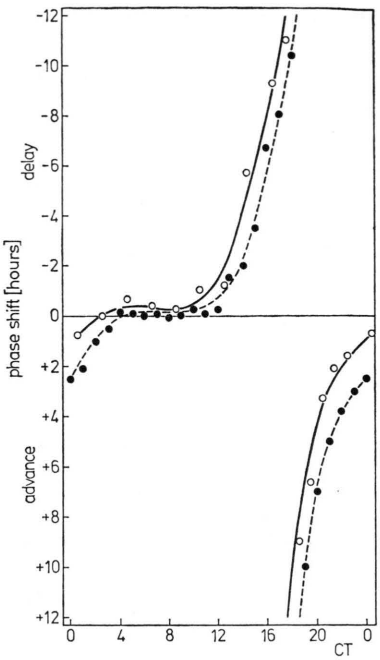

Fig. 3. Phase response curves. Broken curve and filled circles: Responses to light pulses. Solid curve and open circles: Responses to light pulses during low temperature after correcting for the effect of temperature pulses.

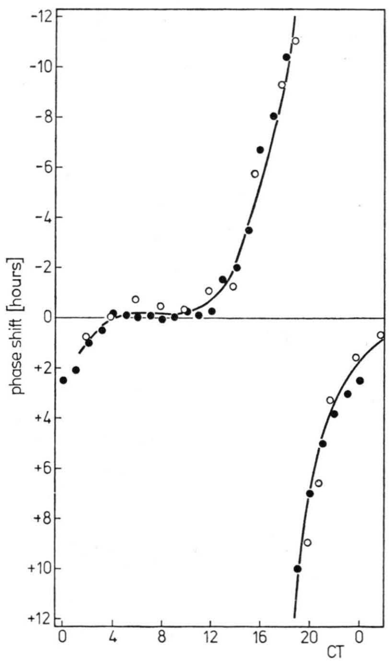

Fig. 4. Phase response curves of light pulses during low temperature pulses after correcting for the effect of temperature pulses and after accounting for their signal delaying action.

would be evoked at a phase 2 hours later in the cycle. If the postulated 2 hour delay of the signal is taken into account and consequently the dotted curve in Fig. 3 is shifted by 2 hours to the right, as has indeed been done in Fig. 4, both phase response curves (for 20 and 6 °C) show much coincidence.

The rhythm attenuating action of the light pulses during low temperature

To test this hypothesis further we exploited an interesting feature of the rhythm discovered by Winfree [8]. He administered a dim light of a certain strength (S^x) at a certain critical phase (T^x) of the *Drosophila* rhythm and abolished the rhythm altogether. This treatment has been interpreted by Winfree to send the oscillation into a state (point) of singularity away from the limit cycle along which the system normally oscillates.

If our interpretation of low temperature delaying the light signal is correct then in order that a light pulse of rhythm-attenuating strength arrives at the critical phase T^x during low temperature it ought to be given some 2 hours earlier. A number of populations were treated in this manner and Fig. 5

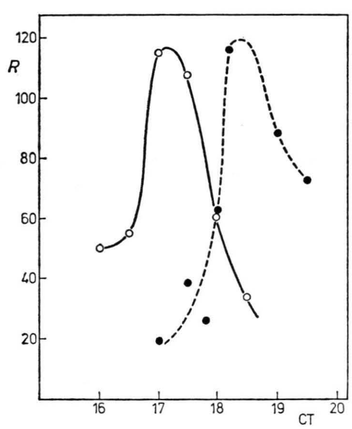

Fig. 5. *R*-values (amount of arrhythmicity) at different circadian times for light pulses. Broken curve and filled circles: 10 sec-light pulses. Solid curve and open circles: Light pulses (10 sec, 100 μW cm^{-2}) during low temperature (2 hours 6 °C).

illustrates the findings. The light pulses were adjusted to the S^x specifications for our *Drosophila* strain [7] (100 μW, 10 sec, 442 nm). Populations exposed to this critical treatment at the specific phase T^x (6 hours after light/dark transition) exhibited later much attenuated rhythms *without* simultaneous low temperature treatment. However, the same light treatment at the same phase *during* a low temperature pulse does not lead to similar arrhythmicity. The critical stimulus S^x has to be administered 1.5 to 2 hours ahead of T^x to attenuate the rhythm when coupled to the low temperature treatment. We consider this additional evidence for our thesis that light signals are delayed by low temperature before reaching the oscillator.

Discussion

We are aware that the events described are complex and that alternative explanations may be just as appropriate as our explanation.

We consider here one other alternative which is based on the findings of Zimmerman *et al.* [9] that the phase shifting action of temperature pulses can be deduced from the phase shifting action of the 2 steps comprising the pulse. Thus in our experiments a series of phase shifts could have resulted in an altered time course. The rhythm could thus be phase shifted by

a. the step down in temperature from 20 to 6 °C,
b. the light pulse occuring 30 min later, and finally
c. the step up in temperature from 6 to 20 °C.

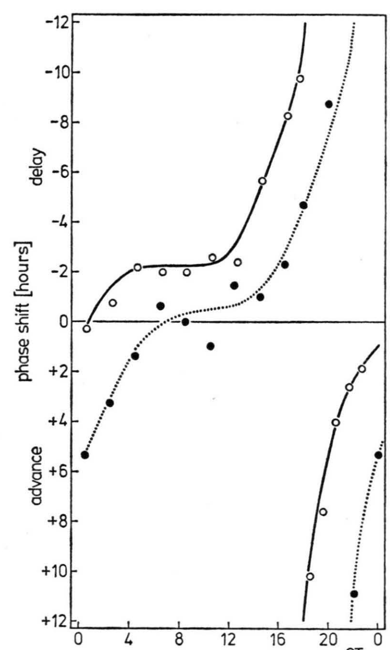

Fig. 6. The resultant phase response curve after all 3 events, the temperature step down, the light pulse and the temperature step up have been taken into account according to the experimental results of Zimmerman *et al.* (1968) (●···●). Actual experimental data (○—○) given for comparison.

Table I. Netto phase shifts obtained under the assumption that the temperature steps are twice as effective as experimentally found by Zimmerman *et al.* (1968). 1st row (CT$\downarrow$T): circadian time at the onset of the temperature step down. 2nd row ($\Delta\varphi\downarrow$T): phase shift brought about by the temperature step down. 3rd row (CT_{LP}): Circadian time at the onset of illumination. 4th row ($\Delta\varphi_{LP}$): phase shift brought about by the light pulse. 5th row (CT$\uparrow$T): Circadian time at the onset of the temperature step up. 6th row ($\Delta\varphi\uparrow$T): phase shift brought about by the temperature step up. 7th row (netto $\Delta\varphi$): netto phase shift as calculated from the 2nd, 4th, and 6th row.

CT $\downarrow$T	00	02	04	06	08	10	12	14	16	18	20	22
$\Delta\varphi\downarrow$T	−0.9	−1.1	−1.3	−1.0	−0.5	−0.2	−0.8	−0.9	−0.7	−0.9	−1.0	−1.2
CT_{LP}	23.6	01.4	03.2	05.5	08.0	10.3	11.7	13.6	15.8	17.6	19.5	21.3
$\Delta\varphi_{LP}$	+2.5	+1.5	+0.4	0.0	0.0	0.0	−0.3	−1.8	−5.3	−9.7	+8.2	+4.8
CT $\uparrow$T	03.6	04.4	05.1	06.9	09.5	11.8	13.5	16.9	22.6	04.8	05.2	03.6
$\Delta\varphi\uparrow$T	+0.9	+0.8	+0.7	+0.5	+0.3	+0.7	+0.6	+0.9	+2.0	+0.8	+0.7	+0.9
netto $\Delta\varphi$	+2.5	+1.2	−0.2	−0.5	−0.2	+0.5	−0.5	−1.8	−4.0	−9.8	+7.9	+4.5

Table II. Netto phase shifts obtained under the assumption that the temperature steps are half as effective as experimentally found by Zimmerman *et al.* (1968).

CT $\downarrow$T	00	02	04	06	08	10	12	14	16	18	20	22
$\Delta\varphi\downarrow$T	−3.6	−4.4	−5.2	−4.0	−2.0	−0.8	−3.2	−3.8	−3.0	−3.8	−4.0	−4.8
CT_{LP}	20.9	22.1	23.3	02.5	06.5	09.7	09.3	10.7	13.5	14.7	16.5	17.7
$\Delta\varphi_{LP}$	+5.4	+3.7	+2.6	+0.6	0.0	0.0	0.0	0.0	−1.7	−3.3	−6.6	9.7
CT $\uparrow$T	03.8	03.3	03.4	04.6	08.0	11.2	10.8	12.2	13.3	12.9	11.4	09.5
$\Delta\varphi\uparrow$T	+3.6	+4.0	+4.0	+2.8	+2.0	+1.8	+1.8	+2.8	+2.4	+2.4	+1.8	+1.2
netto $\Delta\varphi$	+5.4	+3.3	+1.4	−0.6	0.0	+1.0	−1.4	−1.0	−2.3	−4.7	−8.8	+10.8

In order to investigate if such shifts do take place, ideally, temperature step experiments for 6 and 20 °C should be performed in the manner of Zimmerman *et al.* [9] who did so for 20 and 28 °C. Our lower temperature of 6 °C, however, is impractical for rearing the cultures and following up the course of eclosion. We, therefore, use the data of Zimmerman *et al.* [9] in estimating roughly the phase shifts evoked by the temperature steps and the altered phases. Another assumption we make (for want of more precise information) is that the phase shifts of the oscillation are completed after each step rather rapidly before the next step or transition is encountered.

Fig. 6 sets out the actual experimental phase shifts against phase shifts calculated on the basis of the data of Zimmerman *et al.* and the curves formed by the two sets may be seen to be widely divergent. Even attributing a stronger (Table I) or a weaker (Table II) phase shifting capacity to the temperature steps does not lead to phase shifts of the order obtained in our experiments. We, therefore, prefer to stand by our earlier explanation that light signals are delayed by low temperatures on their way to the oscillator and atrtibute this to possible temperature dependent processes occuring between the photoreceptor and the clock.

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