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Entrainment Properties of the Locomotor Activity Rhythm of Drosophila melanogaster Under Different Photoperiodic Regimens

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Abstract

In this paper we report the results of an experiment to assess how closely repeated brief light pulses (LPs) mimic the effects of 12:12 h light/dark (LD) cycles (PPc). The locomotor activity rhythm of individual fruit flies from a laboratory population of *Drosophila melanogaster* was monitored under four different photoperiodic regimens, created using 12 h of light and 12 h of darkness or brief light pulses (LPs). The phase relationship (ψ) and the stability (precision) of the locomotor activity rhythm during entrainment were estimated in order to compare the state of the circadian clocks under the four different photoperiodic regimens. The flies ($n = 72$) were subjected to four different LD cycles: (i) 12 h of light and 12 h of darkness (complete photoperiod, PPc); (ii) a single brief LP of 15 min duration presented close to the onset of activity (SLP-1); (iii) a single brief LP of 15 min duration administered close to the offset of activity (SLP-2); and (iv) two brief LPs administered 12 h apart (skeleton photoperiod, PPs). The locomotor activity rhythm of the flies was first monitored under constant darkness (DD) for about 10 days and then under the four different photoperiodic regimens for about 10 days, and finally in DD for the remainder of the experiment. The ψ of the locomotor activity rhythm and its precision under PPc and PPs did not differ significantly, but they were significantly different from the SLP-1 and SLP-2 conditions. The results provide interesting insights into photoentrainment mechanisms of circadian clocks in *D. melanogaster*, and suggest that skeleton photoperiods, but not single brief LPs, mimic the actions of complete photoperiods.

Keywords: *Drosophila*, locomotor activity, photoperiod, entrainment, phase relationship, precision.

Introduction

The light/dark (LD) cycle of the geophysical environment is one of the most effective time cues that entrain circadian clocks in a variety of organisms ranging from cyanobacteria to humans (Moore-Ede et al., 1982; Dunlap et al., 2003). It is believed that LD cycles entrain circadian clocks by phase resetting them daily by a magnitude that equals the difference between the periodicity of the LD cycles and those of the circadian clocks, τ, the non-parametric model of entrainment (Pittendrigh & Daan, 1976). Thus, according to this model, entrainment of circadian clocks under a 24 h LD cycle depends upon the light-induced phase response curve (PRC) and the τ of the circadian clocks; both PRC and τ are assumed to remain unchanged during the entrainment process (Pittendrigh & Daan, 1976; Daan, 2000; Sharma, 2003). Although the non-parametric model can explain certain aspects of entrainment in *Drosophila* and in mice, it fails to provide an explanation for several key observations such as: the lack of functional relationship between ψ and τ under certain experimental conditions; the occasional lack of entrainment; 'phase jumps' under extremely short and long skeleton photoperiods (PPs), which are never seen under complete photoperiods (PPc); complete compression of activity under longer PPc; and some results from studies on the entrainment of circadian rhythms in a few mammals under natural photoperiods (Pittendrigh & Daan, 1976; Daan, 2000; Sharma, 2003). Therefore, it appears that, though the non-parametric model provides the basic framework necessary for understanding photoentrainment mechanisms of circadian clocks in a wide range of organisms, it does not satisfactorily explain the finer details of entrainment, which probably are much more complex.

Burrowing animals do not experience the entire light phase of a LD cycle, but that does not make them any inferior in keeping time in their natural environment (Roenneberg & Foster, 1997; Daan, 2000; Sharma, 2003). Similarly, several diurnal animals do not expose themselves to the entire profile of LD cycles. It is believed that these animals use brief LPs during twilights, and therefore the entire light phase of LD cycle may be redundant for the photoentrainment of their circadian clocks. Several studies have been carried out to investigate whether brief LPs can mimic effects of complete LD cycles. For example skeleton photoperiods (PPs) created using brief LPs spaced about 12 h apart entrained the eclosion rhythm of fruit flies (Pittendrigh & Minis, 1964), activity rhythm of rodents (Pittendrigh & Daan, 1976; Sharma et al., 1997, 1998a), and sparrows (Binkley & Mosher, 1987). The PPs, however, failed to simulate effects of complete photoperiods which had a light phase much shorter or longer than 12 h.

In spite of such occasional failures, entrainment due to brief LPs have helped us understand a great deal about the photoentrainment mechanisms of circadian clocks (Daan, 2000; Sharma, 2003). However, such photoperiods can be said to mimic complete photoperiods only when some of the key features of entrainment — such as phase control, stability of different phases of the rhythm, and after-effects — match closely the results from complete photoperiods. Although brief LPs have been shown to mimic the entrainment abilities of complete photoperiods in terms of phase con-

trols, such studies have never been extended to investigate the finer aspects of entrainment, such as stability of the different phases of the rhythm and after-effects of entrainment.

An important assumption of the non-parametric model of entrainment is that τ is an invariant property of the circadian clocks. This is not true, however; the τ of circadian rhythms in a few species of insects and mammals are known to vary, often reflecting residual effects of previously experienced environmental conditions, usually referred to as 'after-effects' (Pittendrigh & Daan, 1976; Saunders, 1992). Mice assayed under a DD regimen, after having been exposed to LD cycles of either 20 h or 28 h in period length, continued to exhibit rhythmic locomotor activity for about 100 days with τs close to the periodicity of the LD cycles. In the fruit fly *D. melanogaster*, we reported previously that circadian clocks controlling the locomotor activity rhythm are developmentally plastic. Flies that were reared as pre-adults under a DD regimen had significantly shorter τ compared to those reared under LD or LL regimens (Sheeba et al., 2002). Such after-effects may prove to be advantageous to organisms living under periodic environments, as it might help them maintain a stable ψ, even when the periodic environmental cycles are perturbed due, especially, to cloud cover or behavioural fluctuations (Daan, 2000; Sharma, 2003). The τ of circadian rhythms therefore cannot be taken as an invariant property, as assumed in the tenets of the non-parametric model of entrainment, and it is possible that circadian clocks entrain to periodic LD cycles by changing their τ, either by lengthening or shortening it, in a phase-dependent manner — the parametric model of entrainment (Aschoff, 1963). Hence, just as the PRC is a prerequisite for entrainment in the non-parametric model, the period response curve (τRC), i.e. changes in τ as a function of phase of perturbations, is essential for entrainment in the parametric model. It is possible that circadian clocks use a number of mechanisms for photoentrainment, and thereby enhance their stability in ever-fluctuating environments, some of which include phase and period responses to periodic exposure to light as major components. Therefore, it is also important to estimate the after-effects of entrainment for any assessment of the effect of photoperiodic regimens on circadian clocks. Furthermore, to demonstrate unequivocally the entrainment ability of recurrent brief LPs, it would be necessary to analyze rigorously the pre-entrained, entrained and post-entrained states of circadian clocks under different photoperiodic regimens.

Here we report the results of our experiments on the locomotor activity rhythm of individual fruit flies *D. melanogaster* under four different light conditions: (i) 12:12 h LD cycles (PPc); and (ii) three photoperiodic regimens created using single brief LP (SLP-1 and 2) and two brief LPs presented 12 h apart (PPs), these regimens being aimed at evaluating how closely brief LPs mimic the effects of PPc. We have also compared the ψ and stability of two different phases of circadian rhythms (onset and offset) under PPc, SLP-1, SLP-2 and PPs. The results provide interesting insights into the photoentrainment mechanisms of circadian clocks in *D. melanogaster*.

Materials and Methods

The experimental flies ($n = 72$) were sampled from a large (~1200 breeding adults) outbred single population of *D. melanogaster,* reared at high egg density (~250–300 eggs per vial), on a 21-day discrete generation cycle. The flies were raised under laboratory LD (12:12 h) cycles where temperature and relative humidity were kept constant at 25 (±1°C) and 70 (±5%), respectively. Flies were maintained as pre-adults under LD cycles, and only those males that eclosed during the peak of eclosion were used in the experiments. The flies were then transferred into an activity-monitoring set-up within 24 h of their emergence, and their activity was monitored individually using infrared beams that detected the vertical movements of the flies in a narrow glass tube (0.6 cm inner diameter × 4 cm height) (Sharma, 2003). The light intensity used during the light phase of all four photoperiods was about 100 lux. Red light of wavelength >650 nm was used inside the cubicles for handling flies and other purposes. The locomotor activity of the flies of groups SLP-1, SLP-2, and PPs was first recorded in DD for about 10 days, then under periodic environments for the next 10 days, and finally after transfer into DD. The PPc was created using 12 hours of light and 12 h of darkness ('lights-on' at 08:00 h and 'lights-off' at 20:00 h). The three other photoperiods were created using single brief LPs of 15 min duration at 8:00 h (SLP-1), at 20:00 h (SLP-2) and at 8:00 h and 20:00 h (PPs).

The values of ψ for the onset of locomotor activity rhythm under all four photoperiodic regimens were estimated by calculating the mean time interval between the onsets of locomotor activity and the time of 'lights-on' under PPc (i.e. 8:00 h). Similarly, the ψ for the offset of locomotor activity was estimated by averaging the time intervals between the offsets of activity and the time of 'lights-off' under PPc (i.e. 20:00 h). The ψ values were taken to be positive if the activity onsets (as in the case of ψ onset) and activity offsets (as in case of ψ offset) preceded lights-on or lights-off, respectively, and were taken to be negative if they followed lights-on or lights-off. About 18 flies were used in each photoperiodic regimen.

Statistical analysis

The τ and the ψ of the locomotor activity rhythm was estimated using linear regression lines drawn through the onsets and offsets of locomotor activity. Entrainment of the locomotor activity rhythm was assumed to occur when the lines across both onset and offset of locomotor activity were indistinguishable from vertical. The τ was estimated using activity data for at least five consecutive cycles.

Analysis of variance (ANOVA) was carried out on the ψ of the onsets and the offsets of locomotor activity rhythm using STATISTICA™ software (STATISTICA, 1995) to test for any significant effect of the type of photoperiodic regimen. Post hoc multiple comparisons on the ψ values were done using Tukey's test. Similarly, precision of the onset and the offset of activity under the four different photoperiodic regimens were estimated by the calculating inverse of standard deviation of the daily onset and offset ψs (Sharma & Chandrashekaran, 1999).

The pre- and post- entrainment τ of the locomotor activity rhythm was also sub-jected to ANOVA to evaluate the after-effects of light regimens and post hoc com-parisons were done using Tukey's test.

Results

The locomotor activity rhythm of the flies entrained to all four photoperiodic regi-mens, displaying noticeable differences in ψ and its precision (Figs. 1–4). ANOVA on the ψ of the locomotor activity rhythm revealed a significant effect of photoperi-odic regimen ($F_{3,138} = 12.23$; $p < 0.0001$), and the phase reference point (i.e. onset or
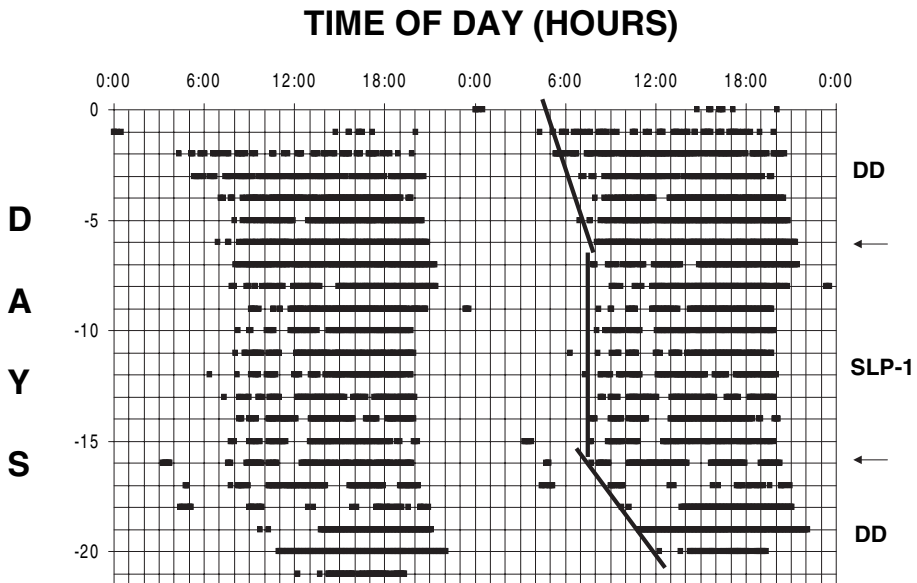
TIME OF DAY (HOURS)

Figure 1.   Locomotor activity record of one fly (out of a group of 15) maintained under con-tinuous darkness (DD). Repeated light pulses (LP) of 10 lux intensity and ~15 min duration were presented at 08:00 h for 10 days (SLP-1). Clock time is plotted along the abscissa and the number of days along the ordinate. Activity data were double-plotted to create a 48-h day length for easier visualization of activity patterns. Presence of dark bars represents activity and their absence, rest. Lines through the onsets of the activity are drawn to give a visual impres-sion of the approximate trend of locomotor activity rhythm during the pre-entrainment, entrain-ment and post-entrainment steady states. The pre- and post-entrainment τ of this fly were 24.30 h and 25.00 h, respectively. The values of ψ for the onset of locomotor activity rhythm under SLP-1 were estimated by calculating the mean time interval between the onsets of locomotor activity and the time of 'lights-on' under PPc (i.e. 8:00 h), and similarly the ψ for the offset of locomotor activity were estimated by averaging the time intervals between the offsets of activity and the time of 'lights-off' under PPc (i.e. 20:00 h). The ψ onset and offset of the activity rhythm during entrainment were +0.93 h and −0.04 h, respectively.
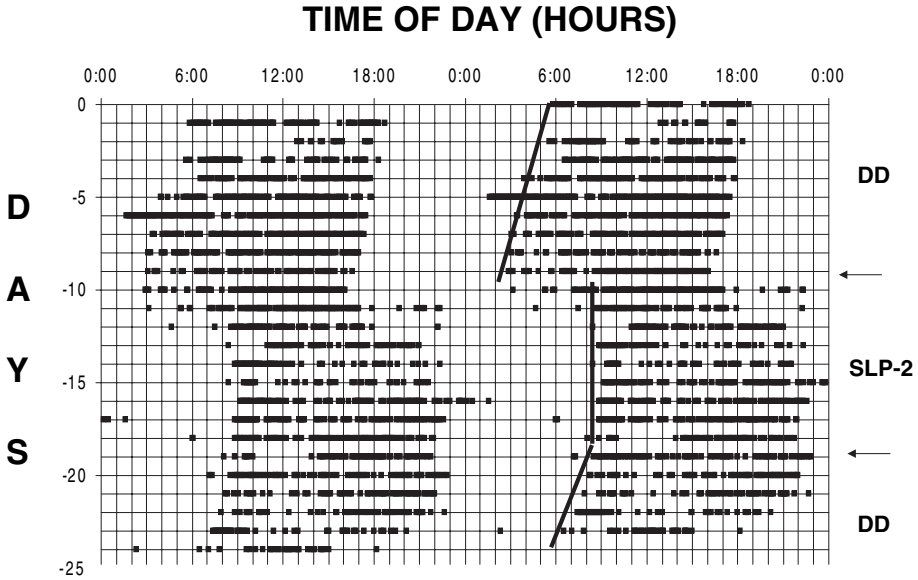
TIME OF DAY (HOURS)

Figure 2. Representative double-plotted actogram of one fly (from a group of 19) maintained in constant darkness. Repeated light pulses (LP) of 10 lux intensity and ~15 min duration were presented at 20:00 h for 10 days (SLP-2). The pre- and post-entrainment τ of this fly were 23.78 h and 23.57 h, respectively. The ψ onset and offset of activity rhythm during entrainment were −0.5 h and −1.35 h, respectively. Other details as in Fig.1.

offset of locomotor activity rhythm) ($F_{1,138} = 8.76$; $p < 0.005$). Post hoc multiple comparisons using Tukey's test showed that the ψ onset under PPc (+0.50 ± 0.11, mean ± SEM) and PPs (+0.35 ± 0.20) were significantly different ($p < 0.001$) from those under SLP-1 (+1.91 ± 0.66) and SLP-2 (+1.11 ± 0.42). The ψ onset under PPc did not differ significantly from those under PPs ($p > 0.05$). The ψ onset of locomotor activity rhythm of flies maintained under SLP-1 and SLP-2 regimens were significantly different ($p < 0.05$) from each other (Figs. 5a and 5b). Similarly, post hoc multiple comparisons showed that the ψ offset under PPc (−0.29 ± 0.09) and PPs (−0.34 ± 0.23) were significantly different ($p < 0.001$) from those under SLP-1 (+1.83 ± 0.41) and SLP-2 (−0.19 ± 0.47). The ψ offset under PPc did not differ significantly from those under PPs ($p > 0.05$). The ψ offset of flies maintained under SLP-1 and SLP-2 regimens were significantly different ($p < 0.05$) from each other (Figs. 5a and 5b).

ANOVA on the precision of ψ revealed a significant main effect of photoperiodic regimen ($F_{3,138} = 13.86$; $p < 0.0001$), but the precision of the locomotor activity rhythm did not differ significantly between the onset and offset of locomotor activity rhythm ($F_{1,138} = 0.40$; $p = 0.53$). Post hoc multiple comparisons showed that the precision of onset of locomotor activity under PPc (+5.07 ± 0.64) and PPs (+5.48 ± 1.27) were significantly greater ($p < 0.05$) than those under SLP-1 (+2.61 ± 0.23) and SLP-2 (+2.98 ± 0.67). The precision of onset under PPc and PPs did not differ significantly
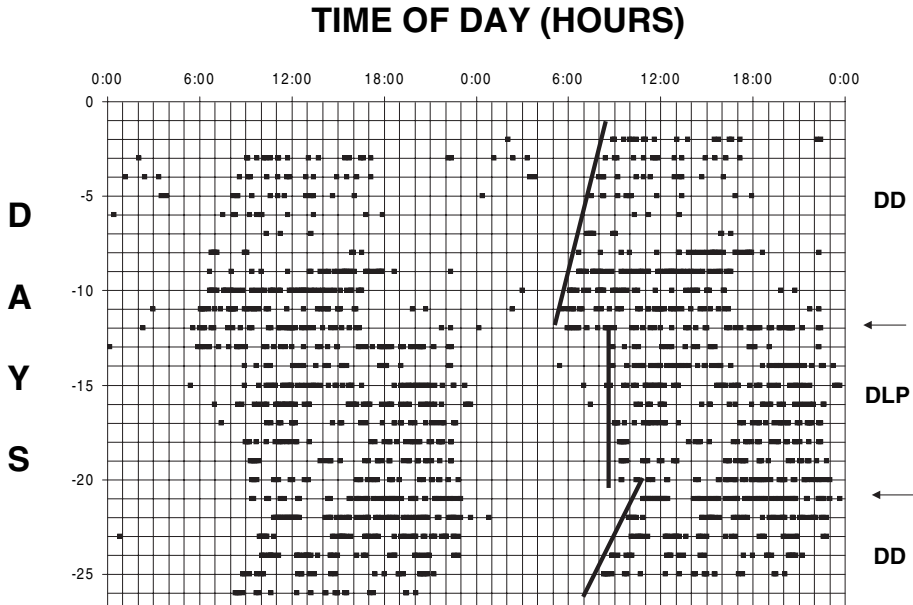
TIME OF DAY (HOURS)

Figure 3. Locomotor activity record of one fly (from a group of 15) maintained under constant darkness. This fly was administered two brief light pulses (LPs) at the beginning (08:00 h) and the end of activity (20:00 h) (PPs). The pre- and post- entrainment τ of the fly were 23.86 h and 23.19 h, respectively. The ψ onset and offset of activity rhythm during entrainment were −0.66 h and −2.64 h, respectively. Other details as in Fig.1.

($p > 0.05$) between each other (Figs. 6a and 6b). Similarly, post hoc multiple comparisons showed that the precision of offset under PPc (+5.45 ± 0.41) and PPs (+5.41 ± 0.82) were significantly greater ($p < 0.05$) than those under SLP-1 (+1.99 ± 0.21) and SLP-2 (+2.24 ± 0.28). The precision of offset under PPc and PPs did not differ significantly ($p > 0.05$) between each other (Figs. 6a and 6b).

ANOVA on the τ of the locomotor activity rhythm showed that the pre- and post-entrainment τs under SLP-1, SLP-2 and PPs did not differ significantly ($F_{1,85} = 0.51$; $p = 0.48$). ANOVA on the post-entrainment τ of the locomotor activity rhythm showed a significant effect of photoperiodic regimen ($F_{3,40} = 4.87$; $p < 0.005$). Post hoc multiple comparisons (Fig. 7) showed that the post-entrainment τ of the flies that experienced PPc (23.34 ± 0.19 h) was significantly ($p < 0.05$) shorter than those which experienced PPs (24.00 ± 0.22 h), SLP-1 (24.42 ± 0.15 h) or SLP-2 (23.91 ± 0.23 h).

Discussion

In the present study we show that the ψ and precision of the locomotor activity rhythm of fruit flies, *D. melanogaster*, observed under PPs did not differ significantly from those under PPc, but differed significantly from the ψ and its precision measured
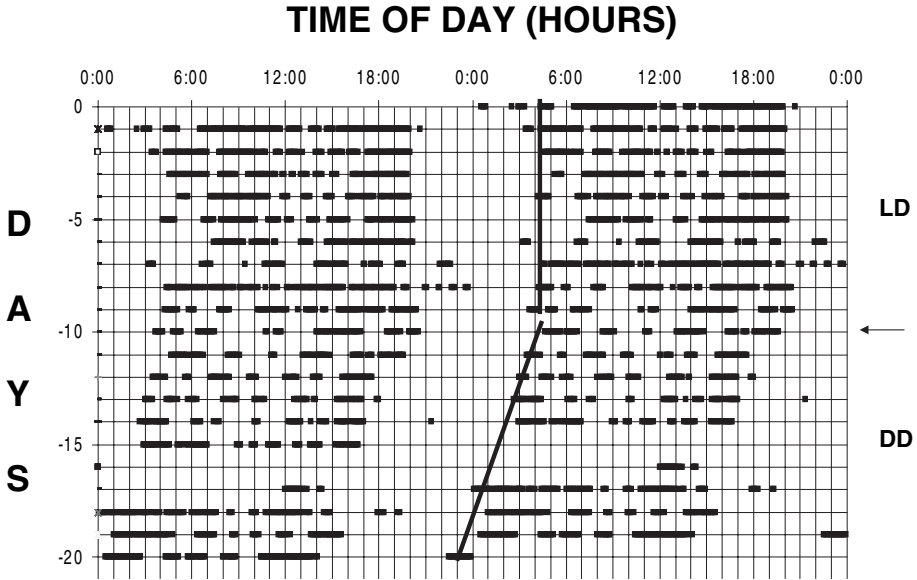
TIME OF DAY (HOURS)

Figure 4. Locomotor activity rhythm of one fly (from a group of 23) maintained under constant darkness. This fly was administered a single light pulses (LPs) of 12 h duration, with 'lights-on' at 08:00 h and 'lights-off' at 20:00 h (complete photoperiod: PPc). The post-entrainment τ of the fly was 23.29 h. The ψ of onset and offset of activity rhythm during entrainment were +3.52 h and −0.01 h, respectively. Other details as in Fig.1.
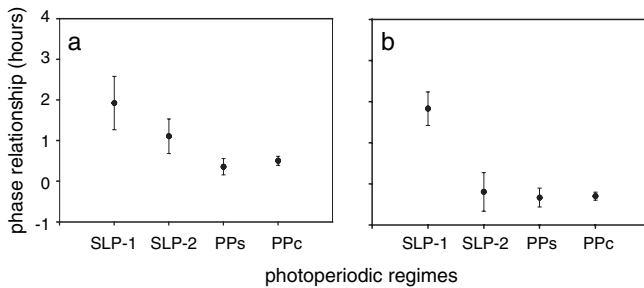

Figure 5. Mean (±SEM) phase-relationship (ψ) of the onset (a) and offset (b) of locomotor activity rhythm of flies relative to 'lights-on' (08:00 h) and 'lights-off' (20:00 h) of 12:12 h light-dark cycles. The phase relationships of onset and offset of locomotor activity rhythms under PPs and PPc were comparable.

under SLP-1 and SLP-2 conditions. These results suggest that PPs can successfully mimic the effects of 12:12 h LD cycles. Although single brief LPs can entrain the circadian locomotor activity rhythm of *D. melanogaster*, such entrainment is significantly different compared to those under PPc or PPs.
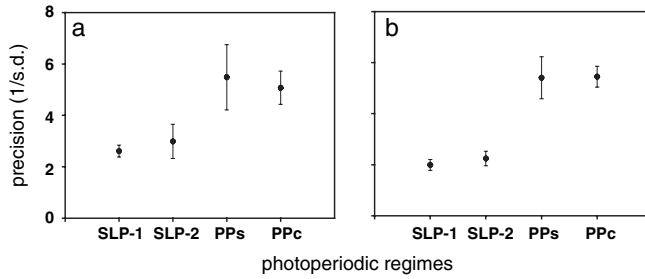
Figure 6. Average (±SEM) precision of the onset (a) and offset (b) of locomotor activity rhythm. Precision of the onset and onset of locomotor activity rhythm was calculated as inverse of the day-to-day standard deviation of the onset and the offset of activity during entrainment. The precision of the onset and the offset of locomotor activity rhythms under PPs and PPc were comparable.
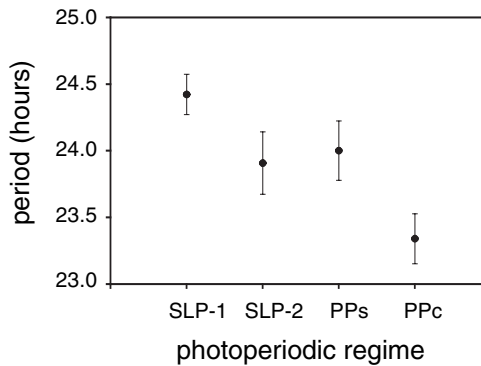

Figure 7. Average (±SEM) post-entrainment free-running period (τ) of the locomotor activity rhythm. The post-entrainment τ of the locomotor activity rhythm of those flies that were kept under PPc was significantly shorter than those under SLP-1, SLP-2, and PPs.

The results of our experiments also indicate that only those flies which experienced PPc showed after-effects of entrainment, as reflected in the post-entrainment value of τ. This suggests that the PPc regimen causes changes in τ besides entraining circadian clocks, exerting both phase and period control. This dual action ensures the stability of the phase of circadian rhythms under fluctuating LD cycles.

Entrainment of circadian rhythms by brief LPs, PPs and PPc has been reported to be comparable in several species of insects and mammals, which implies that it is possible to mimic the effects of PPcs by presenting one or two LPs at the beginning and the end of the light phase of LD cycles (Pittendrigh & Daan, 1976; Pittendrigh, 1981; Saunders, 1992; Sharma, 2003). For example, the circadian locomotor activity rhythm of two species of rodents, *Mesocricetus auratus* and *Peromyscus leucopus,* entrained to a single brief LP of 15 min or 60 min duration, and the ψ followed a

relationship with the τ and the T as predicted by the non-parametric model of entrainment (Pittendrigh & Daan, 1976). When two brief LPs were used per cycle, entrainment was more stable and the animals confined their activity to the longer of the two dark intervals created by the two pulses. Further, the ψ of the locomotor activity rhythm under PPs matched closely that obtained under PPc (Pittendrigh & Daan, 1976). This is similar to the findings in the nocturnal field mouse, *M. booduga*, where the ψ of the locomotor activity rhythm under PPs matched closely that observed under PPc (Sharma et al., 1997). The PPs entrained the locomotor activity rhythm of mice with a τ close to 24 h (Sharma et al., 1997); mice with τs far removed from 24 h did not entrain stably to photoperiods longer than 6 h, but exhibited partial entrainment or oscillatory 'free-run' — relative coordination (Sharma et al., 1998a).

When repeated brief LPs were used instead of PPs, the observed values of ψ showed a sigmoid-shaped relationship with τ. This is predicted by the non-parametric model of entrainment, based on the τ and the light-PRC using LPs of comparable intensity and duration (Sharma & Chidambaram, 2002). The functional nature of the relationship between ψ and τ did not change significantly with increasing light intensity (Sharma & Chidambaram, 2002).

In the fruit fly, *D. pseudoobscura*, PPs entrained the eclosion rhythm, and the ψ of the eclosion rhythm was similar to those seen under PPc (Pittendrigh & Minis, 1964). In this study, the ψ, measured as the time interval between peak of eclosion and the LP, remained stable during the entire duration of entrainment, implying that PPs can simulate the actions of PPc in *D. pseudoobscura*. The simulation of PPc by PPs was almost exact when the intervals between the two LPs were less than 11 h, became less exact when the photoperiod increased to 12 h, and failed completely when photoperiods were longer than 14 h (Pittendrigh, 1966). These results suggest that it is the 'on' and 'off' signals of the photoperiod that are important for entrainment and not the duration of light between such signals. The PPs differ from PPc at least in one respect; under PPs, animals can assume any one of the two dark intervals as subjective night, an ambiguity that does not exist under PPc.

A stable and appropriate ψ would provide an adaptive advantage to organisms living in periodic environments, by enabling them to become efficient foragers, by helping them escape predators, and by enabling them to find suitable mates (Dunlap et al., 2003; Daan, 2000; Sharma, 2003). The ψ of a circadian rhythm is an important parameter of the circadian clocks. It not only describes the temporal niche adopted by any organism but also reflects some key functional aspect of circadian clocks under periodic environments (Sharma, 2003).

Although the results of our experiments clearly demonstrate that PPs, but not brief LPs, mimic the actions of PPc, extensive studies on the effects of natural as well as artificial photoperiods on animals from different ecological conditions are required to decipher the intricate details of the photoentrainment mechanisms. In one of our previous studies on the nocturnal field mice *M. booduga,* we reported that the entrainment of the circadian locomotor activity rhythm by artificial laboratory LD cycles was different from that found under semi-natural LD cycles (Sharma et al., 1998b). The ψ of the locomotor activity rhythm of animals kept under semi-natural LD cycles was negatively correlated with τ, whereas the ψ and τ of the animals maintained under

artificial laboratory LD cycles did not show any significant correlation. Given that most of the studies on circadian rhythms are done under artificial LD cycles with abrupt transitions between light to darkness and darkness to light, it is essential to address whether laboratory LD cycles mimic the effects of the natural LD cycle.

Acknowledgements

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