Twilights Widen the Range of Photic Entrainment in Hamsters

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Abstract The range of entrainment of the circadian rhythm of locomotor activity was compared in four groups of Syrian hamsters (eight animals per group) initially exposed to daily light-dark (LD) cycles with either abrupt transitions between light and darkness (LD-rectangular) or simulated twilights (LD-twilight). Lighting was provided by arrays of white light-emitting diodes; daytime illuminance (10 lux) and the total amount of light emitted per day were the same in the two conditions. The period (T) of the LD cycles was then gradually increased to 26.5 h or gradually decreased to 21.5 h, at the rate of 5 min/day. Under LD-rectangular, the upper and lower limits of entrainment were 25.0 to 25.5 h and 22.0 to 22.5 h, respectively, whereas under LD-twilight, 50% of the animals exposed to the lengthening cycles were still entrained at T = 26.5 h and 50% of those exposed to the shortening cycles were still entrained at T = 21.5 h. In a second experiment, two groups of hamsters were exposed to fixed T = 25 h LD-rectangular (*n* = 15) or LD-twilight cycles (*n* = 7). Only 33% of the animals entrained in LD-rectangular, whereas 86% of the animals entrained in LD-twilight. Free-running periods in constant darkness were longer following successful entrainment to T = 25 h but did not differ between the animals that entrained to LD-rectangular and those that entrained to LD-twilight. The widening of the range of entrainment observed in LD-twilight indicates that twilight transitions increase the strength of the LD zeitgeber. In LD-twilight, successful entrainment to T = 26.5 h was accompanied by an expansion of activity time to 16.52 ± 1.22 h, with activity onsets preceding mid-dusk by 12.56 ± 2.15 h. Together with earlier data showing similar phase response curves for hour-long dawn, dusk, and rectangular light pulses, these results suggest that the effect of twilights on the range of entrainment may involve parametric rather than nonparametric mechanisms.

Key words circadian rhythms, limits of entrainment, twilight, parametric entrainment, hamsters

Twilights are an integral component of the natural daily illumination cycle, providing salient temporal cues for the entrainment of circadian rhythms, yet their contribution to photic entrainment remains uncertain (Boulos et al., 1996a). We have been investigating the effects of dawn and dusk transitions on circadian entrainment in nocturnal and diurnal mammals using twilight simulation technology that

accurately reproduces natural illuminance patterns at any latitude and at any time of the year (Terman et al., 1990, 1991; Boulos et al., 1996a, 1996b).

One such study compared the upper limits of entrainment in Syrian hamsters initially maintained under daily light-dark (LD) cycles with either abrupt (LD-rectangular) or gradual (LD-twilight) transitions, the latter simulating natural dawn and dusk at the summer solstice at 41 °N latitude, but with maximum light intensity set at 10 lux (Boulos et al., 1996c). When the period (T) of the LD cycle was gradually increased from T = 24 h to T = 26 h, all animals in the LD-twilight group remained entrained to the lengthening LD cycle whereas fewer than half of the animals did so in the LD-rectangular group. These results indicated that the inclusion of twilights raises the upper limit of entrainment in hamsters and, thus, appears to increase the strength of the LD zeitgeber. However, the lower limit of entrainment was not examined in that study, and the possibility remained that twilights selectively increase the phase-delaying effects of the LD cycle, or decrease its phase-advancing effects. If so, twilight transitions would only promote entrainment to lengthening LD cycles and could hinder entrainment to shortening cycles.

Another limitation of our earlier study is that the changes in light intensity were produced by varying the voltage to an incandescent lamp, which necessarily entailed a shift toward the red end of the light spectrum as light intensity was decreased at dusk, with the opposite effect occurring at dawn. Changes in spectral distribution occur during natural twilights as well, but these consist of an increase in the relative amounts of both red and blue light and a relative decrease in the yellow to orange range (Rozenberg, 1966; McFarland and Munz, 1975).

The aim of the present study was to examine the effects of simulated twilights on both the upper and lower limits of entrainment using arrays of bright-white light-emitting diodes (LEDs) as light sources to avoid potential confounding effects of spectral shifts. As in our earlier study (Boulos et al., 1996c), the limits of entrainment were studied under LD cycles with gradually changing periods, a procedure previously used in hamsters by Bruce (1960) and in humans by Wever (1983). A second experiment with fixed 25 h LD cycles was also performed in order to compare the aftereffects of entrainment to LD cycles with and without twilight transitions on circadian period.

MATERIALS AND METHODS

Animals and Maintenance

Adult male Syrian hamsters (*Mesocricetus auratus*) (LAK:LVG strain, 90 to 100 g, Charles River Laboratories, Kingston, NY) were individually housed in open-topped compartments (24 × 24 × 30 cm) with black Plexiglas walls and wire mesh floors. The compartments were equipped with running wheels (17 cm diameter). Four compartments were enclosed in each of four ventilated isolation chambers. Temperature in the chambers was maintained at 21 °C to 25 °C, and white noise was played continuously in the experimental room to mask extraneous sounds. Food (LabDiet 5P00) and water were continuously available. Litter was changed weekly, during the light portion of the LD cycles or with the aid of an infrared light source and viewer (FJW Optical Systems, Palatine, IL) when the animals were in constant darkness (DD).

Light Delivery System

Lighting was provided by an array of 20 white LEDs (Type NSPW500BS, Nichia America Corporation, Mountville, PA) mounted on the ceiling of each isolation chamber. The spectral distribution of the light emitted by the LEDs is characterized by a sharp peak at 460 nm in the blue region of the spectrum and a broader and shallower peak at 555 nm in the yellow-green region. The LD cycles were controlled by a twilight simulation system (Naturalistic Illuminator, Medic-Light Inc., Lake Hopatcong, NJ), which was modified for use with LEDs instead of incandescent lamps and equipped with MacLite software (Research Foundation for Mental Hygiene Inc., New York, NY). The software includes a predictive algorithm that specifies the expected momentary illuminance on the earth's surface across the 24-h day on any day of the year and at any latitude. Following lamp calibration, the system adjusts the current delivered to the LED array to produce naturalistic illumination patterns. The term "naturalistic" is used here to indicate that the simulation accurately reproduces the rate of change of illuminance observed during natural twilights up to a user-selected daytime maximum (Terman et al., 1989). Many other studies have examined the effects of artificial twilights (for references, see Boulos et al. 1996a),

but, with rare exceptions (e.g., Kavaliers and Ross, 1981), the rate of change of light intensity used in these studies was arbitrary. Illuminance and irradiance were measured using a Tektronics J16 photometer/radiometer with a cosine-corrected J6511 illuminance probe and a J6512 irradiance probe placed on the cage floor.

Procedure

On arrival in the laboratory, the hamsters were transferred to the isolation chambers and kept under 24 h LD cycles with abrupt transitions between 0 and 10 lux (LD-rectangular) or with artificial twilights simulating summer solstice conditions at a latitude of 41 °N, but with maximum illuminance set at 10 lux (LD-twilight). The 10-lux maximum (which, for white LED light, corresponds to an irradiance of 3.1 μW/cm^2) was chosen to minimize any masking effects of light on locomotor activity, and because preliminary studies by Terman et al. (1990) had shown that rats housed under LD-twilight cycles with a 1000-lux maximum but allowed access to a dark artificial burrow generally avoided exposure to intensity levels higher than about 1 lux.

With the seasonal and latitudinal parameters described above, the day portion of the cycle (10 lux) lasted 962 min (16.03 h), the night portion, defined as the time when light intensity was below 0.001 lux, lasted 304 min (5.07 h), and dawn and dusk (0.001 to 10 lux) each lasted 87 min. The criterion illuminance level of 0.001 lux ($\approx$ 0.0003 μW/cm^2) used to distinguish night from twilight is arbitrary, but was chosen because it is near the lower limit of astronomical twilight and is safely below the threshold for light-induced phase shifts in Syrian hamsters (see Boulos et al., 1996a). In LD-rectangular, the light and dark portions of the cycle lasted 976 min (16.27 h) and 464 min (7.73 h), respectively. These durations equated the total amount of light (in lux min) emitted per day in the two conditions. In LD-rectangular, the lights came on at 0:19 (EST) and went off at 16:35, whereas in LD-twilight, the lights were at 10 lux from 0:26 until 16:28.

Experiment 1: Changing Cycles

A total of 32 hamsters were exposed to the 24 h LD cycle for 24 days. The period of the LD cycle then increased by 5 min/day, from T = 24 h to T = 26.5 h, or

decreased by 5 min/day, from T = 24 h to T = 21.5 h, by proportionate lengthening or shortening of the day, night, and twilight portions of the cycle. The 5 min/day rate of change of the zeitgeber period was chosen because it allows fairly precise estimates of the limits of entrainment in a relatively short period of time (see Wever, 1983). The hamsters were then kept in DD for 11 to 12 days. Sixteen hamsters were studied in each condition, with 8 animals exposed to LD-rectangular and 8 to LD-twilight cycles.

Experiment 2: Fixed T = 25 h Cycles

A total of 24 hamsters were initially exposed to the 24 h LD cycle for 24 days, with 16 hamsters kept under LD-rectangular and 8 under LD-twilight cycles. Over the next 4 days, the period of the LD cycle increased by 15 min per day, from T = 24 h to T = 25 h, then remained fixed at T = 25 h. Due to an unforeseen characteristic of the software, however, the first cycle at the fixed T = 25 h period was advanced by about 1.6 h relative to the preceding cycle, in both LD-rectangular and LD-twilight conditions. After 24 days at T = 25 h, the hamsters were kept in DD for 11 days. One hamster in LD-twilight died during the course of the study, and 1 hamster in LD-rectangular showed a large phase advance 2 days before the end of the 24 h LD condition, apparently in response to cage cleaning. The data from these 2 animals were excluded from all analyses.

Data Collection and Analysis

Wheel-running activity was recorded at 5-min intervals on a personal computer equipped with DataCol 3 hardware and software (Mini-Mitter Company, Bend, OR). Data analysis was performed on a Macintosh computer using Circadia software (Behavioral Cybernetics, Cambridge, MA). Daily wheel-running activity onsets were determined by setting a threshold number of 100 wheel revolutions (except in a few animals with low activity levels where a lower threshold was used) preceded by 6 h of below-threshold activity (ignoring activity bouts associated with cage cleaning). A threshold of five revolutions followed by 6 h of below-threshold activity was used to determine activity offsets. The interval from activity onset to activity offset represented activity time, or alpha. Phase angle differences were calculated between activity onset time and the middle of the dark

portion of the LD cycle (which occurred at the same clock time in LD-rectangular and LD-twilight) and between activity onset time and the time of lights-off or the middle of the dusk twilight, the latter corresponding to a light intensity of 0.018 lux ($\approx 0.0056 \, \mu W /$ cm^2). Group differences were assessed by two-tailed t tests, unless the assumption of homogeneity of variance was violated, as determined by an F test, in which case the Wilcoxon signed rank test was used instead. The level of significance for all tests was set at $p = 0.05$. Entrainment to the fixed 25 h LD cycles was assessed by the periodogram analysis procedure of Dörrscheidt and Beck (1975), which is included in the Circadia software, using a resolution of 5 min. Unless otherwise indicated, data are reported as group means ± SD.

RESULTS

Experiment 1: Lengthening Cycles

Figures 1A through 1C show the wheel-running activity records of three hamsters exposed to lengthening cycles, one in LD-rectangular (Fig. 1A) and two in LD-twilight (Figs. 1B, 1C). The phase angle difference between activity onset time and mid-dark, averaged across the last 5 days under T = 24 h, was slightly more positive in LD-twilight (4.60 ± 0.55 h) than in LD-rectangular (4.22 ± 0.77 h), but the difference was not statistically significant ($t = 1.17$, $p = 0.26$).

Inspection of the activity records indicates that none of the animals in the LD-rectangular group remained entrained to the lengthening LD cycle. The record in Figure 1A, for example, shows that the animal's activity rhythm started to free-run when T reached 25 h, as indicated by the abrupt change in the period of the rhythm observed on that day. The upper limit of entrainment was not always as sharply defined as in that example, but fell roughly between 25.0 and 25.5 h in all cases. In contrast, four of the animals in the LD-twilight group remained entrained throughout the lengthening protocol, with activity onsets occurring progressively earlier relative to dusk as the period of the LD cycle increased from 24 to 26.5 h. In the example shown in Figure 1B, activity onset on the last 5 days under T = 24 h preceded the middle of the dusk twilight by 0.88 h, whereas on the last day of the lengthening cycle, at T = 26.5 h, activity onset preceded mid-dusk by 11.06 h, which represents a net change of +10.18 h in the phase angle of entrainment. One animal appeared to lose entrainment a few days before

the end of the lengthening protocol, at about T = 26.25 h (Fig. 1C), whereas the records of the remaining three animals were indistinguishable from those of the LD-rectangular group.

A more quantitative analysis of the efficacy of the two LD cycles was performed, comparing the total phase displacement resulting from exposure to the lengthening cycle protocol. Total phase displacement was defined as the difference between the mean activity onset time on the last 5 days under T = 24 h and the onset time on the first full day of DD (visual inspection of the activity records allowed unambiguous distinction between a phase displacement of x h and one of $x \pm 24$ h). The results, summarized in Figure 2, show mean phase displacements of –7.01 h (range: +2.00 to –11.93 h) in the LD-rectangular group and –19.70 h (range: –5.93 to –35.75 h) in the LD-twilight group (positive and negative values represent phase advances and phase delays, respectively). The variance was significantly greater in LD-twilight than in LD-rectangular ($F = 0.152$, $p < 0.05$), and the Wilcoxon test was therefore used to compare phase displacement in the two groups; this showed a significantly larger phase delay in LD-twilight than in LD-rectangular ($p < 0.05$). As expected, the four hamsters that remained entrained to the lengthening LD cycle showed larger phase delays than any of the other hamsters in either group.

Free-running periods following termination of the lengthening LD cycles were calculated by fitting a regression line to 10 consecutive activity onsets, beginning on the second day of DD. Free-running periods were longer in the LD-twilight group than in the LD-rectangular group (24.61 ± 0.16 h vs. 24.13 ± 0.03 h, $p < 0.01$) and were highly correlated with total phase displacement ($r = -0.947$, $p < 0.0001$). The longest free-running periods were shown by the four animals that remained entrained to the lengthening LD-twilight cycle (24.95 ± 0.06 h). There was no significant correlation between activity onset time under T = 24 h and total phase displacement ($r = 0.129$, $p = 0.63$).

Experiment 1: Shortening Cycles

The records of three hamsters exposed to shortening LD cycles are shown in Figures 1D through 1F. Under T = 24 h, the phase angle difference between activity onset time and mid-dark was more positive in LD-twilight (4.52 ± 0.33 h) than in LD-rectangular (3.88 ± 0.48 h), and the difference was significant ($t = 2.30$, $p < 0.05$).
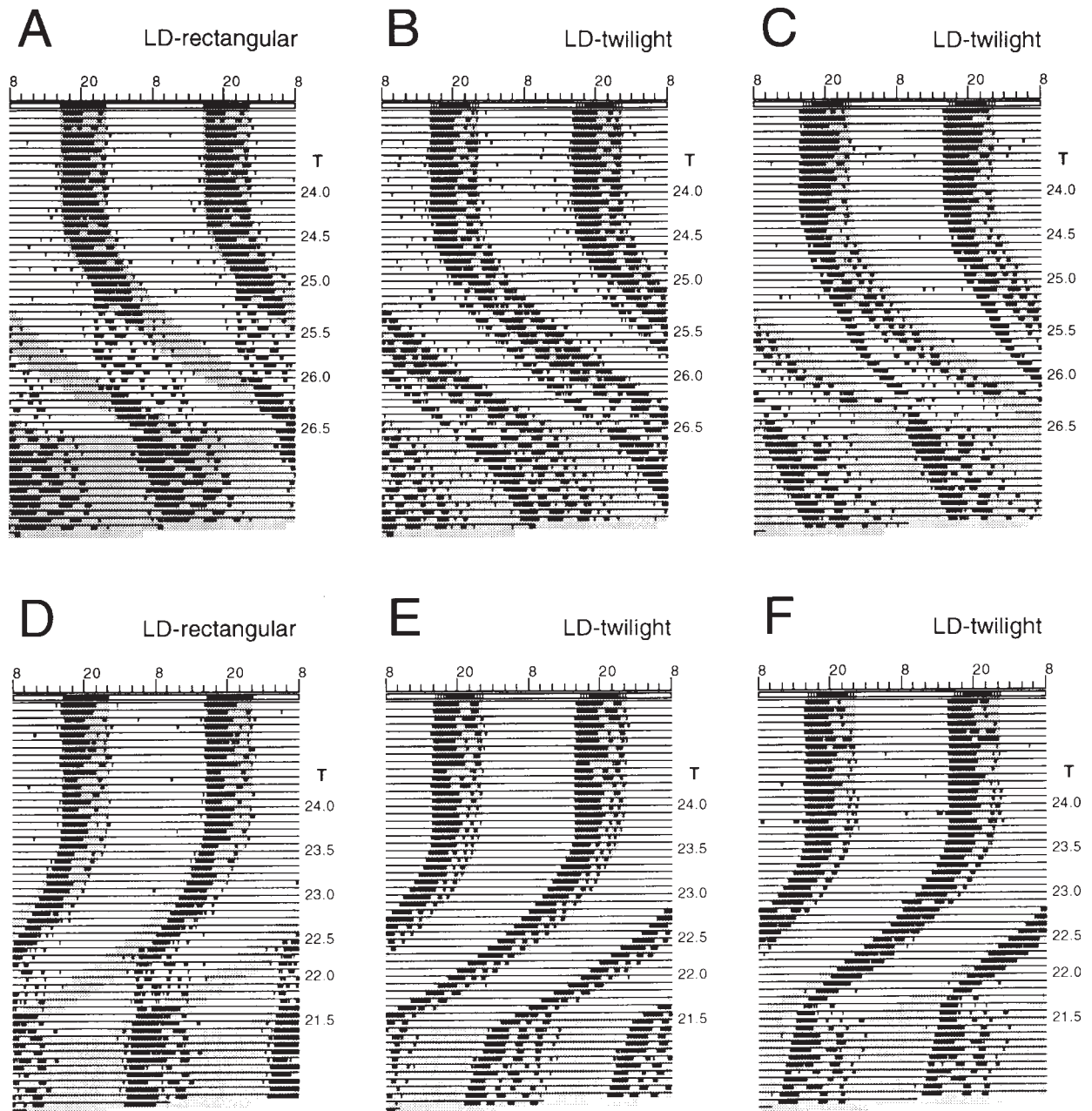
Figure 1. Wheel-running activity records of six hamsters exposed to (A-C) lengthening or (D-F) shortening light-dark (LD) cycles. The records show all 5-min intervals with 10 or more wheel revolutions. The numbers to the left of each record indicate the period (T) of the LD cycle on particular days, starting with the last day under T = 24 h. Horizontal bars above each record depict the initial 24 h LD cycles, with the day (10 lux), night (< 0.001 lux), and twilight (0.001 to 10 lux) portions of the cycles represented by white, black, and striped segments, respectively. Shaded areas of the records indicate times when light intensity was below 10 lux (for LD-twilight cycles, this includes the dawn and dusk transitions). The final 11 to 12 days were spent in constant darkness.

None of the animals in the LD-rectangular group remained entrained to the shortening cycles. Their activity records suggested that, in all cases, entrainment was lost at LD cycle periods ranging from 22.5 to 22.0 h (Fig. 1D). In the LD-twilight group, four hamsters remained entrained throughout the shortening protocol, with progressively later activity onsets relative to dusk. In the example shown in Figure 1E, the phase angle of entrainment (relative to mid-dusk) changed from +1.35 h at T = 24 h to –0.11 h at T = 21.5 h, for a net change of –1.46 h. One hamster appeared to lose entrainment at about T = 21.75 h (Fig. 1F), whereas

the records of the remaining three hamsters were similar to those of the LD-rectangular animals.

Total phase displacements across the shortening cycles are shown in Figure 2. As in the lengthening cycle protocol, the variance was greater in LD-twilight than in LD-rectangular ($F = 0.027$, $p = 0.0001$). The former group showed a mean phase advance of 27.16 h (range: +11.48 to +37.98 h) and the latter of 12.77 h (range: +9.79 to +15.75 h); this difference was significant (Wilcoxon, $p < 0.05$). The largest phase advances were shown by the four hamsters that remained entrained to the shortening LD cycle.

Free-running periods in DD were also shorter following exposure to LD-twilight than to LD-rectangular (23.78 ± 0.14 h vs. 23.96 ± 0.07 h, $p < 0.005$) and were significantly correlated with phase displacement ($r = -0.810$, $p = 0.0001$). The mean period for the four LD-twilight animals that remained entrained to the shortening cycle was 23.71 ± 0.11 h. No significant correlation was found between activity onset time under the 24 h LD cycles and phase displacement ($r = 0.438$, $p = 0.09$).

Experiment 2: Fixed T = 25 h Cycles

Figure 3 shows activity records of six hamsters exposed to fixed T = 25 h cycles, four in LD-rectangular (Figs. 3A-3D) and two in LD-twilight (Figs. 3E, 3F). Under T = 24 h, the phase angle difference between activity onset time and mid-dark did not differ significantly between LD-twilight and LD-rectangular (4.52 ± 0.23 h and 4.35 ± 0.77 h, respectively, $p > 0.05$).

Visual inspection of the activity records indicated that 6 out of 7 (86%) hamsters in LD-twilight entrained to the 25 h LD cycle whereas only 5 out of 15 (33%) hamsters did so in LD-rectangular. Periodogram analysis performed on the last 10 days under T = 25 h confirmed this assessment, showing individual periods for these animals ranging between 24.83 and 25.17 h. Examples of successful entrainment are shown in Figure 3A (LD-rectangular) and Figures 3E and 3F (LD-twilight). None of the remaining animals appeared entrained (Figs. 3B-3D), and all but 1 had periods of 24.58 h or less. The one exception had a period of 24.83 h, but the activity record (Fig. 3C) shows that this animal's rhythm was free-running at a period clearly shorter than 25 h, which continued virtually unchanged in DD. The 24.83 h periodogram value appears to reflect in part the masking component elicited by the onset of darkness. Two LD-rectangular hamsters seemed to be approaching entrainment at the end of
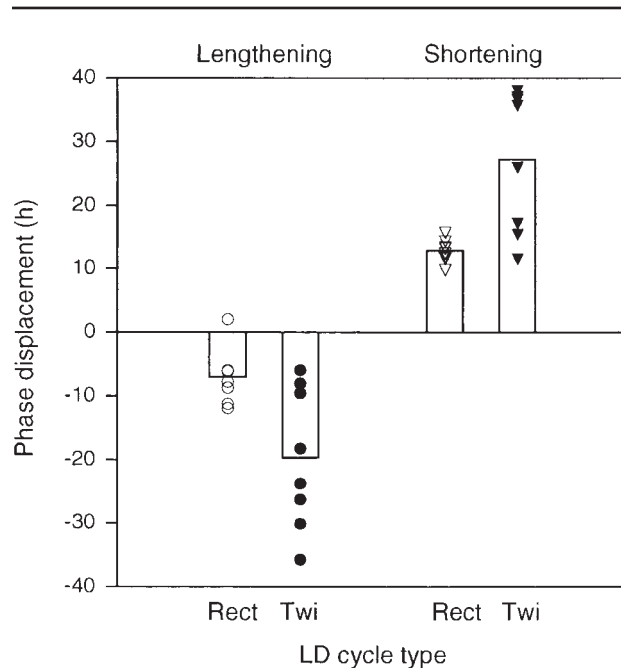

Figure 2. Total phase displacement resulting from exposure to the lengthening and shortening light-dark-(LD)-rectangular (Rect) and LD-twilight (Twi) cycles. Positive and negative values represent phase advances and phase delays, respectively. Histogram bars represent group means. Under both protocols, total phase displacement was significantly greater in LD-twilight than in LD-rectangular ($p < 0.05$), indicating more successful entrainment.

the T = 25 h condition, both with an extreme positive phase angle (the record of one of these animals is shown in Fig. 3D). However, periodogram analysis limited to the last few days of the LD cycle still showed periods of 24.50 and 24.75 h, respectively.

The six LD-twilight and five LD-rectangular hamsters that entrained to T = 25 h did not differ significantly in alpha (12.96 ± 0.76 h vs. 12.09 ± 2.43 h, $p > 0.05$), in phase angle difference relative to mid-dark (10.45 ± 2.41 h vs. 9.73 ± 2.85 h, $p > 0.05$), or in phase angle difference relative to mid-dusk or lights-off (7.09 ± 2.41 h vs. 5.70 ± 2.85 h, $p > 0.05$).

Free-running periods in DD were longer for animals that entrained to T = 25 h than for those that did not (24.62 ± 0.09 h vs. 24.27 ± 0.15 h, $p < 0.0001$) but did not differ significantly between animals that entrained to LD-twilight and those that entrained to LD-rectangular (24.59 ± 0.05 h vs. 24.65 h, $p > 0.05$).

DISCUSSION

The results of experiment 1 indicate that the inclusion of twilights widens the range of entrainment by

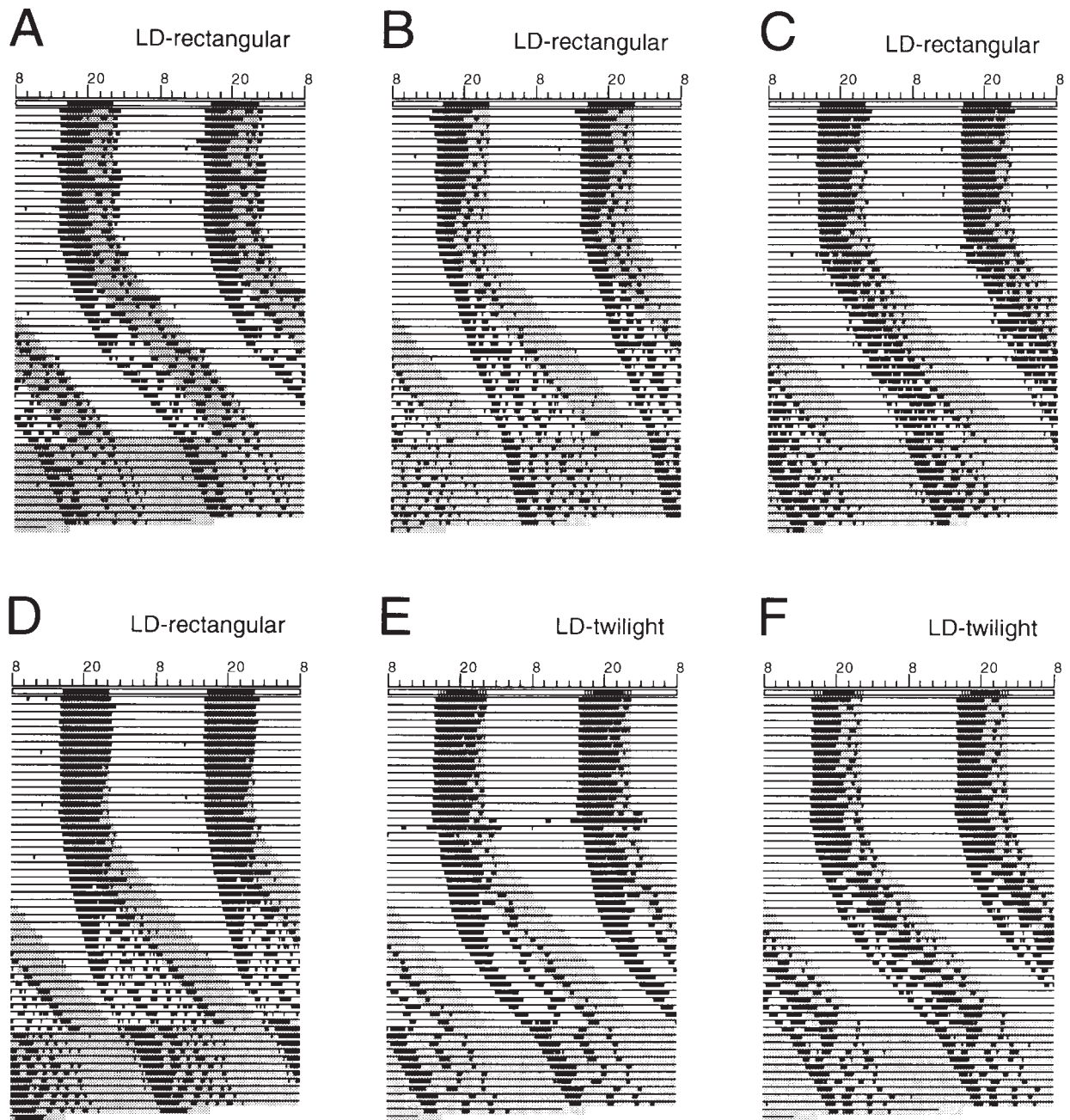**Figure 3. Wheel-running activity records of six hamsters exposed to T = 25 h (A-D) light-dark-rectangular (LD-rectangular) or (E, F) LD-twilight cycles. The period of the LD cycle was increased from 24 to 25 h over 4 days, then remained fixed at 25 h. The first cycle at T = 25 h was advanced by about 1.6 h relative to the preceding cycle. The initial 24-h LD cycles are depicted by horizontal bars above each record, as described in Figure 1.**

raising the upper limit of entrainment as well as by lowering the lower limit: under rectangular LD cycles, entrainment was restricted to period lengths ranging from 22.0 to 22.5 h to 25.0 to 25.5 h, but when the LD cycles included simulated twilights, 50% of the hamsters in the shortening protocol were still entrained at

T = 21.5 h and 50% of those in the lengthening protocol were still entrained at T = 26.5 h. The activity rhythms of these animals in DD started to free-run from the phase held during the preceding LD-twilight conditions (cf. Figs. 1B, 1E), indicating that activity onsets under the lengthening and shortening protocols

reflected the phase of entrainment of the endogenous rhythms rather than any masking effects of light and darkness. Furthermore, under both protocols, the mean phase displacement resulting from exposure to the changing LD cycles was greater in LD-twilight than in LD-rectangular, and the aftereffects of such exposure on free-running periods in DD were more pronounced. These observations represent additional evidence that entrainment was more successful under LD-twilight than under LD-rectangular cycles. The fact that the light sources consisted of LED arrays implies that this effect of twilights is attributable to the gradual changes in light intensity alone, since dimming of LEDs does not alter their spectral characteristics. Whether or not the spectral shifts that accompany natural twilights also affect entrainment remains to be determined.

The range of entrainment of circadian rhythms by a zeitgeber reflects the strength of that zeitgeber. In the case of LD cycles, zeitgeber strength depends on the relative intensities and durations of the light and dark portions of the cycle (Aschoff, 1978; Aschoff and Pohl, 1978). In the present study, maximum light intensity levels were the same under the two LD cycles, as was the total amount of light emitted per cycle. Thus, the wider range of entrainment observed in the LD-twilight groups indicates that twilight transitions increase the strength of the LD zeitgeber.

An increase in the strength of the LD cycle with increasing twilight duration was predicted by Wever (1965), based on a mathematical model of circadian oscillations according to which the effect of a light cycle on the circadian oscillation depends on light intensity levels at different phases of the cycle as well as on the first and second time derivatives of light intensity. Because the time derivatives differ from zero only when light intensity is changing, the model predicts that gradual light transitions will increase the influence of the LD cycle on the circadian oscillation. Wever did not test this prediction directly by examining the effect of twilights on the range of entrainment. Instead, he tested a related prediction, namely, that the phase angle of entrainment will be more positive (or less negative) the longer the duration of twilight. This prediction was confirmed in laboratory studies in diurnal birds and in the nocturnal hamster (Aschoff and Wever, 1965; Wever, 1967). A test of this prediction was also performed under natural illumination by comparing the phase angle of entrainment at two different latitudes around the equinoxes, when photoperiod duration is the same but twilights are longer at the higher latitude (Daan and Aschoff, 1975). However, the predicted increase in the phase angle of entrainment at the higher latitude was not confirmed in any of the three species of birds studied, whereas in hamsters this effect was observed at the spring but not the fall equinox.

Our own data on this issue also have been inconsistent: in the present study, activity onset times under T = 24 h were slightly earlier in LD-twilight than in LD-rectangular, and the phase angle difference between activity onset and the midpoint of the dark segment was therefore more positive. In our earlier study, however, activity onset times occurred later in LD-twilight than in LD-rectangular, even though entrainment to the lengthening LD cycles was more successful in the presence of twilights (Boulos et al., 1996c). Furthermore, there was no significant correlation between activity onset times under T = 24 h and total phase displacement in either study. These results suggest that the phase angle of entrainment under daily LD cycles is not a reliable index of the strength of the LD zeitgeber.

The mechanism underlying the effect of twilights on the range of entrainment is still unknown. Photic entrainment of circadian rhythms is generally attributed to two theoretically distinct processes: nonparametric and parametric (Daan, 1977). In the nonparametric model, entrainment is accounted for by discrete phase shifts, caused by light exposure at dawn and/or dusk, which correct for the difference between the free-running period and the period, T, of the LD cycle. In an earlier study, however, phase response curves (PRCs) for hour-long dawn, dusk, and rectangular light pulses, equated for total light emission, were found to be essentially similar (Boulos et al., 1996d). Thus, the widening of the range of entrainment observed in the present study cannot be accounted for by differences between the phase-shifting effects of gradual and abrupt light transitions.

Aftereffects of entrainment on free-running period, as observed in this and several other studies, are clear evidence that circadian entrainment involves changes in period as well as daily phase shifts (Pittendrigh and Daan, 1976a; DeCoursey, 1989; Beersma et al., 1999). This raises the possibility that, instead of increasing the size of the daily phase shift resulting from exposure to the LD cycle, twilights could affect entrainment by altering the endogenous period of the circadian pacemaker. By making the period of the circadian pacemaker closer to that of the LD cycle, twilights would reduce the size of the required daily phase shift,

allowing entrainment to more extreme LD cycle periods.

One test of this hypothesis would be to compare free-running periods following successful entrainment to non–24 h LD cycles with and without twilights. The results obtained in experiment 1 did not allow such a comparison, because none of the hamsters in LD-rectangular were entrained at the time they were transferred to DD. A fixed LD cycle period of 25 h was therefore used in experiment 2, one that was expected to entrain animals in both lighting conditions. Even at this less extreme LD cycle period, only 33% of the hamsters in LD-rectangular were successfully entrained versus 86% of those in LD-twilight. However, the periods in DD did not differ between the animals that entrained to LD-twilight and those that entrained to LD-rectangular. We conclude that the observed changes in free-running period are the result of successful entrainment to a given LD cycle period, regardless of whether the LD cycle included twilight transitions.

We have also reexamined the data from our earlier study (Boulos et al., 1996c), in which 15 hamsters in LD-twilight and 6 hamsters in LD-rectangular entrained to T = 26 h, the longest LD cycle period used. The results are plotted in Figure 4, along with the data from the T = 25 h experiment. Again, free-running periods in DD did not differ significantly between the two lighting conditions (LD-twilight: 24.62 ± 0.22 h, LD-rectangular: 24.56 ± 0.26 h; $p > 0.05$). Thus, there is no indication to date that twilights promote entrainment by affecting the period of the circadian pacemaker.

Figure 4 also shows that the free-running periods were virtually identical following successful entrainment to T = 25 h and T = 26 h: mean periods after combining the LD-twilight and LD-rectangular groups were 24.62 ± 0.09 h after T = 25 h ($n = 11$) and 24.60 ± 0.11 h after T = 26 h ($n = 21$). These data were obtained with two different protocols—one with a fixed and one with a gradually increasing LD cycle period—and are therefore not strictly comparable, but they suggest that the increase in circadian period obtained with these protocols was at or near the maximum possible in the Syrian hamster. The mean free-running period for the LD-twilight hamsters that successfully entrained to T = 26.5 h in the present study was somewhat longer (24.95 ± 0.06 h), but with only four animals in that group, the reliability of that observation is uncertain.
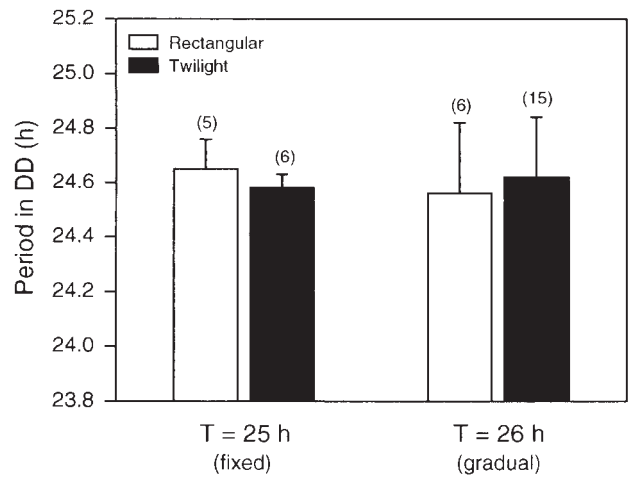

Figure 4. Free-running periods in constant darkness (DD) following successful entrainment to fixed T = 25 h light-dark (LD) cycles (present study) and to LD cycles that were gradually lengthened to T = 26 h (data from Boulos et al., 1996c).

It is clear, however, that although entrainment narrowed the gap between the period of the animals' activity rhythms and that of the LD cycle, sizable daily phase shifts were still required, amounting to average daily phase delays of 0.38 h under T = 25 h, 1.40 h under T = 26 h, and 1.55 h under T = 26.5 h, and an average daily phase advance of 2.21 h under T = 21.5 h.

In nocturnal rodents, entrainment is thought to be accomplished primarily through nonparametric mechanisms because the circadian effects of daily LD cycles with complete photoperiods can be largely reproduced with skeleton photoperiods consisting of one or two brief light pulses per day (Pittendrigh and Daan, 1976b). There is, however, a striking difference between entrainment patterns under single-pulse skeleton photoperiods with non-24-h periods, as described by Pittendrigh and Daan (1976a, 1976b), and those obtained in this study: in the former case, entrainment to LD cycles with periods both shorter and longer than 24 h was accompanied by a compression of activity time, or alpha, whereas in the present study, alpha was compressed under the shortening cycles (Figs. 1E, 1F) but showed a remarkable expansion under the lengthening cycles (Fig. 1B). Indeed, in the four animals that remained entrained to the lengthening LD-twilight protocol, mean alpha increased from 7.95 ± 0.27 h under T = 24 h to 16.52 ± 1.22 h under T = 26.5 h. In these animals, activity onset at T = 26.5 h preceded mid-dusk by 12.56 ± 2.15 h, which means that most of the expanded activity time

occurred during the light portion of the LD-twilight cycle. Substantial expansion of alpha and of the phase angle relative to mid-dusk or lights-off was also observed in the animals that entrained to fixed T = 25 h cycles, in both LD-twilight and LD-rectangular (Figs. 3A, 3E, 3F).

The fact that alpha expansion accompanies entrainment to long LD cycles with complete photoperiods but not to comparable cycles with skeleton photoperiods indicates that the entrainment patterns observed in this study involve a parametric component. Parametric entrainment represents a continuous effect of light on the motion, or angular velocity, of the circadian pacemaker. Thus, light exposure during the delay-sensitive phase of the pacemaker slows down its motion, effectively expanding the delay region of the PRC, whereas light exposure during the advance-sensitive phase speeds up the pacemaker, thereby compressing the advance region of the PRC (Swade, 1969; Daan and Pittendrigh, 1976).

We suggest that near the upper limit of entrainment, only the delay region of the PRC is exposed to light, because light encroaching on the advance region would be expected to shorten the period of the activity rhythm, resulting in a loss of entrainment. Because the delay region of the PRC starts shortly before the time of activity onset, this interpretation would imply that, in the four animals that were still entrained at T = 26.5 h, the delay region of the PRC had expanded to more than 12.56 h, which is the interval between activity onset and the middle of the dusk twilight. In contrast, under the lengthening LD-rectangular cycles, activity duration just before the loss of entrainment was 9.17 ± 0.92 h, with activity onset preceding lights-off by 4.46 ± 2.59 h.

The contribution of parametric entrainment mechanisms under the shortening cycles is more difficult to assess. At the lower limit of entrainment, the advance region of the PRC would be expected to fall entirely within the light portion of the LD cycle, beginning near the start of dawn or lights-on. However, because the end of the advance region cannot be determined from the overt activity rhythm, the compression of the advance region predicted by the parametric model could not be evaluated.

In summary, the results of this study suggest that the inclusion of twilights increases the strength of the LD zeitgeber through parametric rather than nonparametric entrainment mechanisms. How this is accomplished is not yet known, nor is it clear why a widening of the range of entrainment was seen in some but not all of the animals in the LD-twilight groups. Under natural conditions, the rate of change of light intensity at dusk and dawn (and, therefore, the duration of twilight) depends on latitude and on time of year. In laboratory simulations such as the present study, daytime illuminance is also a factor because a higher level (up to the level of natural sunrise) necessarily means a longer twilight duration. A comparison of the range of entrainment under LD-twilight cycles with different seasonal and latitudinal parameters, and with different daytime illuminances, could help determine which features of twilight transitions affect the strength of the LD zeitgeber, and what their relative contributions are.

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