Temperature-Independence of Circannual Variations in Circadian Rhythms of Golden-Mantled Ground Squirrels

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Abstract In golden-mantled ground squirrels, phase angles of entrainment of circadian locomotor activity to a fixed light-dark cycle differ markedly between subjective summer and winter. A change in ambient temperature affects entrainment only during subjective winter when it also produces pronounced effects on body temperature (T_b). It was previously proposed that variations in T_b are causally related to the circannual rhythm in circadian entrainment. To test this hypothesis, wheel-running activity and T_b were monitored for 12 to 14 months in castrated male ground squirrels housed in a 14:10 LD photocycle at 21 °C. Animals were treated with testosterone implants that eliminated hibernation and prevented the marked winter decline in T_b; these squirrels manifested circannual changes in circadian entrainment indistinguishable from those of untreated animals. Both groups exhibited pronounced changes in phase angle and alpha of circadian wheel-running and T_b rhythms. Seasonal variation in T_b is not necessary for circannual changes in circadian organization of golden-mantled ground squirrels.

Key words circannual rhythm, circadian rhythm, body temperature, circadian entrainment, golden-mantled ground squirrel, torpor, hibernation, testosterone

Golden-mantled ground squirrels (Spermophilus lateralis) are small (~250 g), diurnal sciurid rodents indigenous to the mountainous regions of western North America. The squirrels used in the present study were offspring of pregnant females trapped in the Sierra Nevada of Northern California that hibernate from approximately September through April (Bronson, 1979), thereby reducing energy requirements during the winter months. Kenagy et al. (1989) estimate that golden-mantled ground squirrels spend only 15% of their annual energy budget during the 7.5-month hibernation season. Seasonal rhythms in white adipose tissue content account for the striking circannual rhythm of body mass (Dark et al., 1992). Reproduction occurs exclusively during the weeks after arousal from hibernation in May, and litters are weaned in early July each year. In conditions of constant light, circannual rhythms of body mass, reproduction, and hibernation all free-run with a period of less than a year (reviewed in Pengelley and Asmundson, 1974). A circannual rhythm is also evident in the free-running period of the circadian clock; a shorter tau is expressed during subjective summer, coincident with the period of reproductive activity (Mrosovsky et al., 1976; Zucker et al., 1983; Lee et al., 1986). These squirrels also exhibit annual rhythms in the pattern and amount of daily locomotor activity when housed at an ambient temperature (T_a) of 22 to 25 °C and a constant photoperiod of 14 h of light/day (Lee et al., 1986; Lee and Zucker, 1995). During subjective summer,
squirrels maintain a T\(_b\) of 37 °C and remain active (alpha) for approximately 9 to 11 h/day with activity onset approximately 30 min before light onset. During subjective winter, squirrels are hetero- thermic, T\(_b\) intermittently decreases to near T\(_a\) (around 25 °C), alpha is reduced (Lee and Zucker, 1995), and activity onset is delayed by several hours (Lee et al., 1986). Circannual variations in the concentration of gonadal hormones do not drive this rhythm in male or female squirrels (Lee and Zucker, 1995).

The period of the circadian clock is responsive to changes in tissue temperature in ground squirrels during the heterothermic phase of the annual cycle; increases or decreases in T\(_a\) of 10 °C, respectively, shorten and lengthen tau and alter phase angle of entrainment to the light-dark (LD) cycle (Lee et al., 1990a). The same T\(_a\) manipulation is completely ineffective during subjective summer when squirrels defend a T\(_b\) of ~37 °C. It was presumed that a lowering in T\(_b\) induced by the 10 °C reduction in T\(_a\) was sufficient to produce the changes in tau, alpha, and phase angle during winter-type entrainment. Circadian tau in another heterothermic rodent, the Siberian hamster, is also affected by bouts of torpor, although in this species lowered T\(_b\) is associated with a shortening of tau (Thomas et al., 1993). T\(_a\) also modulates circadian entrainment in other vertebrate species, including garter snakes (Heckrotte, 1975) and bats (Erkert and Rothmund, 1981), which are either ectothermic or capable of intermittent bouts of torpor. Thus, the circannual rhythm observed in the pattern of circadian entrainment in golden-mantled ground squirrels may result from the circannual rhythm of heterothermy.

Alternatively, an endogenous circannual rhythm in circadian clock function unrelated to seasonal changes in T\(_b\) regulation may be the primary cause of changes in tau and patterns of entrainment to the LD cycle during the hibernation season; that is, changes in T\(_b\) during the hibernation phase may correlate with rather than cause seasonal variations in the circadian system. In support of this hypothesis, Grahn et al. (1994) demonstrated that alterations in circadian organization occurred prior to the expression of torpor bouts in golden-mantled ground squirrels housed in constant darkness at 5 °C.

Exogenous testosterone (T) supplied via subcutaneous implants eliminates hibernation in golden-mantled ground squirrels (Lee et al., 1990b). On the assumption that testosterone also would prevent squirrels housed at 21°C from lowering T\(_b\) during the heterothermic phase of the circannual cycle, we tested the hypothesis that circannual decreases in T\(_b\) are necessary for and causal of changes in the circadian system during subjective winter.

**METHOD**

**Experiment 1**

Male golden-mantled ground squirrels (*Spermophilus lateralis*) (\(n = 14\)) born in the laboratory during June 1995 to field-caught pregnant dams (trapped in Donner State Park, CA, elevation 1783 m above sea level) were housed from birth in a 14:10 LD cycle (lights on: 0700 PST) at an ambient temperature of 21 ± 3 °C. Animals were moved into cages equipped with running wheels during the first week of 1997, castrated, and Silastic capsules (Dow Corning, i.d. 1.56 mm; od 3.15 mm; length = 10 mm) filled with testosterone (Sigma, \(n = 7\)) or left empty (\(n = 7\)) were implanted under anesthesia induced by ip injection of pentobarbital sodium (12.5 mg/initial 100 g body weight ± 0.5 mg/each additional 10 g body weight). An analgesic solution (60 mg acetaminophen and 6 mg codeine phosphate/100 ml) was present in the drinking water for 4 days after surgery. Tap water and food (Simonsen rat pellets, maintenance diet) were available ad libitum. Locomotor activity was monitored for the next 12 to 14 months, and body weight was recorded weekly. Temperature-sensitive transmitters (Minimitter, Sunriver, OR) were implanted intra-abdominally in each squirrel on 24 October 1997, and T\(_b\) was monitored continuously via computer through 24 December 1997, to assess the ability of the squirrels to lower T\(_b\) during subjective winter.

**Experiment 2**

To better determine the effect of testosterone on T\(_b\), a second group of male squirrels (\(n = 12\)) born in June 1996 to field-caught pregnant females was treated as in Experiment 1, beginning 2 January 1998. The Silastic capsules that were implanted in half the animals were filled with testosterone; the remaining animals received empty capsules. T\(_b\) was monitored continuously throughout the transition from summer to winter patterns of entrainment.

Locomotor activity and T\(_b\) were recorded with Datacol software (Minimitter Co.) for 12 consecutive
months. Transmitters were replaced when their batteries failed. Both locomotor activity and $T_b$ data were analyzed with the Tau program (Minimitter Co.). All procedures were approved by the Animal Care and Use Committee of the University of California at Berkeley.

Alpha and phase angle of activity onset were determined by analyzing 14 to 30 days of data from each animal during the subjective summer and winter phases, respectively. During the winter phase, only days during which squirrels remained euthermic were included in these analyses. Maximal locomotor activity in this species occurs during the phase of body mass gain. High body mass was used as a marker for subjective summer. Similarly, activity decreased during subjective winter, characterized by a steady decline in body mass that was used as a marker for subjective winter. Daily activity onset was defined as the time when activity levels first rose above the daily mean and remained above this value for $\geq 1$ h. Offset of activity was defined as the time that activity fell and then stayed below the mean value for $\geq 1$ h. Alpha was the interval during which activity levels remained higher than the daily mean of total activity; the phase angle of activity onset was defined as the difference in hours between the time of light onset and activity onset. Torpor was said to occur when $T_b$ declined below 30°C for $\geq 1$ h. $T_b$ elevation above 37°C was used as a marker for assessing circadian parameters of the $T_b$ rhythm. To accomplish this, $T_b$ plots were constructed plotting only values above 37°C. The timing of the transition from summer to winter entrainment was determined by drawing eye-fitted lines through the onsets of locomotor activity or elevated $T_b$ and was defined as the date on which onset was first delayed in relation to summer-type entrainment, and remained delayed for $\geq 1$ month. The onset of heterothermy was defined as the date on which the first torpor bout was observed.

Factorial ANOVA and repeated measures ANOVA were performed where appropriate using the Statview for Windows statistics program (V.5.0; SAS Institute, Inc.). Differences were considered significant if $p < 0.05$. Follow-up comparisons were done with Fisher’s PLSD. Analysis of $T_b$ rhythms was restricted to Experiment 2 because only in this study were data obtained during both subjective summer and winter. Where clear $T_b$ data were unavailable due to transmitter or computer failure, statistical analysis was based on less than the full complement of squirrels, as reported in the results.

**RESULTS**

**Circadian Rhythms of Locomotor Activity**

All squirrels, whether treated with empty or testosterone-filled implants, exhibited circannual variations in the pattern of entrainment of the circadian locomotor activity rhythm. No significant differences existed between groups on any of the circadian entrainment measures ($p > 0.05$). Representative actograms (Fig. 1 a,b) indicate that during the subjective summer, phase angle of activity onset was less negative (i.e., occurred earlier) in relation to light onset than during subjective winter. During subjective summer, squirrels became active $0.4 \pm 0.4$ h before light onset, whereas during subjective winter activity began $2.4 \pm 0.2$ h after light onset ($p < 0.001$; Fig. 2). Alpha was longer during the summer than the winter phase ($p < 0.001$), again regardless of implant type (Fig. 3, top). The number of wheel revolutions measured over a 14- to 30-day interval declined during subjective winter as compared to subjective summer for both groups ($p < 0.005$; Fig. 3, bottom). The presence of testosterone capsules eliminated torpor so that none of 13 animals so treated ever entered hibernation; all 10 squirrels (3 from Experiment 1 were excluded due to faulty transmitters) exhibited multiple bouts of torpor over the study period (Fig. 4; top). Control squirrels underwent $16.9 \pm 3.2$ torpor bouts during the winter season, and these first occurred after the transition of circadian entrainment from summer to winter mode (Fig. 1b).

Summer-type circadian entrainment anticipated or coincided with the gain phase of the circannual body mass rhythm (Fig. 1 a,b), and the transition to winter entrainment preceded the onset of the decline in body mass by several weeks.

**Body Temperature Rhythm**

Squirrels exhibited a robust circadian rhythm in $T_b$. Peak $T_b$ occurred during the middle of the light phase and the nadir during the dark phase. The amplitude of
During the heterothermic phase of the annual cycle, the minimum $T_b$ was lower in control squirrels ($32.4 \pm 0.7 \degree C, n = 6$) than those treated with testosterone ($34.6 \pm 0.3 \degree C, n = 6$) even when days on which torpor bouts occurred were excluded from the analysis ($p < 0.02$). Maximum $T_b$ did not differ between groups during either season ($p > 0.05$). Entrainment of the $T_b$ rhythm to the LD cycle varied with subjective season in the same way as did wheel-running activity (Fig. 5). Absolute values of alpha and phase angle of the $T_b$ rhythm did not differ between groups in either season ($n = 6$ for both groups; $p > 0.05$). Animals from both groups were combined for comparisons of summer and winter values. Alpha for $T_b$ (i.e., duration of $T_b$ maintenance above $37 \degree C$) decreased during subjective winter ($n = 12, p < 0.002$; Fig. 6). The onset of peak $T_b$ occurred during the light phase and exhibited a less negative phase angle during the subjective summer than subjective winter ($n = 12, p < 0.006$; Fig. 7).

**Temporal Relation of Torpor and the Circannual Rhythm of Circadian Entrainment**

The transition from summer to winter entrainment occurred on 18 July ± 16 days and 4 August ± 15 days for the locomotor and $T_b$ rhythms, respectively ($p > 0.5$; Fisher’s PLSD). Both transitions preceded the first torpor bout in control animals, which occurred on 9 October ± 23 days ($n = 12$ for activity and $T_b$, and $n = 6$ for torpor; $p < 0.03$; Fig. 8). The transition to winter entrainment preceded the onset of torpor bouts in every squirrel.

Two squirrels implanted with empty capsules were in the winter phase when the experiment began, thus
permitting observation of the transition from winter to summer entrainment and vice versa; in these animals, the last torpor bout occurred on 9 February 1998 and 28 March 1998, respectively. This preceded the transition of the circadian activity rhythm from winter to summer entrainment by 61 and 13 days, respectively. Thus, torpor bouts began after the transition to the winter pattern of entrainment and ended before the transition to summer entrainment.

**DISCUSSION**

Circannual variation in circadian entrainment of locomotor activity persists in golden-mantled ground squirrels even when the marked seasonal decline in body temperature is eliminated. Squirrels that failed to exhibit torpor nevertheless exhibited circannual variations in circadian entrainment indistinguishable from those of control animals that underwent multiple torpor bouts. Bouts of torpor first occurred after the transition to winter-type entrainment (cf. Grahn et al., 1994), and thus cannot be causal in triggering this transition, nor are they required for its subsequent maintenance. The present results provide evidence contrary to the hypothesis that decreases in Tb are necessary for the expression of the circannual rhythm of circadian entrainment, despite previous studies that established their sufficiency (Lee et al., 1990b).

In bats (*Myotis lucifugus*), circadian tau also varies between summer and winter; individuals in the summer condition exhibit a tau < 24 h, whereas those in the winter condition exhibit taus > 24 h, even while maintaining similar Tbs (i.e., 9-10 °C; Menaker, 1961). In contrast to golden-mantled ground squirrels, *Myotis* in summer condition become heterothermic when housed at low Tbs, allowing a direct comparison between summer and winter bats at the same Tb. A similar circannual pattern of circadian clock function independent of tissue temperature may exist in ground squirrels. In another hibernator, the European hamster (*Cricetus cricetus*), clear entrainment of the circadian rhythms of running-wheel activity and Tbs are
evident only during the reproductive phase of subjective summer (Wollnik et al., 1991; Wollnik and Schmidt, 1995); it is not established that this rhythm is truly circannual (cf. Gwinner, 1986), as these studies were carried out under seminatural conditions, which included variations in Ta and/or photoperiod.

Golden-mantled ground squirrels in the present study exhibited circannual variations in the entrainment of circadian locomotor activity and Tb rhythms. We presume that a common circannual substrate controls the expression of both rhythms. During the winter phase of entrainment, squirrels maintain Tb at or above 37°C for fewer hours than during the summer phase. This may be related to the seasonal rhythm of sleep observed in these squirrels. Golden-mantled ground squirrels spend more time asleep during subjective winter than summer (Walker et al., 1980). Perhaps summer-type entrainment requires a minimum duration of elevated Tb that is not met during winter. Results from bats argue against this hypothesis as tau differed between summer and winter bats housed at the same Ta, even though animals maintained similar low Tb's of ~9 to 10°C in both conditions (Menaker, 1961). Changes in Tb from 20 to 30°C resulted in a change in circadian tau of locomotor activity during subjective winter when ground squirrels intermittently reduced Tb below 30°C. Any 10°C increases or decreases in Tb resulted in shortening or lengthening of tau, respectively (Lee et al., 1990a). Fluctuations in Tb, and presumably comparable changes in Tb during subjective winter, were sufficient to alter entrainment of wheel-running activity to the light-dark cycle. The present data indicate that variation in tissue temperature is not necessary for this effect on the circadian clock; rather, circannual modulation in circadian entrainment of locomotor activity appears to be independent of changes in tissue temperature and constitutes a true circannual rhythm in circadian function. A test of this hypothesis awaits technical developments that will permit long-term recording of in vitro electri-
cal activity from SCN neurons of ground squirrels. Records obtained over the course of the several weeks that encompass the transition from subjective summer to winter in vivo should reveal whether seasonal changes in circadian tau are intrinsic to SCN neurons maintained at a constant temperature throughout.

The functional significance of circannual variations in circadian entrainment remains unclear. Winter-type entrainment results in less total activity and a shorter interval during which Tb is defended at or above 37 °C. Hibernation reduces squirrels' energy requirements during winter. The reduced activity exhibited during the hibernation phase, even when squirrels are euthermic, also can be viewed as sparing energy (Walker et al., 1979; Berger, 1984). The transition to winter-type circadian function may be a prerequisite for the expression of torpor or hibernation. This would be consistent with the observation that in this and a previous study (Grahn et al., 1994), the transition to winter entrainment and alpha always preceded the expression of bouts of heterothermy. In this case, the altered function of the circadian clock could be permissive or necessary for the expression of hibernation.

Ablation of the circadian clock in the suprachiasmatic nucleus results in continuous hibernation for more than 2 years in some golden-mantled ground squirrels (Ruby et al., 1996, 1998). Squirrels with SCN ablations have disrupted circadian rhythms (Zucker et al., 1983), thereby implicating circadian clocks in the expression or timing of hibernation.

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The Tb and locomotor activity rhythms show the same pattern of circannual entrainment; during periodic arousals from torpor during the winter, this would help squirrels conserve energy by spending less time maintaining Tb at 37 °C. The circannual adjustment in the circadian clock may be in the service of metabolic damping in a winter environment; during periodic arousals, golden-mantled ground squir-
rels spend more time asleep and at a lower \( T_b \) than during subjective summer (Walker et al., 1980; Trachsel et al., 1991). Altered clock function may also contribute to squirrels sustaining torpor for several circadian cycles per bout (Grahn et al., 1994).

In conclusion, circannual variation in circadian entrainment is not dependent on the correlated circannual rhythm of decreases in tissue temperature. The physiological mechanism that underlies this striking change in circadian organization remains to be identified.

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