10-1-2006

Interval Timer Control of Puberty in Photoinhibited Siberian Hamsters

Ho Park Jin
University of California, Berkeley

Alexander S. Kauffman
University of Washington

Matthew J. Paul
University of California, Berkeley

Matthew P. Butler
University of California, Berkeley

Annaliese K. Beery
University of California, Berkeley, abeery@smith.edu

See next page for additional authors

Interval Timer Control of Puberty in Photoinhibited Siberian Hamsters

Jin Ho Park,*[,1] Alexander S. Kauffman,[§] Matthew J. Paul,* Matthew P. Butler,[†] Annaliese K. Beery,[‡] Ruth M. Costantini,[†] and Irving Zucker*[,†,‡]

*Departments of *Psychology, [†]Integrative Biology, and [‡]Helen Wills Neuroscience Institute, University of California, Berkeley; [§]Department of Physiology and Biophysics, University of Washington, Seattle*

Abstract Puberty, which is markedly delayed in male Siberian hamsters (*Phodopus sungorus*) born into short day lengths, is controlled by an interval timer regulated by the duration of nocturnal melatonin secretion. Properties of the interval timer were assessed by perturbing normal patterns of melatonin secretion in males gestated and maintained thereafter in 1 of 2 short day lengths, 10 h light/day (10L) or 12L. Melatonin secretion of short-day hamsters was suppressed by constant light treatment or modified by daily injection of propranolol to mimic nocturnal melatonin durations typical of long-day hamsters. Constant light treatment during weeks 3 to 5 induced early incomplete gonadal growth in 12L but not 10L hamsters but did not affect late onset of gonadal development indicative of puberty in either photoperiod. Propranolol treatment during postnatal weeks 3 to 5 induced transient growth of the testes and ultimately delayed the timing of puberty by 3 weeks. Similar treatments between weeks 5 and 7 or on alternate weeks for 24 weeks did not affect the interval timer. The first 2 weeks after weaning may constitute a critical period during which the interval timer is highly responsive to photoperiod. Alternatively, the hamsters' photoperiodic history rather than age or developmental stage may be the critical variable. The interpolation of long-day melatonin signals at the time of weaning does not appear to reset the interval timer to its zero position but may reduce timer responsiveness to long-day melatonin signals several weeks later.

Key words puberty, melatonin, hamster, photoperiodism, development, testes

Seasonal changes in day length phase annual patterns of mammalian reproduction (Bronson, 1989). In the field, adult Siberian hamsters (*Phodopus sungorus*) produce offspring during long spring day lengths and cease reproduction during decreasing or short day lengths of autumn and winter (Weiner, 1987). In laboratory studies, long photoperiods sustain the spring phenotype indefinitely, whereas most adult hamsters exposed to short photoperiods become reproductively quiescent. With continuous exposure to short day lengths, however, spontaneous gonadal recrudescence eventually ensues, typically beginning after approximately 15 weeks; several winter traits then revert to the spring-summer phenotype (Reiter, 1980). The onset of gonadal recrudescence during the short day lengths of mid-winter permits reproduction at

1. To whom all correspondence should be addressed: Jin Ho Park, Department of Biochemistry & Molecular Genetics, School of Medicine, University of Virginia, 1300 Jefferson Park Avenue, Charlottesville VA 22908; e-mail: jinhopark@virginia.edu.

JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 21 No. 5, October 2006 373-383
DOI: 10.1177/0748730406292315

the outset of favorable conditions in early spring (Prendergast et al., 2002).

Photoperiod exerts similar effects on reproductive and somatic development of prepubertal Siberian hamsters (Kauffman et al., 2003; Yellon and Goldman, 1984). Pups born into long or increasing day lengths reach sexual maturity at ~5 weeks of age, whereas those born into short or decreasing day lengths delay reproductive maturation by 10 to 15 weeks. For the latter hamsters, an "interval timer," triggered by short day lengths, induces the transformation to the spring gonadal phenotype 15 to 20 weeks later (Anchordoquy and Lynch, 2000; Gorman and Zucker, 1995). The properties of this interval timer are largely unspecified (Prendergast et al., 2002).

Differences in the duration of nocturnal melatonin secretion, which endures for 5 to 6 h in long days and 8 to 12 h in short days, provide a seasonally changing endocrine representation of night length that induces the summer and winter phenotypes of body mass, pelage, and gonadal status (Bartness et al., 1993; Carter and Goldman, 1983; Goldman, 2001; Prendergast et al., 2002). The onset of gonadal growth after prolonged exposure to short days has been attributed to the development of refractoriness of neural substrates to long-duration melatonin signals (Freeman and Zucker, 2001).

Refractoriness to melatonin does not depend in any simple manner on the number of prior long-duration melatonin signals (Prendergast et al., 1998). Once the interval timer is triggered, subsequent disruption of long-duration melatonin signals has variable effects on the course of gonadal growth, depending on the stage of development in which the signals are altered (Anchordoquy and Lynch, 2000). For example, gonadal development was delayed when males born into a September day length of a simulated natural photoperiod were provided with melatonin implants that obliterate endogenous melatonin signaling from 3 to 9 weeks of age; the same treatment given from 9 to 15 weeks of age was ineffective (Gorman, 2003b). This suggests that the age at which Siberian hamsters are exposed to changes in day length is critical. Acute sensitivity to photoperiodic change occurs near the time of weaning (Elliott and Goldman, 1989; Finley et al., 1995; Spears et al., 1990; Whaling et al., 1993); exposure to a single long or longer day between 3 and 5 weeks of age, but not later in life, has enduring effects on the reproductive system (Finley et al., 1995; Spears et al., 1990). Collectively, these studies raise the possibility that the interval timer may be triggered and perhaps labile during the first 3 to 5 weeks of life. Alternatively, the hamsters' photoperiodic history rather than age or developmental stage may be the critical variable.

Juvenile Siberian hamsters gestated and reared in a 10L photoperiod underwent gonadal growth approximately 4 weeks earlier than juveniles gestated and reared in 12L (Gorman and Zucker, 1995). The interval timers of 10L and 12L hamsters may be triggered at the same time but run at different rates (slower in 12L), triggered several weeks later in 12L and run at the same rate, or triggered at different times and run at different rates.

In the present experiments, endogenous melatonin signals of juvenile Siberian hamsters kept in short day lengths were experimentally manipulated. We addressed 3 related questions: 1) Is the interval timer in different short photoperiods equally susceptible to melatonin signal disruption? 2) Is the continuous presence of short-day melatonin signals required for normal time-keeping? and 3) Do short-term perturbations of melatonin signal duration completely reset the timer or only suspend timing during signal disruption?

MATERIALS AND METHODS

Animals

Siberian hamsters were from a local breeding colony, descended from stock originally supplied by Bruce Goldman (University of Connecticut, Storrs, CT) and later outbred to hamsters supplied by Katherine Wynne-Edwards (Queen's University, Kingston, ON).

Adult female hamsters, maintained from birth in a 14L photoperiod (lights-on at 0800 h), were paired with similarly housed males. Beginning on the day of pairing, hamsters were housed in either 10L or 12L (lights-on at 0800 h for both photoperiods) in experiment 1 and in 10L (lights-on at 0200 h) in experiments 2 and 3. No more than 1 male from a single litter was randomly selected to be part of each group in each experiment. When possible, males from 1 litter were randomly distributed into each group in all 3 experiments. Animals from each experiment were tested concurrently. Male offspring were weaned at 18 days of age and then housed individually in polypropylene cages (25 × 14 × 12 cm) containing Tek-Fresh Lab Animal Bedding (Harlan Teklad, Madison, WI). Food (Purina rodent chow 5015, Purina, St. Louis, MO) and water were available ad libitum, and ambient temperature was maintained at 22 ± 2 °C. All methods and procedures were approved by the Animal Care and Use Committee at U.C. Berkeley.

Somatic and Reproductive Measures

At predetermined intervals, hamsters were weighed (±0.1 g) and the length and width of the left testis measured externally (±0.1 mm) under light anesthesia induced with isoflurane vapors. The product of testis width squared times testis length provides a measure of estimated testis volume (ETV) that is highly correlated with testis weight (Gorman and Zucker, 1995). The percent increase in ETV over time and weight gain was calculated from one time point to the next.

Early gonadal growth in *juvenile* hamsters was defined by the 1st increase in ETV ≥20% relative to the previous value; the increase had to be sustained for 2 consecutive measurements before week 11, with each ETV >125.

Onset of *late* gonadal development in photorefractory *adult* hamsters was defined as the 1st time point after week 13 at which ETVs increased by ≥30% and culminated in values ≥400. Substantial numbers of spermatids first appear in the recrudescent testes of adult Siberian hamsters when paired testes weights are ≥400 mg (Schlatt et al., 1995), which corresponds to an ETV of ~400 (Gorman and Zucker, 1995).

Pelage, another photoperiodic trait, was scored at specified weeks in all experiments using the scale of Duncan and Goldman (1984); a score of 1 denotes a brown, summer pelage, and a score of 4 denotes a white, winter pelage.

Nonresponder Exclusions

The majority of Siberian hamsters maintained in short photoperiods retain small undeveloped testes until at least 15 weeks of age, but a minority undergo rapid and substantial testicular growth within several weeks after birth (Hoffmann, 1978, 1982). Animals with ETVs >125 at week 3 were considered photononresponsive and were removed from the study (n = 2 from 10L control, n = 2 from 12L control, n = 1 from 12L-LL, n = 3 from PR3-5, n = 3 from PR5-7, n = 6 from SAL1, n = 1 from PR3/8, n = 3 from PR3-9, and n = 4 from SAL2 groups).

Melatonin Manipulations with Constant Light or Propranolol

Modification of the pattern of endogenous melatonin secretion was achieved by 1) exposing hamsters to constant light (LL; light intensity was approximately 300 lx at cage level), which rapidly abolishes pineal melatonin secretion (Stieglitz et al., 1994), or 2) injecting *D,L*-propranolol (PROP), which acutely depresses melatonin secretion within minutes of injection (Lipton et al., 1981; Fig. 1A). Melatonin production in PROP-treated hamsters remains suppressed for the remainder of the night and recovers its normal pattern the next night (Prendergast et al., 1998). Timed PROP administration given 6 h after dark onset transforms a long-duration melatonin signal (~10 h) to a short melatonin signal (~5 h) that resembles that produced in long days (Fig. 1A). PROP treatment is preferable to treatment with long days because the latter intervention has enduring effects on the circadian system (and melatonin secretion) for an unknown number of days, whereas PROP does not disrupt circadian timekeeping (Prendergast et al., 1998). Fresh PROP stock solution prepared each week was frozen in 1.0-mL aliquots that were thawed nightly and used completely or discarded. All cages were housed in environmental chambers continuously illuminated with dim red light to facilitate injections during the dark phase.

Experiment 1: Impact of LL Treatment from Weeks 3 to 5 on Hamsters Housed in 10L or 12L Photoperiods

Juvenile Siberian hamsters maintained in 10L undergo gonadal development approximately 4 weeks in advance of 12L juveniles (Gorman and Zucker, 1995). To test the impact of elimination of melatonin signals on interval timing, LL treatment was administered for 2 weeks to hamsters gestated and reared in 10L or 12L. This experiment evaluated whether constant light treatment would reset the timer or otherwise change its timing function.

Procedure

Male hamsters gestated and maintained in a 10L or 12L photoperiod were exposed to 2 weeks of LL from 3 to 5 weeks of age (groups 10L-LL and 12L-LL, respectively). Control animals receiving no LL treatment were housed in either 10L or 12L photoperiods throughout (groups 10L and 12L, respectively). ETVs were recorded every 2 to 3 weeks throughout development until week 28. Each treatment group contained 16 to 22 animals.

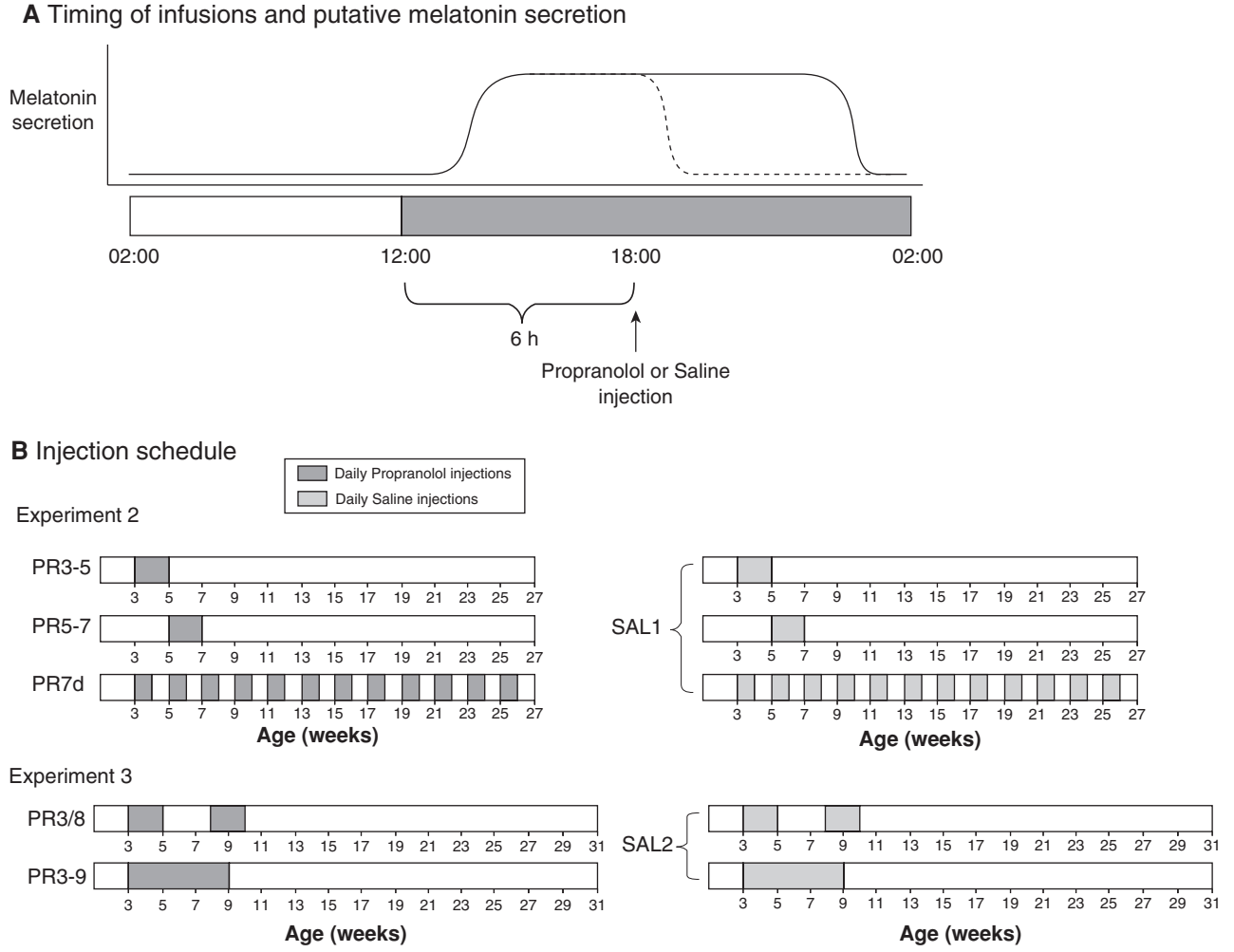

Figure 1. (A) Schematic representation of photoperiods under which hamsters were housed and the timing of the propranolol (PR) or saline (SAL) injections in experiments 2 and 3. The expected patterns of melatonin secretion in a 10L photoperiod by hamsters injected in the nighttime with PR (truncated secretion – dashed line) or saline (solid line). (B) Schematic representation of PR and SAL injection sequences for each treatment group in experiments 2 and 3. Injections were delivered daily from 3 to 5 weeks of age (PR3-5), 5 to 7 weeks of age (PR5-7), for 7 consecutive days every other week beginning on week 3 (PR7d), 3 to 5 and 8 to 10 weeks of age (PR3/8), or 3 to 9 weeks of age (PR3-9). SAL1 hamsters received saline injections either from 3 to 5 or 5 to 7 weeks of age, or for 7 consecutive days every other week beginning on week 3; SAL2 hamsters received saline injections from either 3 to 5 and 8 to 10 or 3 to 9 weeks of age.

Experiments 2 and 3: Impact of Propranolol Treatments during Development on Males Gestated and Reared in a 10L Photoperiod

Experiment 2

We tested the extent to which interval timing is contingent on continuous daily availability of short-day melatonin signals over the course of several weeks. If this is a critical factor, then intermittent substitution of long-day melatonin signals at different stages of development should affect the timing of puberty.

Procedure. Long-day melatonin signals were generated in juvenile hamsters gestated and maintained in 10L by giving them daily subcutaneous injections of PROP (0.5 mg/0.1 mL). Control animals were given daily injections of saline vehicle (0.9% NaCl/0.1 mL). Daily PROP or saline injections were given for a 2-week period beginning when hamsters were either 3 (groups PR3-5 and SAL3-5, respectively) or 5 weeks old (groups PR5-7 and SAL5-7, respectively; Fig. 1B).

Two additional groups of hamsters were given either PROP or saline injections intermittently throughout the entire course of development; these animals received injections for 7 consecutive days every other week, beginning at 3 weeks of age and continuing

through week 27 (groups PR7d and SAL7d, respectively; Fig. 1B). Each group contained 12 to 19 animals (the 3 saline-treated groups were combined into 1 group; SAL1).

Experiment 3

The interval timer was perturbed, and possibly reset, by PROP administration from 3 to 5 weeks of age (experiment 2); if the timer is reset, then interpolation at the appropriate time of 2 more weeks of long-day melatonin signals should produce an equivalent delay in the onset of puberty. Alternatively, the timer could be reset but the response to the 2nd set of signals could be attenuated because of an age-related process that reduces sensitivity to melatonin perturbations. A 3rd possibility is that in the absence of timer resetting, the 2nd long-day treatment may fall during a phase of development during which the timer is unresponsive to melatonin signal changes. This experiment sought to discriminate among these alternatives.

Procedure. Male hamsters gestated and maintained in 10L were injected daily with PROP or saline from 3 to 5 and again from 8 to 10 weeks of age (groups PR3/8 and SAL3/8, respectively; Fig. 1B).

To verify that PROP treatments simulate long-day-like melatonin signals, additional 10L groups were injected daily with either PROP (group PR3-9) or saline (SAL3-9) for 6 consecutive weeks, beginning at 3 weeks of age (Fig. 1B). If this PROP treatment simulates exposure to long days, then it should stimulate early puberty. Experimental and control groups contained 17 to 24 animals; the 2 saline-treated groups were combined into 1 group (SAL2).

Statistical Analysis

Repeated measures analysis of variance (ANOVA) was used to analyze ETV, percentage increase in ETV over time, body mass, and weight gain over time (Statview 5, SAS Institute, Cary, NC). Tukey-Kramer tests were used for post hoc comparisons on specific weeks. Group differences in onset of gonadal development were assessed with between-subjects ANOVAs. Chi-square tests were used to compare group differences in time of onset of gonadal growth. In experiments 2 and 3, reproductive measurements of hamsters that received different saline treatments did not differ significantly. Thus, for statistical analysis, hamsters from experiment 2 injected with saline from weeks 3 to 5, 5 to 7, or for 7 consecutive days every other week were consolidated into 1 control group (SAL1); hamsters from experiment 3 that received saline injections from weeks 3 to 5 and 8 to 10, or weeks 3 to 9 constituted another saline control group (SAL2; Fig. 1B). Differences were considered statistically significant if $p < 0.05$ and are reported as such regardless of the actual value below 0.05.

RESULTS

Experiment 1

Gonadal Development in 12L

12L controls maintained small gonads for the first 3 months of life; ETVs were <200 from weeks 3 to 11 (Fig. 2A). ETVs > 400 were achieved by week 24.

Constant light treatment of 12L hamsters during weeks 3 to 5 resulted in early testicular growth; between weeks 5 and 8, ETVs increased by 258% in 12L-LL hamsters compared to 36% in 12L control hamsters ($p < 0.05$; Fig. 2A). ETVs of 12L-LL animals exceeded those of 12L controls during weeks 8 to 17 ($p < 0.05$ for each time point; Fig. 2A).

Late onset of testicular growth of 12L control and 12L-LL hamsters began at 18.3 ± 0.9 and 18.9 ± 0.9 weeks of age, respectively ($p > 0.05$; Fig. 2B). A majority of 12L-LL hamsters (13 of 19) underwent early testicular growth. Six of these animals underwent gonadal regression over the course of the next 6 weeks (weeks 18-24 or 20-26 or 22-28), whereas 7 others sustained testicular growth until they attained stable adult ETVs >400. The pattern of gradual testicular growth characteristic of 13 of 16 12L control animals from weeks 3 to 28 (Fig. 2A) was generated by only 5 of 19 12L-LL hamsters ($\chi^2 = 10.5$, $p < 0.05$).

Gonadal Development in 10L

Constant light did not significantly affect the pattern of testicular growth in males gestated in 10L (10L-LL; Fig. 2A); after cessation of LL treatment on week 5, ETV values increased slightly and remained elevated until 17 weeks (Fig. 2A), but 10L control and 10L-LL animals did not differ significantly at any time point ($p > 0.05$ between weeks 8 and 28).

Late onset testicular growth occurred at 16.4 ± 0.5 and 17.8 ± 0.7 weeks of age in control and LL groups, respectively ($p > 0.05$; Fig. 2B). Late gonadal development began 2 weeks later in 12L than 10L control

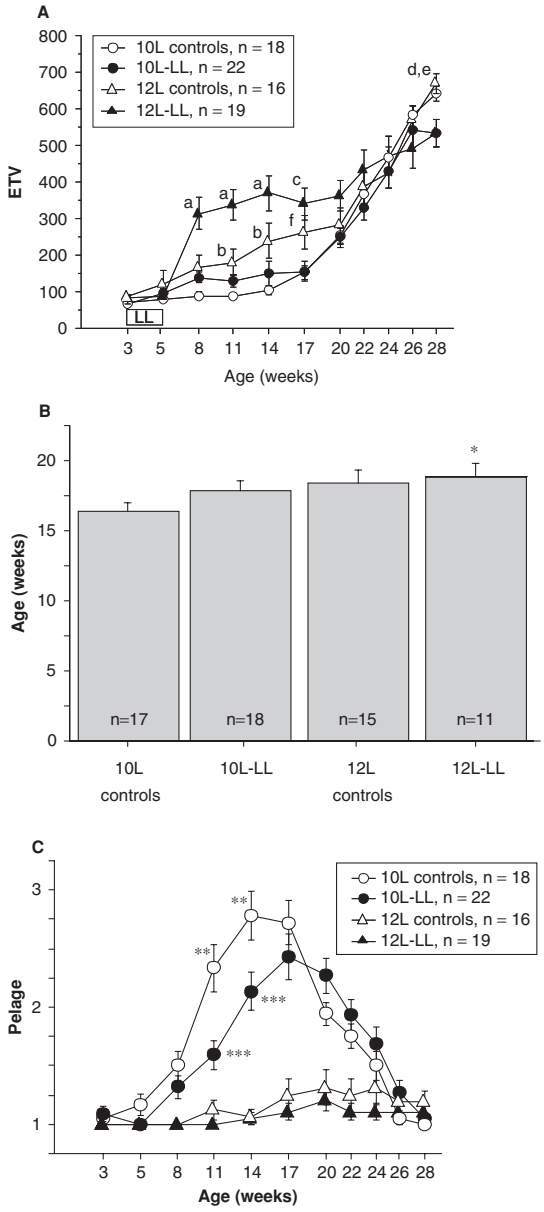

Figure 2. (A) Mean ± SEM estimated testis volumes (ETVs) of 10L control, 12L control, 10L-LL, and 12L-LL hamsters from experiment 1. (B) Mean ± SE age (weeks) at late onset of testicular development after 13 weeks of age. Some hamsters (n = 1 and 8 for 12L and 12L-LL groups, respectively, and n = 1 and 4 for 10L and 10L-LL groups, respectively) were excluded from this analysis either because stable adult ETVs attained early in life were sustained for the remainder of the experiment or testing was concluded before they underwent late onset gonadal development. (C) Mean ± SEM pelage ratings. a: 12L-LL values differ significantly from those of the other 3 groups; b: 12L values significantly different from 10L values; c: 12L-LL values differ significantly from both 10L and 10L-LL values; d: 12L values differ significantly from those of 12L-LL; e: 10L values differ significantly from those of 10L-LL; f: 12L values differ significantly from those of 10L and 10L-LL. *Significantly different from 10L controls. **10L values differ significantly from those of the other 3 groups. ***10L-LL values differ significantly from those of the other 3 groups. Sample sizes for each group are indicated by the values within the bars in panel B.

hamsters (18.3 ± 0.9 vs. 16.4 ± 0.5 weeks, respectively), but the difference fell short of statistical significance ($p < 0.09$).

Pelage in 10L and 12L

The winter pelage (rating > 2) was attained by both the 10L-control and the 10L-LL groups but was delayed by 3 weeks in the latter hamsters (11 vs. 14 weeks of age, respectively; $p < 0.05$; Fig. 2C). The rate at which the pelage reverted back to the summer condition (rating < 2) did not differ significantly between the 10L groups and occurred at approximately week 22 (Fig. 2C). 12L control and 12L-LL hamsters maintained the summer pelage throughout the study (ratings < 2; Fig. 2C).

Experiment 2

Gonadal Development

Testes of 10L control hamsters treated with saline (SAL1) remained undeveloped for the 1st few months of life; mean onset of late gonadal growth occurred at ~17 weeks of age (Fig. 3A, B). Hamsters treated with PROP from weeks 3 to 5 (PR3-5) underwent early testicular growth by week 7 ($p < 0.05$ relative to SAL1; Fig. 3A); thereafter, ETVs declined and were indistinguishable from those of saline-treated controls by week 17 (Fig. 3A). Late gonadal development in the PR3-5 group occurred at ~20 weeks of age and was delayed by 3 weeks relative to that of SAL1 animals ($p < 0.05$; Fig. 3B). In contrast, ETVs of hamsters treated with PROP during weeks 5 to 7 (PR5-7) were indistinguishable from those of SAL1 controls at all time points (Fig. 3A). Onset of late gonadal growth of the PR5-7 group did not differ from that of either the PR3-5 or SAL1 groups ($p > 0.05$ for both comparisons; Fig. 3B).

PROP administration for 7 consecutive days on alternate weeks for 24 weeks (PR7d) resulted in early testicular growth ($p < 0.05$ relative to SAL1 on week 5; Fig. 3A). Mean onset of late testicular growth was not significantly different from that of SAL1 animals but was marginally earlier than that of PR3-5 hamsters ($p < 0.06$).

Pelage

Attainment of the winter pelage in 10L was delayed by 2 weeks in PR3-5 males compared to SAL1 animals (13 ± 1 vs. 11 ± 1 weeks of age, respectively; $p < 0.05$; not illustrated). In contrast, the pattern of pelage changes of hamsters treated with PROP from weeks 5 to 7 did not differ from that of SAL1 controls. Hamsters treated with PROP for

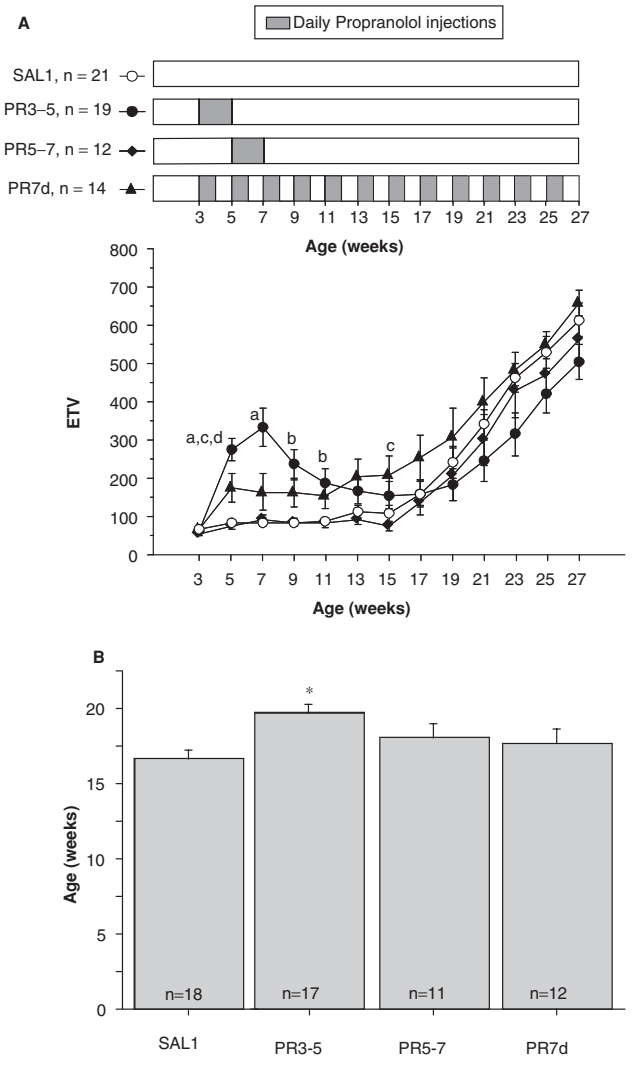

Figure 3. (A) Mean ± SEM estimated testis volumes (ETVs) of PR3-5, PR7d, PR5-7, and SAL1 hamsters. (B) Mean ± SE age at late onset of spontaneous testicular development after 13 weeks of age. Several hamsters (*n* = 3, 2, 1, and 2 for SAL1, PR3-5, PR 5-7, and PR7d, respectively) were excluded for reasons given in Figure legend 2B. a: PR3-5 values significantly different from those of all other groups; b: PR3-5 significantly different from PR5-7 and SAL1; c: PR3-5 significantly different from PR5-7; d: PR7d values significantly different from those of SAL1. *Significantly different from SAL1. Sample sizes for each group are indicated by the values within the bars in panel B.

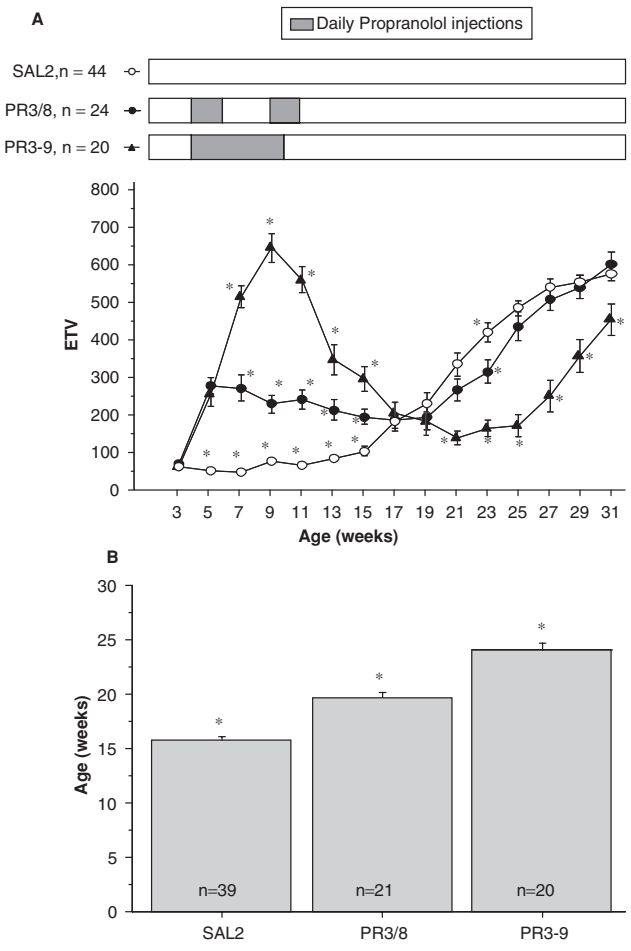

Figure 4. (A) Mean ± SEM estimated testis volumes (ETVs) of PR3/8, PR3-9, and SAL2 hamsters. (B) Mean ± SE age at late onset of spontaneous testicular development after 13 weeks of age. Sample sizes for each group are indicated by the values within the bars in panel B. Five hamsters from SAL2 and 3 from the PR3/8 group were excluded from this analysis, as explained in Figure legend 2B. *Significantly different from all other groups.

7 days on alternate weeks never molted to the winter pelage.

Experiment 3

Gonadal Development

Saline-treated 10L control animals (SAL2) maintained small, undeveloped testes for the first 3 months of life. ETV values first increased substantially from 15 to 19 weeks of age (Fig. 4A) with mean late onset testicular growth occurring at 16 ± 1 weeks of age (Fig. 4B). Early testicular growth was evident in hamsters injected with PROP on both weeks 3 to 5 and 8 to 10 (PROP3/8); this increase was associated with the 1st but not the 2nd course of PROP treatment (Fig. 4A). Mean onset of late gonadal development in PROP3/8 males occurred 4 weeks later than that of SAL2 controls (weeks 20 ± 1 and 16 ± 1, respectively; $p < 0.05$; Fig. 4B).

PROP treatment from weeks 3 to 9 (PR3-9) promoted marked testis growth, first evident on week 5 and continuing on weeks 7 and 9, by which time testes reached adult long-day dimensions (ETV > 400; $p < 0.05$,

relative to SAL2 controls for each time point; Fig. 4A). After PROP treatment ended, PR3-9 hamsters underwent gonadal regression; subsequently, late gonadal growth began approximately 8 weeks later than that of SAL2 controls ($p < 0.05$; Fig. 4B).

Pelage

Attainment of the winter pelage was delayed by 8 weeks in hamsters treated with PROP from weeks 3 to 5 and 8 to 10, and by 14 weeks for those injected with PROP from weeks 3 to 9 ($p < 0.05$ in each case relative to SAL2 controls; not illustrated).

Body Mass

In each of the 3 experiments, the developmental patterns of body mass mirrored those of testicular development.

DISCUSSION

The interpolation of long-day melatonin signals during postnatal weeks 3 to 5, achieved by treating short-day hamsters with PROP, produced a 3-week delay in the onset of pubertal testicular growth several months later. The interval timing mechanism triggered by short day lengths either is arrested, decelerated, or reset by PROP treatment during weeks 3 to 5. In contrast, similar PROP interventions during weeks 5 to 7 were without effect, suggesting an age-specific effect of PROP treatment on timing of puberty. Alternatively, the hamsters' photoperiodic history may be the critical variable; responsiveness at any age may be limited to a brief window immediately before or shortly after the timer has been activated.

Because the increase in testis size induced by the PROP treatment from weeks 3 to 5 was sustained until the onset of spontaneous gonadal growth that began many weeks later, it is unlikely that the delayed onset of puberty reflects inhibitory masking actions of PROP on testicular growth (Fig. 3A, B).

Hamsters treated with PROP during weeks 3 to 5, and again from weeks 8 to 10, delayed puberty by 4 weeks. This differs from the expected 6-week delay if the treatment during weeks 3 to 5 reset the timer. These results suggest that PROP treatment from weeks 3 to 5 does not reset the timer to its zero position. Alternatively, the attenuated delay in late onset gonadal development attributable to additional PROP treatment from weeks 8 to 10 is compatible with timer resetting by the initial treatment, accompanied by reduced responsiveness to short-duration melatonin signals.

The first 2 weeks after weaning constitute a sensitive period during which Siberian hamsters are highly responsive to changes in day length and melatonin (Elliott and Goldman, 1989; Finley et al., 1995; Spears et al., 1990). Two weeks of PROP treatment initiated on week 3 stimulated early gonadal growth in most hamsters, whereas similar treatment at week 5 was without effect. Hamsters treated with PROP on weeks 3 to 5 and again from weeks 8 to 10 did not achieve early ETV values higher than those treated only on weeks 3 to 5, but the testicular decline in the latter group between weeks 7 and 13 was prevented by the supplementary PROP treatment (compare Figs. 3A and 4A). The rapid gonadal growth of hamsters treated with propranolol from weeks 3 to 9 resembled that of hamsters transferred from short to long days (e.g., Hoffmann, 1973). This confirms that melatonin signaling produced by PROP treatment mimics that generated in long days; melatonin signal duration is the salient feature for seasonal control of the reproductive system (Ebling et al., 1995; Gorman, 2003a; Kumar et al., 1993; Prendergast et al., 1998). The delay in spontaneous gonadal recrudescence in these hamsters reflects photostimulatory actions of the propranolol treatment unrelated to modification of interval timer function.

Our results extend earlier findings that Siberian hamsters are highly responsive to changes in day length near the time of weaning (Elliott and Goldman, 1989; Finley et al., 1995; Spears et al., 1990; Whaling et al., 1993). They support the conclusion that once the interval timer is triggered by several weeks of short day lengths, elimination of melatonin signals (experiment 1) has little effect on interval timing (Anchordoquy and Lynch, 2000). Our findings are not entirely consistent, however, with the effects observed when the endogenous melatonin signal is perturbed by continuous-release melatonin implants; when males were provided with melatonin implants from 3 to 9 weeks of age, those born into an August day length of a simulated natural photoperiod attained reproductive maturity at the same time as controls treated with blank capsules, whereas males born into a September day length treated with melatonin implants from weeks 9 to 15 attained reproductive maturity sooner than controls treated with blank capsules (Gorman, 2003b). Differences in duration of treatments (6 vs. 2 weeks), dissimilar methods used to alter the short-day melatonin signal (melatonin

implants vs. constant light and PROP injections), and use of different photoperiods (simulated natural vs. static day lengths) may account for the divergent outcomes. The short melatonin signal (4-6 h) generated nightly by hamsters treated with PROP provides different information to the neuroendocrine system than the complete obliteration of a useful melatonin signal by melatonin implants or pinealectomy; the former more effectively promotes gonadal growth than the latter (Kelly et al., 1994). In addition, simulated natural photoperiods that provide average day-length durations similar to those of a static photoperiod nevertheless elicit different physiological responses (Gorman, 1995; Gorman et al., 1997; Gorman and Zucker, 1997).

Constant light had little or no impact on spontaneous pubertal development in hamsters housed in 10L or 12L. The interval timer was able to bridge a gap of 2 weeks without melatonin signals in hamsters previously maintained in short days from conception to 3 weeks of age. This suggests that once initiated, the interval timer is not perturbed by a several week absence of melatonin signals. The absence of melatonin secretion in constant light promotes less gonadal development than is induced by short-duration melatonin signals (0 vs. 4-6 h/day, respectively) (Carter and Goldman, 1983; Kelly et al., 1994) and does not provide a normal long-day signal.

The change in circadian rhythms of weanling or juvenile hamsters induced by 2 weeks of 300 lx LL treatment is unknown. In any case, this treatment did not affect late onset gonadal development in hamsters housed in either the 10L or 12L photoperiods. The marked increase in early gonadal growth in 12L hamsters treated with LL more likely reflects masking effects of light that obliterate long-duration melatonin signals than changes in circadian organization.

Two weeks of short-duration melatonin signals from weeks 3 to 5, unlike constant light treatment, delayed spontaneous gonadal development many weeks later. Short-duration melatonin signals appear to have a greater impact on the interval timer than the complete absence of melatonin signals.

The onset of spontaneous testicular development was highly variable in hamsters gestated and maintained in 12L; this differs from the synchronous timing of onset of spontaneous gonadal development at ~22 weeks of age in Siberian hamsters maintained in 12L by Gorman and Zucker (1995). Different hamster stocks and different photoperiods during gestation and postnatally (16L vs. 14L) may have contributed to these differences. In both the present and the earlier study, control animals in 10L underwent pubertal gonadal development before the corresponding 12L cohort, although the effect was more pronounced in the earlier study.

Constant light administered between 3 and 5 weeks of age provoked early testicular growth in 12L but not 10L hamsters. Although both of these day lengths are below the critical duration required to stimulate gonadal growth (Duncan et al., 1985), restraint of the hypothalamic-pituitary-gonadal axis apparently is less pronounced in the longer of the 2 short day lengths and is overridden by LL treatment. This represents a masking effect of constant light on gonadotropin secretion rather than differential responsiveness of the interval timer in 10L versus 12L.

Interpolation of long-day melatonin signals on alternate weeks for 24 weeks produced a short-lived early increase in testis growth that did not impact the interval timer (experiment 2, PR7d). A minimum of 2 consecutive weeks of long-day signals appears necessary to influence the pubertal reproductive system; the interval timer bridges repeated 1-week absences of short-day melatonin signals to sustain the short-day gonadal phenotype for several months. In contrast, hamsters subjected to this regimen never developed the short-day winter pelage. Photoperiodic control of pelage, which is mediated by the pars tuberalis and pituitary prolactin secretion (Duncan and Goldman, 1984; Lincoln and Clarke, 1997), has different melatonin-signaling requirements than does the hypothalamic-pituitary-gonadal axis. Two additional findings are consistent with this conjecture: constant light during weeks 3 to 5 delayed attainment of the winter pelage but did not affect onset of pubertal testicular development in 10L hamsters. Also, supplementary PROP treatment during weeks 8 to 10 extended the delay in the onset of spontaneous gonadal growth and molt to the winter pelage by 1 and 6 weeks, respectively, compared to delays recorded for animals treated only during weeks 3 to 5. Hamsters maintained in 12L manifested the winter phenotype of small undeveloped gonads and a summer-like pelage through at least 17 weeks of age. In sharp contrast, 10L hamsters developed the winter pelage. This reinforces conclusions that critical day lengths differ for suppression of gonadotropin secretion, on one hand, and prolactin on the other (Duncan et al., 1985).

The mechanism that times spontaneous testicular development in Siberian hamsters can be triggered by a fraction of the short days that juvenile hamsters experience during the fall and winter (Prendergast

et al., 2000). The present study indicates that this interval timer is affected by changes in day length and melatonin signaling prior to or coinciding with the time of weaning but is unaffected by later manipulations. Also, daily exposure to short-day melatonin signals is not required for normal interval timekeeping. Finally, the timer is affected but does not appear to be reset by changes in melatonin signaling at the time of weaning.

The impact of prenatal and early postnatal melatonin signaling (Tuthill et al., 2005) on interval timer responsiveness to melatonin during and after weaning is not known. The interval timer that mediates spontaneous gonadal recrudescence in adult Siberian hamsters may be similarly influenced by interpolated long-day melatonin signals during the 1st few weeks of short-day treatment. This would indicate that the time around weaning is not the only period during which the interval timer is responsive to melatonin signals. Instead, the hamsters' photoperiodic history would be the critical variable, with interval timer responsiveness limited to a brief window immediately before or shortly after the timer has been activated.

In conclusion, the interval timer that triggers pubertal gonadal development in short-day hamsters is not affected by the complete suppression of melatonin signaling induced by constant light treatment early in development. Furthermore, normal timekeeping is not contingent on the continuous presence of short-day melatonin signals in the months prior to puberty. Also, perturbations of melatonin signaling that delay pubertal gonadal growth did not appear to completely reset the timer but more likely decelerated timing during signal disruption.

ACKNOWLEDGMENTS

We are grateful to Lance Kriegsfeld and 3 anonymous reviewers for comments on an earlier version of the article. Matthew P. Butler is a Howard Hughes Medical Institute Predoctoral Fellow. This research was supported by Grant MH-61171 from the National Institute of Mental Health.

REFERENCES

Anchordoquy HC and Lynch GR (2000) Timing of testicular recrudescence in Siberian hamsters is unaffected by pinealectomy or long-day photoperiod after 9 weeks in short days. J Biol Rhythms 15:406-416.

Bartness TJ, Powers JB, Hastings MH, Bittman EL, and Goldman BD (1993) The timed infusion paradigm for melatonin delivery: what has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses? J Pineal Res 15:161-190.

Bronson FH (1989) *Mammalian Reproductive Biology*. Chicago: University of Chicago Press.

Carter DS and Goldman BD (1983) Antigonadal effects of timed melatonin infusion in pinealectomized male Djungarian hamsters (*Phodopus sungorus* sungorus): duration is the critical parameter. Endocrinology 113: 1261-1267.

Duncan MJ and Goldman BD (1984) Hormonal regulation of the annual pelage color cycle in the Djungarian hamster, *Phodopus sungorus*. I. Role of the gonads and pituitary. J Exp Zool 230:89-95.

Duncan MJ, Goldman BD, Di Pinto MN, and Stetson MH (1985) Testicular function and pelage color have different critical daylengths in the Djungarian hamster, *Phodopus sungorus* sungorus. Endocrinology 116:424-430.

Ebling FJ, Alexander IH, Urbanski HF, and Hastings MH (1995) Effects of N-methyl-D-aspartate (NMDA) on seasonal cycles of reproduction, body weight and pelage colour in the male Siberian hamster. J Neuroendocrinol 7:555-566.

Elliott JA and Goldman BD (1989) Reception of photoperiodic information by fetal Siberian hamsters: role of the mother's pineal gland. J Exp Zool 252:237-244.

Finley CM, Gorman MR, Tuthill CR, and Zucker I (1995) Long-term reproductive effects of a single long day in the Siberian hamster (*Phodopus sungorus*). J Biol Rhythms 10:33-41.

Freeman DA and Zucker I (2001) Refractoriness to melatonin occurs independently at multiple brain sites in Siberian hamsters. Proc Natl Acad Sci U S A 98: 6447-6452.

Goldman BD (2001) Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283-301.

Gorman MR (1995) Seasonal adaptations of Siberian hamsters. I. Accelerated gonadal and somatic development in increasing versus static long day lengths. Biol Reprod 53:110-115.

Gorman MR (2003a) Independence of circadian entrainment state and responses to melatonin in male Siberian hamsters. BMC Physiol 3:10-21.

Gorman MR (2003b) Melatonin implants disrupt developmental synchrony regulated by flexible interval timers. J Neuroendocrinol 15:1084-1094.

Gorman MR, Freeman DA, and Zucker I (1997) Photoperiodism in hamsters: abrupt versus gradual changes in day length differentially entrain morning and evening circadian oscillators. J Biol Rhythms 12:122-135.

Gorman MR and Zucker I (1995) Seasonal adaptations of Siberian hamsters. II. Pattern of change in daylength controls annual testicular and body weight rhythms. Biol Reprod 53:116-125.

Gorman MR and Zucker I (1997) Pattern of change in melatonin duration determines testicular responses in Siberian hamsters, *Phodopus sungorus*. Biol Reprod 56:668-673.

Hoffmann K (1973) The influence of photoperiod and melatonin on testis size, body weight, and pelage colour in

the Djungarian hamster (*Phodopus sungorus*). J Comp Physiol 85:267-282.

Hoffmann K (1978) Effects of short photoperiods on puberty, growth and moult in the Djungarian hamster (*Phodopus sungorus*). J Reprod Fert 54:29-35.

Hoffmann K (1982) The critical photoperiod in the Djungarian hamster *Phodopus sungorus*. In *Vertebrate Circadian Systems: Structure and Physiology*, Aschoff J, Daan S, and Groos G, eds, pp 297-304. New York: Springer.

Kauffman AS, Freeman DA, and Zucker I (2003) Termination of neuroendocrine refractoriness to melatonin in Siberian hamsters (*Phodopus sungorus*). J Neuroendocrinol 15:191-196.

Kelly KK, Goldman BD, and Zucker I (1994) Gonadal growth and hormone concentrations in photoregressed Siberian hamsters: pinealectomy versus photostimulation. Biol Reprod 51:1046-1050.

Kumar V, Lincoln GA, and Tortonese DJ (1993) Effects of excitatory amino acid receptor agonists and antagonists on the secretion of melatonin, luteinizing hormone and prolactin in the ram. J Neuroendocrinol 5:649-654.

Lincoln GA and Clarke IJ (1997) Refractoriness to a static melatonin signal develops in the pituitary gland for the control of prolactin secretion in the ram. Biol Reprod 57:460-467.

Lipton JS, Petterborg LJ, and Reiter RJ (1981) Influence of propranolol, phenoxybenzamine or phentolamine in the *in vivo* nocturnal rise of pineal melatonin levels in the Syrian hamster. Life Sci 28:2377-2382.

Prendergast BJ, Flynn AK, and Zucker I (2000) Triggering of neuroendocrine refractoriness to short-day patterns of melatonin in Siberian hamsters. J Neuroendocrinol 12:303-310.

Prendergast BJ, Nelson RJ, and Zucker I (2002) Mammalian seasonal rhythms: behavior and neuroendocrine substrates. In *Hormones, Brain and Behavior*, Vol. 2, Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, eds, pp 93-156. New York: Elsevier Science.

Prendergast BJ, Zucker I, Yellon SM, Ringold DA, and Gorman MR (1998) Melatonin chimeras alter reproductive development and photorefractoriness in Siberian hamsters. J Biol Rhythms 13:518-531.

Reiter RJ (1980) The pineal and its hormones in the control of reproduction in mammals. Endocr Rev 1:109-131.

Schlatt S, De Geyter M, Kliesch S, Nieschlag E, and Bergmann M (1995) Spontaneous recrudescence of spermatogenesis in the photoinhibited male Djungarian hamster, *Phodopus sungorus*. Biol Reprod 53:1169-1177.

Spears N, Finley CM, Whaling CS, Tuthill CR, and Zucker I (1990) Sustained reproductive responses in Djungarian hamsters (*Phodopus sungorus*) exposed to a single long day. J Reprod Fertil 88:635-643.

Stieglitz A, Gwinner K, Spiegelhalter F, Wicherek M, and Heldmaier G (1994) Urinary 6-sulphatoxymelatonin as an index of pineal function in the Djungarian hamster: influence of photoperiod and ambient temperature. In *Advances in Pineal Research 8*, Moller M and Pevet P, eds, pp 285-291. London: Libbey.

Tuthill CR, Freeman DA, Butler MP, Chinn T, Park JH, and Zucker I (2005) Perinatal influences of melatonin on testicular development and photoperiodic memory in Siberian hamsters. J Neuroendocrinol 17:483-488.

Weiner J (1987) Limits of energy budget and tactics in energy investments during reproduction in the Djungarian hamster (*Phodopus sungorus* sungorus Pallas 1770). Symp Zool Soc Lond 57:167-188.

Whaling CS, Kelly KK, Finley CM, Spears N, Licht P, and Zucker I (1993) Sustained hormonal responses of Siberian hamsters (*Phodopus sungorus*) to a single longer day at weaning. Biol Reprod 49:555-560.

Yellon SM and Goldman BD (1984) Photoperiod control of reproductive development in the male Djungarian hamster (*Phodopus sungorus*). Endocrinology 114:664-670.