

Melatonin Implants Disrupt Developmental Synchrony Regulated By Flexible Interval Timers

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Abstract

Siberian hamsters born into short daylengths near the end of the breeding season are reproductively inhibited from birth and delay gonadal maturation until the following spring. This vernal transition to a reproductive phenotype coincides with an abrupt increase in body weight, and both processes are triggered by an interval timing mechanism that becomes insensitive, or refractory, to short-day inhibition. It was previously demonstrated that hamsters born into simulated natural photoperiods in early August became photorefractory at later ages than hamsters born into September photoperiods. As a consequence of flexibility in the duration programmed by the interval timer, development of seasonal birth cohorts was synchronous with respect to the calendar date simulated by laboratory photoperiod. In the present study, hamsters were born into simulated August or September photoperiods. Hamsters from each cohort were given removable constant release melatonin implants to reversibly obscure the neuroendocrine representation of daylength between 3 and 9 weeks or 9–15 weeks of age. When control hamsters were given beeswax capsules throughout, August-born males were approximately 6 weeks older than September males at the onset of photorefractoriness as assessed by accelerated increases in body weight and testicular size. Females exhibited the same pattern in body weight. These measures were synchronized with respect to calendar date. Synchronization of cohorts was disrupted by melatonin capsules from 3–9 weeks of age but not by later implants. Melatonin implants altered synchronization by influencing the developmental trajectory of September-born hamsters without influencing the August cohort. These results demonstrate that the function of the interval timer underlying photorefractoriness is influenced by photoperiod and by melatonin. The endogenous pattern of melatonin signals adjusts the duration measured by the interval timer to insure that developmental milestones of seasonal cohorts are synchronized with environmental conditions.

Many mammalian species exhibit yearly fluctuations in physiology and behaviour that can be reproduced in the laboratory with simulations of the annual pattern of changing daylength (1, 2). Two categories of seasonality mechanisms have been contrasted (3). In mammals designated as circannual, seasonal rhythms persist even if ambient daylengths are unchanging, but the period of the rhythm generally differs from 12 months. Under natural conditions, the annual light/dark cycle entrains circannual rhythms by phase-advancing or phase-delaying them. Other species, designated as photoperiodic, do not express annual rhythms when ambient conditions are constant (4, 5). Rather, the autumn/winter phenotype may be induced only after daylengths decrease below a critical value. However, the transition to the spring/summer phenotype is driven not by increasing daylengths, but by an endogenously timed process. Initial short-day exposure triggers an interval-timer mechanism that, after approximately

20 weeks of photoperiodic inhibition, prompts animals to revert to the long photoperiod state (6). Photoperiodic processes are generally mediated by the duration of elevated melatonin secreted by the pineal gland: short melatonin signals characteristic of summer nights (e.g. 4–6 h) induce long-day effects, and long melatonin signals of winter (e.g. 10–12 h) mediate short-day effects (7, 8).

The Siberian hamster, *Phodopus sungorus*, is a photoperiodic species that concentrates breeding in spring and summer months, when daylengths are long (9–12). In most adult hamsters, the gonads undergo structural and functional regression as daylengths decrease in late summer. To reduce energetic expenditure, hamsters also reduce body weight, molt to a winter pelage and undergo daily torpor (13, 14). Reproductive and somatic development of prepubertal hamsters is similarly affected by photoperiod (15). Consequently, animals born early in the breeding season mature rapidly and may breed in the season of their birth, whereas young

born late in the breeding season into decreasing daylengths gain weight more slowly and delay reproductive maturation until the following breeding season. Because some adult hamsters continue breeding even after daylengths decrease in late summer, there may be several months in the late summer and early fall in which the newly weaned litters are photo-inhibited (16). For all of these hamsters, puberty is timed by the interval timer and is initiated when daylengths are still very short. If the interval timer operates similarly in cohorts of animals born at different times at the end of the breeding season, these cohorts would be expected to mature asynchronously over several months in late winter/early spring. Alternatively, if the function of the interval timer is to render animals reproductive at the earliest favourable time in spring, then it would need to operate differently in hamsters born into different inhibitory daylengths.

A previous study demonstrated that the duration measured by the interval timer was indeed flexible, so that cohorts born at different phases of the previous breeding season were rendered synchronous in terms of the calendar date of gonadal and somatic development (16). Amongst male hamsters born into an August daylength of a simulated natural photoperiod (SNP) of 50°N latitude, gonadal growth was first detected around a simulated date of January 29 ± 3 days, when hamsters were approximately 25 weeks old. Hamsters born in late September initiated gonadal growth at almost exactly the same date (February 5 ± 3 days). However, because this latter group was born later in the previous summer, they were only 20 weeks old at onset of gonadal growth (16). Body weight trajectories were similarly asynchronous with respect to age, but synchronous with respect to calendar date. Finally, the different durations programmed by the interval timer depended on postnatal rather than prenatal daylength exposure: hamsters born into August daylengths and phase-advanced at birth to September photoperiod conditions more nearly resembled the September-born cohort.

Although of heuristic value, the distinctions drawn between circannual and photoperiodic species may overlook fundamental similarities between these two types of mechanisms. In both cases, photoperiod-sensitive mechanisms result in synchronization of physiological transitions with environmental conditions. Mechanistically, the photoperiodic modulation of the duration programmed by the interval timer in late summer-born cohorts is reminiscent of the entraining action of light and melatonin on circannual rhythms of longer-lived mammals (17–20). The present experiment therefore examined whether manipulations of the endogenous melatonin signal would alter the timing of processes triggered by the interval timer. To create a time-restricted manipulation of melatonin, hamsters were given constant release melatonin capsules that would functionally obscure the endogenous melatonin secretion encoding the daylength signal (21). Capsules could then be removed to restore an intact photoperiodic mechanism. A common property of entrainment mechanisms is that a perturbing stimulus can exert different actions at various phases of the cycle. Accordingly, the role of melatonin implants was assessed at two different time points.

Materials and methods

Siberian hamsters, *Phodopus sungorus*, were housed at 22 ± 2 °C in polypropylene cages (27 × 16 × 13 cm) and provided with food (Mouse Chow #5015, Purina Mills, St Louis, MO, USA) and water *ad libitum* throughout the experiment. Adult female hamsters (n = 39), maintained from birth in 15 h light and 9 h dark daily (15L; lights

out 18.00 h, 100–400 lux light intensity), were paired with similarly housed males. Beginning 12 days later, pairs were exposed to a simulated natural photoperiod of 50°N latitude set for 15 July when daylength = 15 L. On subsequent days, daylengths decreased gradually under the control of Paragon EC71ST Suntracker latitudinal timers (Two Rivers, WI, USA) that varied light onset and offset to match dawn and dusk, respectively (Fig. 1). All dates reported below correspond to photoperiod simulations, and all were approximately 1 month out of phase with the calendar year. Males were removed 16 days after pairing (6 August) and returned to 15 L. Approximately one month later (1 September), males were returned to the SNP50 and paired again for 16 days with the same females as before (Fig. 1).

Offspring of both birth cohorts remained in SNP50 from their respective births until the winter solstice (22 December), at which point the daylength was fixed at the solstice value (approximately 8 L; 7 h 48 min) until the experiment was ended 24 weeks later (Fig. 1). Note that the reference to calendar dates is continued after the winter solstice despite the fact that daylengths were no longer permitted to change (e.g. 1 month after the winter solstice is referred to as 22 January even though daylength remains at 8 L). To assess the role of the endogenously generated, gradually changing pattern of melatonin signals on interval timer function, hamsters were given constant release melatonin implants or beeswax capsules from 3–9 or from 9–15 weeks of age. The two cohorts of offspring were treated identically with respect to their age, but because they were born approximately 6 weeks apart, the daylength at which each manipulation occurred differed between groups. Table 1 summarizes sample sizes and the ambient daylength at the critical stages of the experiment. Male and female offspring were weighed and weaned into same-sexed groups (2–3 per cage) at 21 days of age. Hamsters were anaesthetized with isoflurane vapors (Aerrane; Fort Dodge Animal Health, IA, USA), weighed, and each implanted s.c. with two 8-mm capsules containing melatonin (one-third of hamsters) or beeswax alone (two-thirds of hamsters) intrascapularly. At 9 weeks of age, melatonin capsules were removed and replaced with two blank (beeswax) capsules (Group Mel/Blank). Blank (beeswax) capsules were removed and replaced with two melatonin capsules (one-third of hamsters; Group Blank/Mel) or with two additional blank capsules (1/3 of hamsters; Group Blank/Blank). At 15 weeks of age, all implanted capsules were removed. Litter- and cage-mates were generally assigned different hormone treatments. Males and females were weighed weekly. At 3-week intervals, beginning at weaning, testis width and length was measured in males under light isoflurane anaesthesia. In unanaesthetized females, whether the vagina was open (patent) or closed was assessed weekly from 15 weeks of age.

Melatonin implants were prepared by mixing one part melatonin (Sigma, St Louis, MO, USA) in 24 parts melted beeswax and aspirating the solution into Silastic tubing (1.47 mm inner diameter × 1.96 mm outer diameter; Dow Corning, Midland, MI, USA). Blank capsules filled only with beeswax were similarly prepared. The cooled tubing was cut into 8-mm lengths. The biological efficacy of melatonin implants was verified in a separate controlled study by demonstrating a lack of a photostimulatory response amongst gonadally regressed male hamsters transferred from short (10L) to long daylengths (15L; data not shown).

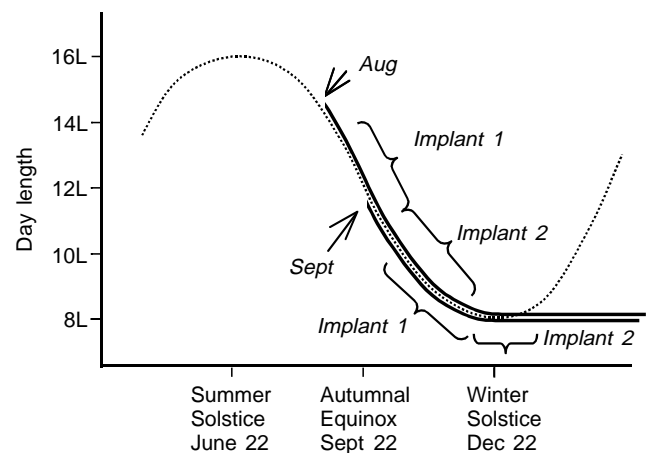


Fig. 1. Schematic representation of photoperiodic stimuli illustrated in the context of the yearly photocycle at 50°N latitude (dashed line). Litters were born into early August (AUG; solid line just above dashed line) or late September (SEPT; solid line just below dashed line). At the winter solstice hamsters remained in 8 L. Brackets indicate times when animals were implanted with melatonin or blank capsules. For exact dates and photoperiods of manipulations, see text and Table 1.

TABLE 1. Simulated Dates and Corresponding Daylengths (DL) of August- and September-Born Hamsters at Times of Experimental Manipulation and Conditions Obscured by Melatonin Implants.

Experimental event	August-born cohort		September-born cohort	
	Date	DL	Date	DL
Date of birth	12 August	14:11	24 September	11:35
First implants (week 3)	02 September	13:02	15 October	10:23
Second implants (week 9)	14 October	10:26	26 November	08:11
Implants removed (week 15)	25 November	08:13	07 January	07:52

Implant group	August-born cohort			September-born cohort		
	Obscured dates	Obscured DLs	n (M/F)	Obscured dates	Obscured DLs	n (M/F)
Blank/Blank	–	–	24/25	–	–	19/22
Mel/Blank	2 Sep to 14 Oct	13:02 to 10:26	24/24	15 Oct to 26 Nov	10:23 to 8:11	28/22
Blank/Mel	14 Oct to 25 Nov	10:26 to 8:13	27/23	26 Nov to 7 Jan	8:11 to 7:52	22/23

M, Males; F, females.

Estimated testis volume (ETV) was calculated as testis length \times width \times width, yielding an index that correlates highly with paired testis weight with correlation coefficients routinely exceeding 0.90 over the range of 80–800 mg (22). Below this range, differences in gonad size are not reliably determined with external measurements.

Nonresponders

Male offspring were considered photononresponsive if the testes failed to regress below 250 units (approximately 225 mg paired testis weight) by the winter solstice and they exhibited a dark summer pelage. This threshold differentiated responders and nonresponders in a study with similar methodology (22). Amongst females, nonresponsiveness was inferred by a dark pelage and patent vagina. Locomotor activity rhythms of all putative nonresponders and four photoresponsive hamsters were monitored to corroborate that the former were atypically entrained to the short daylengths (23). All nonresponders were excluded from subsequent analyses.

Statistical analyses

The data were analysed for acute and long-term effects of hormone manipulation. For acute effects, body weights were compared during each of the two intervals of hormone implants (3–9 and 9–15 weeks of age, respectively). Long-term effects were assessed from raw body weights and from weekly weight gains following removal of implants. For the latter measure, the weekly change in body weight was calculated and smoothed with a 3-week moving average. Compared to raw body weights, which require a large ordinate when plotted over long timeframes (e.g. 30–55 g), weekly weight gain data can be plotted on a more restricted scale (0.0–2.5 g) and more clearly illustrate changes in body weight regulation over time.

The role of melatonin in seasonal timing was assessed using both within-cohort and between-cohort analyses. Within cohorts, a repeated measures ANOVA (Statview 5.0; SAS Institute; Cary, NC, USA) assessed whether melatonin implants altered patterns of somatic and gonadal maturation in each of the two seasonal cohorts. Between-cohort repeated measures ANOVAs were conducted to assess whether the two seasonal cohorts were better synchronized with respect to time of year or with respect to age. This was examined separately for hamsters in each of the experimental endocrine conditions.

As would be expected, body weights and weekly weight gains varied significantly over time in each repeated measures analysis (main effect of time), and so these data are not reported. Also not reported are generally nonsignificant main effects of implant condition or of seasonal cohort. Results of these analyses are reported in the very rare ANOVAs in which these main effects reached statistical significance. Also reported are all significant and non-significant interactions of time \times implant condition and of time \times birth cohort, which reflect group differences (or lack thereof) in the temporal patterning of body weight or weight gain over the analysis interval. Group differences at individual time points were assessed using between-subjects ANOVAs and, where significant, followed by Fisher's PLSD test. Kaplan–Meier survival analysis (Statview 5.0) was used to assess effects of birth cohort and hormone condition on the week of first vaginal opening and on testis growth coded as the first point ETV exceeded 250 units. This size corresponds to the appearance of

substantial spermatids in the recrudescing testis (24). Identical statistical analyses that used a lower threshold, indicative of the onset of testicular growth (ETV >150 units) (16), yielded similar conclusions and are thus not reported.

Results

Breeding and photoresponsiveness

Twenty-seven females (69%) bore litters within a 14-day interval beginning on a simulated 2 August photoperiod. Upon pairing a second time with males, 26 of 39 adult females (67%) gave birth (Group SEPT). Few male offspring were photononresponsive (10 of 154, 6.5%), and these nonresponders were not differentially distributed between birth cohorts (chi squared = 0.02, d.f. = 1, $P > 0.8$) or implant condition (chi squared = 1.1, d.f. = 2, $P > 0.5$). A comparably low incidence was observed amongst female offspring (six of 146, 4.1%) and, again, these were randomly distributed with respect to implant condition (chi squared = 2.9, d.f. = 2, $P > 0.2$). By contrast to males, however, all female nonresponders were born in August photoperiods (chi squared = 5.3, d.f. = 1, $P < 0.05$). All male and female nonresponsive offspring were omitted from subsequent analyses.

Acute actions of melatonin on each cohort

In male hamsters, melatonin had only very minor acute effects on body weight. Amongst the August-born cohort (Fig. 2A), the body weight trajectory of melatonin-implanted hamsters was steeper than that of blank-implanted controls over weeks 3–9 [$F(6,438) = 3.8$, $P < 0.001$], but body weights differed significantly at no single time point. Similarly, a significant time–implant interaction was found over weeks 9–15 [$F(12,432) = 2.6$, $P < 0.01$] driven by significantly different trajectories of Blank/Mel versus Mel/Blank hamsters [$F(4,294) = 4.1$, $P < 0.001$]. Groups differed significantly from one another only at week 10; however, when Mel/Blank hamsters were significantly heavier than Blank/Blank hamsters ($P < 0.01$). Amongst September-born males (Fig. 2B), melatonin capsules had no effect on body weight trajectories during weeks 3–9 [$F(6,402) = 0.4$, $P > 0.8$] or over weeks 9–15 [$F(12,396) = 1.0$, $P > 0.4$]. With the exception of photononresponsive hamsters (see below), testis size was almost universally below

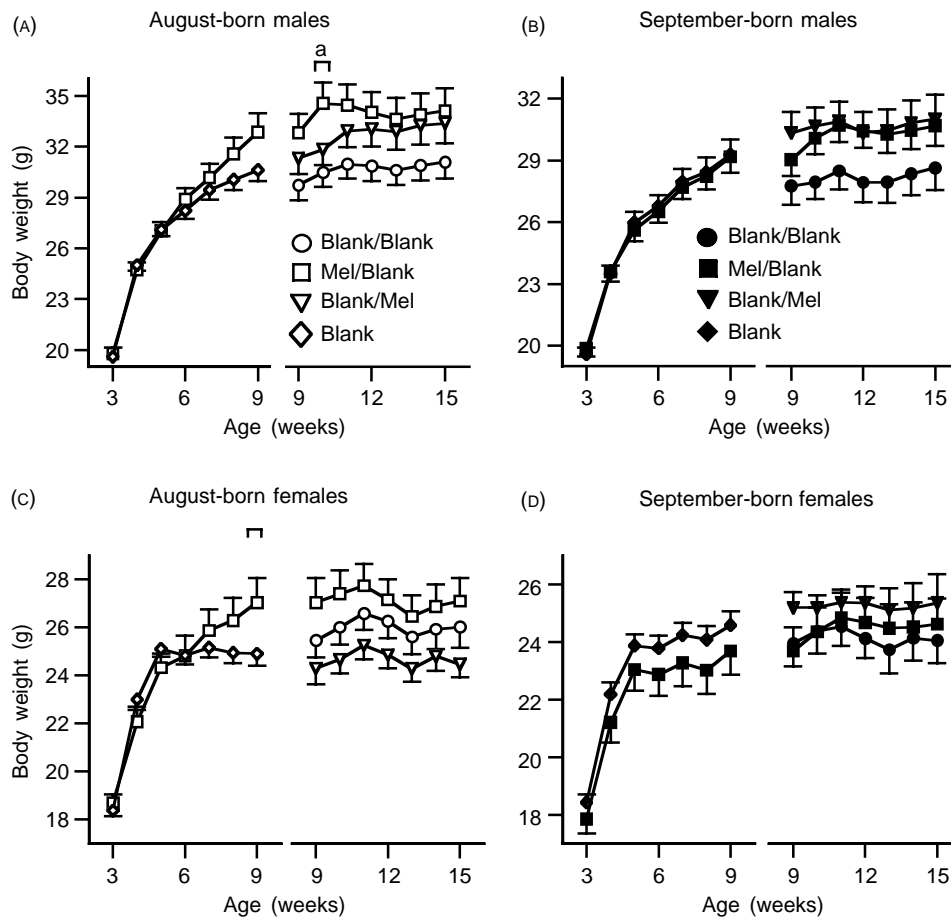


FIG. 2. Acute effects on melatonin or beeswax implants on mean (\pm SEM) body weights of August-born (open symbols) and September-born (filled symbols) male and female hamsters. Brackets above graphed means indicate time points where body weights differ significantly between implant conditions ($P < 0.05$). Letters above brackets indicates which two groups differ significantly (^aBlank/Blank differs from Mel/Blank). Sample sizes ($n = 19$ – 28) are indicated precisely in Table 1.

measurable levels in both seasonal cohorts, and thus no influence of melatonin implants was discernible before week 15 (data not shown).

The acute response of females to melatonin versus blank capsules was very similar to that of males: For August-born females, melatonin-exposed hamsters showed steeper body weight trajectories than did blank-implanted controls (Fig. 2C) [$F(6,420) = 8.2$, $P < 0.001$]. By week 9, those previously implanted with melatonin were significantly heavier than controls ($P < 0.05$). The temporal patterning of body weights of August-born females did not differ with implant condition over weeks 9–15 [$F(12,414) = 0.4$, $P > 0.9$]. Similarly, September-born female hamsters showed no evidence of different patterns of body weight over weeks 3–9 (Fig. 2D) [$F(6,390) = 0.3$, $P > 0.9$] or during weeks 9–15 [$F(12,384) = 0.6$, $P > 0.8$].

Long-term actions of melatonin on body weight of each cohort

Having reached a plateau around 9 weeks of age (Fig. 2), body weights increased again sometime after 15 weeks of age. Figure 3 plots body weights and weekly weight gains of hamsters following the removal of the blank or melatonin capsules at 15 weeks of age. Amongst males born into August daylengths (Fig. 3A), the type of implant had no significant effect on the patterns of body weight

[$F(42,1470) = 0.8$, $P > 0.8$]. When weekly weight gains were plotted and analysed (Fig. 3B), again there was no significant time–implant interaction [$F(40,1400) = 0.7$, $P > 0.9$]. By contrast, absolute body weights as well as weekly weight gains of September-born males were markedly affected by the hormone treatment (Figs 3C,D). Body weights of the three groups differed overall [main effect of implant; $F(2,1386) = 3.4$, $P < 0.05$] and over time [implant–time interaction; $F(42,1386) = 6.8$, $P < 0.001$]. Figure 3(C) identifies statistically significant group differences in body weight from 18–31 weeks of age. The weekly weight gain data more clearly illustrate the differential effects of implants (Fig. 3D) [$F(40,1320) = 7.8$, $P < 0.001$]. In brief, weekly weight gains of Mel/Blank hamsters peaked later than those of Blank/Blank hamsters, whereas Blank/Mel males were most advanced. Weekly weight gains differed significantly between two or more groups throughout most of the interval analysed.

Body weight trajectories of August-born females, similar to those of males, did not differ statistically between hormone treatments (Fig. 3E) [$F(42,1407) = 0.8$, $P > 0.8$]. The same null effect is apparent in the weekly weight gain data (Fig. 3F) [$F(2,1340) = 0.9$, $P > 0.6$]. However, body weight patterns of September-born females were markedly influenced by hormone condition (Fig. 3G) [$F(42,1344) = 2.8$, $P < 0.001$] reflecting the

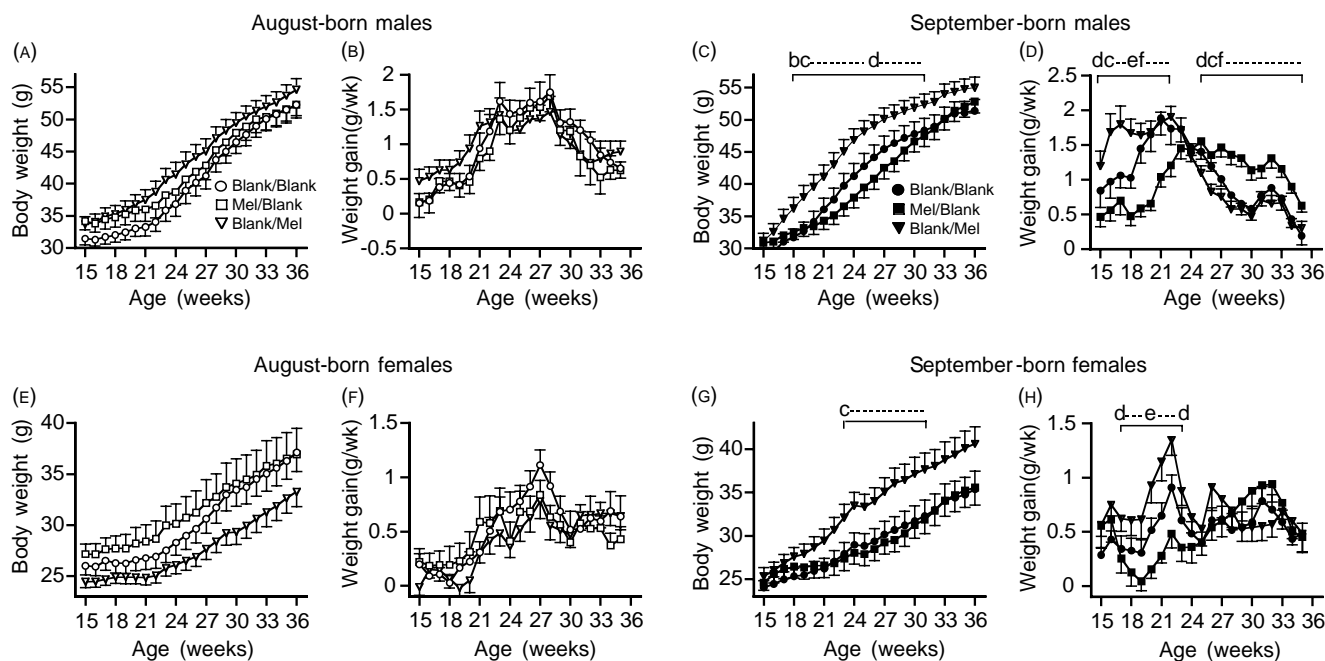


FIG. 3. Long-term effects of melatonin or beeswax implants on mean (\pm SEM) body weight and weekly weight gain of August-born (left) and September-born (right) male (top) and female (bottom) hamsters. Data are plotted from removal of the second implant (15 weeks of age) until the end of the experiment. Sample sizes ($n = 19$ – 28) are indicated precisely in Table 1. Horizontal brackets above data indicate weeks where groups differed significantly from one another. Letters above brackets specify which groups differ significantly ($P < 0.05$; ^aBlank/Blank versus Blank/Mel; ^bBlank/Mel versus other two groups; ^cBlank/Mel versus Mel/Blank; ^dall groups differ; ^eMel/Blank versus other two groups). Dashed line following a letter indicates that the immediately prior group differences persist. For clarity, varying level of statistical significance (e.g. $P < 0.01$ and $P < 0.001$) are not distinguished in the figure. Other conventions are as in Fig. 2.

earlier weight gain of Blank/Mel females compared to other groups. Analysis of weekly weight gains revealed relatively early and late peaks for Blank/Mel and Mel/Blank females, respectively (Fig. 3H) [$F(2,1280) = 2.8$, $P < 0.001$]. Figure 3(H) also indicates time points in which weekly weight gains differed between experimental groups.

Between-cohort synchrony in body weight increases

To assess the role of melatonin signals for between-cohort synchrony of developmental milestones, data from August- and September-born cohorts in each implant condition were compared for synchrony with respect to chronological age and to calendar date (Fig. 4). Because weekly weight gain can be plotted on a more restricted ordinate, only these data are presented here, although analysis of raw body weights leads to similar conclusions. Age-matched control males given only blank capsules attained peak weight gains earlier if they were born into September versus August daylengths (Fig. 4A) [time-cohort interaction: $F(20,800) = 6.7$, $P < 0.001$]. However, adjusted for the season of the year, the weight gain patterns were largely superimposable and not significantly different (Fig. 4D) [$F(20,800) = 1.2$, $P > 0.2$]. Whereas weekly weight gains differed significantly from 15–21, 28–31 and 35 weeks of age, with respect to season of the year, they differed only during the simulated weeks of 2 December and 23 December.

The opposite pattern was observed amongst male hamsters given melatonin implants from 3–9 weeks of age (Figs 4B,E). Despite being born and raised in different photoperiods, patterns of weekly weight gain did not differ significantly between groups matched for age [$F(20,1000) = 1.1$, $P > 0.3$].

Weight gain patterns did differ between cohorts when matched for the season of the year [$F(20,1000) = 7.1$, $P < 0.001$]. Accordingly, weekly growth rates were scarcely different at any age (weeks 32–33), but did differ significantly on the weeks of 2 December, 23 December, 27 January to 24 February and 24 March to 21 April.

Similar to beeswax-implanted controls, males with melatonin capsules from weeks 9–15 gained weight asynchronously with respect to age (Fig. 4C) [$F(20,920) = 6.3$, $P < 0.001$]. Unlike controls, they were not statistically synchronized with respect to time of the year (Fig. 4F) [$F(20,920) = 2.2$, $P < 0.01$]. Weekly weight gains of September-born males exceeded those of August-born from 15–22 weeks of age and were lower from weeks 26–29 and from weeks 34–35. Driving the significant interaction for weight gain data matched for the calendar year, September-born hamsters grew less in December, but were otherwise largely synchronous with August-born hamsters.

August- and September-born females implanted only with beeswax capsules exhibited significantly different weight gain patterns when matched for age (Fig. 4G) [$F(20,900) = 3.0$, $P < 0.001$]. Birth cohorts differed significantly from one another on most weeks from 18–28 weeks of age. However, weight gain patterns were not significantly different when these control females were matched for calendar date (Fig. 4J) [$F(20,900) = 1.4$, $P < 0.1$]. Significantly different weight gains were observed only on the week of 16 December and from 10 February to 3 March.

By contrast, August versus September females given melatonin implants from 3–9 weeks of age exhibited significantly different weight gain patterns regardless of whether they were matched for age (Fig. 4H) [$F(20,860) = 2.1$, $P < 0.01$] or for calendar date (Fig. 4K) [$F(20,860) = 2.4$; $P < 0.001$]. Matched for age,

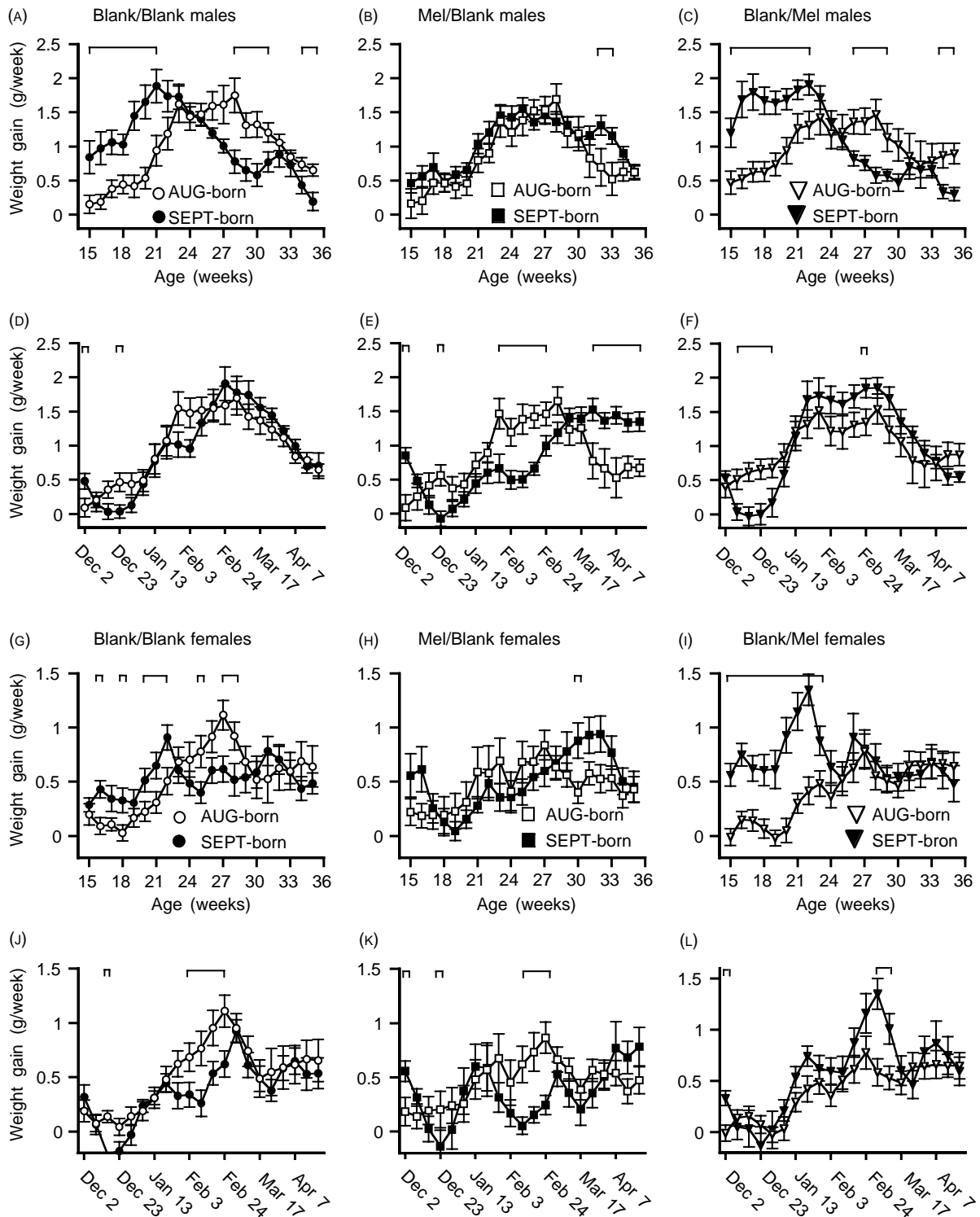


FIG. 4. Mean \pm SEM weekly weight gains of male (top) and female (bottom) hamsters plotted to evaluate synchrony between August- and September-born animals considered with respect to age (A–C; G–I) and to calendar date (D–F; K–M). Shown separately are hamsters implanted with beeswax alone (Blank/Blank; left column), melatonin followed by beeswax (Mel/Blank; centre column), beeswax followed by melatonin (Blank/Mel; right column). Brackets above means indicates that weekly weight gains of August and September cohorts differ significantly at that time ($P < 0.05$).

August- versus September-born cohorts differed significantly only at 30 weeks whereas, matched by simulated calendar date, they differed significantly in the week of 2 December and from 10 February to 3 March.

Amongst Blank/Mel females, patterns of weight gain were asynchronous both with respect to age (Fig. 4I) [$F(20,860) = 4.4, P < 0.0001$] and to calendar date (Fig. 4L) [$F(20,860) = 1.9, P < 0.01$]. Weight gain differed between birth cohorts from

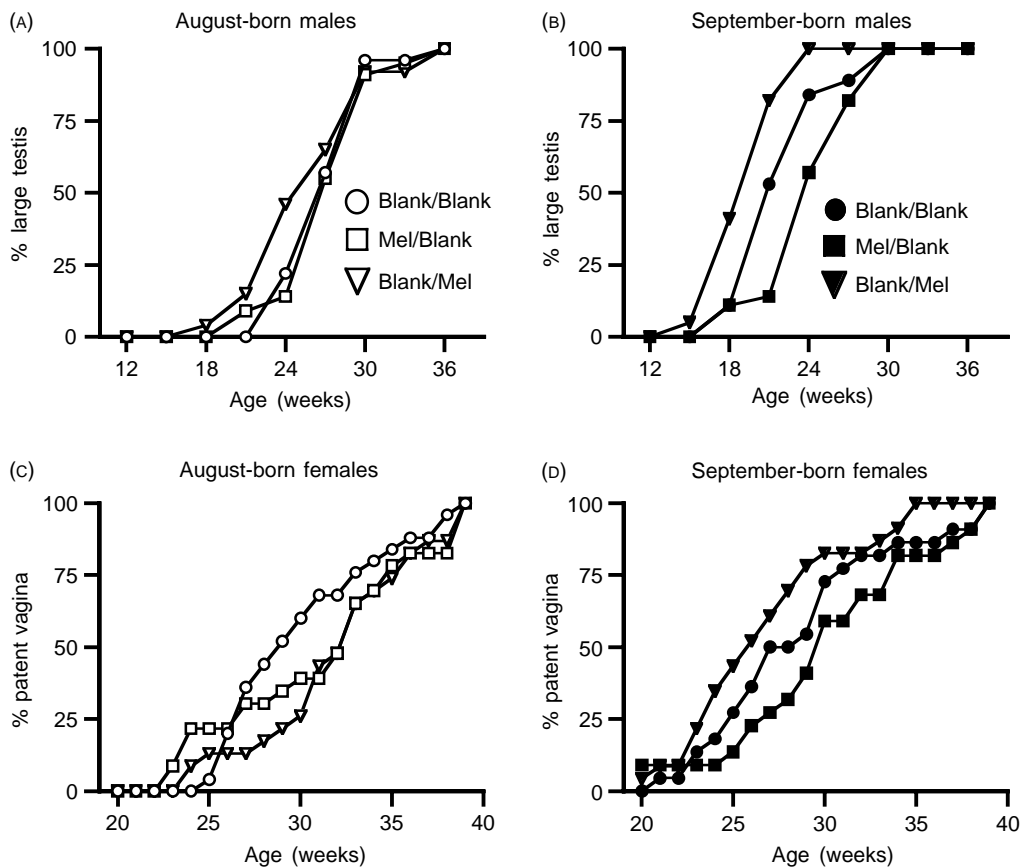


FIG. 5. Cumulative percentage of August-born (A,C) and September-born (B,D) hamsters having attained a threshold level of reproductive maturation plotted as a function of hormone implant condition. For males (A,B), the threshold was an ETV exceeding 250 units. For females (C,D), the vagina needed to be patent.

15–23 weeks of age but, rendered by calendar date, only during the weeks of 2 December and 3–17 March.

Reproductive development

Amongst August-born males, there was no influence of hormone treatment on the timing of gonadal maturation (Fig. 5A) (chi squared = 1.4, d.f. = 2, $P > 0.4$; Mandel-Cox logrank survival analysis). By contrast, September-born hamsters were markedly affected by the capsule content (Fig. 5B) (chi squared = 25.3, d.f. = 2, $P < 0.001$). Relative to blank controls, Mel/Blank capsules delayed gonadal maturation in September-born males (chi squared = 4.5, d.f. = 1, $P < 0.05$) whereas Blank/Mel capsules significantly advanced gonadal growth (chi squared = 7.3, d.f. = 1, $P < 0.01$).

In each of the implant conditions, August-born hamsters developed their gonads at significantly later ages compared to September-born hamsters (Fig. 6A–C) (chi squared = 16.4, d.f. = 1, $P < 0.001$ for Blank/Blank; chi squared = 8.3, d.f. = 1, $P < 0.01$ for Mel/Blank; and chi squared = 27.5, d.f. = 1, $P < 0.001$ for Blank/Mel). However, when development times were expressed as calendar dates, no differences between cohorts was noted for Blank/Blank or Blank/Mel hamsters (Figs 6D,F) (chi squared = 0.8, d.f. = 1, $P > 0.3$; chi squared = 0.6, d.f. = 1, $P > 0.40$, respectively). With Mel/Blank capsules, August-born males were reproductive at an earlier date than the September-born cohort (Fig. 6E) (chi squared = 7.8, d.f. = 1, $P < 0.01$).

In females, the effects of hormone implants on reproductive maturation were also cohort specific. The age of vaginal opening did not vary with implant condition in August-born females (Fig. 5C) (chi squared = 2.4, d.f. = 2, $P > 0.30$) but was affected by hormone treatment in September-born females (Fig. 5D) (chi squared = 7.3, d.f. = 2, $P < 0.05$). In terms of pairwise comparison, Blank/Mel hamsters matured at earlier ages than Mel/Blank females (chi squared = 7.4, d.f. = 1, $P < 0.01$), but no other pairings were significantly different (Fig. 5D).

Amongst control hamsters and those given melatonin from weeks 3–9, cohorts did not differ with respect to age at vaginal opening (Fig. 6G,H) (chi squared = 0.3, d.f. = 1, $P > 0.5$ and chi squared = 0.3, d.f. = 1, $P > 0.6$ for Blank/Blank and Mel/Blank, respectively). However, both groups were asynchronous with respect to calendar date (Fig. 6J,K) (chi squared = 6.2, d.f. = 1, $P < 0.05$ and chi squared = 6.1, d.f. = 1, $P < 0.05$). By contrast, females given melatonin from 9–15 weeks of age were asynchronous in terms of age of vaginal opening (Fig. 6I) (chi squared = 13.3, d.f. = 1, $P < 0.001$) but synchronous in relation to calendar date (Fig. 6L) (chi squared = 0.001, d.f. = 1, $P > 0.99$).

Discussion

Because daylengths were fixed at approximately 8 L after the winter solstice, the transition to a spring/summer phenotype was programmed by an interval timer mechanism that reflects an

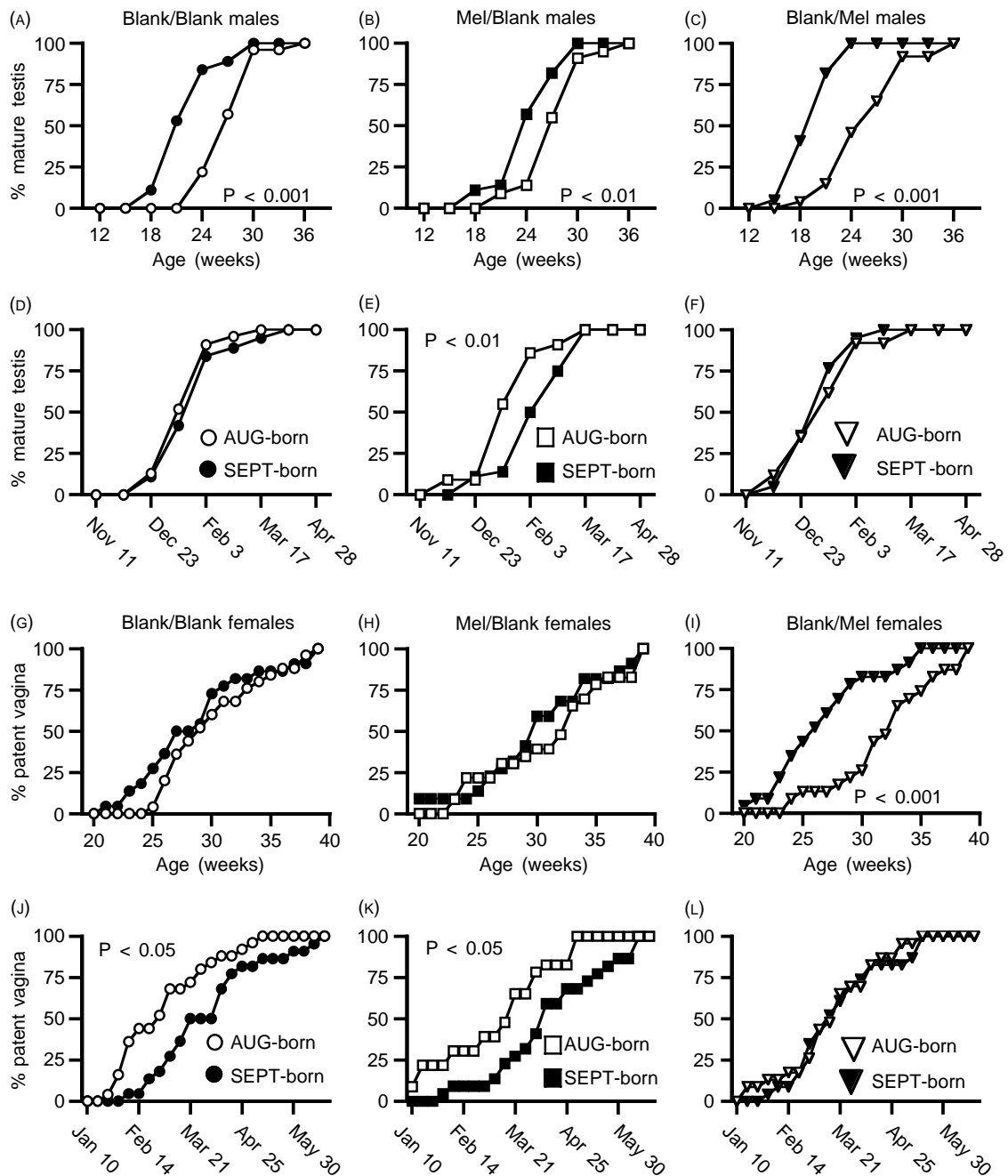


FIG. 6. Cumulative percentage of male (A–F) and female (G–L) hamsters attaining threshold levels of reproductive maturation plotted to evaluate between-cohort synchrony with respect to age (A–C; G–I) and to calendar date (D–F; J–L). Shown separately are control hamsters implanted with only blank capsules, melatonin followed by blank capsules, and blank followed by melatonin capsules. Where present, P values reflect statistically significant time–group interactions (see text for details).

acquired refractoriness to short-day inhibition. Replicating an earlier study (16), August-born males treated only with beeswax initiated gonadal development and resumed somatic growth at a later age than did hamsters born 6 weeks later into September daylengths. The same was true of female body weight in both studies, but first vaginal opening, recorded only in the present report, followed a different pattern as discussed below. The developmental differences with respect to age rendered the August and September cohorts of control hamsters synchronous with respect to calendar date. However, as summarized in Table 2,

the synchronization of cohorts with respect to calendar date or to age could be reversed or disrupted with melatonin implants depending on the time of administration of melatonin. Furthermore, the modulation of the developmental timing was largely a result of plasticity in the September-born cohort for whom the timing of photorefractoriness could be advanced or delayed. By contrast, the interval timer of the August-born cohort was unaffected by melatonin capsules. Thus, the endogenous melatonin signal is critical for seasonal adjustments in the duration programmed by the interval timer.

TABLE 2. Type of Synchronization, If Any, Between August and September Birth Cohorts Observed For Each Dependent Variable.

Treatment	Male somatic growth*	ETV >250 units†	Female somatic growth*	First vaginal opening†
Blank/Blank	Date	Date	Date	Age
Mel/Blank	Age	None	Age	Age
Blank/Mel	Date	Date	Date	Date

*For somatic measures, entry reflects matching procedure (hamster age versus simulated calendar date) that generated fewer weeks with statistically significant differences between birth cohorts. †For reproductive measures, entry reflects matching procedure that yielded no significant time-cohort interaction (see text for details).

In previous studies that employed subcutaneous melatonin implants in pineal-intact hamsters, the marked diurnal variation in serum melatonin titres was abolished as a consequence of elevated daytime levels, but rhythmicity in pineal melatonin content persisted (21). As the duration of elevated circulating melatonin concentrations is the most critical parameter mediating photoperiodic effects in mammals (8), these implants would obscure the neuroendocrine encoding of ambient daylength. Discrimination of ambient daylength is indeed largely abolished after melatonin implants (25) but can be restored following their removal (26), indicating that this manipulation represents a reversible disruption of the photoperiodic time measurement system (specifically the reading of a distinct melatonin signal).

The use of removable implants does not simply 'inactivate' or 'blind' the photoperiodic time measurement system but, like any other manipulation (e.g. pinealectomy, timed melatonin infusions), may constitute a photoperiodic signal of its own. Unlike the case in adult Syrian hamsters where melatonin capsules promote or maintain gonadal regression in photostimulated hamsters (27), gonad size of adult Siberian hamsters is largely unaffected for 2 months following implantation (25). However, among juvenile Siberian hamsters, implants at 15 days of age inhibit gonadal development regardless of photoperiod conditions (28). In the current study, the melatonin implants in 21-day-old hamsters may have conceivably acted either by perpetuating the previous photo-inhibited condition or by directly inhibiting reproductive development.

If all inhibitory photoperiodic signals were functionally equivalent, then hamsters from all groups would be expected to exhibit identical developmental trajectories as a function of age. Seasonal cohorts of hamsters clearly matured at different ages, and melatonin implants advanced or delayed seasonal transitions amongst the September-born cohort. For September-born hamsters, developmental timing was altered when melatonin implants interfered with the encoding of daylengths that were very short and changing modestly, from 10 h 23 min to 8 h 11 min in one case (Mel/Blank) and from 8 h 11 min to 7 h 52 min in another (Blank/Mel). If melatonin implants act as intrinsically inhibitory signals, then they must nonetheless be interpreted differently from the robustly inhibitory melatonin signals generated endogenously during weeks 3–9 or 9–15. A similar challenge to the simple photoperiodic model arises if melatonin implants act by perpetuating the prior photoperiodic state (25). By 9 weeks of age, all hamsters have robust inhibitory histories and yet melatonin implants at this time accelerate developmental trajectories in the September-born cohort. Thus, regardless of the mode of implant action, the patterns of development in the present study cannot be readily explained by a simple photoperiodic model that distinguishes only between melatonin or daylength signals above and below a critical duration.

If a simple photoperiodic model is inadequate, the present results might be better understood in terms of an entraining model of melatonin action. In circannual species, the seasonal transitions from one reproductive and metabolic state to another persist in the absence of annual changes in daylength, albeit with a period generally not equal to 1 year. In both ground squirrels and sheep, the pineal gland is necessary for entrainment of the endogenous rhythm to match the annual photoperiod cycle (19, 29). Moreover, in the latter species, annual rhythms can be entrained by a 3-month pattern of daily melatonin signals that mimics the endogenously generated profile (19, 30), but this hormone replacement of the endogenous signal is not equally effective at every time of the year. Amongst the effective signals, the season of melatonin replacement influences phase angle of entrainment, suggesting that the endogenous rhythm is differentially advanced or delayed as a function of the seasonal signal.

Although the self-sustained circannual rhythms of squirrels and sheep are often considered distinct from the photoperiod driven rhythms of hamsters, which do not persist in constant conditions (2), these classes of rhythms nevertheless share a number of features. First, a functional outcome of entrainment of an endogenous mechanism is synchronization with the environment and with other animals. Given that August- and September-born cohorts are exposed to different daylengths at several developmental stages with known photoperiodic sensitivity (31–33), it is not surprising that blank-implanted hamsters mature at different ages. Why this differential exposure to daylengths should render cohorts synchronous with respect to calendar date is difficult to understand without resorting to a functionalist interpretation. Short-lived rodents are under strong selection pressure to produce litters at the earliest permissive time in spring (34, 35). As development of a functional gonad may take several weeks, this process must be initiated when daylengths are still short and when the climate is still unfavourable. If animals rely on an interval timer to accomplish this transition, animals born over a 2- or 3-month span at the end of the breeding season must adjust the duration of the interval timer or some animals will mature later, or others earlier, than is optimal.

If first vaginal opening is indeed a valid index of fertility, then the functionalist interpretation begs the questions of why this measure was not similarly synchronized with respect to date amongst control hamsters. One possibility is that because activation of the ovary is more rapid than that of the testes, the former process may be initiated when daylengths are somewhat longer, and is therefore not triggered by the interval timer. Sexual dimorphism in seasonality mechanisms has been identified in other rodent species (36, 37), and in the present experiment is suggested by the generally later springtime transitions of females compared to males (Figures 5 and 6). However, despite these differences in timing, somatic growth, if not vaginal development,

is synchronized between cohorts in both sexes. Finally, the fact that vaginal opening was synchronized to calendar date in Blank/Mel hamsters demonstrates that the interval timer of females, similar to males, is plastic and that females retain this capacity for seasonal entrainment at least under certain, if artificial, conditions. Before concluding that reproduction is not synchronized amongst successive cohorts of autumn-born females, the issue should be examined with more direct measures of fertility.

Parallels between circannual rhythms and the interval timer extend to mechanism as well as function. Both timing processes were previously known to be influenced by photoperiod (16, 17, 38), and the present results extend this known similarity to a dependence on melatonin duration. Depending on the context in which they were given, melatonin implants advanced, delayed or had no effect on the seasonal transitions triggered by the interval timer. Are particular age- or season-specific signals critical for modulating the duration of the interval timer mechanism? Although important under certain photoperiod conditions, prenatal differences in photoperiod have been shown to exert little influence in this paradigm (16). Postnatal photoperiodic time measurement mechanisms are functional by day 15, and hamsters are particularly responsive to variations in photoperiod around the time of weaning (33, 39, 40). However, in the present study, exposure to different seasonal signals before 21 days of age was not sufficient to generate different interval timer durations because development was age-synchronized among cohorts implanted with melatonin beginning at week 3. Neither were different photoperiod histories before week 3 necessary for different interval timer durations, as demonstrated by variations among the three September-born groups receiving identical treatment before weaning.

If differential photoperiod exposure before week 3 is neither necessary nor sufficient for effects on interval timer duration, the following weeks would appear to be more important. As stated above, implants from 3–9 weeks of age were sufficient to largely over-ride differences in photoperiod that animals experienced before and after. This synchrony with respect to age derived from a lengthening of the interval timer in the September-born cohort and a corresponding absence of such an effect in the August-born hamsters. Additionally, these August-born hamsters, born 6 weeks earlier, would have been naturally exposed to these same endogenous signals from weeks 9–15 (Table 1), but implants that obscured these endogenous signals were also without effect. Thus, the effects of melatonin capsules are context-specific but not relatable specifically to age or to season. A comparable context-specificity pertains to other known circannual entraining cues for which effects depend both on the entraining stimulus and on the phase of the rhythm in which it is given.

A final parallel with circannual systems exists in the long latency of melatonin action and the dissociation between its acute and long-term effects. The only significant acute effects were seen in body weights of August-born males and females where implants showed no evidence of changes in interval timer function. By contrast, amongst September-born animals, where the largest effects on interval timer function were seen, no acute effects were discernible. Thus, the actions of melatonin on the interval timer mechanism are similar in several respects to those on circannual rhythms where there is unambiguous evidence of an entraining action. While not proving an entraining action specifically, the results do establish that melatonin influences

photoperiodic systems by altering the timing of an endogenous process that mediates the winter/spring seasonal transition. The differences between so-called photoperiodic and circannual systems may instead lie in the robustness or completeness of endogenous processes in the absence of naturalistic environmental input. In this respect, it is noteworthy that weak circannual rhythmicity has been recently reported in rats and in a small fraction of hamsters maintained in short daylengths (41, 42). Further tests of this general view are warranted using alternative manipulations of the melatonin signal.

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