Title: Simulated natural day lengths synchronize seasonal rhythms of asynchronously born male Siberian hamsters

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Running Head: Development of summer-born male Siberian hamsters

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Abstract

Photoperiodism research has relied on static day lengths and abrupt transitions between long and short days to characterize the signals that drive seasonal rhythms. To identify ecologically relevant critical day lengths and to test the extent to which naturally changing day lengths synchronize important developmental events, we monitored 9 cohorts of male Siberian hamsters (*Phodopus sungorus*), born every two weeks from 4 weeks before to 12 weeks after the summer solstice in a simulated natural photoperiod (SNP). SNP hamsters born from 4 weeks before to 2 weeks after the solstice underwent rapid somatic and gonadal growth; among those born 4-6 weeks after the solstice some delayed puberty by many weeks whereas others manifested early puberty. Hamsters born 8 or more weeks after the solstice failed to undergo early testicular development. The transition to delayed development occurred at long day lengths, which induce early puberty when presented as static photoperiods. The first animals to delay puberty may do so predominantly on the basis of postnatal decreases in day length, whereas in later cohorts, a comparison of postnatal day length to gestational day length may contribute to arrested development. Despite differences in timing of birth and timing of puberty, autumn gonadal regression and spring gonadal and somatic growth occurred at similar calendar dates in all cohorts. Incrementally changing photoperiods exert a strong organizing effect on seasonal rhythms by providing hamsters with a richer source of environmental timing cues than are available in simple static day lengths.

**Key Words**: *Phodopus sungorus*, simulated natural photoperiod, puberty, interval timer, critical day length
Introduction

Siberian hamsters exhibit striking seasonal changes in physiology and behavior that are controlled proximately by photoperiod. In adults, shortening day lengths cause gonadal regression, loss of body weight, moult to the white winter pelage, and changes in the endocrine milieu (1, 7). After many weeks of short day exposure, the spring phenotype spontaneously re-emerges, and hamsters are considered refractory to short day lengths (20, 29). Young Siberian hamsters respond similarly; individuals born in long days grow rapidly, and reach puberty at ~5 wks of age, whereas those born in short days gain body weight much more slowly, moult to the winter pelage, and delay puberty until ~5 months of age (19). In the field, the former may breed throughout the summer of their birth (43), whereas those born later in the breeding season likely over-winter as sexually immature juveniles and form the first breeding cohort during the next spring (reviewed in refs. 2, 34).

Early photoperiodism research defined critical day lengths (CDs) as species-specific thresholds that separated long-stimulatory from short-inhibitory day lengths (13h of light/d, 13L, for Siberian hamsters, ref. 18; 12.5L for Syrian hamsters, ref. 6). These values emerged from experiments in which hamsters housed in long or short day lengths were transferred to a variety of photoperiods and monitored for changes in testis size. The CD is a concept of considerable heuristic value, but the use of static photoperiods with transitions from long to short days, or vice versa, in a single day, imposes changes in day length orders of magnitude greater than those encountered on a single day in nature. This type of analysis limits conclusions about the relative salience of different
photoperiodic signals in natural photoperiod conditions (16). In particular, it does not address the potential role of the rate of change of photoperiod, independent of its absolute value.

Working with ewes, Yeates first noted that single day length thresholds could not fully explain the photoperiodic cueing of the breeding season (45). To our knowledge, this result was not incorporated into the early work on rodent photoperiodism, so elaboration of how absolute photoperiod, change in photoperiod, and prior photoperiod exposure interact to control seasonal events was delayed many years. The first significant change to the CD model of photoperiodism was the demonstration that the length of the antecedent photoperiods determined the interpretation of static intermediate photoperiods as either long or short, measured by the stimulatory or inhibitory effect on the gonads (21, 23). This established the importance of photoperiod history. Later, it was shown that small daily changes could drive seasonal transitions even when day lengths never crossed the canonical 13L CD (17). Complete seasonal cycles of gonadal regression and recrudescence initially observed in an SNP (40°N, range 9-15h) were preserved in modified SNPs that had been shifted either up by adding 4h of light per day or down by subtracting 2h light per day so that the new ranges were 13-19h or 7-13h. Even in entirely “long” or “short” day lengths, the patterns of incremental daily changes in day length were sufficient to drive seasonality.

Horton’s early experiments with montane voles showed that photoperiod history was first encoded in gestation (22-24). The interaction of ambient photoperiod experienced by voles postnatally and the photoperiod history they acquired from their mother during gestation, determined whether puberty was accelerated or delayed (22, 24).
Later work replicated and extended these findings in Siberian hamsters (39). Specifically, maternal melatonin imparts a photoperiod history to fetuses during gestation (G) days 13-15 (42). This serves as a reference point for interpreting photoperiods around weaning: day lengths during the 2 weeks from birth to weaning do not affect reproductive development (40, 46), probably because the pups’ own melatonin rhythm, which forms the neuroendocrine representation of day length, only emerges between postnatal days 10 and 15 (P10-15, ref. 41). All of these experiments employed abrupt 2h transitions effected in a single day, so left open is whether photoperiod histories exert any influence on rodents exposed to natural progressions of day length, where G-P differences are less than 120 min (Table 1).

The cues in natural photoperiods that control timing of puberty of Siberian hamsters are unknown, although the decision to advance or delay reproductive maturation may have enormous fitness consequences (2). The minimum difference between the G14 and P15 day lengths that delays development is probably close to one hour: a 120 min transition imposed on the day of birth—16L to 14L—fully inhibits testicular growth (39, 40), whereas a 60 min transition from 16L to 15L causes only partial attenuation of testicular growth (37). In the natural range of Siberian hamsters (47-53°N, ref. 33), the maximum decrease in day length from G14 to P15 occurs at the autumn equinox (79 min; ~4min/d), but as in other rodents for which field data exist, the switch to delayed puberty probably occurs well before the equinox (34). The relevant 19d photoperiod differences (P15 – G14) depend on birth date in the SNP, and in the current study range from +37 to −78 minutes (Table 1 and Methods). After the summer solstice, absolute day length decreases and the magnitude of daily change in day length increases, but the relative
contributions of these two aspects of photoperiod in determining the timing of puberty remain unknown for hamsters maintained in SNPs.

The emergence of the spring phenotype—in both reproductively quiescent adults with regressed gonads, and over-wintering juveniles that have not yet reached puberty—is governed by an endogenous interval timer (IT), not by increasing day lengths (12, 17, 28). The IT that controls puberty in over-wintering juveniles is plastic. Puberty in SNP-housed and photo-inhibited hamsters born 6 weeks apart in either August or September occurred at the same date in the spring (12). This plasticity implicates a mechanism that synchronizes spring development in photo-inhibited hamsters born late in the breeding season, but it remains to be established whether similar synchronization occurs across the entire breeding season and extends to post-pubertal as well as photo-inhibited pre-pubertal hamsters.

The present experiment deployed an SNP that corresponds to the latitude of origin of our founding stock. We assessed the development of hamsters born during the 4 weeks before and the 12 weeks after the summer solstice. We sought to identify the calendar dates and day lengths at which hamsters switched from a pattern of rapid development to one of delayed somatic and gonadal growth. We tested the hypothesis that emergence of the spring phenotype is independent of both date of birth and initial developmental strategy, and controlled instead by the SNP and a plastic interval timer. Finally, we assessed the contributions of prenatal photoperiod history to postnatal development in the SNP.

**Methods**
Nine cohorts of male Siberian hamsters (*Phodopus sungorus*) were born at 2 week intervals beginning 4 weeks before and ending 12 weeks after the summer solstice in a simulated natural photoperiod corresponding to 53°N (SNP: annual range of 7.6-16.9 h of daylight, Fig. 1) generated by a latitudinal timer (EC71ST SunTracker, Paragon Electric Company, Two Rivers, WI). There was no illumination during the dark phase. The last births correspond to the end of the breeding season in the field in mid-September (43). The latitude is close to the northern extent of this species’ natural range and approximates that at which the founder animals of our colony were originally trapped (33, 44). The midpoint of the SNP light phase was matched to the photophase midpoint of our colony (14 h light/d, 14L: lights on 0400 h PST). For each cohort, colony females were transferred to the SNP two weeks before pairing with a colony male. Females were paired with males for one week: two groups of males of proven fecundity were housed in the 14L colony room except during pairing with females. Dams for each cohort were used only once for that cohort. Births were distributed over 4-7d (mean ± S.E., 4.7 ± 0.4), with the first births 1-3d before the target date (Table 1).

Pups were weaned at 23-24d of age, segregated by sex, and housed 3/cage with both littermates and non-littermates of the same age in clear polypropylene cages (18 x 28 x 12 cm), furnished with Harlan Tek-Fresh bedding (Harlan Teklad, Madison, WI). The females offspring were reserved for a separate experiment described elsewhere (M.P. Butler *et al.*, submitted for publication). Throughout the experiment, male hamsters were housed in light-tight boxes that each held 56 cages. Cages of the female offspring were present in the same boxes. Tap water and Lab Diet 5015 Mouse Diet (Brentwood, MO)
were available *ad libitum*. While paired with males and during gestation, females received cotton for nests and sunflower seeds. All procedures were approved by the Animal Care and Use Committee of the University of California at Berkeley.

Beginning on the day of weaning, and at weekly intervals thereafter, body weight and coat color were assessed for all animals until 45 weeks after the summer solstice. Pelage scores were assigned using the 4 point scale of Duncan and Goldman (5), with the addition of half steps. Testis size (estimated testis volume, ETV) and balano-preputial separation (BPS) were recorded in males biweekly until the spring equinox, 39 weeks after the summer solstice. BPS is a reliable external indicator of puberty (10). Until 6 weeks of age, measurements were scheduled so that all hamsters fell in a 2d age window; from 7 weeks on, measurements were all taken on the same day. ETV was assessed with hamsters under light isoflurane anesthesia: the left testis was isolated with forceps in the scrotum and the width (W) and length (L) measured with calipers to ±0.3mm. ETV is defined by $W^2L$, and correlates with testis mass (13). At the end of the present experiment, ETV and paired testes weights (PTW) were measured for 64 hamsters, and were highly correlated ($ETV = 0.94 \times PTW + 85.5$, $R^2 = 0.93$).

**Refractoriness to Short Day Lengths**

To assess whether hamsters were rendered photorefractory by the SNP, 4 hamsters per cohort were transferred to the winter solstice photoperiod (7h, 46min, dark onset held constant) 5 weeks after the winter solstice (Fig. 1), and remained in this day length thereafter. This transfer point was chosen because a previous study had shown that hamsters clamped at the winter solstice photoperiod from the winter solstice became refractory and displayed gonadal development and body weight gain in the 2-8 weeks
after the solstice (11, 12). We wished to confirm that the reported refractoriness was not specifically due to the post-solstice housing in the static short day.

**Activity measures**

Locomotor activity was monitored with passive infrared motion detectors (Quest PIR, Electronics Line USA, Boulder, CO) mounted on the cage lids (described in ref. 26) for all females in the two weeks after transfer to SNP and before pairing with males. Counts per 10 min bin were collected with Dataquest III (Mini-Mitter, Sun River, OR) and analyzed with ClockLab (Actimetrics, Wilmette, IL). Actograms were scored by eye as entrained or not by an independent observer and then verified by estimating the period of circadian locomotor activity in the light dark cycle (τ’) by a χ² periodogram (30). In all cases where τ’ deviated from 24h, this appeared to be due to the small number of days analyzed (7d), and the occasional spike of daytime activity after cage changing. Finally, activity onset and offset were calculated from the average daily activity for the last 7 d of recording. Onset was scored as the first point > 150% of the mean activity after which this level was sustained in 3 of the following 6 bins. Similarly, offset was the last point > 150% of mean activity, before which, activity had exceeded this threshold in 3/6 bins (15). The duration of the daily activity bout (α in h), was offset minus onset time of locomotor activity.

We verified that all females entrained their circadian rhythms to the new photoperiod during these two weeks to ensure accurate transmission of day length information from dams to fetuses in utero. All dams transferred from 14L entrained locomotor activity to the SNP within 1-7d. The dam’s circadian periods ranged from 23.67 to 24.17h with the majority clustered at 24h (23.96 ± 0.01). Average α among the
females of each cohort was correlated with average scotophase in the last week before mating ($r = 0.60$, $n = 86$, $p<0.001$).

**Responsiveness to Short Days**

Two general patterns of responsiveness emerge in summer-bred offspring. Earlier cohorts are characterized by robust somatic and reproductive development, followed by testicular regression, body weight loss, moult to the winter pelage, and loss of balanopreputial separation. Later cohorts delay development of all these characteristics. Nonresponders, well described for this species (9, 15, 32, 38), fail to exhibit the winter phenotype when exposed to short or decreasing day lengths.

Hamsters were classified as nonresponders if at least 3 out of 4 typical short-day traits were absent. These traits are: 1) a decrease of $\geq 48\%$ in ETV from the initial peak before the winter solstice to the subsequent trough (17), measured between the initial peak and the following spring equinox, 2) a decrease of $\geq 12\%$ in body weight (17), using the same timing criteria as above, 3) a pelage score $\geq 2$, and 4) a loss of BPS for more than 4 wks between the fall and spring equinoxes. Among later-born males, some photoresponders have very low initial peak ETV and BW, and thus cannot exhibit large proportional decreases later. Animals without the 48% decrease in ETV or 12% decrease in BW, but satisfying the pelage and BPS criteria, were re-evaluated, and deemed photoresponsive if peak ETV before the winter solstice was $< 350$, implying the absence of fall puberty.

**Timing of puberty**

Male puberty was defined as the age at which ETV $> 350$ and full BPS were both evident.
Timing of regression and recrudescence

An ETV of 350 corresponds approximately to paired testes weights of 300 mg. This point generally falls on the steepest portion of the testicular regression curve, and is the value at which mature spermatids are first detected in the recrudescent testis (35). The time of testicular regression was coded as the week at which ETV fell below 350. Regression onset was identified as the last time point prior to regression before ETV decreased 10% in 2 weeks, or monotonically decreased 20% in 4 weeks. Recrudescence or development time was defined as the week at which ETV increased above 350 and then continued to increase to > 500. The latter value is 2 standard deviations below the mean maximum ETV after the winter solstice. Of the hamsters that underwent recrudescence, all but 1 had an ETV > 500.

Body weight

Peak and trough body weights differ less markedly than corresponding gonadal weights, so threshold analyses are sometimes insufficient. Many individual hamsters had clear body weight increases and decreases, but others required local curve fitting to determine the timing of the fall and spring seasonal transitions (details in Fig. 2). For each hamster, body weight (g) and the acceleration thereof (g/wk²) was estimated at each point by a cubic regression fitted to the 11 week interval centered on that point. The second derivative of this regression defined the body weight acceleration, and this was used to calculate two events of interest. 1) The transition to the winter phenotype was defined as the point of zero acceleration in the period of body weight loss, i.e., the point of inflection in the body weight curve (bw loss). This was scored only for hamsters displaying a 12% decrease in body weight as defined above. 2) Spring development was
defined by maximum acceleration after the autumnal equinox (bw acceleration). Often, winter body weights were not static but continued to increase: the present method nevertheless still identifies relative increases in the body weight gain typical of spring puberty or recrudescence.

In validating this approach, data segment lengths of 5-13 wks for the regressions were tested. Except for the 5 week analysis, the choice of segment length had little effect on the outcome. The relative timing of spring body weight gain between cohorts was preserved, omnibus statistics yielded identical conclusions, and post-hoc pairwise cohort differences were identical save for a single comparison that differed with 9 wk segments compared to the others. To choose among these methods, 20 individuals with clear onsets of spring body weight gain were chosen randomly, and the onset estimated by eye. This subjective measure was then compared to objectively measured acceleration maxima. Variance of the difference between these measures was minimum for 11 week segments, so this interval was selected for all subsequent analyses.

Finally, week to week body weight gains were calculated for all individuals, and smoothed by calculating 3 wk moving averages (11).

**Statistics**

1-way ANOVA or Kruskal-Wallis tests were used where appropriate to test for cohort effects on the timing of developmental and seasonal events. Significant pairwise differences between cohorts were tested with the post-hoc Tukey test, or Dunnett's T3 test when variance was not homogeneous across cohorts. Repeated measures ANOVA was used to analyze effects of time and time by factor interactions. Because we are examining growth curves, all main effects of time (age or date) were significant and are
not reported. All of the above were calculated using SPSS 13.0 (SPSS, Inc., Chicago, IL). Proportions were tested for significance with the $\chi^2$ test for independence (Statview 5.0, SAS Institute, Inc., Cary, NC). Unless otherwise noted, means and standard errors are reported throughout. The significance level was set at 0.05.

To test the competing hypotheses of age versus date synchronization of life history events, linear regressions were calculated for event date as a function of birth date (cohort) using Statview. A slope of 1 indicates a fixed interval between birth and the event date and thus establishes age synchrony. A slope of 0 indicates a fixed interval between the summer solstice and the event date, thus indicating synchronization by day length or calendar date. We defined synchronization to be significant if the 95% confidence interval (CI) contained 1 or 0, indicative of the synchronization being essentially exclusively determined by age or date, respectively. If the slope was in the vicinity of 1 or 0, but 1 or 0 was not included in the CI, then we concluded that synchronization was primarily determined by age or date, respectively, but with secondary modulation by other effects. Note that this is somewhat different than the conventional use of regression analysis, insofar as we are not testing for simple linearity through, e.g., a regression coefficient $R^2$, nor are we testing a single hypothesis through rejection of its null complement. Rather, we note that the competing hypotheses of age versus date synchronization are not exhaustive, and we use proximity of the regression slopes to 1 or 0 as an index of the primacy of the respective mechanisms. For these reasons, $R^2$ and the accompanying p-values for the regressions are not meaningful statistics when assessing synchronization, and we only report CIs in these cases.
Results

Nonresponders: Distribution and Permanence

Of a total of 243 hamsters, 17 were classified as nonresponders. The percentage of nonresponders differed significantly among cohorts and was higher in the earlier cohorts with 13/17 in the 3 cohorts born by the summer solstice (%nonresponder by cohort from K4: 21, 17, 7, 0, 11, 6, 0, 0, 0%; omnibus \( \chi^2 \), \( p<0.01 \), not illustrated). In the classification process, there were four indeterminate animals. One was a “late responder” with a long delayed regression, and three underwent early recrudescence after only 6-8 wks of ETV < 350. Nonresponders and indeterminates were removed from subsequent analyses.

Nonresponsiveness appeared to be permanent. Sixteen of 17 nonresponders survived until the following winter in the SNP: all had ETV > 400 when measured 1 week before the winter solstice. In the previous year, 190 of 192 responders had ETV < 350 by this date (ETV = 72 ± 5).

Timing of Developmental Events in the SNP

Testis Size and Body Weight Over Time. Hamsters held in the SNP displayed two distinct developmental patterns (Fig. 3). Early cohorts (−4 to +2) were characterized by early and rapid testis growth (Fig. 3A, B) and weight gain (Fig. 3C) followed by autumn decreases and spring increases. In those born from 19 July on (cohort +4 and later), body weight gain and testis development were arrested over the autumn and winter months until the advent of spring increases. Figures 3A and 3B contrast the effectiveness of day
length compared to age as a synchronizer of seasonal events. The timing of puberty and the onsets of the winter and spring phenotypes are discussed in detail below.

Repeated measures ANOVAs of ETV as a function of age or date revealed that ETV between cohorts differed significantly (main effects of cohort, p<0.05 for both tests), and that the pattern of change in testis size over time differed between cohorts (cohort × age and cohort × date interactions, p<0.05). When body weight was analyzed as a function of date, the temporal pattern of bw gain differed between cohorts (cohort × date interaction p<0.05), but over-all, bw did not differ between cohorts (main effect of cohort, p>0.2). We therefore examined somatic growth rates at different times (Table 2): there were significant effects of cohort on body weight gain per week from 3-5wks of age (p<0.001), and smaller but still significant effects during the 9 weeks near the winter solstice (p<0.01). Body weights did not differ between cohorts at the winter solstice (p=0.6).

**Puberty.** There were significant cohort effects on the age and date of puberty (Kruskal-Wallis, p<0.05, Fig. 4A, B). Earlier born animals reached puberty at earlier calendar dates for cohorts -4 to +2, while all animals in cohorts +8 to +12 experienced delayed puberty at the same calendar date (Fig. 4B). Cohorts +4 and +6 had strict bimodal distributions, with puberty evident either at 5 wks of age or delayed until at least 25 wks of age (Fig. 4A).

Hamsters were subsequently classified as those that underwent puberty early (by 5 wks of age) or late (≥ 13 wks of age) to test for synchronization by age or date (Fig. 4C). Early puberty was closely correlated with age, with a regression slope of 1.06. This is extremely close to 1.0, the value for which there would be pure synchronization with
age, but the confidence interval (CI = {1.03, 1.09}, n=140) does not include the point 1.0, so there is no significant age synchronization. We conclude that while age is certainly the primary synchronizing variable, there is a minor, but significant modulation of this, most likely reflecting that within this group, earlier born cohorts undergo puberty earlier (cohort -4 reaches puberty significantly earlier than all but cohorts -2 and +2, Dunnett’s test, p<0.05).

The importance of birth date and age disappear in hamsters with delayed puberty, which was synchronized by day length to an absolute calendar date (33.6 ± 0.4 wks after the summer solstice, Figs. 3B, 4). Note that although there were no significant differences between cohorts +8, +10, and +12 in either the age or date of puberty (Dunnett’s test, p>0.05, Fig. 4A, B), there was significant date, but not age, synchronization as assessed by the regression slope confidence interval, (Fig. 4C, CI = {-0.55, +0.16}, n=76).

*Photoperiod History Effects on Pups.* Every hamster beginning with cohort 0 is exposed to decreasing day lengths after birth, and therefore its development is potentially slowed by the photoperiod history effect—the comparison of ambient day length experienced from P15 to the photoperiod history set during gestation (values in Table 1). ETV at 3 wks of age may indicate the degree of photo-inhibition due to this effect. The shorter the day length and the larger the day length decrease from G14 to P15, the smaller the testes: there was a significant effect of cohort (ANOVA, p<0.001, Fig. 5A, B). Full photo-inhibition is evident in the significantly smaller ETVs in cohorts +8, +10, and +12 than in earlier cohorts. Some hamsters born as early as the last weeks of July delay puberty (35% and 52% in cohorts +4 and +6, respectively), and mean ETV in these
cohorts is significantly higher than in cohorts +8 to +12. Surprisingly, this holds true even within the subset of hamsters that delays puberty (Fig. 5C). Among the hamsters delaying puberty in cohorts +4 to +12, there is a significant cohort effect on ETV at 3 wks of age (ANOVA, p<0.001), indicating that early inhibition of testicular growth is a graded response. The effect of a given G14 to P15 decrease depends on the absolute day length. Cohorts +6 and +8 have identical 68 minute decreases from G14 to P15, yet in these cohorts 52% and 100% of hamsters delay puberty, respectively ($\chi^2$, p<0.001). Similarly, cohorts +2 and +4 have equivalent G14 to P15 decreases (−50 and −51 minutes, respectively), yet the percent of hamsters that delay puberty increases from 0 to 35% ($\chi^2$, p<0.001). Over-all, there is much individual variability in the response to decreasing day lengths (Fig. 5D). The combination of photoperiod history and ambient day length may be interpreted by some hamsters as stimulatory and others as inhibitory, even within a single cohort. These figures also show that the hamsters that delay puberty do not exhibit uniformly small testes.

**Onset of the Winter Phenotype.** Testicular regression in autumn was synchronized by date, not age, and occurred 13.0 ± 0.3 wks after the summer solstice (Fig. 6, CIs reported in Legend). Inclusion of the 1 hamster in cohort +10 and 3 hamsters in cohort +12 did not affect this conclusion. The dates of testicular regression for these four hamsters suggest a switch to age synchronization, a conjecture supported by the larger set of body weight data. Body weight loss displayed both date and age synchronization, switching at cohort +2. It was synchronized by date in cohorts −4 to +2 (14.8 ± 0.4 wks post-summer solstice, n=69), but was synchronized by age in cohorts +2 to +6 (13.0 ± 0.3 wks of age, n=44).
Changes in testis size depend on the interaction between birth date and calendar date—there is an interval when testis size was increasing in some cohorts and decreasing in others, despite exposure to the same day length (wks 5-11, Fig. 3B). During the regression phase, the lines for cohorts −2 to +6 are superimposable and all reached the fully regressed state within a 2 week window (Fig. 3B). There was a significant effect of cohort on regression onset (Kruskal-Wallis, p<0.001), but these onsets were not synchronized by either age or date (data not shown).

Onset of the Spring Phenotype. Emergence of the spring phenotype appeared to occur at the same calendar date (Fig. 3B). Overwintering sexually immature juveniles undergoing gonadal growth that culminates in large functional testes did so several weeks earlier in the spring than adult males undergoing testicular recrudescence (32.3 ± 0.4 vs. 35.3 ± 0.4 wks post summer solstice, t-test, p<0.001). Nevertheless, there were no differences in the timing of spring gonadal growth for cohorts +4 and +6, each with substantial representation of both pre- and post-pubertal males (Fig. 7, ANOVA: cohort, strategy, and interaction effects: all n.s.). A similar pattern emerged from the analysis of the body weight data; over-all, body weight acceleration occurred significantly earlier in prepubertal hamsters compared to postpubertal animals (30.3 ± 0.5 vs. 32.5 ± 0.5, t-test, p<0.01), but there were no differences when the analysis was restricted to cohorts +4 and +6 (ANOVA: all effects n.s., not illustrated).

Effects of increasing day lengths on body weight gains

Somatic development in the SNP was compared with that of hamsters transferred at 5 wks after the winter solstice (9h light/day and increasing) to the static winter solstice
The timing of body weight acceleration was independent of photoperiod condition and cohort (n.s. by ANOVA, not illustrated).

The early gains in body weight for SNP and solstice-clamped photoperiod groups were similar for the first 6-8 wks after transfer, but SNP-housed hamsters continued to gain body weight after the static photoperiod-housed hamsters’ body weight had stabilized (repeated measures ANOVA: photoperiod effect n.s.; time and photoperiod×time effect, p<0.001); increasing day lengths were associated with more weight gain, especially after the spring equinox (week 39; Fig. 8). SNP hamsters were significantly heavier at wks 43, 44, and 45 (Tukey test, p<0.05, Fig. 8). Rate of body weight gain was significantly higher in the SNP group at wks 38 and 40-45 (Tukey test: p<0.05). These data underscore the effects of increasing day lengths on late but not initial body weight gain.

**Discussion**

Simulated natural photoperiods (SNPs) are potent organizers of physiological development in Siberian hamsters, determining the immediate postnatal developmental trajectories and synchronizing fall and spring seasonal changes. Puberty occurs soon after birth at a nearly constant age in photostimulated hamsters born in the few weeks before and after the summer solstice. Puberty is markedly delayed in hamsters born later in the calendar year. The subsequent regression and the spring development of all hamsters follow the calendar date, regardless of the reproductive pattern early in life. The latter is an impressive demonstration of the plasticity of the interval timer that
governs the emergence of the spring phenotype. These data replicate and extend previous work showing that the onset of spring growth in the SNP is controlled by the IT (12); the onset of refractoriness, and the beginning of gonadal and somatic growth did not differ between hamsters in increasing versus short static photoperiods.

A main goal of the experiment was to identify ecologically relevant critical day lengths. In the current study, the transition from early to late puberty occurred, as expected, some time after the summer solstice, but before photoperiod decreased to the canonical CD of 13h (18). The transition occurs gradually over 6 weeks (percent showing early puberty in cohorts +2 to +8 were 100, 65, 48, and 0%, respectively). Cohorts +4 and +6, born near the end of July and early August of the SNP, were the critical intermediates with substantial representation of both developmental types (some showing advanced, others delayed puberty). Birth (P0) and weaning (P23) photoperiods were long for both cohorts (15.6-16.2L and 14.1-15.0L, respectively). Any of these photoperiods, if presented statically to Siberian hamsters from birth, would induce puberty by 5 wks of age (18, 39). The smallest magnitude P15 – G14 difference associated with delayed puberty was ~51 minutes, but as discussed below, the effect of this may have been small.

Our data do not definitively establish whether the transition in developmental strategy at 4-6 wks after the summer solstice was driven by the decrease in absolute duration of the photophase during this period, or by the increase in the magnitude of daily changes in day length; these two signals covaried in this experiment. Nevertheless, the 3 week ETV measures indicate that both signals are important (Fig. 5A, B). A given difference between the photoperiod history set in gestation and the day length
experienced postnatally does not solely determine early reproductive development (compare cohorts +2 with +4, and +6 with +8). Indeed, for the last 4 cohorts of this study, the P15 – G14 differences vary by only 10 minutes (−68 to −78 min), and yet puberty is delayed in 55/59 hamsters in cohorts +8 to +12, but only 12/23 hamsters in cohort +6.

The photoperiod history effect on development is not an all or none response (37). Transfer from a 16L gestational photoperiod to 10, 12, 13, or 14L on day 14 of life completely inhibited testicular growth as assessed at 32d of age (paired testes weights < 50mg). Testes of hamsters transferred to 15L, however, were intermediate (~260mg): they were smaller than 16L controls (~470mg), but larger than those of hamsters in shorter postnatal day lengths. Testicular growth occurs equally from birth to 15d of age in both long (16L) and short (10L) photoperiods, at which point paired testes weights are ~50mg (46). Only after this age does testis size diverge between long and short day-housed hamsters. This suggests, therefore, that in the former experiment, the testes of hamsters transferred from 16L to 15L still grew from 15d to 32d of age, but that the growth rate has been slowed by photoperiod history.

Our data also reveal a gradual change in the strength of photoperiod history-dependent inhibition. Most hamsters in cohorts +8, +10, and +12 delay puberty, and have small testes at age 3 wks. We assume that these late August- and September-born hamsters are fully photo-inhibited from birth. Mean ETV at age 23-24d (109 ± 9, n=65) corresponds to a paired testes weight of 26mg, equivalent to the ~25mg testes of 25 day old photo-inhibited hamsters raised in static 10L and much smaller than the ~275mg testes of 16L-housed hamsters (46). Mean ETVs at 3 wks in cohorts +4 and +6 were
much larger, however, both across all the hamsters and among the set of hamsters delaying puberty (Fig. 5). This suggests that the testes are growing from 15 to 23d of age, but that their growth is retarded by their photoperiod history. Decreasing photoperiods subsequently experienced by the pups fully arrest testicular growth and cause regression by 7 wks of age.

The seven hamsters that delayed puberty in cohort +4 are particularly interesting, for photoperiod history effects seem to be small (see Fig. 5C, D). Four of these seven hamsters had ETV > 275 at 23/24d of age, and the minimum ETV of 144 was greater than 51 of the 65 fully photo-inhibited hamsters in cohorts +8 to +12. We note that all hamsters had experienced approximately 8d of endogenous melatonin exposure before the first ETV (41). It is possible that changes in duration of nocturnal melatonin secretion during these 8d of decreasing photoperiod are responsible for the delay in development. The importance of the maternally transmitted photoperiod history to pup developmental trajectory in natural conditions is still unknown. The present work suggests that photoperiod history may play a role in inhibiting development in late born cohorts, but may play a far smaller role in the very first hamsters to delay puberty. Conventional wisdom suggests that the maternal signal is critical in dictating pup development; in the field this would ensure the proper spring versus fall trajectories in intermediate day lengths. To the extent that the SNP in this experiment mimics field photoperiod conditions, the data imply that the first hamsters to delay puberty in the field in July may be relying on ambient photoperiod, and that the photoperiod history effect gains strength in later cohorts.
The different regression onset times in several cohorts (wks 5-13 after the summer solstice, Fig. 3B) also suggest that the photoperiod history mechanism may be insufficient to cause photo-inhibition. Even as the majority of hamsters in earlier cohorts were exhibiting testicular regression, both cohorts +4 and +6 were showing an initial increase in mean ETV from age 3 to 5 wks. Overall, it may require up to 3 wks of melatonin exposure from P15 to P35 before testicular growth is arrested in these cohorts.

Natural populations of rodents employ different pubertal strategies based on the time of birth (reviewed in refs. 2, 3, 34). Because such studies require repeated extensive sampling of marked individuals and encounter difficulties of accurately determining the exact age of trapped individuals, there are few field studies that specify the transition from early to delayed puberty. The July onset of delayed puberty in the present experiment fits with the limited field data documenting a strategy shift midway between the summer solstice and fall equinox, documented in lemmings, voles, and mice (4, 36). In a previous laboratory study of puberty in an SNP, white-footed mice born on 15 July exhibited early puberty whereas those born on 15 September delayed puberty (8).

Our hamsters displayed fall seasonal changes before the autumn equinox, and spring seasonal changes before the spring equinox. Field data document the same patterns, especially in temperate latitudes. Body mass begins to decline in August in meadow voles (25), whereas the onset of reproduction often precedes the spring equinox (4). In voles and *Peromyscus* mice, spermatogenesis begins in December, and the testes mature fully by March (4).

Testicular regression and body weight loss generally occurred at the same calendar date near the autumn equinox for early cohorts (Fig. 6). The body weight loss
data, however, suggest a switch to age synchronization at cohort +2. This is partly reflected in the testicular regression data for the 4 hamsters of cohort +10 and +12, three of which underwent regression at 7 wks of age and one at 9 wks. This age synchronization may represent the minimum time it takes for the gonads and body weight to peak and then decrease again (~7wks and 13wks, respectively). Testicular regression may be a faster process such that once triggered, the testes can be fully regressed by 7 wks of age, while the body weight loss lags.

The emergence of the spring phenotype in all cohorts at similar calendar dates reveals the plasticity of the interval timer, as has been noted previously for overwintering juveniles (12). Whether IT plasticity rests on changes in the interval duration or the timer rate is still unknown. For hamsters undergoing early puberty, regression occurs at similar calendar date, so the IT may be triggered at regression and may run at the same rate and for the same duration in all cohorts. That the delayed regression in cohort K is matched by delayed recrudescence suggests a constant IT for these groups. The similar dates of spring puberty among the over-wintering juveniles is more impressive considering that birth dates range over 8 wks and that there is no obvious common triggering point, like autumn gonadal regression. Moreover, the tendency is not for earlier born cohorts to develop earlier as one would predict based on birth dates, but rather the opposite. Development is delayed in older animals across all cohorts and development strategies. This could reflect aging-related deceleration of the IT or slowed somatic and reproductive development after the onset of photorefractoriness. We favor the former interpretation, because a separate analysis on the time of recrudescence/development onset using an
ETV > 150 threshold instead of the ETV > 350 threshold yielded equivalent results (data not shown).

Cohorts +4 and +6 are of particular interest because both recrudescence and pubertal development occur at the same time (Fig. 7). Synchronized spring development in hamsters with different pubertal phenotypes has been observed in female Turkish hamsters (27). If, within a cohort, the IT is triggered at birth in some hamsters but only at the onset of regression in others, then it must be running at different rates or for different durations in the two groups. Alternatively, the IT may always be triggered at birth and may then run uninterruptedly and independently of whether the hamsters experience early or late puberty.

An IT whose duration is modified by day length and its associated melatonin signals during early life is well supported (11, 12, 28). Constant release melatonin implants, which disrupt the transmission of day length information, affect the IT in Siberian hamsters and prevent synchronization of spring puberty among late summer-born hamsters in an SNP. The melatonin implants are effective during the first 3-9 wks of life but not later (11). The IT also controls the onset of puberty in static short days; the 2 week period between 3-5 wks of age is critical; truncation or elimination of the nocturnal melatonin signals by constant light or by pharmacological means during this time, but not later, affects the timing of puberty (28). These studies do not address the differences between similarly aged animals with distinctly different developmental strategies.

Photo-nonresponsiveness was more prevalent in the early cohorts, especially those born by the summer solstice (13/17, 76%). The increased numbers of
nonresponders in hamsters born earlier in the year (Aug versus Sep) in an SNP has been noted previously (11, 12). This is likely due to environmental induction of nonresponsiveness (15). Very long day lengths render Siberian hamsters nonresponsive to short days by changing the coupling strength between putative morning and evening oscillators; this results in abnormal circadian entrainment to the light-dark cycle and presumably to long-day like melatonin patterns (15). Whether this ever occurs in nature is unknown. Notably, dim light that mimics nocturnal star/moonlight dramatically reduces the incidence of nonresponsiveness (14).

Hamsters do not read and respond exclusively to absolute day length (31, 39). Rather, they attend to incremental changes in day length and respond appropriately based on both absolute day length and direction of change, with different physiological traits responding differently (16). The present data confirm the dominant role of the SNP in organizing somatic and reproductive development patterns. Early pubertal development is halted by small decreases in what would otherwise be interpreted as stimulatory long day lengths. Regardless of time of birth and time of puberty, refractoriness to short days occurs near the same date, ensuring spring growth is restricted to a small window of calendar dates. The relative signal strength of absolute versus changes in day length, and their interaction in governing photoperiodic responses remains to be determined. A more complete analysis will require quantifying the interplay between circadian activity patterns, light sampling behavior, and dawn and dusk transitions in day length measurement.
Acknowledgements

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Figure Legends

1. The simulated natural photoperiod (SNP) is shown schematically with the birth dates of the 9 cohorts (~4 to +12, relative to the summer solstice). Four hamsters from each group were transferred to the winter solstice photoperiod (7h, 46min) at week 31, while all others remained in the SNP.

2. Example of curve fitting of individual hamsters from cohort +4, one with fairly clear seasonal transitions (A) and one in which transitions in body weight (bw) are not obvious in the raw data (B). The bw data (black circles) are fitted by moving cubic regressions of bw on time. Specifically, regression were done over an 11 week time range symmetric at each age. Evaluation of each of the moving regressions at its center point yields the fitted or smoothed bw curve (solid line). Similarly, the bw acceleration (dotted line) is defined by the second derivative of each regression equation at its center point. Filled arrows mark the maximum bw acceleration after the autumnal equinox, and the open arrow marks the point of inflection during fall bw loss (bw acceleration curve crosses 0 here). The time of autumnal bw loss was not scored in B, because this hamster’s bw loss was <12%. The bw acceleration peak in B was greater at 30 than 24 wks of age. Body weight acceleration peaks corresponded well to the time of recrudescence of the testes (diamonds at bottom of each panel). Across all hamsters, the date of maximum body weight acceleration occurred 2.2 weeks earlier than testicular recrudescence (standard deviation = 2.4).
3. ETV as a function of age (A) and of calendar date (B). Beginning 23 wks after the summer solstice, photoperiod rather than age synchronizes testicular regrowth. Body weight is synchronized in a similar manner by the SNP (C). Only responsive hamsters for whom no data were missing were included; values are mean ± SE, n by cohort from −4: 14, 14, 17, 18, 16, 19, 19, 21, 18.

4. Age (A) and date (B) of puberty as a function of cohort (mean ± SE). Points in A show all individual puberty ages: when many overlap, the number of hamsters is indicated next to the point. Different letters indicate cohorts that are significantly different as assessed by Dunnett’s test. Regression analysis on the date of early or late puberty plotted against birth date (C) is a more sensitive measure of synchronization. The regression lines extend only through the data that are included in the regression calculation. Confidence intervals are denoted by the faint grey curved lines above and below each regression line; the confidence interval for early puberty is virtually superimposable on the regression line and difficult to discern in the figure. Numbers per cohort experiencing early and late puberty are shown in parentheses at the bottom and top of the figure. Two hamsters in cohort +4 delayed puberty, but the exact dates of testicular growth were unknown and so are not included. Dotted line shows perfect age synchronization (slope = 1).

5. (A) ETV at 3 wks of age as a function of change in day length from G14 to P15, and (B) of the birth photoperiod. Cohorts are labeled by each data point, and cohorts within a given grey box do not differ significantly. The same significance relations apply to A
and B. N by cohort from −4: 22, 21, 25, 27, 20, 23, 23, 24, 22. (C) ETV at 3 wks for the subset of hamsters that delayed puberty: values with different superscript letters are significantly different; n indicated within each bar. (D) ETV at ages 3, 5, and 7 wks for individual hamsters in the four cohorts that span the transition period from early to delayed puberty. All values except for those in D are displayed as mean ± SE.

6. The timing of the autumnal transition for ETV and body weight (mean ± SE). Date synchronization of testicular regression was significant (cohorts −4 to +6: CI = {−0.22, 0.08}; N by cohort from −4: 16, 23, 22, 24, 14, 12). Body weight loss was synchronized by date or by age, depending on cohort set (all cohorts: CI = {0.38, 0.69}, not plotted; cohorts −4 to +2: CI = {−.42, .23}; cohorts +2 to +12: CI = {0.88, 1.25}; N by cohort from −4: 16, 17, 19, 17, 8, 11, 4, 2, 2). Dotted line shows perfect age synchronization (slope = 1).

7. Timing of testicular development or testicular recrudescence (mean ± SE) in cohorts +4 and +6.

8. Body weight and weekly body weight increases (mean ± SE) for hamsters held in the increasing day lengths of the SNP or in the clamped static winter solstice photoperiod (7h 46min). The transfer date of the hamsters to the static photoperiod is designated by the arrow. The winter solstice (W) and the spring equinox (S) are indicated on the date axis. Significant pair-wise post-hoc comparisons between SNP and static solstice day length groups are indicated for body weight (*) and for body weight gain (†).
### Table 1. Photoperiod and litter information for all cohorts

<table>
<thead>
<tr>
<th>Cohort</th>
<th>D.O.B.</th>
<th># litters</th>
<th>G 0</th>
<th>G 14</th>
<th>P 0</th>
<th>P 15</th>
<th>P 23</th>
<th>P15–G14 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−4</td>
<td>24 May</td>
<td>10</td>
<td>15:32</td>
<td>16:15</td>
<td>16:31</td>
<td>16:52</td>
<td>16:54</td>
<td>+37</td>
</tr>
<tr>
<td>−2</td>
<td>7 Jun</td>
<td>11</td>
<td>16:15</td>
<td>16:49</td>
<td>16:52</td>
<td>17:00</td>
<td>17:00</td>
<td>+11</td>
</tr>
<tr>
<td>0</td>
<td>21 Jun</td>
<td>8</td>
<td>16:49</td>
<td>16:55</td>
<td>16:59</td>
<td>16:41</td>
<td>16:25</td>
<td>−14</td>
</tr>
<tr>
<td>2</td>
<td>5 Jul</td>
<td>8</td>
<td>16:55</td>
<td>16:56</td>
<td>16:43</td>
<td>16:06</td>
<td>15:50</td>
<td>−50</td>
</tr>
<tr>
<td>4</td>
<td>19 Jul</td>
<td>10</td>
<td>16:56</td>
<td>16:23</td>
<td>16:09</td>
<td>15:32</td>
<td>15:01</td>
<td>−51</td>
</tr>
<tr>
<td>6</td>
<td>2 Aug</td>
<td>10</td>
<td>16:23</td>
<td>15:48</td>
<td>15:36</td>
<td>14:40</td>
<td>14:08</td>
<td>−68</td>
</tr>
<tr>
<td>10</td>
<td>30 Aug</td>
<td>10</td>
<td>14:58</td>
<td>14:05</td>
<td>13:53</td>
<td>12:50</td>
<td>12:12</td>
<td>−75</td>
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<tr>
<td>12</td>
<td>13 Sep</td>
<td>9</td>
<td>14:05</td>
<td>13:07</td>
<td>12:53</td>
<td>11:49</td>
<td>11:16</td>
<td>−78</td>
</tr>
</tbody>
</table>

Cohort number designates birth week relative to the summer solstice. Gestation (G) in Siberian hamsters is 18d; males and females were paired on G0, and pups were born on G18 (postnatal day 0, P0), and then weaned on P23. D.O.B. designates the target date of birth for each cohort. In addition to absolute photoperiods, the change in photoperiod from G14 to P15 is shown. Values are relative to the target birth date for each cohort.
Table 2. Body weight gain and winter solstice body weight

<table>
<thead>
<tr>
<th>Cohort</th>
<th>bw gain (g/wk)</th>
<th>wk 26 bw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age 3-5 wks</td>
<td>wks 22–30</td>
</tr>
<tr>
<td>−4</td>
<td>5.5 ± 0.2a</td>
<td>−.17 ± .10a</td>
</tr>
<tr>
<td>−2</td>
<td>5.0 ± 0.2ab</td>
<td>.10 ± .07ab</td>
</tr>
<tr>
<td>0</td>
<td>4.5 ± 0.2b</td>
<td>.37 ± .12ab</td>
</tr>
<tr>
<td>+2</td>
<td>4.5 ± 0.2b</td>
<td>.19 ± .09ab</td>
</tr>
<tr>
<td>+4</td>
<td>3.4 ± 0.2c</td>
<td>.31 ± .12ab</td>
</tr>
<tr>
<td>+6</td>
<td>3.3 ± 0.3c</td>
<td>.25 ± .13ab</td>
</tr>
<tr>
<td>+8</td>
<td>2.3 ± 0.2d</td>
<td>.49 ± .14b</td>
</tr>
<tr>
<td>+10</td>
<td>2.1 ± 0.2d</td>
<td>.56 ± .16b</td>
</tr>
<tr>
<td>+12</td>
<td>2.4 ± 0.2d</td>
<td>.39 ± .16ab</td>
</tr>
</tbody>
</table>

Change in body weight (bw) calculated as the average of week to week changes.

Body weight does not differ between cohorts at the winter solstice (wk 26 post summer solstice). All values are means ± SE; n per cohort ranges from 21 to 27 for ages 3-5, 17 to 24 for wks 22-30, and 15 to 23 for wk 26. Cohorts with different superscripts differ significantly (Tukey test, p<0.05).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

Date of testicular regression or bw loss (wks from summer solstice)

Cohort (Birth Date)

\[ \text{Slope} = 1 \]
Figure 7

Date of Spring Testis Growth (weeks from summer solstice)

- Pre-pubertal
- Post-pubertal

Cohort +4
- n=4
- n=14

Cohort +6
- n=8
- n=10