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Photoperiodic effects on reproductive development in male cavies (*Cavia aperea*)

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HIGHLIGHTS

- Male cavies adjust their timing of maturation to the season.
- Male cavies grow faster in spring than in autumn.
- Patterns of testosterone levels in blood correlate with fecal testosterone metabolite patterns.

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ABSTRACT

Age at maturity is a particularly important life history parameter, as it predicts potential life time reproductive success in many small mammal species. Animals should therefore optimize the timing of maturation by reacting to environmental stimuli that predict future environmental conditions. Photoperiod often reliably predicts ecological conditions. Animals born into a photoperiod indicating favorable conditions (spring, summer) may mature earlier and at a lower weight than animals born into conditions indicating unfavorable conditions (autumn, winter). So far most work was done on small, altricial rodent species and we still lack knowledge about their precocial relatives. Precocial animals are born much further developed than altricials and might show less plasticity in their ontogenetic trajectory than the latter.

We tested the influence of photoperiod simulated by increasing (spring) or decreasing (autumn) light in climate chambers on important life history parameters in a medium sized rodent, the highly precocial cavy (*Cavia aperea*). We wanted to determine whether photoperiod influences timing of maturation and early growth in male cavies and whether patterns of testosterone in blood are reflected by patterns of testosterone metabolites in feces.

Males born into simulated spring grew faster and matured at an earlier age than males born into simulated autumn conditions. Patterns of testosterone in blood correlated with testosterone metabolites measured in feces. Male cavies strongly react to predictive photoperiod cues by adjusting growth and timing of maturation as we found previously for females, corroborating the importance of seasonal cues for adjustments of life history.

1. Introduction

Age at maturity is a particularly important life history parameter as it predicts life time reproductive success to a great extent in many small mammal species [1–3]. The onset of reproduction increases physiological costs as metabolic processes change and behavioral activities like mate search and courtship increase [4,5]. Thereby, the timing of maturation can have profound consequences for reproductive success [6] and animals should optimize the timing of maturation by reacting to environmental stimuli that reliably predict the future environment.

For many species of the temperate zone spring and summer are the most adequate seasons for reproduction, as resources become more abundant and weather conditions more favorable. In many environments, photoperiod is the most reliable predictor of the favorable season and hence photoperiod has become the strongest *Zeitgeber* over evolutionary time [7–9]. Reacting to photoperiodic cues is especially important for small mammals with a long pregnancy. Such species need to start reproduction when conditions are still poor, to insure that juveniles are born when conditions become more favorable. Therefore, seasonally fluctuating environments often influence growth and age at first reproduction [8,10]. Juvenile Siberian hamsters, for instance, attain sexual maturity at an early age when born into long days but delay maturation if born into short days [11]. The same was shown for voles and deer mice in the laboratory [12–14] and in the field [15].

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However, so far most work was done on small, altricial rodent species and we still lack knowledge about their precocial relatives. Precocial animals are born much further developed than altricials [16], thus the former might show less plasticity in their ontogenetic trajectories than the latter. This is especially likely for photoperiodic cues as the circadian system of altricial young develops in the nest after birth [17], while it is active before birth in precocials [9].

Cavies (genus *Cavia*) are medium sized rodents that give birth to few, highly precocial young after a relatively long pregnancy of two months [18]. At birth, offspring are able to locomote independently, have fully developed thermoregulation and sensory system and even start foraging on solid food within the first day [19]. Female offspring born early in the season may begin to reproduce while still substantially growing [20] but female pups born late in the season delay maturation and do not reproduce until the next year ([21] for *C. magna*; [22] for *C. aperea*). In the domesticated relatives, the guinea pig (*C. aperea* f. *porcellus*), static photoperiod simulating summer (16:8 L:D) and winter (8:16 L:D) conditions, did not influence female maturation under laboratory conditions [23] but did influence early growth and caused an earlier onset of maturation in male guinea pigs. A strong increase in testosterone concentration, indicating maturation, was found between 56 and 70 days of age under long days while it was approximately 14 days time delayed under short days [24]. Testosterone plays a key role in the development of male reproductive tissues such as the testis and promotes secondary sexual characteristics. Heightened testosterone levels usually indicate the onset of physiological maturation in mammals [25].

Somatic and reproductive development thus appears even more sensitive to early photoperiod in males than in females as they react also on static photoperiods while females do only react to changing photoperiods. However, Trillmich et al. [24] showed that the onset of puberty in female cavies (*C. aperea*) is strongly influenced by dynamic (increasing or decreasing) photoperiod rather than by static (long days or short days) photoperiod. Female pups matured at about 47 days of age when kept in increasing (from 10:14 to 12.5:11.5 LD) and 79 days when born into decreasing (14.5:9.5 to 12.25:11.75 LD) day length. Thus, one may expect an even greater influence of dynamic photoperiod on male cavies.

We therefore tested the hypotheses (1) that dynamic photoperiod increases the early growth of male cavies and (2) that male cavies born into increasing day length (spring) mature at an earlier age than males born into decreasing day length (autumn) indicated by an earlier increase of testosterone levels. We also tested whether patterns of testosterone metabolites in blood are reflected by patterns of testosterone metabolites in feces.

Usually, testosterone levels are determined by blood sampling procedures, however, circulating hormone levels are affected in response to the stress of capturing, handling and blood sampling which can in turn affect physiological and behavioral parameters investigated [26]. An additional drawback of blood sampling is that samples represent concentrations at a single point in time. Steroid hormones exhibit regular as well as episodic fluctuations over time and hormone levels representing only a narrow time frame may be biased [27–29]. Over the last years, non-invasive techniques like fecal and urinary sampling have been established for many species [30]. Unlike blood samples, fecal hormone concentrations reflect the cumulative secretion and elimination of hormones over several hours. Therefore, they are less affected by short term fluctuations [30–32] and appear to be more suitable for the investigation of long-term processes like maturation.

The general suitability of fecal steroid metabolites for investigating reproductive status was demonstrated before in the closely related guinea pig [32]. By comparing testosterone metabolite levels of intact males, castrated males and females and by assessing intra-individual correlations between fecal and blood patterns of several sex hormones, they demonstrated that the use of fecal testosterone metabolites is a useful alternative to and indeed has several advantages over blood sampling [29,30].

For the current study we monitored both, plasma testosterone levels as well as fecal testosterone metabolites taken on the same day and compared them within individuals. By using a short term and a long term measure of the sex hormone, we are able to draw robust conclusions about the timing of maturation.

2. Methods

2.1. Housing conditions of F0 animals

The wild cavies used for this study were part of a breeding stock derived from Argentina and Uruguay in 1987. Additional animals were introduced every few years to prevent potential effects of inbreeding. Adult females ($n = 20$) were housed singly in 0.8 m^2 standard enclosures containing woodchips for flooring and a shelter. All females were between 1 and 2 years old and had already successfully reproduced before the start of the experiment. Hay, guinea pig pellets (Firma Höveler, Germany) and water were available *ad libitum*. Additionally, vitamin C (1 g/l) was added to the drinking water once a week and diet was supplemented with fresh vegetables every other day.

For acclimatization, females were kept for four weeks under an artificial photoperiod of 12:12 h (L:D) with a medium light intensity of 253 lux (digital luxmeter, Beha, Glottertal) at a temperature of 20–22 °C. Thereafter, the light–dark rhythm was changed to the starting conditions with 10 h light in the "spring" ($n = 10$) and 14.5 h light in the "autumn" ($n = 10$) treatment. At this time a male was introduced into each enclosure and left there for four weeks to ensure successful mating as female cycle length is about 19 days. Nine days after introducing the male we began to simulate the changes in photoperiod. In the spring treatment, the daily light time was increased by 15 min every nine days while it was decreased by 15 min in the autumn treatment. After the approximately 60 day pregnancy, juveniles in both treatments were therefore born under 12 h ± 15 min light (equals 9 changes of daily light time). These light–dark rhythms were chosen as they correspond to natural spring and autumn changes in day-length in the cavies' natural distribution area [24].

The experiment was repeated a second time using the same males and females as breeding pairs. All pairs changed treatment after the first part of the experiment. As the results of the two experimental runs showed no systematic differences with respect to litter size (run 1: 2.8; un 2: 2.8), litter sex ratio (run 1: 0.63 ± 0.19; run 2: 0.49 ± 0.15) or birth weight of juveniles (run 1: 59.5 ± 9.5 g; run 2: 60.6 ± 10.3 g), these two runs were evaluated together.

For biological validation of fecal testosterone metabolites we used 35 additional animals, 10 adult males and females each (age 1 – 2 years), 10 male juveniles below 20 days of age, and five castrated males (castration at least five month ago). Males and females had already reproduced successfully. Females were neither pregnant nor lactating during the experiment. Females and castrates were kept in groups, males in mixed sex pairs and juveniles were kept with their parents. All animals lived in outside enclosures and experienced natural temperature and photoperiod. Concentration of fecal testosterone metabolites of adult intact males, castrated males, females and juveniles differed significantly among groups (Kruskal–Wallis test, $X^2 = 23.4$; df = 3/34; $p = 3.3^{*}10^{-5}$; $median_{intact}$ = 123.8 ng/g feces; $median_{castrate}$ = 39.4 ng/g feces; $median_{juv}$ = 20.9 ng/g feces; $median_{female}$ = 30.3 ng/g feces). Pairwise comparisons with Bonferroni correction showed significantly higher testosterone metabolite concentrations in intact males than in castrated males (adjusted $p = 0.004$), juvenile males (adjusted $p = 0.0003$) and females (adjusted $p = 6.8^{*}10^{-5}$). Testosterone metabolite concentrations of castrated males did not differ significantly from females (adjusted $p = 0.07$) or juvenile males (adjusted $p = 0.3$). Also juvenile males did not differ from females (adjusted $p = 0.45$).

2.2. Housing conditions and experimental procedures of F1 subjects

In the first experimental run, seven litters from the spring treatment and eight litters from the autumn treatment contained at least one male pup. The other litters consisting only of female pups were excluded. In the second experimental run eight litters with male juveniles were born in the spring treatment and nine in the autumn treatment (32 litters in total). Pups remained with their mother and siblings until weaning (19–21 days of age); afterwards they were kept in unfamiliar, unrelated same sex pairs together with juveniles of the same treatment. To ensure individual permanent recognition, each animal was marked with a pit tag (TROVAN, passive transponder system). We used only the heaviest male from each litter as it is known that siblings differ predictably in behavioral and physiological aspects in this species [33]. In the first experimental run we started sampling when animals were 45 ± 2 days of age and continued until males were 120 ± 2 days of age. We chose this time span because the literature states that physiological maturity occurs around 75 days of age in cavies [34]. However, it turned out that testosterone levels of spring males already differed significantly from those of autumn males at 45 days of age. Thus, in the second run of the experiment we additionally sampled males with 14, 25, 32 and 39 days of age and took one final sample when they were 120 days of age to compare testosterone levels with those of the former run.

Animals received dynamic photoperiod throughout the whole experiment. For males born into spring that means they experienced summer conditions (13.5–14 h light) when they were 80 days old, while males born into autumn experienced winter conditions (10–0.25 h light) during that time. When light conditions reached the starting point again (14 h light for spring treatment; 9.5 h light for autumn treatment), treatment conditions were changed so that light time decreased from thereon for the spring treatment and increased for the autumn treatment. Thus, with 120 days of age the former spring males experienced autumn conditions (12.75 – 13 h light) while the former autumn males experienced spring conditions (11 – 11.25 h light).

2.3. Sampling procedure and endocrine analysis

For later analysis of testosterone concentration, we took blood samples of F1 males. Each blood sample was taken without anesthesia between 10 and 11 a.m. by puncturing the marginal ear vein. The vessels were illuminated with a cold-light lamp and vessels were pricked with an injection needle and about 120 μl of blood was collected in heparinized capillary tubes. One experimenter held the animal in his/her lap, while a second collected the sample [35]. To prevent a stress-induced change in blood testosterone concentration, all samples were taken within 5 min after capturing the animal. The plasma was separated by centrifugation for four minutes with 16,060 rcf and plasma was deep frozen (−20 °C) for later analysis.

After collecting the blood sample, each animal was weighed and put into a transparent macrolone box (42 × 27 × 16) for 10 – 30 min to collect feces. The box was placed in the home enclosure of the animal and was checked every 10 min for feces. Feces samples were immediately deep frozen (−20 °C) for later analysis. If an animal did not defecate during that time, it was released and the feces sample was collected in the same way and at the same time one day later.

To determine plasma testosterone concentrations we used a solid phase enzyme-linked immunosorbent assay (ELISA; Testosterone free in Saliva ELISA, Demeditec Diagnostics GmbH, Kiel, Germany). The antibody cross-reacted > 0.1% with relevant steroids as follows: testosterone 100%, 5α-dihydrotestosterone 23.3%, androstenedione 1.6% (for more details see: http://www.immunoassay.co.uk/download/DES6622.pdf). The intra-assay coefficient of variation was 7.3%, the inter-assay coefficient of variation was 7.5%.

The fecal samples were defrosted and subsequently dried for two hours at 80 °C. The dried feces were homogenized and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. In case of a sample weight below 0.05 g, an accordant aliquot of methanol was added [36]. Subsequently, the samples were analyzed with a testosterone enzyme immunoassay as described in detail earlier [32]. Intra- and inter-assay coefficients of variation were between 7.9 and 12.9%.

2.4. Statistical analysis

For comparison of fecal testosterone metabolites in males, females, juveniles and castrates, we conducted a Kruskal–Wallis test followed by pairwise comparisons (with Bonferroni correction) as data were neither comparable in variance nor normally distributed.

As animals sharing the same mother cannot be assumed to represent independent samples and additionally repeated measures were taken from each individual, we analysed all F1 data using linear mixed effect models (LME) including Individual ID nested within mother as random effects. Additionally, we had to correct for differences in variance using the varIdent function (R-package nlme) as variance increased over age for blood samples as well as fecal samples. For all three models (weight development, testosterone level in blood and testosterone level in feces), treatment (spring or autumn) and age (14, 25, 32, 39, 45, 52, 59, 66, 73, 80 and 120 days) were included as categorical fixed effects. As males of both batches were weighed at each time, but blood and feces were only collected at different times for the batches, samples sizes (and hence degrees of freedom) differ between models.

To determine whether fecal testosterone level reliably reflects blood testosterone levels within individuals, we used Pearson correlations. We report effect sizes only, as the declaration of p-values would be quite meaningless (with only 5 points of within-individual comparisons the effect size would have to exceed 0.95 – so all points would have to lie perfectly on a straight line – to become significant). All statistical analyses were conducted using the free software R 2.14.1 (The R Foundation for Statistical Computing).

3. Results

3.1. Body weight development of F1 males

Photoperiodic treatment did not affect male body mass at birth (spring: 66.3 ± 7.5 g; autumn: 61.8 ± 5.1 g; lme, df = 1/186; $p = 0.48$) or body mass until day 45 (Fig. 1). With 45 days of age, spring males tended weakly to be heavier than autumn males (lme, df = 11/186; $t = 1.7$; $p = 0.1$). This difference in body mass increased over time and remained significant until an age of 80 days (Fig. 2; day 52: $t = 1.9$; $p = 0.05$; day 59: $t = 2.6$, $p = 0.01$; day 66: $t = 3.1$, $p = 0.002$; day 73: $t = 2.7$, $p = 0.008$; day 80: $t = 2.0$, $p = 0.02$). However, the final measurement of body mass with 120 days age showed that autumn males had caught up ($t = 1.2$, $p = 0.23$).

3.2. Plasma testosterone concentration and fecal testosterone metabolites in F1 males

Plasma testosterone concentration did not differ between spring- and autumn-born males until an age of 39 days (Fig. 2). Spring-born males first showed a trend towards elevated testosterone concentration when they were 32 days old (df = 10/107, $t = 1.88$, $p = 0.06$). However, at this time they were still not statistically distinguishable from autumn-born males ($t = -1.25$, $p = 0.21$). When males reached an age of 39 days, the spring-born animals showed a significant increase in testosterone concentrations ($t = 3.7$, $p = 0.0003$) that were not distinguishable from testosterone levels of adult males (age: 120 days, $t = -0.94$, $p = 0.35$). Furthermore, they had significantly higher testosterone concentrations than autumn-born males of the same age ($t = -2.2$, $p = 0.03$).

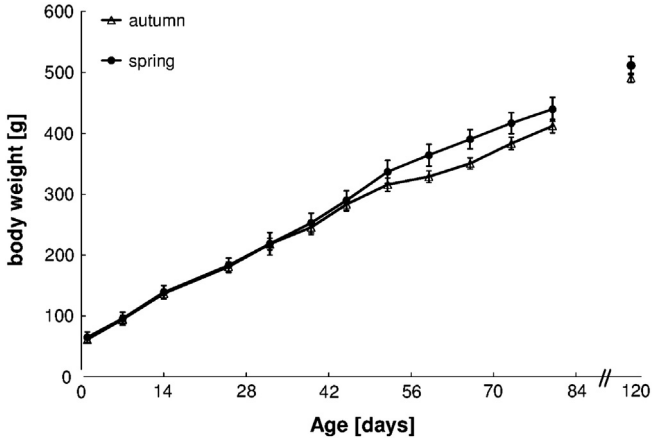

Fig. 1. Body weight (mean ± standard error) of F1 males from birth to 120 days of age. Open triangles represent autumn males (decreasing light period from conception until ~90 days of age, increasing light period until day 120), black dots represents spring males (increasing light period from conception until ~90 days of age, decreasing light period until day 120). Mean weight of males on day 45: spring: 285.7 ± 9 g, autumn: 272 ± 7 g; mean weight on day 66: spring: 389.1 ± 10 g, autumn: 345 ± 6 g; mean weight on day 80: spring: 436 ± 13 g, autumn: 399.3 ± 7 g, mean weight on day 120: spring: 508 ± 15; autumn: 498 ± 7.5. For further details see paragraph 3.1.

Autumn-born males showed a statistically significant increase in testosterone concentration (compared with testosterone concentrations on day 14) only when they were 66 days old (Fig. 2; $t = 3.2$, $p = 0.002$). This was also the first time they did not differ in testosterone levels from spring-born males since day 39 ($t = 1.0$, $p = 0.31$). However, although the first significant increase in testosterone concentration of these autumn-born males occurred with 66 days of age, there were trends for elevated testosterone levels already on days 52 and 59 (52: $t = 2.0$, $p = 0.057$; 59: $t = 1.94$, $p = 0.054$) which points to a slower increase of testosterone levels compared with the steep increase in spring males. Between 66 days of age and 80 days of age both treatment groups did not differ in testosterone concentrations. On day 120 treatment groups did not differ in testosterone concentrations ($t = -0.9$, $p = 0.37$) and there was no batch effect (all males were sampled on day 120 to ensure that males from the first batch were comparable to males of the second batch; df = 1/31, $t = 0.2$, $p = 0.86$).

Analysis of testosterone metabolite concentrations implies a significant increase in testosterone metabolite levels (compared to levels of 14 day old males) in spring males at an age of 45 days ($t = 3.0$, df = 10/107, $p = 0.003$; Fig. 2). Also, on day 45 spring males differed in testosterone metabolite levels from autumn-born males for the first time ($t = -2.2$, $p = 0.03$) and at the same time these levels were no longer distinguishable from adult testosterone metabolite levels ($t = -0.97$, $p = 0.037$). Testosterone metabolite levels remained rather constant from then on to the end of the study.

Autumn-born males first tended to increase in testosterone metabolite levels with 59 days of age ($t = 1.72$, $p = 0.08$) and had significantly increased testosterone metabolite concentrations with 66 days of age ($t = 2.45$, $p = 0.02$). They did not differ from testosterone metabolite concentrations of spring males at this age ($t = 0.3$, $p = 0.76$). Additionally, they did not differ from adult testosterone metabolite levels on day 66 and at later sampling times ($t = 1.14$, $p = 0.25$).

Both, blood testosterone concentrations as well as fecal testosterone metabolites pointed to an earlier onset of maturation in spring born males and show considerable within-individual correlation (mean effect size: $R^2 = 0.7 \pm 0.13$ for $n = 32$ males).

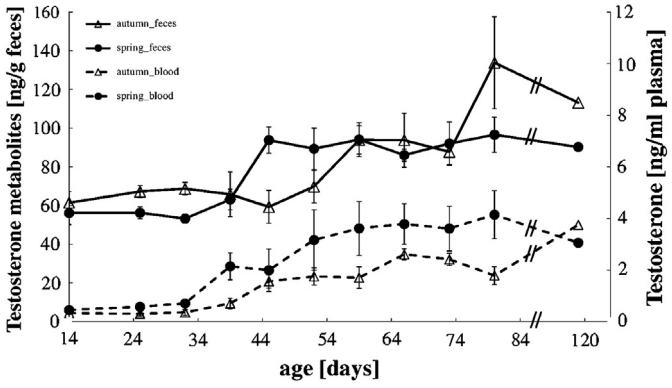

Fig. 2. Concentrations (mean ± standard error) of plasma testosterone and testosterone metabolites in feces of F1 males from birth to 120 days of age. Open triangles represent autumn males, filled dots represent spring males. Solid line indicates testosterone metabolite concentrations in feces, dashed line indicates plasma testosterone levels.

4. Discussion

Different photoperiods during early development strongly affected the somatic and reproductive development of male cavies. Serum testosterone as well as fecal measures of testosterone metabolites indicated an earlier onset of maturity after a steeper increase in testosterone levels in males living under spring conditions.

Differences in photoperiodic conditions during development clearly affected the somatic and reproductive development of young male cavies. Males experiencing different photoperiodic treatments did not differ in body mass at birth and during early growth, but under an increasing photoperiod grew faster during later development, a difference that vanished when they reached adulthood. Differences in patterns of growth related to season of birth have been reported for many small rodent species, mostly in mice and rats. In montane voles, root voles and male fisher rats, long photoperiod stimulates growth while short photoperiod inhibits growth [13,37,38]. In various rat strains, a reduction in food intake under short winter photoperiod was likely to cause the suppression of growth and reproductive development [39]. In our experiment, all animals received ad libitum food, but we did not measure the amount of food eaten. Cavies experiencing autumn photoperiod may have eaten less than animals under spring conditions, but equally likely body composition may differ between the two groups with autumn animals storing more fat [40]. Alternatively, autumn animals may invest more in self maintenance for example by enhancing immune capacity to increase their chances of surviving the winter [41,42]. According to the theory of optimal energy allocation to growth and reproduction, an individual that postpones maturation should allocate all energy to growth in order to gain large body size until the next breeding season [1]. Larger males are better competitors for access to females, therefore, the inhibition of growth in autumn males does not seem optimal at first. The slower growth of autumn males during development could also have an adaptive value for males who have no chance of successful reproduction before winter. Large body size may decrease their chances of survival during winter as demonstrated for several rodent species [43,44]. Lovegrove [45] argued that reduced body weight during winter months resulted in energy conservation and reduced thermoregulatory costs. However, further studies are needed to explain seasonal differences in growth of male cavies.

Both, serum and fecal measures of testosterone levels indicated an earlier onset of testosterone increase in males living in increasing photoperiod than in males living in decreasing photoperiod although the exact times differed slightly between measures.

In mammals, the environmental signal 'day length' is transduced into a physiological signal trough secretion of pineal melatonin during darkness [46]. Melatonin production occurs in the absence of light; the duration of melatonin secretion is inversely proportional to day length [47]. Early work showed that elevated melatonin levels mediated by short day photoperiod inhibits gonadotropin release and causes

slower gonadal development or even gonadal regression in autumn [48,49]. In turn, secretion of sex steroids such as testosterone, are inhibited. Strong effects of photoperiod on testosterone levels have been reported in many seasonally reproducing species, for instance in various species of deer mice, root voles, and rats [38,39,50–52]. Juvenile deer mice and bank voles, for example, that were reared under long days showed a faster testosterone increase than individuals reared under short days [50,53]. Testes of male meadow voles that were reared under long days for five weeks were about twice as heavy as testes of males reared under short days [54]. In prairie voles, plasma testosterone levels of males reared under long days until 35 days of age were even nearly three times as high as testosterone levels of males reared under short days [55] while the same light regime did not cause differences in testosterone levels of golden hamsters with seven weeks of age [56]. Even closely related species do react quite differently upon photoperiodic cues as was demonstrated in several *Peromyscus* species by (52, 57). While three species did not show any differences in testosterone levels when reared into long or short days, three other species developed very pronounced differences (between 3.5 and 8 times higher under the long day treatment). In the precocial cavy, we found the difference in plasma testosterone level on average to be 1.8 times higher in the spring males than in autumn males between 45 and 66 days of age. Thus, while not all tested altricial rodents seem to react upon photoperiodic cues (at least on constant photoperiods), in the species that do react upon these cues, the treatment differences are more clearly pronounced than the differences we found in the current study.

Furthermore, the treatment difference in timing of maturation seems to be more pronounced in altricial species than in the cavy. The difference in maturation between male white-footed mice reared under short or long days was about 80 days [57] while it is only about 14 days in the cavy. Another difference might be that in studies of altricial rodents differences in testosterone levels became even more pronounced with increasing age while this was not the case in our study. We found the most pronounced differences in testosterone levels between 45 and 66 days of age. All differences had disappeared when males were fully adult. Bauer et al. [23] reported in a study on the closely related guinea pig a slow increase in testosterone levels of young males kept under long days until 42 days of age and a more pronounced, steep increase between 56 and 70 days of age. Males that were reared under short days, however, showed a slower increase in testosterone levels (indicated by a shallower elevation) and a peak with 84 days of age. Their results correspond to ours in so far as we also found a slower increase of testosterone levels under autumn conditions. This indicates that the process of maturation itself differs between seasons — fast and early maturation in spring versus slower and later maturation in autumn.

The general timing of testosterone increase in our study fits the timing of maturation given in the literature. Rigaudiere [58] suggested from testosterone titres that male guinea pigs enter maturation around 50 days of age and Young [59] observed most vigorous sexual behavior and first ejaculation between 40 and 84 days of age in guinea pigs. In an earlier study on cavies males were found to reach testosterone levels of adult males kept together with females around 80 days of age [34]. Additionally, males successfully inseminated adult females with 71 ± 3.3 days of age. All males tested in this study were born into and grew up under decreasing natural light conditions in our lab (unpublished data). Hence, these data fit the estimated time of maturation of autumn males in the current study. Our study suggests that it may be worthwhile to study differences between static and dynamic photoperiods in more detail.

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