



Photoperiod-induced differences in uterine growth in *Phodopus sungorus* are evident at an early age when serum estradiol and uterine estrogen receptor levels are not different

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ARTICLE INFO

Article history:

Received 31 August 2009
Received in revised form 15 October 2009
Accepted 15 October 2009
Available online 24 October 2009

Keywords:

Hamster
Uterus
Estradiol
Estrogen receptors
Day length

ABSTRACT

Sexual development is inhibited in Siberian hamsters (*Phodopus sungorus*) in short days (SD), and a small uterus is an obvious indicator of photo-inhibition. The small uterus in SD is presumably due to the delayed onset of estrous cycles. However, in an earlier study, the investigators reported that serum estradiol (E2) concentration was significantly higher in young females raised in SD than in long days (LD), with the highest concentrations measured in SD at 4 weeks of age. These seemingly contradictory findings were investigated in the present study. First, uterine mass and body mass were measured in SD- and LD-reared hamsters from 1 to 12 weeks of age. Uterine mass was significantly greater in LD than in SD by 3 weeks of age and onward. Thereafter, our investigation focused on 4-week-old hamsters. Serum E2 concentrations in LD and in SD were not significantly different and there were no significant LD–SD differences in uterine estrogen receptors (ER), as measured by immunohistochemistry and quantitative real-time RT-PCR. Therefore, alternative explanations for the photoperiodic difference in uterine size in young Siberian hamsters are considered.

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1. Introduction

Age at puberty can vary significantly in short-lived, temperate zone rodents depending on their season of birth. Females born before or near the summer solstice, i.e., when day length is either increasing or near its annual maximum, mature rapidly and produce litters during the year of their birth. Conversely, females born well after the summer solstice are very likely to delay puberty and their first breeding until the following spring (Negus et al., 1986, Butler et al., 2007). One of the hallmarks of delayed puberty in females is the stunted growth of the uterus. In Siberian hamsters, *Phodopus sungorus*, Ebling (1994) measured uterine mass at 5 and 10 weeks of age in females held in either short days (SD) or long days (LD). Whereas uterine mass increased more than two-fold in long day (LD) females, no change in uterine mass was detected in SD females. At both 5 and 10 weeks of age, Ebling (1994) determined that uterine mass in SD hamsters was significantly less than in LD females. Subsequent studies also documented significant uterine size discrepancies in SD and LD hamsters at 10 and 13 weeks of age (Place et al., 2004, Timonin et al., 2006).

Smaller uterine size in SD hamsters has been assumed to result from the indirect effects of the SD melatonin (MEL) signal, with MEL being secreted into the circulation from the pineal gland at night at higher concentrations and for a longer duration than in hamsters held in LD

(Darrow and Goldman, 1985, Hoffmann et al., 1986). Stunted growth of the uterus has been presumed to result from the suppression of gonadotropins in SD (Dodge and Badura, 2002) and the associated reduction in the concentrations of estrogens (e.g., estradiol). To our knowledge, this hypothesis has never been formally tested and this conjecture has been called into question by a study that reported significantly higher serum E2 concentrations in SD than in LD hamsters between 4 and 11.4 weeks of age (van den Hurk et al., 2002). In that study, E2 in SD females was as much as eight-fold higher than in LD hamsters (8 weeks of age), and significantly higher concentrations were noted in SD at each of the ages studied (4, 8, and 11.4 weeks). The highest absolute concentration (~2400 pmol/L or ~654 pg/mL) was measured in SD females at 4 weeks of age. Given the growth promoting effects of E2 on the uterus (Scotti et al., 2007), we found the report of higher E2 in SD hamsters (van den Hurk et al., 2002) to be perplexing. Unfortunately, van den Hurk et al. (2002) did not report uterine mass data, thus the present study is the first to investigate the apparent mismatch between serum E2 concentrations and uterine growth in *P. sungorus*.

To that end, we collected uterine and body mass data at regular intervals from 1 to 12 weeks of age, to more clearly elucidate the differences when females develop in LD or in SD. Earlier investigations that reported the effects of photoperiod on uterine mass in *P. sungorus* evaluated females at only one or two ages (Ebling, 1994, Place et al., 2004, Timonin et al., 2006), and the youngest animals studied were 5 weeks of age (Ebling, 1994). When we determined that the photoperiod-induced differences in uterine size were evident by 3 weeks of age, we then focused our investigation on LD and SD

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hamsters at 4 weeks of age for the following reasons: 1. The highest E2 concentration reported by van den Hurk et al. (2002) was in SD females at this age and the LD–SD difference in serum E2 concentration at 4 weeks was substantial (approximately 5-fold), and 2. Neither LD nor SD females have matured by this age, as indicated by the lack of vaginal patency (Place et al., 2004) and the absence of any signs that ovulations had occurred (van den Hurk et al., 2002). Our investigations of 4-week-old hamsters avoided potentially confounding variables, such as stage of estrous cycle in LD females or LD–SD differences in reproductive state.

Using an assay previously validated for the measurement of E2 in Siberian hamsters (Scotti et al., 2007), we did not replicate the findings of van den Hurk et al. (2002), i.e., that serum E2 concentration is significantly higher in SD than in LD hamsters at 4 weeks of age. However, because we found serum E2 concentrations to be nearly identical in 4-week-old SD and LD females, we looked to the uterus for possible mechanisms to explain the photoperiod-induced difference in uterine size. To test the hypothesis that estrogen receptor (ER) abundance differs in uteri from SD and LD hamsters, we assessed ERs by immunohistochemistry for ER α and quantified mRNA levels by real-time RT-PCR for ER α (*Esr1*) and ER β (*Esr2*).

2. Materials and methods

2.1. Experimental animals

Siberian hamsters from our colony (14 h of light per day, 14L) were transferred to LD (16L) or SD (10L) as breeding pairs to generate experimental LD and SD females. The time of lights-off was synchronized for all animals to 1700 Eastern Standard Time (EST). Animals were originally derived from wild-bred stock obtained from Dr. K. Wynne-Edwards, Queen's University. Experimental females were weaned on postnatal day 18, placed in polypropylene cages (2 to 4 siblings/cage), and maintained in the photoperiod in which they were born. Food (Teklad 8626, Madison, WI, USA) and water were available *ad libitum*. Ambient temperature and relative humidity were held constant at 21 ± 5 °C and $50 \pm 10\%$, respectively.

Different sets of animals were used in Experiments 1 and 2, as well as for different determinations within Experiment 2. All experiments were carried out in accordance with the European Commission legislation on the protection of animals used for experiments (EC Directive 86/609/EEC).

2.2. Experiment 1: Uterine mass and body mass to 12 weeks of age

Measurements of uterine and body mass were made on six to seven SD and LD females at each of the following ages: 1, 2, 3, 4, 6, 8, 10, and 12 weeks. Sibling females were divided across age cohorts, thus siblings were never in the same age group. Body mass was measured just prior to death for each of the predetermined ages. Animals at 1 week of age were killed by decapitation, whereas older animals were given an overdose of pentobarbital sodium. Uteri were removed, dissected free of fat and connective tissue, and weighed on an analytical balance.

2.3. Experiment 2: Determinations at 4 weeks of age

2.3.1. Serum E2 and SHBG

Because female hamsters weigh less than 20 g at 4 weeks of age, serum samples from the entire SD and LD litters had to be pooled (females only) to assure adequate sample volumes. Five SD and six LD litters (three to five female pups per litter) were given an intraperitoneal overdose of pentobarbital sodium and exsanguinated by retro-orbital bleed. Blood was clotted at room temperature for 1 h and centrifuged at 1000g for 20 min in 4 °C. Drawn off serum was pooled by litter, frozen and maintained at -80 °C until assayed for E2. Serum samples were analyzed in duplicate by a radioimmunoassay (RIA) previously validated in Siberian hamsters (Scotti et al., 2007). Briefly, the RIA

used was a solid-phase ^{125}I kit (Diagnostic Products Corporation, [now Siemens], Los Angeles, CA, USA), modified by addition of a pre-assay ether extraction. Following the addition of ^3H -estradiol (50 μL , ~ 1900 cpm) to determine extraction efficiencies, 300 μL serum samples were extracted in diethyl ether, dried under N_2 , and reconstituted in 335 μL of an assay buffer. A 100 μL aliquot was counted on a scintillation counter to calculate recoveries for ^3H -estradiol and separate 100 μL aliquots were added to Coat-A-Count® tubes in duplicate for RIA. The cross-reactivity of the highly specific antibody to other estrogens is less than 2%, save for estrone (10%). A standard curve (15.6–1000 pg/mL) was made by serially diluting an E2 stock solution in the assay buffer. Tubes were incubated at room temperature (23 °C) for 3 h following the addition of ^{125}I -estradiol tracer. Tubes were aspirated of their contents then counted in a gamma counter. Volume and percent extraction recovery specific to each sample were used to calculate concentrations interpolated from the standard curve. The E2 assays met all quality assurance criteria and internal controls were run at the beginning, middle, and end of each assay. The intra- and inter-assay coefficients of variation were $< 10\%$, and the minimum detectable limit (MDL) of the assay was 18 pg/mL. Samples with an estradiol concentration below the level of detection were assigned this value for statistics and graphing.

As a biological validation to our E2 assay, we also measured serum E2 concentration in older hamsters (16–20 weeks of age), when LD females ($n = 12$) are expected to be cycling and should have higher E2 concentrations than immature SD females ($n = 5$). As expected, serum E2 concentration was highly variable in LD females (18.0–125.2 pg/mL), but uniformly low in photo-inhibited SD females (all below the MDL, 18.0 pg/mL). We have found that vaginal cytology does not reliably track the estrous cycle in *P. sungorus*, and no attempt was made to monitor the estrous cycle of LD females. Thus, the sampling likely represents LD females at different stages of the estrous cycle and SD females that were uniformly anestrus.

For analysis of SHBG, blood samples were collected from a separate cohort of LD and SD female hamsters at 4 weeks of age ($n = 12$ and 8, respectively). Competition assays were used to determine the affinities of hamster SHBG for E2 and testosterone relative to 5 α -dihydrotestosterone (DHT). The serum concentration of SHBG was determined by the steroid binding capacity assay, employing ^3H -DHT as the labeled ligand and dextran-coated charcoal as the separation agent (Hammond and Lähteenmäki, 1983).

2.3.2. Histology and immunohistochemistry

A mid-portion of the uterine horn and an ovary from six SD and six LD females at 4 weeks of age were embedded separately in paraffin, sectioned at 6 μm , and mounted on glass slides for standard histology (uterus and ovary) or on Superfrost® Excell slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) for immunohistochemistry (uterus). Sections for standard histology were stained with hematoxylin and eosin (H&E).

For immunohistochemistry, sections from LD and SD uteri were alternately placed on each slide to control for potential staining variability between slides. Adjacent sections were mounted on separate slides for negative controls. After dewaxing and rehydration in a series of ethanols, slides were submerged in Antigen Unmasking Solution (1:100 v/v in H_2O ; H-3300; Vector, Burlingame, CA, USA) and microwave on hi-power for two 15-min bouts. Endogenous peroxides were quenched in hydrogen peroxide (0.5% in methanol) for 20 min. Sections were then incubated overnight at 4 °C in CleanVision™ Blocking solution (ImmunoVision Technologies, Norwell, MA, USA) plus 10% goat serum to block nonspecific binding sites. Monoclonal mouse anti-human ER α antibody (M7047, Dako, Carpinteria, CA, USA) was diluted 1:40 in dilution buffer and incubated with sections for 48 h at room temperature. Sections were incubated with the secondary antibody, biotinylated goat anti-mouse IgG (1:200 in dilution buffer; BA-9200; Vector, Burlingame, CA, USA), for 30 min. Negative controls excluded

Table 1
PCR primers and product sizes.

Gene	Forward primer 5'–3' Reverse primer 5'–3'	Amplicon size (bp)	GenBank accession
ER α (<i>Esr1</i>)	CAGGTGCCCTACTACCTGGA CAGTCTCTCGGCCACTCT	117	EU564705
ER β (<i>Esr2</i>)	TGCAGAACCTCAAAGAGTCC AGCATCCCTCTTGAACCTG	142	EU564704
Aromatase (<i>Cyp19a1</i>)	GATCAGCGGAGAGGAGACAC GAAAGGGCGAATTGTTTCC	178	Pending
<i>Rpl13a</i>	CATGAGGTCGGGTGAAATA AGCTGCTTCTTCCGATAG	105	Pending

the primary or secondary antibody. Immunoreactivities were visualized by incubating sections with Vectastain Elite ABC Solution (Vector) for 30 min and developing with DAB Peroxidase Substrate Solution (Vector) following the manufacturer's instructions. Sections were counterstained with hematoxylin.

For uterine smooth muscle α -actin, the immunohistochemistry protocol followed that for ER α , except for the primary antibody used, which was a monoclonal mouse anti- α smooth muscle actin (A2547 Sigma, St. Louis, MO, USA), at a dilution of 1:2000 and an incubation of 24 h.

We made several attempts to detect ER β by immunohistochemistry, using different primary antibodies and dilutions, without success. Seeing as Jefferson et al. (2000) did not detect ER β in the mouse uterus

on postnatal day 26, or transcripts on postnatal days 5, 12, 19 and 26, we did not pursue this further. However, Jefferson et al. (2000) did detect ER β mRNA (*Esr2*) in postnatal day 1 mouse uteri, thus we measured *Esr2* levels in Siberian hamster uteri at 4 weeks of age.

2.3.3. Quantitative real-time RT-PCR

An additional six to 10 females were generated in each photoperiod as above to harvest ovaries and uteri from 4-week-old individuals to quantify uterine ER α (*Esr1*) and ER β (*Esr2*) and ovarian P450 aromatase (*Cyp19a1*) mRNA levels. Following pentobarbital sodium overdose and exsanguination, ovaries and uteri were removed, dissected free of surrounding adipose and connective tissue, flash-frozen on dry ice, and stored separately at -80°C until RNA extraction. Frozen tissues were homogenized with a Polytron, and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) in accordance to the manufacturer's instructions. RNA was reverse transcribed using Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. RNA samples used for real-time RT-PCR were treated with DNase I (Amp. Grade; Invitrogen) prior to reverse transcription. Quantitative real-time PCR was run under standard conditions using Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The annealing temperature for all genes was 60°C .

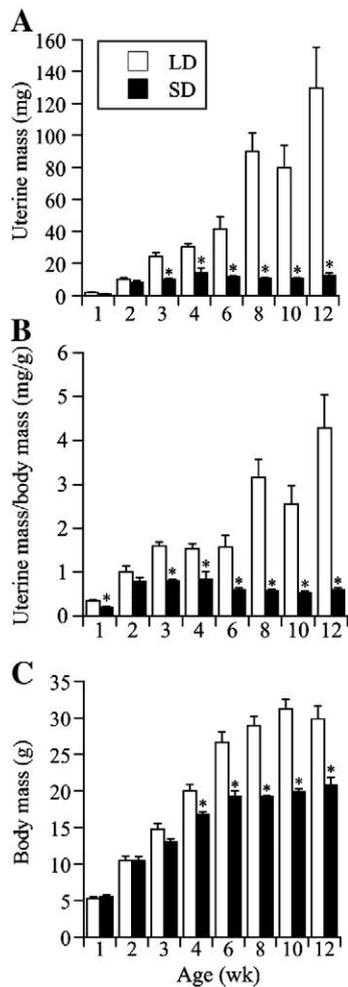


Fig. 1. [A] Mean (+SEM) uterine mass (mg), [B] uterine mass/body mass (mg/g), and [C] body mass (g) in hamsters from 1 to 12 weeks of age when raised in either long days (LD, 16L) or short days (SD, 10L). LD vs. SD differences were significant (*, $p < 0.05$) as follows: uterine mass and uterine mass/body mass at all ages except 2 weeks; body mass at 4–12 weeks. Sample sizes were six to seven animals per age in each photoperiod.

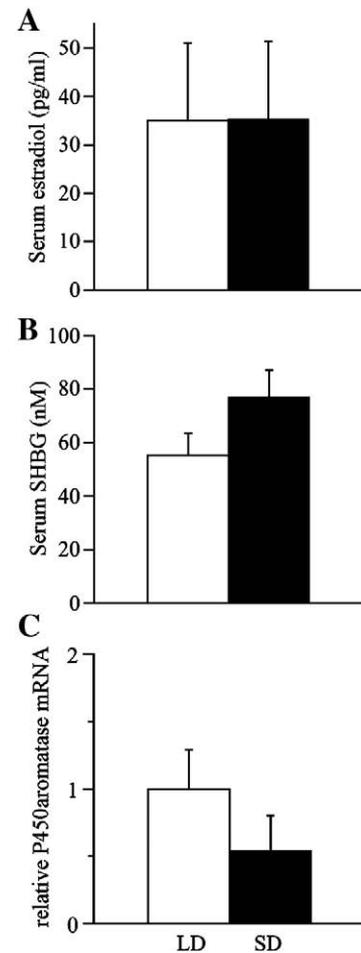


Fig. 2. [A] Mean (+SEM) serum estradiol (pg/mL) and [B] SHBG (nM) concentrations in 4-week-old female Siberian hamsters raised in either LD (16L) or SD (10L). [C] Mean (+SEM) mRNA levels (relative to *Rpl13a*) for ovarian P450 aromatase mRNA (*Cyp19a1*). As an arbitrary point of reference, mean mRNA levels in LD were set to one, and as such, all values were divided by the LD mean. Sample sizes were five to six animals in each photoperiod.

The specificities of the RT-PCR products were confirmed by melting curve analyses. *Esr1*, *Esr2*, and *Cyp19a1* mRNA levels are expressed relative to the levels of Ribosomal protein L13a (*Rpl13a*) mRNA in the same samples. Schroder et al. (2009) noted that *Rpl13a* was expressed at fairly constant levels in uteri under varying conditions, and our pilot data showed comparable results for *Rpl13a* in ovaries. For each target gene and the reference gene, samples were run in triplicate. For quantification of each gene, we used relative standard curves (Cikos et al., 2007). Briefly, standard cDNAs were prepared from other hamster ovaries and uteri, and were amplified along with sample cDNAs in the same PCR run. Using five to seven serially diluted standard cDNAs, standard curves were prepared for both the target (*Esr1*, *Esr2*, and *Cyp19a1*) and reference (*Rpl13a*) genes. For each experimental sample, the amount of the target and reference genes was interpolated from the appropriate standard curve. The mean mRNA levels of LD animals were arbitrarily set to 1.

Primers for the genes of interest were designed from known sequences from other rodent species, including the Syrian hamster (*Mesocricetus auratus*) and Chinese hamster (*Cricetulus griseus*), using Primer3 software (Rozen and Skaletsky, 2000; <http://primer3.sourceforge.net/>). Primers and PCR product sizes are given in Table 1. PCR products from initial experiments were sequenced to confirm their identities. All primers were designed to span at least one intron to ensure that no genomic DNA-based amplicon would confound cDNA-based amplicons.

2.4. Statistical analysis

Results were analyzed with a commercial statistical program (JMP version 7.0.2, SAS Institute, Cary, NC, USA). For uterine mass and body mass from 1 to 12 weeks of age, data were normally distributed but the variances were unequal (Levene's test). Data were log-transformed prior to two-factor ANOVA and posthoc Tukey's HSD tests. Data were back transformed for graphing purposes. Analysis of serum E2 and SHBG concentrations, ovarian *Cyp19a* and uterine *Esr1* and

Esr2 mRNA levels in 4-week-olds were made with *t*-tests. Differences at $p < 0.05$ were considered to be significant.

3. Results

3.1. Experiment 1: Uterine and body mass from 1 to 12 weeks

Uterine mass was significantly greater in LD than in SD females at 3 weeks of age and beyond ($p < 0.05$, Fig. 1A), and a similar pattern was apparent when uterine mass was expressed relative to body mass (Fig. 1B). Uterine mass normalized to body mass was also significantly different at 1 week of age, but the measurement of uterine mass at this age may have been less accurate than at older ages owing to the small size of the pups and their uteri.

Body mass was significantly greater in LD than in SD females at 4 weeks of age and beyond ($p < 0.01$, Fig. 1C). Changes in body mass followed a similar pattern in LD and in SD females, albeit with relatively lower body masses in SD starting at 4 weeks. However, age-associated changes in uterine mass followed a different pattern in LD than in SD females. In SD, uterine mass increased significantly until 4 weeks of age, and then no significant change occurred through 12 weeks of age. Conversely, in LD, uterine mass continued to increase beyond 4 weeks of age. Uterine mass normalized to body mass showed similar growth patterns.

3.2. Experiment 2: Determinations at 4 weeks of age

3.2.1. Serum estradiol, SHBG, and ovarian measures

Serum E2 concentrations in LD and in SD females were not statistically different at 4 weeks of age (Fig. 2A). At this age neither LD nor SD females had achieved sexual maturity, i.e., the vagina remained closed and no peri-ovulatory follicles or corpora lutea were seen (see below). Serum SHBG concentrations in LD and in SD females were also not statistically different at 4 weeks of age (Fig. 2B). Siberian hamster SHBG had a high affinity for DHT and testosterone, whereas the affinity for E2 was relatively low, and only 5% of that for DHT.

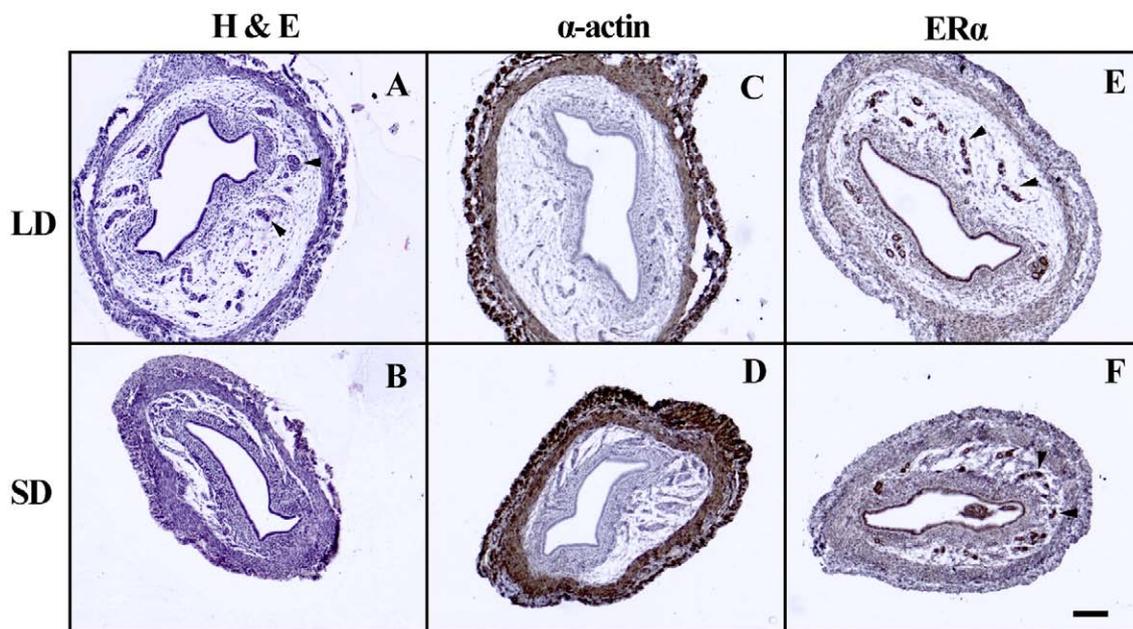


Fig. 3. Representative photomicrographs of uterine horn transverse sections from 4-week-old LD (top) and SD (bottom) females. Left-most panels were stained with hematoxylin and eosin (H&E) and show a more compact uterus in SD as compared to LD. Middle panels show immunohistochemistry for smooth muscle α -actin, which highlights the two outer myometrial layers in uteri from both photoperiods. Right-most panels show immunohistochemistry for ER α , and the staining intensity patterns in each photoperiod are similar (glandular epithelium > luminal epithelium > myometrium). Glandular epithelium (arrowheads in A, E, and F) was readily seen in LD uteri stained by H&E or ER α immunohistochemistry, whereas SD glandular epithelium could only be definitively identified by ER α immunohistochemistry. Bar = 100 μ m, and the same magnification was used in all panels.

Paired ovarian mass (not shown) was significantly greater in LD than in SD females at 4 weeks of age ($p < 0.01$), but this difference disappeared when paired ovarian mass was expressed relative to body mass ($p = 0.24$). Ovarian histology (not shown) was similar in 4-week-old LD and SD females. All follicle classes up to early antral follicles were observed in ovaries from LD and SD females, including primordial, primary, and secondary follicles. Late antral (periovulatory) follicles and corpora lutea were not seen in ovaries from 4-week-old females. The levels of ovarian aromatase (*Cyp19a1*) mRNA did not differ by photoperiod (Fig. 2C).

3.3. Uterine histology and estrogen receptors

The defining histological structures of the uterus (endometrium, endometrial glands, and myometrium) could be readily identified by standard histology (H&E staining) in uteri from 4-week-old LD females, whereas only the endometrium could be identified with confidence in the uteri from age-matched SD hamsters (Fig. 3A and B). The outer layers of the LD and SD uteri were clearly identified as myometrium following immunohistochemistry for smooth muscle α -actin (Fig. 3C and D). The endometrial glands were readily visible following immunohistochemistry for ER α (Fig. 3E and F), and this was especially true for SD uteri (Fig. 3F). The relative intensity of the ER α immunostaining among the different uterine compartments was identical to that reported in mice (glandular epithelium > luminal epithelium > myometrial smooth muscle; Tibbetts et al., 1998). Whereas the intensity of ER α immunostaining may appear to be greater in the SD than in the LD uterus, we refrain from any further attempt to quantify these results, as the impression may simply reflect the more compact nature of the SD uterus.

The mRNA levels of uterine ERs (*Esr1* and *Esr2*) were not significantly different in LD and SD females at 4 weeks of age (Fig. 4A

and B). Generally, the level of ER α (*Esr1*) was much higher than ER β (*Esr2*) (Fig. 4C).

4. Discussion

To our knowledge, the present study is the first to explore the effects of photoperiod on uterine growth and serum E2 concentrations in Siberian hamsters in a single investigation. We have replicated previous observations of a pronounced inhibitory effect of SD on uterine growth (Ebling, 1994, Place et al., 2004, Timonin et al., 2006), and extended those findings to show that the LD–SD divergence in uterine size occurs by 3 weeks of age. This was 2 weeks earlier than had been previously investigated (Ebling, 1994). Because we found no LD–SD difference in serum E2 concentration at an age (4 weeks) when the photoperiod-induced difference in uterine mass has already been established, we looked to the uterus for possible explanations.

Our assessments of uterine estrogen receptors by immunohistochemistry and RT-PCR showed no significant LD–SD differences at 4 weeks of age, and yet, uterine mass was significantly less in SD than in LD. We offer the following as possible explanations: 1. The difference in uterine growth and size could potentially be mediated by differences in MEL secretion, as the pineal MEL rhythm in Siberian hamsters matures before 3 weeks of age (Tamarkin et al., 1980, Yellon et al., 1985). MEL interferes with estrogen signaling pathways by inhibiting E2-induced ER α -mediated transcription via induction of conformational changes in the ER α -calmodulin complex (del Rio et al., 2004). These observations were made in breast cancer cell lines (MCF-7), but Kanishi et al. (2000) also showed an inhibitory effect of MEL on an endometrial cancer cell line (Ishikawa) that expresses ER α . However, no effect was seen in a cell line (SNG-II) that is ER-negative. Because MEL secretion is inhibited by light, it circulates at a higher concentration and for a longer duration in SD than in LD hamsters (Darrow and Goldman, 1985, Hoffmann et al., 1986). Thus it is conceivable that the SD–MEL signal inhibits E2-induced uterine growth in hamsters held in SD. This is not to say that the uterus in SD females will not grow in response to E2, because Scotti et al. (2007) demonstrated substantial uterine growth in E2-implanted SD hamsters. However, the implants in that study resulted in significantly higher serum E2 concentrations than in their LD controls, and Kanishi et al. (2000) abolished the inhibitory effect of MEL by administering E2 to ER-expressing endometrial cells. 2. Somatic growth was inhibited in SD, as evidenced by the divergence in body mass after weaning in hamsters, and modulators of somatic growth (e.g., epidermal growth factor [EGF] and insulin-like growth factor [IGF]) may impact uterine growth by estrogen dependent and independent means (Branham and Sheehan, 1995, Ignar-Trowbridge et al., 1992, Westley and May, 1994). However, Park et al. (2003) found no LD–SD difference in circulating concentrations of IGF-1 in male *P. sungorus*, and to our knowledge, the effect of photoperiod on EGF in Siberian hamsters has not been investigated. Whereas the presumed mechanism for SD inhibition of uterine growth in Siberian hamsters may be mostly correct, i.e., the SD–MEL signal suppresses gonadotropin, and in turn, E2 concentrations, the cause for stunted uterine growth in SD by 3 weeks of age remains to be elucidated.

An explanation for the relatively high E2 values in young SD females as reported by van den Hurk et al. (2002) warrants discussion. van den Hurk et al. (2002) made no mention of a pre-RIA ether extraction of serum samples, thus the method used to measure serum E2 in the present study was significantly different from theirs. Ether extraction of samples prior to RIA for E2 serves three potential benefits: 1. Displacement of E2 from its binding protein (SHBG), 2. Conjugates that may cross-react with the antibody are left in the aqueous phase of the extraction, and 3. Proteins that may interfere with the RIA are removed (Wheeler, 2001). The manufacturer of the RIA states that the antibody for E2 is highly specific, with cross-reactivities to other estrogens being less than 2%, save for estrone (10%). This modest level of

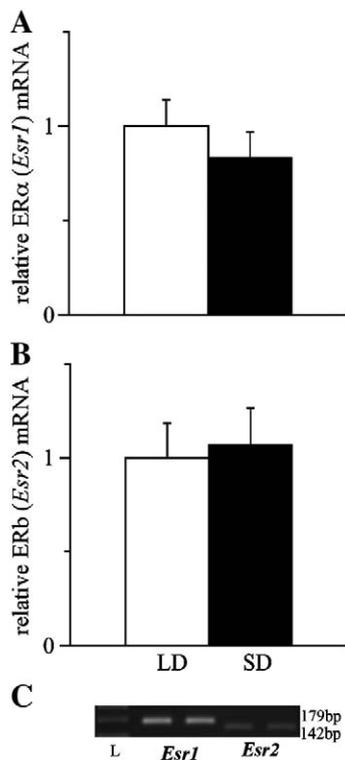


Fig. 4. Mean (+SEM) mRNA levels (relative to *Rpl13a*) for [A] uterine ER α (*Esr1*) and [B] uterine ER β (*Esr2*) from 4-week-old hamsters raised in LD or in SD. As an arbitrary point of reference, mean mRNA levels in LD were set to one, and as such, all values were divided by the LD mean. Sample sizes were nine to 11 animals in each photoperiod. [C] Conventional RT-PCR products for uterine ER α and ER β , run on an ethidium bromide, 1.5% agarose gel, which shows the level of ER α was higher than ER β in uteri from two representative females.

cross-reactivity with estrone is unlikely to explain the discrepancy between extracted and unextracted samples, as estrone (E1) would also be recovered from the ether fraction. In the present study, we also ruled out a LD–SD difference in SHBG concentrations as a possible explanation for the spuriously high E2 concentrations in SD that were reported by van den Hurk et al. (2002). The relatively low binding affinity that Siberian hamster SHBG has for E2 (present study, Gustafson et al., 1989) and for E1 (Gustafson et al., 1989) also argues against SHBG causing high E2 in unextracted serum samples. Alternatively, other substances within the serum could bind non-specifically to the E2 antibody of the RIA, and these yet-to-be-determined substances could be found in higher concentrations in SD than in LD hamsters. Remembering that RIAs work on an inverse relationship basis (less radioactivity means more endogenous hormone), substances that compete with the RIA tracer for antibody binding sites could falsely elevate E2 values. Whatever the offending agent(s) may be, the problem does not seem to be limited to the RIA format, as Scotti et al. (2007) also found higher serum E2 concentrations in SD than in LD hamsters when unextracted samples were analyzed by enzyme immunoassay.

When van den Hurk et al. (2002) sought to explain the high concentration of E2 in SD hamster serum, they logically looked to the ovaries as a potential source. They described the SD ovary as being “highly steroidogenic”, especially the so-called “luteinized atretic follicles”, which showed substantial 3β -hydroxysteroid dehydrogenase (3β -HSD) activity. The so-called “luteinized atretic follicles” are numerous in the SD ovary and are composed of hypertrophied granulosa cells that surround atretic oocytes (Kabithé and Place, 2008). van den Hurk et al. (2002) speculated that these structures were the source of the elevated E2 concentrations in SD. Whereas increased 3β -HSD activity could conceivably provide a mechanism for increased E2 concentrations in SD, by increasing the substrates for P450 aromatase, in the present study we did not find evidence for higher levels of mRNA (*Cyp19a1*) for this enzyme in the SD ovary.

The day length regimes used in the present study and by van den Hurk et al. (2002) were not identical, but variations in the photoperiods used are unlikely to explain the study differences in serum E2 outcomes. In our investigations of development in SD females, we routinely gestate and maintain hamsters in 10L (Place et al., 2004, Timonin et al., 2006, Kabithé and Place, 2008, Place and Cruickshank, 2009) and this was the protocol used in the present study. In contrast, van den Hurk et al. (2002) held females in 16L (LD) during gestation and transferred them to 4L at birth. However, several studies have shown a static SD photoperiod (e.g., 8L or 10L) during gestation and postnatal development was equally effective at inhibiting sexual development as a dynamic transition from prenatal LD to postnatal SD (Hoffmann, 1982, Stetson et al., 1989, Shaw and Goldman, 1995, Goldman and Goldman, 2003). Even though van den Hurk et al. (2002) presented no uterine size data, there is every reason to believe that uterine growth in their SD females was profoundly photo-inhibited, and that the reproductive states of SD females in the two studies were comparable.

In conclusion, we have found that photoperiod-induced differences in uterine growth are evident in Siberian hamsters at an early age when no differences in serum estradiol or uterine estrogen receptors could be detected. The possibility that other mediators of uterine growth (e.g., EGF) are modulated by photoperiod warrants investigation.

Acknowledgements

The authors would like to thank the staff of the Laboratory Animal Services at Cornell University, and Jackie Belliveau in particular, for the exceptional care of our animals. We thank Esther Kabithé and Stella Vincent for technical assistance, Dr. Geoffrey Hammond for the analysis of SHBG, and Dr. Matt Paul and two anonymous referees for their helpful comments on the manuscript. The authors declare that there is no conflict of interest that would prejudice the impartiality of

this scientific work. This study was supported by NIH grant HD-050358.

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