Photoperiod-gonadotropin mismatches induced by treatment with acyline or FSH in Siberian hamsters: impacts on ovarian structure and function

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Abstract

Many seasonal breeders time their reproductive efforts to specific times of the year to ensure adequate resources for the production and care of young. For long-day (LD) breeders, females born before the summer solstice (LDs) reach sexual maturity quickly and often breed that same year, whereas females born after the summer solstice (short days (SDs)) may delay reproductive development to the following spring when environmental conditions are favorable for reproduction. In Siberian hamsters, development in SD is associated with structural and functional differences in the ovary compared with females held in LD, including a greater number of primordial follicles and an abundance of hypertrophied granulosa cells (HGCs), which are immunoreactive for anti-Müllerian hormone. The goal of this study was to determine whether SD-induced gonadotropin suppression is responsible for these phenotypic differences. Gonadotropin levels were suppressed in LD hamsters using the GnRH antagonist acyline. Conversely, to determine whether the SD ovarian phenotype is completely reversed by gonadotropin stimulation, recombinant human FSH (rhFSH) was administered. Our treatments were successful in mimicking FSH concentrations of the opposite photoperiod, but they did not produce a comparable change in the ovarian phenotype. Most notable was the lack of HGCs in the ovaries of acyline-treated LD females. Similarly, HGCs were maintained in the ovaries of SD females treated with rhFSH. Our data suggest that gonadotropins alone do not account for the SD ovarian phenotype. Future studies will determine whether SD-induced changes in other factors underlie these phenotypic changes.

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Introduction

Many long-day (LD) seasonal breeders time their reproductive efforts to the spring and summer months to ensure that adequate resources are available for the production and care of young. Photoperiodic conditions (i.e. day length) at the time of birth and during development may strongly influence life history strategies, specifically the timing of puberty. Females born before or near the summer solstice (LDs) reach sexual maturity quickly and often breed that same year (Bronson 1985). In contrast, females born after the summer solstice, as day length is decreasing (short days (SDs)), may delay reproductive development to the following spring when conditions are more favorable (Butler *et al.* 2007).

The Siberian hamster (*Phodopus sungorus*) is a seasonally breeding rodent that requires LD lengths for reproductive activity (Turek & Campbell 1979, Hoffman *et al.* 1982). When *P. sungorus* females are reared in SD, i.e. SD are initiated at or before weaning, sexual maturation is significantly delayed compared with

hamsters reared in LD. A suite of phenotypic characteristics distinguish hamsters reared in SD vs LD, including coat color (LD, brown and SD, white), body mass (LD > SD), food intake (LD > SD), and vaginal patency (LD, open and SD, closed) (Ebling 1994, Place *et al.* 2004). Moreover, development in SD is associated with significant structural and functional differences in the ovary compared with LD. These SD-induced differences include reduced ovarian mass, greater numbers of primordial follicles, and an abundance of hypertrophied granulosa cells (HGCs), which surround atretic oocytes (Schlatt *et al.* 1993, van den Hurk *et al.* 2002, Place *et al.* 2004). Whereas oocyte atresia also occurs in the ovaries of LD females, it is not accompanied by the hypertrophy of surrounding granulosa cells (Kabithe & Place 2008). More importantly, HGCs within the SD ovary are immunoreactive for anti-Müllerian hormone (AMH), and the abundance of HGCs in SD ovaries likely explains the threefold higher levels of AMH in SD compared with LD ovaries. The broad distribution and abundance of AMH-positive HGCs have been purported to be the mechanism underlying the

Table 1 Predicted effects of photoperiod on the morphology and reproductive phenotype of the Siberian hamster (*Phodopus sungorus*), including expected results of gonadotropin suppression (acyline).

Trait	LD control	SD control	LD acyline
Body mass	High	Low	High
Coat color	Brown	White	Brown
Ovary/uterine mass	High	Low	Low
Vaginal patency	Open	Closed	Closed
Follicle counts	Low	High	High
Corpora lutea	Present	Absent	Absent
HGCs	Absent	Present	Present
Serum AMH	High	Low	Low
Ovarian <i>Amh</i> mRNA	Low	High	High

'Body mass' and 'Coat color' traits are known to be gonadotropin independent, whereas the other traits listed may be modulated by gonadotropins. Bold represents traits that are typical of the short-day (SD) phenotype. LD, long day; rhFSH, recombinant human FSH; HGCs, hypertrophied granulosa cells; AMH, anti-Müllerian hormone. With regard to potential gonadotropin-dependent traits, we predicted that the LD control group would differ from both the SD control and the LD acyline groups, whereas the SD control and LD acyline groups would not differ from each other. Conversely, for gonadotropin-independent traits (i.e. body mass and coat color), the LD acyline group was predicted to resemble the LD control group.

greater numbers of primordial follicles in SD-reared hamsters (Kabithe & Place 2008), as AMH has been shown to inhibit primordial follicle activation as a result of paracrine effects (Durlinger *et al.* 1999, 2002).

The anestrus (anovulatory) state of SD hamsters can be easily accounted for by the suppression of gonadotropins in reduced photoperiod (Schlatt *et al.* 1993). However, we sought to determine whether the pronounced structural and functional changes within SD ovaries (e.g. HGCs) can be induced by pharmacological suppression of gonadotropins, whilst maintaining other elements of the SD phenotype (e.g. decreased body mass and white coat coloration; Table 1). Specifically, gonadotropin levels were suppressed in LD hamsters from weaning (postnatal day 18) to 10 weeks of age using the GNRH antagonist acyline, which blocks GNRH receptor sites in the pituitary and causes an immediate and reversible suppression of gonadotropins (Gottsch *et al.* 2004, Irwig *et al.* 2004, Shahab *et al.* 2005). Control LD and SD females were similarly treated with vehicle. We predicted that if gonadotropins are responsible for the SD ovarian phenotype, then gonadotropin suppression during development in LD females would result in an ovarian morphology similar to that of SD females.

Conversely, to determine whether the LD ovarian phenotype can be produced under SD conditions, long-term gonadotropin supplementation with recombinant human FSH (rhFSH) was administered to SD hamsters from 10–14 weeks of age. A similar regimen of FSH administration in male Siberian hamsters that were held in SD resulted in the restoration of spermatogenesis (Lerchl *et al.* 1993). Control LD and SD females were

similarly treated with vehicle, and an additional group of SD hamsters was transferred and maintained in LD from 10 to 14 weeks of age as a photostimulated control. We predicted that SD hamsters would transition to the LD ovarian phenotype and that hypogonadotropism would be reversed by long-term rhFSH administration (Table 2). If rhFSH treatment does not result in a transition from the SD to LD phenotype, then we would predict that other photoperiod-induced differences contribute to the SD-to-LD transition. Collectively, these studies were meant to elucidate the photoperiod-dependent effects of gonadotropins on ovarian development.

Results

Experiment 1: gonadotropin suppression in LDs

Treatment of LD females with the GNRH antagonist acyline mimicked some, but not all, of the histological features that characterize the SD ovary. Specifically, HGCs were observed in SD control hamsters (7/8, 87.5%), but not in LD control (0/7, 0%) or LD acyline hamsters (0/6, 0%), with significantly more SD control hamsters showing HGCs compared with LD acyline hamsters ($P=0.0002$; Table 3; Fig. 1). These results were confirmed by AMH immunohistochemistry (IHC; Fig. 2), i.e. positive immunostaining for AMH was observed surrounding atretic follicles in SD control hamsters, but not in LD control or LD acyline hamsters. The less intense immunoreactivity (blushed appearance) of HGCs (Fig. 2A) compared with that of granulosa cells within healthy follicles (Fig. 2A, B and C) is a typical

Table 2 Predicted effects of photoperiod on the morphology and reproductive phenotype of the Siberian hamster (*Phodopus sungorus*), including expected results of gonadotropin supplementation (rhFSH).

Trait	SD control	LD control	SD PS	SD FSH
Body mass	Low	High	High	Low
Coat color	White	Brown	Brown	White
Ovary/uterine mass	Low	High	High	High
Vaginal patency	Closed	Open	Open	Open
Follicle counts	High	Low	Low	Low
Corpora lutea	Absent	Present	Present	Absent^a
HGCs	Present	Absent	Absent	Absent
Serum AMH	Low	High	High	High
Ovarian <i>Amh</i> mRNA	High	Low	Low	Low

'Body mass' and 'Coat color' traits are known to be gonadotropin independent, whereas the other traits listed may be modulated by gonadotropins. Bold represents traits that are typical of the short-day (SD) phenotype. LD, long day; PS, photostimulated; rhFSH, recombinant human FSH; HGCs, hypertrophied granulosa cells; AMH, anti-Müllerian hormone. With regard to potential gonadotropin-dependent traits, we predicted that the SD control group would differ from the other groups (i.e. LD control, SD PS, and SD FSH), whereas the SD PS and SD FSH groups would not differ from each other (^aexcluding the presence of corpora lutea, which is dependent on both FSH and LH). Conversely, for gonadotropin-independent traits (i.e. body mass and coat color), the SD FSH group was predicted to resemble the SD control group.

Table 3 Effects of photoperiod and gonadotropin suppression on morphological measurements, hypertrophied granulosa cells, follicle counts, ovarian *Amh* mRNA, and serum hormone levels in female Siberian hamsters (*Phodopus sungorus*).

	LD control	SD control	LD acyline	Planned contrasts	
				LD control vs others	SD control vs LD acyline
Paired ovarian mass (mg)	7.8±0.4	4.7±0.4	1.8±0.5	†	†
Uterine mass (mg)	92.3±11.8	11.7±0.7	18.2±4.4	†	NS
HGCs	0/7 (0%)	7/8 (87.5%)	0/6 (0%)	NS	†
Primordial	83.6±17.3	52.3±7.4	107.8±36.9	NS	NS
Primary	24.3±2.5	16.8±2.7	14.2±5.4	*	NS
Secondary	21.3±1.4	8.8±2.1	13.0±5.9	*	NS
Antral	0.14±0.14	0.00±0.00	0.00±0.00	NS	NS
Corpora lutea	5/7 (71.4%)	0/8 (0%)	0/6 (0%)	†	NS
Serum FSH (ng/ml)	5.3±1.3	2.5±0.7	2.3±0.7	*	NS
Serum AMH (ng/ml)	3.1±0.3	1.8±0.3	1.3±0.4	*	NS
Ovarian <i>Amh</i> mRNA	0.9±0.1	0.8±0.1	1.2±0.2	NS	*

Data are reported as the mean ± S.E.M. or the number (percentage) of individuals with the given trait. Bold represents traits that are hypothesized to resemble the SD phenotype. LD, long day; SD, short day; HGCs, hypertrophied granulosa cells; AMH, anti-Müllerian hormone; NS, nonsignificant. * $P<0.05$; † $P<0.001$. With regard to planned contrasts, LD controls are hypothesized to be significantly different from all other groups (contrast 1), whereas SD controls and LD acyline groups should not differ (contrast 2).

finding in our laboratory. One SD female did not show HGCs; however, this did not affect the significance of the group results. Interestingly, all other SD-typical traits were observed (e.g. low ovarian, uterine, and body mass and white coat coloration).

Acyline induced SD-like changes in some features, such as decreases in ovarian and uterine mass and serum FSH and AMH concentrations. However, primordial follicle numbers and ovarian *Amh* mRNA levels were not significantly different from controls (Table 3). For paired ovarian mass, LD control hamsters had significantly larger ovaries than SD control and LD acyline hamsters ($P<0.0001$). Further, LD acyline hamsters had

significantly smaller ovaries than SD control hamsters ($P<0.0001$). With respect to uterine mass, LD control hamsters had significantly larger uteri than SD control and LD acyline hamsters ($P<0.0001$). As expected, exposure to SD affected vaginal patency; all SD control hamsters had a closed vagina at 10 weeks of age, whereas all LD controls had a patent vagina. Four of seven (57%) LD acyline hamsters had an open vagina by 10 weeks of age. If the three LD acyline hamsters that maintained a closed vagina throughout the experiment are excluded, vaginal patency was initiated at a younger age in LD control than in LD acyline hamsters (7.5 ± 1.2 vs 9.5 ± 0.6 weeks respectively).

Figure 1 Photomicrographs of ovaries from 10-week-old Siberian hamsters held in long days (LD) and treated with vehicle (LD control), held in short days (SD) and treated with vehicle (SD control), or held in LD and treated with acyline (LD acyline). The higher magnification of the boxed region shows oocyte remnants and the cells that surround them. Granulosa cells surrounding atretic oocytes in SD control ovaries have visibly hypertrophied (i.e. hypertrophied granulosa cells (HGCs)). This is in contrast to comparable cells in LD control and LD acyline ovaries. Hematoxylin and eosin staining (H&E); magnification, 100× and 400×.

Figure 2 Immunohistochemistry for anti-Müllerian hormone (AMH); photomicrographs of ovaries from 10-week-old Siberian hamsters held in long days (LD) and treated with vehicle (LD control), held in short days (SD) and treated with vehicle (SD control), or held in LD and treated with acyline (LD acyline). The higher magnification of the boxed region shows oocyte remnants and the cells that surround them. Granulosa cells surrounding atretic oocytes in SD control ovaries have hypertrophied and stain positive for AMH. This is in contrast to comparable cells in LD control and LD acyline ovaries. Magnification, 200 $\times$ and 400 $\times$.

There were no significant differences in primordial or primary follicle counts between any of the groups (Table 3). No antral follicles or corpora lutea were observed in either the SD control or the LD acyline groups compared with the LD control group, suggesting that neither of these groups had ovulated (for corpora lutea, $P < 0.0001$; SD control 0/8 (0%), LD acyline 0/6 (0%), and LD control 5/7 (71.4%)); however, due to the low counts in the LD control group, this difference in antral follicles was not significant.

Serum AMH concentrations were significantly higher in LD control hamsters compared with SD control and LD acyline hamsters ($P = 0.0013$; Table 3). In contrast, there were no statistically significant differences in ovarian *Amh* mRNA between LD control hamsters compared with SD control and LD acyline hamsters; however, LD acyline animals had significantly higher levels of *Amh* mRNA compared with SD controls ($P = 0.029$). Statistically significant differences in serum FSH concentrations were observed between groups, with LD control hamsters having significantly higher FSH levels than SD control and LD acyline hamsters ($P = 0.029$; Table 3).

Body mass and coat color were not affected by acyline treatment, as the LD phenotype was maintained. There were significant effects of treatment ($P = 0.001$), time ($P = 0.0001$), and a time by treatment interaction ($P = 0.028$) on changes in body mass over the course of the experiment (Fig. 3). Specifically, body mass began to plateau at 6 weeks of age in SD control hamsters, whereas body mass continued to increase in LD control and LD acyline hamsters through 9 weeks of age. From

6 weeks of age until the termination of the experiment at 10 weeks of age, mean body mass in LD acyline and LD control hamsters was significantly greater than in SD control hamsters. With respect to coat color, SD control animals displayed the typical white SD coat coloration (9/9 white, 100%), whereas LD control (0/9 white, 0%) and LD acyline (0/7 white, 0%) animals displayed the typical brown LD coat coloration (contrast 1, $P < 0.0001$ and contrast 2, $P = 0.997$).

Experiment 2: long-term rhFSH supplementation in SDs

In contrast to hamsters held in LD (LD control) and SD hamsters transferred to LD at 10 weeks of age

Figure 3 Mean $\pm$ S.E.M. body mass (g) of Siberian hamsters held in long days (LD) and treated with acyline (LD Acy), held in LD and treated with vehicle (LD Ctl), or held in short days (SD) and treated with vehicle (SD Ctl) from weaning until 10 weeks of age. Asterisks (*) indicate significant differences ($P < 0.05$) between groups at individual time points.

Table 4 Effects of photoperiod and gonadotropin stimulation on morphological measurements, hypertrophied granulosa cells, follicle counts, ovarian *Amh* mRNA, and serum hormone levels in female Siberian hamsters (*Phodopus sungorus*).

	SD control	LD control	SD PS	SD FSH	Planned contrasts	
					SD controls vs others	SD PS vs SD FSH
Paired ovarian mass (mg)	4.9 ± 0.5	6.3 ± 0.6	6.9 ± 0.8	7.4 ± 0.7	*	NS
Uterine mass (mg)	18.2 ± 6.7	73.8 ± 8.9	50.3 ± 14.0	9.2 ± 0.9	*	*
HGCs	6/7 (85.7%)	0/6 (0%)	0/6 (0%)	6/6 (100%)	NS	†
Primordial	108.5 ± 22.5	78.3 ± 13.2	115.8 ± 22.0	121.0 ± 18.7	NS	NS
Primary	30.0 ± 4.8	26.3 ± 3.9	27.7 ± 4.6	29.7 ± 2.7	NS	NS
Secondary	20.3 ± 2.2	19.2 ± 2.8	34.0 ± 3.8	32.5 ± 6.3	NS	NS
Antral	0.17 ± 0.17	0.67 ± 0.33	0.71 ± 0.29	0.50 ± 0.34	NS	NS
Corpora lutea	0/7 (0%)	5/6 (83.3%)	1/6 (16.7%)	0/6 (0%)	NS	NS ^a
Serum FSH (ng/ml)	2.2 ± 0.7	3.8 ± 1.2	4.3 ± 1.0	8.6 ± 4.1	*	NS
Serum AMH (ng/ml)	2.6 ± 0.5	3.8 ± 1.7	3.9 ± 0.7	3.6 ± 0.8	NS	NS
Ovarian <i>Amh</i> mRNA	1.1 ± 0.1	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.1	NS	NS

Data are reported as the mean ± S.E.M. or the number (percentage) of individuals with the given trait. Bold represents traits that are hypothesized to resemble the SD phenotype. LD, long day; SD, short day; PS, photostimulated; HGCs, hypertrophied granulosa cells; AMH, anti-Müllerian hormone; NS, nonsignificant. * $P < 0.05$; † $P < 0.001$. With regard to planned contrasts, SD controls are hypothesized to be significantly different from all other groups (contrast 1), whereas SD PS and SD FSH groups should not differ (contrast 2) (excluding corpora lutea as described in Table 2^a).

(SD photostimulated (PS)), HGCs persisted in hamsters treated with rhFSH (SD FSH). Specifically, HGCs were observed in SD control (6/7, 85.7%) and SD FSH (6/6, 100%) hamsters but not in LD control (0/6, 0%) or SD PS hamsters (0/6, 0%), with significantly more SD FSH hamsters showing HGCs compared with SD PS hamsters ($P < 0.0001$; Table 4; Fig. 4), suggesting that rhFSH treatment did not completely recapitulate the LD ovarian phenotype. These results were confirmed by AMH IHC (Fig. 5); positive immunostaining for AMH was observed surrounding atretic follicles in SD control and SD FSH hamsters but not in LD control or SD PS hamsters. One SD control female did not show HGCs. On closer examination, this female displayed SD-like ovarian and uterine masses and a closed vagina but demonstrated a sharp increase in body mass over the final week before killing. It is therefore likely that this female was becoming refractory to the SD signal;

however, this did not affect the significance of the group results.

LD-like increases in ovarian mass and FSH levels were observed with rhFSH treatment, but there were no significant differences in uterine mass, follicle numbers, ovarian *Amh* mRNA, or serum AMH (Table 4). For paired ovarian mass, SD control hamsters had significantly smaller ovaries than LD control, SD PS, and SD rhFSH hamsters ($P = 0.026$), whereas there was no significant difference in paired ovarian mass between SD PS and SD FSH hamsters. In addition, the uterine mass of SD control hamsters was significantly different than LD control, SD PS, and SD FSH hamsters ($P = 0.027$). Interestingly, SD FSH hamsters had significantly smaller uteri than SD PS hamsters ($P = 0.006$). Lastly, SDs delayed puberty as demonstrated by vaginal patency. Animals held in SD did not have an open vagina at the start of treatment, whereas all animals in the LD group

Figure 4 Photomicrographs of ovaries from 14-week-old Siberian hamsters held in short days (SD) and treated with vehicle (SD control), held in LD and treated with vehicle (LD control), raised in short days (SD) and transferred to LD at 10 weeks of age (SD photostimulated (PS)), or held in SD and treated with recombinant human FSH (rhFSH; SD FSH). The higher magnification of the boxed region shows oocyte remnants and the cells that surround them. Granulosa cells surrounding atretic oocytes in SD control and SD FSH ovaries have visibly hypertrophied (i.e. hypertrophied granulosa cells (HGCs)). This is in contrast to comparable cells in LD control and SD PS ovaries. Hematoxylin and eosin staining (H&E); magnification, 100× and 400×.

Figure 5 Immunohistochemistry for anti-Müllerian hormone (AMH); photomicrographs of ovaries from 14-week-old Siberian hamsters held in short days (SD) and treated with vehicle (SD control), held in LD and treated with vehicle (LD control), raised in short days (SD) and transferred to LD at 10 weeks of age (SD photostimulated (PS)), or held in SD and treated with recombinant human FSH (rhFSH; SD FSH). The higher magnification of the boxed region shows oocyte remnants and the cells that surround them. Granulosa cells surrounding atretic oocytes in SD control and SD FSH ovaries have hypertrophied and stain positive for AMH. This is in contrast to comparable cells in LD control and SD PS ovaries. Magnification, 200 $\times$ and 400 $\times$.

had an open vagina by 10 weeks of age (8.4 ± 1.5 weeks). Following transfer to LD, four animals in the SD PS group (44%) had an open vagina by week 14, whereas five animals (56%) maintained a closed vagina until the end of the study. All the SD FSH and SD control animals had a closed vagina at the termination of the study (14 weeks).

There were no significant differences in follicle counts among any of the groups (Table 4). Corpora lutea were observed in LD control (5/6, 83.3%) and SD PS (1/6, 16.7%) hamsters but not in SD control (0/7, 0%) or SD FSH hamsters (0/6, 0%); however, the difference between SD PS and SD FSH hamsters was not significant. There were also no significant differences in either serum AMH concentrations or ovarian *Amh* mRNA levels between any of the groups. In contrast, serum FSH levels were lowest in SD control hamsters compared with LD control, SD PS, and SD FSH hamsters ($P=0.042$), but not significantly different between SD PS and SD FSH hamsters (Table 4).

Body mass and coat color were not affected by rhFSH treatment, as the SD, winter coat phenotype was maintained. There were significant effects of treatment ($P=0.009$) and time ($P=0.001$) on changes in body mass over the course of the experiment (Fig. 6). There was a trend toward a time by treatment interaction; however, this was not statistically significant ($P=0.065$). Specifically, LD control hamsters weighed significantly more than SD control, SD FSH, and SD PS hamsters throughout much of the experiment, and body mass tended to remain constant after about 7 weeks of age. However, body mass began to increase in SD PS hamsters ~3 weeks after they were transferred to LD (between 12 and 13 weeks of age). By 14 weeks of age mean body mass in SD PS females had increased to match the body mass of LD controls. Lastly, SD FSH hamsters maintained the typical white SD coat

coloration (6/6 white, 100%), whereas SD PS hamsters (5/7 white, 71.4%) appeared to be transitioning to the LD brown coat color phenotype; however, this difference was not significant. All SD control animals showed the typical white SD coat coloration (8/8 white, 100%), whereas all LD controls showed the typical LD coat coloration (0/7 white, 0%).

Discussion

In female Siberian hamsters, exposure to SD before the onset of puberty results in the inhibition of reproductive development and numerous structural and functional differences within the ovary (Ebling 1994, van den Hurk *et al.* 2002, Place *et al.* 2004, Timonin *et al.* 2006, Kabithe & Place 2008). Our goal was to elucidate the

Figure 6 Mean $\pm$ S.E.M. body mass (g) of Siberian hamsters held in short days (SD) and treated with vehicle (SD Ctl), held in SD and treated with recombinant human FSH (rhFSH; SD FSH), raised in SD and transferred to long days (LD) at 10 weeks of age (SD photostimulated (PS)), or held in LD and treated with vehicle (LD Ctl) from weaning until 14 weeks of age. The arrow indicates the points at which treatment began (10 weeks of age). Asterisks (*) indicate significant differences ($P<0.05$) between groups at individual time points.

effects of gonadotropins on ovarian morphology and determine whether changes in gonadotropin levels are responsible for the observed photoperiod-dependent alterations in the ovarian phenotype. We found that treatment with the GNRH antagonist acyline induced SD-like decreases in ovarian and uterine horn mass and serum FSH and AMH; however, primordial follicle numbers and ovarian *Amh* mRNA levels were not significantly different than in controls, and HGCs were not observed. Conversely, LD-like increases in ovarian mass and FSH levels were observed with rhFSH treatment, but there were no significant differences in uterine mass, follicle numbers, ovarian *Amh* mRNA, or serum AMH. Additionally, HGCs were maintained in SD FSH hamsters. As expected, GNRH-independent traits, such as body mass and coat color, were not affected by either acyline or rhFSH treatment. Although some characteristics, such as ovarian size and circulating hormone levels, appear to be modulated by gonadotropin concentrations independent of photoperiod, this study provides evidence that the structural and functional characteristics of the SD ovary (e.g. HGCs and AMH) are not solely due to decreases in circulating levels of FSH and LH.

Gonadotropin suppression in LDs

One hallmark characteristic of the SD ovarian phenotype is the presence of HGCs. As described previously, granulosa cells of preantral follicles in the SD ovary persist and hypertrophy following oocyte atresia, and the histology of these 'luteinized' granulosa cells, suggests that they maintain steroidogenic activity (van den Hurk *et al.* 2002, Place *et al.* 2004, Timonin *et al.* 2006). The mechanisms underlying SD-induced changes in granulosa cell atresia are currently unknown but may involve the modulation of apoptotic pathways (Moffatt-Blue *et al.* 2006). Our data suggest that gonadotropin suppression alone is not adequate to promote the development of HGCs but other factors are also involved. Interestingly, although high concentrations of AMH immunoreactive HGCs were observed in the ovaries of SD controls, no differences in *Amh* mRNA were observed among any of the groups. Western blots have shown that AMH protein levels were threefold higher in SD ovaries compared with LD ovaries at 10 weeks of age (Kabithe & Place *et al.* 2008). However, this study is the first to examine SD-induced changes in *Amh* mRNA. These results suggest that protein and mRNA expression levels may be discordant in the ovary. Alternatively, the observed positive immunoreactivity of HGCs for AMH may be the result of prior production by healthy granulosa cells (preceding atresia) as opposed to *de novo* production. Therefore, studies aimed at examining isolated HGCs and quantifying *Amh* mRNA levels are warranted.

The effects of acyline in females, specifically at the level of the ovary, have not been well studied. Interestingly, in this study, there was no effect of acyline treatment on follicle counts. This is in contrast with other GNRH antagonists (e.g. antide and cetrorelix), which have been shown to decrease the number of primordial follicles in mice (Danforth *et al.* 2005). Further, in contrast to previous reports that showed a reduced attrition of primordial follicles in SD (Place *et al.* 2004), we found no effect of SD on primordial follicle counts in any of the treatment groups. One possible explanation for this discrepancy is our photoperiod regimen. In previous studies, hamsters were gestated and reared in SD, whereas our SD controls were gestated and reared in LD and then transferred to SD upon weaning. The above protocol was applied in an effort to balance the treatment groups with respect to relatedness and ensure that treatment times were comparable (i.e. ~7 weeks of either acyline treatment or SD exposure). In addition, perinatal exposure to melatonin does not appear to mediate the effects of day length and melatonin on testes mass in Siberian hamsters (Tuthill *et al.* 2005). However, photoperiodic signals during the perinatal period act as an important source of seasonal information. Specifically, these photoperiodic cues establish and modify the hamster's developmental trajectory via changes in somatic and/or reproductive development. For example, it is well known that photoperiodic information can be transmitted *in utero* via maternal melatonin secretion, and this prenatal signal can affect postnatal photoperiodic responses (Horton 1985, Stetson *et al.* 1986, 1989, Lee *et al.* 1987, Weaver *et al.* 1987, Elliott & Goldman 1989) and the reproductive development of young (Prendergast *et al.* 2000, Kauffman & Zucker 2002, Tuthill *et al.* 2005). More recent studies have shown that perinatal photoperiod can have lasting effects on circadian rhythms expressed by clock neurons, wheel-running behavior (Ciarleglio *et al.* 2011), depressive- and anxiety-like behaviors (Pyter & Nelson 2006), and immune responses (Weil *et al.* 2006). Finally, pilot studies in our laboratory of very young (3-week-old) hamsters gestated and held in LD or SD have demonstrated photoperiod-dependent changes in ovarian gene expression in the absence of morphological or histological differences. Therefore, it is plausible that prenatal and perinatal exposure to LD before the post-weaning transfer of hamsters to SD set a developmental trajectory in motion that countered any SD-induced changes in follicle numbers. Future studies will address the importance of gestational and perinatal photoperiod on ovarian development and specifically on the numbers of primordial follicles.

With regard to potential mechanisms underlying the observed effects, exposure to SD is associated with a number of physiological changes that occur independent of gonadotropins (Duncan & Goldman 1984a, Goldman 2001). As demonstrated in this study, coat color, which is

a prolactin-dependent trait (Duncan & Goldman 1984a), was unaffected by acylone treatment. Prolactin levels are regulated by ambient photoperiod in seasonal mammals via dopamine release in the median eminence. Previous studies have shown increases in prolactin-inhibitory dopamine as soon as 3 weeks after transfer to SD (Benson 1987). Additionally, SD-induced low levels of both serum (Widmaier & Campbell 1981, Timonin *et al.* 2006) and pituitary prolactin have been observed in the Siberian hamster (Reiter 1975). In male hamsters, inhibition of prolactin is necessary for complete testicular regression in SD (Bartke *et al.* 1980, Amador *et al.* 1986). With respect to females, prolactin receptors have been previously characterized across the periovulatory range. Specifically, radiolabeled prolactin was shown to bind to granulosa cells of isolated hamster follicles (Roy *et al.* 1987). Binding was maximal during the early stages of follicular development and dropped considerably with follicle maturation. Prolactin receptors have also been localized to the cell membranes of isolated luteinized granulosa cells obtained from women that were undergoing ovarian stimulation for IVF (Vlahos *et al.* 2001). Further, prolactin has been shown to increase preantral follicle maturation rates (Kiapekou *et al.* 2009) and alter the gonadotropin-induced steroidogenic capacity and apoptosis rates of granulosa cells (Hirakawa *et al.* 1999, Perks *et al.* 2003). Methods for attenuating prolactin release into the periphery in Siberian hamsters have been well characterized and include the use of dopamine agonists such as bromocriptine mesylate and cabergolin (Brooks *et al.* 2005). Therefore, selectively blocking LD-induced changes in prolactin levels in addition to other factors constituting the SD hormonal milieu, such as gonadotropins and melatonin, may help to tease apart the mechanisms underlying the SD phenotype and will be the focus of future studies.

Long-term rhFSH supplementation in SDs

Similar to the effects of gonadotropin suppression in LD, we found that long-term rhFSH supplementation in SD via twice-daily injections of rhFSH did not completely recapitulate the LD ovarian phenotype with respect to the presence of HGCs. Surprisingly, other variables, including follicle numbers and serum AMH, were not significantly different between any of the groups. Although follicle numbers tended to be higher in SD controls compared with LD controls, this difference was not significant and therefore no conclusions can be drawn from these results. The cause for this discrepancy with previous studies is unclear but may be due to the high variability between individuals. With respect to the lack of effect on serum AMH, it is possible that some of the SD hamsters were becoming refractory to the photoperiodic signal and, as a result, this slight variation in refractoriness across individuals may have negated

any photoperiodic effects. Consistent with this hypothesis, one of our SD controls showed a sharp increase in body mass and no evidence of HGCs at the termination of the study.

Collectively, our HGC results are consistent with previous studies showing that FSH treatment alone is not adequate to induce the full LD ovarian phenotype in female Siberian hamsters (Kenny *et al.* 2002), suggesting that additional environmental or hormonal cues are necessary. One potential explanation for the above results is that gonadotropin receptors in the ovary may be downregulated during SD, leading to differential sensitivity compared with LD. To date, steady-state FSH receptor mRNA and protein levels have not been characterized in LD and SD Siberian hamsters. It is possible that gradual changes in the levels of gonadotropins and other pituitary hormones following photostimulation (Meredith *et al.* 1998, Bernard *et al.* 2000) alter a number of factors that may prime the ovary. Moreover, photoperiodic alterations in reproductive axis sensitivity have been previously documented in this species at the level of the hypothalamus and pituitary. Specifically, female Siberian hamsters have been shown to differ in their response to repeated injections of kisspeptin, a positive regulator of GNRH secretion, based on reproductive condition (Mason *et al.* 2007, Greives *et al.* 2011).

Changes in the regulation and expression of GNRH and gonadotropins at the level of the ovary have also been demonstrated in this species. Previous work has shown SD-induced decreases in kisspeptin immunoreactivity within the ovary, with subsequent increases following photostimulation. Further, photostimulation induces significant increases in ovarian kisspeptin mRNA expression (Shahed & Young 2009). Alternatively, although plasma concentrations of gonadotropins are reduced in response to SD exposure (Schlatt *et al.* 1993), intraovarian mRNA expression levels of GNRH, FSH, and LH were shown to increase in SD despite low to no pituitary contribution, suggesting local production of these hormones (Shahed & Young 2011). In addition, the intense eosinophilia of HGCs within SD hamster ovaries suggests that steroidogenesis is occurring. Further, van den Hurk *et al.* (2002) reported strong enzymatic activity for 3 β -hydroxysteroid dehydrogenase in HGCs of 'luteinized atretic follicles'. Collectively, these results suggest that intraovarian levels of GNRH, gonadotropins, and perhaps steroid hormones may be locally involved in SD regression and/or recrudescence and therefore are potentially important in regulating the SD ovarian phenotype.

In addition to photoperiodic differences in reproductive axis sensitivity to gonadotropins and the local production of gonadotropins at the level of the ovary, it is possible that our treatment regimen did not fully mimic natural SD-induced secretion patterns from the pituitary. Gonadotropins are released from the pituitary

in a pulsatile fashion (Dierschke *et al.* 1970, Gay & Sheth 1972, Chappel *et al.* 1984); however, in this study, we administered two bolus injections of rhFSH daily, and rhFSH was administered in the absence of LH. Previous studies have demonstrated that FSH can induce follicular growth in the virtual absence of LH; however, estrogen production is severely impaired (McNatty *et al.* 1979, Schoot *et al.* 1992, Shemesh 2001). Given that serum LH levels are much lower in SD than in LD female hamsters (Darrow *et al.* 1980, Dodge & Badura 2002), it is possible that FSH-dependent estrogen production by granulosa cells is substantially reduced due to a decrease in LH-induced androgen precursor production by theca cells. The observed lack of effect of rhFSH on uterine mass and vaginal patency, which are largely estrogen-dependent traits (Odum *et al.* 1997, van Meeuwen *et al.* 2007, Suzuki *et al.* 2007, Baranda-Avila *et al.* 2009), supports these conclusions. Although LH levels do not immediately increase after transfer to LD (Simpson *et al.* 1982, Meredith *et al.* 1998), LH secretion may be necessary for the complete expression of the LD phenotype. Given that rhFSH produced periovulatory follicles in the ovaries of SD hamsters, studies including the addition of LH or its analog human chorionic gonadotropin to confirm or refute the incidental observation that SD hamsters could not be induced to ovulate with exogenous gonadotropins (Kenny *et al.* 2002) are needed.

Lastly, as described previously, ovarian AMH protein concentrations are threefold higher in SD than in LD hamsters, likely due to the broader distribution of AMH-expressing cells (Kabithe & Place 2008). Previous studies have shown that AMH reduces follicle sensitivity to FSH via reductions in FSH receptor expression in granulosa cells and the inhibition of FSH-stimulated aromatase activity (Pellatt *et al.* 2007). Therefore, the SD ovary may be less sensitive to FSH than the LD ovary, and a higher dose of rhFSH may be necessary to ablate the HGCs that characterize the SD ovary.

Conclusion

Taken together, our data suggest that gonadotropins alone do not account for the SD ovarian phenotype. Future studies will determine whether other factors, such as SD-induced changes in prolactin, underlie these phenotypic changes. Addressing the interactions between gonadotropins and other hormones via the use of antagonists and/or add-back studies may help to better approximate photoperiod-dependent alterations in the hormonal milieu and elucidate the signals that control ovarian structure and function. Collectively, the results of these investigations will provide us with a more thorough understanding of the mechanisms underlying ovarian follicle activation and development in photoperiodic species.

Materials and Methods

Experiment 1: gonadotropin suppression in LDs

Siberian hamsters from our colony (14 h of light per day) were transferred to LD (16 h of light per day) as breeding pairs to generate females for Experiment 1. The time of lights off was synchronized for all animals to 1800 Eastern Standard Time. Animals were originally derived from wild-bred stock obtained from Dr K Wynne-Edwards, Queen's University. Hamsters were weaned on postnatal day 18 and placed individually in polypropylene cages. For all animals, body mass and vaginal patency were recorded weekly starting at weaning and continuing until the end of the experiment. Coat color was scored at the termination of the experiment using the scale described by Duncan & Goldman (1984b). Animals scored as Stage 1 were considered to show the LD (brown) coat coloration, whereas animals scored as Stage 2 and above were considered to show the SD (white) coat coloration. These data are reported as the proportion of animals that showed the white SD coat coloration. Food (Teklad 8626, Madison, WI, USA) and water were available *ad libitum* throughout the experiment. Ambient temperature and relative humidity were held constant at $21 \pm 5^\circ\text{C}$ and $50 \pm 10\%$ respectively. Experimental procedures were approved by Cornell University's Institutional Animal Care and Use Committee and conducted in accordance with the NRC Guide for the Care and Use of Laboratory Animals.

Female *P. sungorus* began treatment at weaning (postnatal day 18), as pilot studies in our laboratory have shown that there are no histological differences between SD and LD ovaries at this time point. Animals were assigned to one of the three treatment groups as follows: 1) maintained in LD and treated with vehicle (LD control), 2) maintained in LD and treated with the GnRH antagonist acyline (LD acyline; Peptide Synthesis Facility, NICHD, Rockville, MD, USA), or 3) transferred to SD and treated with vehicle (SD control). For the groups so designated, 200 μl 5% mannitol (vehicle control) or 50 μg acyline (dissolved in 200 μl 5% mannitol) were injected i.p. every other day until 10 weeks of age, at which point animals in all groups were killed (i.e. after ~ 7.5 weeks of treatment). The 50 μg acyline dose has been shown to effectively suppress FSH in female *P. sungorus* (Mason *et al.* 2007) and alternate-day dosing maintains low FSH levels for at least 48 h (Pareek *et al.* 2007). Further, although acyline treatment suppresses gonadotropin levels, the secretion of other pituitary hormones that are known to vary based on photoperiod, such as prolactin and melatonin, is unaffected, and many aspects of the LD phenotype (e.g. body mass and coat color) are maintained. This allows us to examine the specific role of gonadotropins in mediating the SD ovarian phenotype. With respect to the duration of treatment, previous studies have demonstrated clear structural and functional differences between LD and SD ovaries at 10 weeks of age (Timonin *et al.* 2006, Kabithe & Place 2008). Lastly, SD control hamsters that showed a marked increase in body mass and an open vagina before the termination of the experiment were considered to be refractory to the SD signal and were therefore removed from the analyses. Final sample sizes were seven to ten animals per group.

Experiment 2: long-term rhFSH supplementation in SDs

Siberian hamsters from our colony (14 h of light per day) were transferred to either LD (16 h of light per day) or SD (10 h of light per day) as breeding pairs to generate females for Experiment 2. Experimental females were maintained in the photoperiod in which they gestated, otherwise the general husbandry conditions were identical to Experiment 1.

Female *P. sungorus* began treatment at 10 weeks of age, as the SD ovarian phenotype is fully expressed at this time point in females gestated and held in SD (Timonin *et al.* 2006, Kabithé & Place 2008). Females that were gestated and raised in SD were assigned to one of the three treatment groups at 10 weeks of age as follows: 1) maintained in SD and treated with vehicle (SD control), 2) maintained in SD and treated with rhFSH (SD FSH; Dr A F Parlow, National Hormone & Peptide Program, Torrance, CA, USA), or 3) transferred from SD to LD and maintained in LD (SD photostimulated (SD PS)). Control females that were gestated and raised in LD were maintained in LD and treated with vehicle (LD control). For the groups so designated, 200 µl 10% saline (vehicle control) or 3 IU rhFSH (dissolved in 200 µl 10% saline) was injected s.c. twice a day at the onset of the light phase and just before the onset of the dark phase for 4 weeks (Lerchl *et al.* 1993); photostimulation lasted for the same duration. Long-term treatment with rhFSH has been used successfully in *P. sungorus* without sequelae (Lerchl *et al.* 1993). In SD males, 1 week of rhFSH treatment was shown to reinstate spermatogonial maturation (Meachem *et al.* 2005), and the restoration of spermatogenesis was observed after 33 days (Lerchl *et al.* 1993). Similar to acyline, treatment of SD hamsters with rhFSH maintains other aspects of the SD phenotype (e.g. body mass, coat color, and prolactin levels), which allows us to more thoroughly establish the role of low gonadotropin levels in maintaining SD ovarian morphology.

Upon transfer to LD, circulating FSH levels increase within 3–5 days; conversely, LH increases within 21 days (Simpson *et al.* 1982, Anand *et al.* 2002). Therefore, we opted to supplement SD females with FSH only. Previous studies have shown that hypogonadotropism associated with SD is reversed upon transfer to LD, and ovarian recrudescence from the SD to LD phenotype is completed within ~4 weeks (Anand *et al.* 2002, Salverson *et al.* 2008). Therefore, animals in all groups were killed after 4 weeks of treatment at 14 weeks of age. Finally, SD control hamsters that showed a marked increase in body mass and an open vagina were considered to be refractory to the SD signal and were therefore removed from the analyses. Final sample sizes were seven to nine animals per group.

Blood and tissue collection

For both Experiments 1 and 2, animals from each group were killed by an i.p. overdose of sodium pentobarbital and exsanguination by retro-orbital bleed. All animals were killed during the middle of the light cycle. Blood was clotted on ice for 1 h and centrifuged at 1000 g for 20 min at 4 °C. Drawn off serum was aliquoted, frozen, and stored at –80 °C until assayed for AMH and FSH.

The right ovary was aseptically removed from each animal, dissected free of the surrounding fat, and weighed on an

analytical balance. The ovary was then submerged in RNAlater RNA Stabilization Reagent (Qiagen) and incubated overnight at 2–8 °C. The following day, the ovary was removed from the reagent and stored at –80 °C until RNA extraction. The left ovary was removed from each animal, dissected free of surrounding fat, and weighed on an analytical balance. The ovary was then immersed in 10% buffered formalin for histology, follicle counts, and IHC. Formalin fixation of the left ovary continued overnight at room temperature (RT), followed by serial dehydration into 70% ethanol. Ovaries were imbedded in paraffin and serially sectioned at 6 µm for histology and IHC. Lastly, the uterus was also removed, dissected free of surrounding fat, and weighed on an analytical balance.

Quantification of ovarian follicles

Every tenth section was mounted on a glass slide, stained with hematoxylin and eosin (H&E), and viewed under 400× magnification to count ovarian follicles. Ovarian sections of poor histological quality were replaced with the adjacent section. Ovarian follicles were classified as primordial, primary, secondary, or antral as described previously (Place & Cruickshank 2009). To ensure that follicles were only counted once, visualization of a healthy oocyte nucleus was required for inclusion. In addition, the presence or absence of HGCs and corpora lutea was noted for each section. These data are reported as the proportion of animals that were positive (i.e. presence) for each trait. As described previously, SD ovaries are characterized by an abundance of hypertrophied eosinophilic cells surrounding atretic oocytes, which are termed HGCs (Place *et al.* 2004, Kabithé & Place 2008). As a result of these HGCs, the area surrounding atretic follicles takes on a blushed appearance with eosin staining. In all sections, ovaries were shown to contain either numerous HGCs (scored as positive) or a complete lack of HGCs (scored as negative). All slides were examined by a single investigator who was unaware of the treatment group.

IHC for AMH

Six mid-ovary sections from three animals in each group were immunostained for AMH using a protocol modified from Kabithé & Place (2008). Adjacent sections were mounted on separate slides for use as negative controls, which excluded either the primary or the secondary antibodies. After dewaxing and rehydration in a series of ethanol, slides were submerged in Antigen Unmasking Solution (1:100 v/v in ddH₂O; H-3300; Vector, Burlingame, CA, USA) and microwaved on high for 3 min. Slides were then microwaved on 30% power for 18 min. Following antigen retrieval, slides were allowed to cool at RT for 30 min, and endogenous peroxides were then quenched in hydrogen peroxide (0.3% in methanol) for 30 min. Sections were incubated in 10% normal rabbit serum diluted in dilution buffer (0.5 M sodium chloride, 0.01 M phosphate buffer, 3% BSA, and 0.3% Triton X-100) for 20 min at RT to block nonspecific binding sites. Polyclonal goat anti-MIS antibody (sc-6886; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:1000 in dilution buffer

and incubated with sections for 16 h at 4 °C in a humidified chamber. Sections were then incubated with the secondary antibody, biotinylated rabbit anti-goat IgG (3.3:1000 in dilution buffer; sc-2774; Santa Cruz Biotechnology), for 30 min. Immunoreactivities were visualized by incubating sections with Vectastain Elite ABC Solution (Vector) for 30 min and developed with NovaRed Peroxidase Substrate Solution (Vector) for 5 min. Sections were counterstained with hematoxylin. Previous studies have demonstrated that granulosa cells within the primary and secondary follicles in LD and SD ovaries show the most intense staining for AMH, whereas AMH is less intense in the granulosa cells of antral follicles. AMH staining of moderate intensity is typically observed in HGCs surrounding atretic follicles in SD ovaries. In contrast, granulosa cells that surround atretic oocytes in LD ovaries are neither luteinized nor positive for AMH (Kabithé & Place 2008). Although AMH staining was not quantified in this study, photomicrographs of representative sections were taken and compared with the HGC results (based on H&E staining) to confirm that HGCs surrounding atretic follicles were indeed immunopositive for AMH.

RNA extraction and quantitative real-time PCR

To quantify *Amh* mRNA levels, ovaries were first homogenized with a Polytron, and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. From each group, six to ten ovary RNA samples were used for quantitative real-time PCR (qRT-PCR). RT was performed using Superscript III First-Strand Synthesis System (Invitrogen) with equal amounts of RNA and oligo-dT primer according to the manufacturer's instructions. QRT-PCR was run under standard conditions using Power SYBRGreen 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. Primers and their PCR product sizes are shown in Table 5. For quantification, we used the relative standard curve method (Cikos *et al.* 2007). Ribosomal protein L13a (*Rpl13a*) was used as a reference gene. Standard curves were prepared for both the target (*Amh*) and reference genes using five to seven diluted standard cDNAs. For each experimental sample, the amounts of target and reference genes were interpolated from the appropriate standard curve. Target gene values were then divided by the reference gene values to obtain the relative ratio.

Assay for serum AMH

Serum AMH concentrations were measured in duplicate serum samples using an ELISA manufactured by Diagnostic Systems

Laboratories (Webster, TX, USA). This ELISA was previously validated for the measurement of AMH in Siberian hamsters (Kabithé & Place 2008). Samples from both experiments were randomly assigned to three different plates. The intra- and interassay coefficients of variation (CV) were 7.7 and 9.6% respectively. The minimum detection limit of the assay as reported by the manufacturer was 0.006 ng/ml.

Assay for serum FSH

Serum FSH concentrations were measured in duplicate serum samples using a RIA with rFSH-2 as the standard and anti-rat FSH 11 antibody (provided by Dr A F Parlow, NIDDK, National Hormone & Peptide Program), as previously validated in Siberian hamsters (Wolfe *et al.* 1995, Meredith *et al.* 1998, Paul *et al.* 2006). The degree of cross-reactivity of the anti-rat FSH 11 antibody for rhFSH is not known but is assumed to be <100%. The lower limit of detection was 1.4 ng/ml and the intra- and interassay CV were 8.9 and 8.0% respectively.

Statistical analyses

Results were analyzed using a commercial statistical program (JMP version 9.0.2, SAS Institute, Cary, NC, USA). Normality was verified using a Shapiro–Wilk *W* test and homogeneity of variance was confirmed by Levene's test for all variables, with the test being used after log transformation when indicated. The following measures required log transformation to fit a normal distribution: Experiment 1: serum FSH, uterine mass, and primordial follicles and Experiment 2: uterine mass and serum FSH.

Continuous data were analyzed using an ANOVA with planned (*a priori*) linear contrasts (Sokal & Rohlf 1981). In Experiment 1, we predicted that for gonadotropin-dependent traits, the LD control group would be significantly different than the SD control and LD acyline groups (contrast 1; weights of +2, −1, and −1 for the LD control, SD control, and LD acyline groups respectively). Further, we predicted that the SD control and LD acyline groups would not differ from each other (contrast 2; weights of +1 and −1 for the SD control and LD acyline groups respectively). In this example, the LD control group is the 'true control' group, as there are no *a priori* predictions that the SD control and LD acyline groups will differ. Alternatively, for gonadotropin-independent traits, we predicted that the SD control group would be significantly different than the LD control and LD acyline groups (contrast 1; weights of +2, −1, and −1 for the SD control, LD control, and LD acyline groups respectively), whereas the LD control and LD acyline groups would not differ from each other (contrast 2; weights of +1 and −1 for the LD control and LD acyline groups respectively).

Table 5 Primers for quantitative real-time PCR (qRT-PCR).

Gene	Primer sequences	Product Size (bp)	GenBank accession number
<i>Rpl13a</i>	F: 5'-CATGAGGTCTGGGTGGAATA-3' R: 5'-AGCTGCTTCTTCTCCGATAG-3'	105	JQ820262
<i>Amh</i>	F: 5'-ATGGCCAACTGGACACTATG-3' R: 5'-GATGTGTGTGTGAGGCCCTTG-3'	151	EU564707

In Experiment 2, we predicted that for gonadotropin-dependent traits, the SD control group would be significantly different than the LD control, SD PS, and SD rhFSH groups (contrast 1; weights of +3, -1, -1, and -1 for the SD control, LD control, SD PS, and SD rhFSH groups respectively), whereas the SD PS and SD rhFSH groups would not differ from each other (contrast 2; weights of +1 and -1 for SD PS and SD rhFSH respectively). The SD PS group was used in this comparison rather than the LD control group because the photoperiodic history before treatment was the same between the SD PS and SD FSH groups. As such, if the ovarian phenotype in the SD rhFSH group matches the recovery shown by the PS group, then rhFSH treatment is sufficient to rescue the LD ovarian phenotype.

One exception to the outlined contrasts for gonadotropin-dependent traits in Experiment 2 is the prediction for corpora lutea counts. Specifically, ovulation and corpora lutea formation are dependent on LH, which was not administered with FSH in this study. Therefore, we predicted that the LD control and SD PS groups would show corpora lutea, whereas the SD control and SD rhFSH groups would not (contrast 1; weights of +1, +1, -1, and -1 for the LD control, SD PS, SD control, and SD rhFSH groups respectively). Further, we predicted that corpora lutea counts would differ significantly between SD PS and SD rhFSH groups (contrast 2; weights of +1 and -1 for the SD PS and SD rhFSH groups respectively). For gonadotropin-independent traits, we predicted that SD control and SD rhFSH groups would significantly differ from LD control and SD PS groups (contrast 1; weights of +1, +1, -1, and -1 for the SD control, SD rhFSH, LD control, and SD PS groups respectively), whereas SD control and SD rhFSH groups would not differ from each other (contrast 2; weights of +1 and -1 for the SD control and SD rhFSH groups respectively).

All binomial data (coat color, vaginal patency, corpora lutea, and HGCs) were analyzed using a generalized linear model with a binomial distribution and log-odds (logit) link function using the planned contrasts described earlier. Body mass data, which were recorded weekly from weaning to killing, were analyzed by repeated-measures ANOVA. For all statistical tests, the level of significance (α) was set at $P < 0.05$ and tests were two tailed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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