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Effects of photoperiod and food restriction on the reproductive physiology of female California mice

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ABSTRACT

Many temperate-zone animals use changes in photoperiod to time breeding. Shorter term cues, like food availability, are integrated with photoperiod to adjust reproductive timing under unexpected conditions. Many mice of the genus Peromyscus breed in the summer. California mice (Peromyscus californicus), however, can breed year round, but tend to begin breeding in the winter. Glial cells may be involved in transduction of environmental signals that regulate gonadotrophin releasing hormone I (GnRH) activity. We examined the effects of diet and photoperiod on reproduction in female California mice. Mice placed on either short days (8L:16D) or long days (16L:8D) were food restricted (80% of normal intake) or fed ad libitum. Short day-food restricted mice showed significant regression of the reproductive system. GnRH-immunoreactivity was increased in the tuberal hypothalamus of long day-food restricted mice. This may be associated with the sparing effect long days have when mice are food restricted. The number of GFAP-immunoreactive fibers in proximity to GnRH nerve terminals correlated negatively with uterine size in ad libitum but not food restricted mice, suggesting diet may alter glial regulation of the reproductive axis. There was a trend towards food restriction increasing uterine expression of c-fos mRNA, an estrogen dependent gene. Similar to other seasonally breeding rodents, short days render the reproductive system of female California mice more susceptible to effects of food restriction. This may be vestigial, or it may have evolved to mitigate consequences of unexpectedly poor winter food supplies.

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1. Introduction

Temperate-zone animals commonly use changes in photoperiod (day length) to time their reproduction, which allows physiological parameters to be primed well in advance of favorable breeding conditions. White-footed mice (*Peromyscus leucopus*) are reproductively active under long days (16L:8D) and impaired under short days (8L:16D) [12]. This phenotype is associated with increased gonadotrophin releasing hormone I (GnRH) immunoreactivity in the median eminence (ME) [12]. Given that regular release of GnRH is associated with activation of the reproductive axis [10], these data suggest that the build up of GnRH derives from short day-inhibition of GnRH release as opposed to increased GnRH synthesis [12]. The reproductive system also responds to short-term environmental conditions [31,49]. For example, three weeks of food restric-

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tion increases GnRH immunoreactivity in the preoptic area (POA) and the ME [19] in male prairie voles (*Microtus ochrogaster*). Food availability is often integrated with photoperiod to fine-tune reproductive timing [7,49]. Male deer mice (*Peromyscus maniculatis*) [28], and marsh rice rats (*Oryzomys palustris*) [9] show enhanced sensitivity to food restriction-induced gonadal involution under shorter daylengths whereas hamsters [16] provided carbohydrate supplements and California voles (*Microtus californicus*) [26] provided spinach supplements showed reduced short-day induced gonadal regression. Taken together, these studies suggest that temperate zone rodents may be more susceptible to the effects of nutrition while under daylengths that are less favorable to reproductive activity.

California mice (*Peromyscus californicus*) are not photoperiodic breeders, yet they appear to have annual rhythms in breeding activity. Field observations show that although most pups are born in the winter [37], breeding can occur throughout the year [23,37]. In contrast to other closely related species [43] short days do not inhibit testes size or testosterone levels in male California mice [42]. Despite this flexibility, laboratory studies show that in male California mice luteinizing hormone (LH) is elevated under short days [27]. Moreover, the addition of spinach to the diet under long

Abbreviations: SD-AL, short day-*ad libitum*; SD-FR, short day-food restricted; LD-AL, long day-*ad libitum*; LD-FR, long day-food restricted; ME, median eminence; Arc, arcuate nucleus; POA, preoptic area.

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days increased testes mass and counteracted the long-day induced reduction in sperm count while having no effect in short days [27]. These data suggest that short days may stimulate some aspects of the male reproductive axis. Whether a similar effect occurs in female California mice is unknown. Female mammals are expected to be more likely to suppress reproduction when food is limited [7]. While constrained food intake can alter hypothalamic [1,6,35] and uterine [4] sensitivity to estrogens, food restriction and photoperiod had no effect on estradiol levels in female California mice [40]. Several studies have suggested that glial cells may play an important role in regulation of the female reproductive axis.

Accumulating evidence suggests that glial cells have important effects on GnRH release. For example, work in rats found that during diestrus (when GnRH release is at its nadir), there is increased glial ensheathment of GnRH nerve terminals in the external-zone of the ME [18,32], as well as increased expression of the glial marker, glial fibrillary acidic protein (GFAP) [30]. It is thought that this ensheathment by glial cells physically blocks GnRH release to the pituitary. Accordingly, in the POA a decrease in GFAP immunoreactivity occurs during proestrus when GnRH release peaks, which has been suggested to permit formation of stimulatory synaptic inputs onto GnRH perikarya [17]. Glia may also be important mediators of the environment on reproduction. Hence, glia appear well suited to play a role in regulating hypothalamic–pituitary–gonadal (HPG) axis activity, possibly by altering GnRH release.

We examined how photoperiod and food restriction interact to regulate the reproductive axis of female California mice. Because field data suggests that breeding peaks in winter, we hypothesized that short day-ad libitium (SD-AL) conditions would increase reproductive tissue weights and hypothalamic GnRH expression compared to long day-ad libitum (LD-AL) conditions. We further hypothesized that there would be less suppression of the reproductive axis in short day-food restricted (SD-FR) than long dayfood restricted (LD-FR) mice based on the hypothesis that short photoperiods would provide greater support for reproduction. The study also examined whether changes in glial cells were associated with effects of diet and/or photoperiod on HPG-activity and hypothesized that an increase in hypothalamic GFAP-ir expression would be associated with inhibition of HPG-axis activity. Gene expression for ER- α and the estrogen dependent gene, c-fos [25] was measured to assess uterine estrogen sensitivity.

2. Materials and methods

2.1. Experimental animals

A total of fifty-eight female California mice raised on long day photoperiods (16L:8D, LD) were single-housed in polypropylene cages with careFRESH bedding (Absorption Corp., Ferndale, WA, USA) and randomly placed on either short (8L:16D, SD) or LD photoschedules (lights-off at 1400 h Pacific standard time [PST] in long and short days). Mice were then randomly assigned into one of two groups: restriction to 80% of individual baseline daily food intake, or ad libitum food access (Harland Teklab 2016 rodent diet, Indianapolis, IN, USA). Although this diet is mild compared to other studies [19,52] pilot data showed that 80% ad libitum allowed female California mice to maintain an acceptable and healthy body condition [46]. Baseline daily food intake for each mouse on a restricted diet was assessed by taking the average of the weights of food consumed each day during a one week period. Mice were 90 days old at the start of the experiment, which is about twice the age shown for vaginal introitus and initiation of estrous cyclicity in lab reared California mice [14] and older than the reported mean age of 77 days for introitus in wild mice [23]. Following a week of baseline food intake measurements, mice were maintained for 8-weeks under their assigned conditions. After 8 weeks, long day-ad libitum (LD-AL, n = 21), short day-ad libitum; (SD-AL, n = 13), long day-food restricted (LD-FR, n = 11) and short day-food restricted (SD-FR, n = 13) mice were anesthetized with isoflurane gas (Minirad Inc., Bethlehem, PA, USA) and euthanized by rapid decapitation during the light phase. Brains were fixed overnight in 5% acrolein (Sigma, St. Louis MO, USA) in 0.1 M phosphate buffered saline (PBS) at 4 °C. Brains were transferred to 25% sucrose (Fisher, Pittsburgh, PA, USA) in PBS for 24 h and then frozen at -40 °C. Reproductive tracts were dissected to isolate the uterus, ovaries and oviducts, which were weighed. Uterine tissue was stored overnight at 4 °C in RNA later (Qiagen, Valencia, CA, USA) and then stored at -20 °C. Stage of estrous cycle at the time of euthanasia was determined by vaginal lavage. Introitus was confirmed to have occurred in all mice during the study. However, at the end of the study some mice in the food restricted groups appeared to stop cycling and showed vaginal closure (imperforate vaginal openings), which prevented vaginal lavage. These mice were presumed to be in diestrus because vaginal closure following introitus is indicative of suppressed cycling [48,50]. All procedures were approved by the UC Davis Institutional Animal Care and Use Committee and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Immunohistochemistry

2.2.1. Fluorescent double-label IHC for GnRH and GFAP in the POA and tuberal hypothalamus

Sections were sliced using a microtome (40 μ m) and stored at -20 °C in cryoprotectant (50% v/v phosphate buffer, 30% w/v sucrose, 1% w/v polyvinylpyrrolidone, 30% v/v ethylene glycol). Tissue containing POA was stained every third section for a total of five sections. Tuberal hypothalamic [sections consisting of ME, and arcuate nucleus (Arc)] were chosen by collecting the rostralmost section containing ME and then collecting two additional sections, one every other section (three sections in all over an area of 0.12–0.16 mm). Emphasis was placed on the rostral ME as this region may play a central role in regulating LH release [22]. All treatment groups were run in a single batch.

Tissue was washed in PBS and then incubated for 10 min in 0.1 M sodium borohydride in PBS as an antigen retrieval step. Sections were blocked in PBS with 5% normal goat serum (NGS) and 5% normal donkey serum (NDS). Sections were incubated overnight at 4 °C in mouse anti-GnRH (1:1000, SMI-41, Covance, Princeton, NJ, USA) and rabbit anti-GFAP (1:100, ab7779, Abcam, Cambridge, MA, USA) diluted in PBS with 0.5% Triton X (Tx), 2% NGS and 2% NDS. Next, tissue was washed in PBS and incubated for 2 h at room temperature in DyLight-549 conjugated donkey anti-mouse (1:500, 715-505-150, Jackson ImmunoResearch, West Grove, PA) and biotin-conjugated goat anti-rabbit antibody (1:350, BA-1000, Vector Laboratories, Burlingame, CA, USA) Sections were washed in PBS and then incubated for 30 min in DyLight 488-conjugated streptavidin (1:250, 016-480-084, Jackson ImmunoResearch) in PBS-Tx. After washing in PBS, stained sections were mounted onto Superfrost plus slides (Fisher) and coverslipped using Vectashield Mounting Medium (Vector Laboratories). Both the GnRH [15] and GFAP [40] primary antibodies have been previously validated in Peromyscus.

2.2.2. Single-label immunohistochemistry for ER- α in the ventromedial hypothalamus and Arc

Sections containing the ventromedial hypothalamus (VMH) and Arc were immunostained for ER- α . Five total brain slices were used from each mouse brain and were chosen by collecting the first section before the median eminence started and then taking every third section moving rostrally (area covers about 0.2 mm). All treatment groups were run in a single batch. Immunostaining was performed as previously described [44] and employed a rabbit anti-ER- α primary antibody (1:100,000, C1355, Millipore, Billerica, MA) and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (PI-1000, Vector Laboratories, 1:500). Sections were mounted onto Superfrost plus slides (Fisher, Pittsburgh, PA, USA), and submerged in 100% ethanol and then Histoclear for 3 min each (National Diagnostics, Atlanta, GA, USA). The rabbit anti-ER- α primary has been previously used in *Peromyscus* [44].

2.3. Image analysis

Sections were photographed using an Axiocam MRC camera (Carl Zeiss Meditec) attached to a Zeiss Axioimager (Carl Zeiss Meditec, Inc., Dublin, CA) and images were analyzed using Image J software (NIH, Bethesda, MD, USA). For all regions quantified, the observer was blind to treatment. For the POA and VMH/Arc 4–5 sections were analyzed per mouse while 2–3 sections were analyzed per mouse for the tuberal hypothalamus. For all quantification of GnRH and GFAP, separate images were taken.

2.3.1. Quantification of GnRH and GFAP immunostaining in the ME and Arc

[SD-AL (n = 8), SD-FR (n = 7), LD-AL (n = 7) GnRH, LD-FR (n = 7)].For quantification of the proportion of GnRH immunostaining (percentage staining) in the ME, images were taken at a magnification of $20\times$. Then a 0.022 mm² rectangular box was placed over the photograph in the center of the ME about midway between the base of the third ventricle and the bottom edge of the external zone. Using ImageJ, immunopositive pixels were converted to black by manually setting the threshold for the level where most fibers were picked up but background was minimized. Percentage staining was defined as the percent area occupied by black pixels. GFAP immunoreactivity and number of GFAP-ir fibers were assessed in the external zone of the ME using images photographed at 63× magnification [20]. Analysis was carried out by placing a 0.013 mm² square along the center of the left and right bulbs of the ME. Ouantification was performed as described above. We manually counted fibers (using the cell counter application) that made contact with the edge of the median eminence. GnRH and GFAP immunoreactivity was also quantified in the Arc using a 0.021 mm² rectangular box that was aligned with the bottom of the third ventricle.

2.3.2. Quantification of GnRH and GFAP-ir soma number and percentage staining in the POA

Mice found to be in diestrus/metestrus were examined for GnRH/GFAP expression in the POA [SD-AL (n = 6-7), SD-FR (n = 10), LD-AL (n = 5-6) LD-FR (n = 7)]. Images of the POA were taken on $10 \times$ magnification and quantified bilaterally by placing a 0.378 mm² rectangular box in each side of the POA with the bottom edge against the base of the brain and the side nearest the midline of the brain placed against the midline so that neither box overlapped. The numbers of GnRH-immunoreactive (ir) perikarya and GFAP-ir stained astrocytic soma were hand counted using the cell counter application for image J. Additionally, the area within the box underwent binary contrast enhancement to determine percentage staining of GnRH and GFAP.

2.3.3. ER- α -ir nuclei counts in the VMH and the arc

We examined ER- α expression in the hypothalamus of mice in distrus/metestrus (n = 6 per group) The number of nuclei expressing ER- α was counted in the dorsomedial VMH, the ventrolateral VMH and the Arc using images taken at a magnification of 20×. A rectangle of 0.038 mm² was placed consistently in each of the two VMH regions. A rectangular box of 0.059 mm² was placed in both sides of the Arc by having the bottom of the box line up with

the bottom of the third ventricle and the side of the box nearest to the ventricle line up with the ventricle's edge. For VMH and Arc cells were manually counted using the cell counter application.

2.4. Quantitative real-time polymerase chain reaction

Estrogen dependent gene expression in the uteri of diestrus/ metestrus [SD-AL and SD-FR (n = 4 per group), LD-AL and LD-FR (n = 3 per group)] was analyzed with quantitative real-time polymerase chain reaction. RNA was extracted from uterine tissue using TRIzol (Invitrogen, Carlsbad, CA, USA). 1 µg of RNA per sample was reverse transcribed using an iscript cDNA synthesis kit (170-8891; BIO-RAD, Hercules, CA, USA). California mice c-fos, uterine cDNA was amplified and sequenced using primers based on mouse and rat c-fos sequences (Forward CTCCCGTGGTCACCTG TACT, reverse AATTGGAACACGCTATTGCC; GenBank Accession no. JN601063). Primers and probes for ER- α were based on previously published *Peromyscus* sequences (DQ357060).

The following primers and probes for ER- α and c-fos derive from the sequences which were obtained as described above:

*ER*α forward, 5'-GAACAGCCCCGCCTTGT-3'; *ER*α reverse, 5'-GCATCCAGCAAGGCACTGA-3'; *ER*α probe, 5'-TGACAGCTGACCAGATG-3'; *c-fos* forward, 5'- TGTGTTTCACGCACAGATAAGGT -3'; *c-fos* reverse, 5'- TGGTGCATTTCAGAGAGAGAGAA -3'; *c-fos* probe, 5'- CTCCCTAGGTCTACGGGAACCCTCGAG -3';

The c-fos and ER- α probes were labeled with NED and FAM dyes, respectively. A TaqMan 18S ribosomal RNA primer and Vic-labeled probeset (Applied Biosystems, Foster City, CA, USA) were used to amplify the housekeeping gene. Amplification was performed on an ABI 7500 Fast Real-time PCR Instrument (Applied Biosystems) with the Taqman[®] System (Applied Biosystems). The universal two-step PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression was determined as done in previous studies [33,44]. Average cycle thresholds for duplicate individual samples were compared to standard curves derived from serial dilutions of pooled *P. californicus* uterine cDNA (1:10, 1:100, 1:1000) and then normalized to 18s expression [33,44].

2.5. Statistical analysis

Mice were divided into diestrus/metestrus and proestrus/estrus [3,8,21]. Food restricted mice were more likely to be in diestrus/ metestrus; only one SD-FR mouse and three LD-FR mice were in proestrus/estrus. Therefore, due to low sample size FR mice in proestrus/estrus were excluded from most analyses. Effects of diet and photoperiod on changes in body weight over the course of the experiment as well as final body weight at the end of the experiment were analyzed using two-way ANOVA. We also used food intake data used to calculate FR diets to test whether photoperiod affected food intake. Relative reproductive tissue mass was log transformed prior to analysis by two-way ANOVA followed by planned comparisons between dietary groups. Two-way ANOVA was also used to analyze hypothalamic ER- α protein expression. Treatment group variances were heterogeneous for GnRH expression data in the tuberal hypothalamus, so Mann–Whitney U tests were used to examine effects of diet and photoperiod. Nonparametric tests were also used for examining c-fos and ER-a mRNA uterine expression due to a relatively small sample size and heterogeneous variance. We performed Fisher's exact test to assess whether the effects of food restriction on vaginal closure depended on photoperiod. One-way ANOVA was used to examine differences in reproductive parameters between mice that had imperforate vaginal openings versus that that did not. Uterine c-fos gene in these mice was expression was log transformed for analysis. Nonparametric Spearman correlations were employed to examine the association between relative uterine mass and number of GFAP-ir cells that contacted the edge of the external zone of the ME.

3. Results

3.1. Effects of diet and photoperiod on body weight

FR (n = 24) mice in all estrous stages combined lost weight over the course of the experiment while AL (n = 18) mice gained weight (Fig. 1A, $F_{1,38} = 9.0 \ p < 0.001$). Final weights of FR mice were significantly lower than those of AL mice (Fig. 1B, $F_{1,54} = 19.5$; p = 0.004). There were no main effects of photoperiod nor were there any diet × photoperiod interactions on weight (Fig. 1B, all p's > 0.2). There was no difference in the amount of food supplied to FR mice in one photoperiod versus the other (data not shown, p = 0.77).

3.2. Effects of diet and photoperiod on relative reproductive tissue weights

In diestrus/metestrus mice all reproductive tissues examined comprised a significantly smaller percentage of total body weight (relative weight) when housed under short days as compared with long days (Fig. 2A–D, all *p*'s < 0.025). Food restriction significantly reduced relative oviduct weight (Fig. 2C, *p* = 0.038) and relative combined reproductive tract weight (Fig. 2D, *p* = 0.001) only (all other *p*'s > 0.1). There was a trend towards a photoperiod × diet interaction on relative uterine (Fig. 2A, *p* = 0.057) and ovary weights (Fig. 2B, *p* = 0.052). Planned comparisons revealed that when mice were housed under short days, food restriction reduced relative weights of the reproductive tract (Fig. 2A, B, D, all *p*'s \leq 0.018), with the exception of the oviduct (Fig. 2C, *p* > 0.05). In contrast, food restriction did not affect relative weight of any reproductive tissue examined in mice housed under long days (Fig. 2A–D, all *p*'s > 0.05).

3.3. Environmental effects on hypothalamic expression of GnRH, GFAP and ER- α

Only diestrus/metestrus animals were examined when looking at effects of diet and photoperiod on hypothalamic protein expression.

3.3.1. Tuberal hypothalamic GnRH and GFAP staining

We immunostained the tuberal hypothalamus for GnRH (Fig. 3D). Under long days, food restriction increased expression



Fig. 1. Graphs depicting the percentage change in body weight over the course of the experiment (A) and the final body weight at the time of tissue collection (B). Food restricted mice [short day (n = 13), long day (n = 11)] lost weight while *ad libitum* mice [short day (n = 8), long day (n = 10)] gained weight. Food restricted mice [short day (n = 13), long day (n = 11)] weighed less at the end of the experiment than *ad libitum* mice [short day (n = 13), long day (n = 21)]. *p < 0.004.



Fig. 2. Bar graphs demonstrating the effects of diet and photoperiod on weight of uterus (A), paired ovaries (B), paired oviducts (C) and combined weight of reproductive tissues (D) as a percentage of total body weight (relative weight). Long days caused an overall increase in relative weight of each reproductive structure separately and when combined (2-way ANOVA). In the presence of short days, food restriction reduced the relative weight of the uterus, ovaries and total reproductive tract (planned comparisons). *p < 0.03, *** p = 0.001. Mean ± s.e is given for all data.



Fig. 3. Bar graphs showing percentage of the area stained for gonadotrophin releasing hormone I (GnRH)-immunoreactivity within the median eminence (ME; A), arcuate nucleus (Arc; B) and tuberal hypothalamus (ME + Arc; C) of mice in diestrus/metestrus. Mann–Whitney *U* tests indicated that under long days staining was elevated in the ME and combined tuberal hypothalamus (ME + Arc; of food restricted mice (D, n = 7) as compared with counterparts fed *ad libitum* (n = 7). Diet had no effect under short days (n = 7-8). *p < 0.04, when compared to same photoperiod.). VIII, third ventricle. Scale bars = 100 µm.

of GnRH in the ME (Fig. 3A, p = 0.038) and the combined tuberal hypothalamus (Fig. 3C, p = 0.026) but not in the Arc alone (Fig. 3B, p > 0.05), while no effect of diet occurred for SD mice (Fig. 3A–C, all p's > 0.5).

We also examined tuberal hypothalamic GFAP-expression (Fig. 4). There was no effect of diet or photoperiod on GFAP expression in terms of percentage staining in the Arc or external zone of the ME, nor were there differences in the number of GFAP-ir fibers that appeared in immediate proximity to the edge of the external zone of the ME (Table 1, all p's > 0.1). Nevertheless the relationship between the number of GFAP-ir fibers observed in contact with the edge of the external zone of the median eminence and relative uterine weight was dependent on diet. In AL mice there was a significant negative correlation between these two factors (Fig. 4E, r = -0.58, p = 0.006) while there was no correlation in FR mice (Fig. 4E, r = 0.14, p = 0.61). Moreover, when AL mice were separated by stage of estrous cycle the relationship persisted whether mice were in diestrus/metestrus, (data not shown, r = -0.53, p = 0.043) mice or proestrus/estrus (data not shown, r = -0.089, p = 0.019) mice.

3.3.2. GnRH and GFAP staining in the POA

Two-way ANOVA did not find any main effects of diet or photoperiod on GnRH or GFAP expression (cell count or percentage staining) in the POA, nor was there any interaction (Table 1 and Fig. 5A, B, all p's > 0.15).

3.4. Effects of photoperiod and diet on hypothalamic ER- α expression in the hypothalamus

Only brains from diestrus/metestrus animals were stained for ER- α . Two-way ANOVA showed that there were no main effects nor was there any photoperiod \times diet interaction on ER- α expression in the ARC or either the dorsomedial or ventrolateral VMH (Table 1, Fig. 5C, D, all *p*'s > 0.3).

3.5. Effects of environmental cues on estrogen dependent gene expression in the uterus

A trend emerged toward FR mice exhibiting increased uterine cfos expression (Table 2, p = 0.073). There was no effect of diet on ER- α expression, nor was there any effect of photoperiod on c-fos or ER- α expression (Table 2, P > 0.3).

3.6. Associations between vaginal patency and other reproductive parameters

Food restriction increased the likelihood that vaginal imperforation would occur under both short days (Fisher's exact test, Supplementary data 1, p < 0.001) and long days (Fisher's exact test, Supplementary data 1, p = 0.03). One-way ANOVA indicated that mice with imperforate vaginal openings (n = 4 for mRNA expression, n = 9-10 for everything else) exhibited decreased relative reproductive tract weights (Fig. 6C) and increased expression of hypothalamic GnRH protein (Fig. 6A, B) and uterine c-fos mRNA (Fig. 6D, all p's < 0.05) when compared to mice in diestrus/metestrus that demonstrated vaginal perforation (n = 10 for mRNA expression, n = 18-20 for everything else).

4. Discussion

Although several species of *Peromyscus* are capable of breeding under short days [27,43], less is known about whether photoperiod mediates the effects of food availability on their reproductive axes. In accordance with previous observations in male California mice [27] the effects of photoperiod on the female reproductive axis is limited under *ad libitum* conditions. We had hypothesized that photoperiodic drive to reproduce is greater under short days which would partially override the effects of food restriction on reproductive tract weight. However, our results demonstrate that short days sensitize the female reproductive tract to energy limitations. While this finding contradicts our original hypothesis, it is consistent



Fig. 4. Representative photomicrographs showing gonadotrophin releasing hormone I (GnRH) and glial fibrillary acidic protein (GFAP) expression in the tuberal hypothalamus (A). Higher power images of the boxed area in panel A showing GFAP-immunoreactive fibers (ir) alone (B), GnRH synaptic puncta alone (C) and simultaneous GnRH/GFAP expression (D) in the external zone of the median eminence (ME). Spearman correlations are graphed for the relative uterine weight versus number of GFAP-ir fibers in contact with the edge of the external zone of the ME. There was a significant positive correlation for *ad libitum* mice in all estrous stages combined (E), but no such correlation for food restricted mice (F). Arc, arcuate nucleus; ME, median eminence; PT (pars tuberalis); VIII (third ventricle). (A) scale bar = 100 µm; (B, D) scale bars = 50 µm.

Table 1

Table expressing the mean ± s.e for measures of gonadotrophin releasing hormone I (GnRH), glial fibrillary acidic protein (GFAP) and estrogen receptor (ER)-α-immunoreactivity in various hypothalamic regions. There were no main effects of diet or photoperiod, or any interactions on any of the parameters shown. Short day-*ad libitum*; (SD-AL); short day-food restricted (SD-FR); long day-*ad libitum* (LD-AL); long day-food restricted (LD-FR); preoptic area (POA); median eminence (ME); Arcuate nucleus (Arc); dorsomedial ventromedial hypothalamus; VMHdm, VMHvl, ventrolateral VMH.

GnRH		SD-AL	SD-FR	LD-AL	LD-FR
Neuronal soma/mm ²	POA	2.2 ± 0.4, 7	2.1 ± 0.8, 10	2.5 ± 0.5, 6	2.3 ± 0.6, 7
% Staining	POA	4.3 ± 0.8, 7	5.6 ± 0.8, 10	3.9 ± 0.7, 6	4.4 ± 1.0, 7
GFAP					
# of glial soma/mm ²	POA	11.4 ± 2.0, 6	9.2 ± 1.0, 10	10.5 ± 1.0, 5	9.1 ± 1.0, 7
% Staining	POA	10.3 ± 1.7, 6	9.4 ± 1.4, 10	8.4 ± 1.2, 5	10.2 ± 1.9, 7
# Fibers/mm ²	ME	1263 ± 135, 8	1072 ± 198, 7	1418 ± 167, 7	1143 ± 171, 7
% Staining	ME	24.4 ± 2.0, 8	21.1 ± 2.3, 7	22.0 ± 1.4, 7	19.1 ± 2.3, 7
% Staining	Arc	13.0 ± 3.3, 7	10.68 ± 3.4, 8	15.57 ± 5.9, 7	13.6 ± 4.4, 7
ER-α					
Nuclei/mm ²	VMHdm	3780 ± 137, 6	3516 ± 242, 6	3729 ± 202, 6	3589 ± 194, 6
Nuclei/mm ²	VMHvl	1291 ± 178, 6	1321 ± 163, 6	1313 ± 160, 6	1365 ± 128, 6
Nuclei/mm ²	Arc	1717 ± 127, 6	1633 ± 222, 6	1740 ± 178, 6	1733 ± 108, 6



Fig. 5. Representative microphotographs demonstrating expression of gonadotrophin releasing hormone I (A), and glial fibrillary acidic protein (B) in the preoptic area (POA), as well as estrogen receptor-α protein expression in the arcuate nucleus [Arc, (C)] and the ventromedial hypothalamus [(D) VMH]. OC, optic chiasm; VMHdm, dorsomedial VMH; VMHvl, ventrolateral VMH. (A, B) Scale bars = 200 µm; (C, D) Scale bars = 100 µm.

Table 2

Table shows mean ± s.e, n for c-fos and estrogen receptor (ER)- α mRNA expression in uteri from mice in diestrus/metestrus. There was a trend toward a food restriction-induced increase in c-fos mRNA expression.

	Short day	Short day	Long day	Long day
	ad lib	Restricted	ad lib	Restricted
c-fos (relative to 18S)	0.4 ± 0.15, 4	2.1 ± 0.9, 4	0.7 ± 0.127, 3	1.4 ± 0.92, 3
ER-α (relative to 18S)	1.4 ± 0.23, 4	11 ± 10.48, 3	2.6 ± 1.56, 3	4.4 ± 3.55, 3

with work done in other rodents that are non-obligate photoperiodic breeders including male rats [5] and white-footed mice selected against a photoperiodic response [34], where food restriction reduced gonadal size under short days or in the presence of exogenous melatonin, but not long days alone. Food restriction has been suggested to increase the inhibitory effects of melatonin on reproduction [5]. It is unlikely that the present results were affected by a photoperiod mediated change in metabolic activity, because photoperiod did not influence the effect of FR on body weight.

Food restriction increased GnRH expression in the ME and ME + Arc in LD mice. This result agrees with results showing that food restriction increases GnRH fibers projecting to the ME in voles [19], although that study also reported an increase in GnRH-ir soma in the POA. The authors of this study hypothesized that food restriction caused GnRH staining to increase by inhibiting GnRH expression and release, which in turn contributed to the reproductive impairment associated with constrained food intake [19]. It is

unlikely that this explanation can explain our results because long days ameliorated the effects of food restriction on the reproductive tract. However, future studies will be needed to test whether food restriction decreases GnRH synthesis/release during long days. In contrast, mice that exhibited vaginal imperforation also showed regression of their reproductive tracts. Given these mice appear to be in a reproductively impaired state, the elevated GnRH-immunostaining that they demonstrated in the POA and the tuberal hypothalamus could likely be indicative of a decrease in GnRH release.

An intriguing significant negative correlation emerged between relative uterine weight and number of GnRH fibers observed contacting the external zone of the ME as long as mice were fed *ad libitum*. This correlation was present regardless of stage of estrous cycle and suggests that food restriction may disengage the regulation of GnRH fibers by glia. Parkash et al. [30] described the GFAP-ir processes prevalent in the external zone of the ME as belonging to astrocytes. However, the morphology of the processes that we



Fig. 6. Bar graphs illustrating that food restriction-induced vaginal imperforation corresponds with increased percentage staining of hypothalamic gonadotrophin releasing hormone I (GnRH) protein (A and B), elevated uterine c-fos mRNA expression (D) and decreased relative reproductive tract weight (C) *p < 0.05, **p < 0.001. All data are mean ± s.e.

observed and quantified strongly resembled that of tanycyte endfeet [51]. It is the endfeet of tanycytes that specifically appear to separate GnRH terminals from the portal vasculature during diestrus [32]. Strikingly, tanycytes are also believed to function in the detection of glucose [38] and ATP [11] levels in the brain as well as regulation of the brain's metabolism [29], which readily explains how these cells could play a role in relaying nutritional signals to the HPG-axis.

There was a trend for elevated c-fos mRNA in FR mice. Mice with imperforate vaginal openings had significantly elevated uterine c-fos mRNA expression which may suggest that increased c-fos mRNA could be associated with impaired reproductive cycling. Mitosis of luminal and glandular epithelial cell proliferation in the uterus peaks during diestrus/metestrus and is driven in part by expression of c-fos [24]. Indeed, c-fos mRNA expression reaches its apex during metestrus [24]. The possibility exists that SD-FR mice experienced impaired cyclicity and spent longer in diestrus/ metestrus than SD-AL mice and that this resulted in a greater number of mitotic epithelial cells and increased c-fos gene expression.

We showed that female California mice are more susceptible to the effects of food restriction on their reproductive tracts under short days, whereas a previous study showed that food supplementation augmented reproductive function in males under long days [27]. Of course supplementing a diet with green vegetation such as spinach (a source of phytoestrogens [41]) could be expected to have different effects than food restriction. Still, these results may indeed reflect adaptations to seasonal patterns of food availability. In California short days are associated with the rainy season and, generally, increased productivity and food [47]. It may be that food restriction has a stronger inhibitory effect under short days because food availability is normally increased in winter. In contrast, in unusually wet years (e.g. during El Niño events) abundant food may still be available during the spring and summer. Under such conditions California mice may be more sensitive to augmented food availability (either via ad libitum diet or spinach). This would facilitate the facultative breeding that has been reported in field studies [23,37]. It is also possible that the photoperiodism of California mice reproduction is a consequence of phylogenic constraints [13] related to the long-day breeding ancestral state of its genus [43]. Zebra finches (Taeniopygia guttata) retain a vestigial degree of photoperiodism even though they breed primarily in response to rainfall [2] and California mice, which also use water availability as a major reproductive cue, could be following a similar pattern [27]. Interestingly photoperiod regulates aggression levels in California mice [39,45], even though field data suggest that territories are defended throughout the year [36]. Taken together the results of this study suggest that food restriction enhances regression of the reproductive tract under short days and may alter how the GnRH system is regulated, possibly by changing the degree of involvement of glial cells in this regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2011.12.035.

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