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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.

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 restriction 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 day-food 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 μm) 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 rostral-most 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