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Combinatorial contribution of neuroendocrine variables and food intake to male fertility

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and food intake to male fertility

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Science in Neuroscience from
The College of William and Mary

by

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Introduction

Various neuroendocrine hormones secreted by the hypothalamic-pituitary-gonadal (HPG) axis play a major role in human fertility. By regulating fertility, the HPG axis interacts with nutrition, food intake, body condition, stress and response to season or day length (Roberson et al. 2010). All of these factors must interact correctly to maintain fertility. Not surprisingly, given this complex mechanism, human infertility is frequent, affecting approximately 1 in 10 couples (Matzuk & Lamb 2002). Furthermore, approximately one third of cases of human male infertility remain unexplained (Moskovtsev et al. 2010).

Impaired fertility can be caused by genetic and environmental factors (Achermann & Jameson 1999). Infertility and reproduced fertility can be affected by other neuroendocrine systems such as the thyroid hormones or stress hormones. The most common genetic causes of infertility include mutation of genes involved in the HPG axis (Achermann & Jameson 1999). Many studies have tested DNA sequence variation (Kao et al. 1998). These inherited DNA sequence variations are correlated with infertility, but causation is difficult to show (Nathan et al. 1993). The DNA sequence variants correlated with infertility often have a high false-positive or false-negative error rate. One possible reason is that the combination of multiple genetic variants at multiple genes acts together to cause infertility (Krausz & Giachini 2007). Hence, it would be useful to test combinations of environmental factors and heritable physiological variables to approach the hypothesis.

Peromyscus leucopus

A wild population of *Peromyscus leucopus*, the white-footed mouse, exhibits phenotypic variation in reproductive response to photoperiod (Heideman et al. 1999). Wild-caught *Peromyscus leucopus* from Williamsburg, VA (37°16'N, 76°42'W) were variable in responding to photoperiod. Artificial selection lines of *P. leucopus* were developed for study of variation in the response to short winter-like photoperiod. Approximately 80% of individuals in photoperiod responsive line have small gonads in short day (8L:16D). Individuals in a photoperiod non-responsive line have larger gonads in short day (Heideman et al. 1999; Heideman and Bronson 1991). Reproductive photoresponsiveness was heritable in the population of *P. leucopus* (Heideman et al. 1999).

A recent study also suggested that there is a correlation in the two selected lines of mice between food intake and gonad mass in short winter-like photoperiod. In both short and long photoperiod, males in the non-responsive line consumed 50% more than the photoresponsive mice (Heideman et al. 2005). In SD, Testes mass and food intake were correlated, but in LD Testes mass and food intake were not related. This suggests that reproductive mice in the winter increase food intake to balance the potential cost of winter reproduction.

Significant genetic variation in neuronal GnRH system was found in this population of white-footed mice (Heideman and Pittman 2009). The R line that has suppressed reproduction in short daylength had fewer IR-GnRH neurons than the NR line, which maintains its reproduction in SD (Avigdor et al. 2005). Evidence of additional neuroendocrine variation (Heideman et al. 2010; Mason, Kriegsfeld &

Heideman, unpublished data) suggests that *P. lecuopus* is an appropriate model to examine the combinatorial variation in food intake, gonad size, neuronal systems and hormones and their role on male fertility.

Review of fertility and HPG axis

The HPG axis is comprised of hypothalamic neurons that express the hormone GnRH, the anterior pituitary gland and the gonads (Sower et al. 2008). The HPG axis is activated when the hypothalamus secretes pulses of GnRH. GnRH pulses can be modified by high or low temperature, food intake, and excessive exercise. GnRH neurons are located within the preoptic area in hypothalamus. Secreted GnRH is delivered to the median eminence of the anterior pituitary via hypophysial portal blood vessels (Elias et al. 2008). GnRH binds to GnRH receptors on the surface of cells in the anterior pituitary that secrete LH and FSH (the gonadotropes). GnRH binding to receptors on gonadotropes then elicits the synthesis and secretion of the gonadotropins, LH and FSH.

In males, LH is involved in the production of testosterone, which in turn stimulates spermatogenesis and development of secondary sex characteristics. The circulating LH from blood vessels binds to LH receptor on Leydig cells where it causes production of testosterone (Roberson et al. 2010). Testosterone results in negative feedback effects to inhibit the production of GnRH from the hypothalamus.

Infertility and reproductive impairment can be affected by other neuroendocrine systems such as the thyroid hormones, growth hormone, and biological rhythms. The thyroid gland, daily and seasonal rhythms and gonadal axes interact, affecting fertility. Increased production of thyrotropin-releasing hormone has

been reported to increase pulsatile secretion of GnRH, thus affecting the production of LH and FSH (Poppe et al. 2008). This finding suggests that activation of the thyroid hormone system affects the secretion of gonadotropins at the level of the hypothalamus-pituitary (Krassas and Pontikides 2004). Growth hormone also plays a role in synthesis of androgens by interacting with LH. Pituitary GH binds testicular Leydig cell GH receptors, activates a second messenger system and stimulates the activity of steroidogenic enzymes. GH also increases the response of Leydig cells to LH by increasing LH receptor abundance. Increased LH receptor abundance results in greater stimulation of the activity of several steroidogenic enzymes, resulting in more testosterone synthesis (Harvey and Hull 2000).

GnIH is an inhibitory neurotransmitter and hormone that is proposed to prevent the release of gonadotropins, including LH (Clarke and Smith 2010). The effect of GnIH on gonadotropins allows the HPG axis to control the concentration of secretion of both GnRH and LH effectively. Several studies have indicated that melatonin from the pineal gland induces release of GnIH neuropeptide (Kriegsfeld et al. 2010). Melatonin receptors are expressed in GnIH neurons (Clarke and Smith 2010). Hence, melatonin might induce GnIH release from neurons directly by acting via melatonin receptors. The terminals of GnIH neurons are localized in the median eminence of the hypothalamus. Most GnRH neurons are in the preoptic area (POA) (Kriegsfeld et al. 2010). GnIH receptors (GnIH-R) are expressed in gonadotropin cells that secrete LH and FSH in the anterior pituitary as well as in GnRH neurons in the POA. Thus, GnIH appears to inhibit LH and FSH release and synthesis by binding to GnIH-R directly on gonadotropes in the anterior pituitary. In addition, GnIH also inhibits GnRH release

by acting on GnRH neurons in the POA. Figure. 1 shows the proposed mechanism of GnIH projection on gonadotropin release and synthesis (Kriegsfeld et al. 2010).

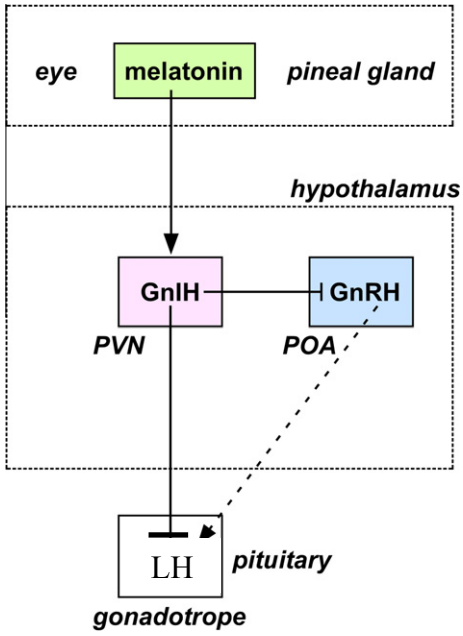

Figure 1. GnIH = Gonadotropin-inhibitory hormone. GnRH = Gonadotropin-releasing hormone. LH = Luteinizing hormone. PVN = Paraventricular nucleus. POA = Preoptic area. Arrowheads indicate stimulatory effect. Bar indicates inhibitory effect.

Combinatorial variation and reproductive phenotype

The combination of genetic variation in regulation of different concentration of hormones might affect infertility. For example, DNA sequence variation at a single gene related to the reproductive system may not cause infertility if redundancy in the pathway, epistasis, or small effects of that gene causes undetectable effects on fertility. However, DNA sequence variation in the combination of many genes might combine to cause impaired fertility. The combination of two or more variables in neuroendocrine traits, along with environmental variables such as food intake and photoperiod could explain the variation in fertility measures. For example, if the level

of LH and the number of GnRH neurons combine to account a measurable 50% of variation in fertility measures, but low or undetectable effects alone, then these traits have a combinatorial effect on fertility measures. If variables are not combining additionally or non additional with each other, then these variables do not have a combinatorial effect.

Recent studies indicate heritable variation among selection lines of a wild-derived population of white-footed *Peromyscus leucopus* (Heideman & Pittman 2009) in the number of immunoreactive GnRH neurons (Avigdor et al 2005). The R line that has suppressed reproduction in short daylength had fewer IR-GnRH neurons than the NR line, which maintains its reproduction in SD (Avigdor et al. 2005). Unpublished data (Mason, Kriegsfeld & Heideman) showed a difference between lines in number of IR-GnIH neurons. Circulating plasma level of LH differed significantly between the selection lines (Heideman et al. 2010). It is not known how combinations of these three traits affect fertility.

The hypothesis proposed here is that the combination of LH level, Food intake and number of IR-GnRH neurons can account for variation in fertility traits better than any of these variables alone. The traits tested to indicate fertility will be testes mass and seminal vesicles mass. In addition, an environmental variable, the effects of photoperiod (LD or SD), and a genetic variable, selection line (NR, R, C), will be tested in the statistical model proposed afterward in the paper. Additional neuroendocrine variables such as the number of IR-GnIH neurons or number of kisspeptins neurons can be included in the model. Then we can build our best-fit model by using all or some of our variables for variation in the male fertility trait.

Alternatively, the null hypothesis of this experiment is that fertility of white-footed mice is not described better by the combined fertility traits than by single variables alone.

My Honors thesis focuses on combinatorial contributions of heritable neuroendocrine variables and food intake to male fertility. I tested for variation in two neuroendocrine hormonal elements: Luteinizing Hormone (LH), and gonadotropin releasing hormone (GnRH). In addition, another honors student from our laboratory will be collecting data on the number of IR-GnIH neurons in 2012-2013 on the same mice. These data will be shared and combined. I studied three selection lines that vary in fertility in short photoperiod. These lines are an unselected control (C) line, non-photoperiod responsive (NR) line and photoperiod responsive (R) line. I included mice raised in short photoperiod (L8:D16;SD) and long photoperiod (L16:D8;LD). In addition to the variables above, I measured ad lib food intake (Heideman et al. 2005). As fertility traits, I measured the mass of testes and mass of seminal vesicles.

Methods

Source of Population

Male mice from two selected lines and one control line of white-footed mice (*P. leucopus*) were used in this experiment. These lines were obtained from a wild population in Williamsburg, VA, in the winter of 1995 (Heideman et al. 1999). Mice were bred for either strong reproductive inhibition in short-day photoperiods or low reproductive inhibition in short photoperiod. The line with suppressed gonadal development in short winter photoperiod was considered responsive to short

photoperiod. The line with normal gonadal development in short winter photoperiod was considered non-responsive to short photoperiod. Therefore most mice from the photoperiod responsive (R) line had suppressed reproductive systems in short photoperiod at age 70 days (Heideman et al. 1999). Most mice from non-photoperiod responsive (NR) line had developed mature reproductive systems in short photoperiod at age 70 days. A control line (C) was unselected and was intermediate in reproductive development at age 70 days (Heideman et al. 2006).

Experimental Design

Experimental mice were either transferred within 2 days of birth from a LD photoperiod to a SD photoperiod or kept in a LD photoperiod. All mice were weaned at age 22 ± 2 days to individual polyethylene cages (27 x16 x 13 cm) with wire tops, with approximately 3 cm depth of pine shavings, and ad libitum access to food (RMH 2000, Southern States Cooperative, Williamsburg, VA) and tap water. After 56 ± 3 days, data on food intake was collected from all selected lines for 2 weeks. Food intake was measured by recording the weight of food in the food hopper three times at one-week intervals, and average daily food intake calculated. Any mouse that ground and discarded food on the cage floor was removed from the experiment; this was approximately 5% of individuals. When mice were 70±3 days old, they were euthanized using gaseous CO_2, and tested for reproductive development. In this experiment, 22 male mice were used (C = 5, R = 3, NR = 3 in each photoperiod).

Perfusion and Sectioning

All mice were perfused when they were between 65 – 75 days old. Mice were anesthetized with an overdose of isoflurane (30% isofllurane in a chamber) and

weighed before the perfusion. All perfusions were started when mice were in respiratory arrest. Mice were perfused through the left ventricle at ~ 4ml/min using a perfusion pump and bled via the right atrium. Perfusion of cold and fresh 0.1M phosphate buffered saline (PBS) at a pH of 7.4 with sodium nitrite was followed by 4% paraformaldehyde and saturated picric acid in PBS (Zamboni's Fixative). Perfusion of Zamboni fixative was performed for 18 to 22 minutes. Brains were then removed and post-fixed overnight at 4°C in Zamboni fixative. After 24 hours, brains were transferred to 30% sucrose in PBS for cryoprotection at 4°C. After perfusion, paired testes and seminal vesicles with prostate were weighted. All brains were sliced within 3-7 days after the perfusion. Frozen brains were sectioned in the coronal plane at 30um on a freezing sliding microtome. Sections were separated into four vials with every fourth section in each vial. Vials were held an antifreeze solution [37.5% sucrose, 37.5% ethylene glycol, and 10g PVP-40 in 500ml 0.02M Tris-buffered saline (TBS)]. Brains were stored at -20°C until ICC.

ICC

Brain sections were rinsed five times for 10 min each in cold 0.02 M TBS in wells for 50 min. Subsequently, the brain tissues were incubated with SMI-41 monoclonal antibody to GnRH at a dilution of 1:20,000 in PBS with 0.25% lambda-carrageenan, 1% bovine serum albumin, and 0.3% Triton X-100 in TBS. SMI-41 is a mouse monoclonal IgG1 antibody. In reacts with the amino acids located near the carbonyl end of the GnRH peptide and the amidation site. Only mature GnRH hormone is recognized by SMI-41 antiserum. After 40-48 hours of incubation, brain sections were rinsed for four-10 min in TBS and incubated in biotinylated horse anti-

mouse IgG at a dilution of 1:500 in TBS for 60 min at room temperature. After three more rinses in 0.02M TBS for 30 min, sections were incubated in alvidin-biotin-peroxidase in TBS for 75 min. Sections were given three rinses in TBS and placed in 1ml of a solution of diaminobenzidine in 0.02 M TBS. The oxidation reaction was continued for 10 min and caused sections to turn to light brownish color. After three 10 min- rinses in TBS, sections were mounted on pre-cleaned slides and air dried overnight. On the next day the slides were cover-slipped with Permount.

Neuron assessment

Neuron assessment was by eye by S.J. Lee with counting conducted blind with respect to line and photoperiod treatment. Approximately 2% of sections were recounted by P.D. Heideman, blind with respect to previous counts, line and photoperiod treatment, which gave similar numbers. The number of GnRH neurons was counted in four sections from each mouse in the medial preoptic area (MPOA) using a compound light microscope. Counting followed the procedure of Heideman et al. (2007). As discussed in Heideman et al. (2007), the highest density of IR-GnRH neurons were in the MPOA. Recent results (Avigdor et al. 2005) have indicated that these four sections have the highest counts of the IR-GnRH neurons in each brain in this species, and are representative of the total number of IR-GnRH neurons. These sections include: the most posterior parts of the vertical limb of the diagonal band of Broca (plate 17 in Paxino and Watson 1986); the first two sections with the medial preoptic area (plate 18 and 19 in Paxinos and Watson 1986), and the most caudal section containing the medial preoptic area (plate 22 in Paxinos and Watson 1986). All brain structures and nuclei referred to here are those given by Paxinos and Watson

(1986). In approximately 25% of brains, one or more of these four sections were either not present or not identified clearly. For these brains, the first four sections that had the highest number of IR-GnRH neurons were assessed.

The Ligand Core lab of the University of Virginia conducted the assay for LH. LH was measured with a two-site sandwich immunoassay effective for laboratory mouse with monoclonal antibodies against bovine LH. This assay was validated previously for use with *P. leucopus* (Heideman et al. 2010)

Statistical Analysis

I used a general linear model (GLM) to test the hypothesis. The GLM determines whether variation in food intake, number of IR-GnIH neurons and number of IR-GnRH neurons, and serum LH combine to predict variation in our fertility measures of testes mass and sperm counts. The general form of the model for variation in fertility can be presented mathematically:

FM = ß0 + ß1FI + ß2GnIHno + ß3GnRHno + ß4LHlevel + ß5EnvLD/SD + Interaction terms + Error

In this model, FM represents testes mass as a fertility measure and the ß terms are constants. Other variables are defined as follows:

FI = ad lib daily food intake
GnIH = number of IR-GnIH neurons
GnRH = number of IR-GnRH neurons
LH level = level of circulating LH
ENV LD/SD = photoperiod (LD or SD)
Interaction terms = the various interactions possible among these variables.

Any correlations found between the fertility measures and other variables could suggest that certain combinations of heritable variables may be responsible for differing levels of male fertility.

The program R-Studio running an R statistical package on a Macintosh computer was used to analyze GLM models. I used correlation analyses to test for relationship between two or more variables. In this thesis, the numbers of IR-GnIH neurons are not included in the model because these data are not yet available from another student.

Results

The highest density of fibers and IR-GnRH neuronal cell bodies were found in the medial preoptic area (Figs. 1, 2 and 3 show typical staining). Generally, NR mice had more IR-GnRH neurons than R and C mice (Fig. 1 and 3).

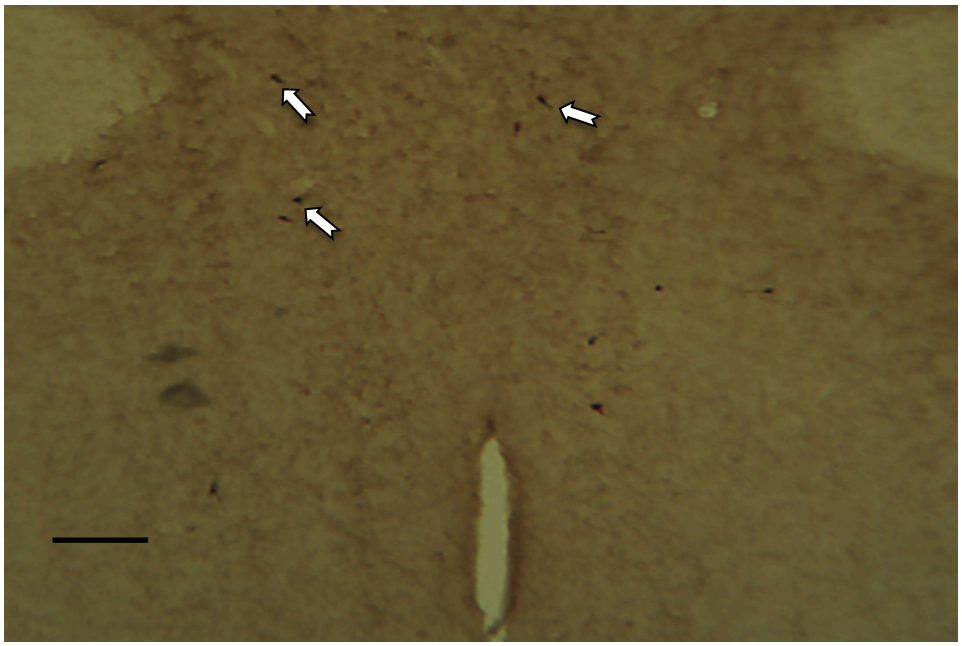

Fig. 1. IR-GnRH neurons found in medial and lateral preoptic area from one NR mouse. Arrows indicate some IR-GnRH neurons. Approximately 10-12 IR-GnRH neurons are on this section. Scale bar: 200 microns.

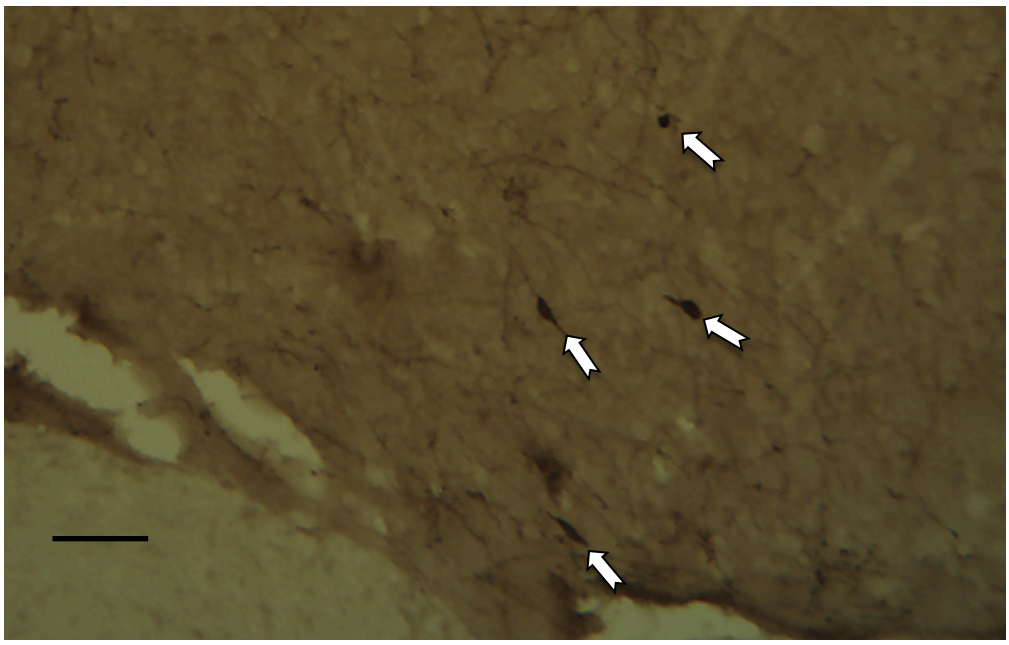

Fig. 2 IR-GnRH neurons found in Medial and Lateral preoptic area from one C mouse. Arrows indicate some IR-GnRH neurons. Scale bar: 50 microns.

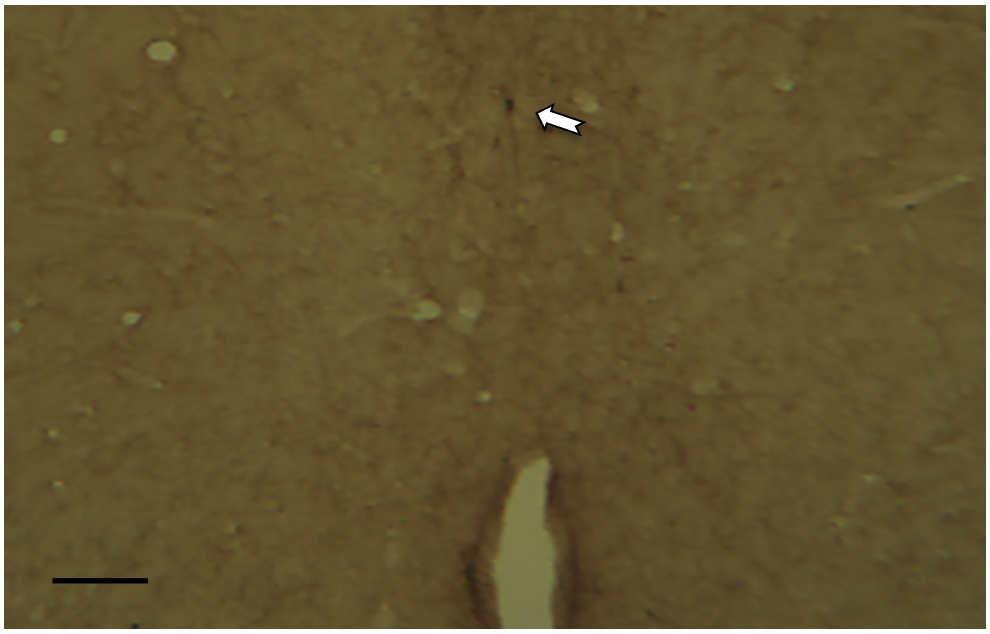

Fig. 3. Medial and lateral preoptic area from one R mouse. An arrow indicates an IR-GnRH neuron. Scale bar: 200 microns

For testes weight, there was a significant effect of line ($F=4.88$; $P<0.014$; Fig. 4) and photoperiod ($F= 43.43$; $P<0.001$; Fig. 5). The photoperiod had a significant effect on the seminal vesicles weight ($F=51.69$; $P<0.001$; Fig. 6). However, the effect of selection line on seminal vesicles weight was marginally insignificant ($F=3.14$;

P<0.058; Fig. 7). There were no significant effects of line or photoperiod on body weight (P>0.05). For testes weight, seminal vesicles weight and body weight, there were no significant interactions between line and photoperiod (P>0.10 for all)

The selection line of mice did not affect the number of IR-GnRH neurons in these data (F=1.597; P<0.22; Fig. 8). Photoperiod did not affect the number of IR-GnRH neurons (F=0.0172; P<0.89; Fig. 9). However there was a significant interaction between line and photoperiod for the number of IR-GnRH neurons (F=5.43; P<0.011). Photoperiod affected the daily food intake of mice significantly (F=7.0; P<0.012; Fig. 10), whereas line (C, NR, and R) did not affect daily food intake (F=1.86; P<0.17; Fig. 11). For daily food intake, selection line had no significant interaction with photoperiod.

Photoperiod affected circulating plasma level of LH (F=7.19; P<0.013; Fig. 12). The circulating LH level had a significant correlation with the number of IR-GnRH neurons (F=3.23; P<0.033; Fig. 13). There was no significant correlation between level of LH and testes weight (F=3.01; P<0.097; Fig. 14). Seminal vesicles weight also was not correlated with plasma level of LH (F=3.27; P<0.085; Fig. 15). Interestingly, daily food intake was significantly correlated with the plasma level of LH (F=5.32; P<0.031; Fig. 16).

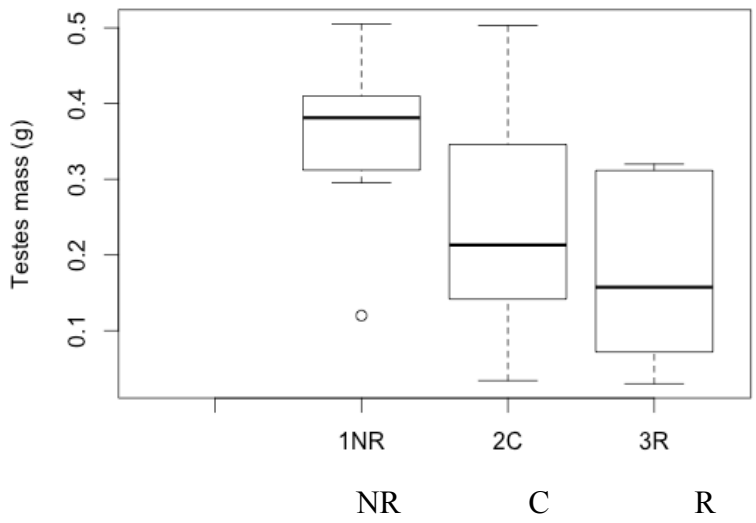

Fig. 4. The effect of line on testes mass in grams (Sample sizes: NR=10; C=16; R=6. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by the R program. NR = Non-responsive; R=Responsive; C=Control.)

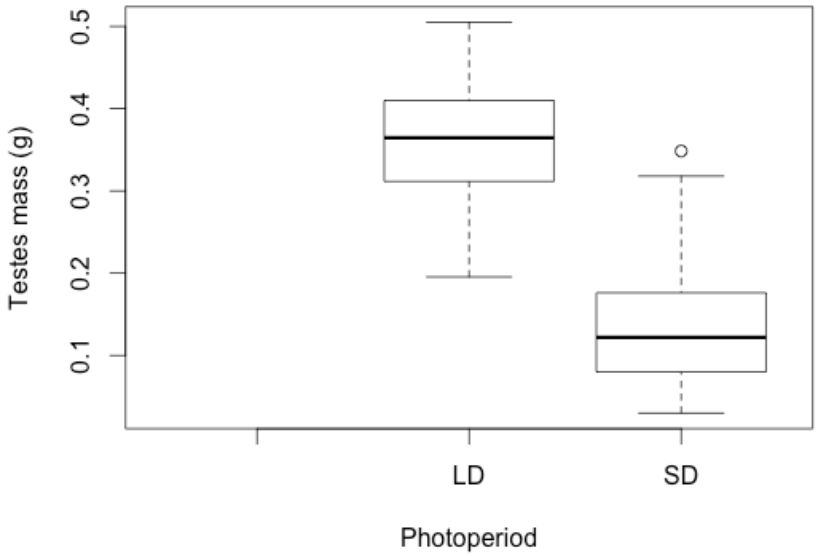

Fig. 5. The effect of photoperiod on testes mass in grams. (Sample size: LD=18; SD=14. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. LD = Long day; SD = Short day.)

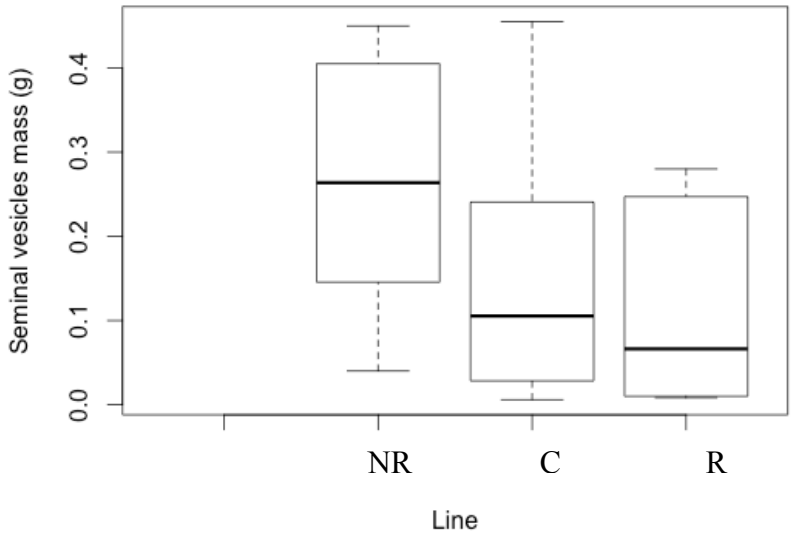

Fig. 6. The effect of line on seminal vesicles mass in grams. (Sample size: NR=10; C=16; R=6. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. NR = Non-responsive; R=Responsive; C=Control.)

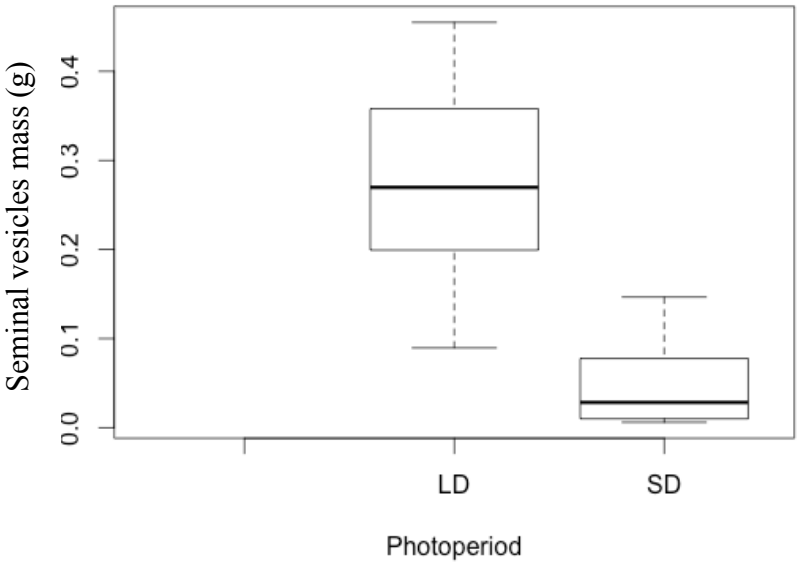

Fig. 7. The effect of photoperiod on seminal vesicles weight (g). (Sample size: LD=18; SD=14. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. LD = long day; SD = short day.)

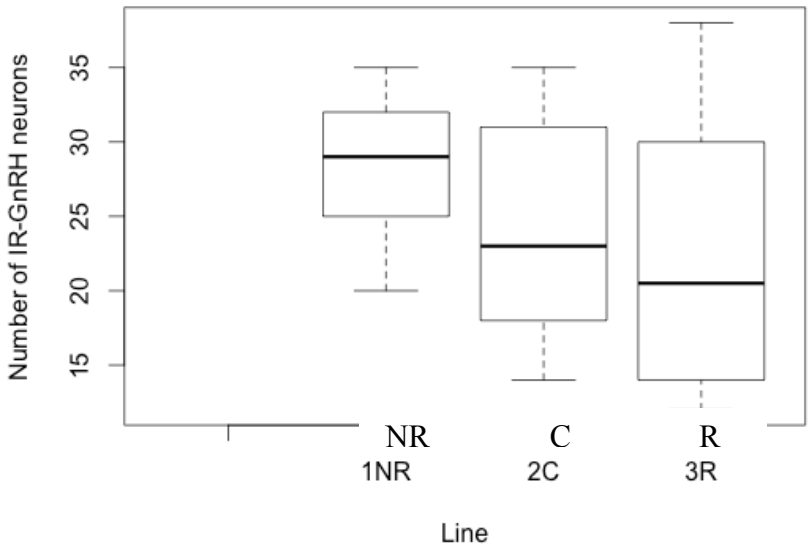

Fig. 8. The effect of line on the number of IR-GnRH neurons. (Sample size: NR=9; C=14; R=6. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. NR = Non-responsive; R=Responsive; C=Control.)

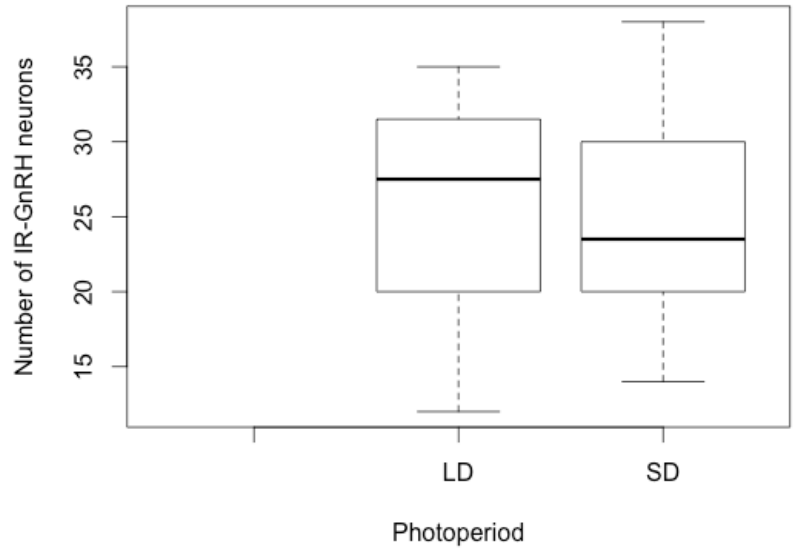

Fig. 9. The effect of photoperiod on number of IR-GnRH neurons. (Sample size: LD=16; SD=14. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. LD =long day; SD=short day.)

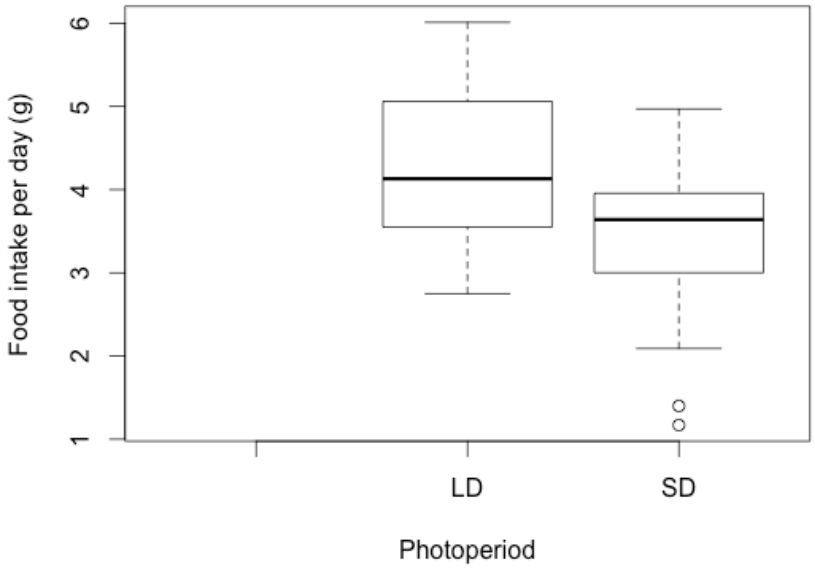

Fig. 10. The effect of photoperiod on daily food intake (g). (Sample size: LD=18; SD=15. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. LD = long day, SD = short day.)

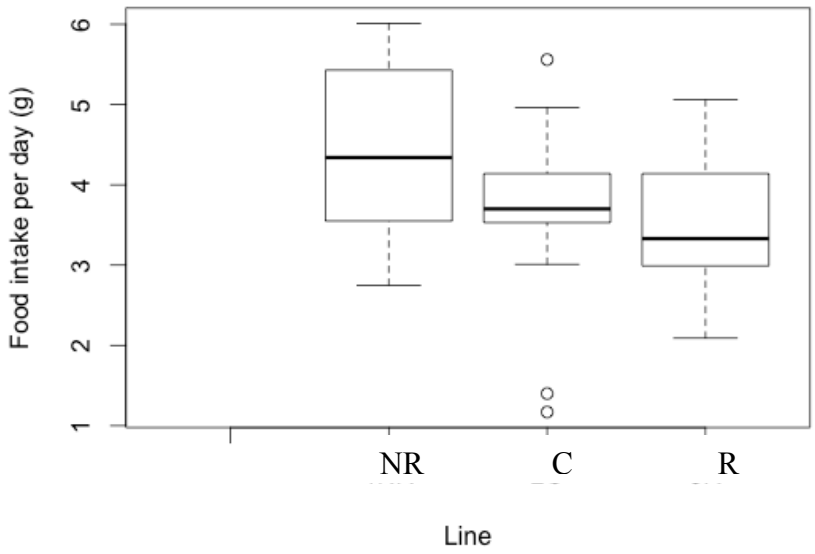

Fig. 11. The effect of line on daily food intake (g). (Sample size: NR=10; C=17; R=6. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. NR = Non-responsive; R=Responsive; C=Control.)

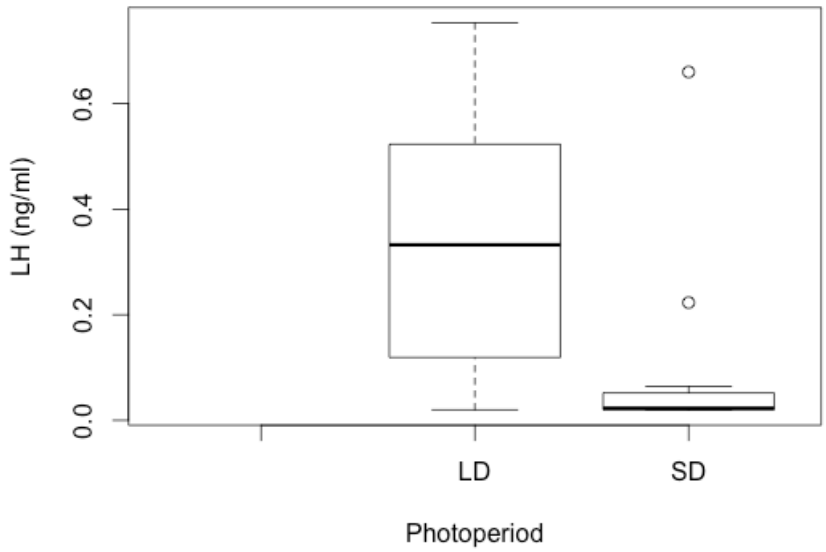

Fig. 12. The effect of photoperiod on LH plasma level (ng/ml). (Sample size: LD=6; SD=5. The thick bar is the mean; the box encloses quartile; dashed lines with whiskers indicate the range. Open circles show extreme data points identified by R. LD = long day, SD = short day.)

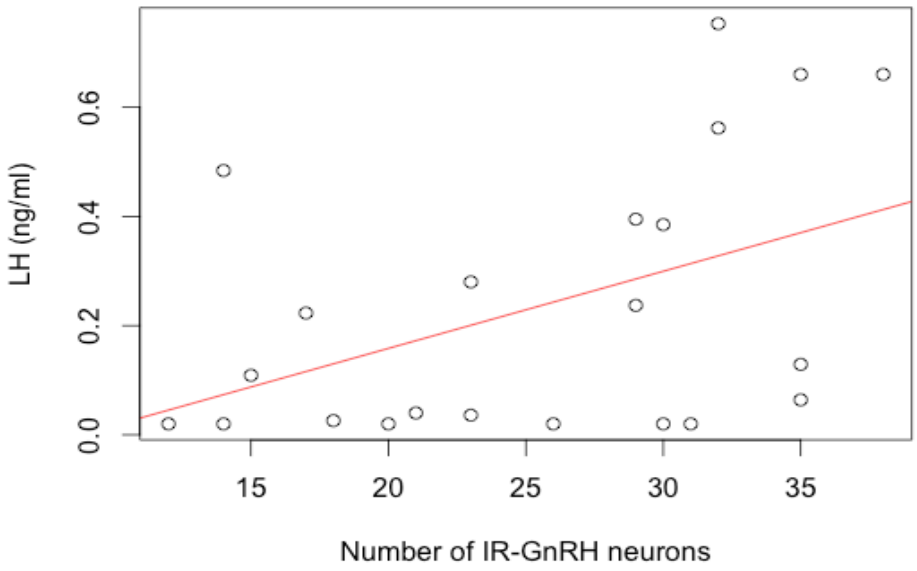

Fig. 13. The relationship between LH plasma level (ng/ml) and number of IR-GnRH neurons. Sample size: N=11. (P = 0.036; R = 0.45).

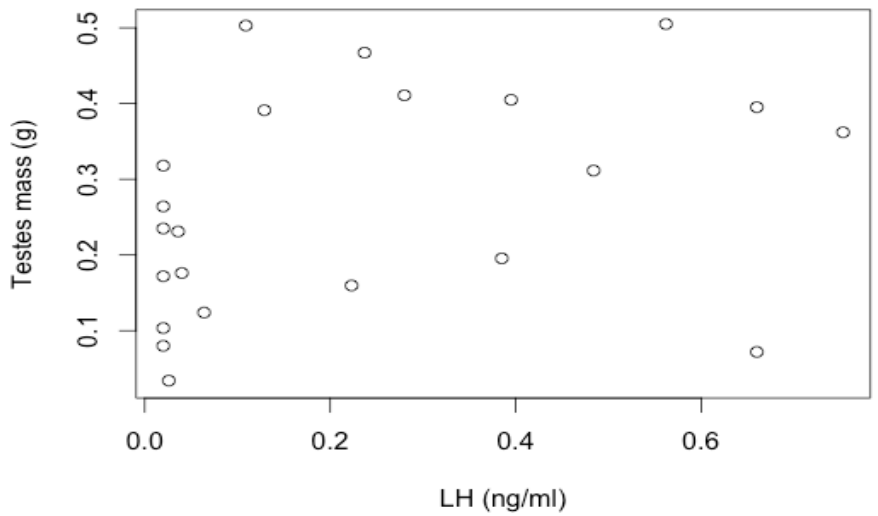

Fig. 14. The relationship between LH plasma level (ng/ml) and Testes mass (g). Sample size: N = 11. (P = 0.58; R = 0.36).

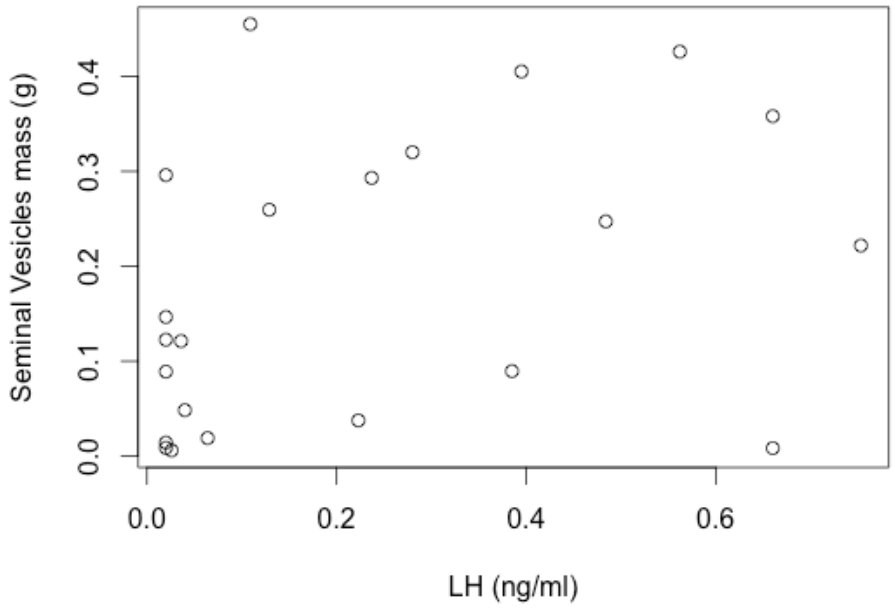

Fig. 15. The relationship between LH serum level (ng/ml) and seminal vesicle mass (g). sample size: N=11. (P = 0.085; R = 0.37)

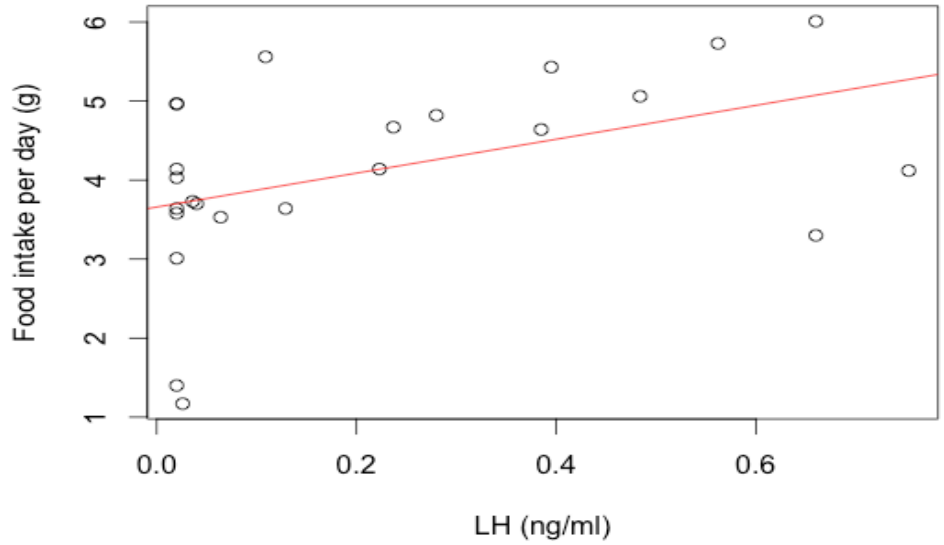

Fig. 16. The relationship between LH plamsa level and daily food intake (g). N=11. (P = 0.03; R = 0.44).

Discussion

These data are the first part of what will become a much larger data set. The results from these data support two conclusions. First, the results replicate some previous findings. Second, they provide new insight into the combinatorial effects of these variables on fertility.

As seen previously by Avigdor et al. (2005), the number of IR-GnRH neurons was higher in the NR line than the R line, though the difference was not significant in my data (Fig. 8). The magnitude of the difference among lines was similar to that from the previous study (Avigdor et al. 2005). A new result is that the C line was intermediate in number of IR-GnRH neurons, though the difference was not statistically significant. Our data were also consistent with the previous experiment by

Avigdor et al. (2005) on the lack of a difference in the number of IR-GnRH neurons in LD and SD photoperiod.

In these data, we found a significant effect of photoperiod on daily food intake. Mice in SD consumed approximately 18% less than those mice in LD. This is consistent with previous observations from Heideman et al. (2005). In contrast to Heideman et al. (2005), the three lines did not differ significantly in daily food intake. However, the magnitude of difference in daily food intake among lines in this study was similar to that in Heideman et al. (2005). In these data, the sample sizes may be too small to show significance with the effect sizes reported in Heideman et al. (2005).

These preliminary data suggest some new insights into the combinatorial effect of our variables on fertility. First, the plasma level of LH and number of IR-GnRH neurons were not correlated. Therefore these two variables may provide independent measures of variability. Interestingly, plasma level of LH was correlated significantly with daily food intake. This result is not surprising, because food intake has been correlated with testes size in previous studies (Heideman et al. 2005). There are several possible explanations. Secretion of corticosterone from the hypothalamic-pituitary-adrenal axis could simultaneously decrease the synthesis of LH by suppressing the GnRH secretion as it suppresses appetite (Gore et al. 2006). In addition, high daily food intake itself might trigger the synthesis of reproductive hormones, including LH. Alternatively, a high level of reproductive hormones could increase appetite and thus increase daily food intake. The correlation between food intake and level of LH should be tested further with larger sample sizes.

We concluded that our fertility measure, testes weight, is best accounted for by the combination of LH plasma level, selection line, photoperiod and daily food intake in the linear models. That combination predicted our best-fit model in which all variables were significant. Clearly, the combination of IR-GnRH neurons with other variables did not predict testes size. Finally, there are many additional variables that could cause variation in reproductive photoresponsiveness, including receptor expression and intracellular pathways stimulated by receptor binding to hormone (Heideman and Pittman 2009).

Future Directions

Data on the both kisspeptin neurons and GnIH neurons might greatly affect our GLM. A study from Moenter et al. (2007) indicated that kisspeptin significantly increases GnRH and LH release and the rate of pulsatile secretion of GnRH in female mice. Another student from our labatory (K.Swanson) studies the role of kisspeptin in regulation of fertility. Further data on kisspeptin from this new data set can be shared and analyzed in a future study. Clarke and Smith (2010) identified that GnIH is a negative regulator of reproduction in mammals. Interestingly, injection of GnIH increased food intake (Clarke and Smith 2010). Since food intake is related to appetite and stress, GnIH should be regarded as one of the central neuroendocrine variables in regulation of stress axis (Clarke and Smith 2010). The number of IR-GnIH neurons is heritable in our mice (Mason, Kingsfold & Heideman; unpublished data). Another student (A. Ives) is currently working develop immunocyotochemistry for GnIH neuron staining. Data on this GnIH study can be analyzed together in a further study.

At a very late stage of experiment, we implemented two-day post fixation instead of one-day post fixation. This minor change in procedure enhanced the quality of brain sections. There were less tissue damages and less tissue shredding near the third ventricles where most of GnRH neurons are located. We anticipate that better tissue quality will reduce the error in counting IR-GnRH neurons or GnIH neurons in future. There were few data on LH. Therefore, any conclusions about levels of LH are weak. The results and statistical analyses from the current data must be improved with a larger sample size. We only had 21 of R, NR and C mice in total that had a complete set of measured variables. Eventually, this data set is planned to be expanded to a total sample of 100 mice.

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