Variation in the Number of Kisspeptin Neurons in a Population of Wild Mice (Peromyscus leucopus)

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Variation in the Number of Kisspeptin Neurons in a Population of Wild Mice
(Peromyscus leucopus)

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by

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ABSTRACT

Neuroendocrine mechanisms give rise to naturally occurring individual variation in life history traits on which natural selection acts. This study explores a possible neuroendocrine mechanism that gives rise to variation in photoperiod responsiveness. In this study, we use a colony of wild-caught P. leucopus at the College of William and Mary. We used two selection lines, one reproductively responsive to photoperiod, the other reproductively non-responsive to photoperiod. Previous research has shown a significant difference in the number of immunoreactive (IR) Gonadotropin Releasing Hormone (GnRH) neurons between photoperiod responsive and non-responsive lines. The photoperiod responsive line had significantly fewer IR-GnRH neurons than the photoperiod non-responsive line. Previous research indicates that the difference between selection lines arises from genetic variation, not photoperiod. GnRH is presumed to be regulated by kisspeptin, the gatekeeper of the hypothalamic-pituitary-gonadal axis. We tested the hypothesis that variation in the number of kisspeptin neurons may contribute to variation in photoperiod responsiveness. Immunocytochemistry specific to kisspeptin was performed on brain sections from the same individuals used in the GnRH study. IR-kisspeptin neurons were counted and compared across selection lines and photoperiod treatments. We detected a significantly higher number of IR-kisspeptin neurons in the photoperiod non-responsive line and a correlation between number of IR-kisspeptin neurons and IR-GnRH neurons. However, we detected no significant difference in number of IR-kisspeptin neurons between photoperiod treatment groups. These results indicate the number of IR-kisspeptin neurons varies genetically in our population of mice. This variation may play a role in responsiveness to photoperiod.
Chapter 1: Literature Review

Variation in Photoperiod Response as a Life History Strategy

Life History Strategies

All organisms face the challenge of allocating limited time and resources toward survival. The ability of an individual to survive and reproduce is known as fitness. Individuals increase their fitness by adopting life history strategies that effectively allocate the individual’s resources throughout three competing demands: growth, survival and reproduction. Reproductive success in some mammals is increased by reproduction at seasonally optimal times. In timing reproduction with the seasons, mammals may optimize the conditions in which they undergo gestation and give birth to progeny. In some mammals, selection favors birth in the spring when temperatures are warmer and there is higher food availability. Higher food availability and warmer temperatures support the increased metabolism needed during lactation and provision of nutrients for newly weaned progeny.

Photoperiod and Seasonality

Seasonal changes are often processed in mammals by measurement of photoperiod, the duration of light to which an organism is exposed in one day. Daily photoperiod is inversely related to the duration of darkness. The duration of darkness is signaled in mammals by the hormone melatonin. Melatonin is secreted by the pineal gland and acts on signaling cascades that control reproductive responses in the organism. Reproductive response is a function of traits such as gonadal development and
reproductive behavior. Seasonal variation in traits such as body weight and gonad development can be due to seasonal variation in the duration of melatonin secretion.

While the duration of secretory melatonin varies by season, not all individuals within a population regulate reproduction by season. This variation in reproductive seasonality is due to variation at the post pineal level, not variation in melatonin secretion by the pineal gland (Blank et al. 1991, Prendergast et al. 2001). Because variability occurs at the post pineal level, it is possible for the same duration of melatonin secretion to affect different signaling pathways in distinct ways (Goldman 2001). These different signaling pathways allow individuals to stimulate some trait, while simultaneously suppressing a different trait (Prendergast et al. 2001). The differential stimulation and suppression of distinct traits allows variation in response to seasonality within a population.

In short winter photoperiods, some individuals may suppress reproduction while others may not, resulting in a variation in response to photoperiod (Prendergast et al. 2001). This variation in response to photoperiod may be a life history trade off (Zera and Harshman 2001, Heideman and Pittman 2009). During years with harsh winters and low food availability, suppressing reproduction in winter might benefit an individual. Reproductive suppression will allow allocation of more energy toward foraging and predator avoidance. For an individual that successfully reproduces, there may be a low probability that their offspring would survive. However, during less harsh winters with high food availability or in a location with high food availability, an individual has the potential to increase their fitness by reproducing in the winter, as their young are more likely to survive. Thus, no single strategy, photoperiod responsiveness or photoperiod
non-responsiveness always has highest fitness. The strategy that results in highest fitness will vary from microhabitat to microhabitat and year to year. This is a potential reason that natural populations have this variation in photoperiod response.

The continuation of a population is dependent on successful reproduction at the individual level. This individual variation has the potential to affect conservation biology of a species by lengthening the breeding season (Nelson 1987). With an increased breeding season, a population and a species have a higher probability of avoiding extinction due to particularly harsh winters or natural disasters. Additionally, if the life expectancy of individuals of a species is particularly short, it may still be beneficial to reproduce in short photoperiod even if there is a high rate of offspring mortality (Heideman et al. 2005).

**The Hypothalamic Pituitary Gonadal Axis**

The hypothalamic-pituitary-gonadal axis (HPG axis) is composed of endocrine glands in the hypothalamus, pituitary gland, and gonads that act in an integrated fashion to regulate processes such as reproduction (Ebling 2005). This set of endocrine glands is regulated by a series of positive and negative feedback loops (Fig. 1)(d’Anglemont de Tassigny and Colledge 2010). Two key regulatory peptides in these feedback loops are kisspeptin and gonadotropin inhibitory hormone (GnIH). GnIH and kisspeptin neurons synapse directly on GnRH neurons. GnRH neurons are inhibited by GnIH neurons (Kriegsfeld et al. 2006). In contrast, kisspeptin neurons stimulate GnRH neurons to secrete a pulse of GnRH release. GnRH pulses promote the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). The mechanism for LH and FSH
release in the hypothalamus is mediated by the binding of GnRH to its receptor, GNRHR-1 in the anterior pituitary. From the anterior pituitary, LH and FSH then travel through the circulatory system to stimulate the gonads to release estrogen, testosterone or progesterone. These hormones then create a feedback loop in which GnIH neurons inhibit GnRH secretion (Fig. 1). In addition to creating an inhibitory feedback loop via GnIH neurons, gonadal hormones create both positive or negative feedback loops on kisspeptin neurons depending where in the brain the kisspeptin neurons are located (Fig. 2).

Kisspeptin neurons are located primarily in two areas of the brain, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (Arc). Gonadal steroids act on kisspeptin neurons in two ways. In the Arc, sex steroids inhibit kisspeptin neurons to produce a negative feedback loop (Popa et al. 2008). This negative feedback loop in the Arc acts to suppress processes such as gonadal development and reproduction. In the second, sustained high levels of estrogen act on kisspeptin neurons in the AVPV to create a positive feedback loop, stimulating both ovum release and estrous sex behavior (Clarkson and Herbison 2009).

The HPG axis and reproduction are altered by environmental and metabolic cues. Mammalian reproductive systems receive input from metabolic factors such as glucose and leptin availability (Roland and Moenter 2010, Smith et al. 2006). A major environmental factor that affects reproduction is photoperiod, as noted above, which signals season in the mammalian reproductive systems. Seasonal changes in the duration of melatonin secretion alter kisspeptin signaling (Popa 2008).
Animal Model

The use of a nontraditional mammalian species allows study of normal variation that exists in nature, but is absent in a laboratory raised species such as the lab rat or lab mouse. The use of a sample derived from a wild-caught population preserves a range of behaviors, patterns of hormone secretion and neuronal variation present in nature. The use of wild-caught mice allows the analysis of variation in neuroendocrine mechanisms that give rise to naturally occurring individual variation in life history traits on which natural selection acts (Smale et al. 2005).

A colony of white-footed mice, *Peromyscus leucopus*, is maintained at the Population and Endocrinology Laboratory at the College of William and Mary. This colony was derived from a population of wild-caught white-footed mice, captured in 1995 in Williamsburg, VA (Heideman et al. 1999).

Two selection lines of white-footed mice, *P. leucopus*, that differ in their responses to photoperiod were developed by our laboratory (Heideman et al. 1999). Photoperiod responsive mice respond to a decrease in photoperiod, mimicking winter, by suppressing reproduction, identified by immature gonads. Photoperiod non-responsive mice respond reproductively not at all or weakly to a winter-like, short photoperiod. The selection lines were developed by selection for or against mature gonads in short photoperiod. Mice were born in long photoperiod, 16 hours of light with 8 hours of darkness, simulating summer conditions. Within 3 days of birth, mice were moved to short photoperiod, 8 hours of light and 16 hours of darkness, simulating winter conditions. Mice were housed in short photoperiod until they reached the age of 70 ± 3
days, at which point mice were grouped/segregated based on responsiveness to short day photoperiod. Males were segregated based on testis size and body weight. Females were segregated based on ovary length, uterine diameter, and body weight (Heideman et al. 1999). They maintain reproduction and gonad size in response to decreased photoperiod (Heideman et al. 1999).

Previous research has shown a significant difference in the number of immunoreactive (IR) Gonadotropin Releasing Hormone (GnRH) neurons between photoperiod responsive and non-responsive selection lines (Avigdor et al. 2005). The photoperiod responsive line had significantly fewer IR-GnRH neurons than the photoperiod non-responsive line. However, there was no significant difference between lines in short day versus long day (Avigdor et al. 2005). This suggests that the difference in number of IR-GnRH neurons between selection lines may arise from genetic variation. This study indicates that genetic variation in photoperiod responsiveness may be partially due to the number of IR-GnRH neurons. An important question is whether variation in the number of IR-kisspeptin neurons may also contribute to variation in photoperiod responsiveness.

**Kisspeptin**

Kisspeptin is a peptide derived from a 145 amino acid propeptide. Kisspeptin was first characterized in Hershey, PA by cancer researchers who characterized it by its ability to suppress the metastasis of cancer cells when cleaved to the length 54 amino acids (Lee et al. 1997). However, it was later discovered that the suppression of metastasis is not the only function of kisspeptin. Either as the 54 amino acid peptide or
with further degradation to the length of 14, 13, or 10 amino acid forms, kisspeptin was identified as a playing a role in the regulation of the human reproduction axis (Popa et al. 2008). The human reproductive system fails to mature and undergo puberty in the absence of a functional kisspeptin receptor (Vogel 2005). This indicates that the both the kisspeptin receptor and kisspeptin play an integral role in the regulation and maturation of the human reproductive system. Subsequent studies have brought kisspeptin to the forefront of reproductive physiology.

Kisspeptin is now presumed to be the gate-keeper of the hypothalamic-pituitary-gonadal (HPG) axis, aiding in the regulation of GnRH (Roa et al. 2011). Kisspeptin secreting neurons are located in close proximity to GnRH neurons. Furthermore, the kisspeptin receptor, GPR54, is a G protein-coupled receptor expressed in GnRH neurons, suggesting that kisspeptin acts directly on the GnRH neurons (Messager et al. 2005). Additionally, administration of kisspeptin has been shown to increase levels of luteinizing hormone (LH) in rhesus maqaque and mouse models (Shahab et al. 2005, Clarkson, et al. 2008). A similar experiment conducted in sheep resulted in increased levels of LH (Messager et al. 2005). The administration of kisspeptin to a group of human males increased levels of LH, FSH, and testosterone (Dhillo et al. 2005). Because the mechanism for LH release in the hypothalamus is mediated by the binding of GnRH to its receptor, it is presumed that increased levels of kisspeptin cause increased levels of LH via stimulation of GnRH release.

Kisspeptin is known to stimulate the reproductive axis at the hypothalamic level via stimulation of GnRH release. Kisspeptin binds to the GPR54 receptor on the surface of GnRH neurons. The GnRH neuron is then depolarized through a pathway involving
activation of phospholipase C (PLC), inositol triphosphate (IP3) and diacylglycerol (DAG). Stimulation of this pathway causes the opening of nonselective cation channels and the inhibition of potassium channels on the cell membrane. This results in sustained depolarization and increased action potentials of the GnRH neurons (d’Anglemont de Tassigny and Colledge 2010). Thus, binding of kisspeptin to its receptor, GPR54 results in increased firing of GnRH neurons and an increase in GnRH release.

As kisspeptin is presumed to act at the hypothalamic level to stimulate GnRH neurons, it is also hypothesized to act on gonadotropes at the level of the anterior pituitary (Luque et al. 2011). Gonadotropes in the anterior pituitary are stimulated by kisspeptin binding to its receptor and activating a pathway involving PLC, phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK). Studies have shown that kisspeptin presence in the anterior pituitary mediates LH release, but not necessarily FSH release. Additionally, studies have linked mammalian target of rapamycin (mTOR) to stimulation of gonadotropes by kisspeptin in the anterior pituitary using the kisspeptin signaling pathway mentioned above (Luque et al. 2011). This suggests a possible point of integration of metabolic cues in kisspeptin signaling.

A study conducted on Siberian hamsters suggests a mechanism for the suppression of reproduction in short day (Revel et al. 2006). In the presence of increased nightly duration of the rise of levels of melatonin, two pathways act that are complementary to each other. The first pathway involves sex steroid negative feedback inhibition of kisspeptin neurons in the brain. The second pathway involves the reinforcement of the sex steroid negative feedback loop that decreases kisspeptin activity (Revel et al. 2006). Thus, kisspeptin neurons have been proposed to play an integral role
in the regulation of photoperiod in Siberian hamsters by two complementary mechanisms.

Because kisspeptin plays a regulatory role in the HPG axis, it is relevant to matters of fertility and reproductive health. Timing of puberty is presumed to be regulated in mammals by kisspeptin (Mayer et al. 2010). Increased expression of the kisspeptin gene in the hypothalamus and the amplification of GnRH activity due to a positive estrogen feedback loop with the ovary is proposed as the mechanism by which kisspeptin triggers puberty (Roa et al. 2011). Delayed onset of puberty and infertility, in males and females, is caused by a condition known as Idiopathic Hypogonadotropic Hypogonadism (IHH). It has been shown that IHH is caused by a lack of GnRH release, and not a mutation that affects hormone sequence. This suggests that hypogonadism and the delayed onset of puberty are due to an inability of GnRH neurons to respond to stimulation (Messager et al. 2005). According to experiments conducted with human and mouse subjects, the cause may be a mutation in the GPR54 kisspeptin receptor gene (Seminara et al. 2003). Further studies demonstrated that IHH can be the result of a heritable loss-of-function mutation in the GPR54 receptor gene, indicating a heritable component to this disorder (Nimri et al. 2010).

Objectives

The purpose of this study is to determine (1) if there is heritable variation in the number of IR-kisspeptin neurons in the brains of *Peromyscus leucopus*, and (2) whether any variation present might contribute to variation in photoperiod responsiveness. Brain sections stored from the study on IR-GnRH neurons by Avigdor et al (2005), were
stained immunocytochemically with a kisspeptin specific primary antibody. IR-kisspeptin neurons were counted and compared across selection lines. If the variation in IR-kisspeptin neurons is not a cause of variation in photoperiod responsiveness, then we would predict no significant difference in the number of IR-kisspeptin neurons in relation to photoperiod responsiveness. However, if the variation in IR-kisspeptin neurons contributes to variation in photoperiod responsiveness, then we would predict a significant difference in the number of IR-kisspeptin neurons between selection lines, with significantly more IR-kisspeptin neurons in the photoperiod nonresponsive line than in the photoperiod responsive line. These are animals that are still reproductive even in inhibitory conditions, which would suggest more kisspeptin neurons in that line.
Chapter 2: Variation in the Number of Kisspeptin Neurons in a Population of Wild Mice (*Peromyscus leucopus*)

INTRODUCTION

The mammalian reproductive axis receives inputs from multiple signaling pathways. These inputs combine to regulate the mammalian reproductive cycle. Thus, a mutation or variation in just one pathway has the potential to alter the reproductive cycle in that individual (Heideman and Pittman 2009). Furthermore, there is potential for multiple points of genetic variation in an individual to cause phenotypic changes in the signaling pathways in that individual (Smith et al. 2006, Mayer et al. 2010). These varied signaling pathways can have a combinatorial effect on the reproductive cycle of an individual (Heideman and Pittman 2009). For this reason, it is important to identify multiple sources of variation in mechanisms by which the mammalian reproductive axis can be upregulated or downregulated. A downregulation of reproduction can lead to infertility. Infertility can be the outcome of variation in just one factor, or it can be the outcome of variation in a combination of factors. Furthermore, the causes of infertility vary between individuals. Unless we address individual variation, we assume that every individual in a species has the same physiological variation that is resulting in infertility, even though each individual varies in its physiology (Steiner and Tuljaparkur, 2012). It is crucial that we study the individual interactions between different combinatorial aspects of physiology in order to better understand the causes of infertility.

As we discover mechanisms of mammalian infertility in research, we can then apply our findings to human systems. We use the model system of the white-footed mouse, *Peromyscus leucopus*, to identify possible mechanisms of infertility in mammals that can be applied to humans. We use *P. leucopus* because they are a wild derived
population that preserves a portion of the variation found in nature (Smale et al. 2005). With this model, we can identify the variation in different physiological systems and then look for potential interactions between physiological systems. It is useful to identify variation in a natural model because physiological variation observed in natural mouse populations may be similar to that in humans. Our research has the potential to suggest complex factors in human physiology that lead to infertility. This might improve therapies to restore fertility in humans.

In order to better understand the combinatorial factors resulting in infertility, we are studying two selection lines of *P. leucopus* (Heideman and Pittman 2009). We developed a reproductively responsive line (R) that responds to winter-like short photoperiod by suppressing reproduction. A reproductively non-responsive line (NR) does not respond to winter-like short photoperiod, continuing reproduction as normal (Heideman et al. 1999). Previous research has shown a significant difference in the number of immunoreactive (IR) Gonadotropin Releasing Hormone (GnRH) neurons between photoperiod responsive and non-responsive lines (Avigdor et al. 2005). The photoperiod responsive line had significantly fewer IR-GnRH neurons than the photoperiod non-responsive line. However, there was no significant difference between individuals in short day versus long day within lines (Avigdor et al. 2005). This indicates that the difference in selection lines arises from genetic variation, not photoperiod, and suggests that genetic variation in photoperiod responsiveness may be partially due to variation in the number or activity of IR-GnRH neurons.

GnRH is presumed to be regulated by the neuropeptide, kisspeptin. Kisspeptin acts directly on GnRH neurons to stimulate GnRH release. Therefore, the kisspeptin
neuronal system is another potential source of variation in fertility. In this study, we compared the number of IR-kisspeptin neurons in the responsive and non-responsive lines of mice. We tested: (1) whether there is heritable variation in the number of IR-kisspeptin neurons in the brains of *Peromyscus leucopus*, (2) whether any variation present might contribute to variation in photoperiod responsiveness. If the variation in IR-kisspeptin neurons is not a cause of variation in photoperiod responsiveness, then we predict there will not be a significant difference in the number of IR-kisspeptin neurons in relation to photoperiod responsiveness. However, if the variation in IR-kisspeptin neurons contributes to variation in photoperiod responsiveness, then we predict there will be a significant difference in the number of IR-kisspeptin neurons between selection lines in a predictable relation to photoperiod responsiveness, with significantly more IR-kisspeptin neurons in the photoperiod nonresponsive line than in the photoperiod responsive line.

**METHODS**

**Animals**

*Peromyscus leucopus* were taken from a colony at the Population and Endocrinology Laboratory at College of William and Mary. The 48 founders of the colony were captured in Williamsburg, VA, 37°16’N. Founders were housed in long day photoperiod and paired, generating a parental generation of the laboratory’s selection lines. Selection lines were created by P. Heideman as previously described (Heideman et al. 1999). The process is summarized as follows. Mice were born in long photoperiod, 16 hours of light with 8 hours of darkness, simulating summer conditions. Within 3 days of
birth, mice were moved to short photoperiod, 8 hours of light and 16 hours of darkness, simulating winter conditions. Mice were housed in short photoperiod until they reached the age of $70 \pm 3$ days, at which point mice were segregated based on their response to short photoperiod. Mice were anesthetized with methoxyflurane (Pittman-Moore, Inc., Mundelein, IL) and body weight and gonad size were obtained. Males were segregated based on testis size. Males with smaller testis ($<24 \text{ mm}^2$) were put into the responsive line, while males with larger testis ($>32 \text{ mm}^2$) were placed in the non-responsive line.

Females were segregated based on ovary length, uterine diameter, and the presence of visible follicles or corpora lutea. Responsive females had ovarian lengths $<2 \text{ mm}$, lacked visible follicles or corpora lutea and had a uterine diameter $\leq 0.5 \text{ mm}$. Non-responsive females had ovarian lengths $>3.5 \text{ mm}$, with large visible follicles or corpora lutea and uterine diameters $>1.0 \text{ mm}$. Individuals in the middle of these ranges were categorized as intermediate and not included as founders in either selection line. Selection was continued within each line for ten generations.

To minimize genetic drift and loss of natural variation, each generation of selection had between 20 and 40 breeding pairs. Additionally, there were no sibling mating pairs were not allowed in order to minimize inbreeding.

Individuals in this study were from the $F_6$, $F_7$, $F_8$ and $F_9$ generations. Mice were housed at the Population and Endocrinology Laboratory at the College of William and Mary. They were singly housed in polyethylene cages with wire tops and pine shavings. Mice were provided food and water ad lib. Males from both lines were housed individually in either long or short photoperiod ($n=12-15$) per treatment until age $70\pm3$ days, at which point they underwent euthanasia and perfusion.
Perfusions and Sectioning

Brains were perfused and sectioned by Mauricio Avigdor. Fixation and initial processing of tissue occurred as previously described (Avigdor et al. 2005) and is summarized as follows. Mice were age 70±3 days at the time of perfusion. Mice were administered an overdose of Isoflourane before perfusion (Abbott Laboratories, North Chicago, IL). Mice were then perfused using 0.1 M phosphate-buffered saline (PBS) at pH 7.4. Following perfusion with PBS, mice were perfused with a solution of 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) and saturated picric acid (Sigma Chemical, St. Louis, MO) in PBS. Brains were then fixed overnight in Zamboni’s Fixative. Before sectioning, brains were cryoprotected in a solution containing 30% sucrose and PBS. 30 μm sections were cut on a freezing sliding microtome and stored in antifreeze solution (37.5% sucrose, 37.5% ethylene glycol, and 10 g PVP-40 in 500 ml 0.02 M Tris-buffered saline) at -20°C until immunocytochemistry. Procedures were approved by the Institutional Animal Care and Use Committee of the College of William and Mary as projects 9837 and 429.

Immunocytochemistry

IR-kisspeptin neurons were detected via indirect avidin-peroxidase complex immunocytochemistry. Each run was balanced across treatment groups, with sections from 6 individuals being treated in each round. All treatments were conducted with gentle agitation on a shaker at room temperature, unless stated otherwise. Brain sections previously stored in an antifreeze solution at -20°C were rinsed in cold 0.2 M Tris-Buffer
Saline (TBS) 3 times for 6 minutes each time. Sections were then incubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidases. Sections were then rinsed in cold TBS 3 times for 10 minutes each, following which, sections were incubated for 36-48 hours at 4°C in polyclonal rabbit anti-kisspeptin specific primary antibody (gift from A. Caraty, Institut National de la Recherche Agronomique, Paris, France) diluted 1:10,000 in TBST(0.3% Triton X-100, 0.25% BSA and 2% NGS). Sections were then washed in cold TBS 5 times for 8 minutes each time. Sections were then incubated for 90 minutes in biotinylated anti-rabbit immunoglobulin (Vector Labs, Burlingame, CA) diluted 1:500 in TBST. Sections were washed in cold TBS 4 times for 8 minutes each, followed by a 90 minute incubation at room temperature in Vector Elite avidin-peroxidase (Vector Labs, Burlingame, CA). Sections were then washed in TBS 3 times for 8 minutes each. Following washes with cold TBS, sections were agitated in Nickel-enhanced diaminobenzidine (Vector Labs, Burlingame, CA) for 4 minutes at room temperature. Finally, sections were washed in cold TBS 3 times for 6 minutes each and mounted on Superfrost Plus Micro Slides (VWR International, Radnor, PA). Slides air dried overnight. Slides were then counterstained with Methyl Green (Vector Labs, Burlingame, CA). After staining with Methyl Green, sections were taken through a series of dehydration steps in acetone, ethanol and xylene. Slides were then coverslipped with Permount (Fisher Scientific, Waltham, MA).

Specificity of the primary antibody to kisspeptin was verified by a control experiment in which staining was performed with and without primary antibody. No significant staining was detected in the absence of primary antibody.
Neuronal Analysis

The location and number of IR-kisspeptin neurons was assessed by K. Swanson, with assistance from Teresa H. Horton and Paul D. Heideman. Neurons were identified and counted by eye on an Olympus CH2 compound light microscope. Counts were carried out blind with respect to treatment group to reduce potential bias in identification of neurons. In addition, 3% of the sections were recounted by a second individual blind to treatment group and previous count to test for consistency.

IR-kisspeptin neurons were counted in the arcuate nucleus (Arc) (Fig. 3). Counts were made from 6 different brain sections at the same approximate cross sectional depth in each area of the brain. Neurons were sampled within a 250 μm by 250 μm ocular grid that overlaid approximately 50% of the arcuate nucleus on one side of the hypothalamus. The grid was consistently positioned with one edge touching the base of the brain and the adjacent edge touching the edge of the third ventricle. A random number generator was used to choose the right or left Arc for counts. If there was damage to a section that might cause an inaccurate count from the side selected at random, the grid was placed over the Arc on the other side. If there was damage to the Arc on both sides of the third ventricle (approximately 2% of sections), counts were not performed on that section. The number of IR-kisspeptin neurons was determined based on the number of immunoreactive cell bodies, not immunoreactive axons. Criteria for identification of IR-kisspeptin neurons included (1) shape and size consistent with the shape and size of neurons, (2) darkness of cell clearly above background staining, and (3) the presence of axonal projections in the same field of focus as the cell body. The first two criteria were necessary for all neurons. The third criterion was confirmatory when shape or size was ambiguous.
Statistical Analysis

Heritable Variation in IR-Kisspeptin Neurons

The distribution in the complete data set on the number of IR-kisspeptin neurons did not meet two statistical assumptions needed to perform parametric statistical analyses (Fig. 4). In one group (R-LD), there was a non-normal distribution of data and there were unequal variances among treatment groups. Therefore, because we could not meet the assumptions of a parametric statistical test with this full data set, we began analysis with a non-parametric Mann Whitney U Test.

The inequality of variances and non-normal distribution of data was due to a single individual in the RLD group with three times the number of IR-kisspeptin neurons than the next highest count. This data point was located 7 standard deviations away from the mean number of IR-kisspeptin neurons for the entire data set, including this individual. We concluded that this data point was a statistical outlier and that it was inappropriate to retain in further statistical analyses. However, we discuss this data point and its implications in the discussion.

We performed a series of correlation analyses with SPSS Statistics 19 software (IBM, Armonk, NY). We also performed a two-way ANOVA using SPSS comparing the number of IR-kisspeptin neurons with relation to photoperiod and selection line.

RESULTS

In the initial analysis, including the outlier in the R-LD group, we applied a Mann-Whitney U Test. There was a significant difference in the number of IR-kisspeptin
neurons between selection lines (p<0.05). In subsequent analysis with the outlier removed, the data set resulted in an approximately normal distribution and similar variances between treatment groups.

We performed a two-way ANOVA comparing the interaction between selection line and photoperiod (Fig. 5). Results of the two-way ANOVA indicate a significant difference in the number of IR-kisspeptin neurons between the responsive and non-responsive lines (F=13.280, p=0.001). There was no significant difference in the number of IR-kisspeptin neurons between short day and long day photoperiod (F=2.115, p=0.153). There was no significant interaction between selection line and photoperiod (F=0.377, p=0.542).

A correlation analysis between the number of IR-GnRH neurons and number of IR-kisspeptin neurons in all individuals (Fig. 6) showed a significant correlation between the number of IR-GnRH neurons and the number of IR-kisspeptin neurons (R=0.358, p=0.011). A correlation analysis on the relationship between the number of IR-kisspeptin neurons and IR-GnIH neurons (Fig. 7) showed no significant correlation between the number of IR-kisspeptin neurons and IR-GnIH neurons (R=0.347, p=0.070). GnIH counts were from unpublished data collected by A. Mason, P. Heideman, and L. Kriegsfeld. A correlation analysis between the number of IR-kisspeptin neurons and body weight (Fig. 8) showed no significant correlation between the number of IR-kisspeptin neurons and body weight (R=0.186, p=0.197). A correlation analysis on the relationship between the number of IR-kisspeptin neurons and testis weight (Fig. 9) showed no significant correlation between the number of IR-kisspeptin neurons and testis weight (R=0.127, p=0.378). A correlation analysis on the relationship between the number of IR-kisspeptin
neurons and seminal vesicle weight (Fig. 10) showed no significant correlation between the number of IR-kisspeptin neurons and seminal vesicle weight \((R=0.101, p=0.479)\).

**DISCUSSION**

The finding that the number of IR-kisspeptin neurons differed between selection lines is consistent with the hypothesis that variation in the kisspeptin neuron system plays a role in variation in responsiveness to photoperiod. In contrast, the exposure to LD or SD photoperiod did not significantly affect the number of IR-kisspeptin neurons counted in an individual. Photoperiod and selection lines did not appear to interact to have a combined effect on the number of IR-kisspeptin neurons.

We did not find a significant correlation between IR-GnIH neurons and IR-kisspeptin neurons, but, we did see a potential trend \((p<0.10)\). In order to improve the assessment of this correlation, we want to increase the accuracy of kisspeptin neuron counts to include more sections counted from each brain.

Individuals from the non-responsive line had more IR-GnIH neurons than those in the responsive line. This is counterintuitive, as GnIH is presumed to inhibit GnRH release (Kriegsfeld et al. 2005). We would expect GnRH release to be higher in a non-responsive individual and thus predict there to be fewer GnIH neurons in the nonresponsive line than in the responsive line. A possible explanation for this phenomenon is that higher immunoreactivity may be a result of higher concentration of GnIH inside the neurons (Bentley et al. 2006). This hypothesis implies that GnIH neurons are stockpiling GnIH and not releasing it. It is not clear why this should be the case.
Multiple studies indicate that kisspeptin stimulates GnRH release (Messager et al. 2005, Roa et al. 2011). As we found significantly higher numbers of IR-kisspeptin neurons in the non-responsive line, we hypothesize that these IR-kisspeptin neurons are detectable because they are producing kisspeptin for release. If the IR-kisspeptin neurons were storing the kisspeptin, then we might expect to see lower fertility in non-responsive mice because they had higher levels of immunoreactivity. Thus, we propose that levels of immunoreactivity in IR-kisspeptin neurons are either not affected by kisspeptin release or intensified by the processes that produce kisspeptin.

Prior to immunocytochemistry, sections in this study were held at -20°C for approximately 8 years. Thus, it is possible that there has been a partial degradation resulting in a loss of immunogenicity for kisspeptin. We assumed that if there has been loss of immunogenicity of kisspeptin neurons, it has been occurring at the same rate in all individual sections. The basis for this assumption is that the sections have been undergone the same treatment and been stored in the same conditions since the time of perfusion and sectioning.

As discussed in the methods, we omitted the statistical outlier from statistical analysis, as it was located 7 standard deviations away from the mean number of IR-kisspeptin neurons for the entire data set, including this individual. This unusual deviation from the mean number of IR-kisspeptin neurons in this individual exists for one of two reasons. First, it is possible that the increased number of IR-kisspeptin neurons is the result of increased immunogenicity in this individual due to inconsistencies in the storage or staining of the sections. However, this seems unlikely, as other individuals stained in the same ICC run and stored in the same way did not exhibit an increased
number of IR-kisspeptin neurons. Second, it is possible that this individual is representative of true variation in our population of mice. In further analysis, we will stain more sections from this individual to see if our counts are consistent with our original counts for this individual. Consistent numbers of IR-kisspeptin neurons after a second round of ICC would suggest that this high number of IR-kisspeptin neurons is not due to an ICC error. It would still be possible that the high number of IR-kisspeptin neurons in this individual is due to variability in section storage and antigen preservation. Finally, it is possible that some individuals in the population have unusually high numbers or activity of kisspeptin neurons.

While we assume that the genetic variation in this population may be representative in some degree of the genetic variation in the natural population, it is important to note that our colony of mice has undergone some genetic drift, a change in the allele frequencies in a population that is due to chance (Heideman et al. 2005). Some genetic drift must have occurred due to the initial founder effect. Our colony is derived from a population of 48 founders, which initially reduced the gene pool from the natural population (Heideman et al. 1999). Additionally, some alleles that are rare and unimportant in the wild will have been increased by chance in one or more of the lines in our colony. Thus, it is reasonable to suggest that the differences between selection lines in our colony are not closely related to reproduction in the gene pool of mice that exists in the wild. However, because we are testing for heritable variation in neuronal traits, the cause of differences between selection lines is interesting, but it is not essential that we distinguish between laboratory selection and genetic drift (Kaseloo, et al. in press).
Our results indicate that the number of IR-kisspeptin neurons varies genetically in this population, and this variation may play a role in responsiveness to photoperiod. Responsiveness to photoperiod may be regulated by an interaction between kisspeptin and GnRH, as previous results indicate the number of IR-GnRH neurons as being variable in this colony of mice (Avigdor et al. 2005).

**FUTURE DIRECTIONS**

An important question for this study is whether there has been degradation of neuropeptides as a result of storage for the eight-year period following preservation of brain tissue. As one method to assess the possibility of degradation, we will compare numbers of IR-GnRH neurons in fresh sections stained by Avigdor et al. (2005) with stored sections stained for GnRH in 2010 as part of the current study (K. Swanson and T. Horton, unpublished data). Comparing the number of IR-GnRH neurons between these two groups of sections will allow us to assess the degree of GnRH peptide degradation that has occurred in our sections overtime. In analyzing these counts, we will be able to estimate an index of degradation that will allow us to make inferences about the effectiveness of the antifreeze solution over time.

In future analysis, we hope to create a general linear model that shows how kisspeptin combines with other variable traits, such as GnRH, GnIH, and environmental factors, such as photoperiod, to result in infertility. The general linear model may provide a better understanding of how variation in kisspeptin interacts with other variable factors in the mammalian HPG axis to regulate fertility. Eventually, it might be possible to add
other potential causes of variation, such as neuropeptide receptor abundance and other potential causes of variation (see Pittman and Heideman 2009).

In this study, we identified the number of IR-kisspeptin neurons as a variable trait in our colony of mice. As our colony represents a subset of the natural gene pool, there may be significantly more variation in the natural population. However, we predict that variation in number of IR-kisspeptin neurons occurs in the natural population. A future study could sample the wild population and test for variation in kisspeptin neurons that is correlated with winter reproduction. By obtaining measurements on the same variables we could test how the results with wild mice compare with our generalized linear model. We could compare the generalized linear model from this study with their measurements in an attempt to get an idea of what fraction of the variation in the wild we successfully captured in our general linear model.

**CONCLUSION**

In this study, we identified the number of IR-kisspeptin neurons as a variable trait in our colony of mice. These results have implications for human reproductive variability. The reproductive axis is similar amongst mammals. Thus, if variation in kisspeptin neurons exists in mice, then it is possible that this variation exists in other species. By examining physiological variation, we may gain the ability to predict levels of fertility in individuals and populations based on variation in neural and hormonal traits. The ability to predict levels of fertility is valuable to conservation and species rehabilitation efforts.

Variation in reproductive phenotypes is present in humans (Kosova et al. 2010). Human variation in reproductive phenotypes may be partially due to variations in the
HPG axis, and a portion of this variation in the HPG axis may be due to varying
expression of kisspeptin. It is possible that varying expression of kisspeptin in the Arc
may partially account for variability of fertility levels in humans. In the future, it may be
possible to predict variation in levels of human fertility through the use of a general linear
model. The use of a general linear model provides a useful way to approach human
physiology using an analysis of variation in combinatorial factors. Many human
conditions or diseases may result from a combination of factors, including diabetes,
autism and infertility. Though our study addresses the factors that result in variation of
fertility, the same basic approach could be applied to other diseases or conditions
affecting humans today.
Fig. 1. The hypothalamic pituitary gonadal (HPG) axis is regulated by a series of feedback loops. Kisspeptin stimulates the release of gonadotropin releasing hormone (GnRH). Gonadotropin inhibitory hormone (GnIH) inhibits the release of GnRH. GnRH surges stimulate luteinizing hormone (LH) release by the anterior pituitary. Gonadal steroids stimulate GnIH. Gonadal steroids both stimulate and inhibit kisspeptin (Modified from Kriegsfeld 2006).
Fig. 2. Positive and negative kisspeptin feedback loops in the hypothalamic gonadal pituitary (HPG) axis. Gonadal steroids form an inhibitory feedback loop with kisspeptin neurons in the arcuate nucleus (Arc). Sustained high levels of estrogen form a stimulatory feedback loop with kisspeptin neurons in the anteroventral periventricular nucleus (AVPV). Kisspeptin binds to its receptor, G protein-coupled receptor 54 (GPR54) and stimulates gonadotropin releasing hormone (GnRH) release. GnRH binds to its receptor Gonadotropin releasing hormone receptor 1 (GnRHR-1) in the anterior pituitary and stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) release, which stimulate the gonads to release gonadal steroids. (Modified from Roseweir and Miller 2009).
Fig. 3. Immunoreactive kisspeptin neurons in the arcuate nucleus (Arc) of mice from the non-responsive line (A) and responsive line (B) showing quality of staining. Scale bar is 20 micrometers.

Fig. 4. Frequency distribution of counts of IR-kisspeptin neurons in the arcuate nucleus from grids of approximately 250 μm by 250 μm.
Fig. 5. Mean number of IR-kisspeptin neurons of R-SD (n=13), NR-SD (n=13), R-LD (n=11), NR-LD (n=13). Error bars show SEM.

Fig. 6. Correlation between the relative number of IR-kisspeptin neurons and number of IR-gonadotropin releasing hormone (GnRH) neurons (n=50). IR-GnRH counts data collected by Avigdor et al. (2005).
Fig. 7. Correlation between the relative number of IR-kisspeptin neurons and the number of IR-gonadotropin inhibitory hormone (GnIH) neurons (n=28). IR-GnIH counts from A. Mason, P. Heideman and L. Kriegsfeld (unpublished data).

Fig. 8. Correlation between the number of IR-kisspeptin neurons and body weight (n=50). Body weight data collected by Avigdor et al. (2005).
Fig. 9. Correlation between the number of IR-kisspeptin neurons and testis weight (n=50). Testis weight data collected by Avigdor et al. (2005).

Fig. 10. Correlation between the number of IR-kisspeptin neurons and seminal vesicle weight (n=50). Seminal vesicle weight data collected by Avigdor et al. (2005).
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