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Combinatorial Contributions of Kisspeptin Neurons and GnRH Neurons to Male Infertility

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

by

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Abstract

Fertility varies within a population due to combinatorial contributions of heritable neuroendocrine variations. A better understanding of these variations can lead to mathematical models that could predict which combination of neuroendocrine traits may improve fertility. Our laboratory has identified neuroendocrine traits responsible for fertility variations within our white-footed mouse population: kisspeptin neuronal count and GnRH neuronal count. The kiss neuron and GnRH neuron, both located in the hypothalamus, regulate the HPG-axis. Each of these traits has been found to be variable, but we do not know the combined effect of the two traits on fertility. This study investigates the combined effect of kisspeptin and GnRH neuronal counts using a correlation study. Correlation between the two neurons would suggest that the variation in one trait is causing variation in the other. No correlation would suggest that the two neuroendocrine traits independently impact fertility. Testes mass and seminal vesicles mass were used as an indicator of fertility level to study the effect of immunoreactive (IR) kisspeptin neuron counts and IR-GnRH neuron counts. First, there was no significant correlation between IR-kisspeptin neuron count and IR-GnRH neuron count, indicating that the two variables may have independent affects on fertility. Second, there was a significant interaction of the two variables in affecting fertility. This suggests that the two variables combined have an effect on fertility that neither has alone. Further statistical analysis and increased sample size is necessary. Overall results suggest that both kisspeptin neurons and GnRH neurons are both significant in determining variation in the level of fertility in this population of Peromyscus leucopus.

Introduction

I. The impact of infertility

Infertility is a condition that affects 6.7 million couples in the United States (CDC 2009). 30% of
the affected couples have no singly identified cause for infertility. While treatments such as in vitro fertilization (IVF) and hormone injections exist, they have relatively low success rates. Only 29.4% of patients who have received IVF carry the offspring to full term (CDC 2009). In many instances, infertility patients do not have one identifiable dysfunction that can be targeted with a treatment. Patients may have a wide range of dysfunctions that cannot be simply treated by single specialized treatment such as GnRH injection. The lack of understanding of factors contributing to infertility makes it a challenging condition to address.

Infertility also has a significant impact on the human agricultural economy. Revenues in the beef industry heavily depend on successful reproduction. Infertility is one of the major problems in the beef industry, and is a leading source of economic loss (Lamb et al. 2011). Cows with a problematic reproductive system that fail to become pregnant during the breeding season fail to produce marketable calves, therefore becoming an economic liability to the manufacturers (Lamb et al. 2011). Infertility causes 4.5% of the U.S. cow herd to be culled annually to prevent further damage to the industry’s revenues (Bellows et al. 2002). Despite the impairing effect of infertility on an organism’s fitness, infertility persists in populations. The persistence of infertility in populations might be explained by polygenic interaction. Many detrimental disorders are caused by single genetic mutations, including genetic disorders such as hemophilia. Due to detrimental effects of the disorder, individuals carrying the mutated allele have low fitness. Therefore, hemophiliac phenotypes cannot proliferate in a population. Unlike single gene disorders, infertility often is not attributed to one single genetic mutation. Because of these polygenic interactions, infertility may persist in a population despite being detrimental to fitness.

Many genes and varying alleles contribute to polygenic conditions. As a result, it is possible that two infertile individuals may have completely different sets of alleles that
contribute to infertility. In addition, some of those genes may interact with the environment (G × E) to produce different phenotypes in different environments. Other genes associated with fertility may have variable epigenetic markers that transcriptionally repress or activate genes. The combination of multiple factors may allow infertility to persist in populations. This poses a challenge for understanding the genetic causes of infertility.

II. New approach to studying infertility

Current research efforts focus on individual genes and mechanisms associated with infertility. However, a relatively high percentage of the infertile population does not have a single dysfunction that can be targeted using current knowledge. Our laboratory’s novel approach to addressing fertility may provide valuable insight to how certain heritable reproductive traits combine to affect fertility in a natural population. There are two main goals:

1. Identify heritable and variable traits that lead to fertility variation within a population
2. Understand how these traits combine to affect the level of fertility in an individual. Identifying heritable variation related to fertility is important because heritable variation persists to affect multiple generations.

Many heritable variable traits have been found to affect the level of fertility. To characterize the combinatorial effect of these traits, we must understand the magnitude of interaction between the traits. First, all heritable variable traits may affect fertility separately and independently. Second, a particular heritable variable trait may influence the level of fertility but also cause another heritable variable trait to vary in a similar pattern. By simply observing trait variability in a population, one cannot make a conclusion that variation in any particular trait is caused by another trait or is independently affecting fertility. Therefore, it is important to test for correlations among variable reproductive traits. If two heritable variable traits do not show
correlation, then the two traits may independently influence fertility. However, if two heritable traits are correlated, the relationship suggests that there may be a potential mechanism that induces variation in one trait by the other trait. Independent heritable variable traits should be included in the final fertility measure model, but it would be redundant to incorporate correlated traits into the model.

**Background Information**

I. The hypothalamic-pituitary-gonadal axis

The HPG axis links the brain to the gonads via a neuroendocrine pathway. Various HPG axis endocrine signals regulate gametogenesis, sexual maturation, hormonal surges, and events associated with reproduction. One of the gatekeeper elements of the HPG axis is the population of GnRH neurons, which secretes GnRH to the anterior pituitary. GnRH stimulates the anterior pituitary (AP) to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH) to gonads. Testes and ovaries require a supply of FSH and LH for gametogenesis to produce sperm and ova and for secretion of gonadal steroids, including progesterone (P), testosterone (T), and estrogen (E). These steroids have a negative feedback effect to inhibit the HPG axis by traveling through the vascular system to the hypothalamus to influence the entire HPG axis to reduce its overall activity.

Early hypotheses about the negative feedback mechanism behind the reduced activity of HPG axis did not correlate with the molecular evidence found in GnRH neurons. The initial hypothesis proposed that GnRH neurons decreased the GnRH peptide output due to a direct negative feedback interaction with P, T, and E. However, receptor studies demonstrated that GnRH neurons express only one gonadal steroid receptor, estrogen receptor β (ERβ), which does
not play a role in HPG axis feedback (Roseweir et al. 2009). Therefore, another unknown regulator was presumed to be a mediator between gonadal steroids and GnRH neurons.

II. Kisspeptin neurons

a. Kisspeptin neurons interact with GnRH neurons

The recent discovery of kisspeptin neurons and kisspeptin peptides elucidated a more complete HPG pathway. Kisspeptin is a neuropeptide translated from the gene KISS-1 located on human chromosome 1q32 (Roseweir et al. 2009). Due to post-translational modification, multiple length kisspeptin peptides (10, 13, 14 amino acids) exist as a part of a larger protein family known as RFamides. All cleaved kisspeptin fragments retain a C-terminal decapeptide, which is essential for biological activity, such as their agonist role for kisspeptin receptors (Kiss1r) (Clements et al. 2001, Kotani et al. 2001, Ohtaki et al. 2001). A G protein-coupled receptor, Kiss1r (formerly known as Gpr-54), is a receptor specific to kisspeptin peptides (Lee et al. 1999). The KISS-1 gene and Kiss1r are highly conserved across most mammalian species (Clements et al. 2001; Kotani et al., 2001). The binding of a kisspeptin peptide to a Kiss-1r will elicit phosphorylation of ERK1/2 and p38MAPK, cellular reorganization of stress fibers, and induction of focal adhesion kinase to inhibit cell movement (Kotani et al. 2001).

Kisspeptin neurons are in close apposition with GnRH neurons, which express Kiss1r (Clarkson & Herbison 2006). Kisspeptin input to GnRH neurons is critical for GnRH secretion and normal reproductive functions. Upon administration of kiss peptides in mice, GnRH neurons increase the amplitude and frequency of GnRH secretion. Such a response is not observed in kiss receptor knockout mice (kiss1r/-), demonstrating the necessity of kisspeptin in increasing GnRH pulses (d’Anglemont de Tassigny et al. 2008). Furthermore, higher doses in kisspeptin injections
in mice results in an increased expression of c-fos, a marker signifying neuronal activation, in GnRH neurons. Inactivating Kiss1r with an antagonist attenuates GnRH firing rate, which confirms the interaction of kisspeptin peptide with GnRH neurons via kisspeptin receptors (Roseweir et al. 2009).

Kisspeptin is found in the nervous system (PNS and CNS) as well as in other parts of the HPG-axis, such as the testis, ovary, and anterior pituitary. In the mammalian central nervous system, both Kiss1 mRNA and kisspeptin peptides are highly expressed in the hypothalamus, specifically in the arcuate nucleus (ARC), anteroventral periventricular nucleus (AVPV), and periventricular nucleus (PVN) (Gottsch et al. 2004). Other organs, such as pancreas and small intestine, placenta, and breast tissue express kisspeptin (Richard et al. 2008). The abundance of regions containing kisspeptin suggests there may be more functions of kisspeptin peptides beyond HPG axis regulation. Female fertility seems to be detrimentally associated with increased level of kisspeptin-10. Kisspeptin-10 is overexpressed in patients with trophoblastic neoplasia, but decreases in level after chemotherapy (Dhillo et al. 2006). Moreover, different types of cancer have shown significant difference in kisspeptin expression. In breast cancer patients, kisspeptin and kisspeptin receptor mRNA are overexpressed in breast tissues (citation to be added). However, lung cancer patients have significantly lower expression of kisspeptin and kisspeptin receptor mRNA (citation to be added). The function of kisspeptin signaling in these diseases is still under investigation. While a majority of kisspeptin research focuses on its role in reproduction and puberty, there are other roles of kisspeptin that should be explored in the future.

b. Kisspeptin neurons mediate gonadal steroid feedback in the hypothalamus

Gonadal steroids exert positive and negative regulatory effect on production and release
of GnRH. Until the discovery of kisspeptin, kisspeptin neuron and kiss1r, the gonadal steroid feedback pathway was incompletely understood because while gonadal steroids impacted the level of GnRH, GnRH neurons do not express progesterone receptor, androgen receptor, and estrogen receptor alpha (Roseweir 2009). After the discovery of kisspeptin, however, it became evident that the kisspeptin neuron is the major mediator of gonadal steroid feedback in the hypothalamus. Kisspeptin neurons in different regions of the hypothalamus have contrasting functions in the HPG-axis. ERα -expressing kisspeptin neurons in the AVPV cause positive feedback of estrogen on GnRH neurons at the time of the LH surge and ovulation. In contrast, ERα -expressing kisspeptin neurons in the ARC are part of the negative feedback pathway to reduce GnRH production and release in response to estrogen during other parts of the estrus cycle.

c. Kisspeptin neurons secrete multiple peptides and mediate other environmental inputs

Kisspeptin neurons express additional neuropeptides in addition to kisspeptin peptides. The expression of the neuropeptides neurokinin B (NKB) and dynorphin in kisspeptin neurons are conserved in mammals (Hameed et al. 2011). Both NKB and dynorphin are also associated with regulation of GnRH expression, suggesting there are additional signals other than gonadal steroids that regulate the HPG axis. Dynorphin is associated with progesterone-mediated negative feedback regulation of GnRH release. GnRH neurons express tachykinin neurokinin 3 receptor (NK3R), a receptor for NKB. NKB dramatically increases LH release by directly stimulating GnRH neurons (Hameed et al. 2011).

d. The role of kisspeptin in pubertal development

During puberty, juvenile mammals develop physical and endocrinal characteristics that
enable reproduction. Kisspeptin neurons and kisspeptin play a critical role in inducing puberty in mammals, including monkeys, ewes, humans, and mice (Hameed et al. 2011). Knockout mice (kiss1r/- and kiss1/-) fail to reach puberty, while kisspeptin administration to juvenile rats induced increases uterine weight and raises levels of LH and estradiol (Hameed et al. 2011). The natural development of kisspeptin neurons suggests that puberty is induced by the development of kisspeptin neurons in the correct regions and numbers. From postnatal day 25 (PND 25), kisspeptin neurons become apparent in locations close to GnRH neurons. From PND 25 to onset of puberty (PND 31), the number of detectable kisspeptin neurons continues to rise until reaching the adult level (Clarkson & Herbison 2006). This increase in number and synaptic specificity between kisspeptin neurons and GnRH neurons is thought to increase GnRH release pulse frequency and amplitude (Hameed et al. 2011).

e. The role of kisspeptin in seasonal reproduction

Many mammalian species reproduce in certain seasons but suppress reproduction in other seasons. In the temperate zones, this seasonality in breeding is attributed to changes in photoperiod. However, GnRH neurons are not directly stimulated by photoperiodic cues. Kisspeptin neurons may be upstream mediators that receive seasonal photoperiodic cues and relay the information to the HPG axis. In sheep, kisspeptin neurons seem to play an important role in a non-steroid-dependent circannual rhythm (Clarke & Caraty 2013). In mice in short-day winter-like photoperiods, there is a decrease in kisspeptin function. However, the decrease in kisspeptin and reduction in fertility can be counteracted by administration of exogenous kisspeptin peptides (Clarke & Caraty 2013). This supports the evolutionary adaptation of seasonal breeding to prevent costly reproduction during harsh seasons. Interestingly, kisspeptin neurons do not express the melatonin receptor, a part of the critical photoreception pathway.
(Clarke & Caraty 2013). While kisspeptin mediates interactions between photoperiod and the HPG axis, the mechanism involved to connect photoperiod and kisspeptin activity has not been identified.

f. Role of kisspeptin neurons in mediating nutritional signals

The reproductive ability of an organism is partially dependent on nutrition. Kisspeptin neurons have the ability to receive information about nutrition and regulate the HPG-axis accordingly. Leptin, a hormone produced by adipocytes, relays information about the amount of stored fat to various regions of the body, including the HPG axis. Administration of leptin in immature mice induces early onset of puberty, whereas insufficient leptin results in delayed onset of puberty or infertility (Hameed et al. 2011). This leptin-induced infertility has similar phenotypic consequence to hypogonadotrophic hypogonadism induced by chronically low GnRH. Insufficient leptin causes symptoms including low testis mass and low testosterone levels (Carnegie 2004).

While leptin plays a significant role in reproductive regulation, GnRH neurons do not express the leptin receptor (Ob-Rb) (Hameed et al. 2011). The mammalian hypothalamus is receptive to leptin due to leptin receptor expression in kisspeptin neurons in the arcuate nucleus (Smith et al. 2006). The level of Kiss1r mRNA depends upon availability of food. In a nutrient poor setting, rats decrease hypothalamic expression of kisspeptin mRNA (Castellano et al. 2005; Hameed et al. 2011). In a prolonged nutrient poor setting, the onset of puberty is delayed in mammals, while this delayed puberty can be corrected with administration of kisspeptin peptides (Castellano et al. 2005). An overview of the role of kisspeptin neurons in the HPG axis is described in Figure 1 from (Hameed et al. 2011).
III. Life history background

a. Fitness

Survival and reproduction contribute to the ability of an organism to increase fitness. Individuals with high fitness will contribute more alleles to the gene pool in the next generation, while individuals with low fitness have a low chance of passing of alleles to the next generation. Over multiple generations, alleles passed on by the fit individuals comprise the majority of alleles in the population.

Some of the alleles that determine the fitness of an individual affect resource allocation. Three components of resource allocation are integral to achieving high fitness: those affecting growth, survival, and reproduction. Individuals inheriting alleles that provide effective allocation strategies in relation to their environment will produce offspring with those same alleles. Individuals with alleles that cause allocation strategies poorly matched to their environment will have a low chance of survival and reproduction. Over time, populations become composed of individuals with alleles for allocation strategies that maximize fitness.

b. Life history

Resource allocation strategies that result in high fitness are an important element of life history theory. Life history theory explains how selective pressures shape individuals to optimize survival and reproduction in the face of ecological challenges posed by the environment (Stearns 2000). These life history traits cause an individual to allocate specific proportions of resources to growth, survival, and reproduction. By examining the fitness components of an organism that are life history traits, life history theory allows prediction of fitness under different environmental pressures. Any combination of life-history traits may be referred to as a life-
c. Life history strategies can be optimized, but cannot reach perfection.

Ideally, populations should adapt until all individuals have a life-history strategy that allows optimal growth, survival, and reproduction. Over time, life-history strategy evolves to optimize fitness under specific ecological factors that affect the probability of growth, survival and reproduction. Those factors may include a high number of predatory organisms, spatial and resource limitation, and harsh weather conditions. However, organisms have constraints that may prevent achievement of the fittest life-history strategy. These constraints may be extrinsic ecological or environmental constraints or intrinsic physiological constraints. Given these extrinsic and intrinsic constraints, selection may act to increase fitness, but individuals might still never achieve an ideal life history strategy.

d. Trade-offs in reproduction vs. survival

The process of fitness optimization requires organisms to allocate their limited resources to growth, survival, and reproduction. The differential allocation of resources to growth, survival, and reproduction is often referred to as a trade-off. A trade-off exists when increasing fitness in one life history trait is coupled with a decrease in fitness due to effects on another life history trait, so that the fitness benefit through increasing trait 1 is balanced against a fitness cost through decreasing trait 2 (Fabian & Flatt 2012). An important life-history trade-off is the cost of reproduction, which has two major components of costs: survival cost and future-reproduction cost (Stearns 1989). Individuals with alleles causing delayed reproduction may reduce their immediate fitness, but this decrease in immediate fitness may be balanced by the increased possibility of future reproduction. In the same population, an individual with a different genotype
that includes alleles for early reproduction or year-long reproduction may increase immediate fitness, but this individual may have a fitness cost due to loss of resources for long-term survival. Genotypic variation in a population in alleles that affect the timing of reproduction and other life history traits results in variation in life-history strategy within a population (Fig. 2).

e. Phenotypic plasticity in life history traits

Genetic variation is not the sole determinant of variation in life history strategy. Varying magnitudes of an environmental pressure can influence a single genotype to express a wide range of phenotypes. This phenomenon is called phenotypic plasticity, the ability of a single genotype to produce different phenotypes across different environments (Stearns 1992). In a population, phenotypic plasticity may vary among different genotypes.

A form of phenotypic plasticity relevant to life-history strategy is variable reproductive responsiveness to seasonal changes in photoperiod. In many rodent species, winter-like long dark periods suppress reproduction while summer-like long light periods activate reproduction. If there is variation in alleles for seasonal changes in response to photoperiod, then a wide range of reproductive phenotypes may be found during different seasons of a year (Fig. 3).

f. Seasonality adds more trade-offs

In the temperate zones, environmental pressures associated with winter may include a higher probability of predation, lower food availability, and higher thermal losses. Gamete production, mate searching, embryo support, and offspring care require extra resources in winter. These reproductive requirements increase energetic costs. Winter heightens these challenges by typically decreasing food supply and lowering temperature. Moreover, lower temperature requires extra resources for cellular maintenance, thermoregulation, and locomotor costs.
Therefore, winter introduces a potential fitness trade-off. Increasing foraging behavior in winter is necessary for reproduction, but increases the risk of predation and lowers probability of survival. In contrast, spring or summer may have lower costs and risks associated with reproduction because of lower probability of predation, higher food availability, and minimal thermal losses. In such conditions, successful phenotypes can be those that decrease immediate fitness in winter, delaying reproduction until spring when costs of reproduction are lower.

g. Microhabitats annual differences introduce additional variation in life-history of a population

In the temperate zone, different microhabitats and different years may differ in resources and costs of reproduction. If selection pressures are variable over time and space, then a wide range of phenotypes and genotypes may be favored or disfavored, depending on the season, year, or location because (Heideman & Pittman 2009). In nutrient-rich years, a rodent population encounters abundant food that is easy and safe to obtain, making costs of reproduction low. In a following nutrient-poor year or in a nutrient-poor location, individuals may need to forage much further and at more dangerous sites to obtain food, making costs of reproduction high. For example, in acorn mast years with high food production, an individual under a masting oak tree may find sufficient food within minutes each day, and with a low risk of predation. Other individuals at different sites or other years might require long periods of foraging in more open areas for small and scarce seeds or other food. Such spatial and temporal heterogeneity might result in variable selection on life history strategies, including reproductive timing (Heideman & Pittman 2009). In such conditions, there may be two or more alternative successful winter
phenotypes: (a) reproductive delay in winter versus (b) reproduction throughout the winter, with the potential cost of an increase in foraging time in order to the gain resources to maintain reproduction.

IV. Life History of *Peromyscus leucopus*

a. Genetic variation

Wild *Peromyscus* populations can contain individuals with variable seasonal timing of reproduction (Heideman 2004). Some individuals may have strong reproductive inhibition in short photoperiod while other individuals have little or weak reproductive inhibition. This variation in reproductive timing has been shown to respond to artificial selection, indicating that at least some of the natural variation in seasonal reproduction is genetically based (Bronson & Heideman 1994).

Some *P. leucopus* have combinations of alleles that induce little or no reproductive inhibition in short photoperiod, while others have combinations of alleles that completely inhibit reproduction in short photoperiod. In nature, the reproductively nonresponsive individuals will vary in fitness from year-to-year or among locations due to differences in resource availability. In a resource-rich space or time, such as under oak trees with abundant acorns, these individuals attain higher resources to meet winter costs associated survival and reproduction and can have high fitness. In a resource-poor space or time, these same nonphotoresponsive individuals may not attain resources to meet winter costs associated with both survival and reproduction, and thus have low fitness. Therefore, in resource-poor microhabitats or times, reproductively nonphotoresponsive individuals will be disfavored by natural selection. As a result of this variation in selection, nonphotoresponsive mice will persist in the most favorable winter
microhabitats scattered in a mosaic of favorable and unfavorable winter microhabitats.

In the same population of *P. leucopus*, other individuals have combinations of alleles that induce strong inhibitory reproductive responses to short photoperiod; these individuals are considered photoperiodically responsive. In nature, these responsive individuals also will vary in fitness due to differences in resource availability. In a resource-rich space or time, the responsive individuals would have access to high nutrients to meet winter costs associated with winter reproduction and survival. However, their genotypes prevent their reproductive physiology from benefitting from extra nutrients. In this scenario, photoperiod responsive mice may be disfavored by natural selection by failing to reproduce in winters when reproduction is possible. In a resource-poor space or winter, in contrast, these responsive mice are more likely to maximize fitness because they require resources only for survival, and not for reproduction. As a result of this variation in selection, responsive mice will persist in the least favorable winter microhabitats in a mosaic of favorable and unfavorable microhabitats.

b. Variations in fertility are partially attributed to variations in the HPG-axis

The substantial genetic neuroendocrine variation in reproductive suppression in response to SD photoperiod could be attributed to variations in very many possible neuroendocrine locations. Heideman *et al.* (1991) hypothesized that individual variation in the HPG axis or photoperiod pathway causes variation in reproductive response to photoperiod. Day length information reaches the HPG axis from the eyes through the suprachiasmatic nucleus, superior cervical ganglia, pineal gland, and melatonin (Heidman *et al.*1991). Variation in any of these elements could lead to variation in reproductive inhibition, as could variation in the regulation of energy balance, metabolism, and other systems. However, because some of these elements are
critical for other physiological pathways, some forms of variation may have pleiotropic effects that may be disfavored by natural selection in a natural population (Heideman & Pittman 2009). Therefore, the most plausible variable traits would be elements that have a specific direct impact on the reproductive system through the HPG axis, with little or no effect on nonreproductive traits.

The evidence available suggests that natural genetic variation in seasonal reproductive regulation originates from elements of the photoperiod pathway specific to reproductive regulation (Smale et al. 2005). First, the amount of melatonin binding in the medial preoptic area differs between individuals with strong reproductive inhibition and individuals with weak reproductive inhibition (Heideman et al. 1999). Second, the number of immunoreactive neurons, GnRH neurons in the hypothalamus vary among individuals (Avigdor et al., 2005). Third, serum luteinizing hormone level varies within a population (Heideman 2010). In addition, voluntary food intake and basal metabolic rate of individuals vary in correlation with fertility level (Kaseloo et al. 2012), indicating that certain metabolic traits may have a significant role in variability in reproduction. There are also fertility traits that vary genetically independently of photoperiod, such as testis mass and seminal vesicle mass. Combinations of these traits may determine the level of fertility.

The highest levels of underlying physiological variation leading to variation in fertility level may occur at the top of the HPG-axis pathway. Individual variation in the numbers of GnRH neurons or their level of activity could lead to variation in fertility. If there are fewer GnRH neurons in some individuals, then the sum of stimulatory inputs might no longer be sufficient to support reproduction in short photoperiods (Heideman & Pittman 2009). With more GnRH neurons, however, the hypothalamus might have the potential to release more GnRH even
in the low stimulatory input of short photoperiod. Consistent with this prediction, in responsive *P. leucopus* there were fewer immunoreactive GnRH neurons than in nonresponsive *P. leucopus* (Avigdor *et al.* 2005).

c. Interactions of kisspeptin neurons and GnRH neurons

One can consider effects of phenotype × environment interactions with three hypothetical phenotypically distinct mice: A mouse with a higher number of kisspeptin neurons than GnRH neurons (Fig. 4a); a mouse with equal numbers of kiss neurons and GnRH neurons (Fig. 4b); a mouse with a lower number of kisspeptin than GnRH neurons (Fig. 4c). Although all three mice express both types of neurons, the proportion of each neuronal population may dictate the strength of GnRH secretion and thus the level of fertility. The most fertile phenotype would activate as many Kiss-1r on GnRH neurons as possible (Fig. 4a). Conversely, the mice expressing the lowest number of kiss neurons (Fig. 4c) will be most likely to have low fertility in all times and places. The mice expressing intermediate numbers of kiss neurons will most likely display intermediate fertility (Fig. 4b).

Another possibility is that coordination of the development of kiss and GnRH neurons results in a specific proportion of one type to the other. Hypothetically, kiss neurons might survive during development only by making sufficient connections with enough GnRH neurons. If so, then a mouse with low numbers of GnRH neurons might retain low numbers of kiss neurons. A correlation between the numbers of kisspeptin neurons and GNRH neurons may suggest that natural selection has favored specific proportions of the two neurons.

If a correlation between GnRH neurons and Kiss neurons exists, then a few known mechanisms may be attributed to the correlation of two neurons. Without meeting the neuronal
developmental specifications as GnRH neurons develop, mice fail to reach puberty and become infertile (Schwarting et al. 2007). It is possible that kisspeptin neurons exert a chemical signal to determine the number of GnRH neurons or vice versa. Developmental mechanisms involved in adjusting neuron structure and neurons may be inducing a specific proportion to occur in the GnRH and kisspeptin neurons involved in fertility.

During developmental stages of rodent models, both kisspeptin neurons and GnRH neurons seem to affect the ontogeny of each other. During the embryonic stage of a mouse (E12.5-18.5), kisspeptin neurons appear and increase to reach a number close to half that of the adult kisspeptin population (Desroziers et al. 2012). However, unlike the case for GnRH neurons, the detectable kiss neurons markedly decrease from E22.5 to postnatal day (PND) 0. The early increase is hypothesized to function as the initial guidance factor for fetal mouse GnRH neuron development (Desroziers et al. 2012). The following sudden drop in the detectable kisspeptin neurons may function to lower the kisspeptin secretion to delay reproductive development until a stronger kisspeptin stimulus required for puberty and maturation of the reproductive system.

GnRH neurons continue to be immunoreactive as they migrate and remodel from the embryonic stage until puberty. Many major events of GnRH neuron migration and development coincide with kisspeptin developmental events. Evidence suggests that potential regulatory signals from kisspeptin neurons affects the developmental pattern of GnRH neurons. In mice, approximately 1200 GnRH neurons migrate from olfactory placode to the hypothalamus between E11 and E15 (Schwarting 2007). The neuronal migration is regulated by a series of proteins, some of which are associated with the extra cellular matrix and cytoskeleton, such as NELF, Ark/Axl, and Anosmin-1 (MacColl et al. 2002). These factors initiate the GnRH neuron migration, but cannot inhibit migration once the neurons are in the appropriate hypothalamic
region. The migratory inhibition is prompted by another series of proteins, one of which is the kisspeptin peptide. The kisspeptin peptide activation of Kiss1r in cancer cells have been reported to mediate decreased cell motility (Ohtaki et al. 2001). Moreover, some studies have reported apoptotic activity of kisspeptin and kiss1r by activating proapoptotic genes (Kauffan & Smith 2013). The early embryonic release of kisspeptin may have a similar function in the in regulating the ontogeny of GnRH neurons (Wierman 2011).

In addition to the embryonic migration and potential neural apoptosis, GnRH neurons undergo changes in dendritic architecture near puberty. In the hypothalamus of prepubertal mice, there is a sudden increase in the number of GnRH neurons as well as an increase in the number of kisspeptin neurons. The increase in neuronal population occurs simultaneously with architectural changes in the GnRH neurons. Maturing GnRH neurons display somatic size decrease and simplification of the structure of dendrites (Hemond et al. 2013). As a result, GnRH neurons become more responsive to changes in synaptic input. It is possible that the efficiency of the remodeling mechanism may vary between individuals. In this case, the variation could lead to GnRH pulse variation, ultimately resulting in variation in fertility (Heideman & Pittman 2009). The development of GnRH neurons seems to be heavily influenced by kisspeptin neurons. However, the ontogeny of kisspeptin neurons and mechanisms associated with their development is less well known. Therefore, there are fewer known mechanisms that might determine the number of kisspeptin neurons with respect to GnRH neurons. One known developmental mechanism that occurs in prepubertal mice is kisspeptin apoptosis induced by apoptotic protein Bax. Bax plays a role in inducing apoptosis in kisspeptin neurons to achieve lower number of kisspeptin neurons in males than females (Kauffman & Smith 2013). While male mice undergo higher frequency of kisspeptin neuron apoptosis, both males and females express Bax, which
suggests both sexes undergo kisspeptin neuronal count adjustment during pre-puberty. It is plausible to infer that kisspeptin neurons could use this mechanism to correlate numbers of kisspeptin neurons with GnRH neuronal count.

**Goal of this study**

Previous studies have implicated critical roles of kisspeptin neurons and GnRH neurons in regulating the HPG axis. Separate studies have demonstrated that kisspeptin neuron count and GnRH neuron count vary between the R line and NR line. However, the interaction between kisspeptin neurons and GnRH neurons has not been studied. There are two hypotheses. First, one neuroendocrine trait may be causing variation in the other neuroendocrine variable. This would suggest that one neuroendocrine variable in itself has no effect in contributing to fertility variation in a population. Second, the two neuroendocrine traits may be independently determined, combinatorially contributing to the level of fertility.

The main objective of this thesis is to characterize the potential combinatorial effect of kisspeptin neurons in relation to other variables. First, the immunoreactive (IR-) GnRH neuron count was assessed for heritability and variability with respect to the selection lines. Second, the IR-kisspeptin neuron count was also assessed for its heritability and variability with respect to the selection lines. Third, IR-kisspeptin neuron counts and IR-GnRH neuron counts were assessed for the strength of correlation. Fourth, analysis of covariance tests were used to test the combined effect of the neuroendocrine variables on other reproductive traits such as seminal vesicle mass, testis mass, body weight, and food intake.

A second goal of this thesis is to assess potential correlations among non-neuroendocrine variables, including seminal vesicles mass, testis mass, body weight, and food intake. First, each
trait was assessed for its heritability and variability with respect to selection lines. Second, combinations of traits were assessed for the strength of correlation.

Methods

To test the correlation between kisspeptin neurons and GnRH neurons, I conducted perfusions, cryosectioning, immunohistochemistry, and kisspeptin neuron counting on 39 male mice of age 70 ± 3 days.

I. Animal model

In 1995, Heideman and collaborators captured 48 founder Peromyscus leucopus in Williamsburg (37º16’N) (Heideman et al., 1999). The founding colony members were separated into one control line and two experimental lines. Randomly chosen individuals from the founding colony were used to represent the population variation in photoresponsiveness in a control line (C). The remaining individuals of the founding colony were artificially selected in two groups with different responsiveness to SD photoperiod: Nonresponsive (NR) and Responsive (R). The NR line represents individuals from the wild population that display weak reproductive inhibition under SD photoperiod. The R line represents individuals from the wild population that display strong reproductive inhibition under SD photoperiod.

The colony was artificially selected for either strong reproductive inhibition or weak reproductive inhibition in SD photoperiods, as indicated by gonad size (Heideman et al., 1999). Male mice with testis index less than 24mm² (length x width) were selected to found the R selection line and those with testis index 32mm² or greater were selected to be NR. Female mice with ovarian length greater than 3.5mm, uterine diameter greater than 1.0mm, and a visible follicles or corpora lutea were selected to be NR. Female mice with ovarian length of less than
2 mm, a uterine diameter of less than 0.5 mm, and no visible follicles or corpora lutea were selected to be R. These initial founders served as parental stock for the establishment of generations of the two lines. The unselected C line was maintained as an outbred line. Each line had at least 20 and generally more than 40 breeding pairs per generation to minimize genetic drift and the loss of natural variation.

For the purpose of this study, a total of 39 male individuals were used (NR=10 R=8 C=21). The target for the final sample size for the entire project is at least 100 individuals, including both males and females.

II. Perfusion

Before perfusion, mice were retro-orbitally bled to collect serum for analysis of luteinizing hormone serum for a separate study. After blood collection, mice were anesthetized in a glass chamber with ½ ml of isoflurane (producing up to 30% isoflurane anesthetic in the chamber) until respiratory arrest. The mice were then transcardiacally perfused through the left ventricle with 0.1 M phosphate buffered saline (PBS) for 2-3 minutes, then with 4% paraformaldehyde and 10% picric acid in 0.1M PBS (Zamboni’s fixative) for 7-8 minutes at fluid velocity of 6 ml/ml, then with the same Zamboni’s fixative for 12-13 minutes at 3 ml/min. Brains were dissected out of the cranium and then post-fixed for 24 hours in a new Zamboni’s fixative with agitation at 4°C. Brains went through a second 24 hour post-fixation without agitation in 4°C in a new Zamboni’s fixative. After the entire post-fixation procedure, brains were placed in 10% sucrose in 0.1M PBS for 24 hours and then 30% sucrose in 0.1M PBS for 24 hours. The sucrose solution protected brain tissue from damage due to ice crystal formation during the cryostat protocol. The incubation in graduated sucrose concentrations allowed gradual
brain density change observable as a transition from floating in solution to complete submersion of brain. Many of the perfusions and tissue preparations were conducted by L. Kroese and M.L. Brazer.

III. Cryosectioning

Brains were coronally sectioned using a Thermo Sliding Microtome with a freezing stage. Brains were rapidly frozen for 7-8 minutes at -27°C until visibly frozen, and then the temperature was lowered to -18°C for sectioning. Brains were sectioned from the anterior to posterior until all known nuclei containing kisspeptin neurons and GnRH neurons were collected. Sectioned slices were then stored in glass vials filled with antifreeze solution (37.5% sucrose, 37.5% ethylene glycol, and 10g PVP-40 in 500ml 0.02M tris-buffered saline) then stored at -20°C until immunohistochemistry on kisspeptin neurons.

IV. Immunohistochemistry

Kisspeptin neuron immunohistochemistry was made possible by a protocol adapted from Dr. Theresa Horton of Northwestern University.

On day 1, brain sections immersed in antifreeze were washed clean of antifreeze using 0.02M tris-buffered saline (TBS) three times on a shaker at room temperature, lasting 6 minutes in duration for each wash. The sections were incubated in 3% hydrogen peroxide (CVS pharmacy) for 10 minutes on a shaker at room temperature (RT) to eliminate any peroxidases that could contribute to nonspecific staining. Wells containing H₂O₂ and the sections were wrapped in foil to block any light sources. The sections were washed again with 0.02M TBS 3 times for 10 minutes each to wash off H₂O₂. Then the sections were incubated for 90 minutes in a blocking solution of 0.02M TBS with normal goat serum (NGS) and λ-carrageenan (2% NGS, 30% λ-
carrageenan) to avoid non-specific background staining. Primary antibody carrier solution was made using TBS with the detergent triton-X-100 (TBST) using 5%TBS, 45% distilled water, 50% λ-carrageenan, 2% NGS, 250% protease-free BSA (Sigma, A3059-10G), and 0.3% triton X-100. The solution was put on a shaker at RT until all reagents were dissolved, especially Triton X-100 and bovine serum albumin (BSA). After the sections had finished incubating in the blocking solution, they were placed in tissue vials filled with 1ml of the primary antibody carrier solution. 1:10,000 Polyclonal rabbit anti-kisspeptin antibody (Kiss 566; Dr. A. Caraty, Physiologie de la Reproduction et des Comportements, Nouzilly, France) was added to the tissue vial. The sections were incubated in TBST and Kiss566 antibody for 36-48 hours on a rotating incubator at 4°C.

On day two, the sections were washed clean of the primary antibody and blocking solution using 0.02M TBS six times, each wash lasting 8 minutes. Then the sections were incubated in biotinylated goat anti-rabbit secondary antibody at 1:500 and secondary antibody carrier solution (0.1% TBS, 0.9% distilled water, 250% protease-free BSA, 0.3% Triton X-100) for 90 minutes on a room temperature shaker. After the secondary antibody incubation, the sections were washed in 0.02M TBS 6 times, each wash lasting 8 minutes. In the meanwhile, an avidin-biotin-complex (ABC) solution was made and incubated on a RT shaker for 30 minutes at 1:400, diluted in 0.01M TBS. The sections were then immediately incubated in the pre-incubated with Vectastain Standard Elite ABC kit (1:500) (Vector labs, PK-6100) for 90 minutes on a shaker at RT. The sections then were washed with 0.02M TBS six times, each wash lasting 8 minutes. The sections were chromogenically stained using nickel-enhanced Diamobenzoiil (DAB) (Vector labs, SK-4100) (5 ml H2O, 2 drops of buffer solution, 4 drops of diaminobenzidine (DAB) reagent, 2 drops of H2O2, 2 drops of nickel solution). After the reagents were mixed, the
sections were immersed in the solution and incubated on a RT shaker for 5 minutes. The sections were washed in 0.02M TBS three times, each wash lasting 6 minutes.

The stained sections were mounted using 3% gelatin solution in distilled water. The dry mounted slides were dehydrated in xylene for 10 minutes. The slides were then immediately cover-slipped using Permount (Fisher Chemicals, SP15-100).

V. GnRH IHC

GnRH neurons also were detected by using the ABC-DAB method. GnRH immunostaining was conducted by Brazer and Kroese. (For the complete GnRH IHC protocol, refer to Avigdor et al. 2005)

VI. Neuron Assessment

The goal of the project was to obtain relative estimates of immunoreactive (IR) kiss neurons and IR-GnRH neurons. We chose five sections from five specific locations in the brain for counts of kisspeptin neurons. Counts of kisspeptin neurons from these areas have been sufficient to detect variation in kisspeptin neurons (Swanson, 2012).

The location and number of kiss neurons were assessed by eye with assistance from the imaging software, Image J (NIH). Images of the IR-kisspeptin neurons were taken with a Canon EOS 5D Mark II camera attached to an Olympus BH-2 light microscope and images were uploaded to Image J. The software allowed adjustment of image contrast for a better visualization of stained cell bodies. It also allowed measurement of cell body diameter. We set an image detection threshold to be 200 nm. We measured the diameter of structures assessed as cell bodies and recorded counts only those that met the image detection criterion. Five identical
sections of the brain were sampled across the 39 mice to represent relative kisspeptin neuronal count for each mouse. At a later date, a repeat measurement of all of the counted sections was conducted to test for within-observer variation in the counting technique. Neurons were counted blind with respect to selection line to ensure the absence of bias. The same assessment technique was used to quantify GnRH neurons (n=36), but without the digital assistance of Image J. Due to some poor perfusions and consequent brain tissue damage, three samples were eliminated from the final dataset for GnRH neurons. Repeat measurement of 20 samples for counts of IR-GnRH neurons were conducted by L. Kroese to test for inter-observer variation in the neuron counts.

VII. Statistical Analysis

Data were analyzed using paired t-tests, ANOVA, correlation analysis, and ANCOVA using a Microsoft Data Analysis Add-on, MATLAB, and Minitab.

Results

From each mouse, data on several reproductive traits were collected. The collected data were: Kisspeptin neuron count, GnRH neuron count, seminal vesicles mass, testes mass, body mass, and food intake. First, each trait’s heritability and variability was assessed. Then, the correlation between kisspeptin neuron count and GnRH neuron count was assessed. The combinatorial effect of the two neuroendocrine variables on fertility was assessed using ANCOVA. The correlation between other non-neuroendocrine variables was assessed. These traits were also assessed for combinatorial effects on the level of fertility using ANCOVA.

I. GnRH and Kisspeptin neuron count validation

Counts of IR-GnRH and IR-kisspeptin neurons were assessed for accuracy and precision.
Some sections were counted for IR-GnRH neurons twice by separate individuals, both blind to selection line and previous counts. Individual 1 (FH) counted neurons in the entire sample (n=38) while individual 2 (PL) counted 20 samples. The counts resulted in an average of 5.56% difference between two counters. Prior to counting, we had set a standard of 10% difference between each independent count as a threshold of reliability. A paired t-test was used to determine that the mean difference between the two independent counts was significantly less than 10% ($H_0 = \mu_d < 10\%$; $\alpha = 0.05$; $t = 1.73$; $P = 3.34E-09$) (Fig. 13, 14).

Kisspeptin neuron analysis was difficult due to high fiber density which obstructed countable somas. Although additional protocols were implemented for stringent counting criteria, the kisspeptin neuron analysis did not reach the precision of GnRH neuron analysis. Kisspeptin neuron analysis employed a single independent counter blind to prior counts and counted all designated sections on two separate occasions. The independent counts resulted in an average of 18.63% difference between counts. Kisspeptin neuron analysis failed to meet the 10% difference standard. A paired t-test determined that the mean difference between the two independent counts was marginally insignificant ($P = 0.09$; $t = 1.69$; $\alpha = 0.05$). However, repeated measurements with an average percent difference of less than 20% were deemed to be useable for this preliminary analysis. Results from the statistical analyses of the double-counting suggest that GnRH neuron count was precise and accurate. However, kisspeptin neuron count was precise but not accurate. Results of the correlation study would not be affected by some inaccuracy, as long as the relative precision is maintained.

II. Effects of line, photoperiod, and interaction (photoperiod/line) on kisspeptin neuron counts and GnRH neuron counts
If a trait is contributing to variations in fertility level, then the trait must vary within a population and be inherited from a parent to the offspring. Kisspeptin heritability and variability was assessed by comparing the neuron counts between lines (NR vs. C vs. R). It is possible that the number of IR-kisspeptin neurons may be different in different photoperiods, and IR-kisspeptin neuron count in relation to photoperiod may be variable among lines. Therefore, the effect of photoperiod (LD vs. SD) and the effect of photoperiod and line interaction (NR-LD vs. NR-SD; C-LD; C-SD; R-LD; R-NR) were also assessed. ANOVA was used to examine the effects of line, photoperiod, or the interaction. The number of IR-kisspeptin neurons in the Arc nucleus was significantly affected by line (P=0.006; F=5.86) and the interaction (P=0.039; F=3.66), but not by photoperiod (P=0.789; F=.073) (Table 2; Fig. 5).

If GnRH neuron count contributes to genetic variation in fertility, then the GnRH neuron count should also display variability and heritability as well. The effects of line, photoperiod, and interaction on the number of IR-GnRH neurons counted were assessed using ANOVA. Contrary to past studies (Avigdor et al. 2005; Heideman et al. 2007), IR-GnRH neuron count showed no significant difference between lines (P=0.982; F=0.019) in this sample. The effect of photoperiod (P=0.420; F=0.667) and interaction (P=0.364; F=1.156) were also not significant (Table 2; Fig. 6).

The effects of line, photoperiod, and interaction on the testis mass were assessed. There was a marginally nonsignificant effect of line on testes mass (F=2.711; P=0.080). Photoperiod had a significant effect on testis mass (F=11.674; P=0.002), and the interaction between line and photoperiod also had a significant effect on testis mass (F=5.088; P=0.001). Seminal vesicles mass was also assessed for the effect of line, photoperiod, and interaction. Line, photoperiod, and their interaction had highly significant effects on seminal vesicles (Line: F=1.803; P=0.180 |
Photoperiod: F=23.684; P=2.257E-05 | Interaction: F=3.706; P=0.09) (Table 2; Fig. 7, 8).

The effects of line, photoperiod, and interaction on food intake were assessed. Line had a marginally nonsignificant effect on food intake (F=2.467; P=0.10). Neither photoperiod nor the interaction had a significant effect on food intake (Photoperiod: F=0.0221; P=0.963 | Interaction: F=0.972; P=0.449) (Table 2; Fig. 9).

The effect of line, photoperiod, and interaction on body mass was assessed. Line had a significant effect on body mass (F=18.831; P=2.530E-06). Photoperiod did not have a significant effect on body mass (photoperiod: F=0.0352; P=0.852), but the effect of the interaction on body mass was significant (F=7.328; P=0.000) (Table 2; Fig. 10).

Comparisons of the reproductive variables among lines demonstrate that most traits are heritable and variable, with the exception of a few traits that were marginally nonsignificant. However, the IR-GnRH count among the three lines was non-significant, in contrast with past published results. The contrasting result from the current study may be ascribed to either the low sample size or the number of brain sections counted from each brain; in addition, the inclusion of the control line, which is intermediate, reduces the overall variance in the sample.

III. Correlations among IR-kisspeptin neurons, IR-GnRH neurons, and other reproductive traits

Past studies demonstrated that IR-kisspeptin neuron count and IR-GnRH neuron count both heritable and variable traits. Correlation analysis of IR-kisspeptin neuron count and IR-GnRH neuron count indicated no significant correlation (r=0.402; P=0.144) (Table 3; Fig. 11e). The results suggest that the two neuroendocrine traits are independently inherited.
IV. Analysis of Covariance of kisspeptin and GnRH neuron counts

Analysis of covariance (ANCOVA) was used to analyze the combined effects of the two neuroendocrine variables on the level of fertility. An appropriate reproductive variable that reflects the level of fertility is testis mass and seminal vesicles mass. Studies have shown high testes mass positively correlates with sperm count, and seminal vesicles mass is positively correlated with the level of testosterone. Analyzing the effect of kisspeptin neuron count and GnRH neuron count on testes mass and seminal vesicles mass should indicate which neuron might be associated with different aspects reproductive functions.

The effect of kisspeptin neuron count and GnRH neuron count on testes mass was tested using ANCOVA. Kisspeptin neuron count alone did not have an effect on testes mass. GnRH neuron count alone also had no effect on testes mass. However, the combination of kisspeptin neuron count and GnRH neuron count had a significant effect on testes mass (Table 5a).

The effect of kisspeptin neuron count and GnRH neuron count on seminal vesicles mass was tested using ANCOVA. As with testes mass, neither kisspeptin neuron count or GnRH neuron count independently had an effect on seminal vesicles mass. However, the combination of kisspeptin neuron count and GnRH neuron count had a significant effect on seminal vesicles mass (Table 5b). Together, these statistical analyses suggest that kisspeptin neuron count and GnRH neuron count interact together to have an effect on the level of fertility.

V. Magnitude of correlations between other reproductive variables

The second objective aimed to assess potential correlations among other reproductive traits. The magnitude of correlation may demonstrate whether one variable is responsible for determining the level of fertility or the two traits combinatorially contribute to the level of
fertility. Correlations between kisspeptin neuron count and seminal vesicle mass, testis mass, body weight, food intake, and GnRH neuron count were assessed. None of the variables had a significant correlation with kisspeptin neuron count (Table 3; Fig. 11 a-e).

Correlations between GnRH neuron count and seminal vesicle mass, testis mass, body weight, food intake were assessed. There was no significant correlation between GnRH neuron count and any of the four variables (TM: R=0.025, P=0.883; SVM: R=-0.157, P=0.369; BM=-0.152, P=0.375; FI: R=-0.127, P=0.461) (Table 3; Fig. 12 a-d). The overall correlation results suggest that kisspeptin neuron count and GnRH neuron count are not directly associated with the variation in other reproductive variables.

Correlations among seminal vesicle mass, testis mass, body weight, and food intake were assessed. The magnitude of correlation may demonstrate whether one variable is responsible for determining the level of fertility or the two traits combinatorially contribute to the level of fertility. As expected, testes mass and seminal vesicles mass displayed a very strong correlation (R=0.867; P<0.001).

The remaining variables showed no correlations among each other. Correlation between seminal vesicle mass and food intake, seminal vesicle mass and body mass, body mass and testis mass, body mass and food intake, and food intake and testis mass were all non-significant (Table 4; Fig. 13b-d). The results suggest that most reproductive variables may be independently inherited and that there are separate mechanisms that cause variations in these traits. However, the strong correlation between testes mass and seminal vesicles suggest that there may be a mechanism that induces high seminal vesicles if an organism has inherited high testes mass or vice versa (Fig. 13a).
VI. Analysis of covariance on reproductive neuroendocrine traits

I tested for more complex covariation among neuron counts and reproductive traits in relation to line and photoperiod using ANCOVA models. This is important because the effect of line or photoperiod on a variable of interest may be masked by another variable. ANCOVA can account statistically for the effect of a particular variable (the covariate), while simultaneously isolating the effect of treatment groups on the variable of our interest. Results of ANCOVA can be used to analyze specific reproductive variables that may have an impact on variable of interest. Reproductive traits showing even a slight correlation with another trait were assessed using ANCOVA (Table 6a,b).

The effect of line on IR-GnRH neuron count (covariate= kisspeptin neuron count) was assessed. The effect of line on IR-GnRH neuron count remained nonsignificant (F=0.970; P=0.389). In contrast, the effect of line on IR-kisspeptin neuron count (covariate = IR-GnRH neuron count) became more highly significant when the effect of IR-GnRH neuron count was included in the model ($P_{ANCOVA}=0.004; P_{ANOVA}=0.006$) (Table 6a).

The effect of line on seminal vesicle mass (covariate=testis mass) was assessed; seminal vesicle mass was not affected by line when the effect of testis mass was included in the model ($P_{ANCOVA}=0.962; P_{ANOVA}=0.180$).

The effect of line on testis mass was assessed using three different covariates (covariate$_1$= seminal vesicle mass; covariate$_2$= body mass; covariate$_3$= food intake). The effect of line on testis mass was not significant when the effect of seminal vesicle was included in the model ($P_{ANCOVA}=0.498; P_{ANOVA}=0.080$). However, after controlling the testis mass variation due to body mass, the testis mass difference among the three lines remained significant (F=4.910;
When controlling for variation in testis mass due to food intake, the effect of line on testis mass remained significant (F=4.91; P=0.033). (Table 6a).

The effect of photoperiod on seminal vesicles mass was assessed (covariate=testis mass). Seminal vesicles mass continued to be significantly affected by photoperiod (P_ANCOVA=0.004; P_ANOVA=0.001) (Table 6b). The effect of photoperiod on testis mass was assessed (covariate=seminal vesicle mass). The effect of photoperiod on testis mass was not significant when the effect of seminal vesicle mass was included (P_ANCOVA=0.363; P_ANOVA=0.002) (Table 6b).

The effect of photoperiod on food intake remained nonsignificant even after controlling the food intake variation in relation to kisspeptin and GnRH neuron count (covariate1= kisspeptin neuron count; covariate2=GnRH neuron count) (F_kiss=0.00, P_kiss=0.987; F_GnRH=0.02, P_GnRH=0.884) (Table 6b).

The effect of photoperiod on testis mass was assessed (covariate1= kisspeptin neuron count; covariate2=GnRH neuron count). The effect of photoperiod on testis mass remained significant after controlling the effect of kisspeptin neurons (F=11.29; P=0.002). The effect of photoperiod on testis mass remained significant after controlling the effect of GnRH neurons (F=22.64; P=0.00) (Table 6b).

Overall, these analyses confirm that line and photoperiod affected testes mass and seminal vesicles mass. In addition, even when covariates were taken into account, the neuron counts showed similar relationships to line and reproductive organ mass.
Discussion

This thesis had two objectives: 1. Characterize the relationship of kisspeptin neuron count and GnRH neuron count in contributing to the level of fertility 2. Identify other correlations among other reproductive traits that may have interactions among themselves.

Before discussing the results further, it is important to emphasize that staining technique and counting technique must be validated. The kisspeptin neuron immunohistochemistry technique resulted in stained sections that were difficult to quantify. The subjectivity problem was at least partially addressed using stringent criteria for consistency in counting. The kisspeptin criteria seemed effective in counting relative numbers of kisspeptin neurons. When compared with the first counting event, the second counts on average identified about 10 additional neurons. Thus, while the independent counting events were not accurate, they were precise. For this preliminary correlation study between kisspeptin neurons and GnRH neurons, relative numbers should produce useful results.

Comparison of IR-kisspeptin neurons among lines suggests that kisspeptin neuron count is heritable and variable within this population. This is consistent with unpublished data from another study (Swanson 2013) that also used individuals derived from the same population of *Peromyscus leucopus* and the same immunohistochemistry technique. These results suggest that photoperiod does not affect the IR-kisspeptin neuron count. While photoperiod may be affecting kisspeptin neuron function, the changes were not apparent in this IHC study.

Interestingly, the average IR-GnRH neuron count was not significantly different among the lines. Past studies, most with larger sample sizes from the R and NR lines in the same animal population and with the same IHC technique have demonstrated differences between the R and
NR lines and heritable variation within the control line (Avigdor et al. 2005; Heideman et al. 2009; Heideman & Swanson n.p.). The current sample size is lower than in these earlier studies, and may be inadequate for a powerful statistical test. Attaining the full sample size of 100 individuals or counting more sections from each sample may show differences among lines.

Both a previous unpublished result and results from the current study have demonstrated heritability and variability of kisspeptin neuron count and GnRH neuron count. The absence of a correlation between kisspeptin neuron count and GnRH neuron count suggests that the two neuroendocrine variables are independently inherited traits. Contrary to the correlated trait hypothesis, the numbers of one neuron type is not solely responsible for determining the counts of the other neuron. Moreover, the lack of correlation suggests there is no specific proportion of kisspeptin neurons to GnRH neurons in the P. leucopus population. As the ANCOVA analyses demonstrate, both neurons combinatorially contribute to the level of fertility in an organism. This was true both for testes mass, which is related primarily to gametogenesis, and for seminal vesicles mass, which related primarily to levels of testosterone. This suggests that reduced fertility in an individual that inherited low GnRH neuron count may be rescued if it inherited a higher number of kisspeptin neuron numbers. Further investigation of these interactions is important. Testes mass is positively correlated with testosterone level and seminal vesicles mass is positively correlated with increase spermatogenesis. Testosterone is necessary for activation of genes in Sertoli cells, which promotes spermatogenesis. An organism that inherits high testis mass may determine the mass of seminal vesicles mass. Therefore, these traits are not independent in relation to fertility.

The second objective aimed to examine potential correlations among other reproductive and nonreproductive variables. Testes mass, seminal vesicles mass, food intake, and body mass
were tested for heritability and variability. Among these other variables, only testes mass and seminal vesicles mass showed a strong and significant correlation. Most variables were marginally non-significant or significantly affected by line. Line had no effect on seminal vesicles mass. This is contradictory to multiple past studies, which have demonstrated that there are significant differences in seminal vesicles mass between NR and R. Again, completing the full sample collection may alter this result.

In the current study, the number of IR-kisspeptin neurons and food intake was not correlated. A previous pilot study demonstrated a significant correlation between the two variables (r=0.739, P=0.023, n=10 ; Heideman & Ives n.p.). A connection is possible because of evidence of the leptin receptor in kisspeptin neurons. Kisspeptin release is increased when more adipose tissue is stored inside the body, and leptin can have this effect. This pathway signals the animal about the availability of resources and the appropriateness of breeding condition. Despite the pilot results above, the current study did not show a correlation between the two variables. Completing the full sample collection or increasing the number of brain sections counted may alter this result.

Conclusion

The overall results suggest that kisspeptin neuron count and GnRH neuron count are independently inherited, contrary to the hypothesis that kisspeptin neurons may be exerting developmental influence on GnRH neuron and numbers during the embryonic and pubertal stage in rodents. Moreover, the two neuroendocrine variables may interact to affect the level of fertility in this population. Fertility level, as indicated by testes mass and seminal vesicles mass, was affected by the combination of kisspeptin neuron count and GnRH neuron count.
**Future directions**

The project needs to be completed by increasing the sample size to the target of 100 individuals. The additional statistical power may clarify some of the relationships that were ambiguous in this study.

Another contributor for weak statistical power could be the relative subjectivity of kisspeptin neuron analysis. Improvement in a few aspects of kisspeptin neuron protocols would allow higher accuracy in data on kisspeptin neurons. The subjectivity of the neuron analysis was attributed to the extremely high fiber density that obstructed the view of kisspeptin cell bodies. One solution would be to investigate an alternative marker specific to kisspeptin neurons that could also serve to identify these cells. Another solution may be to perform in-situ hybridization of kiss1 mRNA. These methods would reduce the high fiber density in tissue samples and increase accuracy. If the correlation between the number of IR-GnRH and IR-kisspeptin neurons remains when more samples are obtained, it would be interesting to compare GnRH and Kisspeptin neuron development between the R and NR lines.

Previous study has suggested that neuron number variation and neuron architecture variation is not mutually exclusive (Avigdor et al. 2005). These qualities are determined during developmental stages of mice. It is possible to that there may be underlying developmental mechanism variation in GnRH neurons and kisspeptin neurons that may be contributing to the variation in fertility. For example, variation in kisspeptin neuron developmental genes may lead to weak or strong synaptic activity, causing variations in GnRH neuron firing rate. It would be interesting to investigate the potential genetic variation in developmental genes between the R and NR lines in the laboratory population of *Peromyscus leucopus*. Finally, studies have shown
there is genetic variation in neuronal firing rate, size, and synaptic activity even within the same cell line (Hemond et al. 2013). It would be interesting to establish protocols that might provide results that reflect the kisspeptin neuron firing rate, size, and secretion activity, and other variables that could impact differential stimulation of GnRH neurons.

Acknowledgements

The research students in the laboratory have been essential in aiding with my data collection. I would like to especially thank Leah Brazer and Lani Kroese for undertaking time consuming protocols including perfusions, food collections, and GnRH IHC. I would like to thank Lani Kroese for assisting as counter number two for IR-GnRH neuron double counting. I would like to thank Phoebe for assisting me with kisspeptin neuron IHC. I would like to thank the animal facilities staff and Donna Bucci for maintaining the population of Peromyscus leucopus for the study. I would like to thank Melissa Proffitt for maintaining the lab space and aiding with ANCOVA.

I would like to thank Dr. A. Caraty at the Physiologie de la Reproduction et des Comportements in Nouzily, France for providing kisspeptin primary antibody. I would like to thank the members of my committee for constructive suggestions to improve the project. Finally, I would like to thank Dr. Paul Heideman for his excellent guidance through the span of the project. His mentorship has been an excellent guidance throughout my entire college career.

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Figure 1. An overview of the Hypothalamus-Pituitary- Gonadal Axis (Hamieed et al. 2011) Abbreviations are as in the text.
Figure 3. Genotypic difference in life-history strategy. Some individuals may be genetically constrained to allocate more energy to survival and less on reproduction. Others may be genetically constrained to devote more resources towards reproduction and less on survival.

Figure 2. Hypothetical reproductive phenotypic plasticity as a function of photoperiod. In this hypothetical data set, one genotype has a different reaction norm than another genotype, illustrated by the non-parallel slopes of the line. In this case, genotypes that are nonresponsive to photoperiod are less affected by changes in photoperiod than the responsive genotype.
Figure 4. Three hypothetical models with varying proportion of GnRH neuron and kisspeptin neuron expression. (A) has the highest number of kisspeptin neurons thereby having the most stimulatory inputs into GnRH neurons. (B) has an equal number of kisspeptin neurons and GnRH neurons, thereby having an intermediate input into GnRH neurons. (C) has the least number of kisspeptin neurons, thereby having the least input into GnRH neurons. The evolutionarily preferred proportion may not be (B); there may be a different proportion of GnRH neuron and Kisspeptin neurons that produce intermediate fertility. (Image modified from P. Heideman)
Table 1. Kisspeptin neuron and GnRH neuron counts affected by date or counter

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<th>Kisspeptin neuron count</th>
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<th>GnRH neuron count</th>
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<tr>
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<td>Date 1 vs. Date 2</td>
<td>Counter 1 vs. Counter 2</td>
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<tr>
<td><strong>P</strong></td>
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<td>6.68E-09**</td>
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H₀: The percent difference between two counts is greater than 10%. Data are organized by t and P values from paired t-test. *signifies marginally insignificant P-values and ** signifies P-values <0.05.

Table 2. Effect of line, photoperiod, and interaction on Kisspeptin neurons and GnRH neurons

<table>
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<td>GnRH neurons</td>
<td>1.101</td>
<td>0.345</td>
<td>0.667</td>
</tr>
<tr>
<td>Testis mass (g)</td>
<td>2.711</td>
<td>0.080*</td>
<td>11.674</td>
</tr>
<tr>
<td>Seminal vesicle mass (g)</td>
<td>1.803</td>
<td>0.180</td>
<td>23.684</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>2.467</td>
<td>0.099*</td>
<td>0.002</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>18.831</td>
<td>&lt;0.001**</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Data are organized by the F and P values from ANOVA using “Line” (C vs. NR vs. R), “Photoperiod” (LD vs. SD), and “Interaction” (NR-LD vs. NR-SD vs. R-LD vs. R-SD).

Table 3. Correlations for kiss neurons and GnRH neurons

<table>
<thead>
<tr>
<th></th>
<th>Kisspeptin neuron</th>
<th>GnRH neuron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>r</strong></td>
<td><strong>P</strong></td>
</tr>
<tr>
<td>Seminal vesicle mass (g)</td>
<td>-0.053</td>
<td>0.752</td>
</tr>
<tr>
<td>Testis mass (g)</td>
<td>-0.048</td>
<td>0.774</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>-0.014</td>
<td>0.409</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>-0.113</td>
<td>0.494</td>
</tr>
<tr>
<td>GnRH neuron</td>
<td>0.144</td>
<td>0.402</td>
</tr>
</tbody>
</table>

Data are organized by correlation coefficient (r) and P-value. Correlation analysis was conducted between kisspeptin neuron count and reproductive variables and between GnRH neuron count and reproductive variables. The x variables were kisspeptin neuron and GnRH neuron. The y variables were seminal vesicle mass, testis mass, body mass, food intake, and GnRH neuron. No correlation was significant.
Table 4. Correlations for other reproductive traits

<table>
<thead>
<tr>
<th></th>
<th>Testis mass</th>
<th>Food intake</th>
<th>Body mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
<td>r</td>
</tr>
<tr>
<td>Seminal vesicle mass (g)</td>
<td>0.867</td>
<td>&lt;0.001**</td>
<td>0.246</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>0.243</td>
<td>0.136</td>
<td>0.174</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>0.149</td>
<td>0.366</td>
<td></td>
</tr>
</tbody>
</table>

Data are organized by correlation coefficient and P-value. Correlation analysis was conducted among testis mass, food intake, and body mass. ** denotes P-value < 0.05.

Table 5. Analysis of Covariance on testes mass and seminal vesicles mass examining the effects of kisspeptin neuron count, GnRH neuron count, and kisspeptin and GnRH neuron count on a. testes mass and b. seminal vesicles mass

A. Response: testes mass

<table>
<thead>
<tr>
<th></th>
<th>t value</th>
<th>Pr (&gt;t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kisspeptin</td>
<td>-0.42</td>
<td>0.67</td>
</tr>
<tr>
<td>GnRH</td>
<td>-0.09</td>
<td>0.93</td>
</tr>
<tr>
<td>Kisspeptin: GnRH</td>
<td>2.75</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

B. Response: seminal vesicles mass

<table>
<thead>
<tr>
<th></th>
<th>t value</th>
<th>Pr (&gt;t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kisspeptin</td>
<td>-0.38</td>
<td>0.71</td>
</tr>
<tr>
<td>GnRH</td>
<td>-0.84</td>
<td>0.41</td>
</tr>
<tr>
<td>Kisspeptin: GnRH</td>
<td>2.58</td>
<td>0.01**</td>
</tr>
</tbody>
</table>

ANCOVA examines the effects of kisspeptin neuron count, GnRH neuron count, and kisspeptin and GnRH neuron count on A) testes mass and B) seminal vesicles mass.
<table>
<thead>
<tr>
<th>Response variable</th>
<th>Covariate</th>
<th>Factor</th>
<th>F</th>
<th>( P_{\text{ANCOVA}} )</th>
<th>( P_{\text{ANOVA}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seminal vesicle mass</td>
<td>Testis mass</td>
<td>Photoperiod (LD vs. SD)</td>
<td>9.530</td>
<td>0.004**</td>
<td>0.001**</td>
</tr>
<tr>
<td>Testis mass</td>
<td>Seminal vesicle mass</td>
<td></td>
<td>0.850</td>
<td>0.363</td>
<td>0.002**</td>
</tr>
<tr>
<td>Testis mass</td>
<td>Kisspeptin</td>
<td></td>
<td>11.290</td>
<td>0.002**</td>
<td>0.002**</td>
</tr>
<tr>
<td>Food intake</td>
<td>Kisspeptin</td>
<td></td>
<td>0.000</td>
<td>0.987</td>
<td>0.963</td>
</tr>
<tr>
<td>Testis mass</td>
<td>GnRH</td>
<td></td>
<td>22.640</td>
<td>0.000**</td>
<td>0.002**</td>
</tr>
<tr>
<td>Food intake</td>
<td>GnRH</td>
<td></td>
<td>0.020</td>
<td>0.884</td>
<td>0.963</td>
</tr>
</tbody>
</table>

Data are organized by F and P values from Analysis of covariance. Response variables were reproductive traits that we aimed to isolate. Covariates were reproductive traits that may be influencing the variation of response variable. ANCOVA eliminated the variation of response variable caused by the covariate. Factors were the experimental groups of the study. PANOVA and ANOVA are displayed together to show the effect of eliminating covariates. * denotes marginal insignificance. ** denotes P-values < 0.05.
Figure 5. Mean total IR-kiss neurons between lines and photoperiods. C-LD (n=11), C-SD (n=10), NR-LD (n=4), NR-SD (n=4), R-LD (n=3), R-SD (n=7). Standard error bars signify errors of LD and SD, not individual line and photoperiod group.

Figure 6. Mean total IR-GnRH neurons between lines and photoperiods C-LD (n=9), C-SD (n=10), NR-LD (n=3), NR-SD (n=4), R-LD (n=3), R-SD (n=7). Standard error bars signify errors of LD and SD, not individual line and photoperiod group.
Figure 7. Mean seminal vesicle mass between lines and photoperiods. C-LD (n=11), C-SD (n=10), NR-LD (n=4), NR-SD (n=4), R-LD (n=3), R-SD (n=7). Standard error bars signify errors of LD and SD, not individual line and photoperiod group.

Figure 8. Mean testes mass between lines and photoperiods. C-LD (n=11), C-SD (n=10), NR-LD (n=4), NR-SD (n=4), R-LD (n=3), R-SD (n=7). Standard error bars signify errors of LD and SD, not individual line and photoperiod group.
Figure 9. Mean daily food intake between lines and photoperiods. C-LD (n=11), C-SD (n=10), NR-LD (n=4), NR-SD (n=4), R-LD (n=3), R-SD (n=7). Standard error bars signify errors of LD and SD, not individual line and photoperiod group.

Figure 10. Mean body mass between lines and photoperiods. C-LD (n=11), C-SD (10), NR-LD (n=4), NR-SD (n=4), R-LD (n=3), R-SD (n=7). Standard error bars signify errors of LD and SD, not individual line and photoperiod group.
**Figure 11a.** Scatter plot showing the relationship between kisspeptin neuron count and seminal vesicle mass (n=37).

**Figure 11b.** Scatter plot showing the relationship between kisspeptin neuron count and testis mass (n=39).
Figure 11c. Scatter plot showing the relationship between kisspeptin neuron count and body mass (n=38).

Figure 11d. Scatter plot showing the relationship between kisspeptin neuron count and food intake level (n=39).
Figure 11e. Scatter plot showing the relationship between kisspeptin neuron count and GnRH neuron count (n=36).

Figure 12a. Scatter plot showing the relationship between GnRH neuron count and seminal vesicles mass (n=36)
Figure 12b. Scatter plot showing the relationship between GnRH neuron count and testes mass (n=36)

Figure 12c. Scatter plot showing the relationship between GnRH neuron count and body mass (n=36)
Figure 13a. Scatter plot showing the relationship between testes mass and seminal vesicles mass (n=38)

Figure 11. Scatter plot showing the relationship between seminal vesicles mass and food intake (n=38)
Figure 12d. Scatter plot showing the relationship between GnRH neuron count and food intake level (n=36)

Figure 13d. Scatter plot showing the relationship between body mass and food intake (n=39)
Figure 13e. Scatter plot showing the relationship between food intake and testes mass (n=39)

Figure 13f. Scatter plot showing the relationship between body mass and testes mass (n=39)
Figure 14. Brain section at 100X magnification (top) and 400x magnification (bottom). The tissue is stained for IR-kisspeptin neurons. Image J was used to measure cell body diameters in order to minimize subjectivity of kisspeptin neuron assessment. Red arrows signify counted cell bodies.
Figure 15. Brain section at 100x magnification (top) and 400x magnification (bottom). Tissues were stained for IR-GnRH neurons. There is less subjectivity in neuron assessment for GnRH neurons. Arrows indicate the counted cell bodies.
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