Evaluation of the Hypothalamic Kisspeptin System Throughout the Estrous Cycle and During the Attainment of Puberty in Gilts

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EVALUATION OF THE HYPOTHALAMIC KISSPEPTIN SYSTEM THROUGHOUT THE ESTROUS CYCLE AND DURING THE ATTAINMENT OF PUBERTY IN GILTS

BY

ERIC STEVEN JOLITZ

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Animal Science

South Dakota State University

2016
EVALUATION OF THE HYPOTHALAMIC KISSPEPTIN SYSTEM THROUGHOUT THE ESTROUS CYCLE AND DURING THE ATTAINMENT OF PUBERTY IN GILTS

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Jeffrey S. Clapper, Ph.D. Thesis Advisor

Date

Joe Cassady, Ph.D. Department Head, Animal Science

Date

Dean, Graduate School Date
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Thank you all!
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<tbody>
<tr>
<td>AP</td>
<td>anterior pituitary</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>AVPV</td>
<td>anteroventral periventricular nucleus</td>
</tr>
<tr>
<td>β-actin</td>
<td>beta-actin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>d</td>
<td>day(s)</td>
</tr>
<tr>
<td>ºC</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E₂</td>
<td>estradiol-17beta</td>
</tr>
<tr>
<td>ER-α</td>
<td>estrogen receptor-alpha</td>
</tr>
<tr>
<td>ER-β</td>
<td>estrogen receptor-beta</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
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<tr>
<td>GnRHR</td>
<td>gonadotropin releasing hormone receptor</td>
</tr>
<tr>
<td>GPR54</td>
<td>kiss1 derived protein receptor</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>HH</td>
<td>hypogonadotrophic hypogonadism</td>
</tr>
<tr>
<td>HPD</td>
<td>hypothalamo-pituitary-disconnect</td>
</tr>
<tr>
<td>IGF-I</td>
<td>insulin-like growth factor I</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram(s)</td>
</tr>
<tr>
<td>Kiss1</td>
<td>kisspeptin1 gene</td>
</tr>
<tr>
<td>Kiss1R</td>
<td>kisspeptin1 receptor gene</td>
</tr>
<tr>
<td>Kp-234</td>
<td>kisspeptin-234</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>m</td>
<td>meter(s)</td>
</tr>
<tr>
<td>MBH</td>
<td>mediobasal hypothalamus</td>
</tr>
<tr>
<td>ME</td>
<td>metabolizable energy</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomized</td>
</tr>
<tr>
<td>P4</td>
<td>progesterone</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SAS</td>
<td>statistical analysis system</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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Forward and reverse primers for Real-Time PCR for porcine mRNA

Forward and reverse primers for Real-Time PCR for porcine mRNA
ABSTRACT

EVALUATION OF THE HYPOTHALAMIC KISSPEPTIN SYSTEM THROUGHOUT THE ESTROUS CYCLE AND DURING THE ATTAINMENT OF PUBERTY IN GILTS

ERIC STEVEN JOLITZ

2016

Kisspeptin has been demonstrated to affect reproductive cyclicity and the attainment of puberty in multiple species, presumably through its actions on GnRH and LH, as demonstrated in other species. Kisspeptin administration causes increased plasma concentrations of LH in pigs, sheep, and rats. Two experiments were conducted to evaluate 1) the hypothalamic kisspeptin system throughout various days of the estrous cycle and 2) the hypothalamic kisspeptin system during the peripubertal period in gilts. In experiment 1, 40 crossbred gilts (191 d, 121 kg) were administered an intramuscular injection of PG600. Twelve days after the administration of PG600, gilts were fed 15 mg of altrenogest daily for 15 d to synchronize estrus. Following estrus synchronization, estrus detection was performed by exposing gilts to a mature boar and the first day gilts stood immobile was denoted d 1 of the estrous cycle. Blood samples were collected via jugular venipuncture on d 1, 4, 7, 9, 14, 16, and 19 of the estrous cycle. Ten animals were slaughtered on d 1, 9, 14, and 21 of the estrous cycle when MBH, AP, and blood were collected. Relative expression of hypothalamic Kiss1, Kiss1R, ER-α, ER-β and β-actin was determined using real-time reverse transcriptase PCR. Fold changes in relative
expression were determined using the Relative Expression Software Tool. Relative expression of Kiss1 was increased \((P=0.006)\) 3.2 fold on d 1 versus d 21 and \((P=0.003)\) 2.3 fold on d 9 versus d 21 of the estrous cycle. Relative expression of Kiss1 was not different \((P>0.05)\) among the remaining days. Relative expression of ER-\(\beta\) was decreased \((P=0.05)\) 0.8 fold on d 9 versus d 21 and \((P=0.005)\) 0.7 fold on d 14 versus d 21. Relative expression of ER-\(\beta\) was not different \((P>0.05)\) among the remaining days. Relative expression of Kiss1R and ER-\(\alpha\) were each not different \((P>0.05)\) among days. In experiment 2, two studies were performed, each with 25 crossbred gilts (151 d, 105 kg). In study 1 gilts were slaughtered on d 7 and in study 2 gilts were slaughtered on d 8. Gilts were relocated and exposed to a mature boar throughout study to naturally induce puberty. Gilts that stood immobile within 24 hours of slaughter were considered to have attained puberty \((n=8/{\text{study}})\). Plasma samples were collected on d 1, 3, and 7 of the first study and d 2, 4, 6, and 8 of the second study. When gilts were slaughtered in both studies, MBH, AP, and blood were collected. Relative expression of MBH Kiss1 and \(\beta\)-actin and AP GnRHR, LH-\(\beta\), and \(\beta\)-actin was determined using real-time reverse transcriptase PCR. Fold changes in relative expression were determined using the Relative Expression Software Tool. Hypothalamic and AP content of kisspeptin were determined by RIA and differences were determined using the GLIMMIX procedure of SAS. Relative expression of Kiss1 was increased \((P=0.005)\) 2.2 fold in the gilts that had attained puberty in the first study, however, was not different \((P=0.16)\) when comparing treatments in the second study. Relative expression of GnRHR was not different \((P>0.05)\) between treatments in both studies. Relative expression of LH-\(\beta\) tended to be decreased
(P=0.09) 0.8 fold in the first study but was not different (P=0.19) in gilts that attained puberty in the second study. AP concentrations of LH were not different (P=0.35) between treatments in the first study and were decreased (P=0.01) in gilts that attained puberty in the second study. AP concentrations of kisspeptin were not different (P=0.43) in the first study but were increased (P=0.04) in gilts that had attained puberty in the second study. MBH concentrations of kisspeptin were increased (P=0.03) in gilts that had attained puberty in the first study but were not different (P=0.68) between treatments in the second study. These data further support the notion that MBH expression of Kiss1 and concentrations of kisspeptin and AP concentrations of kisspeptin influence reproductive cyclicity and the attainment of puberty in the gilt.

Keywords: Kisspeptin; Kisspeptin Receptor; Hypothalamus; Pituitary; Pig
Chapter 1

Literature Review

*Kisspeptin background*

Kisspeptin has been demonstrated to play a pivotal role in many physiological functions, including reproduction and the growth of proliferating cells. First discovered in 1996 in Hershey, PA, the Kisspeptin1 gene (Kiss1) was termed a metastasis-suppressor gene due to its anti-oncogenic growth characteristics in the human (Lee et al., 1996). Since 1996, kisspeptin has been extensively demonstrated to also play a major role in reproduction through its actions on GnRH in a number of mammalian and non-mammalian species (Abbara et al., 2013; Clarkson et al., 2008; Colledge and d'Anglemont de Tassigny, 2010; Decourt et al., 2008; Elizur, 2009; Franceschini et al., 2006; Hrabovszky et al., 2007; Seminara et al., 2003; Tobari et al., 2010). Its actions on GnRH may be of importance during the attainment of puberty, estrous cycle and may have effects during pregnancy, parturition, lactation, and the postpartum recovery period in pigs (Caraty et al., 2007; Castellano et al., 2005; Clarkson, 2013).

Past research has indicated that the Kiss1 and the Kiss1-receptor gene (Kiss1R) are highly expressed throughout many tissues in pigs (Li et al., 2008). Li et al. (2008) analyzed 18 tissues for the presence of Kiss1R transcripts and found it was particularly abundant in the adrenal, prostate, testis, thymus, pituitary, and hypothalamus. Because the Kiss1R is present in so many tissues throughout the body it is likely that kisspeptin affects many physiological processes throughout the body.
The Kiss1 gene encodes the pre-prohormone Kisspeptin-234 (Kp-234), which is biologically inactive in mammalian and non-mammalian species (Ronnekleiv and Kelly, 2013). Kisspeptin-234 is cleaved into one of four biologically active forms that can bind to the Kiss1-receptor. The four biologically active forms of kisspeptin in mammalian species are Kisspeptin-54 (Kp-54), Kisspeptin-14 (Kp-14), Kisspeptin-13 (Kp-13), and Kisspeptin-10 (Kp-10). Amino acid sequences of these four proteins are highly conserved among mammalian species (Kotani et al., 2001; Ohtaki et al., 2001). The carboxy-terminus is the most highly conserved region among all mammalian species and confers the biological activity of kisspeptins (Colledge, 2008; Pasquier et al., 2014). Also, the 2, 4, 6, 7, 8, and 9 amino acids are completely conserved across mammalian species sequenced to date (Pasquier et al., 2014). The kisspeptin isoforms vary in length among mammalian species (De Bond and Smith, 2014).

Kisspeptins bind to their cognate receptor with high affinity. The Kiss1R gene encodes the Kiss1 derived protein receptor (GPR54), which is 398 amino acids in length. Kisspeptin1 derived protein receptor is a G-protein coupled receptor (GPR) (Pasquier et al., 2014) and acts through the classical GPR pathway. When the biologically active kisspeptins bind to GPR54, intracellular metabolic pathways including protein kinase are activated (Glanowska and Moenter, 2015; Liu et al., 2008). All of the four biologically active forms of kisspeptins can bind to GPR54.
**Site of action of kisspeptin and its actions on GnRH**

Gonadotropin releasing hormone (GnRH) is important for successful reproduction in numerous mammalian and non-mammalian species. This decapeptide has been shown to have actions in the hypothalamus through ultra-short feedback, however its actions are primarily in the AP causing the synthesis and release of the gonadotropins LH and FSH (Cai and Zhang, 2005; Jackson et al., 2013; Karsch and Evans, 1996). Gonadotropin releasing hormone neurons are highly abundant in the hypothalamus in many species including the pig (Silverman et al., 1987; Silverman et al., 1979). In most mammalian species including the pig, there are two particular regions of interest: the surge center and the tonic center, which control the release of GnRH.

Though it is plausible that kisspeptin elicits the release of hormones in many tissues in the body, it is thought that its main actions are within the hypothalamus to cause the release of GnRH and subsequent gonadotropin release. In the presence of a GnRH antagonist the stimulatory effects of kisspeptin on gonadotropin release are attenuated in female mice, rats, sheep, and monkeys (Matsui et al., 2004; Navarro et al., 2005; Plant et al., 2006; Roseweir et al., 2009; Smith et al., 2011). Peripheral administration of kisspeptin-54 has been shown to activate GnRH neurons in the hypothalamus (Caraty et al., 2007). Peripheral administration of kisspeptin to sheep caused an increase in GnRH concentrations in the cerebrospinal fluid (CSF) in sheep (Matsui et al., 2004; Messager et al., 2005).
Data also support the implication that kisspeptin acts on GnRH neurons due to the anatomical intimacy between GnRH neurons and kisspeptin receptors. The neurohormone kisspeptin is thought to elicit the release of GnRH via an intimate connection of kisspeptin neuronal axons associated with dendrites of GnRH neurons (Hrabovszky et al., 2007). This was discovered through double-immunostaining for kisspeptin and GnRH neurons (Hrabovszky et al., 2007). Research has also indicated that GPR54 is expressed by GnRH neurons and is directly stimulated by kisspeptin to cause GnRH release (Muir et al., 2001; Navarro et al., 2004). More than 70% of GnRH neurons are co-expressed with kisspeptin receptor mRNA in the rat hypothalamus (Irwig et al., 2004). In the rat, it has also been demonstrated that kisspeptin immunoreactive nerve elements directly adjoin to GnRH nerve elements in the hypothalamus (Uenoyama et al., 2011). It has also been shown that kisspeptin stimulated the release of GnRH from rat hypothalamic explants (Thompson et al., 2004). These data indicate that kisspeptin may stimulate the release of GnRH from GnRH neurons in certain areas of the hypothalamus (Uenoyama et al., 2011).

While it is thought that the predominant actions of kisspeptin are in the hypothalamus through its actions on GnRH neurons, it has also been thought that kisspeptin may act directly on the anterior pituitary gland (AP). In AP cell cultures from adult female baboons, kisspeptin elicited the release of LH in a dose-dependent manner (Luque et al., 2011). Kisspeptins have been demonstrated to elicit the release of LH and FSH from the AP in nonmammalian vertebrates (Elizur, 2009; Tobari et al., 2010), rodents (Revel et al., 2006), ungulates (Decourt et al., 2008; Franceschini et al., 2006; Li
et al., 2008; Magee et al., 2009; Ohkura et al., 2009; Pompolo et al., 2006), and primates (Hrabovszky et al., 2007; Seminara et al., 2003). It is thought that kisspeptins act through direct association with GnRH neurons as well as kisspeptins release into the hypothalamohypophyseal vascular plexus to associate with the gonadotrophs directly (Dungan et al., 2006; Ezzat et al., 2010). Additionally, estradiol-17β (E₂) increased the release of LH from AP cells when treated with kisspeptin (Luque et al., 2011). Similarly, in the rat, another study indicates that when rat anterior pituitary cells were incubated with kisspeptin-10, LH was increased in a dose dependent manner (Navarro et al., 2005). On the contrary, Thompson et al. (2004) demonstrated that there was no release of LH from cultured anterior pituitary cells during treatment of pharmacological doses of kisspeptin in the rat. Another study in female and male rats indicated that when kisspeptin-54 was incubated with cultured anterior pituitary cells there was no in vitro LH or FSH release, even after the pituitary cells were primed with E₂ (Matsui et al., 2004). Though kisspeptins have some effect on the release of the gonadotropins from cultured anterior pituitary cells, research seems to be equivocal as to whether kisspeptin acts on the AP directly.

Lents et al. (2008) administered kisspeptin-10 peripherally and centrally to pigs and reported that it caused an increase in circulating concentrations of LH. Anterior pituitary cultures from the bovine and porcine species elicited LH release in response to kisspeptin in a dose dependent manner, however, pharmacological doses were required (Suzuki et al., 2008). Suzuki et al. (2008) also demonstrated that LH release occurred within cultured AP cells in response to physiological doses of GnRH. This supporting
evidence suggests that in addition to GnRH, kisspeptin may also increase the release of LH through the GnRH pathway (Messager, 2005).

Kisspeptin-54 is a key hormone influencing the regulation of reproductive function and development (Adachi et al., 2007; Gottsch et al., 2004). Centrally administered kisspeptins stimulate the release of GnRH in prepubertal and pubertal female mice and rats (Gottsch et al., 2004; Kinoshita et al., 2005). There is also supporting evidence that in Kiss1R knockout mice, kisspeptin is not able to stimulate the release of FSH and LH which is thought to be through the GnRH pathway (Messager et al., 2005). Additionally, during treatment with acyline, a potent GnRH antagonist, the kisspeptin-mediated release of LH was completely inhibited (Gottsch et al., 2004).

During ICV treatment with kisspeptin there was an increase in the release of GnRH in the mouse (Messager et al., 2005). When kisspeptin is incorporated with GnRH neurons in vitro, there is a potent increase in GnRH neuronal firing (Han et al., 2005; Zhang et al., 2008). These data support the notion that the stimulatory actions of kisspeptin seem to be mainly on GnRH neurons.

Over the past decade there have been a multitude of publications pertaining to the influence of Kiss1 and kisspeptins and the regulation of GnRH secretion (Gottsch et al., 2004; Messager et al., 2005; Novaira et al., 2009). In addition to the association between Kiss1, kisspeptin, and GnRH, there have also been studies describing the regulation of the expression of hypothalamic Kiss1 in correspondence with E2 in mice and rats (Oakley et al., 2009; Smith et al., 2005a). An increase in E2 causes an increase in the expression of hypothalamic Kiss1 in the anteroventral periventricular nucleus (AVPV) but decreases
the expression of hypothalamic Kiss1 in the arcuate nucleus (ARC) in the female mouse (Smith et al., 2005a; Smith et al., 2006b). The location in which the expression of hypothalamic Kiss1 is up-regulated is of importance because the positive feedback effects of E2 are primarily exhibited on the AVPV in rodent species (Petersen and Barraclough, 1989; Wiegand et al., 1980).

Although correspondence of the expression of hypothalamic Kiss1 and E2 is true in mice and rats, the concept in sheep is somewhat different. In sheep, the primary region that receives positive feedback of E2 is the preoptic area (POA) indicating that the basal and mediobasal hypothalamus (MBH) may be sufficient in maintaining the steroid-mediated release of GnRH (Caraty et al., 1998). Regardless of the region of regulation of the expression of hypothalamic Kiss1 by E2 in the brain, kisspeptin potently excites GnRH neurons in all instances indicating that specific areas within the hypothalamus may be influenced by kisspeptin and involved in the release of GnRH and LH.

Mechanisms by which E2 and progesterone (P4) feed back to the hypothalamus and AP are also of great importance in the control of GnRH and the LH surge. For the GnRH surge to occur there have to be threshold concentrations of E2 in the absence of P4 (Clarke and Cummins, 1984; Everett and Sawyer, 1950; Harris et al., 1999; Moenter et al., 1993; Richter et al., 2001). Past research is equivocal as to whether GnRH neurons express E2- and P4-receptors in mammalian species. Gonadotropin releasing hormone neurons do express estrogen receptor-beta (ER-β) in humans (Hrabovszky et al., 2007). This supports the notion that E2 can directly affect GnRH neurons thus changing the animals’ endocrine status. However, it is thought the ER-β receives the signals for
negative estrogen feedback when positive feedback through estrogen receptor-alpha (ER-α) is needed for the GnRH surge to occur in the mouse (Glidewell-Kenney et al., 2007). In the female mouse there are positive, but not negative, feedback actions of E2 through ER-α in kisspeptin neurons (Dubois et al., 2015). It has not been demonstrated that GnRH neurons express ER-α (Herbison and Theodosis, 1992; Lehman and Karsch, 1993). Therefore, other neurons upstream of GnRH neurons that possess ER-α have been considered as possible mediators of steroidal effects on GnRH release.

Dual immunofluorescence revealed that neurons containing kisspeptin-10 expressed ER-α (Franceschini et al., 2006). The majority of cells expressing kisspeptin neurons co-expressed progesterone receptors (PR) in the ARC in sheep (Smith et al., 2007). Regulation of the expression of hypothalamic Kiss1 is likely to be a mediator of negative feedback in multiple species (Adachi et al., 2007; Franceschini et al., 2006; Goodman et al., 2007; Smith et al., 2005a; Smith et al., 2005b). There is also supporting evidence that reduced activity of kisspeptin neurons in the hypothalamus of many mammalian species is responsible for the relay of negative feedback effects of E2 to GnRH neurons. Castrated male and ovariectomized (OVX) female mice had increased levels of the expression of hypothalamic Kiss1 versus gonadally intact female mice (Smith et al., 2005a; Smith et al., 2005b). There was also an increase in the expression of hypothalamic Kiss1 in OVX sheep and primates versus controls (Pompolo et al., 2006; Rometo et al., 2007; Shibata et al., 2007). During estrogen administration to OVX female sheep, the expression of hypothalamic Kiss1 returns to levels not different from control animals after not being administered estrogen (Pompolo et al., 2006; Rometo et al., 2007;
Shibata et al., 2007). The expression of ARC Kiss1 was increased, however the expression of POA Kiss1 was decreased in OVX ewes (Smith et al., 2007). Intact ewes that exhibited normal steroid feedback had decreased expression of hypothalamic Kiss1 during times of anestrous (Smith et al., 2007). Moreover, the number of kisspeptin positive cells in the ARC of OVX ewes was reduced by treatment of E₂ and P₄ (Smith et al., 2007). Kisspeptin neurons are associated with ER-α, PR, and androgen receptors and therefore have the potential to relay steroidal feedback effects onto GnRH neurons (Dubois et al., 2015; Roseweir and Millar, 2009). Though exact mechanisms are unknown, these data suggest that steroids can regulate the expression of hypothalamic Kiss1, thereafter stimulating GnRH neurons.

**Effects of acute kisspeptin administration on LH secretion**

Luteinizing hormone, and the preovulatory LH surge are of utmost importance for mammals to successfully reproduce. In most instances during peripheral, intravenous (i.v.), and central acute administration, kisspeptin has been shown to stimulate gonadotropin release. Though kisspeptin has been shown to act primarily in the hypothalamo-pituitary axis with very low circulating levels in the blood, peripheral administration of kisspeptin has been demonstrated to consistently stimulate the release of gonadotropins in a number of mammalian species.

The plasma half-life of Kisspeptin-10 *in vivo* is very short - only 4 minutes in the human (Jayasena et al., 2011). Kisspeptin-54 also has a short *in vivo* plasma half-life of about 25 minutes in the human (Dhillon et al., 2005). Therefore, i.v. administration of
kisspeptin-10 would be expected to have a short duration of effects and/or may need to be administered at pharmacological levels to elicit the release of gonadotropins. Matsui et al. (2004) demonstrated that a subcutaneous administration of 6.7 nmol of human kisspeptin-54 in pre-pubertal female rats elevated plasma LH levels 10-fold from baseline levels 2 hours after injection. Additionally, administration of kisspeptin-54 caused ovulation in a large percentage of follicles, similar to the effects of human chorionic gonadotropin (hCG) (Matsui et al., 2004). It has also been demonstrated that male rats (10 weeks of age) that were administered subcutaneous equimolar doses of kisspeptin-54 and GnRH responded to the two hormones in almost an identical fashion, indicating that kisspeptin-54 and GnRH are equally effective at stimulating the release of LH (Thompson et al., 2009).

During central and peripheral administration of kisspeptin-10 in GnRH-primed, castrated rhesus monkeys, researchers observed that 100 µg of kisspeptin-10 administered both centrally and peripherally induced the release of LH (Shahab et al., 2005). During both ICV and i.v. infusions plasma concentrations of LH increased more than 20-fold by 30 minutes after the start of kisspeptin-10 infusion and continued to be elevated for approximately 2 hours (Shahab et al., 2005). However a smaller dose of 30 µg kisspeptin-10 stimulated LH secretion in a lesser manner (Shahab et al., 2005).

The effects of i.v. administration of kisspeptin-10 were also studied in livestock species. Human kisspeptin-10 was infused intravenously into pubertal, female goats in the luteal phase in a dose dependent manner. Circulation concentrations of LH increased greater than 4-fold 20 to 30 minutes following infusion (Hashizume et al., 2010).
Similarly in cattle, an intravenous and intramuscular bolus of kisspeptin-10 at 100 and 200 pmol/kg increased plasma LH levels 9.7- and 3-fold, respectively (Whitlock et al., 2008). Additionally, an intravenous infusion of kisspeptin-10 at doses of 0.77, 1.54, and 3.84 μmol to pre-pubertal gilts stimulated an increase in LH to a similar degree following all doses (Lents et al., 2008). Therefore, kisspeptin-54 and -10 are effective in stimulating LH secretion whether given centrally, intravenously, intramuscularly, or subcutaneously to a number of species, including the pig.

**Kisspeptin and the estrous cycle**

The average estrous cycle of the pig is approximately 21 d in length of which the follicular phase and luteal phase are approximately 5 d and 16 d in length, respectively. The follicular phase is the time during which concentrations of E₂ are increased and P₄ are decreased. Additionally, the GnRH and LH surge and ovulation occur during this period. During the luteal phase, serum concentrations of P₄ are increased and serum concentrations of E₂ are decreased. Corpora lutea (CL) are producing P₄ during this phase and concentrations of E₂ are decreased since there is a decrease in the pulsatility and amplitude of LH. Therefore, the follicular phase of the estrous cycle is of particular interest because it is a time coincident with increased hypothalamic concentrations of kisspeptin, the GnRH and LH surge, and the subsequent ovulation.

The amplitude and frequency of LH release are of importance during normal reproductive cyclicity in mammals. In order for ovulation to occur LH has to reach a threshold level – this being termed the preovulatory LH surge. Past research indicates that
kisspeptin plays a pivotal role in the generation of the LH surge. During ICV infusion of kisspeptin-10 in the pig there was a LH surge (Pineda et al., 2010). Caraty et al. (2007) demonstrated that constant i.v. infusion of kisspeptin-10 for 8 h beginning 30 h after P4 withdrawal, resulted in an earlier LH surge compared to ewes treated with a placebo infusion (Caraty et al., 2007). Intravenous infusion of kisspeptin-10 also stimulates an LH surge in anestrous ewes (Sebert et al., 2010). Ewes treated with Kiss1-receptor antagonist p-271 did not exhibit an E2 induced LH surge (Smith et al., 2007).

Kisspeptin is thought to exert most of its actions in certain areas of the hypothalamus through the GnRH pathway. *In vitro* studies have shown kisspeptin to act at the level of the AP, but not *in vivo* studies. Cultures of ovine anterior pituitary cells responded to kisspeptin treatment exhibiting LH release in a dose dependent manner, however pharmacological doses of kisspeptin were required to elicit this response (Suzuki et al., 2008). Hypothalamo-pituitary-disconnected (HPD) ewes did not respond to kisspeptin-10 treatment as there was no difference in concentrations of LH versus ewes that did not have a HPD (Smith et al., 2008). There was an increase in the number of kisspeptin positive cells in the ARC and an increase in the expression of ARC and POA Kiss1 during the follicular phase in the ewe, a time coincident with ovulation (Estrada et al., 2006; Smith et al., 2007). Smith et al. (2009), also demonstrated that there was an increase in the percentage of kisspeptin cells in the ARC during acute administration of E2 in intact ewes versus OVX ewes administered a chronic dose of E2 the response in intact ewes was greater (Smith et al., 2009). These data indicate that kisspeptin is seemingly obligatory for the generation of the GnRH surge and the subsequent LH surge.
However, it is unclear if kisspeptin acts at the level of the AP, *in vivo*, under normal physiological conditions.

**Kisspeptin and puberty**

The attainment of puberty and hormonal patterns that coincide with the attainment of puberty are important concepts to understand. There are many definitions indicative of puberty: the first time a female ovulates, the first time a female exhibits estrus, the first time the preovulatory LH surge occurs, and the first time a female can carry an embryo/fetus to term without it having deleterious effects to her body. Though there are advantages and disadvantages to each of these definitions, the first time a female stands in a natural estrus is one of the easiest to use from a research standpoint.

In the gilt, age at puberty is positively correlated with age at first boar exposure (Filha et al., 2009). For best results, gilts are brought to boars where they interact with the boar through sight, sound, smell, and tactile stimulation. Past studies have shown that relocation followed by exposure to a mature boar can induce puberty in gilts without the use of exogenous hormones. Gilts also have to be of proper physiological maturity in order to attain puberty naturally. Puberty in the gilt, as described by the first preovulatory LH surge, is attained when there is a decrease in the sensitivity to the negative feedback effects of E₂ which allows the LH surge to occur. During the first GnRH surge and subsequent LH surge the gilts hypothalamus shifts from being controlled in a negative to positive steroid feedback fashion. During relocation and boar exposure, this physiological shift can be achieved causing the subsequent natural attainment of puberty. As the gilt
attains puberty there is also an increase in FSH, which causes the development of follicles that synthesize microgram amounts of E2. These physiological factors contribute to the attainment of puberty in the gilt although the exact mechanisms by which these hormones are released is not fully understood.

There are an abundance of hormones and growth factors that contribute to the attainment of puberty. Of those hormones and growth factors, kisspeptin and hormones associated with kisspeptin are of interest. Neurokinin B (NKB) is a tachykinin and has been demonstrated to have effects on the secretion of GnRH in the ewe (Goodman et al., 2014). Neurokinin B is co-expressed with kisspeptin in a large number of hypothalamic cells in sheep indicating NKB may also play a role in the initiation of puberty (Nestor et al., 2012). When prepubertal ewes were injected with senktide, a NKB agonist, there was an immediate pulse of LH versus animals that were administered vehicle (Nestor et al., 2012). Additionally, there was an increase in kisspeptin positive cells in the hypothalamus after the attainment of puberty in intact ewes as well as in prepubertal OVX ewes versus intact prepubertal ewes (Nestor et al., 2012). Peripheral administration of kisspeptin-10 caused an increase in circulating plasma concentrations of GH in prepubertal heifers (Nestor et al., 2012). Insulin-like growth factor I has been demonstrated to have positive effects on the LH surge in a number of species (Adam et al., 2000; Stewart et al., 1996). Insulin-like growth factor-I can also induce an increase in hypothalamic Kiss1 mRNA expression during the onset of puberty in the rat, although the signaling pathway by which this occurs remains elusive (Hiney et al., 2009). Hiney et al. (2009) demonstrated that when rats were administered alcohol, an IGF-I inhibitor, there
was a decrease in the expression of hypothalamic Kiss1. Rats that were administered IGF-I with saline instead of alcohol showed an increase in the expression of hypothalamic Kiss1 (Hiney et al., 2009). These data indicate that several physiological factors influence the hypothalamic kisspeptin system which may have implications during the attainment of puberty.

Many mammalian species exhibit different hormonal patterns during central and peripheral infusion of when kisspeptin antagonists. Extensive in vitro and in vivo kisspeptin research has been conducted in a number of mammalian species, including the pig. During ICV infusion of kisspeptin antagonist (p-234) there was a decrease in LH pulse amplitude in the ewe – the level of LH was decreased to the extent in which a pulse no longer occurred (Roseweir et al., 2009). There was also a decrease in plasma concentrations of LH (Roseweir et al., 2009). Central infusion of p-234 during the peripubertal period in female rats caused delayed vaginal opening, and decreased uterine and ovarian weights during the expected time of puberty (Pineda et al., 2010). Chronic ICV administration of p-234 for 4 d prevented the subsequent LH and FSH surge during the time of the expected GnRH surge (Pineda et al., 2010). Systemic administration of labeled p-234 attenuated the acute release of LH and FSH while females were also administered kisspeptin-10 systemically (Pineda et al., 2010). Additionally, through the administration of the Kiss1-receptor antagonist p-271 there was a decrease in LH concentrations in OVX ewes (Smith et al., 2011). Cultures of mouse MBH explants that were incubated with kisspeptin in a dose dependent manner for 1 h elicited GnRH release, whereas this did not occur in GPR54 null animals (de Tassigny et al., 2008).
These data demonstrate that kisspeptin antagonists have a negative effect on reproductive biology and normal reproductive cyclicity.

Kisspeptin is also important during the attainment of puberty and normal reproductive cyclicity in the human. One major area of concern is that in which humans suffer from hypogonadotrophic hypogonadism (HH). Hypogonadotrophic hypogonadism is characterized by a decrease in the function and release of the gonadotropins from the AP and a decrease in the function and weight of the gonads. Multiple studies have indicated that a decrease in the expression of hypothalamic Kiss1 causes HH in the human and the mouse (d'Anglemont de Tassigny et al., 2007; Topaloglu et al., 2012). Additionally, there is also evidence that mutations in the Kiss1R and GPR54 decrease the effects that kisspeptins have on the hypothalamo-pituitary-gonadal axis (Semple et al., 2005). After central and peripheral administration of kisspeptins in rats there was a dose dependent increase in circulating plasma levels of LH (Thompson et al., 2004). These data indicate that kisspeptin and GPR54 have added a new critical dimension to the physiological actions within the HPG axis.

Kisspeptin restores normal reproductive cyclicity in humans and this moves one to believe that kisspeptin could also be used in a similar fashion in livestock species. Although GnRH is currently used and effectively works to cause ovulation, there may be other associations kisspeptin has on the hypothalamo-pituitary-gonadal axis. Not only may kisspeptin be used to treat animals with reproductive disorders but it could also be used as a diagnostic tool. Previous research has lead scientists to believe that if there are
decreased peripheral concentrations of kisspeptin the animal may be inferior to its cohorts.

**Other physiological signaling associated with kisspeptin**

A majority of the work involving kisspeptin has focused on its interaction with and control of the release and regulation of GnRH. Although this has been proven to be true, kisspeptin is also thought to be integrated with metabolism and other hormones critical in the regulation of reproduction, pregnancy, and lactation. Metabolic signals and energy balance are important factors that influence the success of the attainment of puberty and reproductive cyclicity and any disruptions in such aspects may be linked to reproductive failure. Because the interactions between reproduction and metabolism are multifaceted, it is highly likely that kisspeptin plays a role in metabolic processes in the mammalian body.

To date, there has been extensive research that links leptin, growth hormone, and IGF-I to the expression of hypothalamic Kiss1 and plasma concentrations of kisspeptin, however it must be stressed that a majority of these studies have used *in vitro* experiments and the *in vivo* experiments that have been performed have used pharmacological doses to elicit a response. Although precautions should be taken as to whether these responses occur physiologically it is plausible that some of them may and it is worthwhile mentioning the association of kisspeptin and metabolic factors. In gonadectomized ob/ob mice there was a suppression of the expression of hypothalamic Kiss1 when leptin was absent (Smith et al., 2006a). Also, during the re-administration of
leptin there was a restoration of the expression of hypothalamic Kiss1 (Smith et al., 2006a). Central infusion of leptin in a dose dependent manner to male rats with uncontrolled diabetes and hypogonadotrophic hypogonadism has been shown to normalize the expression of hypothalamic Kiss1 (Castellano et al., 2006). It has also been documented that leptin administration increased the expression of ARC and POA Kiss1 mRNA (Backholer et al., 2010). It was also demonstrated that the leptin receptor gene was expressed in Kiss1 neurons in the arcuate nucleus and preoptic area (Backholer et al., 2010). These data support the notion that kisspeptin may be a modulator, through leptin status, of GnRH neurons and release which promotes proper function of the reproductive axis.

There is a correlation between plasma levels of GH and LH, therefore it is plausible that there is a like between kisspeptin and GH. Cultured bovine anterior pituitary cells that were directly treated with kisspeptin-10 in a dose-dependent manner responded accordingly with a dose dependent increase in the secretion of GH (Kadokawa et al., 2008). Similarly, cultured rat anterior pituitary cells incubated with kisspeptin-10 responded similarly (Gutierrez-Pascual et al., 2007). Cultured anterior pituitary cells from nonhuman primates also exhibited the release of GH in response to treatment of kisspeptin-10 (Luque et al., 2011). This evidence is suggestive of a link between kisspeptin and GH, however, research is equivocal. During the administration of kisspeptin to prepubertal cattle and prepubertal female pigs, there was no increase in circulating concentrations of GH (Ezzat Ahmed et al., 2009; Lents et al., 2008). Although research is equivocal as to whether physiological levels of kisspeptin increase the release
of GH, further exploration of the concept is necessary to conclude the exact mechanisms by which kisspeptin and GH interact.

Another hormone that may influence kisspeptin signaling is IGF-I. Although insulin infusion does not appear to control the regulation of the expression of hypothalamic Kiss1 in diabetic rats, there are implications that IGF-I and other growth factors may regulate the expression of hypothalamic Kiss1 (Luque et al., 2007). In addition to in vivo regulation of the expression of hypothalamic Kiss1, hypothalamic cell cultures (N6) that were treated with insulin did not exhibit an increase in the expression of hypothalamic Kiss1. Hiney et al. (2009) reported that IGF-I stimulated Kiss1 gene expression in the hypothalamus 6 hours post administration. During that experiment it is also worthy of noting that plasma concentrations of E2 did not change during or after the infusion of IGF-I, therefore E2 did not cause the change in the expression of hypothalamic Kiss1 (Hiney et al., 2009). There was a marked increase in the release of LH, presumable through the GnRH pathway, in ovariectomized female rats that were injected with IGF-I or an IGF-I inhibitor during intramuscular administration of kisspeptin. (Neal-Perry et al., 2014). Under these conditions, IGF-I also increased the kisspeptin-induced LH release without an increased number of GnRH immunoreactive cells (Neal-Perry et al., 2014).

Collectively, these data indicate that there may also be a link between the IGF-I and kisspeptin systems in the control and regulation of the release of GnRH and LH.

A link may exist between pregnancy and lactation specific hormones and kisspeptin. During intravenous kisspeptin administration to non-pregnant female rats plasma concentrations of oxytocin were increased (Kotani et al., 2001). During
pregnancy in the human there is a dramatic increase in plasma concentrations of kisspeptin. From baseline, there is approximately an 850-fold increase in plasma concentrations of kisspeptin in the first trimester and approximately a 7,000-fold increase in plasma concentrations of kisspeptin in the third trimester (Dhillo et al., 2007). The main source of the increased plasma concentrations of kisspeptin during pregnancy may be stemming from the placenta because the expression of Kiss1 and kisspeptin have been found in the syncytiotrophoblastic cells of the placenta (Horikoshi et al., 2003). The exact reason for the increase in kisspeptin during pregnancy is unknown and it would be worthwhile further investigating this concept in other species.

**Summary and Conclusion**

Kisspeptin plays an important role in the regulation of GnRH and LH during the estrous cycle and attainment of puberty. It has been demonstrated that central and peripheral infusion of kisspeptin stimulate the release of GnRH and/or LH. *In vitro*, kisspeptin has also been shown to elicit the release of LH from anterior pituitary cells. During the attainment of puberty, prepubertally, and after ovariectomy, peripheral administration of kisspeptin-10 increased circulating plasma levels of LH in the bovine species. There are also implications that kisspeptin is associated with metabolic factors as well as pregnancy and lactation specific hormones. Although the mechanisms of kisspeptins actions are not fully understood and future studies may provide more insight into the matter, it is widely accepted that kisspeptin and the expression of hypothalamic Kiss1 is highly associated with hypothalamic signaling in reproductive physiology. Kisspeptin has been demonstrated to play a role in reproductive cyclicity throughout the
estrous cycle and during the attainment of puberty in the gilt, however, changes in the hypothalamic kisspeptin system and other related reproductive hormones have not been determined throughout the estrous cycle and during the peripubertal period in the gilt. Therefore, the objectives of the following studies was to ascertain the relationship between peripheral concentrations of E₂ and P₄, AP concentrations of kisspeptin, LH, and IGF-I, MBH concentrations of kisspeptin, AP expression of GnRHR and LH-β, and MBH expression of Kiss1, Kiss1R, ER-α, and ER-β.
Chapter 2

EVALUATION OF THE HYPOTHALAMIC KISSPEPTIN SYSTEM THROUGHOUT THE ESTROUS CYCLE IN GILTS

Introduction

Kisspeptin administration has been found to increase serum concentrations of LH in a number of species. Kisspeptin has also been found to cause the release of LH from AP cell cultures and/or cause release of AP LH in cattle (Whitlock et al., 2008), sheep (Sebert et al., 2010), and pigs (Lents et al., 2008); presumably through the GnRH pathway. Intravenous administration of kisspeptin-10 caused a 4-fold increase in plasma concentrations of LH within 30 minutes in the goat (Hashizume et al., 2010). Similarly in cattle, during intravenous and intramuscular infusion of kisspeptin-10, there was a 9.7- and 3-fold increase in plasma concentrations of LH, respectively (Whitlock et al., 2008). Pineda et al. (2010) found that there was a LH surge during ICV infusion of kisspeptin-10 in pigs. Intravenous infusion of increasing doses of kisspeptin-10 to pre-pubertal gilts also stimulated an increase in LH to a similar degree (Lents et al., 2008). Therefore, these studies indicate that the administration of kisspeptin via ICV and intravenous infusion caused an increase in peripheral concentrations of LH in a number of mammalian species.

Anterior pituitary cell cultures from adult female baboons released LH in response to kisspeptin in a dose dependent manner (Luque et al., 2011). In the rat, LH was released in a similar fashion to that of the baboon upon kisspeptin treatment (Navarro
et al., 2005). The release of LH from anterior pituitary cultures has also been demonstrated in the horse (Decourt et al., 2008), fish (Elizur, 2009), and sheep (Franceschini et al., 2006).

Recently, research has been performed to ascertain the relationship between the expression of hypothalamic Kiss1 and plasma concentration of estradiol-17β (E2) (Oakley et al., 2009; Smith et al., 2005a). An increase in circulating concentrations E2 caused an increase in the expression of anteroventral periventricular nucleus (AVPV) Kiss1 but decreased the expression of arcuate nucleus (ARC) Kiss1 in the female mouse (Smith et al., 2005a; Smith et al., 2006b). In the rat, the expression of AVPV Kiss1 peaks at a time coincident with the pre-ovulatory LH surge and Kiss1 neurons express c-Fos induction at a coincident time (Adachi et al., 2007; Smith et al., 2006b). Clarkson et al. (2008) reported that mice bearing deletions in the Kiss1R appear to lack the capacity to exhibit an LH surge versus mice that were ovariectomized and then treated with estrogen and progesterone. Hence, it is plausible that an increase in the expression of hypothalamic Kiss1 may contribute to the pre-ovulatory GnRH/LH surge in the pig.

It is widely accepted that there is a pre-ovulatory LH surge around d 19 of the estrous cycle in the pig (Henricks et al., 1972). Leading to the time of the pre-ovulatory LH surge, there is a decrease in plasma concentrations of P4 followed by an increase in plasma concentrations of E2 (Henricks et al., 1972). These events then lead to a standing estrus which occurs at a time temporally related to ovulation. Smith et al. (2009) demonstrated that the number of identifiable Kiss1 mRNA expressing cells and the Kiss1 mRNA expression per cell in the pre-optic area (POA) were greater during the late
follicular phase versus the luteal phase in the sheep. This was also true for the number of identifiable Kiss1 mRNA expressing cells in the caudal and middle parts of the ARC (Smith et al., 2009). Smith et al. (2009) also demonstrated that there was a 2-fold increase in kisspeptin-immunoreactive cells in the ARC during the late-follicular phase of the estrous cycle versus the luteal phase of the estrous cycle. These data indicate that there is a link between kisspeptin and Kiss1 mRNA expression during the events that occur during estrus in mammals.

Kisspeptin has been demonstrated to play a critical role in the regulation of reproduction through multiple endocrine pathways, however, the changes in kisspeptin and related reproductive hormones throughout the estrous cycle in the gilt are yet to be determined. Therefore, the objective of this research was to determine serum concentrations of E₂ and P₄, anterior pituitary concentrations of LH, kisspeptin, and IGF-I, and hypothalamic expression of Kiss1, Kiss1R, ER-α, and ER-β and concentrations of kisspeptin on various days of the porcine estrous cycle.

**Materials and Methods**

**Animals**

Forty crossbred (Duroc x Large White x Landrace) gilts of similar age (191 ± 11.6 d) and weight (120.7 ± 32.8 kg) were used in this experiment. Gilts were penned (1.83 m x 2.44 m) into groups of four and received ad libitum access to a corn-soy based diet that contained 3.4 Mcal ME/kg, 18% protein, and 0.9% lysine and water. Each gilt was administered an intramuscular injection of PG600 (400 IU equine chorionic
gonadotropin and 200 IU human chorionic gonadotropin) two days after they were penned into groups of four. Twelve days after the administration of PG600 gilts were orally administered 15 mg of altrenogest (Matrix; Merck Animal Health, Kenilworth, NJ) each day and continuing for 15 d to synchronize estrus. Gilts were exposed to a mature boar twice daily beginning the fourth day after the cessation of the altrenogest treatment and continuing for 4 d to detect estrus. The first day gilts stood immobile in the presence of the boar was designated as d 1 of the estrous cycle.

Ten gilts were slaughtered at the South Dakota State University Meat Lab on days 1, 9, 14, and 21 of the estrous cycle at which time blood samples, anterior pituitaries (AP), mediobasal hypothalami (MBH), and reproductive tracts were collected. Blood samples (10 mL) were collected from all gilts on d 1 of the estrous cycle and remaining gilts that had not yet been slaughtered on days 4, 7, 9, 14, 16, and 19 of the estrous cycle via jugular venipuncture. Blood samples were allowed to clot overnight at 4ºC then serum was collected by centrifugation (1,500 x g for 30 minutes at 4ºC) and stored at -20ºC. Anterior pituitary glands and MBH were trimmed of connective tissue, bisected midsaggitally, wrapped in aluminum foil, snap frozen in liquid nitrogen, and stored at -80ºC. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at South Dakota State University.

**Estradiol-17β**

Serum concentrations of E₂ were determined in duplicate in all blood samples by RIA. Estradiol-17β (E8875; Sigma Life Science, St. Louis, MO) was the standard and
radioiodinated E₂ (#07138228; MP Biomedicals, Solon, OH) was the tracer. Antisera (GDN#244 anti-estradiol-17β-6-BSA; Fort Collins, CO) was used at a dilution of 1:425,000. Sera (250-μL) were extracted with a 4-mL volume of methyl tert-butyl ether. Recovery of [¹²⁵I]estradiol-17β added to porcine serum before extraction averaged 96 ± 2%. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay and inter-assay coefficients of variation were 9.2% and 18.6%, respectively. Sensitivity of the assay was 0.2 pg/tube.

**Progesterone**

Serum concentrations of progesterone were determined in duplicate in all blood samples by RIA. Progesterone (P0130; Sigma Life Science; St. Louis, MO) was the standard and radioiodinated progesterone (#07-170126; MP Biomedicals, Solon, OH) was used as the tracer. Antisera (#111.2C7.3; Enzo Life Sciences, Farmingdale, NY) was used at a dilution of 1:700,000. Samples were diluted 1:10 prior to assay. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay coefficient of variation was 12.1%. Sensitivity of the assay was 0.33 ng/tube.

**Insulin-Like Growth Factor-I**

Anterior pituitary gland concentrations of IGF-I were determined in duplicate by RIA (Echternkamp et al., 1990; Funston et al., 1995). One half of each anterior pituitary gland was homogenized in a 15-mL polypropylene tube with 2-mL of homogenization buffer (1% cholic acid, 0.1% SDS, 200 μM phenylmethylsulfonylfluoride, 100 μM EDTA, 1 μM leupeptin, and 1 μM pepstatin) and homogenized on ice with a T25 Ultra-
Turrax tissue dispenser (IKA Works, Wilmington, NC) for 30 s at 20,500 rpm. Anterior pituitary glands were then diluted to 100 mg of AP tissue/mL with homogenization buffer. Homogenates were centrifuged at 12,000 x g for 10 min at 4°C and the supernatant was removed and stored at -20°C. Protein content of the AP homogenates (1:20 dilution) was determined by the Bradford method using reagents provided by Bio-Rad (Hercules, CA). Insulin-like growth factor binding proteins were extracted from all homogenized anterior pituitary gland samples with a 1:17 ratio of sample to acidified ethanol (12.5% 2 N HCl:87.5% absolute ethanol; (Daughaday et al., 1980)). Recombinant human IGF-I (GF-050; Austral Biological, San Ramon, CA) was used as the standard and radioiodinated antigen. Antisera (UB2-495; National Hormone and Peptide Program, NIDDK) was used at a dilution of 1:62,500. Recovery of $[^{125}\text{I}]$IGF-I added to porcine serum before extraction averaged 91 ±3.2%. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay coefficient of variation was 14.8%. Sensitivity of the assay was 10.9 pg/tube.

**Luteinizing Hormone**

Anterior pituitary gland concentrations of LH were determined in triplicate by RIA (Clapper et al., 1998). Porcine LH (AFP3881A; National Hormone and Peptide Program, NIDDK) was used as the radioiodinated antigen and standard. Luteinizing hormone antiserum (AFP15103194; National Hormone and Peptide Program, NIDDK) was used at a dilution of 1:200,000. Anterior pituitary homogenates were diluted 1:25,000 in 0.01 M PBS-0.1% gelatin prior to assay. Inhibition curves of increasing
amounts of sample were parallel to standard curves. Intra-assay coefficient of variation was 9.9%. Sensitivity of the assay was 0.12 ng/tube.

**Kisspeptin-54**

Hypothalamic and AP concentrations of kisspeptin-54 were determined in duplicate by RIA. Human kisspeptin (1443; Tocris Bioscience, Ellsville, MO) was used as the radioiodinated antigen and standard. Kisspeptin antiserum (GQ2; provided by Waljít Dhillo, Imperial College, London, England, UK) was used at a dilution of 1:50,000. The GQ2 kisspeptin antibody cross reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with any other related human RF amide protein (Dhillo et al., 2005). Hypothalami and AP homogenates were diluted 1:30 in 0.01 M PBS-0.1% gelatin prior to assay. Inhibition curves of increasing amounts of sample were parallel to standard curves. Inter-assay coefficients for MBH and AP assays were 9.4% and 11.2%, respectively. Intra-assay coefficients of variation for MBH and AP assays were 4.6% and 8.0%, respectively. Sensitivity of the assay was 9.68 pg/tube.

**Isolation of RNA**

Total RNA was isolated from one half of each AP and MBH using TriReagent (TR118; Molecular Research Company, Cincinnati, OH). Tissue was homogenized in a 15-mL polypropylene tube containing 1-mL of TriReagent with a T25 Ultra-Turrax tissue dispenser (IKA Wroks, Wilmington, NC) for 30 s at 20,500 rpm. The resulting homogenates were diluted to 5-mL with TriReagent. One milliliter of the homogenate was removed from the 15-mL polypropylene tube and pipetted into a 1.5-mL
polypropylene tube. One hundred microliters of 1-bromo-3-chloropropane (BCP; #BP151, Molecular Research Center, Cincinnati, OH) was added to the tubes containing 1-mL of homogenate, mixed, and incubated at room temperature for 5 min. Samples were centrifuged (12,000 x g for 15 min at 4°C) and the resulting aqueous layer was removed. Isopropanol (500 µL) was added to the aqueous layer to precipitate the RNA. The resulting RNA pellet was washed twice with 75% ethanol, centrifuged, dried, and suspended in nuclease-free water. Concentration of RNA was determined via spectrophotometer (Nanodrop, Thermo Scientific, Washington, DE). Purity of RNA was determined by measuring the $A_{260}/A_{280}$ ratio. The ratio of all samples ranged from 1.8 to 2.0.

**cDNA Synthesis**

Any contaminating DNA was removed from 10 µg of AP RNA and 5 µg of MBH RNA for each sample using the Turbo DNA-Free™ Kit (#AM1907; Ambion, Austin, TX) following the manufacturer’s protocol. Two micrograms of the resulting RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit containing random hexamer primers (#4374966; Applied Biosystems, Foster City, CA) following the manufacturer’s protocol.

**Porcine Primers**

Previously published mRNA sequences for the gene of interest and porcine β-actin were used to design specific forward and reverse primers. Primers were designed
using software provided by Integrated DNA Technologies (IDT, Coralville, IA; Table 2.1).

**Real-Time PCR**

Real-time semi-quantitative PCR was used to measure the quantity of MBH ER-\(\alpha\), ER-\(\beta\), Kiss1, and Kiss1R, and AP LH-\(\beta\) mRNA relative to the amount of porcine \(\beta\)-actin mRNA in each sample. Measurements of the relative quantity of the cDNA of interest was carried out using RT\(^2\) Real-Time™ SYBR Green/ROX PCR Master Mix (SuperArray Bioscience Corp., Foster City, CA). Twenty-five microliter reactions were measured using the Stratagene MX3005P quantitative real-time PCR instrument (Agilent Technologies, Foster City, CA). Thermal cycling conditions recommended by the manufacturer (40 cycles of 30 sec at 95°C, 1 min at 55°C, and 1 min at 72°C) were used for all genes except Kiss1, in which the following thermal cycling conditions were used; 45 cycles of 30 sec at 95°C, 1 min and 3 sec at 55°C, and 1 min at 72°C. Concentrations of forward and reverse primers used for the genes of interest were 300 nM, except Kiss1R, in which concentrations of forward and reverse primers were used at a concentration of 800 nM. A linear response was obtained when these concentrations of primer pairs were used with increasing amounts of cDNA. Dissociation curve analysis was performed after each real-time PCR run to confirm that a single amplicon of appropriate melting temperature was present. Additionally, all amplicons were electrophoresed through a 2% agarose gel and stained with ethidium bromide to visualize that only amplicons of the appropriate size were present in each sample.
Table 2.1

<table>
<thead>
<tr>
<th>Gene and Accession Number</th>
<th>Primer</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKiss1 (NM_001134964.1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward: 5’ – GGCAGCTGATGTCTTCTTTCTTT – 3’&lt;br&gt;Reverse: 5’ – CGGGCCTGTAGATCTAGGATT – 3’</td>
<td>89 bp</td>
</tr>
<tr>
<td>pKiss1R (NM_001044624.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Forward: 5’ – CAGGGAACTGACTTGGGATCTCTT – 3’&lt;br&gt;Reverse: 5’ – GCACAGCAGAAACGTCAAATC – 3’</td>
<td>110 bp</td>
</tr>
<tr>
<td>pER-β (NM_001001533)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Forward: 5’ – GGACAGGGATGAAGGGAAATG – 3’&lt;br&gt;Reverse: 5’ – CATGGCCTGGACACAGAGATAC – 3’</td>
<td>124 bp</td>
</tr>
<tr>
<td>pER-α (NM_214220)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Forward: 5’ – GAATGTTGAAGCACAAGCGCCAGA – 3’&lt;br&gt;Reverse: 5’ – ACCGGGCTGTTCCTTCTTAGTGTT – 3’</td>
<td>91 bp</td>
</tr>
<tr>
<td>pLH-β (NM_214080.1)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Forward: 5’ – ATGCTCCAGAGACTGCTTGTGT – 3’&lt;br&gt;Reverse: 5’ – TGCTGGTGTTAAAGGTGATGCAGA – 3’</td>
<td>151 bp</td>
</tr>
<tr>
<td>pβ-actin (U07786.1)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Forward: 5’ – TCAGGCGAGGATGCAAGGAAGG – 3’&lt;br&gt;Reverse: 5’ – AGGTGGACAGCAGGGCCAGGAT – 3’</td>
<td>129 bp</td>
</tr>
</tbody>
</table>

<sup>a</sup>porcine Kiss1; <sup>b</sup>porcine Kiss type 1 receptor; <sup>c</sup>porcine estrogen receptor-beta; <sup>d</sup>porcine estrogen receptor-alpha; <sup>e</sup>porcine luteinizing hormone subunit beta; <sup>f</sup>porcine beta-actin
**Statistical Analysis**

To determine the effect of day of the estrous cycle on serum concentrations of estradiol-17β and progesterone, hypothalamic concentrations of kisspeptin-54, and anterior pituitary concentrations of kisspeptin-54, IGF-I and LH, statistical analyses were performed using the Mixed Procedure of SAS 9.3 (SAS 9.3, SAS). The model for determining differences in serum concentrations of estradiol-17β and progesterone was

\[ Y_{ijkl} = \mu + \text{Pig}_i + \text{Treatment}_j + \text{Pig}_i(\text{Treatment})_j + \text{Day}_k + \text{Treatment}_j \times \text{Day}_k + \text{Pig}_i(\text{Treatment})_j \times \text{Day}_k + e_{ijkl} \]

with repeated measures. The model for determining differences in hypothalamic concentrations of kisspeptin-54, and anterior pituitary concentrations of kisspeptin-54, IGF-I, and LH was

\[ Y_{ijkl} = \mu + \text{Pig}_i + \text{Treatment}_j + \text{Pig}_i(\text{Treatment})_j + \text{Day}_k + \text{Treatment}_j \times \text{Day}_k + \text{Pig}_i(\text{Treatment})_j \times \text{Day}_k + e_{ijkl} \]

Pig within treatment by day was the subplot error term used to test pig, day, and treatment by day effects.

Fold differences in expression of MBH Kiss1, Kiss1R, ER-α, and ER-β and AP LH-β among treatments was determined using the Relative Expression Software Tool (REST; Corbett Research & M. Pfaffl, Technical University Munich). The expression of a target gene is standardized by a non-regulated gene. Relative expression is based on the expression ratio of a target gene versus a reference gene. The expression ratio results of the investigated transcripts were tested for significance by Pair Wise Fixed Reallocation Randomized Test (Pfaffl et al., 2002).
Results

Mean serum concentrations of E₂ were greatest (P<0.05) on d 19 of the estrous cycle and were not different (P>0.05) on days 4, 7, 9, and 14 of the estrous cycle (Figure 2.1). On d 1 of the estrous cycle, mean serum concentrations of estradiol-17β were greater (P<0.05) than on days 4 and 21 of the estrous cycle (Figure 2.1). Mean serum concentrations of estradiol-17β were greater (P<0.05) on d 16 compared to d 21 of the estrous cycle (Figure 2.1).

Serum Estradiol-17β

![Graph showing mean serum concentrations of estradiol-17β](image)

Figure 2.1. Mean serum concentrations of estradiol-17β in gilts throughout various days of the estrous cycle. Means are expressed as least square means ± SEM. a,b,c,d Means with different letters differ (P<0.05) by day.
Mean serum concentrations of progesterone followed the typical pattern observed throughout the estrous cycle in gilts (Figure 2.2). Mean serum concentrations of P4 increased from d 1 through 14 and were greatest (P<0.05) at d 14 of the estrous cycle (Figure 2.2). From d 14 to d 21 of the estrous cycle mean serum concentrations of P4 decreased (P<0.05) (Figure 2.2).

![Serum Progesterone](image)

**Figure 2.2.** Mean serum concentrations of progesterone in gilts throughout various days of the estrous cycle. Means are expressed as least-square means ± SEM. Means with different letters differ (P<0.05) by day.
Mean AP concentrations of IGF-I were greater (P<0.05) on d 21 compared to d 1 of the estrous cycle (Figure 2.3). No other differences among days of the estrous cycle were detected (P>0.05) (Figure 2.3).

**AP IGF-I**

![Graph showing AP IGF-I concentrations across days of the estrous cycle](image)

**Figure 2.3.** Mean AP concentrations of IGF-I in gilts (n=10/d) on d 1, 9, 14, and 21 of the estrous cycle. Data are expressed as least-square means ± SEM. *ab* Means with different letters differ (P<0.05) by day.
Mean AP concentrations of LH were greatest (P<0.05) on day 14 of the estrous cycle (Figure 2.4). Mean AP concentrations of LH were not different (P>0.05) on days 1 and 9, and 1 and 21 of the estrous cycle (Figure 2.4), however mean AP concentrations of LH were greater (P<0.05) on day 9 versus 21 of the estrous cycle (Figure 2.4).

**Figure 2.4.** Mean AP concentrations of LH in gilts (n=10/d) on d 1, 9, 14, and 21 of the estrous cycle. Data are expressed as least-square means ± SEM. abc Means with different letters differ (P<0.05) by day.
No differences were detected (P>0.05) in mean AP concentrations of kisspeptin throughout the estrous cycle (Figure 2.5).

Figure 2.5. Mean AP concentrations of kisspeptin in gilts (n=10/d) on d 1, 9, 14, and 21 of the estrous cycle. Data are expressed as least-square means ± SEM.
No differences were detected (P>0.05) in mean MBH concentrations of kisspeptin throughout the estrous cycle (Figure 2.6).

**Figure 2.6.** Mean MBH concentrations of kisspeptin in gilts (n=10/d) on d 1, 9, 14, and 21 of the estrous cycle. Data are expressed as least-square means ± SEM.
Mean relative expression of MBH ER-α did not differ (P>0.05) in gilts throughout the estrous cycle (Figure 2.7).

**Figure 2.7.** Mean relative expression of MBH ER-α in gilts (n=10/d) on days 1, 9, 14, and 21 of the estrous cycle. Total RNA was DNase treated and analyzed for the level of ER-α by semi-quantitative real-time reverse transcriptase PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the values in pigs on d 1 of the estrous cycle.
Mean relative expression of MBH ER-β was up-regulated ($P<0.05$) on days 9 and 14 compared to day 21 of the estrous cycle (Figure 2.8).

**Figure 2.8.** Mean relative expression of MBH ER-β in gilts ($n=10/d$) on days 1, 9, 14, and 21 of the estrous cycle. Total RNA was DNase treated and analyzed for the level of ER-β by semi-quantitative real-time reverse transcriptase PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the values in pigs on d 1 of the estrous cycle. Means with different letters differ ($P<0.05$) as determined by the relative expression software tool.
Mean relative expression of MBH Kiss1 was greatest (P<0.05) on d 21 of the estrous cycle (Figure 2.9). Mean relative expression of MBH Kiss1 was down-regulated (P<0.05) on days 1 versus 14 and 21 and 9 versus 21 of the estrous cycle (Figure 2.9).

**Figure 2.9.** Mean relative expression of MBH Kiss1 in gilts (n=10/d) on days 1, 9, 14, and 21 of the estrous cycle. Total RNA was DNase treated and analyzed for the level of Kiss1 by semi-quantitative real-time reverse transcriptase PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the values in pigs on d 1 of the estrous cycle. abc Means with different letters differ (P<0.05) as determined by the relative expression software tool.
Mean relative expression of MBH Kiss1R did not differ (P>0.05) in gilts throughout the estrous cycle (Figure 2.10).

**Figure 2.10.** Mean relative expression of MBH Kiss1R in gilts (n=10/d) on days 1, 9, 14, and 21 of the estrous cycle. Total RNA was DNase treated and analyzed for the level of Kiss1R by semi-quantitative real-time reverse transcriptase PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the values on d 1.
Discussion

Kisspeptin, a protein produced by most mammalian species, has been found in many tissues in the body (Li et al., 2008). Kisspeptins are small, neurohormones encoded by the Kiss1 gene (Kotani et al., 2001). After ICV and intra-ARC infusion of kisspeptin antibodies there was a profound decrease in serum concentrations of LH in the rat (Li et al., 2009). Kisspeptin has been shown to elicit the release of LH via ICV, intramuscular, and intravenous infusion in a number of mammalian species (Lents et al., 2008; Sebert et al., 2010; Whitlock et al., 2008). Kisspeptin-54 has also been demonstrated to stimulate the hypothalamo-pituitary gonadal axis in humans (Chan et al., 2012; Dhillo et al., 2005).

Although these studies demonstrated that exogenous kisspeptin can cause the release of LH, it is also thought that endogenous kisspeptin released by the hypothalamus of mammals can cause the release of LH.

In the present study, estradiol-17β and progesterone followed patterns that were indicative of a normal porcine estrous cycle. Henricks et al. (1972) demonstrated that during the luteal phase (from d 4 to 16 of the estrous cycle) plasma concentrations of estrogen were low (< 20 pg/mL/animal). Following that, plasma concentrations of estrogen started to increase and reached their peak (> 50 pg/mL/animal) between 1 and 2 days before estrus (Henricks et al., 1972). Mean plasma concentrations of progesterone increased dramatically from d 2 to 6 of the estrous cycle and continued until they peaked by d 12 of the estrous cycle (Henricks et al., 1972). In the present study, serum concentrations of estrogen started to increase only after plasma concentrations of
progesterone started to decrease, which was temporally related with the next expected LH surge.

Insulin-like growth factor-I has been shown to positively associate with estrogen, in that E$_2$ caused an increase in peripheral and AP concentrations of IGF-I in the pig (Hilleson-Gayne and Clapper, 2005; Rempel and Clapper, 2002). Estrogens are synthesized from androgens via aromatase enzymes (Simpson et al., 1994). Anastrozole is a non-steroidal aromatase-inhibiting compound that has been shown to dramatically reduce the synthesis of estrogens and reduce plasma concentrations of estrogen by 86% in women (Geisler et al., 2001; Mauras et al., 2000). Hilleson-Gayne and Clapper (2005) demonstrated that serum concentrations of IGF-I were greater in boars that were untreated versus boars that were treated with anastrozole. Estrogen has also been demonstrated to affect concentrations of AP IGF-I in the pig as well. Rempel and Clapper (2002) reported that estrogen implanted barrows had greater AP concentrations of IGF-I versus untreated barrows and boars. These data support the notion that E$_2$ could also affect the AP concentrations of IGF-I during various days of the estrous cycle. Gilts slaughtered on the last day of the estrous cycle and at a time directly following greater serum concentrations of E$_2$ (day 19) had greater AP concentrations of IGF-I versus gilts that had lesser serum concentrations of E$_2$ directly following a standing estrus on d 1 of the experiment. Insulin-like growth factor-I has been demonstrated to stimulate the release of LH from cultured AP cells in rats and pigs (Soldani et al., 1994; Whitley et al., 1995). Adam et al. (1998) also demonstrated that peripheral administration of IGF-I can acutely stimulate an increased in plasma concentrations of LH in sheep. Therefore, it is
plausible that IGF-I may affect the AP LH system and work in concert with kisspeptin to cause a greater release of LH.

Mean AP concentrations of LH were greatest on d 14 of the estrous cycle and least on d 1 and 21; a time coincident with a standing estrus and a time temporally related with the next expected estrus, respectively. These data coincide with the patterns of E₂ and P₄. Henricks et al. (1972) demonstrated that serum concentrations of LH decreased after a standing estrus until just before the subsequent estrus when LH increased to its peak. During the peak in LH, serum concentrations of estrogen were increased and serum concentrations of progesterone remained decreased (Henricks et al., 1972). Additionally, Baird and Scaramuzzi (1976) found that basal peripheral concentrations of LH decreased to a level similar to peripheral concentrations of LH during the luteal phase of the estrous cycle following subcutaneous P₄ implantation in ewes. In the present study, it is plausible that increased P₄, in the face of decreased E₂, was providing negative feedback to the MBH and AP during the luteal phase of the estrous cycle causing a buildup of AP LH prior to the pre-ovulatory LH surge.

Past research has shown that there is a correlation between circulating concentrations of sex steroids and hypothalamic concentrations of kisspeptins and expression of Kiss1 (Kauffman et al., 2007; Smith et al., 2005a; Smith et al., 2005b). In the present study, there was no difference in MBH and AP concentrations of kisspeptin-54 throughout various days of the estrous cycle, however there was an increase in relative MBH Kiss1 gene expression versus the first day of the estrous cycle compared to the last day of the estrous cycle. Increased relative MBH Kiss1 gene expression occurred at a
time coincident with greater serum concentrations of E_{2} and greater AP concentrations of LH. Cui et al. (2015) performed an experiment measuring Kiss1 and GnRH gene expression and protein concentration in multiple areas of the hypothalamus in ovariectomized (OVX) and rats treated with E_{2}. During the onset of puberty, kisspeptin-immunoreactive cells increased in the ARC, periventricular nucleus, and preoptic areas (Cui et al., 2015). Levels of both hypothalamic Kiss1 and GnRH gene expression were greater in OVX + E_{2} and/or intact + E_{2} compared to OVX and intact animals that were not treated with E_{2} (Cui et al., 2015). Furthermore, hypothalamic expression of Kiss1 mRNA during estrus was decreased versus other stages of the estrous cycle in the rat (Salehi et al., 2013). These data support the notion that an increase in hypothalamic Kiss1 mRNA and hypothalamic kisspeptin concentration play a role in modulating the activity of estrogen during the time an animal expresses estrus. Though the timing of sample collection impacts the outcome greatly, the present study parallels with past research. During expression of estrus, when serum concentrations of E_{2} were greater, there was a decrease in hypothalamic expression of Kiss1 compared to the last day of the estrous cycle when serum concentrations of E_{2} were lesser.

Kisspeptins bind to their cognate receptor, GPR54, with high affinity (Messager et al., 2005). There is evidence that GnRH neurons possess kisspeptin receptors and after kisspeptin binds to GPR54 there is an increase in hypothalamic concentrations of GnRH (Messager et al., 2005). The Kiss1R gene has been demonstrated to be highly abundant in multiple tissues in the body, including the hypothalamus and anterior pituitary gland (Li et al., 2008). In the present study, hypothalamic Kiss1R gene expression was measured
on various days of the estrous cycle but no differences among days were found. Kisney-Jones et al. (Kinsey-Jones et al., 2009) reported that there was a down-regulation of hypothalamic expression of Kiss1R during a stress-induced suppression of LH in the female rat. Although there were no differences among days of the estrous cycle it is plausible that variations in the expression of the Kiss1R gene and the number of kisspeptin receptors may influence the GnRH pathway and endocrine status of the animal at a time coincident with a standing estrus.

No differences were detected in mean relative expression of MBH ER-α, however mean relative expression of MBH ER-β was decreased on d 21 of the estrous cycle compared to d 9 and 14 of the estrous cycle and tended to be lesser on d 21 compared to d 1 of the estrous cycle. In the rat, selective blockade of ER-α in gonadally intact, cyclic females decreased the release of LH in response to kisspeptin, inhibited the preovulatory LH surge, and blocked ovulation (Roa et al., 2008). Roa et al. (2008) also demonstrated that after selective activation of ER-α there was an increase in LH secretion in response to kisspeptin. This was not the case when ER-β was activated (Roa et al., 2008).

Additionally, when rats were treated with an ER-β antagonist the preovulatory LH surge still occurred, however there was a decrease in acute LH responses to kisspeptin (Roa et al., 2008). Although there are alterations in the MBH expression of ER-α and ER-β in the present study, GnRH neurons may not be direct targets for the actions of E₂ (Herbison and Theodosis, 1992; Shivers et al., 1983). Kisspeptin is a potent secretagogue of GnRH (Dhillo et al., 2005; Gottsch et al., 2004). Additionally, kisspeptin possess estrogen receptors (Franceschini et al., 2006; Smith et al., 2005a; Smith et al., 2006b), and AVPV
expression of Kiss1 is up-regulated at a time coincident with the LH surge (Adachi et al., 2007; Smith et al., 2006b). Therefore, the hypothalamic kisspeptin system and ER-α and –β are closely integrated and alterations in hypothalamic expression of ER-α and –β may cause increased release of kisspeptin, the subsequent GnRH/LH surge, and the attainment of puberty in mammalian species.

In conclusion, kisspeptin has been demonstrated to cause the release of LH in vivo and in vitro in a number of mammalian species, including the gilt (Lents et al., 2008; Sebert et al., 2010; Whitlock et al., 2008). Recent research has also described the relationship between increased peripheral concentrations of E2 and increased hypothalamic concentrations of Kiss1 (Oakley et al., 2009; Smith et al., 2005a; Smith et al., 2006b). In the present study, there were variations in hypothalamic Kiss1 gene expression at key time points in the estrous cycle; particularly just before gilts expressed estrus. Kisspeptin may be an important hormone driving normal reproductive cyclicity, however the timing at which samples are collected is critical and needs to be further investigated.
Chapter 3

EVALUATION OF THE HYPOTHALAMIC KISSPEPTIN SYSTEM DURING THE ATTAINMENT OF PUBERTY IN GILTS

Introduction

Activation of gonadotropin releasing hormone (GnRH) neurons and the release of GnRH is critical for the attainment of puberty, however, research is equivocal as to whether GnRH neurons lack estrogen receptors. Therefore other endocrine pathways that are plausible to cause the preovulatory luteinizing hormone (LH) surge are of interest. Several researchers have hypothesized that kisspeptin works at the level of the hypothalamus to activate GnRH neurons and start the onset of puberty. Through the administration of exogenous kisspeptin it has been shown that kisspeptin increased hypothalamic concentrations of GnRH at puberty in a number of species including primates (Skorupskaite et al., 2014), rodents (Messager et al., 2005), and sheep (Li et al., 2012). Central administration of kisspeptin increased plasma concentrations of LH in prepubertal female rats versus controls (Navarro et al., 2004). Repetitive administration of kisspeptin to prepubertal female lambs increased plasma concentrations of sex steroids and caused a LH surge (Redmond et al., 2011). Therefore, kisspeptin has been found to increase hypothalamic concentrations of GnRH and plasma concentrations of LH during the attainment of puberty in multiple mammalian species.
The expression of hypothalamic Kiss1 is associated with the attainment of puberty in mammalian species. Navarro et al. (2004) reported that immediately before the onset of puberty there was a dramatic increase in the expression of hypothalamic Kiss1 in female and male rats. This same phenomenon has been reported in gonadally intact female monkeys (Shahab et al., 2005). It was also shown that hypothalamic Kiss1 mRNA expression was up regulated in ovariectomized (OVX) and intact rats that were administered E2 compared to OVX and intact rats that received no estradiol-17β (E2) (Cui et al., 2015). Navarro et al. (2004) showed that there was an increase in hypothalamic expression of Kiss1 around the time of puberty in male and female rats. Similarly, there was also an increase in the MBH expression of Kiss1 mRNA during the attainment of puberty in intact female rhesus monkeys (Shahab et al., 2005). Nestor et al. (2012) also showed that the number of kisspeptin neurons in the ARC was greater in post-pubertal ewes versus pre-pubertal lambs. Therefore, it is evident that an increase in hypothalamic Kiss1 gene expression is correlated with hypothalamic concentrations of kisspeptin and an increase in the production of GnRH at a time coincident with the attainment of puberty in mammals.

It is widely accepted that exogenous and endogenous kisspeptin partially control the LH surge (Lents et al., 2008; Luque et al., 2011). A multitude of research has also been done demonstrating the effects of the administration of a kisspeptin antagonist and its effects on the hypothalamo-pituitary-gonadal axis. During ICV infusion of a kisspeptin antagonist in the ewe there was no pulsatile release of LH detected compared to controls (Roseweir et al., 2009). Smith et al. (2011) demonstrated that through the
administration of a Kiss1-receptor antagonist there was a decrease in plasma concentrations of LH in OVX ewes. Intracerebroventricular infusion of a kisspeptin antagonist also caused delayed vaginal opening, decreased uterine and ovarian weights, and prevented the LH and FSH surge in female rats (Pineda et al., 2010). Hence, through the administration of kisspeptin antagonists, it has been proven that kisspeptin is a pivotal hormone that causes the release of LH in sheep, rats, and pigs (Lents et al., 2008).

Kisspeptin has been proven essential in puberty through its intimate relationship with GnRH neurons (Hrabovszky et al., 2007). Measurement of hypothalamic Kiss1 mRNA expression and hypothalamic concentrations of kisspeptin along with other hormones may provide insight into possible answers regarding kisspeptins exact association with the attainment of puberty. Previous research has shown that kisspeptin plays an integral role in the regulation of reproduction, however, changes in the hypothalamic kisspeptin system and related reproductive hormones during the peripubertal period in gilts has not been determined. Therefore, the objectives of the current experiments were to determine 1) plasma concentrations of E$_2$ and P4, 2) AP concentrations of LH, IGF-I and kisspeptin, and 3) MBH expression of Kiss1 and concentrations of kisspeptin during the peripubertal period in the gilt.

**Materials and Methods**

**Animals**

Two experiments were conducted with twenty five crossbred gilts (Duroc x Large White x Landrace) in each experiment. On d 1 of both experiments, gilts were received
and penned (1.83 x 2.44 m) into groups of 3 and 4. Gilts were of similar age (151.5 ± 4.5 d) and weight (104.9 ± 0.3 kg) in both experiments. Gilts were given ad libitum access to water and a corn-soy based diet that contained 3.4 Mcal ME/kg, 18% protein, and 0.9% lysine. On day 1 of both experiments gilts were exposed to a mature boar twice daily. Gilts were exposed to a mature boar for 6 d in the first experiment and 7 d in the second experiment. Gilts in the first experiment were slaughtered on d 7 and gilts in the second experiment were slaughtered on d 8. In the first experiment, blood samples (10 mL) were collected from gilts via jugular venipuncture on d 1 and 3 into sodium heparin tubes. In the second experiment, blood samples (10 mL) were collected from gilts via jugular venipuncture on d 2, 4, and 6 into sodium heparin tubes. All samples were collected on ice, centrifuged (1,500 x g, for 30 min at 4ºC), and frozen within 60 min to prevent protein degradation.

Estrus detection was performed twice daily at 12 h intervals in both experiments. The day gilts stood immobile in the presence of the boar was considered the day they attained puberty (n=8/study). The remaining gilts (n=17/study) that did not stand in estrus within 24 hours of slaughter were considered to be peripubertal. On d 7 of the first experiment and d 8 of the second experiment all gilts were slaughtered at the South Dakota State University Meat Lab when blood, anterior pituitaries (AP), mediobasal hypothalami (MBH), and reproductive tracts were collected. Anterior pituitary glands and MBH were trimmed of connective tissue, bisected midsaggitally, wrapped in aluminum foil, snap frozen in liquid nitrogen, and stored at -80ºC. Following slaughter, reproductive tracts were subjectively evaluated for edema and tone and ovaries were evaluated for
structures including follicles, corpora hemmoragica, corpora lutea, and corpora albicantia to determine if gilts had cycled prior to the experiment. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at South Dakota State University.

_Estradiol-17β_

For both experiments plasma concentrations of E₂ were determined in duplicate by RIA in the same assay. Estradiol-17β (E8875; Sigma Life Science, St. Louis, MO) was the standard and radioiodinated E₂ (#07138228; MP Biomedicals, Solon, OH) was the tracer. Antiserum (GDN#244 anti-estradiol-17β-6-BSA; Fort Collins, CO) was used at a dilution of 1:425,000. Sera (250-μL) were extracted with a 4-mL volume of methyl tert-butyl ether. Recovery of [¹²⁵I]estradiol-17β added to porcine plasma before extraction averaged 94.6 ± 2.5%. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay and inter-assay coefficients of variation were 11.4% and 15.7%, respectively. Sensitivity of the assay was 0.5 pg/tube.

_Insulin-Like Growth Factor I_

Anterior pituitary gland concentrations of IGF-I were determined in duplicate by RIA (Echternkamp et al., 1990; Funston et al., 1995). One half of each anterior pituitary gland was homogenized in a 15-mL polypropylene tube with 2-mL of homogenization buffer (1% cholic acid, 0.1% SDS, 200 μM phenylmethylsulfonylfluoride, 100 μM EDTA, 1 μM leupeptin, and 1 μM pepstatin) and homogenized on ice with a T25 Ultra-Turrax tissue dispenser (IKA Works, Wilmington, NC) for 30 s at 20,500 rpm.
pituitary glands were then diluted to 100 mg of AP tissue/mL with homogenization buffer. Homogenates were centrifuged at 12,000 x g for 10 min at 4°C and the supernatant was removed and stored at -20°C. Protein content of the AP homogenates (1:20 dilution) was determined by the Bradford method using reagents provided by Bio-Rad (Hercules, CA). Insulin-like growth factor binding proteins were extracted from all homogenized anterior pituitary gland samples with a 1:17 ratio of sample to acidified ethanol (12.5% 2 N HCl:87.5% absolute ethanol; (Daughaday et al., 1980)). Recombinant human IGF-I (GF-050; Austral Biological, San Ramon, CA) was used as the standard and radioiodinated antigen. Antisera (UB2-495; National Hormone and Peptide Program, NIDDK) was used at a dilution of 1:62,500. Recovery of $[^{125}\text{I}]$IGF-I added to porcine serum before extraction averaged 83 ± 1.5%. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay coefficient of variation in the first experiment was 14.8%. Intra-assay and inter-assay coefficients of variation in the second experiment were 3.2% and 7.9%, respectively. Sensitivity of the assay for both experiments was 11.9 pg/tube.

**Luteinizing Hormone**

Anterior pituitary gland concentrations of LH were determined in triplicate by RIA (Clapper et al., 1998). Porcine LH (AFP3881A; National Hormone and Peptide Program, NIDDK) was used as the radioiodinated antigen and standard. Luteinizing hormone antiserum (AFP15103194; National Hormone and Peptide Program, NIDDK) was used at a dilution of 1:200,000. Anterior pituitary homogenates were diluted 1:25,000 in 0.01 M PBS-0.1% gelatin prior to assay. Inhibition curves of increasing
amounts of sample were parallel to standard curves. Intra-assay coefficient of variation in the first experiment was less than 1%. Intra-assay and inter-assay coefficients of variation in the second experiment were 16.5% and 15.8%, respectively. Sensitivity of the assay for both experiments was 0.10 ng/tube.

**Kisspeptin-54**

Hypothalamic and AP concentrations of kisspeptin-54 were determined in duplicate by RIA. Human kisspeptin (1443; Tocris Bioscience, Ellsville, MO) was used as the radioiodinated antigen and standard. Kisspeptin antiserum (GQ2; provided by Waljit Dhilllo, Imperial College, London, England, UK) was used at a dilution of 1:50,000. The GQ2 kisspeptin antibody cross reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with any other related human RF amide protein (Dhillo et al., 2005). Hypothalami and AP homogenates were diluted 1:30 in 0.01 M PBS-0.1% gelatin prior to assay. Inhibition curves of increasing amounts of sample were parallel to standard curves. Intra-assay coefficients of variation for MBH and AP assays for the first experiment were 4.6% and 8.0%, respectively. Intra-assay coefficient of variation for all samples of the second experiment was 7.2%. Sensitivity of the assay for both experiments was 10.1 pg/tube.

**Isolation of RNA**

Total RNA was isolated from one half of each AP and MBH using TriReagent (TR118; Molecular Research Company, Cincinnati, OH). Tissue was homogenized in a 15-mL polypropylene tube containing 1-mL of TriReagent with a T25 Ultra-Turrax tissue
dispenser (IKA Works, Wilmington, NC) for 30 s at 20,500 rpm. After homogenization the resulting homogenates were diluted to 5-mL with TriReagent. One milliliter of the resulting homogenate was removed from the 15-mL polypropylene tube and pipetted into a 1.5-mL polypropylene tube. One hundred microliters of 1-bromo-3-chloropropane (BCP; #BP151, Molecular Research Center, Cincinnati, OH) was added to the polypropylene Eppendorf tubes containing 1-mL of homogenate, mixed, and incubated at room temperature for 5 min. Samples were centrifuged (12,000 x g for 15 min at 4ºC) and the resulting aqueous layer was removed. Isopropanol (500 µL) was added to the aqueous layer to precipitate the RNA. The resulting RNA pellet was washed twice with 75% ethanol, centrifuged, dried, and suspended in nuclease-free water. Concentration of RNA was determined via spectrophotometer (Nanodrop, Thermo Scientific, Washington, DE). Purity of RNA was determined by measuring the A$_{260}$/A$_{280}$ ratio. The ratio of all samples ranged from 1.8 to 2.0.

**cDNA Synthesis**

Any contaminating DNA was removed from 10 µg of AP RNA and 5 µg of MBH RNA for each sample using the Turbo DNA-Free™ Kit (#AM1907; Ambion, Austin, TX) following the manufacturer’s protocol. Two µg of the resulting RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit containing random hexamer primers (#4374966; Applied Biosystems, Foster City, CA) following the manufacturer’s protocol.
Porcine Primers

Previously published mRNA sequences for the gene of interest, porcine β-actin, was used to design specific forward and reverse primers. Primers were designed using software provided by Integrated DNA Technologies (IDT, Coralville, IA; Table 3.1).

Real-Time PCR

Real-time semi-quantitative PCR was used to measure the quantity of MBH Kiss1 and Kiss1R and AP LH-β and gonadotropin releasing hormone receptor (GnRHR) mRNA relative to the amount of β-actin mRNA in each sample. Measurements of the relative quantity of the cDNA of interest was carried out using RT² Real-Time™ SYBR Green/ROX PCR Master Mix (SuperArray Bioscience Corp., Foster City, CA). Twenty-five microliter reactions were measured using the Stratagene MX3005P quantitative real-time PCR instrument (Agilent Technologies, Foster City, CA). Thermal cycling conditions recommended by the manufacturer (40 cycles of 30 sec at 95°C, 1 min at 55°C, and 1 min at 72°C) were used for all except Kiss1, in which the following thermal cycling conditions were used; 45 cycles of 30 sec at 95°C, 1 min and 3 sec at 55°C, and 1 min at 72°C. Concentrations of forward and reverse primers used for the genes of interest were 300 nM, except Kiss1R, in which concentrations of forward and reverse primers were used at a concentration of 800 nM. A linear response was obtained when these concentrations of primer pairs were used with increasing amounts of cDNA. Dissociation curve analysis was performed after each real-time PCR run to confirm that a single amplicon of appropriate melting temperature was present. Additionally, all amplicons
were electrophoresed through a 2% agarose gel and stained with ethidium bromide to visualize that only amplicons of the appropriate size were present in each sample.

Table 3.1.

<table>
<thead>
<tr>
<th>Gene and Accession Number</th>
<th>Primer</th>
<th>Amplicon Size</th>
</tr>
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| pKiss1 (NM_001134964.1) | Forward: 5’ – GGCAGCTGATGTTCTTTCTTTTCTTTG – 3’  
Reverse: 5’ – CCGGCCTGATCTCAGATT – 3’ | 89 bp |
| pLH-β (NM_214080.1) | Forward: 5’ – ATGCTCCAGAGACTGCTGTGTG – 3’  
Reverse: 5’ – TGCTGGTGGTAAAGGTGAGA – 3’ | 151 bp |
| GnRHR (NM_214273.1) | Forward: 5’ – AGCCACACTCTGGGAGACTCTGT – 3’  
Reverse: 5’ – AGCTGAGGACTTTGGCATGAGA – 3’ | 101 bp |
| pβ-actin (U07786.1) | Forward: 5’ – TCGCGACAGGAGCTGAGAAGG – 3’  
Reverse: 5’ – AGTGGACAGCGAGCCAGGAG – 3’ | 129 bp |

*a* porcine Kiss1;  
*b* porcine Kiss type 1 receptor;  
*c* porcine luteinizing hormone subunit beta;  
*d* porcine gonadotropin releasing hormone receptor;  
*e* porcine beta-actin
Statistical Analysis

The same statistical analyses were used in both experiments. To determine the effect of day on plasma concentrations of estradiol-17β, hypothalamic concentrations of kisspeptin-54 and anterior pituitary concentrations of kisspeptin-54, IGF-I, and LH, statistical analyses were performed using the GLIMMIX Procedure of SAS 9.3 (SAS 9.3, SAS) to compare pubertal and peripubertal gilts. The model for determining differences in estradiol-17β was $Y_{ijkl} = \mu + \text{Pig}_i + \text{Pubertal Status}_j + \text{Pig}_i(\text{Pubertal Status})_j + \text{Day}_k + \text{Pubertal Status}_j \times \text{Day}_k + \text{Pig}_i(\text{Pubertal Status})_j \times \text{Day}_k + e_{ijkl}$ with repeated measures. Pig within treatment by day was the subplot error term used to test pig, day, and pubertal status by day effects. The model for determining hypothalamic concentrations of kisspeptin and anterior pituitary concentrations of kisspeptin, IGF-I, and LH was $Y_{ijk} = \mu + \text{Pig}_i + \text{Pubertal Status}_j + \text{Pig}_i(\text{Pubertal Status})_j + e_{ijk}$. Pig within pubertal status was the whole plot error term used to test the effect of treatment.

Fold differences in expression of MBH Kiss1 and Kiss1R and AP LH-β and GnRHR among treatments were determined using the Relative Expression Software Tool (REST; Corbett Research and M. Pfaffl, Technical University Munich). The expression of a target gene is standardized by a non-regulated gene. Relative expression is based on the expression ratio of a target gene versus a reference gene. The expression ratio results of the investigated transcripts were tested for significance by Pair Wise Fixed Reallocation Randomized Test (Pfaffl et al., 2002).
Results – Experiment 1

To confirm that gilts had not cycled before both experiments, ovaries were examined post-slaughter and no corpora lutea, corpora albicantia, or corpora hemmorhagica were found.

Mean plasma concentrations of E₂ were not different (P>0.05) in pubertal versus peripubertal gilts on d 1 and 3 of the experiment and were greater (P<0.05) within both groups of gilts comparing d 1 to 3 of the experiment (Figure 3.1). Mean plasma concentrations of E₂ were greater (P=0.02) in pubertal versus peripubertal gilts on d 7 of the experiment (Figure 3.1).

**Serum Estradiol 17-β**

![Graph showing serum estradiol concentrations](image)

**Figure 3.1.** Mean plasma concentrations of estradiol-17β in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least square means ± SEM. abc Means with different letters differ (P<0.05) according to pubertal status and/or day.
No differences were detected (P>0.05) in mean AP concentrations of IGF-I in pubertal versus peripubertal gilts (Figure 3.2).

**Figure 3.2.** Mean AP concentrations of IGF-I in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM.
No differences were detected (P>0.05) in mean AP concentrations of LH in pubertal versus peripubertal gilts (Figure 3.3).

**Figure 3.3.** Mean AP concentrations of LH in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM.
Mean MBH concentrations of kisspeptin were greater (P=0.04) in pubertal gilts compared to peripubertal gilts (Figure 3.4).

**Figure 3.4.** Mean hypothalamic concentrations of kisspeptin in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM. 

\( \text{ab} \) Means with different letters differ (P=0.04) by group.
Mean relative expression of MBH Kiss1 was up-regulated (P=0.005) approximately 2-fold in pubertal gilts compared to peripubertal gilts (Figure 3.5).

**Figure 3.5.** Mean relative expression of hypothalamic Kiss1 in gilts that had (n=8) and had not (n=17) attained puberty. Total RNA was DNase treated and analyzed for the level of Kiss1 by quantitative reverse transcriptase real time PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the pigs that had not attained puberty. ab Means with different letters differ (P=0.005) as determined by relative expression software tool.
No differences were detected (P>0.05) in mean AP concentrations of kisspeptin in pubertal versus peripubertal gilts (Figure 3.6).

**Figure 3.6.** Mean anterior pituitary concentrations of kisspeptin in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM.
Mean relative expression of AP LH-β did not differ (P>0.05) in pubertal versus peripubertal gilts (Figure 3.7).

![Bar chart showing mean relative expression of AP LH-β in gilts](image)

**Figure 3.7.** Mean relative expression of anterior pituitary LH-β in gilts that had (n=8) and had not (n=17) attained puberty. Total RNA was DNase treated and analyzed for the level of LH-β by quantitative reverse transcriptase real time PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the pigs that had not attained puberty.
Mean relative expression of AP GnRHR did not differ (P>0.05) in pubertal versus peripubertal gilts (Figure 3.8).

Figure 3.8. Mean relative expression of anterior pituitary GnRHR in gilts that had (n=8) and had not (n=17) attained puberty. Total RNA was DNase treated and analyzed for the level of GnRHR by quantitative reverse transcriptase real time PCR with normalization for the corresponding level of beta-actin. Data are expressed as a fold change in expression relative to the pigs that had not attained puberty.
Results – Experiment 2

Mean plasma concentrations of E<sub>2</sub> were not different (P>0.10) in pubertal versus peripubertal gilts on d 2, 4, and 8 of the experiment (Figure 3.9). Mean plasma concentrations of E<sub>2</sub> tended to be greater (P=0.06) on d 4 and were greater (P=0.02) on d 6 in pubertal versus peripubertal animals (Figure 3.9).

**Serum Estradiol 17-β**

![Graph showing serum estradiol concentrations](image)

**Figure 3.9.** Mean plasma concentrations of estradiol-17β in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least square means ± SEM. ab Means with different letters differ (P<0.05) according to pubertal status and/or day.
No differences were detected (P>0.05) in mean AP concentrations of IGF-I in pubertal versus peripubertal gilts (Figure 3.10).

**Figure 3.10.** Mean AP concentrations of IGF-I in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM.
Mean AP concentration of LH were less (P=0.01) in pubertal versus peripubertal gilts (Figure 3.11).

**Figure 3.11.** Mean AP concentrations of LH in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM. Means with different letters differ (P=0.01) by group.
No differences were detected (P>0.05) in mean MBH concentrations of kisspeptin in pubertal versus peripubertal gilts (Figure 3.12).

**MBH Kisspeptin**

![Bar chart showing MBH Kisspeptin concentrations](image)

**Figure 3.12.** Mean hypothalamic concentrations of kisspeptin in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM.
Mean relative expression of MBH Kiss1 did not differ (P>0.05) in pubertal versus peripubertal gilts (Figure 3.13).

**Figure 3.13.** Mean relative expression of hypothalamic Kiss1 in gilts that had (n=8) and had not (n=17) attained puberty. Total RNA was DNase treated and analyzed for the level of Kiss1 by quantitative reverse transcriptase real time PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the pigs that had not attained puberty.
Mean AP concentrations of kisspeptin-54 were greater (P=0.04) in pubertal versus peripubertal gilts (Figure 3.14).

Figure 3.14. Mean anterior pituitary concentrations of kisspeptin in gilts that had (n=8) and had not (n=17) attained puberty. Data are expressed as least-square means ± SEM. abMeans with different letters differ (P=0.04) by group.
Mean relative expression of AP LH-β did not differ (P>0.05) in pubertal versus peripubertal gilts (Figure 3.15).

**Figure 3.15.** Mean relative expression of anterior pituitary LH-β in gilts that had (n=8) and had not (n=17) attained puberty. Total RNA was DNase treated and analyzed for the level of LH-β by quantitative reverse transcriptase real time PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the pigs that had not attained puberty.
Mean relative expression of AP GnRHR did not differ (P>0.05) in pubertal versus peripubertal gilts (Figure 3.16).

**Figure 3.16.** Mean relative expression of anterior pituitary GnRHR in gilts that had (n=8) and had not (n=17) attained puberty. Total RNA was DNase treated and analyzed for the level of GnRHR by quantitative reverse transcriptase real time PCR with normalization for the corresponding level of beta-actin. Data are expressed as a fold change in expression relative to the pigs that had not attained puberty.
Discussion

Plasma concentrations of E\textsubscript{2} increase as animals approach puberty, which occurs at a time around standing estrus. This increase in E\textsubscript{2} causes the preovulatory GnRH and LH surge. Gonadotropin releasing hormone neurons are scattered throughout the hypothalamus and are considered to be the gatekeepers for the attainment of puberty, however, new research indicates there may be several factors controlling the attainment of puberty, including kisspeptin (Silverman et al., 1987; Silverman et al., 1979). Because research is equivocal as to whether GnRH neurons possess estrogen receptors it is thought that other factors contribute to the attainment of puberty.

Kisspeptin is a neurohormone that is produced by the Kiss1 gene (Kotani et al., 2001). Kisspeptin and kisspeptin neurons have been demonstrated to associate intimately with GnRH neurons in the hypothalamus (Hrabovszky et al., 2007). It was reported that deletions or mutations in GPR54 in the mouse resulted in infertility and the failure to attain puberty (de Roux et al., 2003; Seminara et al., 2003). Additionally, deletions and mutations in the Kiss1 gene in humans and mice have been associated with difficulties in the attainment of puberty (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). These data support the notion that the hypothalamic kisspeptin system is associated with the attainment of puberty in multiple mammalian species.

Plasma concentrations of estradiol-17\beta have been demonstrated to be increased during the attainment of puberty in gilts (Karlbom et al., 1982). In the present studies, plasma concentrations of E\textsubscript{2} were the same among all animals upon their arrival in both
experiments. By the end of experiment 1, gilts that were considered to be pubertal by exhibiting a standing estrus response had greater plasma concentrations of E₂ than gilts that were considered peripubertal. Gilts in experiment 2 were slaughtered a day later, however, there was no difference in plasma concentrations of E₂, but plasma concentrations of E₂ were greater on d 6 (2 days prior) in gilts that had attained puberty versus gilts that were peripubertal. It is worth noting that this difference in plasma concentrations of E₂ may influence MBH and AP concentrations of hormones comparing the puberty experiments. Therefore, it is apparent that the timing of sample collection and which day gilts are slaughtered greatly affected plasma concentrations of E₂.

In both experiments there was no difference in AP concentrations of IGF-I in gilts that pubertal compared to gilts that were peripubertal. Clapper and Taylor (2011) demonstrated that on d 19 of the estrous cycle AP concentrations of IGF-I were greater than on d 7, 13, or 22 of the estrous cycle. Insulin-like growth factor I has also been shown to have positive effects on the LH surge in a number of species (Adam et al., 2000; Stewart et al., 1996). In addition to its effects on LH, IGF-I has also been shown to induce an increase in hypothalamic expression of Kiss1 mRNA during the onset of puberty in rats (Hiney et al., 2009). Hiney et al. (2009) also demonstrated that when rats were administered an IGF-I inhibitor there was a decrease in hypothalamic Kiss1 mRNA expression in the rat. Therefore, it is plausible that IGF-I may influence hypothalamic concentrations of kisspeptin and expression of Kiss1 mRNA, however, the exact mechanism has not been determined. Previous research has indicated that the effects of IGF-I are modulated by insulin-like growth factor binding proteins (IGFBP) (Bourner et
al., 1992; Ling et al., 1993). Insulin-like growth factor binding proteins can have either inhibitory or stimulatory effects (Mohan and Baylink, 2002), which can also be tissue specific (Hoeflich et al., 2000; Slootweg et al., 1995). Additionally, direct actions of IGFBP that are independent of IGF-I may exist (Andress, 1998). It is plausible that IGFBP may contribute in the regulation of the hypothalamic kisspeptin system, however they were not quantified in the present experiments.

Puberty in the gilt can be defined as the first time the gilt expresses a standing estrus. It is widely accepted during the attainment of puberty the preovulatory LH surge occurs followed by ovulation approximately 24-36 h later (Driancourt et al., 2013). Although the LH surge was not measured in this experiment it occurs during the time before a standing estrus in the gilt. In the first experiment there was no difference in AP concentrations of LH in pubertal versus peripubertal gilts. However, in the second puberty experiment there were decreased AP concentrations of LH in gilts that had attained puberty versus peripubertal gilts. Comparing this with MBH and AP concentrations of kisspeptin and hypothalamic Kiss1 mRNA expression, there is an apparent difference in hormone levels when gilts are slaughtered one day later with respect to the start of boar exposure. In the first experiment both hypothalamic Kiss1 mRNA expression and MBH concentrations of kisspeptin were greater in gilts that had attained puberty compared to peripubertal gilts. In the second puberty experiment there were no differences in hypothalamic Kiss1 mRNA expression and MBH concentrations of kisspeptin comparing pubertal and peripubertal gilts. During the attainment of puberty there is an increase in hypothalamic Kiss1 mRNA expression in rodents and sheep.
(Navarro et al., 2004; Shahab et al., 2005), which correlates with the first puberty experiment. Although the second experiment does not correlate with previous research, we think it is due to timing of sample collection.

There was no difference in AP concentrations of kisspeptin between pubertal and peripubertal gilts in the first experiment, however AP concentrations of kisspeptin were greater in gilts that were pubertal compared to gilts that were peripubertal in the second study. When gilts were slaughtered a day later there was a decrease in AP concentrations of LH in pubertal gilts versus peripubertal gilts as well as an increase in AP concentrations of kisspeptin. Navarro et al. (2004) demonstrated that central administrations of kisspeptin increased plasma concentrations of LH in female rats compared to controls. Also, repetitive administration of kisspeptin to prepubertal female lambs increased plasma concentrations of sex steroids as well as caused the preovulatory LH surge (Redmond et al., 2011). In experiment 1 there was an increase in plasma concentrations of E_2 in pubertal gilts versus peripubertal gilts on the day gilts were slaughtered. On the same day, there was also an increase in hypothalamic concentrations of kisspeptin and MBH expression of Kiss1, however, there was no difference in AP concentrations of kisspeptin and LH comparing pubertal to peripubertal gilts.

In experiment 2 there was no difference in plasma concentrations of E_2 between treatments on the day gilts were slaughtered (d 8), however, plasma concentrations of E_2 were greater on d 6 in pubertal animals. Additionally, there was no difference in hypothalamic concentrations of kisspeptin and expression of Kiss1 between treatments, however, there was an increase in AP concentrations of kisspeptin and a decrease in
concentrations of LH in pubertal gilts in the second study. A plausible mechanism could be that hypothalamic expression of Kiss1 is increasing at a time temporally related with an increase in release of AP LH (Lents et al., 2008; Sebert et al., 2010; Whitlock et al., 2008). At a time coincident with an increase in hypothalamic expression of Kiss1 there was an increase in hypothalamic concentrations of kisspeptin in the present study. Kisspeptin receptor expression and kisspeptin receptors have been found to be co-expressed with GnRH neurons in the hypothalamus and in the AP (Hrabovszky et al., 2007; Irwig et al., 2004; Li et al., 2012). Additionally, peripheral administration of kisspeptin-54 has been shown to activate GnRH neurons in the hypothalamus (Caraty et al., 2007). Therefore, an increase in MBH and/or AP concentrations of kisspeptin may increase kisspeptin receptor binding in the MBH and/or AP potentially causing the increased release of GnRH and/or LH, respectively. Although the exact mechanism of the interaction of AP LH and MBH and AP kisspeptin in the pig is not fully understood, these data indicate the existence of this possible mechanism.

Although no differences were found in AP expression of LH-β and GnRHR mRNA between treatments in the present studies, it is plausible that during the attainment of puberty, which is coupled with an increase in peripheral concentrations of E₂, there is an up-regulation of AP expression of LH-β and GnRHR. During the time of the preovulatory LH surge AP LH-β and GnRHR mRNA expression are up-regulated in the rat (Haisenleder et al., 1988; Seong et al., 1998). Luteinizing hormone subunit-beta confers the biological activity of LH and at times during the preovulatory LH surge AP LH-β mRNA expression is up regulated nearly 3-fold in the rat (Haisenleder et al., 1988).
Under the influence of both progesterone and estrogen, rats exhibited an increase in AP expression of GnRHR mRNA compared to rats that were only under the influence of estrogen (Seong et al., 1998). Turzillo et al. (1994) found that E₂ caused an increase in AP expression of GnRHR mRNA in the ewe. Although no differences in AP expression of GnRHR and LH-β were found in the present experiments, we believe that the timing of sample collection and/or species differences may have impacted our results. Haisenleder et al. (1988) found differences in the rat, however, our timing was not as precise as theirs due the rat having a shorter estrous cycle.

Timing is critical in detecting differences in hormone concentrations that occur during the peripubertal period. Following relocation and boar exposure, which caused gilts to attain puberty, there were differences in MBH and AP concentrations of kisspeptin and Kiss1 mRNA expression in gilts slaughtered 7 days versus gilts slaughtered 8 days following the start of boar exposure. Although there were variations in hypothalamic, anterior pituitary, and peripheral levels of hormones in the present studies due to timing, changes within the hypothalamic kisspeptin system are consistent with what is found in other mammalian species.
Chapter 4

Summary and Conclusions

The results of these experiments provide evidence that MBH and AP concentrations of kisspeptin and Kiss1 mRNA expression differ throughout the estrous cycle and during the peripubertal period. The levels of MBH, AP, and plasma hormones seemed to be equivocal comparing both of the puberty experiments. Timing of sample collection is important when measuring tissue hormone levels throughout the estrous cycle and during the attainment of puberty. It is plausible that kisspeptin may be a driving factor in the GnRH and LH surge, which is a time temporally related with a standing estrus.

During the estrous cycle there were increased levels of AP LH on d 14 versus d 21 of the estrous cycle, indicating that the AP released LH between those times and the preovulatory LH surge had occurred. At a time temporally related with the released of AP LH, hypothalamic Kiss1 mRNA expression was 2.5-fold greater versus when AP concentrations of LH were increased. Relative expression of MBH ER-β mRNA was also down regulated at a time coincident with increased MBH Kiss1 mRNA expression and a standing estrus. This decrease in expression may have been caused by kisspeptin or other reproductive hormones during a standing estrus, which is temporally related with the preovulatory LH surge.

During the first experiment of the puberty study gilts were slaughtered on d 7 and there was increased hypothalamic expression of Kiss1 and increased MBH concentrations
of kisspeptin in pubertal versus peripubertal gilts. This occurred during the time when gilts stood in estrus; a time temporally related with the preovulatory LH surge. During the second experiment of the puberty study gilts were slaughtered on d 8 and there were no differences in hypothalamic Kiss1 expression and MBH concentrations of kisspeptin between treatments, however, AP concentrations of kisspeptin were greater in pubertal gilts compared to peripubertal gilts. Pubertal gilts also had decreased AP concentrations of LH versus peripubertal gilts. This indicates that an increase in AP concentrations of kisspeptin and a decrease in AP concentrations of LH occur at a time coincident with each other and during a standing estrus. It is plausible that this occurred because kisspeptin was released from the hypothalamus into the hypothalamo-pituitary portal plexus, traveled to the AP, and in concert with GnRH, caused the release of LH.

Therefore, these studies agree with previous research and support the notion that during the time of a standing estrus and the expected preovulatory LH surge, there was an increase in hypothalamic Kiss1 expression and/or an increase in MBH and/or AP concentrations of kisspeptin.
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