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Evaluation of the Hypothalamic Kisspeptin System throughout the Estrous Cycle in Gilts

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Abstract

Kisspeptin has been demonstrated to affect reproductive cyclicity and the attainment of puberty in multiple species, presumably through its actions on gonadotropin releasing hormone and luteinizing hormone. Kisspeptin administration causes increased plasma concentrations of LH in pigs, sheep, and rats. The objective of this experiment was to evaluate changes in the hypothalamic kisspeptin system throughout the estrous cycle in gilts. Estrus was synchronized in forty crossbred gilts (191 d, 121 kg) and estrus detection was performed by exposing gilts to a mature boar. 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 medial basal hypothalami, anterior pituitary glands, and blood were collected. Relative expression of hypothalamic kisspeptin (KISS1), kisspeptin receptor (KISS1R), estrogen receptors-a, anterior pituitary gland GnRH receptor, β-actin, and GAPDH 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 2.3 fold (P = 0.003) on d 9 versus d 21 of the estrous cycle, but was not different (P > 0.05) among the remaining days of the estrous cycle. Relative expression of estrogen receptor-b 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, but was not different (P > 0.05) among the remaining days. Relative expression of anterior pituitary gland GnRH receptor was increased (P < 0.01) on d 1 and 21 versus d 9 and 14. These data support the notion that medial basal hypothalamic expression of KISS1 changes throughout the estrous cycle and may influence reproductive cyclicity in the gilt.

Keywords

Kisspeptin, Gilts, Estrous Cycle, GnRH

1. Introduction

Gonadotropin releasing hormone (GnRH) is a decapeptide hormone essential to reproduction through its actions on the release and synthesis of gonadotropins [1] [2] [3]. Circulating concentrations of gonadotropins fluctuate throughout the estrous cycle and stimulate ovarian follicular development, while lutenizing hormone (LH) in particular is luteotrophic in many species and is responsible for causing ovulation. It is widely accepted that progesterone and estradiol −17 b control the release of gonadotropins through positive and negative feedback, however, the exact mechanisms by which this occurs are not clear. It would be expected that GnRH neurons bear estrogen receptors, however, data remains equivocal as to the presence of estrogen receptors on the GnRH neurons [1] [2] [3]. Thus, the release of GnRH may be controlled by other hormones, one of which is kisspeptin.

Kisspeptin may elicit the direct release of GnRH because it has been found that kisspeptin neuronal axons are associated with the dendrites of GnRH neurons [2]. It has also been shown that the kisspeptin receptor, GPR54, is expressed by GnRH neurons and is directly stimulated by kisspeptin to cause the release of GnRH [4].

Recently, research has been performed to ascertain the relationship between the expression of hypothalamic kisspeptin (KISS1) and plasma concentrations of estradiol-17β (E_2) [5] [6]. An increase in circulating concentrations of E_2 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 [6] [7]. In the rat, 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 [7] [8]. Clarkson *et al.* [9] reported that mice bearing deletions in the kisspeptin receptor 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.

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 estradiol-17β and progesterone, anterior pituitary gland (AP) concentrations of LH, kisspeptin, and insulin like growth factor-I (IGF-I), medial basal hypothalamus (MBH) concentrations of kisspeptin and MBH expression of KISS1, kisspeptin receptor (KISS1R), estrogen receptor

alpha (ER-α), estrogen receptor beta (ER-β) and AP GnRH receptor (GnRHR) throughout the porcine estrous cycle.

2. Materials and Methods

2.1. Animals and Procedures

Forty crossbred (Duroc × Large White × Landrace) gilts of similar age (191 ± 11.6 d) and weight (120.7 ± 8.8 kg) were used in this experiment. Gilts received ad libitum access to water and a corn-soy based diet that contained 3.4 Mcal ME/kg, 18% protein, and 0.9% lysine. Each gilt was administered an intramuscular injection of PG600 (Merck Animal Health, Summitt, NJ) 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 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), 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 4˚C then serum was collected by centrifugation (1500 ×g for 30 minutes at 4˚C) and stored at −20˚C. Anterior pituitary glands and MBH were trimmed of connective tissue, bisected midsaggitally, wrapped in aluminum foil, snap frozen in liquid nitrogen, and stored at −80˚C. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at South Dakota State University.

2.2. Hormone Assays

Serum concentrations of estradiol-17β were determined in duplicate in all blood samples by radioimmunoassay (RIA). Estradiol-17β (E8875; Sigma Life Science, St. Louis, MO) was the standard and radioiodinated estradiol-17β (#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-mL) 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.

Serum concentrations of progesterone (P_4) 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.

Anterior pituitary gland concentrations of IGF-I were determined in duplicate by RIA [10] [11]. One half of each anterior pituitary gland was homogenized in homogenization buffer (1% cholic acid, 0.1% SDS, 200 mM phenylmethylsulfonylfluoride, 100 mM EDTA, 1 mM leupeptin, and 1 mM pepstatin). Anterior pituitary glands were diluted to 100 mg of AP tissue/mL with homogenization buffer. Homogenates were centrifuged at 12,000 ×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; [12]). 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 [125I] IGF-I added to porcine serum before extraction averaged 91% ± 3.2%. Intra-assay coefficient of variation was 14.8%. Sensitivity of the assay was 10.9 pg/tube.

Anterior pituitary gland concentrations of LH were determined in triplicate by RIA [13]. 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.

Hypothalamic and AP concentrations of kisspeptin 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 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 [14]. 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 9.7 pg/tube.

2.3. Reverse Transcriptase Polymerase Chain Reaction (PCR) and Real-Time PCR

Total RNA was isolated from one half of each MBH and AP using TriReagent (TR118, Molecular Research Company, Cincinnati, OH). Purity of RNA was determined by measuring the A260/A280 ratio. The ratio of all samples ranged from 1.8 to 2.0. The integrity of RNA was confirmed by agarose gel electrophoresis. Samples were treated with DNase according to the manufacturer's protocol (TurboDNA-free kit, Applied Biosystems, Foster City, CA, USA). Reverse transcriptase PCR was used to measure the abundance of each specific mRNA relative to the abundance of porcine β-actin and porcine GAPDH in the total RNA isolated from MBH and AP tissue. Expression of β-actin and GAPDH did not differ among days. Primer pairs used for specific amplification of MBH expression of KISS1, KISS1R, ER-α, ER-β, AP GnRHR, β-actin and GAPDH are listed in Table 1. Reactions were measured using the Stratagene MX3000P quantitative real-time PCR instrument (Agilent Technologies, Foster City, CA) using 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). 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 800 nM due to its low abundance. 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 and confirmed 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.

2.4. Statistical Analysis

The effect of day of the estrous cycle on serum concentrations of estradiol-17β, and progesterone, was analyzed by the Proc Mixed procedure with repeated measures using the JMP 8.0 program (Statistical Analysis System [SAS], Cary, NC, USA). The effect of day of the estrous cycle on AP concentrations of IGF-I, LH, kisspeptin and MBH concentrations of kisspeptin was analyzed by one-way analysis of variance of SAS. Fold changes in relative expression of MBH ERα, ERβ, KISS1, KISS1R, and AP GnRHR were determined using the Relative Expression Software Tool [15].

3. Results

Mean serum concentrations of E_2 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 1(a)). 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. Mean serum concentrations of estradiol-17β were greater ($P < 0.05$) on d 16 compared

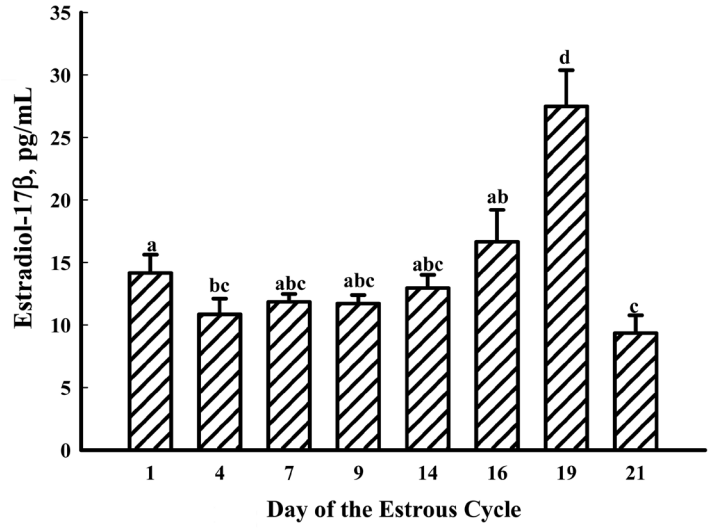
Table 1. Forward and reverse primers for real-time PCR for porcine mRNA.

Gene and Accession Number	Primer	Amplicon Size
pKISS1 (NM_001134964.1)[a]		
Forward	5'-GGCAGCTGATGTTCTTTCTTTG-3'	89 bp
Reverse	5'-CGGGCCTGTAGATCTAGGATT-3'	
pKISS1R (NM_001044624.2)[b]		
Forward	5'-CAGGGAACTCACTTGTCATCTT-3'	110 bp
Reverse	5'-GCACAGCAGAAACGTCAAATC-3'	
pER-β (NM_001001533)[c]		
Forward	5'-GGACAGGGATGAAGGGAAATG-3'	124 bp
Reverse	5'-CATGGCCTTGACACAGAGATAC-3'	
pER-α (NM_214220)[d]		
Forward	5'-GAATGTTGAAGCACAAGCGCCAGA-3'	91 bp
Reverse	5'-ACCGGGCTGTTCTTCTTAGTGTGT-3'	
pGnRHR (AF017079.1)[f]		
Forward	5'-AGCCAACCTGTTGGAGACTCTGAT -3'	101 bp
Reverse	5'-AGCTGAGGACTTTGCAGAGGAACT-3'	
pGAPDH (AF017079.1)[f]		
Forward	5'-GCAAAGTGGACATTGTCGCCATCA-3'	105 bp
Reverse	5'-TGACTGTGCCGTGGAATTTGCCAT-3'	
pβ-actin (U07786.1)[g]		
Forward	5'-TCGCCGACAGGATGCAGAAGGA-3'	129 bp
Reverse	5'-AGGTGGACAGCGAGGCCAGGAT-3	

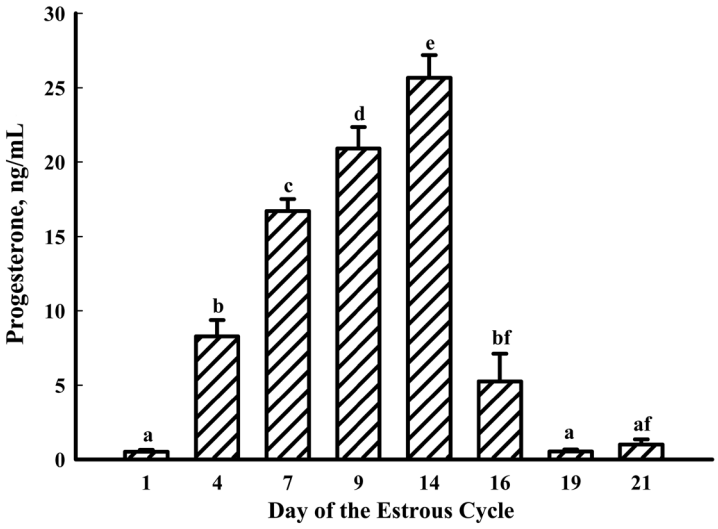
[a]porcine KISS1; [b]porcine KISS1 type 1 receptor; [c]porcine estrogen receptor-beta; [d]porcine estrogen receptor-alpha; [e]porcine GnRHR; [f]porcine glyeraldehyde 3-phosphate dehyrdogenase; [g]porcine beta-actin.

to d 21 of the estrous cycle. Mean serum concentrations of progesterone followed the typical pattern observed throughout the estrous cycle in gilts (**Figure 1(b)**). Mean serum concentrations of P_4 increased from d 1 through 14 and were greatest ($P < 0.05$) at d 14 of the estrous cycle. From d 14 to d 21 of the estrous cycle mean serum concentrations of P_4 decreased ($P < 0.05$).

Mean AP concentrations of IGF-I were greater ($P < 0.05$) on d 21 compared to d 1 of the estrous cycle (**Figure 2(a)**). No other differences among days of the estrous cycle were detected ($P > 0.05$). Mean AP concentrations of LH were greatest ($P < 0.05$) on day 14 of the estrous cycle (**Figure 2(b)**). Mean AP concentrations of LH were not different ($P > 0.05$) on days 1 and 9, and 1 and 21 of



(a)



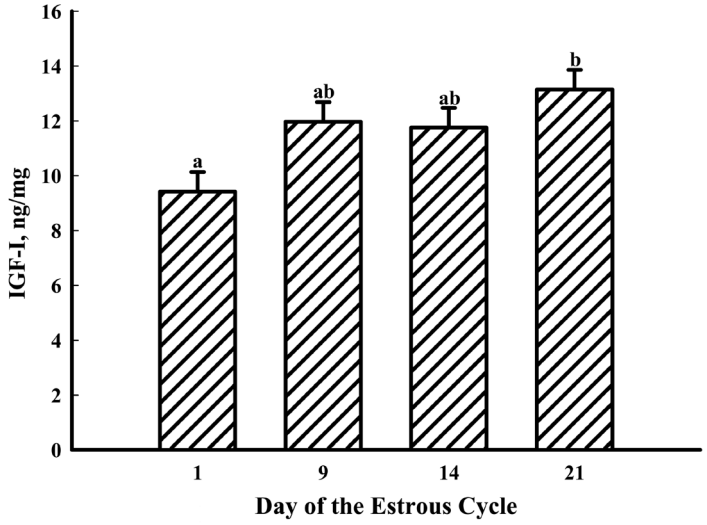
(b)

Figure 1. Mean serum concentrations of estradiol-17β (a) and progesterone (b) in gilts throughout various days of the estrous cycle. Means are expressed as least square means ± SEM. [abcdef]Means with different letters differ ($P < 0.05$) by day.

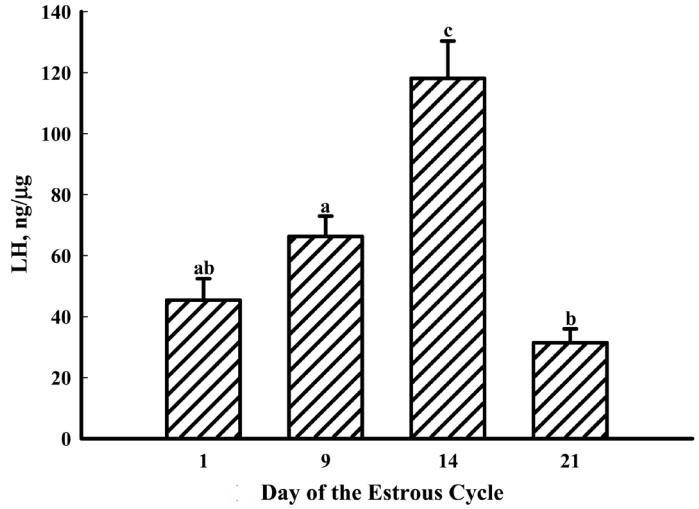
the estrous cycle, however, mean AP concentrations of LH were greater ($P < 0.05$) on day 9 versus 21 of the estrous cycle.

No differences were detected ($P > 0.05$) in mean AP concentrations of kisspeptin throughout the estrous cycle (**Figure 3(a)**). No differences were detected ($P > 0.05$) in mean MBH concentrations of kisspeptin throughout the estrous cycle (**Figure 3(b)**).

Mean relative expression of MBH ER-α did not differ ($P > 0.05$) in gilts throughout the estrous cycle (**Figure 4(a)**). 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 4(b)**). Mean relative expression of AP GnRHR was up-regulated ($P < 0.01$) on d 1 and 21 versus d 9 and 14.



(a)



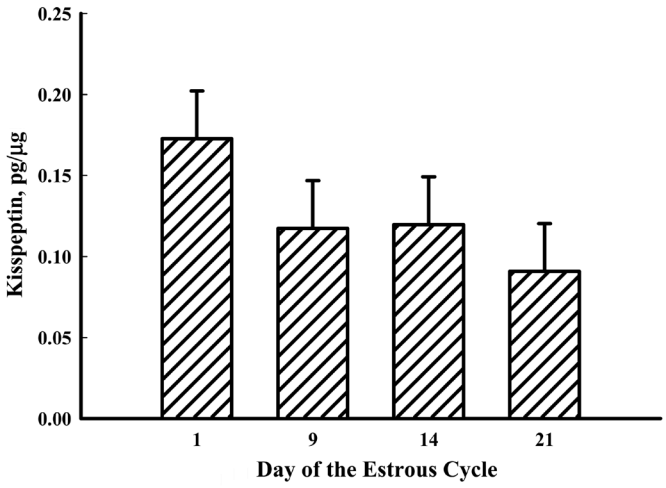
(b)

Figure 2. Mean AP concentrations of IGF-I (a) and 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.

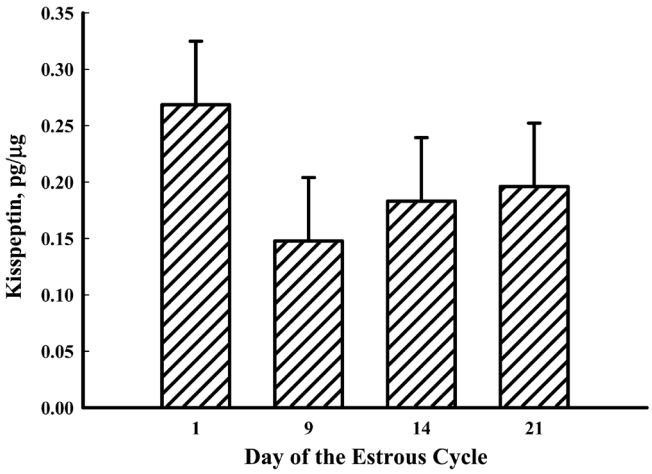
Mean relative expression of MBH KISS1 was greatest ($P < 0.05$) on d 21 of the estrous cycle (**Figure 5(a)**). Mean relative expression of MBH KISS1 was down-regulated ($P < 0.05$) on days 1 versus 14 and 21 and 9 versus 21 of the estrous cycle. Mean relative expression of MBH KISS1R (**Figure 5(b)**) did not differ ($P > 0.05$) in gilts throughout the estrous cycle.

4. Discussion

Kisspeptin, a protein produced by most mammalian species, has been found in many tissues throughout the body [16]. Kisspeptins are small, neurohormones encoded by the KISS1 gene [17]. After ICV and intra-ARC infusion of kisspeptin antibodies there was a profound decrease in serum concentrations of LH in the



(a)

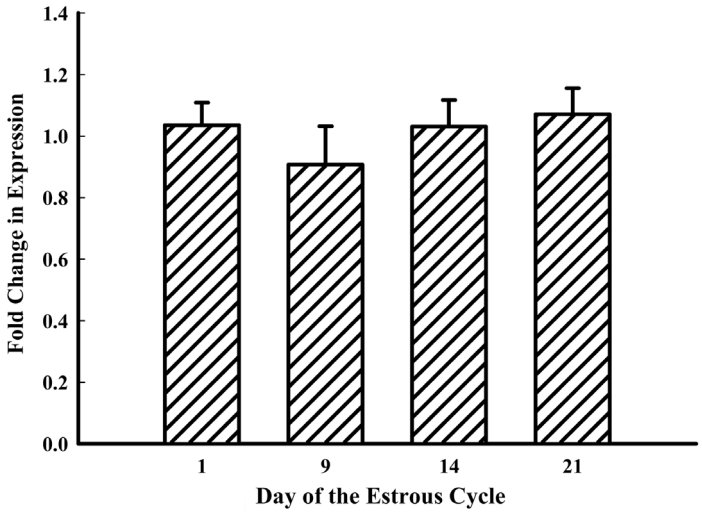


(b)

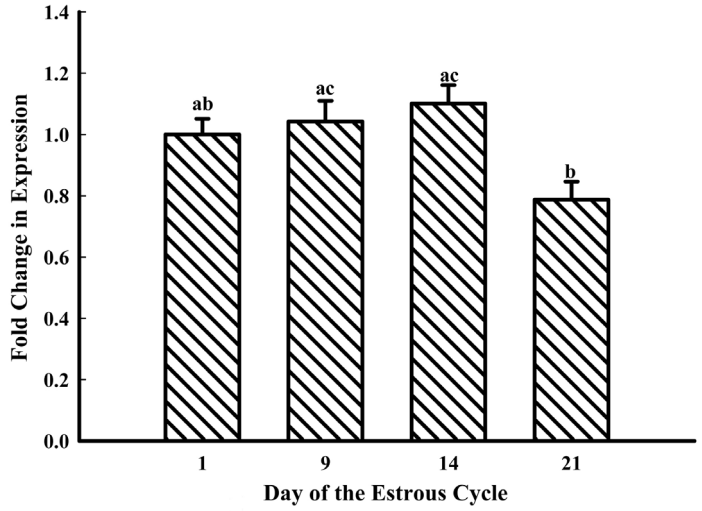
Figure 3. Mean AP (a) and MBH (b) concentrations of kisspeptin in gilts (n = 10/d) on d 1, 9, 14, and 21 of the estrous cycle. Data are expressed as least-square means ± SEM.

rat [18]. Kisspeptin has been shown to elicit the release of LH via ICV, intramuscular, and intravenous infusion in a number of mammalian species [19] [20] [21]. Kisspeptin-54 has also been demonstrated to stimulate the hypothalamo-pituitary gonadal axis in humans [14] [22]. 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.* [23] 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 [20]. Mean plasma concentrations of progesterone increased dramatically from d 2 to 6 of the estrous cycle



(a)

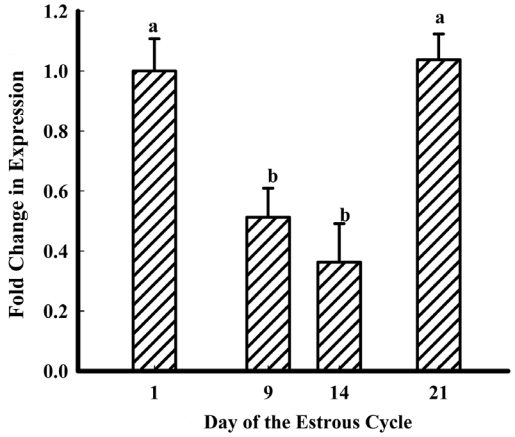


(b)

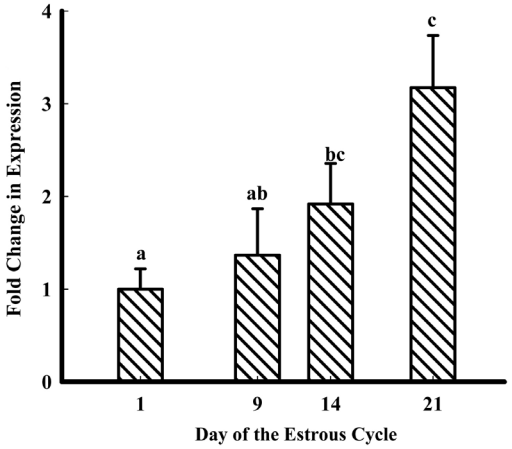
Figure 4. Mean relative expression of MBH ER-α (a) and ER-β (b) in gilts (n = 10/d) on days 1, 9, 14, and 21 of the estrous cycle. Total RNA was DNase treated and analyzed for the level of ER-α by semi-quantitative real-time reverse transcriptase PCR with normalization for the corresponding level of β-actin and GAPDH. 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.

and continued until they peaked by d 12 of the estrous cycle [23]. In the present study, serum concentrations of estrogen started to increase only after plasma concentrations of progesterone started to decrease, which was temporally related to the next expected LH surge.

Insulin-like growth factor-I has been shown to be positively associated with estrogen, in that E_2 caused an increase in peripheral and AP concentrations of IGF-I in the pig [24] [25]. Estrogens are synthesized from androgens via aromatase enzymes [26]. Anastrozole is a non-steroidal aromatase-inhibiting compound



(a)



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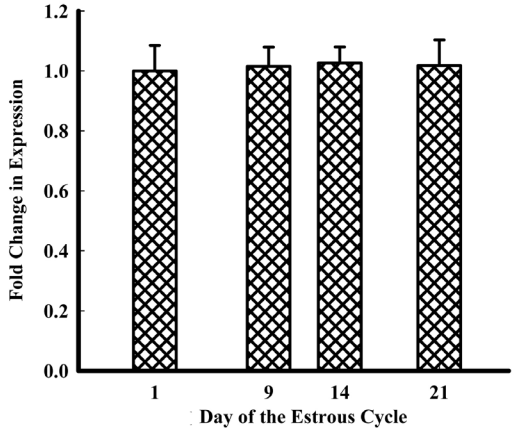

(c)

Figure 5. Mean relative expression of GnRHR (a), MBH KISS1 (b), and KISS1R (c) 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, KISS1R, and GnRHR by semi-quantitative real-time reverse transcriptase PCR with normalization for the corresponding level of β-actin and GAPDH. 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.

that has been shown to dramatically reduce the synthesis of estrogens and reduce plasma concentrations of estrogen by 86% in women [27] [28]. Hilleson-Gayne and Clapper [24] 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 [25] 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 [29] [30]. Adam *et al.* [31] also demonstrated that peripheral administration of IGF-I can acutely stimulate an increase 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 E_2 and P_4. Henricks *et al.* [23] 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 [23]. Additionally, Baird and Scaramuzzi [32] 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 P_4 implantation in ewes. In the present study, it is plausible that increased P_4, in the face of decreased E_2, 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 kisspeptin and expression of KISS1 [6] [33] [34]. 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 on 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.* [35] performed an experiment measuring KISS1 and GnRH gene expression and protein concentration in multiple areas of the hypothalamus in ovariectomized (OVX) rats treated with E_2. During the onset of puberty, kisspeptin-immunoreactive cells increased in the ARC, periventricular nucleus, and

preoptic areas [35]. 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 [35]. Furthermore, hypothalamic expression of KISS1 mRNA during estrus was decreased versus other stages of the estrous cycle in the rat [36]. 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 less.

In the present study relative expression of AP GnRHR was increased during the periovulatory period of the estrous cycle as has been previously documented [37]. This, coupled with the fact that MBH KISS1 expression was increased at a coincident time, may reflect a mechanism by which kisspeptin and GnRH receptors work together to cause the preovulatory LH surge. Kisspeptin could cause the increased release of GnRH and, in turn, the increased GnRH receptors could respond to cause a surge release of LH to cause ovulation.

Kisspeptins bind to their cognate receptor, GPR54, with high affinity [38]. There is evidence that GnRH neurons possess kisspeptin receptors and after kisspeptin binds to GPR54 there is an increase in hypothalamic concentrations of GnRH [38]. The KISS1R gene has been demonstrated to be highly abundant in multiple tissues in the body, including the hypothalamus and anterior pituitary gland [16]. 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.* [39] 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 less on d 21 compared to d 1. 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 [40]. Roa *et al.* [40] 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 [40]. 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 [40]. 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 E_2 [1] [41]. Kisspeptin is a potent secretagogue of GnRH [14]. Additionally, kisspeptin possesses estrogen receptors [6] [7] [41], and AVPV expression of KISS1 is up-regulated at a time coincident with the LH surge [7] [8]. 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 ovulation in mammalian species.

5. Conclusion

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 [19] [20] [21]. Recent research has also described the relationship between increased peripheral concentrations of E_2 and increased hypothalamic concentrations of KISS1. 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 investigated further.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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