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Evaluation of the Hypothalamic Kisspeptin System during the Attainment of Puberty in Gilts

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Abstract

Background: Kisspeptin has been demonstrated to affect the attainment of puberty in multiple species, presumably through its actions on GnRH and LH. It has previously been found there is an increase in expression of hypothalamic in the rat and the monkey coincident with puberty. Whether a similar phenomenon occurs in the pig remains to be determined. The objectives of the current experiments were to determine 1) Plasma concentrations of estradiol-17β; 2) Anterior pituitary gland concentrations of LH, IGF-I, kisspeptin, and anterior pituitary gland expression of LH-β and GnRH receptor; 3) Mediobasal hypothalamus expression of Kiss1 and concentrations of kisspeptin during the peripubertal period in the gilt. **Methods:** Two experiments were performed, each with 25 crossbred gilts (151 d, 105 kg). Gilts were relocated and exposed to a mature boar beginning on d 1 and continuing throughout the experiments to naturally induce puberty. Gilts that stood immobile within 24 hours of slaughter were considered to have attained puberty. Plasma samples were collected on d 1, 3, and 7 of the first experiment and d 2, 4, 6, and 8 of the second experiment. Gilts were slaughtered on d 7 (experiment 1) or d 8 (experiment 2), when mediobasal hypothalamus (MBH), anterior pituitary glands (AP), and blood were collected. Relative expression of MBH Kiss1 and β-actin and AP GnRH receptor, LH-β, 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. **Results:** Relative expression of Kiss1 was increased ($P = 0.005$) 2.2 fold in the gilts that had attained puberty in the first experiment, however, those that had attained puberty in the second were not different ($P > 0.05$) from gilts that had not. Relative expression of GnRH re-

ceptor was not different ($P > 0.05$) between treatments in both experiments. Relative expression of LH-β tended to be decreased ($P = 0.09$) 0.80 fold in gilts that attained puberty in the first experiment but was not different ($P > 0.05$) in gilts that attained puberty in the second experiment. AP concentrations of LH were not different ($P > 0.05$) between treatments in the first experiment and were decreased ($P = 0.01$) in gilts that attained puberty in the second experiment. AP concentrations of kisspeptin were not different ($P > 0.05$) in the first experiment but were increased ($P = 0.04$) in gilts that had attained puberty in the second experiment. MBH concentrations of kisspeptin were increased ($P = 0.03$) in gilts that had attained puberty in the first experiment but were not different ($P > 0.05$) between treatments in the second experiment. **Conclusions:** These data further support the role that MBH expression of Kiss1 and concentrations of kisspeptin and AP concentrations of kisspeptin fluctuate during the peripubertal period and may play a role in the attainment of puberty in the gilt.

Keywords

Kisspeptin, Kisspeptin Receptor, Hypothalamus, Pituitary, Pig

1. Introduction

Many factors including genetics, stress, social interactions, season, housing, and nutrition can affect the age of puberty in gilts [1]. The key to the effects of all these factors is the effects on the hypothalamic-pituitary-gonadal axis, specifically on the release of gonadotropin releasing hormone (GnRH). 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 possess estrogen receptors. 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 [2], rodents [3], and sheep [4]. Central administration of kisspeptin increased plasma concentrations of luteinizing hormone (LH) in prepubertal female rats versus controls [5]. Repetitive administration of kisspeptin to prepubertal female lambs increased plasma concentrations of sex steroids and caused a LH surge [6]. 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.* [5] 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 re-

ported in gonadally intact female monkeys [7]. It was also shown that hypothalamic Kiss1 mRNA expression was up regulated in ovariectomized (OVX) and intact rats that were administered E_2 compared to OVX and intact rats that received no estradiol-17β (E_2) [8]. It has also been reported that there was an increase in the mediobasal hypothalamus (MBH) expression of Kiss1 mRNA during the attainment of puberty in intact female rhesus monkeys [7]. Nestor *et al.* [9] also showed that the number of kisspeptin neurons in the arcuate nucleus 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 [10] [11]. A multitude of research has also demonstrated the effects of the administration of a kisspeptin antagonist and its effects on the hypothalamo-pituitary-gonadal axis. During intracerebroventricular infusion of a kisspeptin antagonist in the ewe, there was no pulsatile release of LH detected compared to controls [12]. Smith *et al.* [13] 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 [14]. Hence, through the administration of kisspeptin antagonists, it has been proven that kisspeptin plays a pivotal role in the release of LH in sheep, rats, and pigs [10].

Kisspeptin has been proven to play an essential role in puberty through its intimate relationship with GnRH neurons [15]. Measurement of hypothalamic Kiss1 mRNA expression and hypothalamic concentrations of kisspeptin along with other hormones may provide insight into kisspeptin's association with the attainment of puberty. Therefore, the objectives of the current experiments were to determine 1) Plasma concentrations of E_2; 2) Anterior pituitary gland concentrations of LH, IGF-I, kisspeptin, and anterior pituitary gland expression of LHβ and GnRH receptor; 3) Mediolbasal hypothalamus expression of Kiss1 and concentrations of kisspeptin during the peripubertal period in the gilt.

2. Materials and Methods

2.1. 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 placed into pens (1.83 × 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 expe-

riment 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 (1500 × g, for 30 minutes at 4˚C), and frozen within 60 minutes to prevent protein degradation. Estrus detection was performed twice daily at 12 hour intervals in both experiments. The day gilts first stood immobile in the presence of the boar was considered the day they attained puberty. 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 pituitary glands (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 −80˚C. Following slaughter, reproductive tracts were subjectively evaluated for edema and tone and ovaries were evaluated for structures including follicles, corpora hemmorhagica, 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.

2.2. Estradiol-17β

For both experiments plasma concentrations of E_2 were determined in duplicate by radioimmunoassay (RIA) in the same assay. Estradiol-17β (E8875; Sigma Life Science, St. Louis, MO) was the standard and radioiodinated E_2 (#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 [^{125}I]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.

2.3. Insulin-Like Growth Factor I

Anterior pituitary gland concentrations of IGF-I were determined in duplicate by RIA [16] [17]. 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 × g for 10 min at 4˚C and the supernatant was removed and stored at −20˚C. Protein content of the AP ho-

mogenates (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 [18]). 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}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.

2.4. Luteinizing Hormone

Anterior pituitary gland concentrations of LH were determined in triplicate by RIA [19]. 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.

2.5. Kisspeptin

Hypothalamic 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. Hypothalami 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 coefficient of variation for the first experiment was 4.6%. Intra-assay coefficient of variation in the second experiment was 7.2%. Sensitivity of the assay for both experiments was 10.1 pg/tube.

2.6. Real-Time PCR

Total RNA was isolated from one half of each AP and MBH using TriReagent (TR118; Molecular Research Company, Cincinnati, OH). 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. Integrity of the RNA was confirmed by gel electrophoresis. Samples were treated with DNase according to the manufacturer's protocol (Turbo DNA-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 AP and MBH tissue. Expression of β-actin and GAPDH did not differ among days. Two micrograms of total RNA were reverse transcribed using random hexamer primers (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA) to produce cDNA. One hundred micrograms of cDNA were used in each reaction. Twenty-five-microliter PCR reactions were performed using RT2 SYBR Green/ROX qPCR Master Mix (SuperArray Bioscience Corp., Foster City, CA). Primer pairs used for specific amplification of AP LHβ and GnRHR and MBH expression of KISS1, 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. 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.

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'	
pLH-b (NM_214080.1)[b]		
Forward	5'-ATGCTCCAGAGACTGCTGTTGT-3'	151 bp
Reverse	5'-TGCTGGTGGTAAAGGTGATGCAGA-3'	
GnRHR (NM_214273.1)[c]		
Forward	5'-AGCCAACCTGTTGGAGACTCTGAT-3'	101 bp
Reverse	5'-AGCTGAGGACTTTGCAGAGGAACT-3'	
pGAPDH (AF017079.1)[d]		
Forward	5'-GCAAAGTGGACATTGTCGCCATCA-3'	105 bp
Reverse	5'-TGACTGTGCCGTGGAATTTGCCAT-3'	
pb-actin (U07786.1)[e]		
Forward	5'-TCGCCGACAGGATGCAGAAGGA-3'	129 bp
Reverse	5'-AGGTGGACAGCGAGGCCAGGAT-3'	

[a]Porcine Kiss1; [b]Porcine luteinizing hormone subunit beta; [c]Porcine gonadotropin releasing hormone receptor; [d]Porcine glyceraldehyde phosphate dehydrogenase, [e] porcine beta-actin.

2.7. 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 IGF-I, and LH, statistical analyses were performed using the GLIMMIX Procedure of SAS 9.3 (SAS 9.3, SAS) to compare gilts that had attained puberty with gilts that had not. The model for determining differences in estradiol-17β was $Y_{ijkl} = \mu + Pig_i + Pubertal\ Status_j + Pig_i\ (Pubertal\ Status)_j + Day_k + Pubertal\ Status_j \times Day_k + Pig_i\ (Pubertal\ Status)_j \times Day_k + e_{ijkl}$ with repeated measures. Pig within treatment by date was the subplot error term used to test pig, date, and pubertal status by day effects. The model for determining hypothalamic concentrations of kisspeptin and anterior pituitary concentrations of IGF-I, and LH was $Y_{ijk} = \mu + Pig_i + Pubertal\ Status_j + Pig_i\ (Pubertal\ Status)_j + e_{ijk}$. 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 AP LH-β and GnRH receptor (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 [20].

2.8. Results—Experiment 1

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

Mean plasma concentrations of E_2 were not different ($P > 0.05$) in gilts that had and had not attained puberty on d 1 and 3 of the experiment but were greater ($P < 0.05$) within both groups of gilts on d 1 versus d 3 of the experiment (**Figure 1**). Mean plasma concentrations of E_2 were greater ($P = 0.02$) in gilts that had attained puberty compared to gilts that had not on d 7 of the experiment (**Figure 1**).

No differences were detected ($P > 0.05$) in mean AP concentrations of IGF (**Figure 2(a)**) or LH (**Figure 2(b)**) in gilts that attained puberty versus gilts that had not. However, mean MBH concentrations of kisspeptin were greater ($P = 0.04$) in gilts that attained puberty compared to those that had not (**Figure 3(a)**). No difference was detected ($P > 0.05$) in mean AP concentrations of kisspeptin in gilts that had attained puberty compared to those that did not (**Figure 3(b)**).

Mean relative expression of MBH KISS1 was up-regulated ($P = 0.005$) approximately 2-fold in gilts that attained puberty compared to those that did not (**Figure 4(a)**). Mean relative expression of AP LH-β **Figure 4(b)** and GnRHR (**Figure 4(c)**), each did not differ ($P > 0.05$) in gilts that attained puberty versus those that did not.

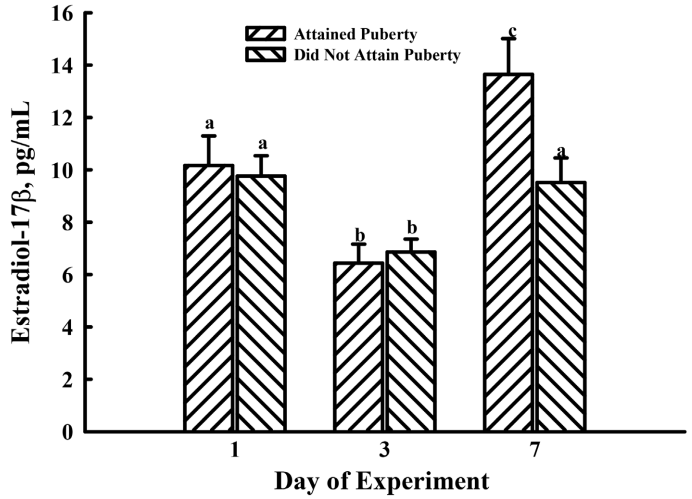

Figure 1. Mean plasma concentrations of estradiol-17β in gilts that had (n = 8) and had not (n = 17) attained puberty. Data are expressed as least square means ± SEM. [abc]Means with different letters differ ($P < 0.05$) according to pubertal status and/or day.

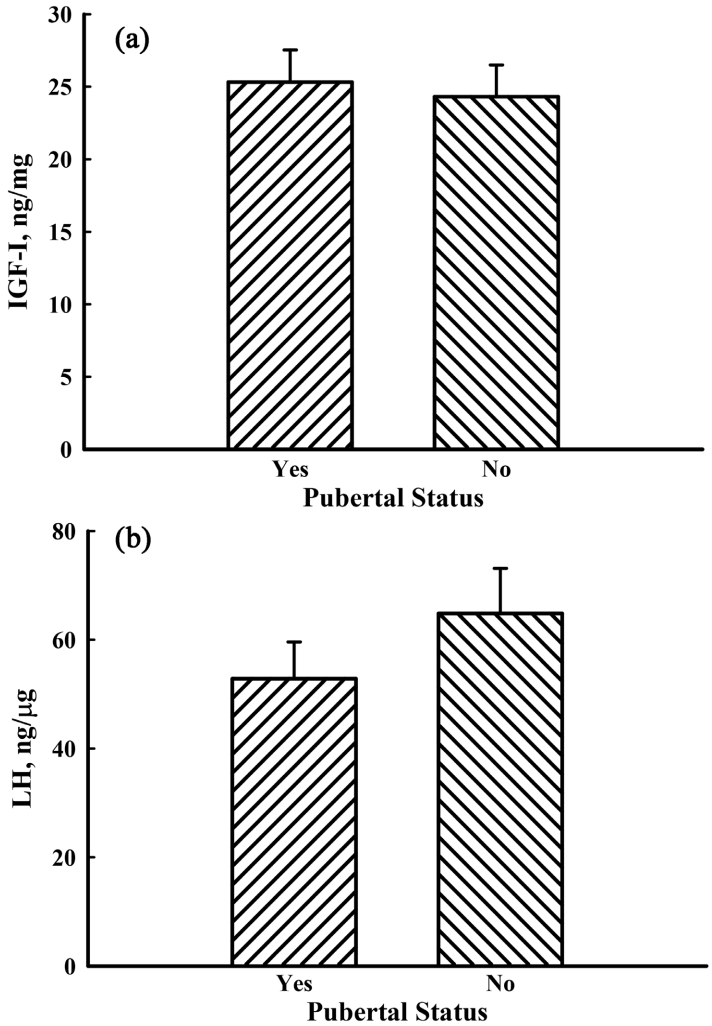

Figure 2. Mean AP concentrations of IGF-I (a) and LH (b) in gilts that had (n = 8) and had not (n = 17) attained puberty. Data are expressed as least-square means ± SEM.

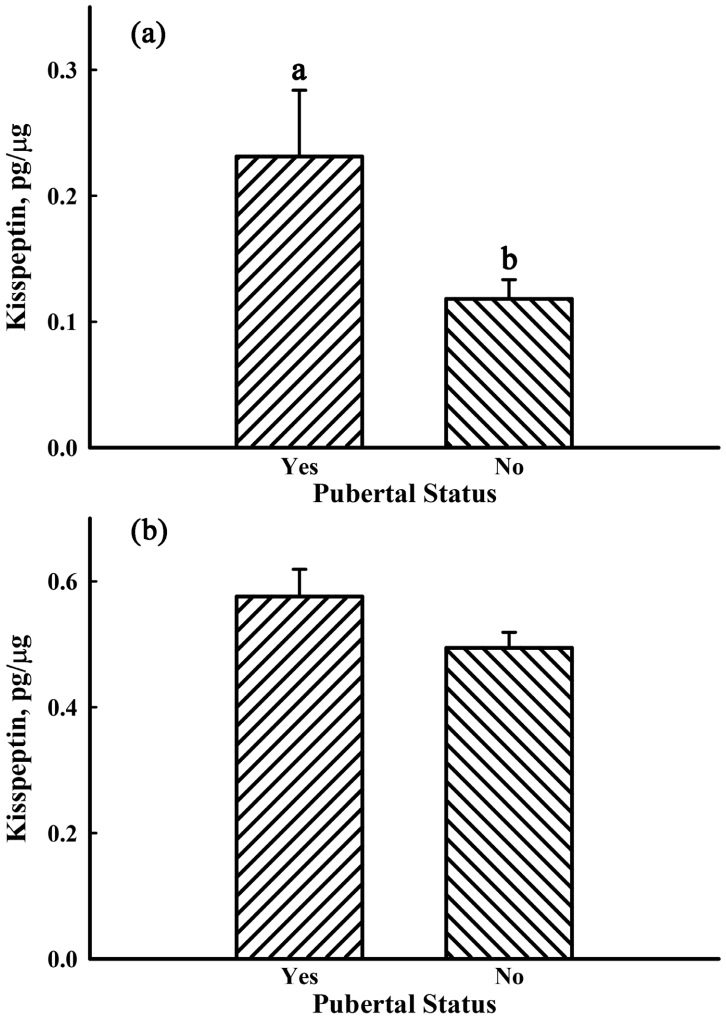

Figure 3. Mean MBH (a) and AP (b) concentrations of kisspeptin in gilts that had (n = 8) and had not (n = 17) attained puberty. [ab]Means with different letters differ ($P < 0.05$). Data are expressed as least-square means ± SEM.

2.9. Results—Experiment 2

Mean plasma concentrations of E_2 were not different ($P > 0.05$) in gilts that attained puberty compared to those that did not on d 2, 4, and 8 of the experiment (Figure 5). Mean plasma concentrations of E_2 tended to be greater ($P = 0.06$) on d 4 and were greater ($P = 0.02$) on d 6 in animals that had attained puberty compared to animals that had not (Figure 5).

No differences were detected ($P > 0.05$) in mean AP concentrations of IGF-I in gilts that attained puberty versus those that did not (Figure 6(a)). However, mean AP concentrations of LH were less ($P = 0.01$) in gilts that attained puberty compared to those that did not (Figure 6(b)). Mean AP concentrations of kisspeptin were greater ($P = 0.04$) in gilts that attained puberty versus those that did not (Figure 7(a)). No differences were detected ($P > 0.05$) in mean MBH concentrations of kisspeptin in gilts that attained puberty compared to gilts that did not (Figure 7(b)).

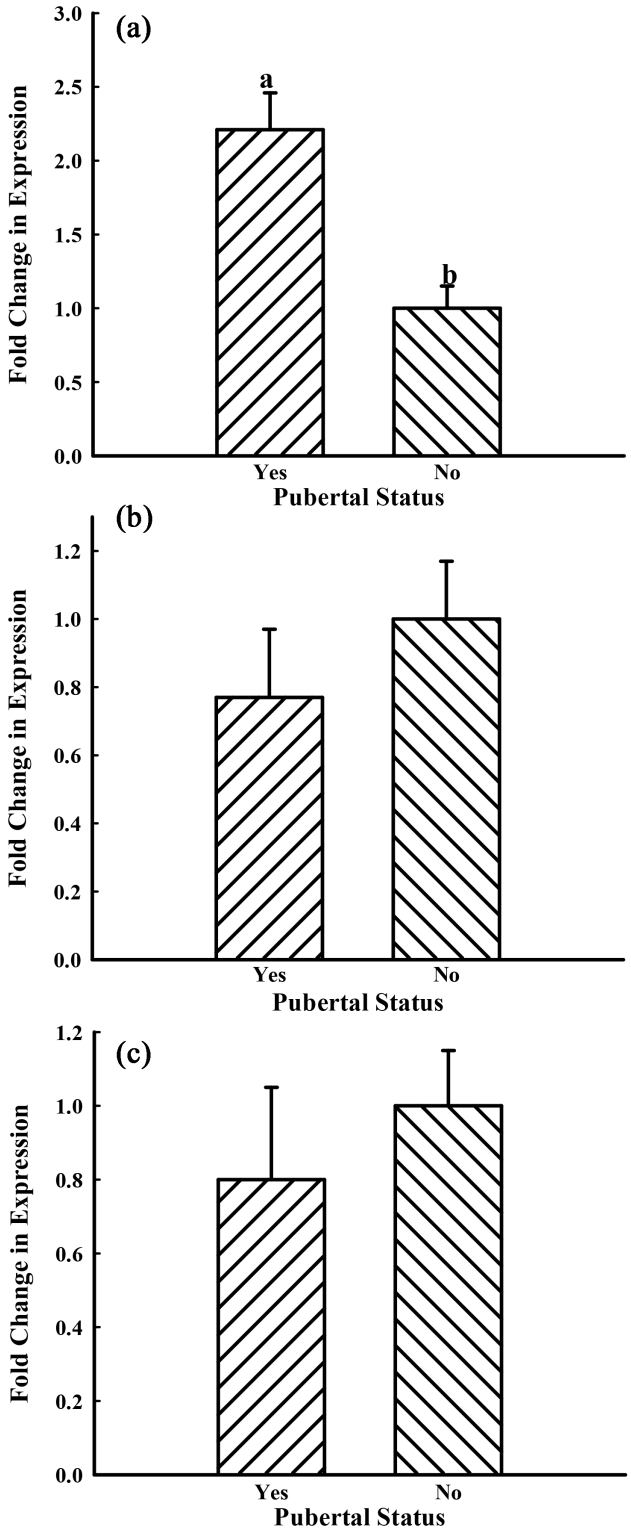

Figure 4. Mean relative expression of MBH Kiss1 (a), AP LHβ (b), and AP GnRHR (c) in gilts that had (n = 8) and had not (n = 17) attained puberty. Total RNA was DNase treated and analyzed for the level of Kiss1 by semi-quantitative reverse transcriptase real time PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the gilts that had not attained puberty. [ab]Means with different letters differ ($P < 0.05$).

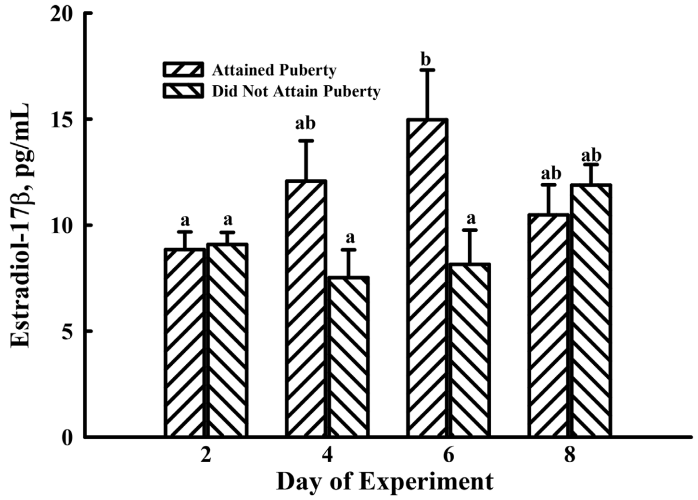

Figure 5. Mean plasma concentrations of estradiol-17β in gilts that had (n = 8) and had not (n = 17) attained puberty. Data are expressed as least square means ± SEM. [ab]Means with different letters differ ($P < 0.05$) according to pubertal status and/or day.

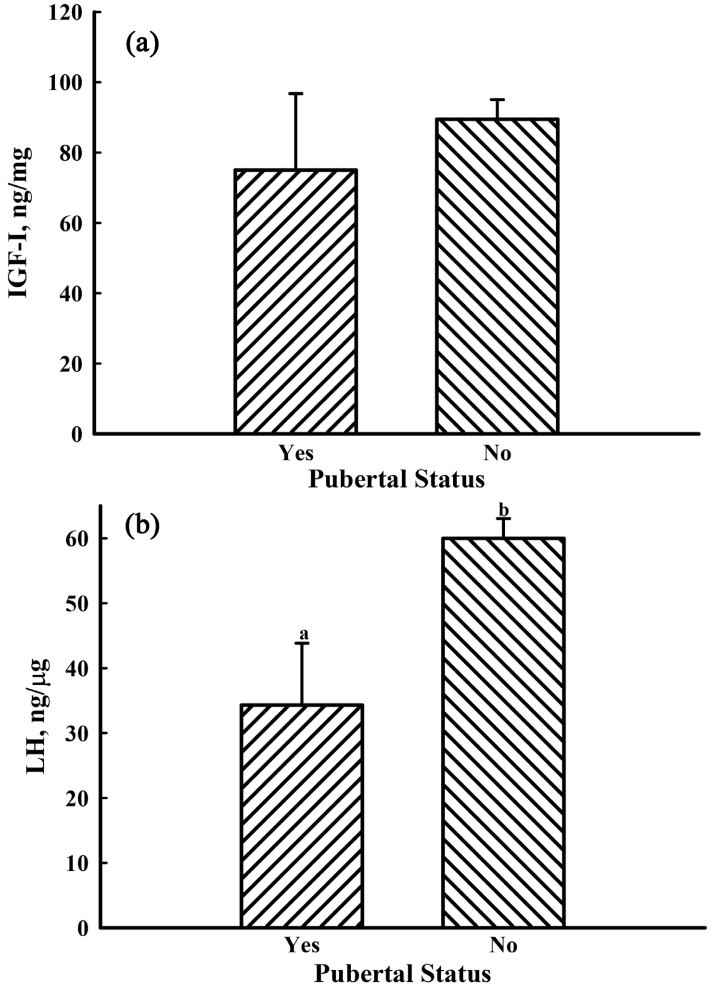

Figure 6. Mean AP concentrations of IGF-I (a) and LH (b) in gilts that had (n = 8) and had not (n = 17) attained puberty. Data are expressed as least-square means ± SEM. [ab-]Means with different letters differ ($P < 0.05$).

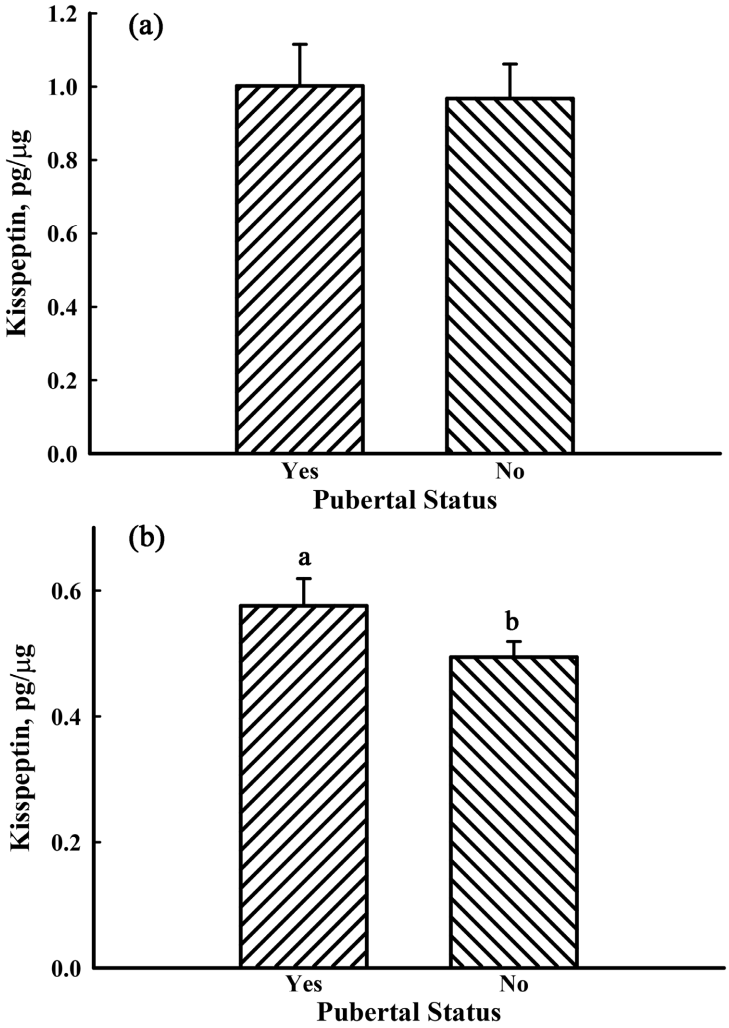

Figure 7. Mean MBH (a) and AP (b) concentrations of kisspeptin in gilts that had (n = 8) and had not (n = 17) attained puberty. Data are expressed as least-square means ± SEM. [ab]Means with different letters differ ($P < 0.05$).

Mean relative expression of MBH KISS1 did not differ ($P > 0.05$) in gilts that attained puberty compared to those that did not (**Figure 8(a)**). Mean relative expression of LHβ (**Figure 8(b)**) and GnRHR (**Figure 8(c)**), each, did not differ ($P > 0.05$) in gilts that attained puberty versus those that did not.

3. Discussion

Plasma concentrations of E_2 increase as animals approach puberty, which occurs at a time around standing estrus. This increase in E_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 [21] [22]. Because research is equivocal as to whether GnRH neurons possess estrogen receptors it is thought that other factors contribute to the attainment of puberty.

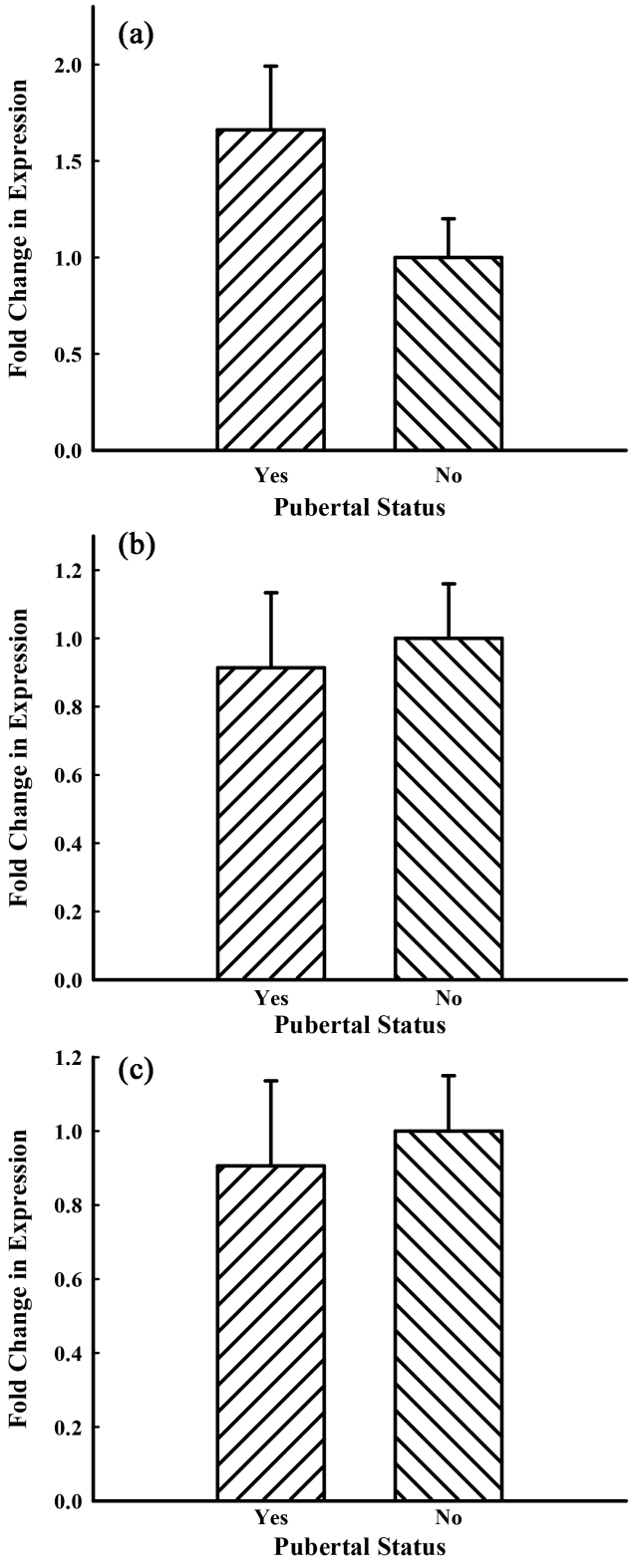

Figure 8. Mean relative expression of MBH Kiss1 (a), AP LHβ (b), and AP GnRHR (c) in gilts that had (n = 8) and had not (n = 17) attained puberty. Total RNA was DNase treated and analyzed for the level of Kiss1 by semi-quantitative reverse transcriptase real time PCR with normalization for the corresponding level of β-actin. Data are expressed as a fold change in expression relative to the gilts that had not attained puberty.

Kisspeptin is a neurohormone that is produced by the KISS1 gene [23]. Kisspeptin and kisspeptin neurons have been demonstrated to associate intimately with GnRH neurons in the hypothalamus [15]. It was reported that deletions or mutations in GPR54 in the mouse resulted in infertility and the failure to attain puberty [24] [25]. Additionally, deletions and mutations in the Kiss1 gene in humans and mice have been associated with difficulties in the attainment of puberty [26] [27]. 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-17β have been demonstrated to be increased during the attainment of puberty in gilts [28]. In the present studies, plasma concentrations of E_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 E_2 than gilts that were not considered pubertal. Gilts in experiment 2 were slaughtered a day later, however, there was no difference in plasma concentrations of E_2, but plasma concentrations of E_2 were greater on d 6 (2 days prior) in gilts that had attained puberty versus gilts that had not. It is worth noting that this difference in plasma concentrations of E_2 may influence MBH and AP concentrations of hormones when comparing the experiments. Therefore, it is apparent that the timing of sample collection and which day gilts are slaughtered greatly affected plasma concentrations of E_2.

In both experiments there was no difference in AP concentrations of IGF-I in gilts that had and had not attained puberty. Clapper and Taylor [29] demonstrated than 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 [30] [31]. 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 [32]. Hiney *et al.* [32] 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) [33] [34]. Insulin-like growth factor binding proteins can have either inhibitory or stimulatory effects [35], which can also be tissue specific [36] [37]. Additionally, direct actions of IGFBP that are independent of IGF-I may exist [38]. 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. The timing of puberty in the gilt can be greatly affected by exposure of the gilt to mature boars, with many of the gilts reaching puberty within 5 to 10

days post exposure [1]. It is widely accepted during the attainment of puberty the preovulatory LH surge occurs followed by ovulation approximately 24 - 36 h later [39]. 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 gilts that had and had not attained puberty. However, in the second puberty experiment there were decreased AP concentrations of LH in gilts that had attained puberty versus gilts that had not. 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 gilts that had not attained puberty. In the second puberty experiment there were no differences in hypothalamic Kiss1 mRNA expression and MBH concentrations of kisspeptin comparing gilts that had and had not attained puberty. During the attainment of puberty there is an increase in hypothalamic Kiss1 mRNA expression in rodents and sheep [5] [7], which correlates with the first puberty experiment. Although the second experiment does not correlate with previous research, it may be indicative of the need for more precise timing with regard to when samples are obtained.

There was no difference in AP concentrations of kisspeptin between gilts that had and had not attained puberty in the first experiment, however AP concentrations of kisspeptin were greater in gilts that had attained puberty compared to gilts that had not in the second experiment. When gilts were slaughtered a day later in the second experiment there was a decrease in AP concentrations of LH in gilts that attained puberty as well as an increase in AP concentrations of kisspeptin. Navarro *et al.* [5] 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 [6]. In experiment 1 there was an increase in plasma concentrations of E_2 on the day gilts were slaughtered, which may have been caused by increased kisspeptin because. On the same day, there was also an increase in hypothalamic concentrations of kisspeptin and MBH expression of Kiss1 on the same day.

In experiment 2 there was no difference in plasma concentrations of E_2 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, however, there was an increase in AP concentrations of kisspeptin and a decrease in concentrations of LH. 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 [10] [40] [41]. 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 [4] [15] [42]. Additionally, peripheral administration of kisspeptin-54 has been shown to activate GnRH neurons in the hypothalamus [43]. 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 in the present studies, it is plausible that during the attainment of puberty, which is coupled with an increase in peripheral concentrations of E_2, 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 [44] [45]. 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 [44]. 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 [45]. Turzillo *et al.* [46] found that E_2 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.* [44] found differences in the rat, however, our timing was not as precise as theirs due the rat having a shorter and more predictable estrous cycle.

4. Conclusion

In conclusion, changes in the kisspeptin system occur during the attainment of puberty in the gilt, however, 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 of sample collection, changes within the hypothalamic kisspeptin system are consistent with what is found in other mammalian species during the attainment of puberty.

Authors' Contributions

The experiments were designed and conducted by Jeffrey Clapper and Eric Jolitz. Experimental data were collected, analyzed, and the manuscript was prepared by Jeffrey Clapper, Eric Jolitz, and LeAnn Hall. Development of the kisspeptin radioimmunoassay and kisspeptin antibody was performed by Waljit Dhillo.

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Availability of Data and Materials

All data generated or analyzed during this study are included in the manuscript.

Declarations

Ethics Approval and Consent to Participate

All experimental procedures involving animals were approved by the South Dakota State University Institutional Animal Care and Use Committee.

Conflicts of Interest

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

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