EFFECTS OF FEED RESTRICTION ON PHYSIOLOGIC AND METABOLIC PARAMETERS AND LEPTIN EXPRESSION IN ADIPOSE TISSUE IN OVARIECTOMIZED PREPUBERAL GILTS.

by

HEATHER ANNE LANGFORD HART

(under the direction of C. Richard Barb)

ABSTRACT

Ovariectomized prepuberal gilts were either fed to appetite (FA) or feed restricted to one-third of FA diet (RST) for 7 seven days. On day 8 blood samples were collected. Serum concentrations of insulin ($P<0.07$), triiodothyronine ($P<0.08$), and thyroxine ($P<0.04$) were reduced in RST pigs. There was a treatment by time interaction ($P<0.04$) for non-esterified fatty acids levels, but β-hydroxybutyrate, luteinizing hormone (LH), and leptin concentrations were unaffected by RST. RST failed to effect backfat leptin, long form leptin receptor, adipocyte fatty acid binding protein, CCAAT/enhancer binding protein-α, or peroxisome proliferator activated receptor-γ 2 gene expression. FA gilts gained ($P <0.001$) more BW than RST gilts but BF thickness was unchanged. These results may be related to the failure of RST of this duration to influence subcutaneous BF. Thus, the leptin and LH response to RST may require energy levels and (or) BF reduction reaching a putative inhibitory threshold.

INDEX WORDS: Prepuberal Gilt, Leptin, Luteinizing Hormone, Feed Restriction

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DEDICATION

To my wonderful family who have given me the wings to fly and find my place in life. To the good Lord for abundantly blessing me and allowing me the opportunity to complete a higher degree.

In honor of my loving husband, Darrell Hart, who has sacrificed and supported me in my long journey. You are my concrete pillar and I hope to spend the rest of my life showing you my appreciation.

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TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

CHAPTER 1

INTRODUCTION

The effect of inadequate nutrition on reproductive function in the female pig can range from delayed puberty, delayed return to estrus, decreased ovulation rate, and inhibition of gonadotropin secretion. Therefore, understanding the mechanism which modulates energy metabolism and the reproductive system is imperative. Leptin is a 16 kDa protein consisting of 146 amino acids which is synthesized primarily by adipose tissue and is secreted in the bloodstream after cleavage of the 21 amino acid signal peptide. Leptin has been shown to play a role in energy balance, feed intake, neuroendocrine axis, reproduction, and immunological processes [1-3]. Numerous studies have shown a strong relationship between the reproductive function and leptin [4-7]. Leptin increased LH secretion from porcine pituitary cells in vitro and GnRH release from hypothalamic tissue in vitro [2]. Feed restriction in the mature ovariectomized gilt reduced LH pulse frequency and mean serum leptin concentrations [8]. The effects of feed restriction on leptin gene expression in the pig are unclear. The objective of the current study was to determine the effects of feed restriction on leptin expression and secretion and the relationship between serum LH and leptin concentrations in the restricted prepuberal gilt.

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CHAPTER 2

REVIEW OF LITERATURE

Prepuberal gilt and the HPG axis

Demand for leaner pork has forced swine breeders to select for animals with reduced body fat while continuing to select for improved growth rate and reproduction. Onset of puberty in gilts is associated with attainment of a critical age or body weight [1] and a minimum ratio of adipose: lean tissue [2]. In prepuberal gilts, inadequate nutrition delayed puberty and resulted in delayed first mating [1,3,4]. In the postpartum animal, inadequate nutrition delayed the return to estrus after weaning, decreased ovulation rate, inhibited gonadotropin secretion, and decreased embryonic survival [5,6]. Therefore, adequate nutrition is necessary for optimal reproductive performance.

Little is known about the mechanisms within the brain which brings the various components of the hypothalamic-pituitary-gonadal axis (HPG) into proper temporal relationship to initiate puberty. Gonadotropin releasing hormones (GnRH), released from the hypothalamus stimulates pituitary luteinizing hormone (LH) into the peripheral circulation, which in turn regulates gonadal activities. Introceptive and extroceptive factors detected by the CNS are translated by the neuroendocrine system into signals that alter the pattern of GnRH and LH secretion.

Gonadotropin releasing hormone is released at the median eminence and travels to the adenohypophysis (anterior lobe of the pituitary gland) via portal vessels. GnRH binds to GnRH

receptors on gonadotropes to stimulate the synthesis and secretion of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). FSH is primarily involved in stimulating the growth of ovarian follicles, while LH controls ovulation and steroidgenesis. Wetsel et al. [7] showed immortalized hypothalamic neurons secrete GnRH in a pulsatile fashion. Pressing et al. [8] and Lutz et al. [9] demonstrated that hourly administrations of GnRH induced estrus and ovulation in intact prepuberal gilts. Leshin et al. [10] demonstrates that both GnRH and LH were secreted in pulsatile patterns with peaks of GnRH often, but not always occurring coincident with or just preceding LH pulse in the ovariectomized gilt. In lactating anestrous sows, pulsatile GnRH administration resulted in induced estrus and ovulation [11]. In nutritionally-anestrous gilts hourly administration of exogenous LH or GnRH reinitiated follicular growth [12]. These data suggest that the mode of GnRH and LH secretion is required for normal ovarian function.

An LH pulse and LH surge generator are located within the CNS of pigs [13]. There are two modes of LH secretion in pigs based on rhythmic GnRH release: pulsatile secretion and surge secretion. Surge secretion is characterized by prolonged elevation resulting from high frequency of LH pulses [13]. The surge secretion is unique to the female and involves periodic massive releases of LH and to a lesser extent FSH. It is generally accepted that surge secretion of LH is a result of positive feedback action of elevated serum estrogen concentrations from preovulatory follicles. Surge secretion of LH from the pituitary at approximately the onset of estrus is crucial for ovulation.

Pulsatile secretion consists of low amplitude episodes of LH release with the frequency and amplitude influences by steroid hormones. The pulsatile release of LH varies with the stage of the estrous cycle. LH pulse frequency is low, but amplitude is high during the luteal phase

when estrogen secretion is relatively low and progesterone secretion is relatively high. During the follicular phase, estrogen secretion is high and progesterone secretion is low, while LH pulse frequency is high and amplitude is low [14]. In concert with GnRH secretion, surge and pulsatile LH secretion are both necessary for normal estrous cycles and ovulation in the female pig.

Negative and positive feedback actions of ovarian steroids on LH secretion occur at the CNS in pigs [13]. Estradiol is the primary sex steroid produced by the follicle. Estradiol stimulates the preovulatory LH surge in pigs by acting primarily on the CNS [13]. The rising concentration of estradiol secreted by developing preovulatory follicles stimulates the preovulatory surge of LH. In the ovariectomized (OVX) gilt, estradiol treatment resulted in a preovulatory-like LH surge approximately 48-72 hours after treatment [15]. At times of decreased follicular growth, estrogen has a negative feedback effect on GnRH at the hypothalamus and LH at the anterior pituitary. Kesner et al. [16] demonstrated in pigs estradiol treatment induced a preovulatory-like LH surge only after serum LH concentrations were suppressed for forty eight hours. These results demonstrate the importance of estradiol in driving the secretory pattern of LH and FSH in the female.

In prepuberal animals, the gonads are quiescent and plasma steroid levels are low. The 'gonadostat' hypothesis proposes that the mechanism by which the hypothalamus governs the release of gonadotropin from the pituitary of prepuberal animals is via negative feedback action of ovarian estradiol to suppress gonadotropin secretion. As the animal matures, the gonadostat is reset and the hypothalamus becomes less sensitive to the negative feedback of estradiol. As a result, gonadotropin secretion increases to adult levels resulting in follicular development and ovulation [14]. Lutz et al. [9] demonstrated in the gilt, the onset of puberty is immediately

preceded by a significant increase in serum LH concentrations characterized by an accelerated frequency (approximately 1 peak per hour) of lower amplitude LH pulses. In addition, this occurred concomitantly with increased estrogen secretion. The HPG axis and its components are critical in establishing and maintaining normal estrous cycles.

Influences of feed restriction

The GnRH/LH pulse generator is sensitive to numerous blood born metabolites and metabolic hormones. Undernutrition due to fasting, illness, or heat stress inhibits reproductive activity by acting on the hypothalamic-pituitary axis [2,17,18]. Metabolic changes inhibit the HPG system in part by increasing the sensitivity to the negative feedback effects of estrogen [19]. Feed restriction in the pig [12,20,21], heifer [22], and lamb [23] suppressed pulsatile LH secretion resulting in a quiescent gonad. In feed restricted gilts, diameter and follicular volume were decreased compared to fed controls [24,25]. Pulsatile LH and FSH secretion, follicular development and ovulation can be reinstated by pulsatile treatment with GnRH in feed-restricted gilts, and primiparous, lactating sows [12,26]. In prepuberal gilts, feed restriction delayed onset of puberty and ovulation [27,28]. These results suggest that fasting or feed restriction negatively affects the reproductive axis.

Leptin

Leptin, a 16 kDa protein consisting of 146 amino acids, is synthesized primarily by adipose tissue and is secreted in the bloodstream after cleavage of the 21 amino acid signal peptide. Leptin is described as cytokine-like peptide due to similarities based upon the structural similarity between the leptin receptor and IL-6 receptor [29]. Leptin contains four anti-parallel

alpha helices connected by two long crossover links and one short loop arranged in a left handed twisted helical bundle. It also contains a single disulfide linkage connecting the CD loop to the carboxy terminus. Leptin is mainly secreted by adipocytes; however, research has shown leptin is secreted by the placenta in humans [30-32], the rodent [33], and in the pig [34]. Under conditions of unrestricted food intake, leptin is a static index of the amount of triglycerides stored in adipose tissue [35].

Zhang et al. [36] identified leptin as the gene product deficient in the obese ob/ob mouse by positional cloning. Treatment of ob/ob mice with recombinant leptin resulted in reduced feed intake, reduced body weight, and improved fertility [37-39]. The porcine ob gene was cloned by Bidwell [40] and shares 95%, 92%, and 89% sequence homology to bovine, human, and murine leptin.

The leptin receptor family consists of, the long or "signaling form" (OB-rb), several short forms which exhibit weak signal transducing capabilities (OB-ra, OB-rc, OB-rd, and OB-rf), and the secreted form, which is soluble and can act as a binding protein (OB-re) [41,42]. The Ob-rb is highly expressed in the hypothalamus in areas responsible for appetite regulation, and energy balance in sheep [43], rodents [44], and pigs [45,46]. In the rodent, Mercer et al. [44] found the Ob-rb gene expression in the nucleus of the solitary tract, lateral parabranchial nucleus, and medullary reticular nuclease. In the pig, the Ob-rb has been found in the hypothalamus, anterior pituitary, ovary, uterine body, and adipose tissue [45]. In addition, to a central site of action, the porcine leptin receptor is also expressed in granulosa and luteal cells in the pig ovary [47]. These results support the idea that leptin regulates appetite, energy balance, and reproductive function.

Leptin plays a role in regulating energy balance, feed intake, neuroendocrine axis, reproduction, and immunological processes [29,48-51]. The most well-known function of leptin is to regulate feed intake. Injection of recombinant leptin resulted in a reduction of feed intake in the pig [52-54], the ob/ob mouse [38], the rat [37], and the ewe [55]. In addition, leptin provides a molecular basis for the lipostatic theory which proposes that body energy reserves are "sensed" by peripheral signals which transmit energy status signals to the brain, in order to maintain a positive energy balance [56]. The ability to monitor energy stores must be central to the link between reproduction and energy balance.

Leptin secretion is associated with changes in energy balance. However, both circadian and ultradian variations in plasma leptin levels have been reported independent of changes in energy in the pig [53,57] and human [58]. Daniel et al. [58] reported episodic leptin secretions, but circadian variation were absent in fat and thin ewes.

Plasma leptin concentrations are associated with body fatness in the sheep [56,60], rodent [61], human [62], and the pig [63]. Greater body fat content coincided with greater leptin mRNA expression in pigs [40,63,64], sheep [65], rats [66] and humans [67,68]. Leptin mRNA concentrations were greater in adipose tissue from genetically obese pigs compared to leaner pigs [69]. Lin et al. [70] found leptin mRNA expression in back fat was lower in 3.5 month old gilts than in 7 day old gilts and hypothalamic Ob-Rb expression was greater at 3.5 and 6 months of age then compared to the 106 day old fetus and 7 day old pig. In addition, Zhou et al. [71] found expression of Ob-Rb mRNA in the hypothalamus decreased gradually after birth and then increased until weaning in pigs. Qian et al. [72] demonstrated that leptin mRNA expression was not increased by age in estrogen treated 90 and 150 day old OVX gilts.

However, by 210 day of age, leptin mRNA expression was 2.5 fold greater in estrogen treated pigs compared to control animals.

Shillabeer [66] reported that rats feed restricted diets (67% of control diet) had lower mRNA leptin expression compared to ad libitum counterparts. In pigs, Spurlock [73] demonstrated that fasting for three days decreased leptin mRNA concentrations; however, severe to maintenance level feed restriction for 28 days did not alter mRNA in adipose tissue. In addition, McNeel and Mersmann [74] saw no difference in leptin transcript concentration in either obese or lean young pigs when feed restricted for 5 weeks. Scholz et al. [67] reported that long term intake restriction uncouples serum leptin from fat mass such that a deficiency of leptin may develop relative to adiposity in humans. This may explain the reason long term restriction shows no changes in leptin mRNA concentrations compared to fasting animals.

Leptin and reproduction

Numerous studies demonstrated a relationship between the reproductive system and serum leptin concentrations [75-78]. In rodents, leptin plays a role in regulating gonadotropin secretion. Genetically obese (ob/ob) mice, which are leptin deficient due to a mutation in the gene encoding leptin, are infertile and have atrophic reproductive organs [39]. Leptin treatment of the ob/ob mouse restored circulating concentrations of LH and fertility in these mice [39,79].

Leptin increased LH secretion from pig pituitary cells in culture and GnRH release from hypothalamic tissue in vitro [50]. Yu et al. [80] demonstrated that leptin treatment stimulated LH secretion after acute intracerebroventricular (i.c.v.) injection in estrogen primed OVX rats, stimulated GnRH release from cultured median eminence-arcuate explants, and stimulated LH and FSH release from rat pituitaries in culture. Injected recombinant leptin restored plasma LH

concentrations and prolactin concentration following a 48 hour fast in the rat [81-83]. In sheep, leptin treatment increased LH secretion in feed restricted ewes [55,84]. Leptin treatment prevented fasting induced reductions in the frequency of LH pulses in prepuberal heifers [85]. In addition, Nagatani and associates [86] reported that subcutaneous administration of leptin increased serum LH concentrations during fasting in estrogen treated wethers. In the prepuberal gilt, serum LH concentrations were unaffected by i.c.v. leptin treatment [49]. The authors suggested that the inability of leptin treatment to increase LH secretion may have been due to stage of sexual development during a period of heightened negative feed back action of estrogen on LH secretion [49]. Collectively, these results indicate that leptin plays a role in regulating the GnRH/LH axis.

Leptin and puberty

Onset of puberty is linked to attainment of a critical body weight or a minimum percentage of body fat [87]. Leptin controls appetite and energy metabolism and thus acts as a "lipostat", regulating body fat storage [87]. Initiation of puberty may be influenced by unidentified factors of peripheral origin [49]. Leptin accelerated reproduction, vaginal opening, onset of first estrous cycle, and maturation of reproductive tissues in feed restricted and ad libitum rodents [88,89]. In addition, chronic leptin treatment not only reduced food intake but restored fertility in the infertile ob/ob mice [89]. Qian et al. [72] reported that in the OVX prepuberal gilt, estrogen induced leptin mRNA expression in the adipose tissue occurred at the time of puberty in comparison of intact contemporaries. Furthermore, long form leptin receptor (Ob-Rb) mRNA expression in the hypothalamus increased by 3.5 months of age and remained elevated at 6 months of age in the prepuberal gilt demonstrating an age dependant increased in Ob-Rb

expression [72]. In prepuberal heifers, serum leptin concentrations, leptin gene expression, and IGF-1 increase as puberty approaches [90].

However, there exists a matter of controversy as to the exact role of leptin in the onset of puberty. Leptin administration failed to advance puberty onset in well nourished female mice [91]. In addition, several reports demonstrated that circulating leptin concentrations remain unchanged during prepuberal development in the female mouse and rat [91,92]. These results may suggest leptin does not serve as a triggering signal but may play a permissive role.

Leptin and feed restriction

The swine industry has selected animals for maximum growth rate to achieve greatest growth and feed utilization in the growing animal. However, the pig does not eat to gut fill. The pig will adjust its intake to maintain a constant energy intake [93]. The regulation of feed intake is complex in pigs and many theories have been developed. One hypothesis is that leptin acts on the hypothalamus to reduce feed intake and increase energy expenditure to regulate body weight [94].

Leptin functions to promote the partitioning of energy away from lipid accretion within porcine adipose tissue by promoting lipolysis directly or indirectly by reducing insulin-mediating inhibition of lipolysis [95]. It has been suggested that the hypothalamus may be insensitive to elevated leptin levels but would sense low leptin levels such as during fasting. This would activate a survival response that would conserve energy resources by shutting down, for example, the reproductive system [96]. The response to fasting can be rescued by leptin injections, demonstrating that leptin mediates the starvation-induced response [97]. Feed restriction in the gilt [21,98], lactating sow [26], cow [99], horse [100], heifers [101], and

human [35] resulted in decreased serum leptin concentrations. However, McNeel et al. [69] showed no differences in plasma leptin concentrations in feed restricted obese or lean pigs compared to ad libitum fed controls.

Barb et al. [57] reported a reduction in leptin pulse frequency within 24 to 28 hours after feed removal in gilts. Whisnant and Harrell [21] reported that feed restriction in the mature OVX gilt decreased serum leptin, insulin, and LH concentrations. Re-feeding the OVX gilts reversed this pattern. In addition, Cosgrove et al. [102] demonstrated in prepuberal ovary-intact gilts seven day feed restriction decreased pulsatile LH secretion and refeeding within 8 hours restored LH secretion. Collectively, these results suggest leptin plays a role in signaling the energy status to the brain which in turns activates the reproductive system.

Regulators of leptin

Leptin signals nutritional status to key regulatory centers in the hypothalamus via mediators in the brain [103]. Insulin may mediate the action of leptin within the hypothalamus. Leptin receptors have been found on pancreatic beta cells and inhibited beta cell secretion of insulin [29]. Leptin decreased in response to insulin deficiency and increased in response to insulin treatment in mice [104]. In addition, leptin treatment of ob/ob mice enhanced insulin sensitivity [105]. During the luteal phase of the estrous cycle in gilts, feed restriction increased follicle atresia and decreased ovulation rate, which is counteracted by insulin treatment [106]. Leptin has been shown to have receptors located on granulosa and theca cell of the porcine ovary [45]. In the OVX gilt, leptin pulse frequency decreased within 24 to 28 hours after feed removal. This occurred concomitantly with a decrease in serum insulin concentrations [57].

Leptin gene expression can be influenced by hormones such as insulin and glucocorticoids [51]. Leptin expression increased after peak insulin secretion during the feeding cycle in the human [35]. Insulin directly stimulated leptin expression in rodent adipocyte cultures [104]. Fasting reduced leptin expression in epididymal and inguinal fat pads, and insulin treatment restored leptin mRNA to control levels in rats [107]. These results suggest that insulin may play a role in mediating leptin's actions.

Leptin suppresses Neuropeptide Y (NPY) expression and secretion in the arcuate nucleus [56]. NPY is a strong stimulator of food intake and is involved in the regulation of various pituitary hormones [108]. NPY has been proposed as the primary mediator of leptin action on feed intake and LH secretion in the rodent [109] and the pig [50]. Co-localization of leptin receptor mRNA with NPY gene expression in the rodent supports this idea [75]. In ob/ob mice with a homozygous null mutation for NPY, fertility was only partially restored by leptin treatment [110]. In the pig, i.c.v. administration of NPY increased GH secretion, feed intake, and antagonized the action of leptin on intake [48,53].

These data suggest that leptin effects on feed intake may be mediated by NPY neurons. It has been suggested that leptin reduces food intake by upregulating anorexigenic (appetite-reducing) neuropeptides and down regulating orexigenic (appetite-stimulating) neuropeptides factors [111]. Orexin, a hypothalamic neuropeptide that regulates feeding behavior in rats, also regulates LH in OVX rats [112]. Orexigenic peptides, such as AGRP, MCH, and orexins A and B, are inhibited by leptin. I.c.v. injection of orexin B increased food intake in castrated male sheep [113]. Anorexigenic peptides are stimulated by leptin, such as POMC, alpha-MSH, CART, and CRH [111]. These hypothalamic neuropeptides are another pathway leptin may regulate feed intake.

Conclusion

The effect of inadequate nutrition on reproductive function in the female pig can range from delayed puberty, delayed return to estrus, decreased ovulation rate, and inhibition of gonadotropin secretion. Therefore, understanding mechanism which modulate energy metabolism and the reproductive system is imperative. Leptin, an adipocyte secreted hormone, has been shown to play a vital role in feed intake, energy regulation, and the reproductive system. Leptin plays a role in signaling the energy status to the brain which in turn regulates the reproductive system. Leptin has been shown to increase LH secretion in the food restricted animal. Continuing research is necessary to determine the role of leptin in regulating reproductive function.

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CHAPTER 3

FAILURE OF SHORT-TERM FEED RESTRICTION TO EFFECT LUTEINIZING HORMONE OR LEPTIN SECRETION AND SUBCUTANEOUS ADIPOSE TISSUE EXPRESSION OF LEPTIN OR LONG FORM LEPTIN RECEPTOR (OB-R) IN THE PREPUBERAL GILT[1].

[1]H. A. Hart, M. J. Azain, G. J. Hausman , D.E. Reeves and C.R. Barb to be submitted to *Domestic Animal Endocrinology*.

Abstract

Ovariectomized (OVX) prepuberal gilts averaging 164 days of age and 79.19 ± 3.84 kg body weight (BW) were either fed to appetite (FA; n = 6) or feed restricted (RST; 33% of FA diet; n = 6) for 7 seven days. On day 8, blood samples were collected every 15 min for 8 h. Serum concentrations of glucose, β-hydroxybutyrate (B-HBA), triiodothyronine (T3), thyroxine (T4), non-esterified fatty acids (NEFAs), insulin, luteinizing hormone (LH) and leptin were determined. Real-time PCR was performed on total RNA extracted from subcutaneous backfat (BF) collected on day 0 (start RST) and day 9. FA gilts gained ($P < 0.001$) more BW (8.18 ± .59 kg) than RST (-2.45 ± .59 kg) gilts; however, BF was unchanged. A treatment x time interaction was detected for serum glucose ($P<0.009$) and NEFA ($P<0.04$) concentrations. Serum insulin ($P<0.07$), T3 ($P<0.08$), and T4 ($P<0.04$) concentrations were reduced and BHBA concentrations were unchanged in RST gilts. Serum leptin and LH concentrations, pulse amplitude, and pulse frequency were not affected by RST. RST failed to effect BF leptin, long form leptin receptor (Ob-Rb), AFABP (adipocyte fatty acid binding protein), C/EBPα (CCAAT/enhancer binding protein α), or PPARγ2 (peroxisome proliferator activated receptor-γ2) transcript expression compared to FA gilts. These results may in part be related to the failure of RST of this duration to influence subcutaneous BF. Thus, the leptin and LH response to RST may require energy levels and/or BF reduction reaching a putative inhibitory threshold.

1. Introduction

Onset of puberty is linked to attainment of a critical body weight and/or a minimum percentage of body fat [1]. Leptin is a 16 kDa protein mainly produced by adipocytes and plays a role in regulating energy balance, feed intake, neuroendocrine axis, reproduction, and immunological processes [2-6]. Leptin controls appetite and energy metabolism and thus acts as a "lipostat", regulating body fat storage [1]. Initiation of puberty may be influenced by unidentified factors of peripheral origin such as leptin [3]. Leptin treatment accelerated vaginal opening, onset of first estrous cycle, and maturation of reproductive tissues in feed restricted and ad libitum fed rodents [7,8]. In addition, chronic leptin treatment not only reduced food intake but restored fertility in the infertile leptin deficient (ob/ob) mice [8].

Qian et al. [9] reported that in the ovariectomized (OVX) prepuberal gilt, estrogen-induced leptin mRNA expression in adipose tissue occurred at the time of expected puberty. Moreover, backfat (BF) thickness was positively associated with leptin mRNA expression [5] and the onset of puberty in the pig [10]. Furthermore, long form leptin receptor (Ob-Rb) mRNA expression in the hypothalamus increased by 3.5 months of age and remained elevated at 6 months of age in the prepuberal gilt demonstrating an age dependant increased in Ob-Rb expression [9]. Leptin increased LH secretion from pig pituitary cells in culture and GnRH release from hypothalamic tissue in vitro [4]. The above reports support the idea that leptin and BF may play a role in the timing of puberty. The objective of this study was to examine the effects of short term feed restriction on serum LH and leptin concentrations, and leptin, Ob-rb, and transcription factors gene expression in subcutaneous adipose tissue in OVX prepuberal gilts.

2. Materials and Methods

2.1 Experimental Procedure

Twelve crossbred gilts, 79.19 ± 3.84 kg body weight (BW) and 164 days of age, were OVX two weeks before the start of the study. Gilts were individually penned in an environmentally controlled building and exposed to constant temperature of 21 ˚C and artificial 12:12 light:dark photoperiod. During the two weeks, the gilts were fed ad libitum a corn-soybean meal ration (Table 1.) supplemented with vitamins and minerals, according to the National Research Council guidelines [11]. On day 0 gilts were weighed, BF samples were taken, and 10th rib BF thickness was assessed using ultrasound (Renco Lean Meter, Renco Corp., Minneapolis, MN), and animals were randomly allotted to either feed restriction (RST; one-third of FA diet; n=6) or fed to appetite (FA; n=6). The FA group received 3 kg per day of the corn-soybean meal ration and the RST group received 1 kg of the same ration. Both groups were fed twice daily at 8:30 and 16:30 h with one half of the ration provided at each feeding. All feed was consumed by both treatments throughout the study. Water was available ad libitum. On day 7, gilts were fitted with an indwelling jugular vein cannula [12]. On day 8, blood samples were collected every 15 min for 8 h starting at 8:00 h and ending at 16:00 h. Pigs were fed at 8:30 h on Day 8. Samples were allowed to clot and then stored overnight at 4 ˚C, centrifuged and serum stored at -20 ˚C until assayed. On day 9, pigs were euthanized and animal BW, BF samples, and 10th rib BF measurements were taken. Backfat samples were flash frozen in liquid nitrogen and stored at -20 ˚C until RNA isolation. All procedures were approved by the Richard B. Russell Agriculture Research Center Committee on Animal Care and Use.

2.2 Metabolite and hormone assays

Samples were assayed for glucose every 15 minutes from 8:00-9:00 h and at 10:00, 10:30, 11:00, 12:00, 14:00, and 16:00 h using a glucose oxidase kit (Sigma Chemical, St. Louis, MO). The 8:00, 12:00, and 16:00 h samples were assayed for T3 and T4 using T3 solid phase component system kit and T4 monoclonal solid phase radioimmunoassay kit (RIA; MP Biomedicals, Orangeburg, NY). Non-esterified fatty acids (NEFAs) were measured on hourly samples using a colormetric procedure (WAKO Chemicals, Richmond VA). Insulin was determined on hourly samples using Linco Porcine RIA kit (Linco, St. Louis, MO). Plasma ß-hydroxybutyrate was determined on samples collected at 8:00, 12:00, and 16:00 h using an enzymatic assay kit (R-Biopharm, Marshall, MI).

Serum concentrations of LH [13] and leptin ([9]; Multi-Species Leptin Assay XL-85 kit, Linco Research, St. Louis, MO) were quantified by a RIA as previously described. Sensitivities of the assays were 0.15 ng/ml and 0.1 ng/ml for LH and leptin, respectively. Intra-assay and inter-assay CV for LH were 14.4% and 22.7% and 7.87% and 10.2% for leptin, respectively.

2.3 Gene Expression

Total RNA was isolated from the tissues using Qiazol reagent and RNA Maxi Kits (Qiagen, Valencia, CA) according to the manufacturer's procedure. Total RNA quality was determined using spectrophotometry at the UV absorbency of 260 nm by microfluidic technology on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). Quantity of the RNA was determined by spectrophotometry on a µquant Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Real-time PCR was performed by the University of

Georgia Functional Genomics Resource Facility and carried out using the Applied Biosystems 7900HT Sequence Detection System. Porcine sequences for the genes of interest were obtained from Genbank and were subjected to bioinformatics analysis using website tools publicly available at the National Center for Biotechnology Information, the Institute for Systems Biology, and the Institute for Genomic Research. Porcine 18s rRNA was amplified as an endogenous control. Two µl of total RNA was transcribed into complementary deoxyribonucleic acid (cDNA) in a volume of 20 µl with the ABI cDNA archive kit (4322171) following manufacturer's protocol. Thermal cycling parameters were as follows: 1 cycle 95˚C for 15 min, followed by rest at 95˚C for 15 sec, and 60˚C for 1 min.

2.4 Statistical Analysis

To determine the effect of treatment on serum LH, leptin, and metabolite concentrations, data were subjected to a split plot-in-time analysis of variance using the general linear model procedure of the Statistical Analysis System [14]. Treatment, pig, and time were discrete (class) variables. For each gilt, mean serum concentrations, basal concentrations, number of pulses, and pulse amplitude was determined for LH and leptin by PULSAR analysis, using a 1% criterion of variation [15]. Data were then subjected to a one-way analysis of variance procedure.

Differences in gene expression between treatment were performed by using group means for statistical significance by the pairwise fixed allocation randomization test, which was performed with the relative expression software tool (REST© version 2) [16,17]. All means were normalized to the endogenous control 18S rRNA expression.

3. Results

FA gilts gained ($P < 0.001$) more BW (8.18 ± 0.59 kg) than RST (-2.45 ± 0.59 kg) gilts; however, BF thickness did not change (Table 3). A treatment x time interaction ($P<0.02$) was detected for serum glucose concentrations (Fig. 1). Serum insulin ($P< 0.07$, Fig. 2), T3 ($P<0.08$, Fig. 3), and T4 concentrations ($P< 0.04$, Fig.4) were reduced in the RST animals compared to the FA gilts. A treatment by time interaction was detected for serum NEFA concentrations ($P< 0.04$, Fig. 5). There was no effect of RST on serum BHBA concentrations, LH (Fig.6), or Leptin (Fig. 7). Feed restriction failed to influence indices of LH secretion (Table 4) except for LH pulse amplitude, which was greater in RST animals ($P < 0.01$, Table 4) compared to controls, and the indices of leptin secretion (Table 5). Feed restriction in the RST gilts failed to affect BF leptin, Ob-rb, AFABP, C/EBPa, or PPARγ2 transcript expression compared to FA gilts (Table 6).

4. Discussion

In the present study, it was expected that the feeding regime employed would alter metabolic status and subsequent LH and leptin secretion and BF gene expression. Overall, the results indicate that indeed short term feed restriction did alter the metabolic state. First, RST suppressed growth. Second, serum concentrations of insulin, T3, and T4 were decreased and NEFA concentrations were elevated. These are all indicators of negative energy balance and agree with previous reports in the prepuberal and mature gilt [18-24]. Although RST reduced growth, no difference in BF was detected. Research has shown that in growing and finishing

pigs when intake is reduced or animals are subjected to marginal amino acid restrictions, adequate energy is available to maintain protein deposition at the expense of lipid deposition [25,26]. This data suggest weight loss may be due to an increase in lipid metabolism instead of a decrease in protein accretion.

Numerous studies have demonstrated the effect of RST on circulating LH concentrations in the sexually mature pig (for review see [27]). However, the influence of feed restriction on LH secretion is not as clear in prepuberal animals. Booth et al. [22] demonstrated a reduction in mean plasma LH concentration following 14 day RST in intact prepuberal gilts. In contrast, other studies reported no change in mean plasma LH concentrations using similar RST paradigm [21,23]. For example, no difference in LH secretion was observed in 160 day old intact prepuberal gilts fed 47% of control diet for 80 days. However, by 210 days of age RST decreased LH secretion [23]. Interestingly, the present study demonstrated that RST, at approximately 33% of control for 7 days, failed to reduce serum LH concentrations. In addition, serum LH pulse amplitude was higher in the restricted animals in this study. In prepuberal ovary-intact gilt, Barb et al. [28] demonstrated that a 28 hour fast decreased serum leptin concentrations but not LH secretion, where as Whisnant and Harrell [18] reported that a 7 day feed restriction at 33% of control diet decreased mean serum leptin concentrations and LH pulse frequency in OVX mature gilts. This dichotomy may in part be due to the differences in energy restriction and amount of BW and BF lost. Whisnant and Harrell [18] fed their gilts equal amounts of feed as the current study. However, the gilts were heavier and therefore, had a larger energy deprivation compared to the current study. Whisnant and Harrell [18] did not report BF measurements in their study.

Thus the relationship between leptin and LH remains unclear. Genetically obese (ob/ob) mice, which are leptin deficient due to a mutation in the gene encoding for leptin, are infertile and have atrophic reproductive organs [29]. Leptin treatment restored circulating concentrations of LH and fertility in the ob/ob mouse [29,30]. Treatment with recombinant leptin restored plasma LH concentrations and prolactin concentration following a 48 hour fast in the rat [31-33]. In sheep, leptin treatment reversed the feed restriction-induced suppression of LH secretion [34-36]. In the present study, serum leptin concentrations were not decreased by RST. This is in agreement with McNeel et al. [37], who observed no effect of RST on serum leptin concentrations in animals that received 50% of the control diet for a period of 5 weeks. In addition, Estienne et al. [38] demonstrated no change in serum leptin levels in pregnant gilts fed a high energy diet (6882 kcal ME) or a low energy diet (5221 kcal ME) even though differences in BF thickness were observed. In a different experiment, Estienne et al. [39] demonstrated that in the lactating sow with higher BF thickness at farrowing exhibited greater serum leptin concentrations compared to sows with less BF. However, the same sows at weaning had similar serum leptin concentrations even though BF thickness remained different. In contrast, Whisnant and Harrell [18] showed a decrease in serum leptin concentrations in OVX gilts following a seven day feed restriction. This dichotomy between Whisnant and Harrell's study and others may be due to the increase in metabolic rates between younger and older animals. Young animals can continuously break down lipid stores in order to maintain euglycemia and possibly uncoupling leptin's response to short term energy deprivation [28]. These data suggest that in pigs, leptin synthesis and/or secretion is not affected by short term restriction in nutrient availability.

Greater body fat content coincided with greater leptin mRNA expression in pigs [40-42], sheep [43], rats [44], and humans [45,46]. Leptin mRNA concentrations were greater in adipose tissue from genetically obese pigs compared to leaner pigs [37]. Restriction of feed intake to 33% of ad libitum intake for 7 days did not change the concentration of transcripts studied. Similarly, Spurlock et al. [47] reported that pigs allowed only submaintenance intake (restricted to 23% of the maintenance intake) lost 14.4% of their initial BW but had no significant effect on leptin mRNA expression compared to ad libitum fed pigs. Furthermore, McNeel et al. [37] observed no effect on porcine adipocyte expressing C/EBPa, PPARγ, aP2 (AFABP), and leptin in genetically obese and lean pigs fed 50% of the control diet. In addition, Ajuwon et al. [48] demonstrated that intermediate and high doses (0.01 and 0.03 mg/kg/day) of leptin injected intramuscularly to young pigs depressed adipose expression of PPARγ 1 and 2. However, leptin injection at a high dose (0.05 mg/kg/day) for 14 days in 53 kg castrated barrows upregulated PPARγ2 gene expression.

These data indicate that 67% feed restriction was not severe enough to overcome the homeostatic mechanisms of the prepuberal animal to produce a measurable effect. However, in the present study leptin expression was evaluated only in a single adipose depot. Research has demonstrated adipose dependant differences in leptin expression [49]. Therefore, results from the present study suggest that the ability of the prepuberal gilt to maintain euglycemia during RST may account for the failure of RST to effect LH or leptin secretion. It may also be that the animals used in this study which were younger than those in previous work [18] did not have a fully functioning leptin system. Thus, the leptin and LH response to RST may require energy levels and/or BF reduction reaching a putative inhibitory threshold.

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Table 1. Composition of Diet.	
Ingredients (%)	Amount
Corn	81.57
Soybean Meal	12.39
Fat	3.15
Dical. Phosphate	1.21
Limestone	0.90
Salt	0.35
Lysine HCL	0.20
Vitamin PreMix	0.13
Trace Mineral Mix	0.10
Calculated Analysis	Amount
Crude Protein (%)	12.98
DE (kcal/kg)	3586
Lysine (%)	0.75
Calcium (%)	0.65
Phosphorus (%)	0.50

Table 2. Primer pairs for each selected gene, probe sequences and Genbank accession number from which the primer pairs were selected.

Gene	Porcine probe	Probe size, bp	Probe sequences	Primer sequences	Source for primers	Genbank accession number
Leptin (Ob)	LEPTIN-1-144	68	CACATGCAGTCTGTCTCC	Forward-GACGATTGTCACCAGGATCAGT Reverse-CGGTGACCCTCTGTTTGGA	Porcine	U59894
Leptin Receptor (Ob-r)	LEPR-1-1602	74	TTTCACCACGGAATCAG	Forward-GATTCTCCACCAACATGTGTCATTC Reverse-ATTTCTGCTTTCACACTGGATGGA	Porcine	AF092422
CCAAT/enhancer binding protein alpha - (C/EBP alpha)	CEBPA-1-196	81	CATCTTCCGCCAGCTGC	Forward-CAACTGAGCCGCGAACTG Reverse-GTTGCCCATGGCCTTGAC	Porcine	AF103944
Adipocyte Fatty Acid Binding Protein (AFABP)	AFABP-1-166	72	CAGGAAAGTCAAGAGCACC	Forward-GGCCAGGAATTTGATGAAGTCACT Reverse-GGCGCCTCCATCTAAGGTTAT	Porcine	AF102872
Peroxisome Proliferator Activated Receptor Gamma 2 (PPAR gamma 2)	PPARG2-1-1180	72	TCTCAGTGGAGACCGCC	Forward-GACCTGGCGATATTTATAGCTGTCA Reverse-GATGGGCTTCACATTCAGCAAAC	Porcine	AF103946
18S ribosomal	18S-506	71	CAGCCACCCGAGATTG	Forward-AGGGCATCACAGACCTGTTATTG Reverse-CCCCAACTTCTTAGAGGGACAAG	Porcine	NR_002170

Table 3. Mean ±SE Body weight and 10th rib backfat measurements for prepuberal gilts fed to appetite (FA; n=6) and feed restricted (RST; n=6).

	Treatment	
	FA	RST
Day 0 Weight (kg)	79.19 ± 3.84 [a]	76.87 ± 4.82 [a]
Day 9 Weight (kg)	87.39 ± 3.96 [a]	74.43 ± 3.76 [b]
Day 0 BF (cm)	1.25 ± 0.22 [a]	1.40 ± 0 06 [a]
Day 9 BF (cm)	1.43 ± 0.06 [a]	1.28 ± 0.26 [a]

[a,b] Means in row with different superscripts are different (P< 0.002)

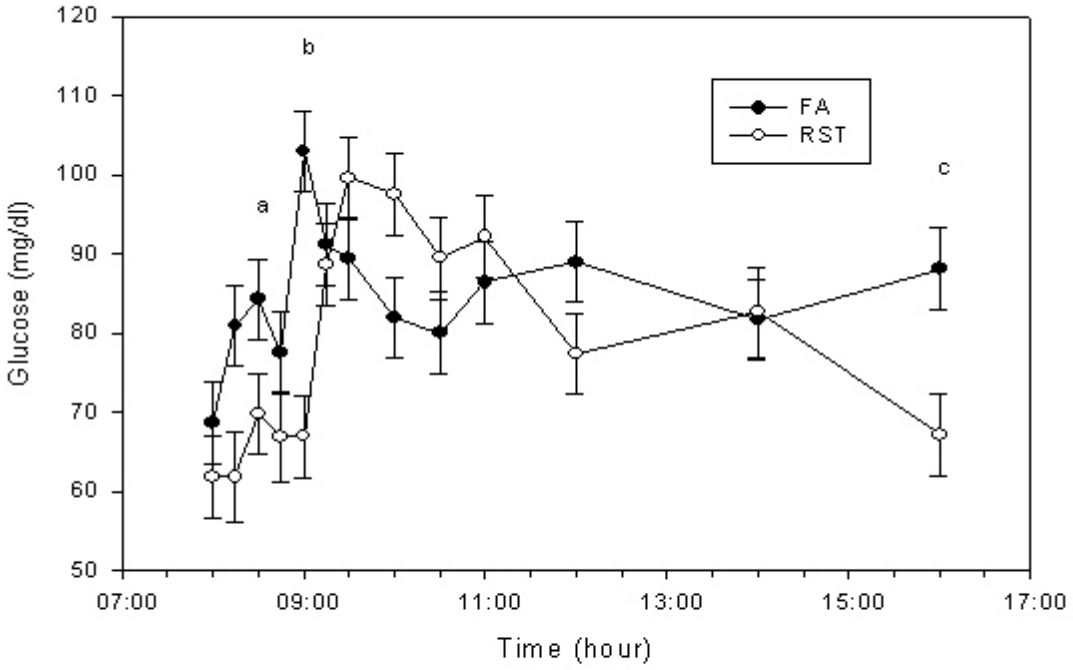

Fig. 1 Mean ±SE serum glucose concentrations for prepuberal gilts fed to appetite (FA; n=6) and feed restricted (RST; n=6). Times at which effects of treatment were different [a] ($P < 0.02$), [b] ($P < 0.05$),[c] ($P < 0.005$).

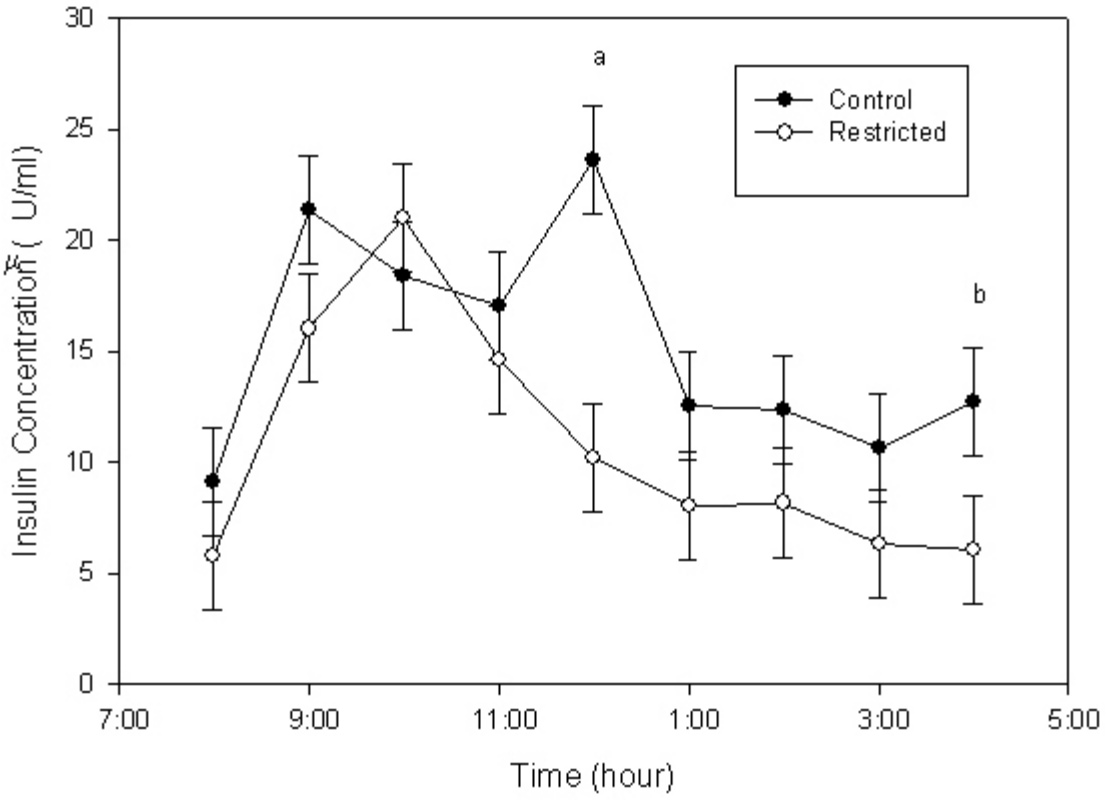

Fig. 2. Mean ±SE serum insulin concentrations for prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6). Times at which effects of treatment were different [a] ($P < 0.002$), [b] ($P < 0.05$).

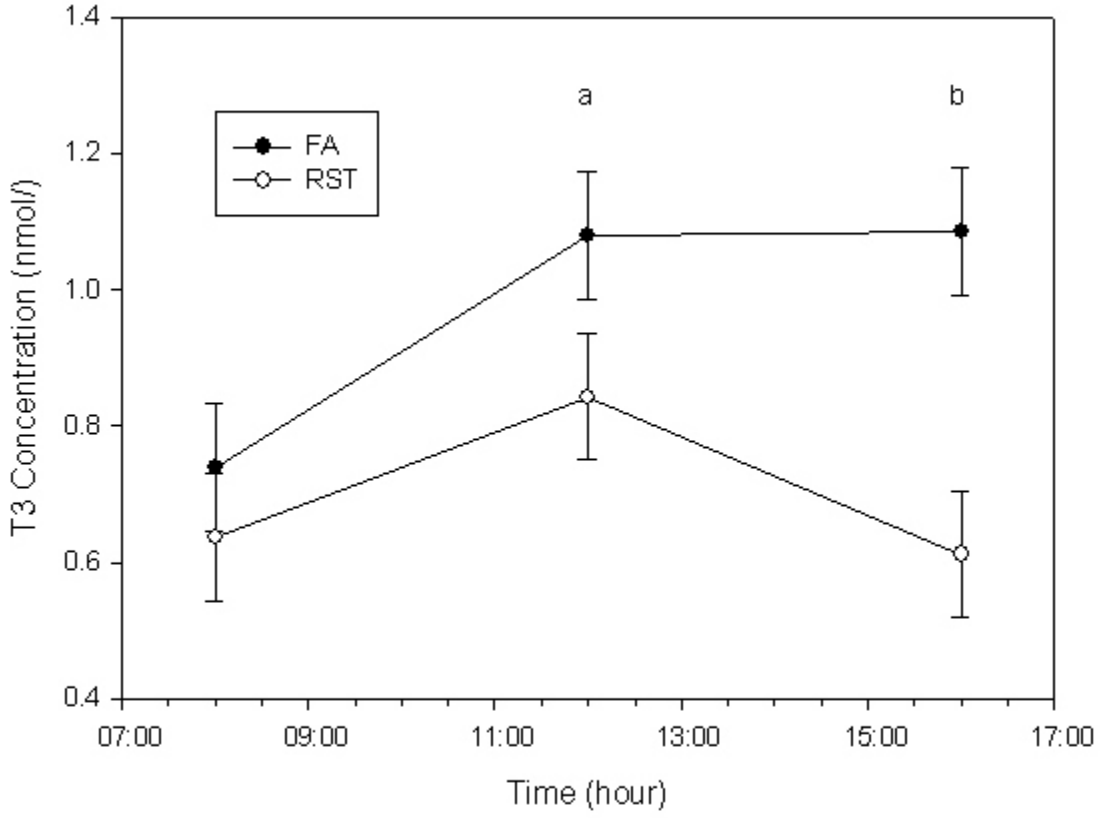

Fig. 3. Mean ±SE serum T3 concentrations for prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6). Times at which effects of treatment were different [a](P<0.09) and [b](P<0.002).

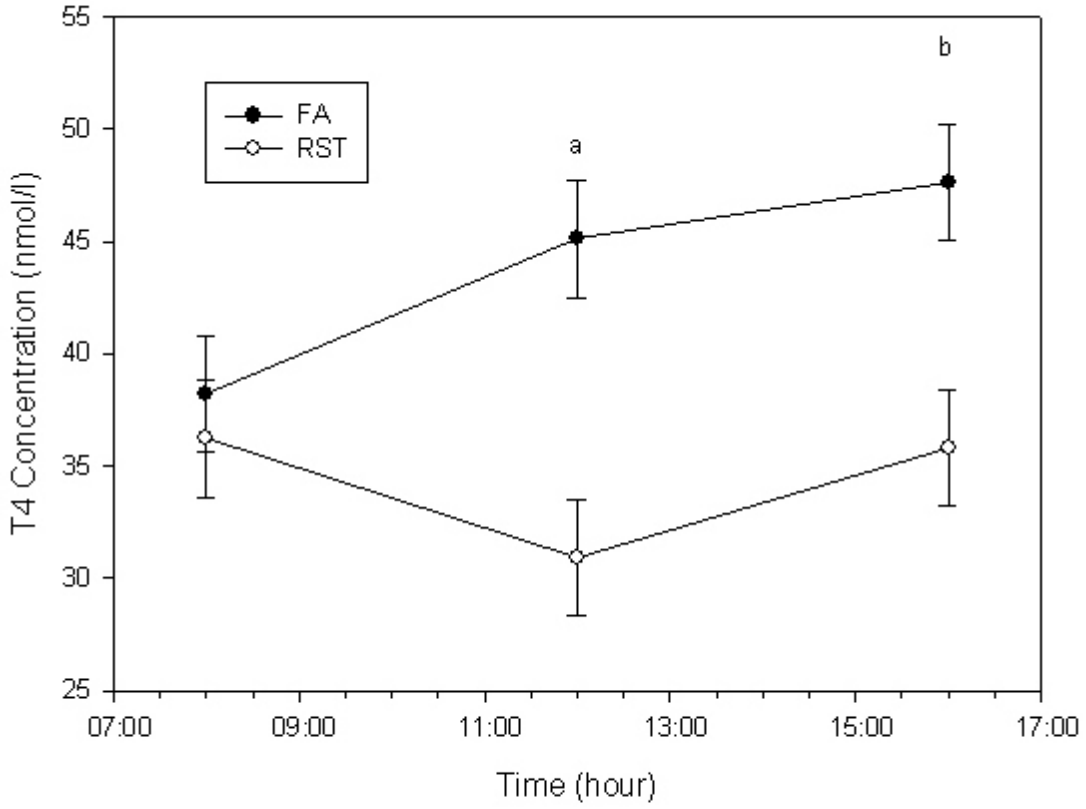

Fig. 4. Mean ±SE serum T4 concentrations for prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6). Times at which effects of treatment were different [a](P<0.01) and [b](P<0.004).

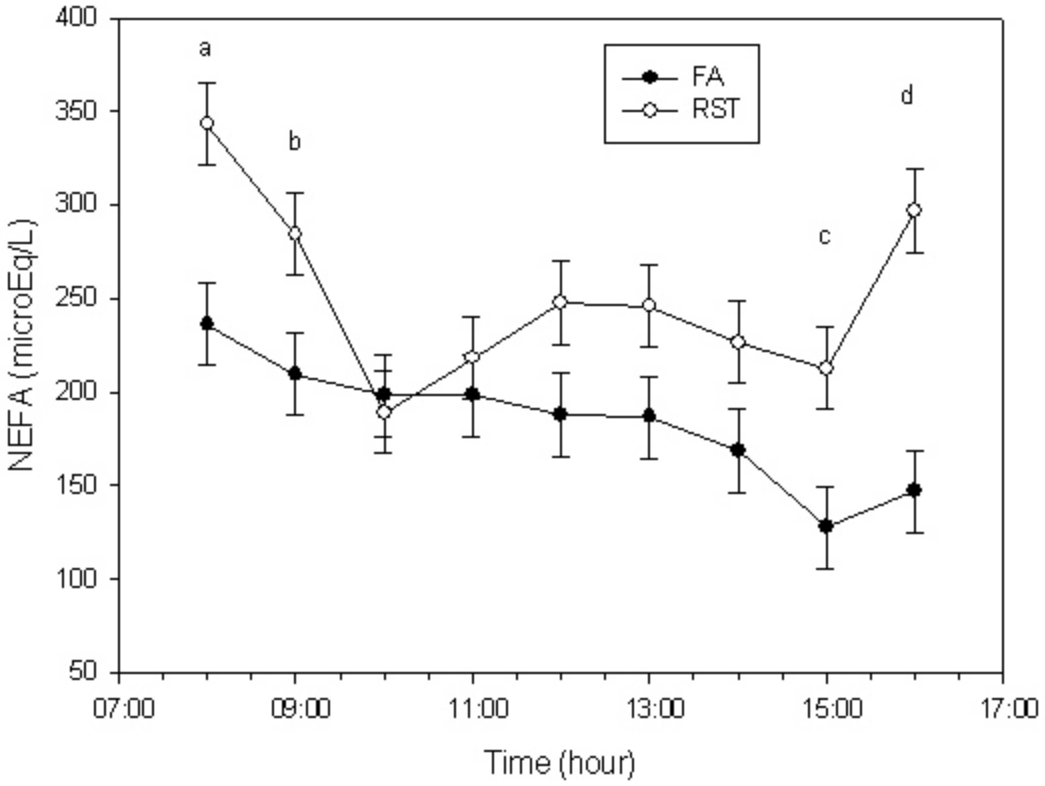

Fig. 5. Mean and ±SE serum NEFAs concentrations for prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6). Times at which effects of treatment were different [a](P<0.009), [b](P<0.02), [c](P<0.008), and [d](P<0.001).

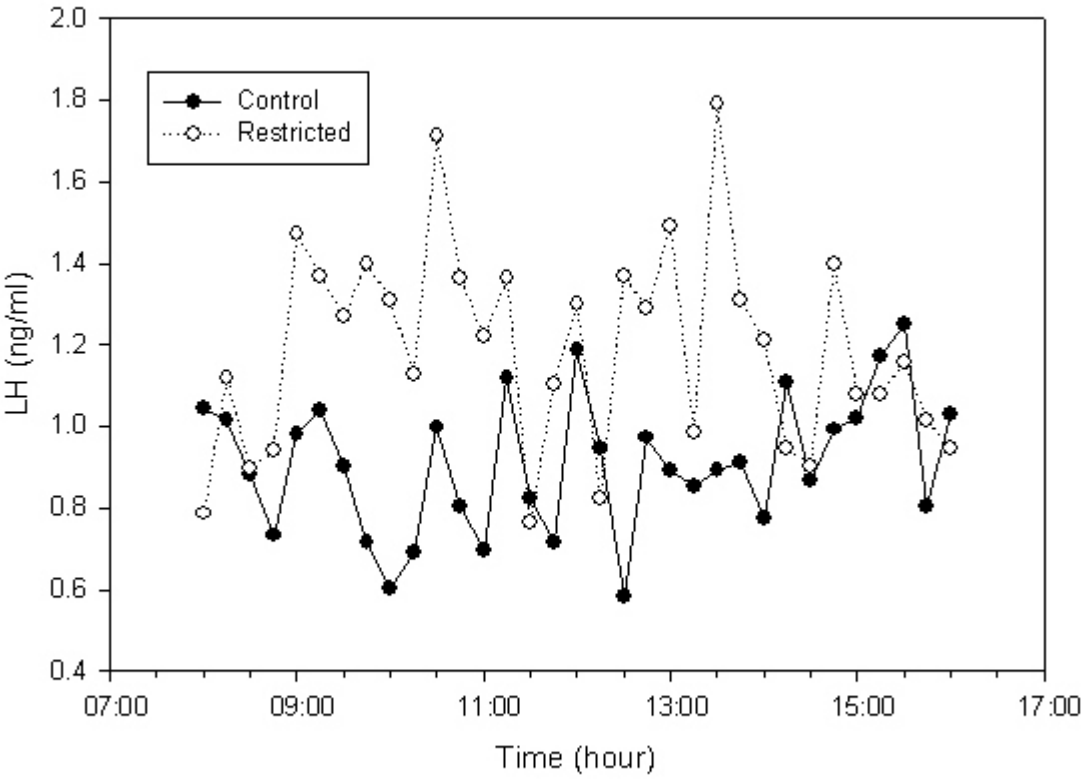

Fig. 6. Mean ±SE serum LH concentrations for prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6). Pooled SE of 0.022.

Table 4. Mean and baseline serum LH concentrations, LH pulse frequency, and LH pulse amplitude for prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6)[a].

	FA	RST
Mean LH (ng/ml)	0.910 ± 0.075 [b]	1.20 ± 0.22 [b]
Baseline LH (ng/ml)	0.757 ± 0.077 [b]	0.847 ± 0.195 [b]
Pulse (per hour)	0.834 ± .042 [b]	0.89 ± .051 [b]
Pulse Amplitude (ng/ml)	0.683 ± 0.043 [b]	1.21 ± 0.162 [c]

[a] Value expressed as mean ±SE.

[b,c] Means in a row with different superscripts are different ($P < 0.01$)

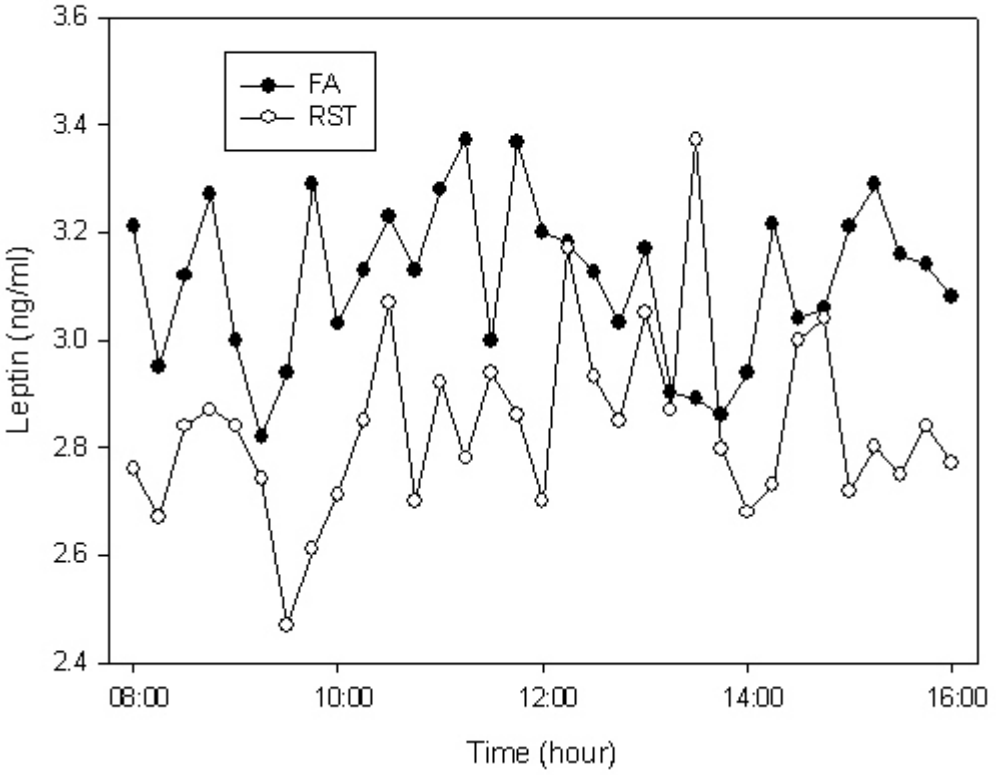

Fig. 7. Mean ± SE serum leptin concentrations in prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6). Pooled SE of 0.03.

Table 5. Mean and baseline serum leptin concentrations, leptin pulse frequency, and leptin pulse amplitude for prepuberal gilts fed to appetite (FA; n = 6) and feed restricted (RST; n = 6)[a].

	Treatment	
	FA	RST
Mean Leptin Conc. (ng/ml)	3.11 ± 0.32	2.84 ± 0.33
Baseline (ng/ml)	2.99 ± 0.36	2.69 ± 0.31
Pulse (per hour)	0.687 ± 0.071	0.79 ± 0.042
Pulse Amplitude (ng/ml)	0.57 ± 0.13	0.59 ± 0.11

[a] Value expressed as mean ±SE.

Table 6. Pair Wise Fixed Reallocation Randomisation Test © for Gene Expression Analysis (samples are normalized to 18S control; n=18)

	18S	Leptin	Leptin Receptor	AFABP	C/EBPα	PPARγ2
FA Means ± SE (cycles)	11.89 ± 0.14	29.87 ± 0.34	32.44 ± 1.04	22.13 ± 0.11	27.41 ± 0.22	28.49 ± 0.04
RST Means ± SE (cycles)	11.81 ± 0.21	29.75 ± 0.24	32.11 ± 0.22	22.13 ± 0.14	27.20 ± 0.17	28.20 ± 0.04
Expression Ratios		1.023	1.181	0.944	1.092	1.155
P-Values		0.902	0.753	0.542	0.460	0.458

CHAPTER 4

CONCLUSION

The adipocyte hormone, leptin, appears to play a role in reproductive function. The results the present study suggest that the ability of the prepuberal gilt to maintain euglycemia during feed restriction may account for the failure of feed restriction to effect luteinizing hormone (LH) or leptin. Thus, the leptin and LH response to restriction may require energy levels and/or back fat reduction reaching a putative inhibitory threshold.