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Effects of Development, the Estrous Cycle and 17β-Estradiol on Adipocyte Leptin, Erα and Erβ Gene Expression in Gilts

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Abstract: The effect of development, stage of the estrous cycle and estradiol on adipocyte leptin, ERα and ERβ gene expression was examined. Blood was collected twice weekly from 24 gilts before and after 1st estrus and analyzed for serum leptin by RIA. Serum concentrations of leptin increased ($p = 0.02$) to puberty. Subcutaneous adipose tissue was collected from 12 prepubertal (PP) gilts (121±1.2 d of age, 50.4±1.5 kg) and 12 Pubertal (PE) gilts (235±2.6 d of age, 137±3.3 kg) at first estrus (D 0) and during their mid-luteal (ML; D 8) stage of the respective estrous cycle for tissue mRNA analyses and cell culture. Isolated adipocytes were cultured for 18 h with 17β-estradiol (0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M; n=3 wells/dose/pig). Leptin, ERα, ERβ and cyclophilin mRNA were analyzed using real-time reverse transcription-polymerase chain reaction (RT-PCR). Leptin mRNA was found to be in greater abundance ($p = 0.05$) in adipose tissue from PE than PP or ML gilts. Estrogen receptor subtype gene expression was similar in adipose tissue from PP and PE gilts; however, ERβ tended ($p<0.1$) to be higher in adipose tissue from PE than ML gilts. *In vitro* studies revealed that 17β-estradiol had no effect on leptin gene expression in PP, PE, or ML adipocytes. Estrogen receptor α gene expression decreased ($p<0.05$) in *in vitro* estradiol-treated PP and ML adipocytes; however, ERβ only decreased in estradiol-treated adipocytes from ML gilts. Estradiol did not appear to affect leptin, ERα, or ERβ gene expression in PE adipocytes. Although the synthesis and secretion of leptin increased as puberty approached and appeared to change during the estrous cycle, these data do not attribute the developmental and / or reproductive cycle changes in leptin synthesis and secretion to a direct effect of estradiol.

Key words: Leptin, estradiol, adipocyte, gilt

INTRODUCTION

Leptin is a potent satiety hormone that is synthesized and secreted by adipocytes and it participates in the regulation of food intake, energy homeostasis and various reproductive processes[1,2]. Deficiencies in leptin result in morbid obesity and infertility[1,2], which has been reversed or corrected with exogenous leptin[3]. Moreover, leptin treatment reduces age at puberty in normal wild-type female mice[4,5] however, the mechanisms through which leptin influences pubertal development and other reproductive processes are not known.

Estrogen is an ovarian hormone that plays a critical role in all reproductive processes of the mammalian female including pubertal development and reproductive cycles. Interestingly, estrogen has been reported to influence the synthesis and secretion of leptin. Serum concentrations of leptin are lower in ovariectomized (OVX) rats compared to intact controls, which is reversed with the administration of estradiol[6]. Moreover, estradiol activates the leptin promoter in transfected MCF-7 breast cancer and JEG-3 choriocarcinoma cells[7]. This apparent relationship between estrogen and leptin may be influenced to some extent by stage of development. Qian *et al.*[8] reported higher concentrations of leptin mRNA in adipose tissue from 210-d old OVX + estradiol-implanted gilts than in either contemporary untreated OVX gilts or 90-or 150-d old OVX + estradiol-implanted gilts. This developmentally-regulated association may be attributed to Estrogen Receptor (ER) subtypes, α and/or β, both of which have been identified in adipose tissue.

Estrogen receptor subtypes are reported to differ in both concentration and ratio in various tissue types during different stages of development[9,10] and the reproductive cycle[11], which may account for age related

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changes in sensitivity of tissue to estrogen. Moreover, the ER subtypes often exhibit opposing transcriptional activities[12,13]. Therefore, leptin's influence on various reproductive processes may be associated with estrogen and ER subtype. Hence, the current study was conducted to:

- characterize leptin, ERα and ERβ gene expression in adipose tissue collected from gilts: a) prior to puberty, b) at first pubertal estrus and c) during the luteal cycle and
- utilizing adypocytes collected and cultured from these three time points, assess the effects of estradiol treatment on adypocyte leptin, ERα and ERβ gene expression.

MATERIALS AND METHODS

Animals: Twenty-four Yorkshire gilts (121±1.2 d of age, 50.4±1.5 kg) from the Texa A and M University-Kingsville Farm were utilized. Twelve of the gilts were allocated for Prepubertal (PP) sample collection and analysis. The remaining 12 gilts were allocated for sample collection at first standing Pubertal Estrus (PE) and during the Mid-Luteal (ML) stage of the estrous cycle. The PE gilts were exposed to a boar daily for 30 min beginning at 150 d of age. Puberty was defined as gilts displaying classical behavioral estrus in response to boar exposure and subsequent ovulation, which was confirmed by serum progesterone concentration = 2 ng mL^{-1}. All gilts were housed in concrete pens (7 x 13 m) in an outdoor, sheltered facility. Ad libitum access to water and a corn-soybean based diet were provided throughout the experiment. All animals utilized were in pre-accordance with the approved institutional animal care and use committee protocol.

Blood collection and serum analyses: Blood samples were collected via jugular venipuncture from the PE group of gilts twice weekly from 150 d of age until 8 d following first pubertal estrus. Serum was harvested and stored at -80°C until analysis for circulating concentrations of leptin and progesterone. Serum concentrations of progesterone were quantified by Radioimmunoassay (RIA) using Coat-A-Count® assay kit (Diagnostics Product Corporation, Los Angeles, CA). Use of these kits have been previously validated for porcine plasma[14,15]. Sensitivity of the assay was 0.1 ng mL^{-1} with an intraassay coefficient of variation of 6%. Serum concentrations of leptin were determined using a leptin RIA validated for porcine serum[16] having inter- and intraassay coefficients of variation < 10%.

Tissue collection: Subcutaneous adipose tissue (~2-3 g) from the cervical vertebral region was collected from PP gilts (121±1.19 d of age, 50.42±1.52 kg) upon initiation of the study, from the PE gilts at first estrus (D 0 of the estrous cycle, 235±2.6 d of age, 137±3.3 kg) and during the ML (D 8) stage of the estrous cycle. All tissue samples were collected following anesthetization by intravenous administration of sodium thiopental (2.5%; Butler Animal Health Supply, Dublin, OH) using a Butterfly (25G x 19 mm needle) tubing (30 cm) infusion set (Abbott Laboratories, North Chicago, IL). Approximately 1 g of harvested tissue was snap frozen in liquid nitrogen and stored at -80°C for leptin, Erα and Erβ mRNA analysis using real-time RT-PCR. The remaining tissue was placed in Hank's Balanced Salt Solution (HyClone, Logan, UT), transported to the laboratory in a pre-warmed (37°C) container and prepared for adipocyte isolation and cell culture.

Adipocyte isolation and cell culture: Adipose tissue was divided into 100 mg pieces, minced and placed in digestion solution [phenol-free Dulbecco's Modified Eagle's Medium Nutrient Mixture F12 Ham [(DMEM; Sigma, St. Louis, MO), 1.5 mg mL^{-1} collagenase (Type II, Sigma)] for 90 min at 37°C in a shaking waterbath (125 rpm). Digested tissue was filtered through a 60-mesh screen to remove undigested tissue. Filtered cells were centrifuged for 1 min at 28 x g (550 rpm). The infranatant was removed from the floating adipocytes, 1 ml of pre-warmed (37°C) DMEM was added to rinse cells free from digestion solution and were centrifuged for 1 min at 28 x g (550 rpm). Adipocyte concentration was determined using a hemocytometer. Approximately 3 x 10^5 cells mL^{-1} were cultured in 1.5 ml of culture media [phenol-free DMEM (Sigma), 0.1 mg mL^{-1} streptomysin and 100 U mL^{-1} penicillin (HyClone), 1.5% BSA (Sigma), 2.5 mM mL^{-1} L-Glutamine (HyClone)] with or without 17β-estradiol (Sigma, 0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} M; n = 3 wells/dose/pig) in a 12-well culture plate (Corning Inc., Corning, NY). Adipocytes were incubated at 37°C in an atmosphere of 5% CO_2 in air with 95% humidity for 18 h. Upon termination of the incubation, cells were placed in denaturing solution [double distilled water, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) N-laurylsarkosine, 4M guanidine thiocyanate] with 2-mercaptoethanol (7 uL mL^{-1}; EM Science, Gibbstown, NJ) and stored at -80°C until analysis for leptin, Erα and Erβ mRNA using real-time RT-PCR.

Gene expression analyses: Leptin, ERα and ERβ mRNA levels were analyzed by real-time RT-PCR using the DNA Engine Opticon® II System (MJ Research, Waltham, MA). All RT-PCR reactions were performed using Quantitect® SYBR® Green one-step RT-PCR kit (QIAGEN Inc., Valencia, CA). The RT-PCR mixture contained 2X Quantitect SYBR Green RT-PCR Master mix,

Table 1: Primer sequences used for PCR

Gene	Forward	Reverse	Product Size (bp)	Annealing temperature (°C)	GenBank	Reference:
Leptin	ACAGAGGGTCACCGGTTTGG	TAGAGGGAGGCTTCCAGGAC	258	56°C	AF026976	Lin *et al.* 1998
ERα	TCCGTATGATGAATCTCCAG	TTGGCCATCAGGTGGATCAA	171	50°C	AF267736	
ERβ	TACAACTGCAGTCAATCTGT	CCAAGGACTCTTTTGAGGT	267	50°C	Z37167	
Cyclophilin	TGCCATCCAACCACTCAG	TAACCCCACCGTCTTCTT	369	50°C	AF14571	Dozois *et al.* 1997

25 pmol of forward and reverse primers, 1 μg of template RNA, 0.25 μL of Quantitect RT mix and brought to volume (25 μL) with RNase free H_2O. Porcine specific leptin, ERα, ERβ and cyclophilin primers were utilized to amplify the target cDNA products Table 1. All primers were synthesized by IDT Inc. (Coralville, IA). Relative quantities of leptin, ERα and ERβ were calculated from a relative standard curve (1 μg to 10 pg of total RNA) obtained from real-time RT-PCR of cyclophilin mRNA in porcine liver tissue[17]. Real-time RT-PCR was conducted in one step as follows; reverse transcription at 50°C for 30 min and 95°C for 15 min to deactivate the reverse transcriptases (Sensiscript® and Omniscript®) and activate HotStarTaq® DNA polymerase. The cycling stage consisted of 94°C for 1 min, 50-55°C anneal for 30 sec and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was incorporated to allow for completion of product extension. Melting curve analysis was performed following a cycling protocol of 1°C temperature increment/1 sec holding time from at 65°C to 99°C to verify the identity and homogeneity of PCR products. Each real-time RT-PCR run was performed in duplicate to reduce experimental errors.

Cycle threshold (C_t), the cycle at which the florescence amplification curve crosses a threshold, was set at 18.5 for ERα and ERβ and 19.5 for leptin to standardize each PCR run. Tissue gene expression values, i.e., values obtained from the relative standard curve with the adjusted C_t, were transformed to Log_{10} and normalized with cyclophilin, a housekeeping gene to account for variability in sample preparation. Adipocyte gene expression values were determined as described for tissue; however in addition, the Log_{10} values were transformed to a percent of 0M (100%). Estrogen receptor α and ERβ PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide, extracted, purified and cloned using the pGEM®-T Vector System (Promega, Madison, WI). Cloned products were sequenced using Gene Gateway Service (Hayward City, CA) to specify the target sequence of the ERα and ERβ primers.

Statistical analyses: Mean serum concentrations of leptin from 54 d prior to puberty until detection of pubertal estrus (D 0) were evaluated using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) for repeated measures. Day was the source of variation and gilt within day was used as the subject to account for correlated variation within animal. One gilt was eliminated from serum leptin analysis due to a high degree of behavioral stress during blood sample collection, which is associated with increased concentrations of circulating glucocorticoids, a hormone reported to stimulate leptin production[18]. The effect of stage of development (PP vs. PE) and the estrous cycle (PE vs. ML) on leptin, ERα and ERβ mRNA expression in adipose tissue were analyzed using the MIXED procedure of SAS. Sources of variation included either stage of development or the estrous cycle. The effect of 17β-estradiol on leptin, ERα and ERβ mRNA expression in cultured adipocytes were analyzed within group (PP, PE, ML) using the PROC MIXED procedure of SAS. Source o f variation in the model included treatment. Gilt was the RANDOM variable to account for variation within animals. Upon detection of significance in the model, differences in treatment dose means were determined using the ESTIMATE procedure of SAS. Values were represented as LS means±SEM. A P value = 0.05 was considered to be statistically significant.

RESULTS

Serum concentrations of leptin increased as puberty approached ($p = 0.01$, Fig. 1), peaking ~19 d prior to pubertal estrus. Leptin gene expression in adipose tissue was higher ($p<0.01$) in PE vs. PP gilts Fig. 2. However, leptin decreased ($p = 0.05$) in ML adipose tissue compared to PE tissue Fig. 3 . No difference in ERα or ERβ gene

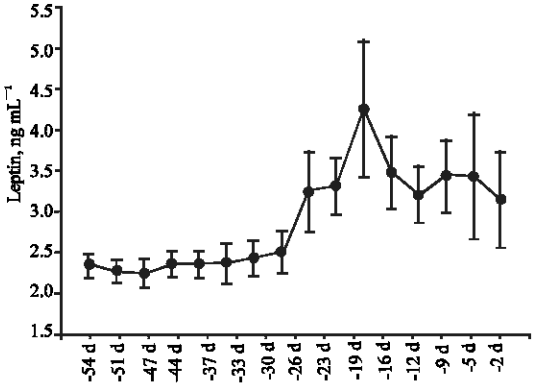

Fig. 1: Mean (± SEM) serum concentrations of leptin (ng mL^{-1}) relative to days (d) prior to pubertal estrus (d 0; PP) in gilts (n=11). Serum concentrations of leptin increased ($p<0.05$) as puberty approached, peaking 19 d prior to first estrus

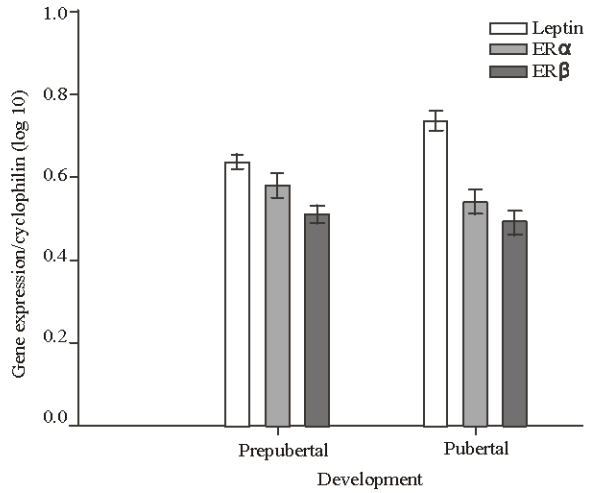

Fig. 2: Leptin, ERα and ERβ mRNA expression (± SEM) in subcutaneous adipose tissue from prepubertal (PP; n=11) and pubertal (PE; n=12) gilts. Values were obtained using relative quantitative real-time RT-PCR. Relative quantities of leptin, ERα and ERβ were generated from a relative standard curve (1 μg to 10 pg of total RNA) obtained from RT-PCR of cyclophilin mRNA from porcine liver tissue. Relative quantities were transformed to Log_{10} and normalized with cyclophilin (the housekeeping gene for each sample). *Means differ ($p<0.01$) significantly between stages of development

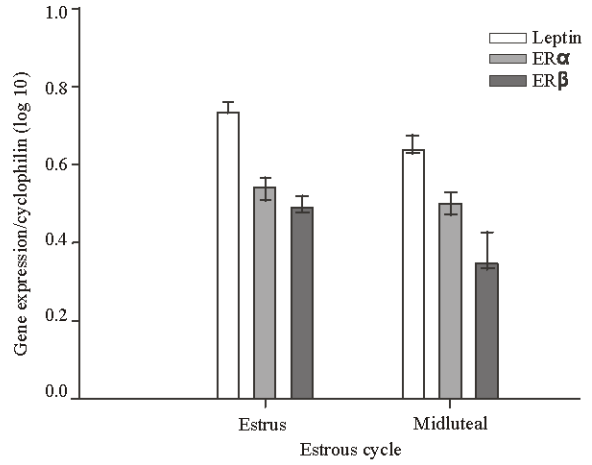

Fig. 3: Leptin, ERα and ERβ mRNA expression (± SEM) in subcutaneous adipose tissue from gilts at pubertal estrus (PE; d 0; n=12) and during the mid-luteal stage of estrous cycle (ML; d 8; n=12). Values were obtained using relative quantitative real-time RT-PCR. Relative quantities of leptin, ERα and ERβ were generated from a relative standard curve (1 μg to 10 pg of total RNA)obtained from the RT-PCR of cyclophilin mRNA in porcine liver tissue. Relative quantities were transformed to Log_{10} and normalized with cyclophilin, the housekeeping gene for each sample. *Means differ ($p<0.05$) significantly between estrus (PE) and the mid-luteal (ML) stage of the estrous cycle. [a]Superscript represents means that tended ($p<0.1$) to differ significantly between estrus and the mid-luteal stage of the estrous cycle

expression was detected in subcutaneous adipose tissue relative to the stage of development (Fig. 2); but, ERβ gene expression tended ($p<0.1$) to be higher at estrus than during the ML stage of the estrous cycle (Fig. 3). Estradiol treatment did not affect leptin gene expression in PP, PE, or ML adipocytes (Fig. 4-6). However, ERα was lower ($p<0.05$) at all doses of estradiol in PP and ML adipocytes. Gene expression of Erβ only decreased ($p<0.05$) in the ML adipocytes Fig. 6. Estradiol treatment did not appear to affect leptin, ERα, or ERβ gene expression in adipocytes from PE gilts Fig. 5.

DISCUSSION

As anticipated, leptin gene expression was higher in adipose tissue from PE gilts than PP gilts. Subsequently, serum concentrations of leptin increased as puberty approached. Leptin production is highly correlated with both BW and adiposity in humans, rodents, cattle, sheep and swine; increasing as BW and adiposity increase[19-22]. Therefore, an increase in the synthesis and secretion of leptin, accompanied with development, is associated, in part, with an increase in BW and fat accumulation. However, circulating concentrations of leptin appear to peak ~19 d prior to pubertal estrus, decreasing thereafter, but remaining relatively high. Recabarren *et al.*[23] reported similar results in ewe lambs after observing a significant increase in serum leptin 42-14 d prior to puberty.

Additionally, these authors reported a simultaneous increase in mean serum concentrations of Luteinizing Hormone (LH). Although serum LH was not analyzed in this study, increases in LH and FSH pulse frequency occur in prepubertal gilts around the period of time[24] when serum leptin increased in the current study. Interestingly, leptin gene expression decreased in adipose tissue during the ML stage of the estrous cycle compared to estrus, which is a period of time when pulsatile secretion of LH and FSH are low. Collectively, the results support the supposition that leptin may be associated with physiological events involved in both the onset of puberty and the estrous cycle.

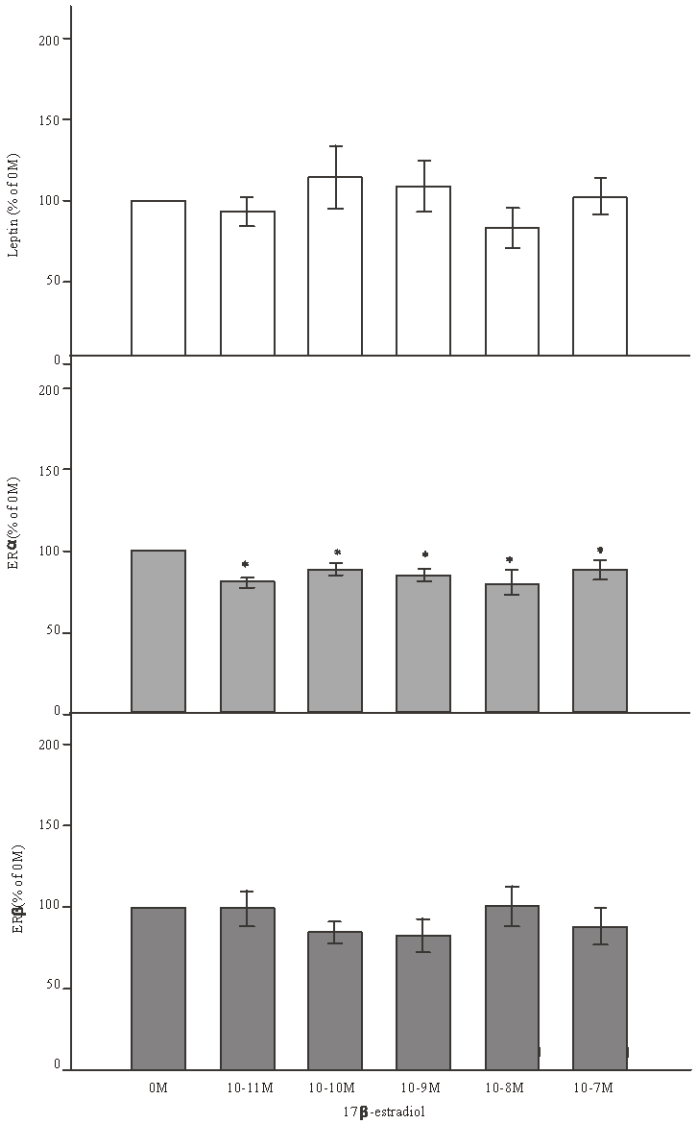

Fig. 4: Leptin (n=12), ERα (n=12) and ERβ (n=11) mRNA expression (± SEM) in subcutaneous adipocytes from prepubertal (PP) gilts. Cells were treated with or without 17β-estradiol (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 0 M). Values were obtained using relative quantitative real-time RT-PCR. Relative quantities of leptin, ERα and ERβ were generated from a relative standard curve (1 μg to 10 pg of total RNA) obtained from the RT-PCR of cyclophilin mRNA in porcine liver tissue. Relative quantities were transformed to Log_{10} and normalized with cyclophilin, the housekeeping gene for each sample. Leptin, ERα and ERβ values were represented as a percentage of 0 M values. *Means differ ($p<0.05$) significantly from control (0M)

Estrogen is an ovarian hormone that also plays a critical role in pubertal development in addition to its role in reproductive cycles[25,26]. As circulating concentrations of estrogen increase, the inhibitory / negative feedback effect of estradiol on LH secretion diminishes allowing an increase in the pulsatile release of LH[25]. Hence, the sharp increase in serum leptin may be more associated with changes in estrogen rather than LH. In support, ER hasbeen identified in both human and rodent adipose tissue[27,28] and the presence of both ER subtypes, α and β, were confirmed in porcine adipose tissue in the present study. Additionally, estradiol has been reported to directly activate the leptin promoter in transfected MCF-7 and JEG-3 choriocarcinoma cells[7]. Moreover, Machinal *et al.*[29] reported an increase in leptin mRNA in estradiol-treated adipocytes from mature OVX female rats. In contrast, the current study provides evidence that estradiol does not influence leptin gene expression

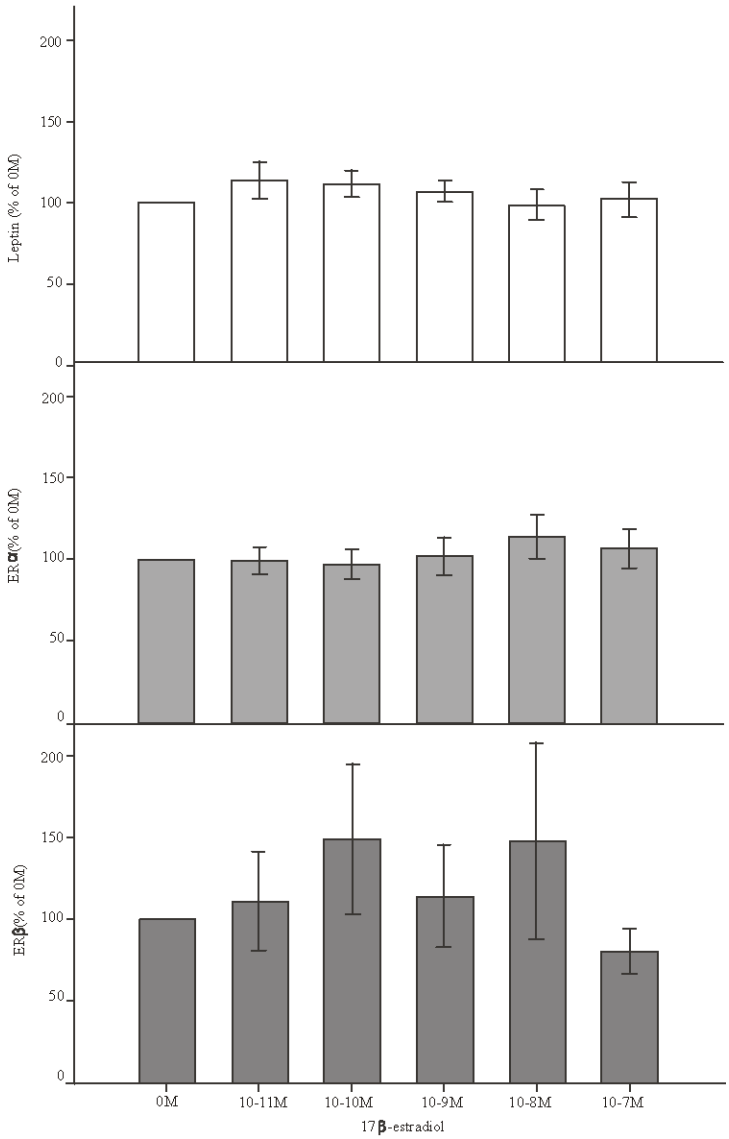

Fig. 5: Leptin (n=12), ERα (n=12) and ERβ (n=11) mRNA expression (±SEM) in subcutaneous adipocytes from (PE) gilts exhibiting pubertal estrus. Cells were treated with or without 17β-estradiol (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 0 M). Values were obtained using relative quantitative real-time RT-PCR. Relative quantities of leptin, ERα and ERβ were generated from a relative standard curve (1 μg to

pg of total RNA) obtained from the RT-PCR of cyclophilin mRNA in porcine liver tissue. Relative quantities were transformed to Log_{10} and normalized with cyclophilin, the housekeeping gene for each sample. Leptin, ERα and ERβ values were represented as a percentage of 0 M values. *Means differ ($p<0.05$) significantly from control (0M) relative to development or the reproductive cycle. The apparent lack of responsiveness of estradiol-treated adipocytes may be attributed to ER subtype since they are reported to differ in concentration in various tissue types during development[9,10] and the reproductive cycle[11], which would alter tissue sensitivity. However, ERα and ERβ gene expression did not differ in PP adipose tissue compared to PE tissue and only tended (ERβ only) to differ between PE and ML tissue. Moreover, ERα gene expression decreased in estradiol-treated PP and ML adipocytes, a hallmark of responsiveness to estradiol[12,13,30]. Hence, in comparison to the previously reported studies, the differences reported herein are likely attributed to specie specific variation and experimental model (i.e.,ovariectomized vs. intact females). Unlike ERα, ERβ gene expression did not consistently decrease in

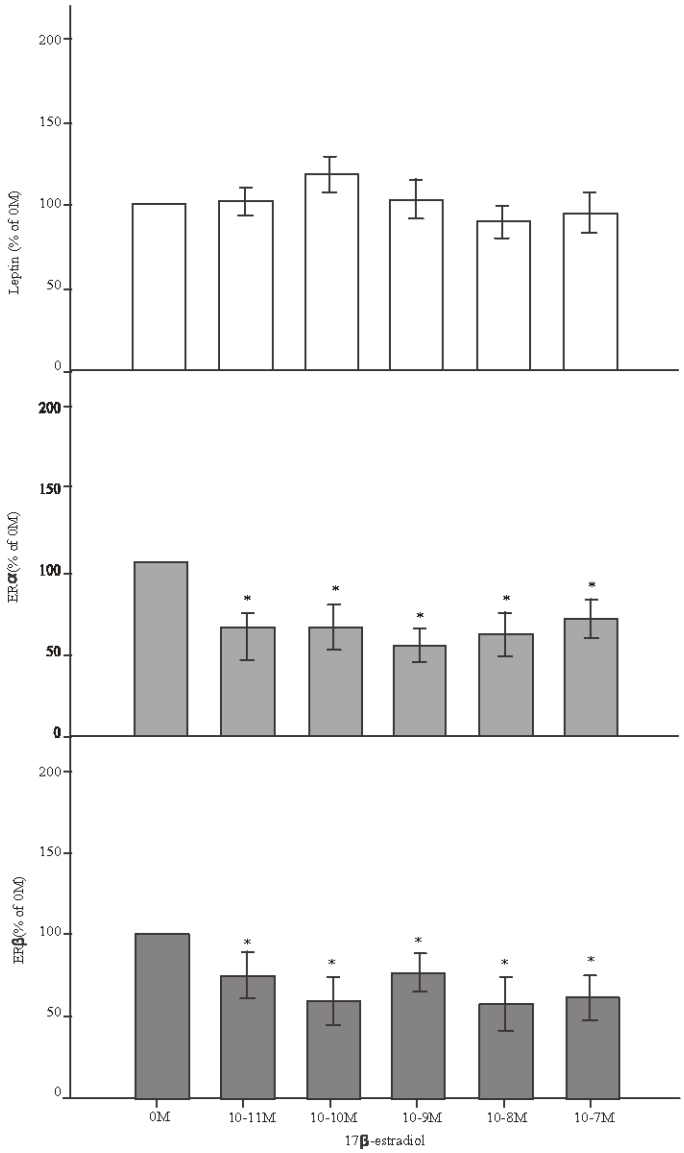

Fig. 6. Leptin (n=12), ERα (n=12) and ERβ (n=10) mRNA expression (± SEM) in subcutaneous adipocytes from gilts during the mid-luteal stage of the estrous cycle (ML). Cells were treated with or without 17β-estradiol (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 0 M). Values were obtained using relative quantitative real-time RT-PCR. Relative quantities of leptin, ERα and ERβ were generated from a relative standard curve (1 μg to 10 pg of total RNA) obtained from the RT-PCR of cyclophilin mRNA in porcine liver tissue. Relative quantities were transformed to Log_{10} and normalized with cyclophilin, the housekeeping gene for each sample. Leptin, ERα and ERβ values were represented as a percentage of 0 M values. *Means differ ($p<0.05$) significantly from control (0M)

response to estradiol, as observed for ERα in PP and ML adipocytes. Instead, ERβ gene expression decreased in response to estradiol only in ML adipocytes. Despite the apparent discrepancy in estradiol's ability to regulate ER subtype gene expression, differential regulation by estradiol is not a novel concept and has been reported to occur in various tissues including the ovary[31], mammary[32] and brain[33]. However, the estrogen-ER regulatory relationship in adipose tissue is not well defined. In humans, estradiol increases both ERα and ERβ gene expression in subcutaneous adipocytes[27], which is in contrast to the study reported herein. However, in the human study, the samples were obtained from pre-menopausal women averaging 42 years of age and the tissue samples may have been harvested during a stage of the reproductive cycle different from the stages in the

current study. Hence, further investigation will need to be conducted in order explore the estrogen-ER subtype relationship in adipose tissue.

In contrast to PP and ML estradiol-treated adipocytes, PE adipocytes did not appear to be responsive to estradiol since no effect on leptin, ERα, nor ERβ gene expression were detected. The lack of adipocyte responsiveness is attributed to overexposure to estrogen. In support, samples were collected on the first day of behavioral estrus, during which time circulating concentrations of estradiol typically ranges from 70 to 80 pg mL^{-1} in gilts [26].Moreover, estradiol is a fat soluble compound that accumulates in adipose tissue[34,35]. Furthermore, long-term exposure to estrogen is known to cause a refractory response in cells[36,37]. Therefore, adipocytes isolated from gilts at estrus were perhaps desensitized to estradiol in culture treatments and were not able to respond.

CONCLUSION

In conclusion, the present study provides evidence that leptin gene expression changes in adipose tissue in gilts during different stages of development and the reproductive cycle without concurrent changes in ER subtypes. These differences were not attributed to estrogen since estradiol did not appear to influence leptin gene expression *in vitro*. Estradiol's inability to influence leptin gene expression is not attributed to a lack of cell responsiveness since ER α and/or β decreased in estradiol-treated cells, which is a hallmark of estrogenic effects. Therefore, other factors, presumably associated with pubertal development and the estrous cycle, influence leptin gene expression during these two different reproductive processes.

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