Body condition of late pregnant gilts affects the expression of selected adipokines and their receptors in mammary fat and backfat tissues¹

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ABSTRACT: The impact of body condition in late gestating gilts on gene expression of selected adipokines and their receptors in backfat and mammary fat tissues was studied. The presence of associations between mammary gland composition variables and the mRNA abundance of selected genes and serum concentrations of adiponectin and leptin was also investigated. A total of 45 gilts were selected at mating based on their backfat depth and were allocated to three groups: (1) low backfat (**LBF**; 12–15 mm; $n = 14$), (2) medium backfat (**MBF**; 17–19 mm; $n = 15$), and (3) high backfat (**HBF**; 22–26 mm; $n = 16$). Gilts were fed different amounts of a conventional diet to maintain differences in backfat depth throughout the gestation period. Blood samples were collected at day 109 of gestation to measure adiponectin and leptin serum concentrations. Gilts were slaughtered on day 110 of gestation, and mammary glands were collected to determine mammary composition. Mammary fat and backfat tissues were also sampled to measure the mRNA abundance of selected genes. In mammary fat tissue, there was an effect of body condition on the *prolactin* (**PRL**; $P = 0.01$), *adiponutrin* (**PNPLA3**; $P < 0.10$), and *prolactin receptor long*

form (**PRLR-LF**; $P < 0.10$) genes. There was a greater *PRL* mRNA abundance in mammary fat tissue from HBF than LBF or MBF gilts ($P < 0.05$). The *PNPLA3* mRNA abundance was lower in HBF than in MBF gilts ($P < 0.05$), and that of *PRLR-LF* was lower in LBF than in HBF gilts ($P < 0.05$). In backfat, body condition affected the mRNA abundance of *leptin* ($P < 0.05$) and *PNPLA3* ($P < 0.01$), with the greatest expression levels being observed in HBF gilts for both genes. Association analyses suggest a detrimental effect of high circulating leptin concentrations on gilts mammary development, as reflected by the negative correlations between serum leptin and protein percent ($r = -0.66$, $P < 0.01$), and concentrations of DNA ($r = -0.62$, $P < 0.01$) and RNA ($r = -0.60$, $P < 0.01$) in mammary parenchyma. Current results show that body condition of gilts at the end of gestation can affect the expression of adipokines in mammary fat and backfat tissues, with a different regulation of transcript abundance being observed in these two fat depots. Results also suggest that circulating leptin is strongly associated with mammary gland composition of late pregnant gilts, whereas locally synthesized leptin from mammary fat tissue is not.

Key words: adipokines, adipose tissue, gene expression, gestation, gilt, mammary development

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INTRODUCTION

Accumulating evidence suggests that the gilt's body condition in gestation can affect its mammary development (Farmer et al., 2016a,b). However, little is known about the mechanisms

of action. One possible avenue is that the synthesis and secretion of adipose-derived factors, such as adipokines, may regulate mammary gland development and function (Palin et al., 2017). The white adipose tissue is an endocrine organ that produces and secretes numerous compounds with diverse biological functions (Coelho et al., 2013). The mammary fat tissue is described as a complex environment mainly composed of adipocytes, and of other cell types such as macrophages and lymphocytes, that can interact with mammary epithelial cells (MECs) (Hovey et al., 1999) through the synthesis and secretion of hormones, growth factors, and adipokines (Celis et al., 2005; Hovey and Aimo, 2010). In fact, the mammary fat tissue secretes more than 350 adipokines (Celis et al., 2005), with leptin and adiponectin being the most widely studied. In livestock species, the presence of leptin and adiponectin receptors in MEC, along with the capacity of leptin to regulate the action of prolactin in MEC, suggested that these two adipokines may affect mammary growth and function (Palin et al., 2017). Because of the key roles of adipokines, such as leptin and adiponectin, in maintaining metabolic homeostasis (Stern et al., 2016), it could be hypothesized that they act as energy sensors to regulate the metabolic adaptations of mammary glands occurring during late pregnancy and lactation in gilts.

The effect of fat-derived adipokines, either produced locally by mammary fat tissue or produced by peripheral fat depots, on mammary gland development has never been investigated in pigs. Therefore, our objectives were (1) to determine if body condition of gilts in gestation affects the mRNA abundances of selected adipokines and their receptors in backfat and mammary fat tissues; (2) to look for possible associations between mammary gland composition and the mRNA abundances of selected genes and serum concentrations of adiponectin and leptin; and (3) to determine whether selected genes present different expression profiles in backfat and mammary fat tissues.

MATERIAL AND METHODS

The experiment was conducted according to the recommended Canadian code of practice (CCAC, 2009), and procedures were approved by the Institutional Animal Care Committee of the Sherbrooke Research and Development Centre of Agriculture and Agri-Food Canada.

Animals and Treatments

Samples were obtained as part of a trial where three groups of pregnant gilts differing in body

condition were created to look at the impact on mammary development (Farmer et al., 2016a). A total of 45 Yorkshire × Landrace gilts were fed ad libitum during the growing and finishing phases and were selected at mating based on their body condition to form three groups, namely low backfat (LBF, 12–15 mm; $n = 14$), medium backfat (MBF, 17–19 mm, $n = 15$), and high backfat (HBF, 22–26 mm; $n = 16$). The gilts were bred via AI using pools of semen from Duroc boars of proven fertility. Throughout the gestation period, LBF, MBF, and HBF gilts received different amounts of a conventional diet (12.68 MJ/kg DE, 11.80% CP, and 0.59% lysine) to maintain similar backfat thicknesses from mating until the end of gestation (day 110). Details on the feeding management are provided in Farmer et al. (2016a).

Body Condition Measurements and Tissue Collection

Gilts were weighed and their backfat thicknesses measured ultrasonically at P2, being 5 cm from the midline at the level of the last rib (Vetkoplus, NOVEKO, Lachine, QC, Canada), at mating and throughout the gestation period (days 30, 50, 70, 100, and 109) in order to adjust the amount of feed provided so that backfat could remain stable. Preprandial blood samples (40 mL) were collected from the jugular vein in the morning on day 109 of gestation and were assayed for adiponectin and leptin. Vacutainer tubes for serum (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for blood collection and left at room temperature for 4 h, followed by an overnight period at 4 °C. Serum was then harvested after a centrifugation at $1,800 \times g$ (4 °C) for 12 min. Serum adiponectin concentrations were measured using a porcine ELISA Kit (Cusabio Biotech Co., College Park, MD). Parallelism was demonstrated with different serum pool dilutions (101.2%) and average recovery rate (106.9%) was calculated by adding different amounts of adiponectin standard to serum samples. Assay sensitivity was 1.88 µg/mL and intra- and interassay CV were 3.26% and 5.58%, respectively. For serum leptin, a multispecies commercial RIA Kit (EMD Millipore Corporation, Etobicoke, ON, Canada) was used. Parallelism (98.5%) and average recovery rate (98.2%) were calculated as described above. Sensitivity was 1.0 ng/mL, intra- and interassay CV were 6.00% and 0.59%, respectively (Farmer et al. 2016a).

Gilts were slaughtered on day 110 of gestation to collect mammary glands from both sides of the abdominal wall. The udder was separated in halves along the medial suspensory ligament and the side

with the largest number of functional glands was dissected to determine mammary gland composition, as described in [Farmer et al. \(2016a\)](#). For the other side of the udder, a sample (filling a 15-mL tube) of extraparenchymal tissue (mammary fat tissue) was collected from the fourth teat to measure the relative mRNA abundance of genes known or suspected to affect mammary development. Selected genes included adipokines [*adiponectin* (*ADIPOQ*) and *leptin* (*LEP*)] and adipokine receptors [*adiponectine receptors 1 and 2* (*ADIPOR1*, *ADIPOR2*), the long form of the *leptin receptor* (*LEPR-LF*)], as well as *adiponutrin* (*PNPLA3*), *prolactin* (*PRL*), and the long form of the *prolactin receptor* (*PRLR-LF*). Expression levels of *PRL* and its receptor were investigated because the *PRL* transcript was detected in adipose tissue from the human breast ([Zinger et al., 2003](#)) and from human subcutaneous fat layer ([Hugo et al., 2008](#)). Moreover, *PRL* was reported to affect adiponectin and leptin release from adipose tissue in rodents ([Ben-Jonathan and Hugo, 2015](#)). Adiponutrin was selected because it is expressed in the pig's subcutaneous adipose tissue ([Chen et al., 2011](#)), because its expression was shown in the rat mammary gland ([Russo and Russo, 2013](#)) and because leptin may be implicated in regulating *PNPLA3* gene expression ([Lake et al., 2005](#)). Subcutaneous backfat tissue was also collected for gene expression analyses of *ADIPOQ*, *LEP*, *PNPLA3*, and *PRL*. Adipose tissue samples were frozen in liquid nitrogen within 15 min after slaughter and were then stored at -80°C .

RNA Extraction and cDNA Synthesis

Total RNA was extracted from extraparenchymal and backfat tissues using TRIzol reagent (Thermo Fisher Scientific, Burlington, ON, Canada) according to the manufacturer's recommendations. Extracted RNA was dissolved in sterile water and its concentration, quality, and integrity were determined using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies Inc., Wilmington, DE) and a 1% agarose gel electrophoresis. One microgram of total RNA was treated with DNase I (1 U/ μg ; Amplification grade, Thermo Fisher Scientific) to remove contaminating genomic DNA. Efficiency of the DNase I treatment was verified by performing a PCR amplification of the *GAPDH* gene using primers that are interrupted by an intron. Total RNA was reverse transcribed to cDNA using a SuperScript II Reverse transcriptase (200 U/ μL ; Thermo Fisher Scientific) and oligo(dT)12 to 18 primers.

Expression Profile of Selected Genes in Backfat and Mammary Fat Depots

The relative mRNA abundance of selected genes in mammary fat tissue (*ADIPOQ*, *ADIPOR1*, *ADIPOR2*, *LEP*, *LEPR-LF*, *PNPLA3*, *PRL*, and *PRLR-LF*) and in backfat (*ADIPOQ*, *LEP*, *PNPLA3*, and *PRL*) was determined using real-time PCR amplification. Forward and reverse primers used are listed in [Table 1](#). Reactions were performed in a 10- μL reaction volume, except for *LEPR-LF* and *PRL* in extraparenchymal tissue and *PRL* in backfat (25 μL). The PCR reactions included corresponding concentrations of forward and reverse primers ([Table 1](#)), $1\times$ SYBR Green Master Mix final concentration (PE Applied Biosystems, Foster City, CA), 3 μL of $15\times$ diluted cDNA (7.5 μL for the 25 μL reaction) and 0.01 unit/ μL of uracil N-glycosylase AmpErase (PE Applied Biosystems). Cycling conditions started with a 2 min of AmpErase activation at 50°C followed by 10 min at 95°C . Forty repetitive cycles of 15 s at 95°C and annealing and polymerization for 45 s at 60°C were then performed. Amplifications were performed in triplicate in an ABI 7500 Fast Real-Time PCR System (PE Applied Biosystems) and specificity of primers used to amplify fragments was verified with the melting curve analysis. For each gene, standard curves were made with serial dilutions of cDNA pools ([Labrecque et al., 2009](#)) and were assayed in duplicate to obtain relative mRNA quantification using the standard curve method (User Bulletin #2; [Applied Biosystems, 1997](#)). Since two independent real-time PCR analyses were performed for the backfat and mammary adipose tissues, one cDNA pool was made for each tissue. Each 96-well plate included a standard curve (in duplicate) to account for experimental variations across plates. Five [*actin beta* (*ACTB*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *peptidylprolyl isomerase A – Cyclophilin A* (*PPIA*), *ubiquitin C* (*UBC*), and *vesicle-associated membrane protein-associated protein B/C*] and four (*ACTB*, *GAPDH*, *PPIA*, and *UBC*) reference genes (RG) were also amplified in the mammary fat and backfat tissues, respectively. The *PPIA* RG was identified as the least affected by treatments as determined by the NormFinder algorithm ([Andersen et al., 2004](#)). Relative quantification values were then obtained using *PPIA* as a normalization factor for both the extraparenchymal fat and backfat tissues. The relative quantity ratios were calculated by dividing the relative quantity units of studied genes by those of *PPIA*. Statistical analyses were then performed using the mean values from triplicates.

Table 1. Primer sequences used for real-time PCR amplifications of studied and reference genes in mammary fat and backfat tissues

Genes ¹		Primer sequence (5'–3') ²	GenBank accession no.	Product size (bp)	Primer (nM)		Amplification efficiency (%) ³
					Mammary fat	Backfat	
Studied genes							
<i>ADIPOQ</i>	(F)	GCTGTACTCTTCACCTACGACCAGTA	NM_214370.1	96	300	150	97.2
	(R)	CCAGACTTGGTCCCCCTTCT			900	900	
<i>ADIPOR1</i>	(F)	GCCATGGAGAAGATGGAGGA	NM_001007193.1	74	900	—	98.0
	(R)	AGCACGTCGTACGGGATGA			300		
<i>ADIPOR2</i>	(F)	TGTTCGCCACCCCTCAGTAT	NM_001007192.1	71	300	—	96.8
	(R)	AATGATTCCACTCAGGCCCA			900		
<i>LEP</i>	(F)	AGGGTCACCGGTTTGGACTT	NM_213840.1	101	300	300	96.3
	(R)	AGACTGGTGAGGATCTGTTGGTAGA			900	300	
<i>LEPR-LF</i>	(F)	AAGAGTGCGGTGCCCATTC	NM_001024587.1	71	300	—	104.2
	(R)	GTGAACAGCTCTCCTGGAGGAT			900		
<i>PNPLA3</i>	(F)	CTGAGGGAAGCAGCTAGAAACC	NM_001146126.1	133	300	300	96.2
	(R)	GTCTCTGGACCACAGCAATCG			900	900	
<i>PRL</i>	(F)	TGGAATCAAGGAGAATGAGGTCTAC	NM_213926.1	88	900	900	99.7
	(R)	AAAAGCAAAAAGGCGAGTGTCT			900	900	
<i>PRLR-LF</i>	(F)	CCACATCCTGGTGTTAGTGCA	NM_001001868.1	68	900	—	101.7
	(R)	CCTTGGTGGTTCTTCAAACG			900		
Reference genes							
<i>ACTB</i>	(F)	CATCACCATCGGCAACGA	XM_003124280.4	128	900	900	99.5
	(R)	GGATGTCGACGTCGCACTT			300	150	
<i>GAPDH</i>	(F)	CCCCAACGTGTCGGTTGT	NM_001206359.1	91	300	300	103.8
	(R)	CTCGGACGCCTGCTTCAC			900	900	
<i>PPIA</i>	(F)	GCACTGGTGGAAGTCCAT	NM_214353.1	71	300	300	100.6
	(R)	AGGACCCGTATGCTTCAGGA			300	300	
<i>UBC</i>	(F)	GGTGGGATGCAGATCTTCGT	XM_003483411.3	98	150	150	101.8
	(R)	TGGATCTTCGCCTTGACATTC			900	300	
<i>VAPB</i>	(F)	CTCTGGACGACACCGAAGTGA	NM_001123213.1	118	150	—	96.2
	(R)	CTCATCCGAAGGCCGTCTT			300		

¹*ADIPOQ* = adiponectin; *ADIPOR1* = adiponectin receptor 1; *ADIPOR2* = adiponectin receptor 2; *LEP* = leptin; *LEPR-LF* (*Ob-Rb*) = long form of the leptin receptor; *PNPLA3* = patatin-like phospholipase domain containing 3 (adiponutrin); *PRL* = prolactin; *PRLR-LF* = long form of the prolactin receptor; *ACTB* = actin beta; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *PPIA* = peptidylprolyl isomerase A; *UBC* = ubiquitin C; *VAPB* = vesicle-associated membrane protein-associated protein B/C.

²F = forward and R = reverse primers.

³Amplification efficiency (*E*) was calculated with $E = 10^{[-1/\text{slope}]}$.

Comparison of Expression Profiles of Selected Genes Between Backfat and Mammary Fat Tissues

A comparison of the relative mRNA abundance of *ADIPOQ*, *LEP*, *PNPLA3*, and *PRL* genes between mammary and backfat depots was also conducted using real-time PCR amplifications, as described above. This experiment was conducted independently of the abovementioned gene expression analyses because a distinct standard curve was needed to be able to compare backfat and mammary fat tissues. Reactions were performed in a 10-μL reaction volume, with the exception of *PRL* that was adjusted to 25 μL in both fat depots. Concentrations of forward and reverse primers were the same as those used for backfat (Table 1). Standard curves were composed of serial dilutions of cDNA pools using both fat depots (Labrecque et al., 2009). The *GAPDH*,

PPIA, and *UBC* RG were also amplified. The relative quantity ratios were determined by dividing the relative quantity units of studied genes by those of the *PPIA* gene, which was found to be the most stable RG. Amplifications were performed in triplicates and mean values were used for statistical analyses.

Statistical Analyses

Statistical analyses were carried out using the MIXED procedure of SAS (SAS Inst. Inc., 2002). The univariate model (with three body condition levels) was used to test the effect of treatment on the mRNA abundance of selected genes, with the residual error used as the error term to test the main effects of treatment. When significant differences ($P < 0.05$) or tendencies ($0.05 < P < 0.10$) were found on the global ANOVA, all-pairwise multiple comparisons

were performed with a Tukey adjustment for which significance was set at $P < 0.05$. Spearman correlation analyses between mammary fat mRNA abundance of selected adipokines and mammary gland composition variables, as well as between adiponectin and leptin serum concentrations and mammary gland composition variables were calculated using the PROC CORR procedure of SAS. To compare the *ADIPOQ*, *LEP*, *PNPLA3*, and *PRL* mRNA abundance between backfat and mammary fat tissues, an analysis of variance with a 3×2 (treatment $\times$ tissue type) arrangement of treatments was used to analyze the data. Since both tissues were measured on the same animal, the factor tissue was considered as a repeated measure.

RESULTS

Impact of Body Condition on mRNA Abundance of Selected Genes in Backfat and Mammary Fat Tissues

Body condition affected the BW of gilts, with means of 123.8, 134.7, and 143.2 kg (SEM 2.9) at mating ($P < 0.05$), and 165.3, 190.2, and 211.7 (SEM 3.0) on day 109 of gestation ($P < 0.01$) for LBF, MBF, and HBF gilts, respectively (Farmer et al., 2016a). Backfat thickness was also affected

by body condition with means of 14.1, 18.1, and 22.9 mm (SEM 0.4) at mating ($P < 0.01$), and 13.7, 18.6, and 23.5 mm (SEM 0.3) on day 109 of gestation ($P < 0.01$) for LBF, MBF, and HBF gilts, respectively (Farmer et al., 2016a). In the extraparenchymal fat tissue, there was an effect of body condition for the *PRL* gene ($P = 0.01$, Table 2), with a higher *PRL* mRNA abundance in HBF than in LBF and MBF gilts ($P < 0.05$). In the same tissue, there were tendencies for a treatment effect on the expression of the *PNPLA3* and *PRLR-LF* genes ($P < 0.10$). The *PNPLA3* mRNA abundance was lower in HBF than in MBF gilts ($P < 0.05$), and that for the *PRLR-LF* gene was lower in LBF than in HBF gilts ($P < 0.05$). There was no effect of body condition ($P > 0.10$) on the mRNA abundance of *ADIPOQ*, *ADIPOR1*, *ADIPOR2*, *LEP*, and *LEPR-LF* genes in mammary fat depot.

In backfat, there was a body condition effect for the *LEP* ($P < 0.05$) and *PNPLA3* ($P < 0.01$) genes (Table 2). Multiple comparison analyses showed a lower *LEP* mRNA abundance in LBF than in HBF gilts ($P < 0.05$). In the same tissue, the mRNA abundance of *PNPLA3* increased from LBF to MBF to HBF gilts ($P < 0.05$). There was

Table 2. Relative mRNA abundance of selected genes in mammary fat and backfat tissues of gilts maintaining a low (LBF, 12–15 mm), medium (MBF, 17–19 mm), or high (HBF, 22–26 mm) backfat depth from mating to day 110 of gestation

Genes ¹	Groups			SEM max	P-value
	LBF <i>n</i> = 14	MBF <i>n</i> = 15	HBF <i>n</i> = 16		
Mammary extraparenchymal fat ²					
<i>ADIPOQ</i>	1.30	1.38	1.01	0.21	0.38
<i>ADIPOR1</i>	0.84	0.91	0.94	0.05	0.32
<i>ADIPOR2</i>	1.00	1.15	0.90	0.17	0.55
<i>LEP</i>	0.88	1.20	0.97	0.21	0.55
<i>LEPR-LF</i> ³	0.87	0.90	1.60	0.29	0.11
<i>PNPLA3</i>	1.23 ^{a,b}	1.38 ^a	0.84 ^b	0.17	0.06
<i>PRL</i> ³	0.75 ^b	0.82 ^b	1.19 ^a	0.11	0.01
<i>PRLR-LF</i>	0.54 ^b	0.72 ^{a,b}	0.88 ^a	0.10	0.07
Backfat ²					
<i>ADIPOQ</i>	1.16	1.01	1.11	0.13	0.68
<i>LEP</i>	0.72 ^b	0.95 ^{a,b}	1.12 ^a	0.11	0.04
<i>PNPLA3</i>	0.67 ^c	0.96 ^b	1.52 ^a	0.11	<0.01
<i>PRL</i> ³	0.72	0.68	0.85	0.15	0.67

^{a-c}Means within a row with different superscripts differ at $P < 0.05$ based on a Tukey adjustment.

¹*ADIPOQ* = adiponectin; *ADIPOR1* = adiponectin receptor 1; *ADIPOR2* = adiponectin receptor 2; *LEP* = leptin; *LEPR-LF* (*Ob-Rb*) = long form of the leptin receptor; *PNPLA3* = patatin-like phospholipase domain containing 3 (adiponutrin); *PRL* = prolactin; *PRLR-LF* = long form of the prolactin receptor.

²Relative mRNA abundances using *PPIA* as a normalization factor.

³*LEPR-LF* and *PRL* in mammary extraparenchymal fat and *PRL* in backfat were amplified using more cDNA (7.5 μ L) in a 25- μ L reaction volume compared with (3 μ L) in a 10- μ L reaction volume for the other genes. These genes also had high mean cycle threshold (C_T) values (31.8 for *LEPR-LF* and 30.9 for *PRL* in mammary fat; 32.4 for *PRL* in backfat) which indicate low expression levels.

no effect of body condition ($P > 0.10$) on transcript abundance of *ADIPOQ* and *PRL* in backfat tissue.

Associations Between mRNA Abundance of Selected Genes in Mammary Fat Tissue and Mammary Gland Composition

Mammary gland composition of gilts used in this study was previously reported (Farmer et al., 2016a). In the overall gilt population encompassing the three treatment groups, there was a negative correlation between the *PNPLA3* mRNA abundance in extraparenchymal tissue and the percentage of mammary parenchymal fat ($r = -0.30$, $P < 0.05$), whereas a positive correlation was found with the percentage of protein ($r = 0.32$, $P < 0.05$; Table 3). The mRNA abundance of *PRL* in extraparenchymal tissue was positively correlated with the percentages of dry matter ($r = 0.42$, $P < 0.01$) and fat ($r = 0.37$, $P < 0.05$) in parenchymal tissue. A negative correlation was observed between *PRL* mRNA abundance and parenchymal tissue protein ($r = -0.31$, $P < 0.05$). There was no correlation between any variables of mammary gland composition and the mRNA abundance for the *ADIPOQ* and *LEP* genes in extraparenchymal tissue (Table 3).

Associations Between Mammary Gland Composition and Serum Concentrations of Adiponectin and Leptin

In the overall gilt population, there was no significant correlation between mammary gland

composition variables and serum adiponectin levels (Table 4). Positive correlations were found between serum leptin concentrations and extraparenchymal tissue weight ($r = 0.39$, $P < 0.05$), as well as percentages of dry matter ($r = 0.73$, $P < 0.01$) and fat ($r = 0.65$, $P < 0.01$) in parenchymal tissue. Serum leptin concentrations were negatively correlated with protein percent ($r = -0.66$, $P < 0.01$), and concentrations of DNA ($r = -0.62$, $P < 0.01$) and RNA ($r = -0.60$, $P < 0.01$) in mammary parenchyma (Table 4).

Comparison of the Expression Profile of Selected Genes Between Mammary and Backfat Depots

There was a tissue $\times$ treatment interaction for *LEP* ($P < 0.01$) and *PNPLA3* ($P < 0.001$) mRNA abundances (Table 5). In LBF, MBF, and HBF gilts, the *LEP* mRNA abundance was always higher in backfat than in mammary fat tissue, but the backfat to mammary fat ratio differed, being 2.8 for LBF, 1.8 for MBF, and 3.5 for HBF gilts. Differences in *PNPLA3* mRNA abundance between fat tissues were only significant in HBF gilts ($P < 0.001$), with a 2.7-fold higher *PNPLA3* transcript abundance observed in backfat than in mammary fat tissue. There was no tissue $\times$ treatment interaction for *ADIPOQ* and *PRL* mRNA abundance. The mRNA abundance of *ADIPOQ* was 2.2-fold higher in backfat than in mammary fat depots ($P < 0.001$, Table 5). Contrarily, the *PRL* mRNA abundance

Table 3. Overall correlation coefficients between the mRNA abundance of *ADIPOQ*, *LEP*, *PNPLA3*, and *PRL* in the mammary fat tissue and mammary composition variables of gilts ($n = 45$)

Item ¹	mRNA abundance			
	<i>ADIPOQ2</i>	<i>LEP</i>	<i>PNPLA3</i>	<i>PRL</i>
Extraparenchymal tissue, g	-0.22	-0.07	-0.18	0.22
Parenchymal tissue, g	-0.29	-0.25	0.08	-0.18
Dry matter, %	-0.01	0.09	-0.24	0.42**
Fat ³ , %	-0.05	0.06	-0.30*	0.37*
Fat, g	-0.28	-0.18	-0.06	0.05
Protein ³ , %	0.08	-0.03	0.32*	-0.31*
Protein, g	-0.21	-0.20	0.14	-0.17
DNA ³ , mg/g	0.10	-0.04	0.23	-0.24
DNA, g total	-0.24	-0.27	0.11	-0.17
Protein/DNA	0.06	0.12	0.19	-0.06
RNA ³ , mg/g	0.06	-0.03	0.25	-0.27
RNA, g total	-0.28	-0.28	0.09	-0.18
RNA/DNA	-0.01	0.07	0.10	-0.22

Values represent Pearson's correlation coefficients.

¹Mammary gland composition values are reported in Farmer et al. (2016b).

²*ADIPOQ* = adiponectin; *LEP* = leptin; *PNPLA3*, patatin-like phospholipase domain containing 3 (adiponutrin); *PRL* = prolactin.

³Expressed on a dry matter basis.

* $P < 0.05$ and ** $P < 0.01$.

in mammary fat was 1.9-fold higher than in backfat tissue ($P = 0.0001$, Table 5).

DISCUSSION

The essential role of the mammary extraparenchymal fat tissue for mammary growth and function is well established (Hovey et al., 1999); however, we still have limited knowledge on the adipose-derived factors involved and their mechanisms of action. Moreover, we still do not know whether locally produced adipokines have functions similar to those of circulating adipokines in terms of mammary development.

Table 4. Overall correlation coefficients between circulating adiponectin and leptin and mammary composition variables of gilts ($n = 45$) maintaining a low, medium, or high backfat depth from mating to day 110 of gestation

Item ¹	Adiponectin, $\mu\text{g/mL}^2$	Leptin, ng/mL^2
Extraparenchymal tissue, g	0.14	0.39*
Parenchymal tissue, g	-0.12	-0.20
Dry matter, %	0.13	0.73**
Fat ³ , %	0.17	0.65**
Fat, g	-0.07	0.22
Protein ³ , %	-0.20	-0.66**
Protein, g	-0.14	-0.25
DNA ³ , mg/g	-0.14	-0.62**
DNA, g total	-0.17	-0.29
Protein/DNA ratio	-0.04	0.08
RNA ³ , mg/g	-0.09	-0.60**
RNA, g total	-0.15	-0.27
RNA/DNA	0.10	-0.06

Values represent Pearson's correlation coefficients.

¹Mammary gland composition values are reported in Farmer et al. (2016b).

²Circulating concentrations of adiponectin and leptin.

³Expressed on a dry matter basis.

* $P < 0.05$ and ** $P < 0.01$.

Among the genes that were selected in this study, leptin is a well-known adipokine that can act as a metabolic sensor and is involved in lipid metabolism and glucose homeostasis. The presence of leptin and leptin receptor transcripts and proteins in mammary parenchymal and extraparenchymal tissues of various livestock species suggest that leptin may also affect mammary development and function (Palin et al., 2017).

Current results suggest that circulating leptin at the end of gestation may affect mammary composition in gilts, and that this does not seem to be the case for leptin produced locally in mammary extraparenchymal fat. The absence of an effect from locally produced leptin is suggested from the observation that *LEP* mRNA abundance in extraparenchymal fat was not affected by gilts body condition, and also by the absence of significant correlations between *LEP* mRNA abundance in extraparenchymal fat and mammary composition variables. The observation that gilts with greater serum leptin had less parenchymal cells (i.e., DNA, mg/g) and reduced metabolic activity of these cells (i.e., RNA, mg/g) suggests detrimental effects of high serum leptin on mammary gland development. In agreement with these results, the addition of leptin to bovine MEC decreased cell number by 59.3% when compared with cells without leptin (Kamikawa et al., 2009). In prepubertal heifers, leptin was also found to decrease MEC proliferation when using an in vivo intramammary IGF-I infusion model (Silva et al., 2008). However, these findings contrast with those of Thorn et al. (2006) who rather reported no effect of leptin on MAC-T cells and bovine primary mammary epithelial cells. The positive correlations in the current study between serum leptin and percentages of parenchymal fat and dry matter, and the negative correlation with the percentage of protein in parenchymal tissue may reflect an increase

Table 5. Effect of fat depot and body condition on relative mRNA abundance of selected genes in gilts at day 110 of gestation

Genes ¹	Fat depot						SEM ³	<i>P</i> -values		
	Mammary			Backfat						
	LBF ²	MBF ²	HBF ²	LBF	MBF	HBF				
	(14)	(15)	(16)	(14)	(15)	(16)				
<i>ADIPOQ</i>	0.59	0.73	0.47	1.42	1.19	1.36	0.19	<0.001	0.844	0.266
<i>LEP</i>	0.46	0.70	0.55	1.27	1.25	1.89	0.16	<0.001	0.060	0.009
<i>PNPLA3</i>	0.81	1.06	0.70	0.92	1.20	1.87	0.15	<0.001	0.020	<0.001
<i>PRL</i>	1.29	1.15	1.85	0.79	0.58	0.89	0.25	<0.001	0.074	0.456

¹*ADIPOQ* = adiponectin; *LEP* = leptin; *PNPLA3* = patatin-like phospholipase domain containing 3 (adiponutrin); *PRL* = prolactin.

²LBF = 12 to 15 mm, MBF = 17 to 19 mm, and HBF = 22 to 26 mm backfat depth. The number of animals used for each treatment is indicated in parenthesis.

³Maximum value.

of embedded adipocytes in the parenchyma relative to MEC. These correlations may also reflect an effect of leptin on MEC lipogenesis because [Feuermann et al. \(2004\)](#) demonstrated that adding leptin to bovine mammary explants increases fatty acid synthesis in the presence of prolactin.

In the present study, body condition affected *LEP* mRNA abundance in backfat, with higher expression levels in HBF than in LBF gilts. Such results are in agreement with those of [Robert et al. \(1998\)](#) who reported higher *LEP* mRNA abundance in the backfat tissue of fat ($27.4 \text{ mm} \pm 5.5$) versus lean ($12.4 \text{ mm} \pm 1.6$) barrows. Other authors reported higher leptin levels in pelvic than in subcutaneous and kidney adipose tissues of mature gilts ($224 \pm 7.4 \text{ d}$), whereas there was no difference in prepubertal gilts ($132.2 \pm 4.1 \text{ d}$) ([O’Gorman et al., 2010](#)). Current findings also suggest a different regulation of leptin transcription in various adipose tissue compartments since *LEP* mRNA abundance was lower in mammary extraparenchymal than in backfat adipose tissues. Our results also demonstrate that the gilt’s body condition at the end of gestation can affect the backfat to mammary extraparenchymal fat ratios of *LEP* mRNA abundance. Collectively, previous papers and current findings suggest that *LEP* transcript abundance in different fat depots may differ according to sex, age (i.e., puberty), and animals’ body condition.

[Feuermann et al. \(2006, 2008\)](#) previously demonstrated that the secretion of leptin from mammary fat explants increases the prolactin-induced mammary tissue proliferation and MEC α -casein synthesis, suggesting a paracrine action of locally produced leptin. However, this may not be the case in pigs since the *LEPR-LF* transcript was barely detectable in parenchymal tissue of late pregnant gilts ([Farmer et al., 2016b](#)). Conversely, the presence of *LEP* and *LEPR-LF* transcripts in mammary extraparenchymal tissue from gilts suggests a possible autocrine and paracrine effect of locally produced leptin in that tissue.

The essential role of prolactin for swine mammary development was demonstrated by [Farmer et al. \(2000\)](#). It is now well established that adipocytes from different fat compartments can express *PRL* in humans ([Ben-Jonathan and Hugo, 2015](#)), including the breast adipose tissue that synthesizes and secretes prolactin ([Zinger et al., 2003](#)). In the current study, *PRL* and *PRLR-LF* transcripts were also detected in the mammary extraparenchyma, with *PRLR-LF* showing a greater mRNA abundance than *PRL* (mean cycle threshold (C_T) values of 28.2 and 30.9, respectively). These two

genes were both affected by the body condition of gilts. Interestingly, gilts injected with prolactin had greater *PRLR* mRNA abundance in mammary parenchyma than control gilts ([Farmer and Palin, 2005](#)). Prolactin was also found to increase *PRLR* transcript abundance in 3T3-442A preadipocytes ([Brandebourg et al., 2007](#)). Although these two papers suggest a possible regulation of *PRLR* transcription by prolactin, further work is needed to determine whether the observed increase of *PRLR-LF* mRNA abundance in extraparenchymal tissue of HBF gilts is due to the simultaneous increase of *PRL* gene expression in that same tissue. The *PRL* mRNA abundance was 1.88-fold higher in mammary extraparenchymal fat than in backfat, which may suggest a fat depot-specific regulation of *PRL* transcription. Such depot-specific regulation was indeed demonstrated by [Brandebourg et al. \(2007\)](#) who reported a decrease in prolactin release from subcutaneous fat of obese versus non-obese subjects, whereas there was no difference in visceral fat. Our results differ from those of [Brandebourg et al. \(2007\)](#) since we report greater *PRL* mRNA abundance in mammary extraparenchyma of HBF compared with LBF gilts, and no effect of body condition in backfat tissue. Therefore, it appears that prolactin synthesis and secretion from adipose tissue may vary according to the species and fat compartments.

The synthesis and secretion of prolactin by the mammary extraparenchymal tissue ([Zinger et al., 2003](#)) and the presence of *PRLR* transcripts ([Farmer and Palin, 2005](#)) in parenchymal tissue from gilts suggest a possible paracrine action of prolactin on the MEC. In this study, the positive correlations between *PRL* mRNA abundance in extraparenchyma and percentages of parenchymal fat and dry matter suggest a possible effect of prolactin on mammary lipid metabolism. Such an effect was demonstrated in previous studies reporting that prolactin can decrease lipogenesis in human adipose tissue ([Nilsson et al., 2009](#)) and 3T3-L1 adipocytes ([Hogan and Stephens, 2005](#)). Prolactin also reduces the activity of lipoprotein lipase in human abdominal adipose tissue, thus decreasing the fatty acid supply to adipocytes ([Ling et al., 2003](#)). In the present case, the positive correlations found between *PRL* mRNA abundance in extraparenchyma and parenchymal fat and dry matter percentages may suggest an increase of lipogenesis in MEC or a redirection of fatty acids from the adipocytes to the MEC. This was observed in rodents, where MEC induced lipolysis in nearby adipocytes, possibly through the action of lipoprotein lipase, in

order to support MEC de novo lipogenesis during lactation (Elias et al., 1973; Clegg, 1981).

Adiponutrin was initially described as a membrane-associated protein that is highly expressed in mice adipose tissue (Baulande et al., 2001), and it was later detected in several human tissues (Wilson et al., 2006). In pigs, *PNPLA3* transcripts are observed in different tissues with its greatest expression in backfat (Chen et al., 2011). Russo and Russo (2013) found *PNPLA3* transcripts in rat mammary glands, but they did not look for its presence in the mammary fat pad. When compared with virgin female rats, there was a downregulation of *PNPLA3* gene expression in parenchymal tissue of late pregnant rats, suggesting a different transcriptional regulation between pregnant and non-pregnant rats (Russo and Russo, 2013). In human subcutaneous adipose tissue, the gene expression of *PNPLA3* is regulated by insulin, glucose (Moldes et al., 2006), and energy balance (Liu et al., 2004). Moreover, its gene expression increases in 3T3-L1 cells during differentiation into adipocytes (Baulande et al., 2001). These observations are common features of many adipokines. Adiponutrin has been considered as a nonsecreted protein for many years, but Winberg et al. (2014) recently reported its presence in human plasma where it circulates as a high molecular weight multimeric protein. These authors also demonstrated secretion of adiponutrin from HepG2 hepatocytes, but they did not investigate adiponutrin secretion from adipocytes. In the present study, *PNPLA3* transcripts were detected in mammary extraparenchymal and backfat tissues of late pregnant gilts. In backfat, the higher *PNPLA3* mRNA abundance of HBF compared with LBF and MBF gilts corroborates results of Steinberg et al. (2007) who reported an increase in *PNPLA3* mRNA abundance in subcutaneous fat of obese compared with lean humans. Wieser et al. (2017) rather observed an increase of *PNPLA3* mRNA abundance in subcutaneous fat of severely obese patients after weight loss provoked by bariatric surgery. Adiponutrin gene expression in inguinal fat increased in obese *falfa* Zucker rats, whereas it decreased in adipose tissue of obese *ob/ob* mice (Baulande et al., 2001; Lake et al., 2005). Since *falfa* Zucker rats are leptin resistant due to a mutation in the leptin receptor and *ob/ob* mice are leptin deficient, it was suggested that leptin may be involved in the regulation of adiponutrin. In this study, the *LEP* gene expression profile in backfat of LBF, MBF, and HBF gilts is similar to that of *PNPLA3*, suggesting a possible transcriptional co-regulation. However, this does not seem to be

the case in mammary extraparenchyma because of the tendency for a body condition effect on mRNA abundance of *PNPLA3* but not of *LEP*. Moreover, *PNPLA3* mRNA abundance in extraparenchyma was lower in HBF than in LBF gilts and the opposite was true for backfat. Differences in *PNPLA3* gene expression in various fat depots were also reported in rats (Caimari et al., 2007; Oliver et al., 2012), suggesting different transcriptional regulatory mechanisms depending on the fat depot. In this study, *PNPLA3* mRNA abundance was higher in backfat than in mammary fat, which may suggest a different transcriptional regulation in these two fat depots.

Present results suggest that locally produced *PNPLA3* has a limited effect on mammary gland composition in late pregnant gilts. Indeed, although significant correlations were found with percentages of parenchymal fat and protein, none of the other mammary composition variables were associated with *PNPLA3* gene expression. When using in vitro cell culture models, adiponutrin exhibits triacylglycerol lipase and acylglycerol transacetylase activities (Jenkins et al., 2004; Lake et al., 2005), thus suggesting an effect in mobilization of energy and storage in adipocytes. However, there was no effect of *PNPLA3* knockdown on the body fat content or BW of mice (Chen et al., 2010). Therefore, the exact functional role of adiponutrin in adipose tissue is still a matter of debate and requires further investigation.

CONCLUSIONS

Results from this study show that the body condition of late pregnant gilts can affect the transcript abundance of leptin, adiponutrin, and prolactin and that their expression can be modulated differently in backfat and mammary extraparenchymal tissues. Although *ADIPOQ* mRNA abundance was lower in mammary extraparenchyma than in backfat, body condition did not affect its expression level in both fat tissues. Moreover, the absence of correlation between mammary composition variables and serum adiponectin and *ADIPOQ* mRNA abundance in mammary extraparenchyma suggests a limited role of adiponectin for mammary gland development in late pregnant gilts. Among studied adipokines, leptin was the one showing the strongest associations with mammary composition variables. These associations were found with circulating leptin but not with leptin produced locally from the mammary extraparenchymal tissue. Further work is needed to better understand the exact function of

leptin produced in the mammary gland of gilts and determine whether it affects the actions of prolactin in swine mammary parenchymal tissue.

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