# REGULATION OF AMINO ACID TRANSPORT EFFICIENCY IN THE PORCINE MAMMARY GLAND

**BY**

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**A DISSERTATION**

**Submitted to**
**Michigan State University**
**in partial fulfillment of the requirements**
**for the degree of**

**DOCTOR OF PHILOSOPHY**

**Animal Science**

**2011**

## ABSTRACT

### REGULATION OF AMINO ACID TRANSPORT EFFICIENCY IN THE PORCINE MAMMARY GLAND

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Imbalances created by excesses or deficiencies of dietary amino acids (AA) reduce the efficiency of dietary protein utilization, limiting milk protein synthesis and litter growth, and increasing nitrogen (N) losses to the environment. The overall goal of the dissertation research described herein was to evaluate the effect of dietary AA availability on AA utilization by the porcine mammary gland during lactation. The main hypothesis was that the efficiency of AA transport by the porcine mammary gland increases in response to dietary crude protein (CP) reduction with subsequent crystalline (C) AA inclusion, and that this increase is mediated via an increase in mRNA abundance of mammary expressed genes encoding for AA transporter proteins. To test this hypothesis, three objectives were addressed: (1) to select suitable reference genes for reverse transcriptase quantitative PCR (RT-qPCR) analysis of AA transporter and milk proteins in porcine mammary gland; (2) to measure mRNA abundance of genes encoding for mammary lysine (Lys) transporters at different stages of mammary physiological activity, and determine the relationship between their expression and that of genes encoding for milk proteins; and (3) to test whether dietary AA availability modulates the efficiency of Lys utilization by the mammary gland and the expression of genes encoding for mammary Lys transporters and milk proteins.

For objective 1, mammary tissue was collected by biopsy from 4 sows on d 110 of gestation, d 5 and d 17 of lactation, and d 5 after weaning. Expression of potential reference genes was measured by RT-qPCR, and their invariance was assessed using a novel analytical model that corrected gene expression for mammary RNA and DNA concentrations. For objective 2, in addition to the mammary tissue collected from sows used in objective 1, samples were taken from 3 additional sows at d 2 and 5 of lactation. Expression of AA transporter genes *SLC7A9, SLC7A7, SLC7A6, SLC6A14, SLC7A1* and *SLC7A2*, and milk protein genes *CSN2* and *LALBA* was measured by RT-qPCR and the relationship between AA transporter and milk protein gene expression was determined. For objective 3, 18 sows were allocated to one of three diets containing 9.5 %, 13.5% and 17.5% CP levels. Mammary tissue and arterial and mammary venous samples were collected on d 7 and 17 of lactation. Expression of *SLC7A9*, *SLC7A6*, *SLC6A14*, *SLC7A1*, *SLC7A2*, *CSN2* and *LALBA* was measured by RT-qPCR and AA transport efficiency was measured by AA arterio-venous (AV) difference across the mammary gland relative to the arterial AA concentration. Results of the described studies showed that 1) changes in expression of selected reference genes during lactation are related to variation in RNA and DNA mammary concentration, and therefore such genes can be used as normalization factors for RT-qPCR analysis; 2) mRNA abundance of *SLC7A6*, *SLC6A14*, *SLC7A1* increases with milk demand and iwas positively related to mRNA abundance of *CSN2* and *LALBA;* and 3) dietary CP reduction with CAA inclusion improves the efficiency of dietary Lys transport across mammary gland and dietary AA utilization for litter growth, but does not change the expression of mammary AA transporter gene during lactation. These results are a novel contribution to our understanding of AA nutrition of the lactating sow and to the critically needed tools for development of mechanistic models of nutrient utilization for milk production.

## DEDICATION

To my parents and sister

## ACKNOWLEDGMENTS

I am extremely grateful to many people that have contributed to the thesis presented here. In particular, I would like to thank Dr. Nathalie Trottier for providing me the opportunity to undertake these studies and her continued support, advice and education. I would also like to sincerely thank the other members of my committee, Dr. Patty Weber, Dr. Roy Kirkwood, Dr. Juan Pedro Steibel and Dr. Cathy Ernst for their support and advice during my research. I would also like to express my gratitude to the staff at the Center for Animal Functional Genomics for their support and training. A special acknowledgment goes to the Michigan State University Swine Teaching and Research Center staff, Al Snedegar and Kevin Turner, and undergraduate students Emma Wilsplotz and Nathanael Taylor for assistance in animal care and handling. We gratefully acknowledge National Pork Board for funding this research. I would also like to acknowledge Barb Sweeney, Kathy Tatro and Dr Steven Bursian for their assistance throughout my graduate career at MSU. Last but not least, I would like to thank Jim Liesman for always being available to solve problems and provide support.

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## KEY TO ABBREVIATIONS

AA = amino acid

AV = arterio-venous

BCAA = branched chain amino acids

EAA = essential amino acid

N = nitrogen

NEAA = nonessential amino acid

RT-qPCR = reverse transcription quantitative PCR

# INTRODUCTION

Extensive research has been conducted over the past 20 years to determine an optimal amino acid (AA) profile in the diet to maximize milk production of lactating sows and litter growth rate (Dourmad et al., 1998; NRC, 1998; Touchette et al., 1998). However, lactation performance responses from these studies have been inconsistent due to the lack of understanding behind the physiological mechanisms linking an optimal AA profile to increased utilization of dietary AA and improved milk production. The overall goal of this dissertation research was to investigate physiological mechanisms by which an optimal dietary AA profile improves the efficiency of utilization of AA by the lactating sow. The efficiency of dietary AA utilization depends on both AA transport into the mammary cells and the utilization of intracellular AA for milk protein synthesis. The intracellular availability of dietary AA is controlled by a coordinated activity of AA protein-carriers located in the cellular membranes and responsible for channeling AA into the cells (Shennan and Peaker, 2000, Palacín et al., 1998; Broër et al., 2008). Lysine is the first limiting AA for milk protein synthesis, particularly when diets are based on corn and soybean meal as the main protein sources (Richert et al., 1997). Likewise, Lys has the highest extraction rate of all essential AA by the lactating sow mammary gland (Trottier et al., 1997). As a consequence, regulation of Lys transporters may control Lys utilization via modulation of Lys uptake by mammary cells and thus may impact the global efficiency of dietary protein utilization during lactation.

The main hypothesis was that the efficiency of Lys transport across the porcine mammary gland increases in response to dietary crude protein (CP) reduction with crystalline AA (CAA) inclusion and that this increase is mediated via an increase in mRNA abundance of mammary genes encoding for Lys transporters. To test this hypothesis, three different objectives were addressed: 1) selection of reference genes for analysis of Lys transporter

gene expression by RT-qPCR; 2) selection of AA transporters involved in Lys uptake by the mammary gland; and 3) determination of the effect of an optimal AA profile in the diet on the transport of Lys across the mammary gland and the expression of genes encoding for Lys transporters.

Expression of genes encoding for AA transporters and milk proteins was measured using reverse transcription quantitative PCR (RT-qPCR). The accuracy of the gene expression analysis by RT-qPCR is dependent on the availability of a set of reference genes that remain constant across different stages of mammary physiological activity (Bustin, 2000). However, recent studies in bovine and porcine mammary tissue have shown that up-regulation of genes involved in milk synthesis causes a large increase in transcript abundance of those genes, thus diluting the mRNA abundance of reference genes that actually maintain stable amount of mRNA per cell during lactation (Bionaz and Loor, 2007; Tramontana et al., 2008). Thus, the first objective of the thesis was to assess the invariance of reference gene expression across different stages of mammary physiological activity and is addressed in Chapter 1. A protocol for RT-qPCR analysis accounting for changes in mammary RNA and DNA concentration is described and compared to an available method for reference gene selection (i.e., geNorm; Vandesompele et al., 2002). Despite that the protocol was shown to be a valid alternative for selection of reference genes for RT-qPCR analysis, its final acceptance for publication in the Journal of Dairy Science was posterior to the acceptance for publication of results pertaining to those of objectives 2 and 3, respectively presented in Chapters 2 and 3. Hence, geNorm software, a broadly accepted protocol in the scientific community, was used for selection of a set of suitable reference genes for normalization of candidate gene mRNA expression under objectives 2 and 3.

Previous studies demonstrated that genes *SLC7A1*, *SLC7A2* and *SLC6A14*, respectively encoding for Lys transporters CAT-1, CAT-2b and $ATB^{0,+}$ were expressed in porcine mammary gland during lactation (Pérez-Laspiur et al, 2004; 2009). However, it remains unknown whether genes *SLC7A7*, *SLC7A6* and *SLC7A9*, respectively encoding for lysine transporters $y^+$LAT1, $y^+$LAT2 and $b^{0,+}$, highly expressed in intestine and kidneys (Bröer et al, 2008), are also expressed in mammary gland. Furthermore, it is unknown whether the expression of these genes is related to that of mammary synthesized milk proteins. Thus, the second objective of the thesis was to determine the effect of mammary physiological activity on transcript abundance of genes *SLC7A9*, *SLC7A7*, *SLC7A6*, *SLC6A14*, *SLC7A1* and *SLC7A2* using RT-qPCR. In addition, the relationship between mRNA abundance of these genes with that of genes *LALBA* and *CSN2*, known to encode for sow milk proteins β-casein and α-lactalbumin respectively, is also discussed. This objective was critical in order to first assess the stage of lactation at which expression of genes encoding for candidate Lys transporter was maximized, and second, to select Lys transporter genes expressed in porcine mammary gland and associated with mammary synthesized milk proteins to be used in Objective 3.

Finally , the third objective presented in Chapter 3 was to determine the effect of a dietary AA challenge designed to optimize daily litter gain, under 3 different levels of CP, on AA transport efficiency across the mammary gland and mRNA abundance of the same candidate genes selected under objective 2. Mammary AA transport efficiency was defined as the percentage of circulating AA taken by the mammary gland at each blood pass. This latter objective was achieved by surgically placing catheters in the carotid artery and mammary vein of lactating sows, and collecting arterial and venous blood samples at two stages of lactation. In addition, western blot and immunohistochemistry analysis were performed to

assess localization of CAT-1 in mammary parenchymal tissue, with results presented in Appendix D. Finally, it is acknowledged that milk protein synthesis is highly dependent on glucose availability and lactopoietic hormones. Hence, the relationship between milk protein synthesis and expression of lactopoietic hormones and glucose transporters in the lactating sow mammary gland was also examined and included in Appendix A.

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# CHAPTER 1

## SIMPLE AND ANALYTICAL PROCEDURE FOR SELECTION OF REFERENCE GENES FOR RT-qPCR NORMALIZATION DATA

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## Abstract

Variation in cellular activity in a tissue induces changes in RNA concentration, which affect the validity of gene mRNA abundance analyzed by reverse transcription quantitative PCR (RT-qPCR) analysis. A common way of accounting for such variation consists of the use of reference genes as normaliser. Programs such as geNorm may be used to select suitable reference genes, although a large set of genes that are not co-regulated must be analyzed to obtain accurate results. The objective of this study was to propose an alternative experimental and analytical protocol to assess the invariance of reference genes in porcine mammary tissue using mammary RNA and DNA concentrations as correction factors. Mammary glands were biopsied from 4 sows on d 110 of gestation (pre-partum), on d 5 (early) and 17 (peak) of lactation, and on d 5 after weaning (post-weaning). Relative expression of seven potential reference genes, *API5*, *MRPL39*, *VAPB*, *ACTB*, *GAPDH*, *RPS23* and *MTG1*, and one candidate gene, *SLC7A1*, was quantified by RT-qPCR using a relative standard curve approach. Variation in gene expression levels, measured as cycles to threshold at each stage of mammary physiological activity, was tested using a linear mixed model fitting RNA and DNA concentrations as covariates. Results were compared to those obtained with geNorm analysis and genes selected by each method were used to normalize

*SLC7A1*. Quantified relative mRNA abundance of *API5*, *GAPDH* and *MRPL39* remained unchanged ($P > 0.15$) across stages of mammary physiological activity after accounting for changes in tissue RNA and DNA concentration with the proposed analytical protocol. In contrast, geNorm analysis selected *MTG1*, *MRPL39* and *VAPB* as best reference genes. However, when target gene *SLC7A1* was normalized with either genes selected by our proposed protocol or those selected by geNorm, fold change in mRNA abundance did not differ. In conclusion, the proposed analytical protocol assesses expression invariance of potential reference genes by accounting for variation in tissue RNA and DNA concentration and thus represents an alternative method to select suitable reference genes for RT-qPCR analysis.

**Introduction**

Reverse transcription quantitative PCR (RT-qPCR) is the most common and reliable method for rapid quantification of mRNA (Pfaffl, 2001; Wong and Medrano, 2005). However, variations in RNA extraction yield, reverse-transcription yield, loading cDNA and efficiency of RT-qPCR amplification make the analysis prone to errors (Bustin et al., 2009, 2010). The reliability of the RT-qPCR analysis can be improved by amplifying, simultaneously with the target gene, a reference gene that serves as internal control against which other mRNA values can be normalized (Bustin et al., 2000; 2002). However, the identification of a reference gene whose mRNA copy number per cell remain constant across treatments or samples represents a challenge, as the expression of all genes is regulated during the cell cycle to control diverse cellular functions. A feasible method for selection of suitable reference genes is to statistically test for differences in relative gene mRNA abundance between treatments. However, testing differential expression of the reference gene requires quantification of mRNA abundance of the gene in question, which in itself poses a

circular problem (Pfaffl et al., 2004). The search for a suitable reference gene becomes more difficult when the expression of target genes is compared between tissues with different cell growth and proliferation, such as the lactating mammary gland (Tucker, 1987; Capuco et al., 2001). In bovine and porcine mammary tissue, Bionaz and Loor (2007) and Tramontana et al. (2008) have shown that an increase in total cellular RNA between pregnancy and lactation caused an artificial dilution of mRNA across all mammary genes tested, including that of reference genes. As a result, the same authors proposed the use of geNorm software (Vandesompele et al., 2002) to perform pairwise ratio-stability comparisons between multiple unrelated genes for selection of the best reference genes. However, such a statistical method relies on the assumption that the genes tested are not co-regulated (Derveaux et al, 2010; Vandesompele et al., 2002). An alternative approach would be to correct gene expression by accounting for the variation in mRNA abundance resulting from total cellular RNA changes. However, expression of reference gene mRNA abundance relative to an increasing amount of RNA would be subjected to dilution, as previously acknowledge by Bustin (2000; 2002).

Therefore, the objectives of the present study were: 1) to propose an analytical protocol to assess invariance of reference genes by accounting for the artifactual dilution effect in gene expression, and 2) to validate the proposed protocol by comparing expression results of a target gene normalized using the reference genes selected either with the proposal analytical protocol or with geNorm. Material and Methods

## Material and Methods

### *Animal and Tissue Collection*

The study was performed in accordance with the Institutional Animal Care and Use Committee at Michigan State University. Four sows (all parity 5, Landrace × Yorkshire) were

selected at 107 d of pregnancy and moved to farrowing crates in a room maintained at $20^0$C. Animals were fed a corn and soybean meal-based diet meeting all nutrient requirements for lactating sows nursing ten piglets with a predicted piglet average daily gain of 250 g (NRC, 1998). Sows were fed a maximum of 5 kg/d to ensure equal dry matter (DM) intake. Litters were equalized to 10 piglets within 48 h after farrowing and piglets weaned at d 21 of lactation. Mammary parenchymal tissue was biopsied in the morning, following a 12-h overnight fast, from the first and second thoracic glands according to procedure previously described by Kirkwood et al. (2007). Mammary tissue was sampled at four different physiological stages, i.e., d 110 of gestation (pre-partum), d 5 (early) and d 17 (peak) of lactation, and d 5 after weaning (post-weaning). Mammary tissue was flash frozen in liquid $N_2$ and stored at $-80^0$C. For mammary tissue collection, piglets were isolated in an adjacent pen equipped with a heat lamp. Three hours after biopsy, piglets were returned to sows and allowed to nurse.

***Gene Expression Analysis***

*RNA Extraction and Complementary DNA Synthesis.*

Ribonucleic acid was extracted from mammary tissue using the PerfectPure RNA Cell and Tissue Kit according to the manufacturer's instructions (5 PRIME, Gaithersburg, MD). Isolated RNA was tested for purity by spectrophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, DE) and for quantity and integrity using the Agilent Bioanalyzer 2100 with the RNA 6000 Nano Labchip (Agilent Technologies, Palo Alto, CA). The $A_{260}/A_{280}$ ratio ranged from 2.06 to 2.11, whereas the RNA integrity number (RIN) values ranged from 8.7 to 10. Complementary DNA (cDNA) was synthesized using 2 µg of total RNA from each sample as template in reverse transcription reactions using Superscript III

reverse transcriptase and oligo$(dT)_{15-18}$ primer (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. Final complementary DNA concentration was quantified by spectrophotometry (NanoDrop 1000), diluted to a working stock containing 10 ng/µL and stored at -20$^{o}$C.

*Primer Design*

Primer sequences for 7 potential reference genes and candidate gene *SLC7A1* are presented in Table 1.1. Potential reference genes were selected based on previous studies (Bionaz and Loor, 2007; Tramontana et al., 2008), but the primers used were different from those published to optimize the efficiency of the RT-qPCR reaction in our samples. Primers were designed based on publicly available swine cDNA and expressed sequence tag (EST) sequences deposited in the National Center for Biotechnology and Information database using Primer Express software (v. 3.0, Applied Biosystems, Foster City, Ca) with default settings. Designed primers were blasted against published swine (*Sus scrofa*), human (*Homo sapiens*), bovine (*Bos taurus*) and rat (*Rattus norvegicus*) genome sequences; pairs that showed significant alignment (i.e., high query coverage) with nucleotide sequences other than the protein of interest in any of the species mentioned were discarded. Amplicons from the primer pair were not sequenced. Evaluation of primer-dimer formation was performed by determining the presence of a single peak in the dissociation curve after the RT-qPCR reaction. Primers were not designed to span exon/exon junctions, however, the method used to extract RNA provided a step for DNA digestion by performing on-column DNase treatment (PerfectPure RNADNase, Gaithersburg, MD) in order to eliminate genomic DNA.

Primer pairs were optimized for concentration using a primer optimization matrix (Mikeska and Dobrovic, 2009), and a relative standard curve was used to determine the

efficiency (Yuan et al., 2006). The standard curve was constructed using cDNA synthesized from a RNA pool made of all samples using the following amounts of cDNA (in duplicate): 40 ng, 20 ng, 10 ng, 5 ng and 2.5 ng. Efficiency of the RT-qPCR reaction for each gene was calculated from the slope of the standard curve using the formula $(10^{(-1/\text{slope})} - 1) \times 100$, as described by Yuan et al. (2006). Specific hybridization of the primers was validated by the presence of a unique peak in the dissociation curve at the end of the RT-qPCR amplification. Non-template controls were included in all RT-qPCR plates to validate that primers were not amplifying contaminating DNA.

*RT-qPCR Assay.*

A total of 3µL (30ng) of template cDNA, 12.5 µL of SYBR Green master mix (Applied Biosystems, Foster City, California), 6µL each of 10 µM forward and reverse primers and 3.5 µL DEPC-treated and nuclease-free water (Fisher Scientific, Fair Lawn, New Jersey) were employed in each RT-qPCR reaction. Reactions were performed in MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems) that were sealed with sealing foil, centrifuged at 400 × *g* for 1 min and loaded into an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Cycles to threshold values were obtained in duplicate for each sample on the analyzer. Coefficient of variation was calculated for all duplicates and 0.1 used as cut-off value. Conditions for amplification and quantification included 2 initial steps (50°C for 2 min and 95°C for 10 min) followed by an amplification program (step 3) repeated for 40 cycles (95°C for 15 s and 60°C for 1 min). Data was calculated with the 7000 RQ Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). A relative standard curve was used as the RT-qPCR method to correct for differences on RT-qPCR reaction efficiency between plates (Larionov et al., 2005). Normality of the residuals was tested using the

Shapiro-Wilk test under the UNIVARIATE procedure of SAS (SAS Institute Inc., Cary, North Carolina).

***Total RNA and DNA quantification.***

Mammary DNA and RNA were extracted from mammary tissue using cold perchloric acid (Sigma-Aldrich, St Louis, MO) following the procedure described by Labarca and Paigen (1980) and Capuco et al. (2001), respectively. DNA concentration was quantified using bisbenzimide (Hoechst 33258, Sigma-Aldrich) and a Bio-Tek FL600 plate reader with 360/460 nm filter set (Bio-Tek Instruments, Inc., Winooski, VT), while RNA concentration was determined by spectrophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, DE). Both DNA and RNA concentrations are reported in $\mu g \times mg^{-1}$ mammary tissue.Values for each sample were log base 2 transformed for further analysis as described below.

***Analytical models***

*Analytical Protocol for Reference Gene Selection.*

Three consecutive steps were used (Figure 1.3), i.e., 1) log transformation of the relative amounts from the standard curve for each gene (Steibel et al., 2009), 2) a lack of fit test to assess the linear relationship between expression of reference genes and tissue RNA and DNA concentration, and 3) a linear mixed model to assess invariance of each reference gene expression across samples after correction with RNA and DNA concentrations.

*1. Log-transformation of the relative amounts from the standard curve*

Duplicate relative RNA amounts from the standard curve for each sample were log-transformed and averaged (Equation 1.1):

$$\mathrm{Log_2 Y_{ij}} = \mathrm{Log_2(Q_{1ij})} + \mathrm{Log_2(Q_{2ij})/2} \quad [1.1]$$

where $\mathrm{Log_2 Y_{ij}}$ is the value of potential reference genes corrected by the standard curve and log base 2 transformed for each sow (j) at each stage of lactation (i), and $\mathrm{Q_{1ij}}$ and $\mathrm{Q_{2ij}}$ are the quantities from the termocycler software corrected by the standard curve for each gene (i.e. duplicates).

*2. Lack of fit test*

The relationship between the values of potential reference genes corrected by the standard curve and log base 2 transformed and tissue RNA and DNA concentrations was analyzed by the lack-of-fit sums of squares test using a linear mixed model procedure (PROC MIXED) in SAS (Figure 1.4). The model included the random effect of sow and the continuous effect of tissue RNA and DNA concentration. The statistical model is as follows (Equation 1.2):

$$\mathrm{Log_2 Y_{ij}} = \mu + \beta_1 x_{1\mathrm{ij}} + \beta_2 x_{1\mathrm{ij}}^2 + \beta_3 x_{2\mathrm{ij}} + \beta_4 x_{2\mathrm{ij}}^2 + \mathrm{b_j} + \mathrm{e_{ij}} \quad [1.2]$$

where $\mathrm{Log_2 Y_{ij}}$ is the value of potential reference genes corrected by the standard curve and log base 2 transformed for each sow (j) at each stage of lactation (i), $\mu$ is the overall mRNA mean, $\beta_1$ and $\beta_2$ are the regression coefficients relating tissue RNA concentration to the potential reference gene for each sow (j), $\mathrm{x_{1ij}}$ is the tissue RNA concentration for the $\mathrm{j^{th}}$ sow within the $\mathrm{i^{th}}$ stage of lactation, $\beta_3$ and $\beta_4$ are the regression coefficients relating tissue DNA concentration to the potential reference gene, $\mathrm{x_{2ij}}$ is the total DNA for the $\mathrm{j^{th}}$ sow

within the $i^{th}$ stage of lactation, $b_j$ is the random effect of the sow, and $e_{ij}$ is the experimental error.

*3. Linear model*

Test for invariance expression of reference genes with linear relationship between gene mRNA abundance and tissue RNA and DNA concentration was performed using a linear mixed model in SAS (Figure 1.3). The model included stage of lactation as a fixed effect, sow as random effect, and DNA and RNA concentrations in mammary tissue as continuous variables. The statistical model is as follows (Equation 1.3):

$$Log_2Y_{ij} = \mu + \alpha_i + \beta_1 x_{ij} + \beta_2 x_{2ij} + b_j + e_{ij} \quad [1.3]$$

where $Log_2Y_{ij}$ is the value of potential reference genes corrected by the standard curve and log base 2 transformed for each sow (j) at each stage of lactation (i), $\mu$ is the overall mRNA mean, $\alpha_i$ is the fixed effect of the $i^{th}$ level of stage of lactation, $\beta_i$ is the regression coefficient relating tissue RNA concentration to the potential reference gene, $x_{1ij}$ is the tissue RNA concentration for the $j^{th}$ sow within the $i^{th}$ stage of lactation, $\beta_2$ is the regression coefficient relating tissue DNA concentration to the potential reference gene, $x_{2ij}$ is the total DNA for the $j^{th}$ sow within the $i^{th}$ stage of lactation, $b_j$ is the random effect of the sow, and $e_{ij}$ is the experimental error.

*Validation of Protocol for Reference Gene Selection.*

Validation of the analytical protocol was performed by comparing expression results of a target gene normalized using the reference genes selected either with the proposal analytical protocol or with geNorm.

*1. Selection of Best Reference Genes with geNorm*

To determine the most stable genes among the set of potential reference genes, relative mRNA amounts from the standard curve were entered directly into the geNorm software, as previously described by Vandesompele et al. (2002). The gene expression stability (M) value for each gene and the numbers of reference genes that should be used as normalization factors were then calculated by geNorm. Briefly, M was determined as the average pairwise variation of each gene with all other reference genes, whereas the number of reference genes that should be used was calculated by analysis of the pairwise variation ($V_n/V_{n+1}$) between 2 sequential normalization factors ($NF_n$ and $NF_{n+1}$). Such normalization factors ($NF_n$) are based on the geometric mean of the expression levels of n and n+1 best reference genes (Vandesompele et al., 2002).

*2. Target gene normalization and statistical analysis*

Normalization of relative gene expression values from a standard curve was made by dividing the relative mRNA amounts of each target gene by the geometric mean of relative mRNA amounts of several reference genes (Equation 1.4):

$$Y_{ij} = \frac{Q_{Cij}}{\sqrt[3]{Q_{R1ij} \times Q_{R2ij} \times Q_{R3ij}}} \quad [1.4]$$

where $Y_{ij}$ are target gene normalized relative mRNA amounts for the $j^{th}$ sow within the $i^{th}$ stage of lactation, while $Q_{Cij}$ and $Q_{Rij}$ are target and reference gene quantities from the termocycler software corrected by the standard curve, respectively. Then the quotient between target and reference gene quantities (Equation 1.4) was log-transformed, so gene expression values become additive (Equation 1.5):

$$\mathrm{Log}_2 Y_{ij} = \mathrm{Log}[Q_C/(Q_{R1} \times Q_{R2} \times Q_{R3}\ )\text{^}(1/3)] =$$

$$\mathrm{Log}\left(Q_C\right) - \mathrm{Log}\ (Q_{R1} \times Q_{R2} \times Q_{R3}\ )\text{^}(1/3) =$$

$$\mathrm{Log}\left(Q_C\right) - \frac{1}{3}\left(\mathrm{Log}\ Q_{R1} + \mathrm{Log}\ Q_{R2} + \mathrm{Log}\ Q_{R3}\right) \quad [1.5]$$

where $\mathrm{Log}Y_{ij}$ is the value of target gene corrected by the standard curve, log base 2 transformed and normalized for the $j^{th}$ sow within the $i^{th}$ stage of lactation, $Q_{Cij}$ and $Q_{Rij}$ are target and reference gene quantities from the thermocycler software corrected by the standard curve, respectively. Both normalization methods (Equation 1.4 and 1.5) are mathematically equivalent. Thus, target gene fold changes calculated with data obtained from either Equation 4 or 5 are identical. However, log-transformed quantities are additive, improving normality of the residuals and homogeneity of the variance (Steibel et al., 2009).

Changes in target gene normalized values in response to physiological phases of mammary activity were assessed using a linear mixed model procedure of SAS that included the fixed effect of stage of lactation and the random effect of sow (Equation 1.6):

$$\mathrm{Log}_2 Y_{ij} = \mu + \alpha_i + b_j + e_{ij} \quad [1.6]$$

where $Log_2Y_{ij}$ is the expression value of target gene corrected by the standard curve, log base 2 transformed and normalized for the $j^{th}$ sow within the $i^{th}$ stage of lactation, $\mu$ is the overall mean of target gene, $\alpha_i$ is the fixed effect of the $i^{th}$ stage of lactation, $b_j$ is the random effect of the $j^{th}$ sow, and $e_{ij}$ is the experimental error.

Results were presented as target gene fold changes. Briefly, least square means at pre-partum were subtracted from least square means at early lactation, peak lactation, and post-weaning, and then 2 was raised to the power of the differences (Equation 1.7).

$$Y = 2^{(\text{LogY early lactation} - \text{LogY prepartum})} \quad [1.7]$$

where Y is the target gene fold change. *P*-values associated to the fold changes were the same as those from least square mean differences, and therefore were taken directly from the SAS output. Intervals of standard error bars for each fold change were calculated using the following formula (Equation 1.8):

$$Z = 2^{\mu \pm SE} \quad [1.8]$$

where Z are the upper and lower limits of standard error bars for fold change, μ is the least square mean difference, and SE is the standard error of the least square mean difference obtained from the SAS output. Multiple comparisons were accounted for with Bonferroni adjustment, and $P < 0.05$ was used for determining significance.

## Results

### *Analysis Protocol for Reference Gene Selection.*

The lack-of-fit test showed that a linear regression provided a reasonable fit ($P < 0.05$ for linear fit and $P > 0.15$ for quadratic fit) to the regression between tissue RNA concentration and the relative mRNA abundance of *API5*, *GAPDH*, *ACTB* and *MRPL39* (Table 1.2). Although tissue DNA concentration was included in the lack of fit test to correct for changes in cell number, the *P*-value associated to the relationship between DNA concentration and the relative mRNA abundance of genes was not used to select for potential reference genes (See Discussion). The linear mixed model fitting tissue RNA and DNA concentrations as regression variables to assess invariance of each reference gene expression across stages of mammary physiological activity showed that relative mRNA abundance of *API5*, *MRPL39* and *GAPDH* remained unchanged ($P > 0.15$), whereas that of *ACTB* was significantly different ($P \leq 0.15$) (Table 1.2).

### *Validation of Protocol for Reference Gene Selection.*

GeNorm analysis showed that *MTG1*, *MRPL39* and *VAPB* had the lower average expression stability parameter (M), and therefore were selected as the most stable set in porcine mammary gland (Figure 1.1). Analysis of the pairwise variation between sequential normalization factors showed that the optimal number of reference genes was 3, as their pairwise variation was below the 0.15 cut-off value proposed by Vandesompele et al. (2002), and the inclusion of a fourth gene increased (V2/3 = 0.11 and V3/4 = 0.12) the pairwise variation (Figure 1.2).

Expression of target gene *SLC7A1* was significantly different across stages of mammary physiological activity when normalized with the geometric mean of the relative

mRNA abundance of reference genes selected with the proposed analysis protocol (i.e. *API5*, *MRPL39* and *GAPDH*; $P < 0.0001$) or that of geNorm (i.e. *MRPL39*, *MTG1* and *VAPB*; $P = 0.0001$). Fold change ± SE and *P*-value for the different comparisons are represented in Figure 1.4. Compared to pre-partum, *SLC7A1* fold change increased at early ($P < 0.01$) and peak lactation ($P < 0.001$), irrespective of the normalizing gene set used.

**Discussion**

Previous studies have shown that upregulation of genes involved in milk synthesis causes a large increase in transcript abundance of those genes, resulting in an artificial dilution of mRNA abundance of reference genes that actually maintain a stable amount of mRNA per cell during lactation (Bionaz and Loor, 2007; Tramontana et al, 2008). In support of these authors' work, results from the present study indicated a significant increase ($P < 0.05$; Figure 1.5) in RNA per mg of mammary tissue over pre-partum to peak lactation while relative mRNA abundance of all potential reference genes decreased over the same time period ($P < 0.05$; Figure 1.6, 1.7 and 1.8) (see statistical analysis of tissue RNA and DNA concentration, and relative mRNA abundance of reference genes, in Appendix C). As an extreme example of this paradox, data analysis showed an AA transporter, known to be upregulated during lactation (Chapter 2), as the only gene whose expression remained unchanged over pre-partum to post-weaning period ($P > 0.1$; Figure 1.8). Consequently, validation of reference gene expression across different stage of mammary physiological activity depends on an approach that accounts for artifactual changes in expression due to variation in tissue RNA concentration.

Mammary tissue undergoes continuous changes in cell proliferation during lactation, which can be quantified by analysis of mammary DNA concentration (Tucker, 1969, 1987; Capuco et al., 2001). Accordingly, Kim et al. (1999) showed a linear increase in the amount

of DNA in porcine mammary gland during lactation ($P < 0.05$), suggesting cellular hyperplasia in the tissue. In the present study there was a significant decrease ($P < 0.05$) in DNA concentration post-weaning (Figure 1.6), probably due to apoptosis of mammary cells (Ford et al., 2003). Variation in cell number is likely accompanied by changes in tissue RNA concentration, which do not cause an artifactual dilution of mRNA. Thus, if mammary DNA concentration differs between samples, the use of mammary RNA concentration alone as a correction factor for reference gene selection will lead to misleading results. Conversely, utilization of both RNA and DNA concentrations will provide the exact amount of RNA causing the artifactual dilution effect. Consequently, in order to protect against tissue RNA changes unrelated to gene expression, mammary DNA concentration is also included as covariate in the analytical model. Noteworthy to mention, tissue DNA concentration was used to correct gene expression in both the lack of fit test and the linear mixed model, but it was only used to select reference genes in the linear mixed model. By doing this, the analytical method can be used to select reference genes in tissues with no changes in cell number, and hence with non significance *P*-value associated to the relationship between DNA concentration and relative gene mRNA abundance.

The proposed analytical protocol and geNorm selected different reference genes, but *SLC7A1* fold changes did not differ when using genes obtained under either method. These results indicate that among the analyzed genes, there is more than one set of suitable reference genes. Differences between both analyses are due to the statistical approach used. GeNorm selected as best reference genes those whose changes in mRNA abundance were more related to each other, whereas our protocol selected those reference genes whose expression levels were not different across stages of mammary physiological activity, after accounting for variation in tissue RNA and DNA concentrations. Noteworthy to mention, results from geNorm analysis in the present study coincided only partially with those

previously reported by Tramontana et al. (2008), despite the fact that mammary tissue from lactating animals was used in both studies. As such, *MTG1* was previously discarded as one of the least stable gene, whereas in the present study *MTG1* was selected as the most stable gene, along with *MRPL39*. Variation between both analyses is likely due to the inclusion of few different genes and one additional stage of mammary physiological activity (i.e., post-weaning) in the present study. These results point out the importance of testing for invariance of potential reference genes on every experiment, rather than relying on reference genes previously published.

There are several differences between geNorm and the proposed analytical protocol. In contrast to geNorm, the proposed analytical method does not account for analytical errors inherent to the RT-qPCR reaction. However, the artifactual dilution effect is not an analytical error. The method presented herein uses tissue RNA and DNA concentrations, which are directly related to reference gene quantities, to account for such artifactual dilution effect. Noteworthy to mention, the same dilution apply to all genes, so normalizing the target gene with suitable reference genes cancels out the dilution effect. As a result, the proposed method is a tool to uncover appropriate reference genes, but normalization of RT-qPCR data with reference gene expression corrected by tissue RNA and DNA is not recommended. On the other hand, geNorm analysis relies on current knowledge of interactions between the selected genes, which to date are only partially understood. Conversely, the proposed analytical model does not assume lack of co-regulation between potential reference genes, as invariance analysis of each gene tested is independent of expression values of every other gene in the data set.

Finally, the proposed analytical method quantifies the exact number of genes that remain stable. Thus, all reference genes in the set will be discarded if significant differences

in relative mRNA remain after accounting for tissue DNA and RNA variation. Likewise, a single reference gene can also be selected. However, normalization against a single reference gene is not acceptable. The reference gene corrects for all other transcripts and thus, even though its mRNA abundance remains invariant under the experimental conditions described, an error on reference gene expression analysis will drastically affect the results. In fact, Vandesompele et al (2002) demonstrated an error associated with the use of only 1 or even 2 reference genes, thus in order to account for inherent variation in expression of reference genes a minimum of 3 different control genes is recommended (Bustin et al., 2009).

In summary, the proposed analytical protocol is composed of three consecutive steps in order to accurately test for reference gene invariance. First, it requires the log base 2 transformation of relative quantites for analysis of gene expression values in a linear model. Second, it assesses the relationship between tissue RNA and DNA concentrations and reference gene expression with a lack-of-fit test, as only a linear relationship between mRNA abundance of reference genes and the external parameters to the RT-qPCR reaction support a dilution effect on gene expression. Finally, it challenges the invariance of reference genes using a linear mixed model that accounts for variation in tissue RNA and DNA concentrations between samples. In conclusion, the proposed analytical protocol assesses expression invariance of potential reference genes by accounting for the artifactual dilution effect in mRNA abundance. As a result, it represents a valid alternative to select suitable reference genes for RT-qPCR analysis.

**Table 1.1.** Primer information for reverse transcription quantitative PCR (RT-qPCR) assays

| Accesion Number[1] | Gene | Protein | Primer[2] | Primer (5'-3') | E[3] (%) |
|---|---|---|---|---|---|
| CV872150.1 | *API5* | Apoptosis inhibitor 5 | F. 502<br>R. 568 | CTGGAGTGGTGGCAATAATCTCT<br>CCAAGGGAGCTCAGGTTTAGC | 99.4 |
| AY610067.1 | *MRPL39* | Mitochondrial ribosomal protein L39 | F. 540<br>R. 601 | TCGCTGGAGCTTTCTGCTATG<br>TGTTGGCATCCACTCATCAAG | 103.5 |
| NM_001123213.1 | *VAPB* | Vesicle-associated membrane protein-associated protein B/C | F. 1012<br>R.1072 | TGGCGCTGGTGGTTTTG<br>CCTACAAGGCGATCTTCCCTATG | 101.9 |
| DQ452569.1 | *ACTB* | β-Actin | F. 746<br>R. 803 | TGCGGGACATCAAGGAGAA<br>GCCATCTCCTGCTCGAAGTC | 111.8 |
| AF017079.1 | *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | F. 376<br>R. 429 | CGTCCCTGAGACACGATGGT<br>CCCGATGCGGCCAAAT | 97.6 |
| XM_001927465.1 | *RPS23* | Ribosomal protein 23 | F. 52<br>R.115 | CCACCGACGGGACCATAA<br>CAGGGCTGTGCCCAAATG | 99.6 |
| XM_001927648.1 | *MTG1* | Mitochondrial GTPase 1 | F:463<br>R:525 | GGCAAGTCCTCGCTCATCAA<br>CTTGGTGGCTTTTCCTTTCCT | 102.7 |
| NM_001012613.1 | *SLC7A1* | CAT-1 | F. 1172<br>R.1234 | GGGCTGCTGTTTAAGTTTTTGG<br>CGTGGCGATTATTGGTGTTTT | 100.4 |

[1]Accesion number corresponds to the complementary DNA (cDNA) or the expressed sequence tag (EST) sequence deposited in the National Center for Biotechnology and Information database from which the primers were designed.

[2]Direction (F = forward; R = reverse) and hybridization position for each primer (5′–3′) within the nucleotide sequence from which the primers were designed.

[3]Primer pair efficiency (E) was calculated as follow: $E = -1+10^{(-1/slope)} \times 100$. The $R^2$ values for all standard curves for reference and candidate genes were > 0.98, indicating excellent linear relationships between quantities of serially diluted cDNA and Ct when RT-qPCR was performed

**Table 1.2.** Lack-of-fit test and linear mixed model (fitting tissue RNA and DNA concentration) used to assess invariance of *API5*, *MRPL39*, *VAPB*, *GAPDH*, *ACTB*, *MTG1*, *RPS23* and *SLC7A1* in porcine mammary tissue over pre-partum to post-weaning periods

| Gene Symbol | Linear[a] RNA | Quadratic[b] RNA | Stage[c] *P*-value |
|---|---|---|---|
| *API5* | < 0.001 | 0.28 | 0.36 |
| *GAPDH* | 0.01 | 0.83 | 0.22 |
| *MRPL39* | 0.02 | 0.43 | 0.16 |
| *ACTB* | < 0.001 | 0.15 | 0.03 |
| *MTG1* | 0.06 | 0.51 | NA[d] |
| *VAPB* | 0.35 | 0.25 | NA |
| *RPS23* | 0.07 | 0.06 | NA |
| *SLC7A1* | 0.47 | 0.10 | NA |

[a] *P*-value for testing linear term in Equation 1.
[b] *P*-value for testing quadratic term in Equation 1.
[c] *P*-value for stage factor model with DNA and RNA concentration in mammary gland in equation 2.
[d] Non-applicable

**Figure 1.1.** Average expression stability values (M) of potential reference genes *API5*, *MRPL39*, *VAPB*, *MTG1*, *RPS21*, *ACTB* and *GAPDH* plotted from least stable (left) to most stable (right).

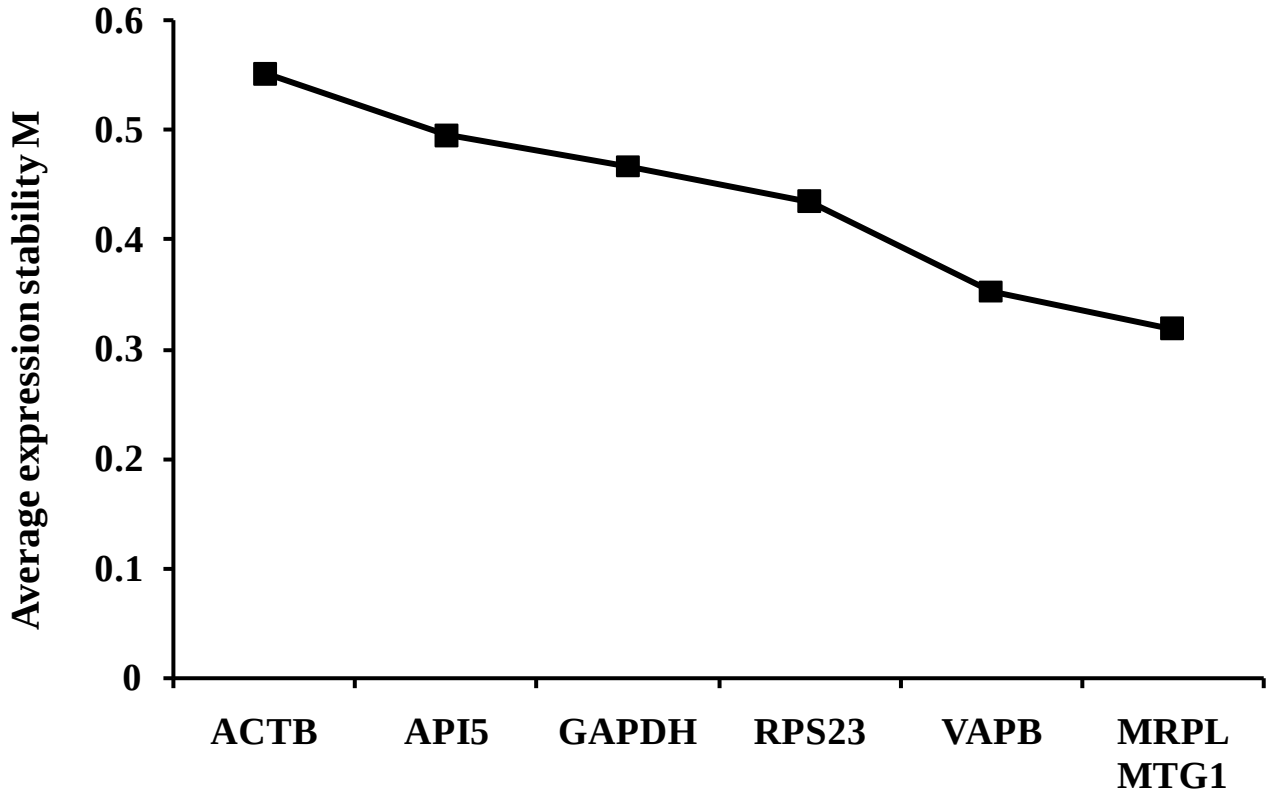


**Figure 1.2.** Pairwise variation ($V_n/V_n + 1$) between the normalization factors $NF_n$ and $NF_{n+1}$ to determine the optimal number of reference genes for normalization.

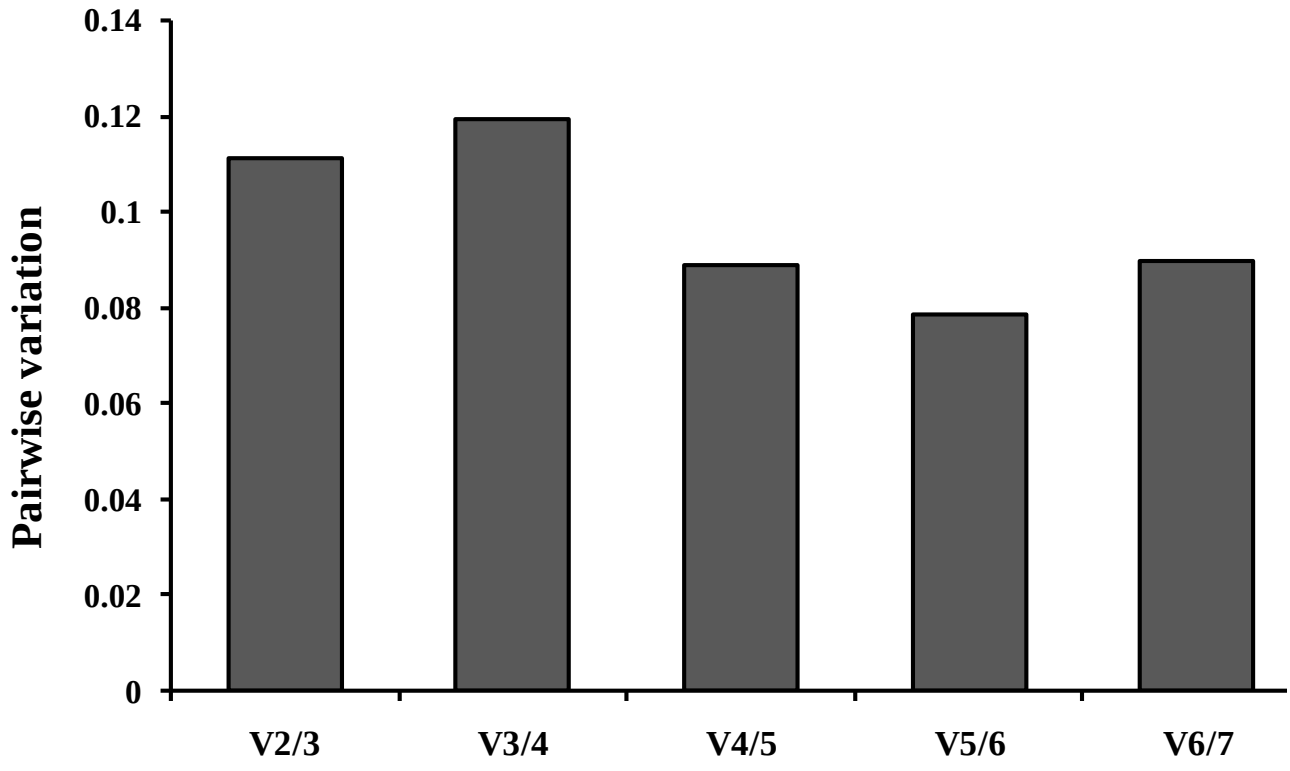


**Figure 1.3.** SAS code to assess relationship between relative mRNA abundance of reference genes and tissue RNA and DNA concentrations, and to assess invariance of each reference gene expression across samples. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

```
proc mixed data=exp1;
  by gene;
  class sow stage;
  model logamt= logRNA logDNA logRNA2 logDNA2/htype=1;
  random sow;
run;

proc mixed data=exp1;
  by gene;
  class sow stage;
  model logamt= stage logRNA logDNA;
  random sow;
  lsmeans stage/pdiff;
run;
```

**Figure 1.4.** Fold changes of target gene *SLC7A1* in porcine mammary tissue at early lactation, peak lactation and post-weaning compared to pre-partum. Target gene was normalized using the geometric mean of reference genes selected either with the proposed analytical protocol or with geNorm.; $^{**}P < 0.01$; $^{***}P < 0.001$.

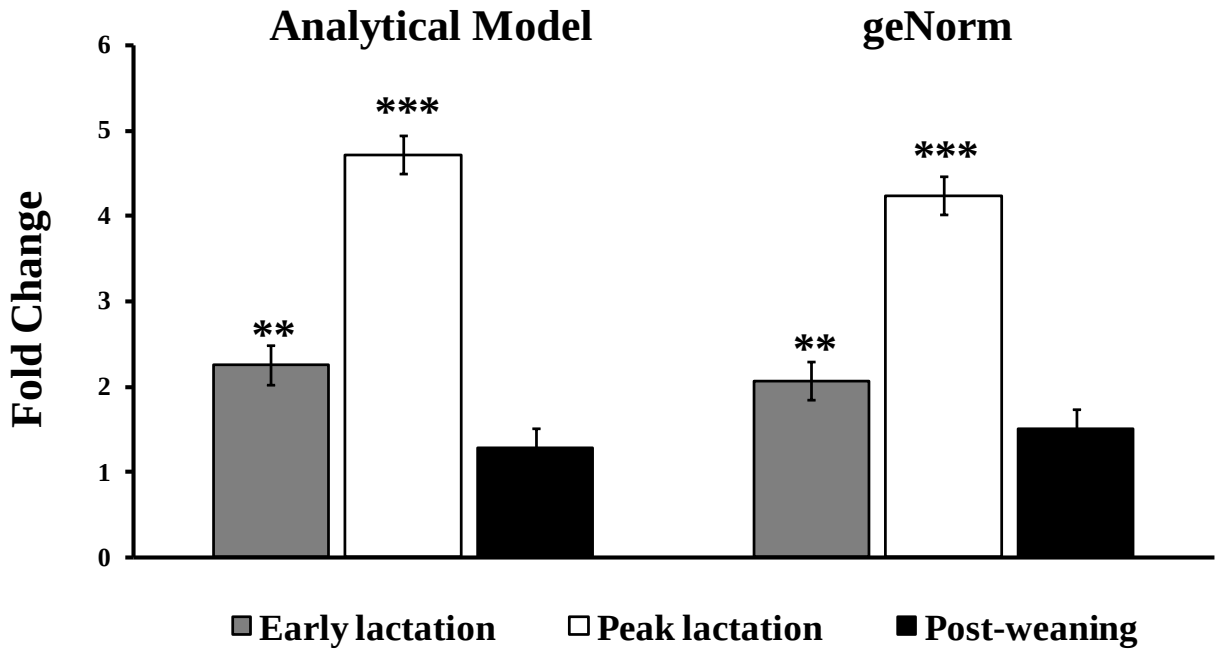


**Figure 1.5.** Log-transformed RNA and DNA concentrations in sow mammary tissue. Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation and d 5 after weaning (post-weaning) and peak lactation. Statistical analysis is shown in Appendix C. $^{\dagger}P < 0.1$, $^{***}P < 0.001$.

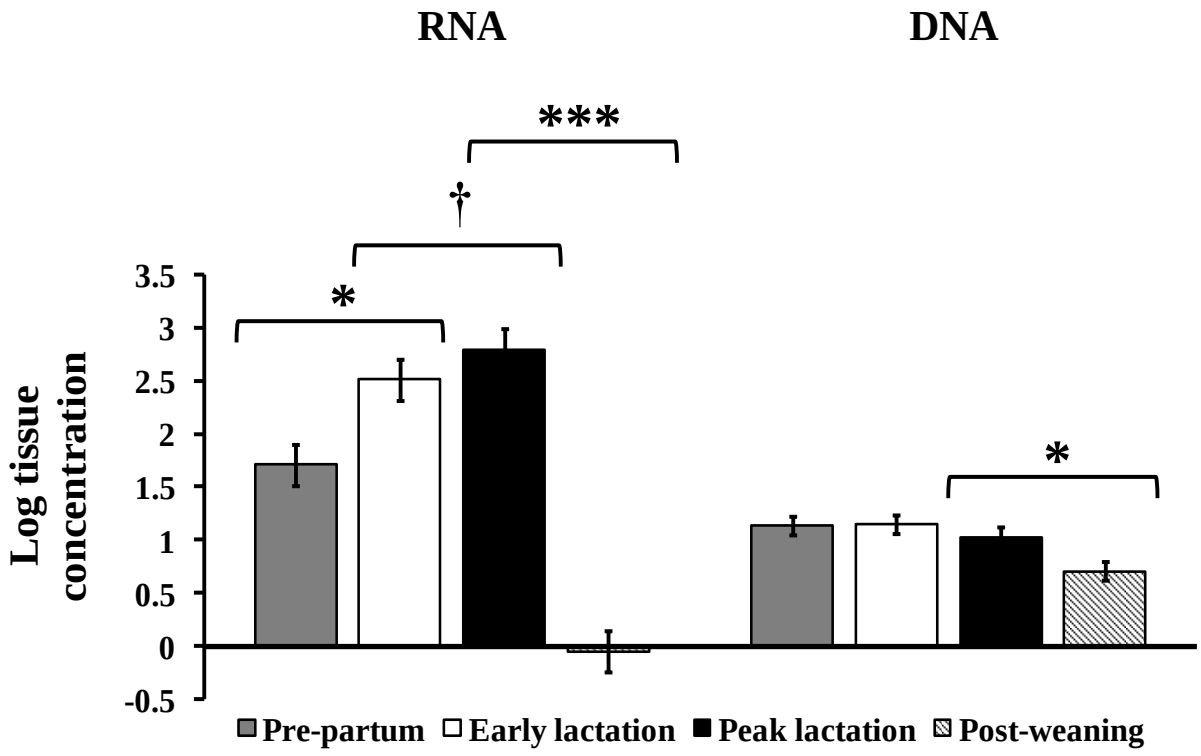


**Figure 1.6.** Log-transformed relative mRNA abundance of *API5*, *VAPB*, *MRPL39* in sow mammary tissue measured by reverse transcription quantitative PCR (RT-qPCR). Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation and d 5 after weaning (post-weaning) and peak lactation. Statistical analysis is shown in Appendix C. **P* < 0.05; ***P* < 0.01

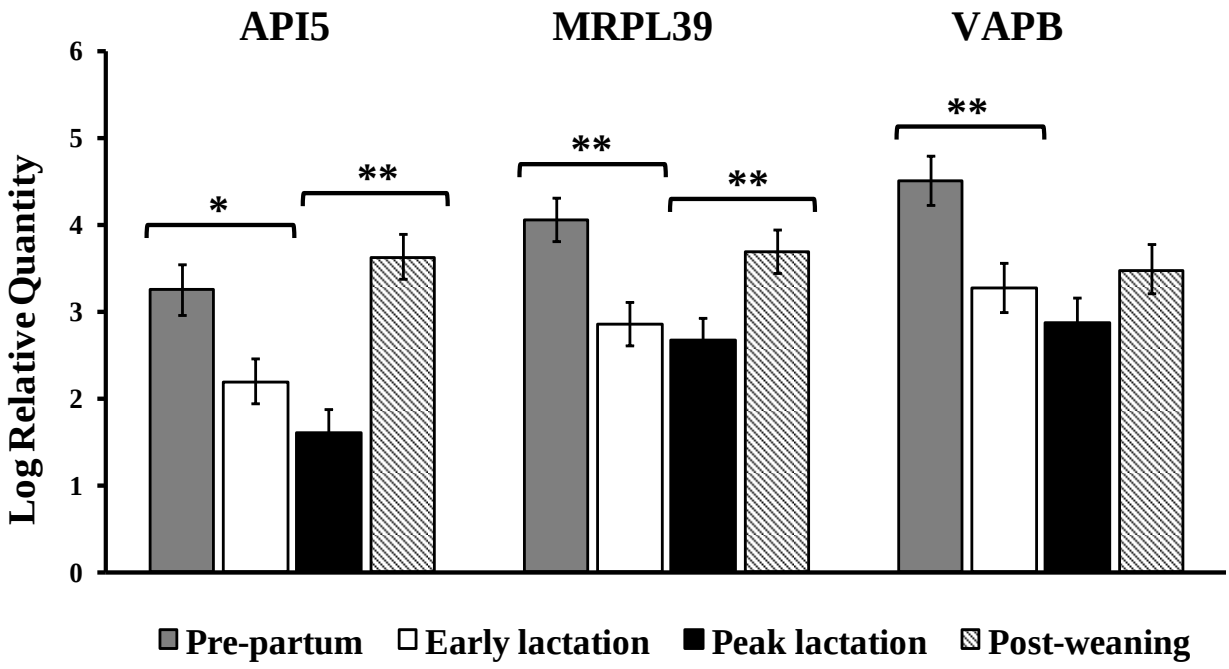


**Figure 1.7.** Log-transformed relative mRNA abundance of *ACTB, GAPDH* and *MTG1* in sow mammary tissue measured by reverse transcription quantitative PCR (RT-qPCR). Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation and d 5 after weaning (post-weaning) and peak lactation. Statistical analysis is shown in Appendix C.*$P < 0.05$; **$P < 0.01$

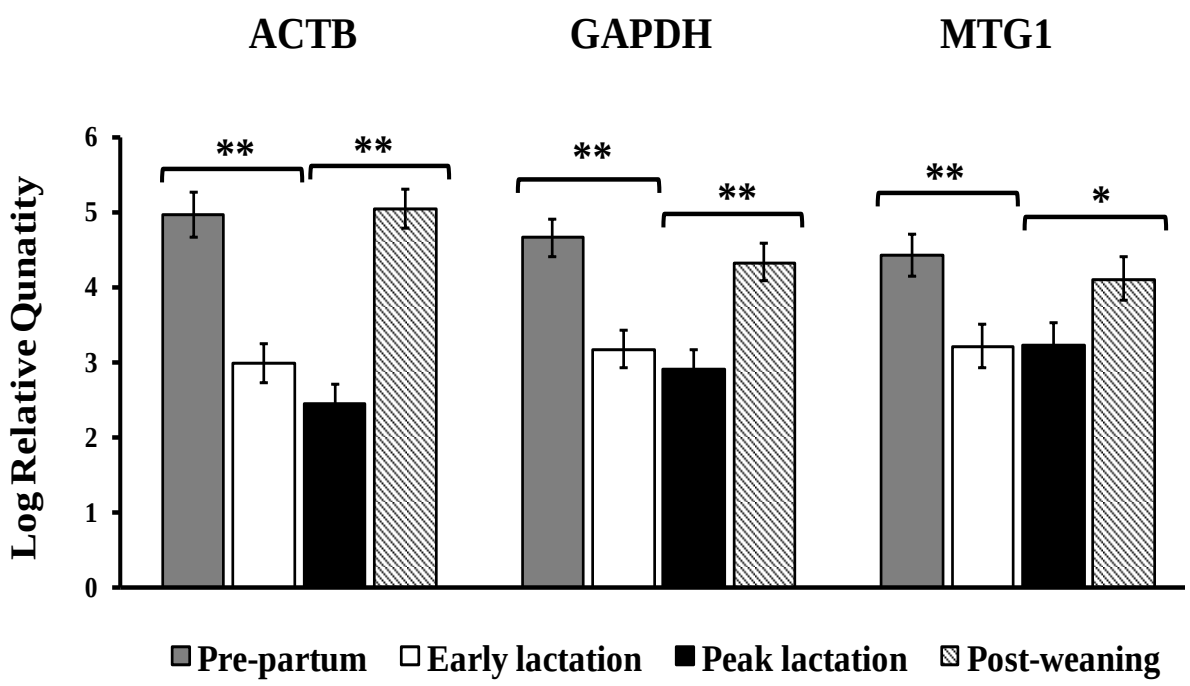
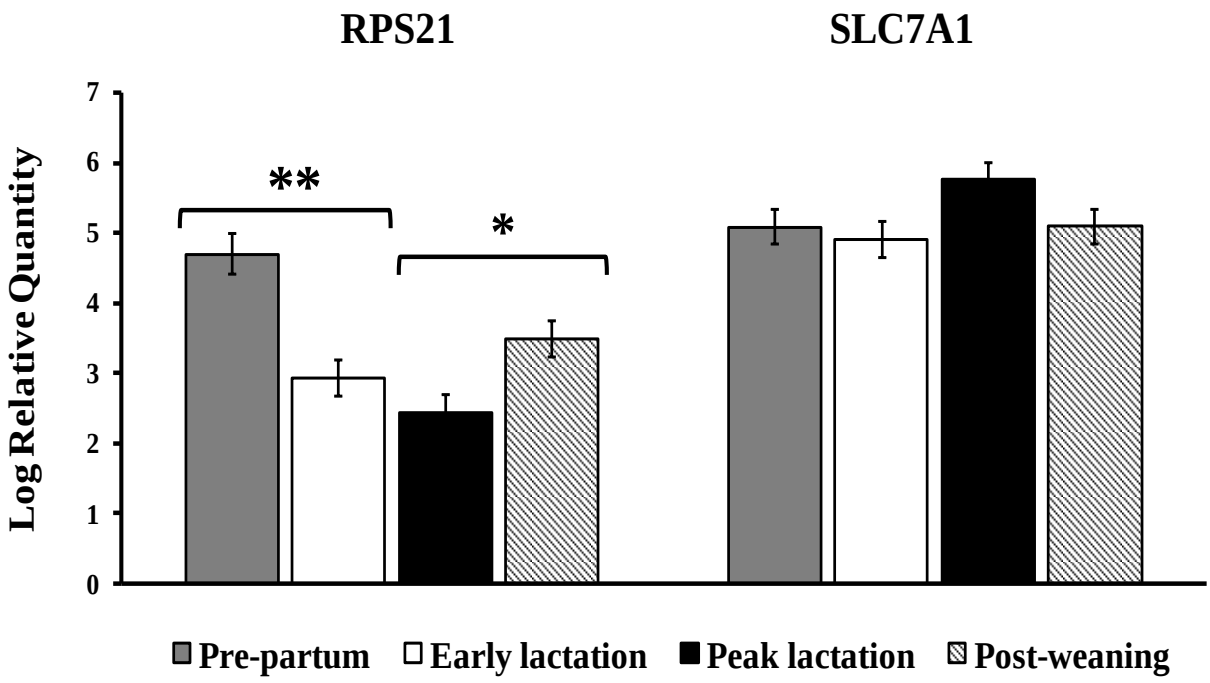


**Figure 1.8.** Log-transformed relative mRNA abundance of *RPS21* and *SLC7A1* in sow mammary tissue measured by reverse transcription quantitative PCR (RT-qPCR). Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation and d 5 after weaning (post-weaning) and peak lactation. Statistical analysis is shown in Appendix C. **P* < 0.05; ***P* < 0.01



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# CHAPTER 2

## TRANSCRIPT ABUNDANCE OF AMINO ACID TRANSPORTERS, β-CASEIN AND α-LACTALBUMIN IN MAMMARY TISSUE OF PRE-PARTUM, LACTATING AND POST-WEANED SOWS

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### Abstract

The objective of these experiments was to test the hypothesis that transcript abundance of cationic amino acid transporter- and milk protein-encoding genes increase in the porcine mammary gland in response to higher lactation demand. Genes of interest included those encoding for the milk proteins α-lactalbumin and β-casein (*CSN2* and *LALBA*, respectively), and AA transporter $b^{0,+}$AT, $y^+$LAT1, $y^+$LAT2, $ATB^{0,+}$, CAT-1 and CAT-2b (*SLC7A9*, *SLC7A7*, *SLC7A6*, *SLC6A14*, *SLC7A1* and *SLC7A2*, respectively). Mammary tissue was biopsied from 4 sows on d 110 of gestation (pre-partum), on d 2 (early post-partum), on d 5 (early) and d 17 (peak) of lactation, and on d 5 after weaning (post-weaning), and mRNA of target genes quantified by reverse transcription quantitative PCR (RT-qPCR). Compared to pre-partum, CAT-1, $ATB^{0,+}$, $y^+$LAT2, β-casein and α-lactalbumin mRNA abundance was higher at early lactation, whereas compared to early lactation, only CAT-1 and α-lactalbumin mRNA abundance was higher at peak lactation. CAT-2b, $y^+$LAT1 and $b^{0,+}$AT mRNA abundance did not differ

when comparing either pre-partum or peak lactation to early lactation. Compared to peak of lactation, post-weaning mRNA abundance of CAT-1, $ATB^{0,+}$, α-lactalbumin and β-casein decreased, $y^+$LAT2, CAT-2b and $b^{0,+}$AT remained unchanged, and $y^+$LAT1 increased. Messenger RNA abundance of $y^+$LAT2 increased from early post-partum to early lactation, and remained unchanged for CAT-1, $ATB^{0,+}$ α-lactalbumin and β-casein. From pre-partum to peak of lactation, mRNA abundance of CAT-1, $y^+$LAT2 and $ATB^{0,+}$ was positively correlated with that of β-casein and α-lactalbumin. In conclusion, expression of genes encoding for $y^+$LAT1, CAT-2b and $b^{0,+}$AT remained unchanged in porcine mammary gland over pre-partum to peak lactation period, whereas expression of genes encoding for CAT-1, $ATB^{0,+}$ and $y^+$LAT2 was upregulated and positively correlated to expression of genes encoding for the mammary synthesized milk proteins β-casein and α-lactalbumin.

**Introduction**

During the second week of lactation an increase in milk demand imposed by the growing piglet exceeds the ability of the sow to produce milk, and cationic and neutral AA including Lys, Thr and Val may become limiting for milk protein synthesis (NRC, 1998). Therefore, numerous studies have focused on optimizing dietary Lys (NRC, 1998; Dourmad et al., 1998; Touchette et al., 1998), Val (NRC, 1998, Gaines, 2006) and Thr (Cooper et al., 2001) intake by the sow to maximize litter growth. One critical step of AA utilization involves cellular AA transport, a process mediated by transporter proteins located in cellular membranes (Shennan and Peaker, 2000; Broër, 2008). However, little to none is known of the AA transporters, their regulation and

the potential that they are coordinately regulated along with milk proteins. Proteins known to facilitate cationic and neutral AA transport into mammalian epithelial cells (Palacín et al., 1998) include those of the $y^+$ system of AA transporter family specific for cationic AA, two of which have been identified in lactating porcine mammary tissue, namely CAT-1 and CAT-2b (Pérez-Laspiur et al., 2004; 2009). Cationic and neutral AA may also be transported using shared systems with other AA, such as system $B^{0,+}$, of which protein $ATB^{0,+}$ was also shown to be abundantly expressed in porcine mammary tissue (Pérez-Laspiur et al., 2004; 2009). However, their expression and that of more novel cationic and neutral AA transporters $b^{0,+}$ and $y^+LAT$ in relation to genes encoding for the milk proteins β-casein and α-lactalbumin, has not been determined. Furthermore, it is unknown when and if these genes change in expression relative to the well characterized milk production curve in sows (Duncan and Garton, 1966; Nielsen et al., 2002). This information would provide novel insight in the regulation of intracellular AA availability for milk protein synthesis. We hypothesized that cationic AA transporter and milk protein genes increase in expression with higher lactation demand. To test this hypothesis, our objectives were to 1) profile mRNA abundance of genes encoding for cationic AA transporter proteins and for mammary synthesized milk proteins at distinct stages of mammary physiological activity, and 2) to determine the relationship between mRNA abundance of each respective candidate AA transporter and mammary synthesized milk protein genes.

## Material and Methods

### *Animals and Tissue Collection*

Studies were performed with the approval of the Institutional Animal Care and Use Committee at Michigan State University (AUF#10/08-162-00). All sows were fed a corn-soybean meal-based diet meeting all nutrient requirements for lactating sows nursing piglets with a predicted average daily gain of 250 g/d (NRC, 1998; Table 2.1). Sows selected on d 107 of pregnancy were moved to farrowing crates in a room maintained at 20 $^{o}$C and fed 3 kg/d divided into two meals. The day after farrowing was considered d 1 of lactation and sows were fed 1 kg twice a day (0800 and 1600). On d 2 and 3 of lactation, sows were fed 3 and 4 kg, respectively, in two equal meals. For the remainder of the study, sows were fed a maximum of 5.5 kg/d to ensure equal dry matter (DM) intake among all sows. Sow feed intake was recorded daily throughout lactation. Fresh water was freely available at all times.

In experiment 1, four sows (all parity 5, Landrace × Yorkshire) were used. Litters were equalized to 10 piglets within 48 h after farrowing and litter weight was recorded on d 2 and 17 of lactation. Piglets were weaned on d 21. Mammary parenchymal tissue was biopsied from the first and second thoracic glands (Kirkwood et al., 2007) in the morning following a 12-h overnight fast on d 110 of gestation (pre-partum), d 5 (early) and d 17 (peak) of lactation, and d 5 after weaning (post-weaning). For mammary tissue sampling, piglets were isolated in an adjacent pen equipped with a heat lamp. Immediately following the biopsy, mammary tissue was flash-frozen and kept in liquid $N_2$ until later stored at -80$^{0}$C. Three hours after biopsy, piglets were returned to sows and allowed to nurse.

In experiment 2, four sows (parity 2 (n=2), parity 4 (n=1) and parity 5 (n=1); Landrace × Yorkshire) were selected and managed as above, except that litter size was equalized to 8 and mammary tissue was biopsied on d 2 and 5 of lactation only.

***Gene Expression Analysis***

Primer sequences for reference and target genes are presented in Table 2.2. In order to facilitate the readership throughout the remainder of the manuscript, target genes will be referred as the common name of the AA transporter and milk proteins that they encode for. Hence *SLC7A9* will be referred to as $b^{0,+}$AT, *SLC7A7* as $y^{+}$LAT1, *SLC7A6* as $y^{+}$LAT2, *SLC6A14* as $ATB^{0,+}$, *SLC7A1* as CAT-1, *SLC7A2* as CAT-2b, *CSN2* as β-Casein and *LALBA* as α-Lactalbumin. Reference genes *API5*, *VAPB* and *MRPL39* were selected based on a previous study that support their stability in porcine mammary gland during lactation (Tramontana et al, 2008), although primers utilized were different from those published to optimize the efficiency of the reverse transcription quantitative PCR (RT-qPCR) reaction in our samples. RNA extraction and cDNA synthesis, primer design and optimization, RT-qPCR assay and normalization methods are as described in Chapter 1.

***RT-qPCR data normalization***

Analysis of target gene expression was made by subtracting from each of the target gene Ct the arithmetic mean of the 3 selected reference genes Ct (Equation 2.1)

$$\Delta Ct_{ij} = Ct_{Cij} - \left[\frac{1}{3}\left(Ct_{R1ij} + Ct_{R2ij} + Ct_{R3ij}\right)\right] \quad [2.1]$$

where $\Delta Ct_{ij}$ is the difference between Ct of target and reference gene for the $j^{th}$ sow within the $i^{th}$ stage of lactation, and $Ct_{Cij}$ and $Ct_{Rij}$ are the cycles to threshold of target and reference gene for the $j^{th}$ sow within the $i^{th}$ stage of lactation.

***Statistical Analysis***

Changes in ΔCt for each target gene in response to physiological phases of mammary activity were assessed using a linear mixed model procedure (PROC MIXED) of SAS (SAS Institute Inc., Cary, North Carolina) that included the fixed effect of time and the random effect of sow (Equation 2.2):

$$Y_{ij} = \mu + \alpha_i + b_j + e_{ij} \quad [2.2]$$

where $Y_{ij}$ is ΔCt of target gene for the $j^{th}$ sow within the $i^{th}$ stage of lactation, $\mu$ is the overall ΔCt mean, $\alpha_i$ is the fixed effect of the $i^{th}$ stage of lactation, $b_j$ is the random effect of the $j^{th}$ sow, and $e_{ij}$ is the experimental error. In experiment 1, ΔCt for each target gene were compared between early lactation and pre-partum, peak lactation and early lactation and post-weaning and peak lactation. In experiment 2, ΔCt of target genes were compared between d 2 and 5 of lactation. Multiple comparisons in Experiment 1 were accounted for with Bonferroni adjustment. Gene expression results reported as ΔCt values may often be confusing, as increasing ΔCt reflect decreasing mRNA abundance. Thus, subtracting ΔCt values from a constant value prior to or after statistical analysis allows for an easier interpretation of the data, i.e., increasing ΔCt reflects increasing mRNA abundance, without altering the *P*-value or the

standard error. To further simplify the interpretation of results, this constant was selected to be an entire number higher than any ΔCt values among all genes, so that the values presented in the figures are all positive. Thus, mRNA abundance of individual genes is reported as 13 - ΔCt ± SEM. Differences between physiological stages of mammary activity were considered at $P \leq 0.05$.

To investigate the relationship between mRNA abundance of each respective AA transporter and mammary synthesized milk protein genes, correlations between mRNA abundance (ΔCt) of AA transporter genes and that of milk protein genes were determined using the PROC CORR procedure of SAS. Pearson's correlation coefficient (r) was considered significant at $P \leq 0.05$.

**Results**

***Experiment 1***

Lactation performance between d 2 and 17 of lactation was uniform among sows, as evidenced by a piglet average daily gain (ADG) of 257.8 ± 9 g and a daily sow feed intake of 4.7 ± 0.3 kg. Compared to pre-partum, CAT-1 mRNA abundance increased ($P = 0.02$) at early lactation and increased further ($P = 0.02$) from early to peak lactation. Compared to peak of lactation, CAT-1 mRNA abundance was lower post-weaning ($P = 0.001$; Figure 2.1). Compared to pre-partum, $ATB^{0,+}$ and $y^{+}LAT2$ mRNA abundance increased ($P < 0.0001$ and $P = 0.001$, respectively) at early lactation, but did not differ ($P > 0.1$) at peak compared to early lactation (Figure 2.2). Compared to peak of lactation, $ATB^{0,+}$ and $y^{+}LAT2$ mRNA abundance decreased

post-weaning ($P < 0.0001$; Figure 2.1). Messenger RNA abundance of CAT-2b, $y^+$LAT1 and $b^{0,+}$AT remained unchanged over pre-partum to peak lactation periods ($P > 0.1$; Figures 2.1 and 2.2). Compared to peak of lactation, post-weaning CAT-2b and $b^{0,+}$AT mRNA abundance did not differ and that of $y^+$LAT1 increased ($P < 0.0001$) (Figures 2.1 and 2.2). Both β-casein and α-lactalbumin mRNA abundance were higher ($P = 0.003$ and $P < 0.0001$, respectively) in early lactation compared to pre-partum but did not differ at peak compared to early lactation. Compared to peak of lactation, β-casein and α-lactalbumin mRNA abundance decreased ($P < 0.0001$) post-weaning (Figure 2.3). Over the pre-partum to peak lactation periods, mRNA abundance of $ATB^{0,+}$, CAT-1 and $y^+$LAT2 was positively correlated to β-casein mRNA abundance ($r = 0.76$ and $P = 0.0034$; $r = 0.78$ and $P = 0.0023$; $r = 0.71$ and $P = 0.008$, respectively) and to α-lactalbumin mRNA abundance ($r = 0.87$ and $P < 0.001$; $r = 0.71$ and $P = 0.008$; $r = 0.86$ and $P < 0.001$, respectively). Messenger RNA abundance of CAT-2b, $y^+$LAT1 and $b^{0,+}$AT was not correlated with that of either β-casein or α-lactalbumin ($P > 0.1$).

***Experiment 2***

In experiment 2, piglet ADG and daily sow feed intake between d 2 and 5 of lactation was 357.2 ± 41.4 g and 4.0 kg, respectively. Compared to d 2 of lactation, mRNA abundance of $y^+$LAT2 was higher ($P = 0.005$) (Figure 2.4) on d 5. There was no difference in CAT-1, $ATB^{0,+}$, α-lactalbumin and β-casein mRNA abundance between d 2 and 5 of lactation ($P > 0.1$; Figures 2.4 and 2.5). Between d 2 and d 5, mRNA abundance of CAT-1 and $y^+$LAT2 was correlated to that of β-casein ($r = 0.76$ and $P = 0.003$; $r = 0.67$ and $P = 0.01$, respectively) and of α-

lactalbumin (r = 0.82 and $P = 0.04$; r = 0.83 and $P = 0.03$, respectively). Messenger RNA abundance of $ATB^{0,+}$ was not correlated with either that of β-casein or α-lactalbumin ($P > 0.1$).

**Discussion**

Previous studies performed in lactating rats using mammary tissue explants have indicated that Lys is transported into mammary cells by cationic AA transporters of the $y^+$ system (Shennan et al., 1997; Shennan and Peaker, 2000). In the current study, genes encoding for the cationic-specific AA transporters CAT-1 and CAT-2b, and for the shared cationic-neutral AA transporters $ATB^{0,+}$, $b^{0,+}AT$, $y^+LAT1$ and $y^+LAT2$, were expressed at the transcription level in porcine mammary tissue. Amino acid transporters CAT-1 and CAT-2b (system $y^+$) are $Na^+$-independent and highly specific for Lys and Arg (Deves and Boyd, 1998), whereas $ATB^{0,+}$ (system $B^{0,+}$) is $Na^+$-dependent and mediates intracellular transport of both cationic and neutral AA in intestinal epithelial cells (Broër, 2008). In this study, mRNA of CAT-1 and of $ATB^{0,+}$ was highly abundant in porcine mammary tissue collected during lactation relative to that of other AA transporters. Furthermore, CAT-1 and $ATB^{0,+}$ mRNA abundance increased with the expected increase in piglet growth and was highly related to changes in β-casein and α-lactalbumin expression at the mRNA abundance level. Both transporters were highly abundant and up- regulated prior to or at the onset of lactation, since there was no increase in mRNA as early as between d 2 and 5 of lactation. In contrast, AA transporter CAT-2b was low in abundance and remained unchanged between pre-partum and post-weaning. Pérez-Laspiur et al. (2009) reported that both CAT-1 and CAT-2b were unaffected by increase in milk demand

between d 7 and 18 of lactation, and suggested that these transporters may be up-regulated earlier in lactation. In the present study, mammary biopsies were taken no later than d 5, and CAT-1 increased from d 5 to 17 of lactation. In humans, CAT-1 and CAT-2b isoforms demonstrate clear differences in their functionality with CAT-1 having a 3-fold higher substrate affinity and being more sensitive to trans-stimulation than CAT-2b (Closs et al., 1997). The higher dependence of CAT-1 on substrate concentration at the trans-side of the membrane may facilitate cationic AA uptake into mammary epithelial cells.

Similarly to CAT-2b, AA transporter $b^{0,+}$AT (system $b^{0,+}$) was relatively low in abundance and demonstrated no change in mRNA abundance between the pre-partum and post-weaning periods. In contrast to mammary tissue, $b^{0,+}$AT is now recognized as the major protein mediating cationic AA transport across small intestinal and kidney tubular cells. In those organs, $b^{0,+}$AT-mediated transport is a $Na^{+}$-independent process localized to the apical rather than basolateral membrane; in those cells, $b^{0,+}$AT is highly abundant and functions to reabsorb and absorb, in the renal tubules and intestinal epithelial cells, respectively, cationic AA in exchange for neutral AA (Chillaron et al., 1996; Sperandeo et al., 2008, Broër, 2008). Thus, $b^{0,+}$AT may also be localized to the apical aspect of mammary cells rather than to the basolateral side. Its role in porcine mammary cells during lactation is unclear; it may function to recover free cationic AA from the alveolar lumen where free milk AA concentration constitutes a very small fraction of total milk nitrogen (Wu and Knabe, 1994). With these notions in mind, $b^{0,+}$AT mRNA abundance would not be expected to change in relation to that of β-casein and α-lactalbumin.

Amino acid transporters $y^+$LAT1 and $y^+$LAT2 (System $y^+$L) have similar properties (Bröer et al., 2000; Pfeiffer et al., 1999). Both transporters are $Na^+$-independent and shown to be localized to the basolateral membrane of kidney and intestinal epithelial cells, where they mediate the uptake of circulating neutral AA in exchange for intracellular cationic AA (Bröer, 2008; Closs et al., 2004). Thus, both transporters are likely to be expressed on the basolateral aspect of mammary epithelial cells. However, mRNA abundance of $y^+$LAT1 was unrelated to that of the milk protein genes, as it remained unchanged from pre-partum to peak lactation, whereas that of $y^+$LAT2 was upregulated with the onset of lactation and highly correlated to β-casein and α-lactalbumin mRNA. Therefore, it may be possible that both transporters contribute similarly to neutral AA uptake during late gestation in porcine mammary gland, with only $y^+$LAT2 subjected to further upregulation with the onset of lactation to ensure adequate leucine, valine and isoleucine uptake for milk protein synthesis.

Increasing milk protein synthetic demand with advancement of lactation decreases arterial concentration of Lys (Trottier et al., 1997), the most limiting AA in the diet of lactating sows (NRC, 1998). Consequently, Lys transporters such as CAT-1, $ATB^{0,+}$ and $y^+$LAT2 may play a critical role in adapting to fluctuating plasma Lys levels to ensure intracellular Lys concentrations are maintained at high levels to keep tRNA fully charged, as suggested by the high correlation found between their mRNA abundance and that of β-casein and α-lactalbumin. We acknowledge that gene expression of both α-lactalbumin and β-casein is a complex pathway regulated by numerous hormones and growth factors. In fact, gene expression of milk proteins α-lactalbumin and β-casein was also highly correlated to that of specific lactopoietic hormone

receptors, mTOR related kinases and glucose transporters in porcine mammary gland (See Appendix A). As such, the correlation found between specific AA transporters and milk protein only suggests a potential role of CAT-1, $ATB^{0,+}$ and $y^+LAT2$ in providing AA for milk protein synthesis, but not a regulatory mechanism for milk protein gene expression. Based on the marked increase in expression of CAT-1, $ATB^{0,+}$ and $y^+LAT2$ from the pre-partum to early lactation periods, we further quantified their transcript abundance, along with α-lactalbumin and β-casein, during the very early stage of lactation, i.e., between d 2 and d 5. However during that short time period, only expression levels of CAT-1 and $y^+LAT2$ were correlated to those of α-lactalbumin and β-casein, but only mRNA abundance of $y^+LAT2$ increased on d 5. It is possible that increase in mRNA abundance of $y^+LAT2$ occurs after farrowing, whereas CAT-1 and $ATB^{0,+}$ increase before farrowing in preparation for galactopoiesis.

Earlier studies have shown that as lactation progresses in the sow, arterial extraction rate increases for the majority of indispensable AA, likely to support the increasing need for milk protein synthesis (Trottier et al., 1997; Nielsen et al., 2002). In the present study however, only CAT-1 mRNA abundance increased between early and peak of lactation. It is well known that an increase in mRNA abundance does not always translate into increase in protein level. However, despite that CAT-1 protein levels were not measured, *in vitro* (Aulak et al., 1999) and *in vivo* (Pérez-Laspiur et al., 2009) studies showed parallel changes in CAT-1 protein and transcript abundance in porcine mammary tissue. Therefore, it is possible that an increase in extraction efficiency by CAT-1 is supported by an increase of mRNA and protein abundance ($V_{max}$ effect), whereas uptake regulation of neutral AA by $y^+LAT2$ and $ATB^{0,+}$ is mediated by a

change in binding affinity of the transporters ($K_m$ effect). Alternatively, it is possible that CAT-1 carries out more functions in the mammary gland than merely providing AA to the cells. Increased CAT-1 mRNA abundance during lactation may play a key role in porcine mammary cell growth and turnover by sensing changes in extracellular Arg and Lys concentrations; for instance, in the fat body of mosquitoes, active uptake of cationic AA via specific transporters activated vitellogenic genes through TOR pathway (Attardo et al., 2006). In addition, CAT-1 may offer a unique port of entry for Arg in endothelial cells. Arginine plays a pivotal role in enhancing blood flow in mammary gland during lactation as it is the substrate for synthesis of the vasodilator nitric oxide (Mann et al., 2003). Indeed, arterial Arg extraction rate by the mammary gland increases with lactation (See Chapter 3), and others have shown that CAT-1 is located on the basolateral membrane of endothelial cells (Mann et al., 2003; Woodard et al., 1994).

In conclusion, changes in mRNA abundance indicate that expression of selected AA transporters is regulated, in part, at a pre-translational level. Messenger RNA abundance of CAT-1, $ATB^{0,+}$ and $y^+LAT2$ was upregulated between pre-partum and peak of lactation, and positively correlated to expression of genes encoding for the mammary synthesized milk proteins β-casein and α-lactalbumin. As such, CAT-1, $ATB^{0,+}$ and $y^+LAT2$ may be involved in transport of limiting AA in cells of the mammary parenchyma tissue, constituting molecular targets for improving sow milk production during lactation. Further studies are critically needed to determine the regulatory mechanisms that control the expression of these genes.

**Table 2.1.** Ingredient and nutrient composition of lactation diet (as-fed basis)

| Item | % |
|---|---|
| Ingredients | |
| Yellow corn | 61.16 |
| Soybean meal | 25.38 |
| Dried beet pulp | 5.0 |
| Choice white grease | 4.0 |
| Calcium phosphate | 1.48 |
| Limestone | 0.77 |
| Vitamin premix[1] | 0.60 |
| Trace mineral premix[2] | 0.50 |
| Sow pack[3] | 0.30 |
| MTB-100 Alltech[4] | 0.10 |
| Natuphos 600[5] | 0.08 |
| Vitamin E | 0.01 |
| Salt | 0.50 |
| Calculated analysis | |
| Dry matter | 89.71 |
| Metabolizable energy, Mcal/kg | 3,374 |
| Crude protein | 17.86 |
| Lysine, apparent digestible | 0.85 |
| Threonine, apparent digestible | 0.57 |
| Fat | 6.49 |
| Neutral detergent fiber | 10.25 |
| Acid detergent fiber | 4.30 |
| Calcium | 0.80 |
| Available phosphorus | 0.40 |
| Sodium | 0.31 |
| Chloride | 0.35 |

[1]Provided the following per kg of diet: 4500 IU vitamin A, 458 IU vitamin $D_3$, 55 IU vitamin E, 11 mg vitamin K, 3.66 mg menadione, 0.0275 mg vitamin $B_{12}$, 3.66 mg riboflavin, 14.67 mg D-panthotenic acid, 22 mg niacin, 0.913 mg thiamine, 0.825 mg pyridoxine.

[2]Provided the following per kg of diet: 335 g Ca,5 g Fe, 5 g Zn, 5 g Cu, 150 μg Se and 75 μg I.

[3]Provided the following per kg of diet: 2755.7 IU vitamin A, 220.5 μg biotin, 385.8 mg choline, 1.65 mg folic acid.

[4]Non-viable dried yeast of *Saccharomyces cerevisiae* used as anti-caking agent. Each kg contains 22% crude protein, 0.05 crude fat, and 11% crude fiber (Alltech, Nicholasville, KY)

[5]Provided 500 phytase units of activity per kg of diet (500 FTU/kg; BASF Corporation, Florham Park, NJ)

**Table 2.2.** Primer information for real time quantitative PCR (RT-qPCR) assays

| Accesion Number[1] | Gene | Protein | Primer[2] | Primer (5'-3') | E[3] (%) |
|---|---|---|---|---|---|
| CV872150.1 | *API5* | Apoptosis inhibitor 5 | F. 502<br>R. 568 | CTGGAGTGGTGGCAATAATCTCT<br>CCAAGGGAGCTCAGGTTTAGC | 99.4 |
| AY610067.1 | *MRPL39* | Mitochondrial ribosomal protein L39 | F. 540<br>R. 601 | TCGCTGGAGCTTTCTGCTATG<br>TGTTGGCATCCACTCATCAAG | 103.5 |
| NM_001123213.1 | *VAPB* | Vesicle-associated membrane protein-associated protein B/C | F. 1012<br>R.1072 | TGGCGCTGGTGGTTTTG<br>CCTACAAGGCGATCTTCCCTATG | 101.9 |
| NM_001110421.1 | *SLC7A7* | $y^+$LAT1 | F. 51<br>R. 108 | TTTGGTTCCCAAGGTTGCA<br>GCAGCTTCCTGGCATTGC | 115.6 |
| CX064558.1 | *SLC7A6* | $y^+$LAT2 | F. 114<br>R. 172 | CTGCCGCCTGCATGTGT<br>TGTGCCCCACTTGACATAGG | 101.9 |
| NM_001012613.1 | *SLC7A1* | CAT-1 | F. 1172<br>R. 1234 | GGGCTGCTGTTTAAGTTTTTGG<br>CGTGGCGATTATTGGTGTTTT | 100.4 |
| NM_001110420.1 | *SLC7A2* | CAT-2b | F. 1723<br>R. 1071 | GCCCCAGAATCAGCAAAAAGTA<br>GATGCTGAAGGCTGGCAAAA | 110.7 |
| NM_001166042.1 | *SLC6A14* | $ATB^{0,+}$ | F. 162<br>R. 226 | CCGTGGTAACTGGTCCAAAAA<br>CCAATCCCACTGCATATCCAA | 101 |
| NM_001110171.1 | *SLC7A9* | $b^{0,+}$AT | F. 138<br>R. 205 | CCAGAGCACTGAACCCAAGAC<br>TGATGCAGATGCCACTGAACA | 97.1 |
| NM_214434.1 | *CSN2* | β-casein | F. 333<br>R. 398 | CTGTGGTGGTGCCTCTTCTTC<br>GAACAATGGTCTCCTTAGCTTTGG | 96.4 |
| NM_214360.1 | *LALBA* | α-lactalbumin | F. 433<br>R. 495 | CCCGCTGTCTTGCTGCTT<br>AGGTAGCCTTAGGGAAGAGGAGTT | 94.5 |

[1]Accesion number corresponds to the complementary DNA (cDNA) or the expressed sequence tag (EST) sequence deposited in the National Center for Biotechnology and Information (NCBI) database from which the primers were designed.

[2]Direction (F = forward; R = reverse) and hybridization position for each primer (5′–3′) within the nucleotide sequence from which the primers were designed.

**Table 2.2 (cont'd)**

[3]The reverse transcription quantitative PCR (RT-qPCR) efficiency (E) was calculated as follow: $E = -1+10^{(-1/slope)} \times 100$. The $R^2$ values for all standard curves for reference and target genes were > 0.98, indicating excellent linear relationships between quantities of serially diluted cDNA and cycles to threshold (Ct) when RT-qPCR was performed.

**Figure 2.1.** Messenger RNA abundance of amino acid transporters $ATB^{0,+}$, $y^+LAT1$ and $y^+LAT2$ in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 13 - ΔCt ± SE (n = 4). Data was compared between: d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (post-weaning) and peak lactation. $^{**}P < 0.01$; $^{***}P < 0.001$.

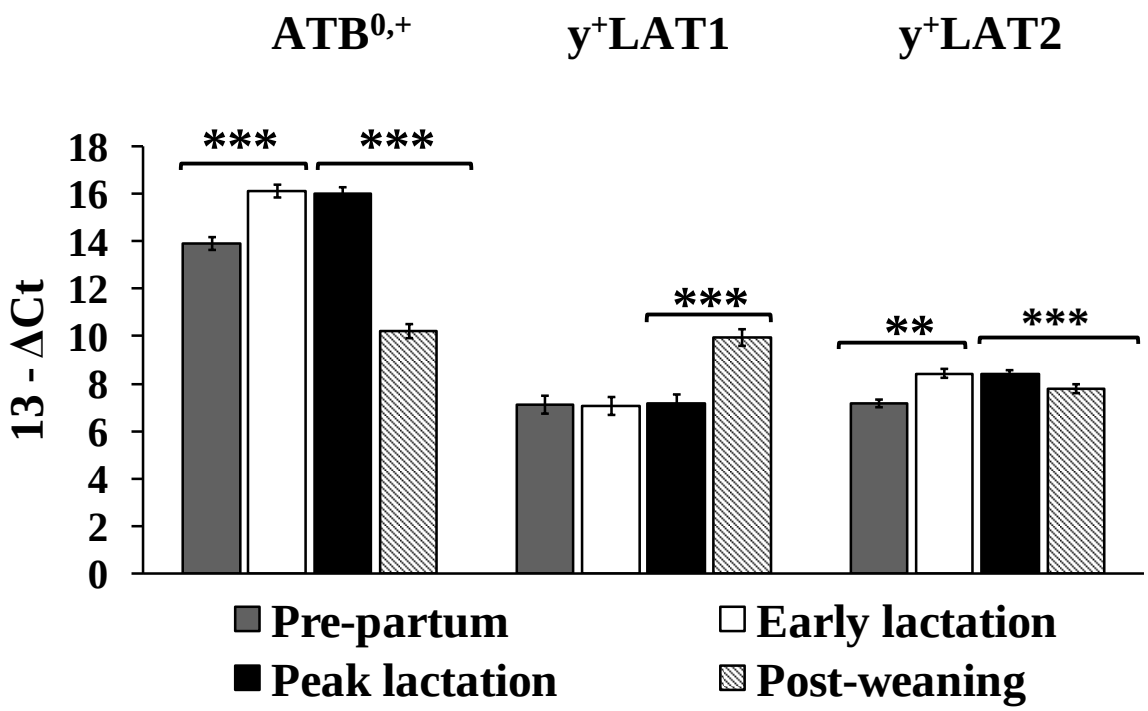


**Figure 2.2.** Messenger RNA abundance of amino acid transporters CAT-1, CAT-2b and $b^{0,+}AT$, in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 13 - ΔCt ± SE (n = 4). Data was compared between: d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (post-weaning) and peak lactation. $^{*}P < 0.05$; $^{***}P < 0.001$.

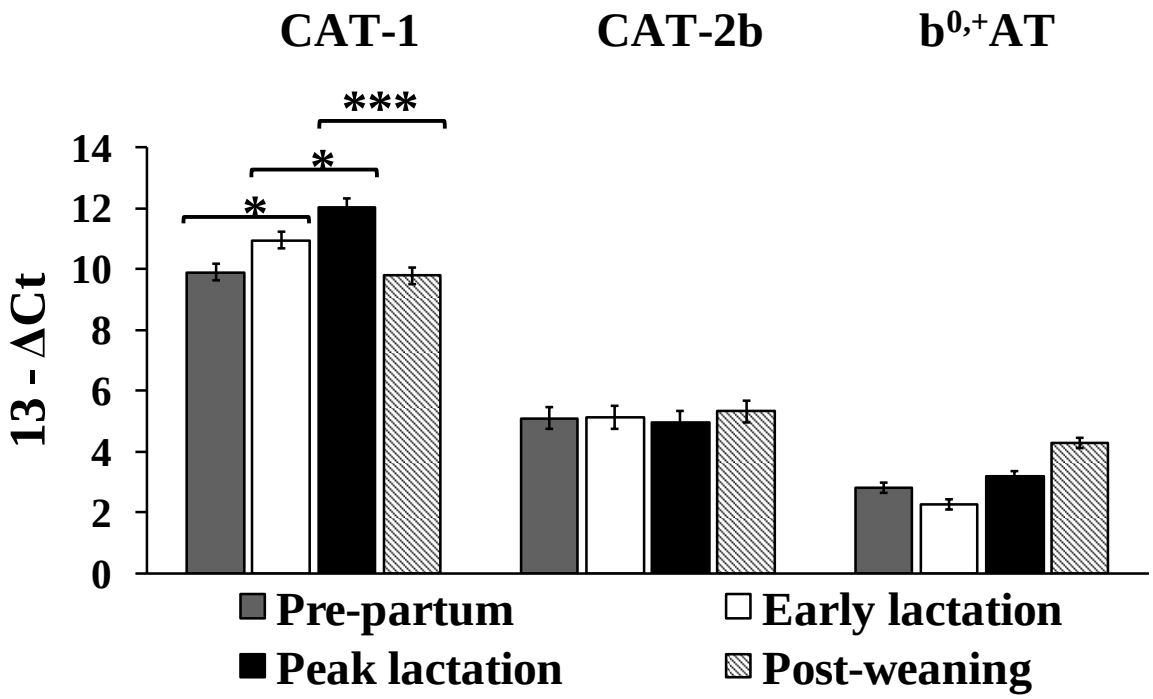


**Figure 2.3.** Messenger RNA abundance of milk protein genes β-casein and α-lactalbumin in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 13 - ΔCt ± SE (n = 4). Data was compared between: d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (post-weaning) and peak lactation. $^{***}P < 0.001$.

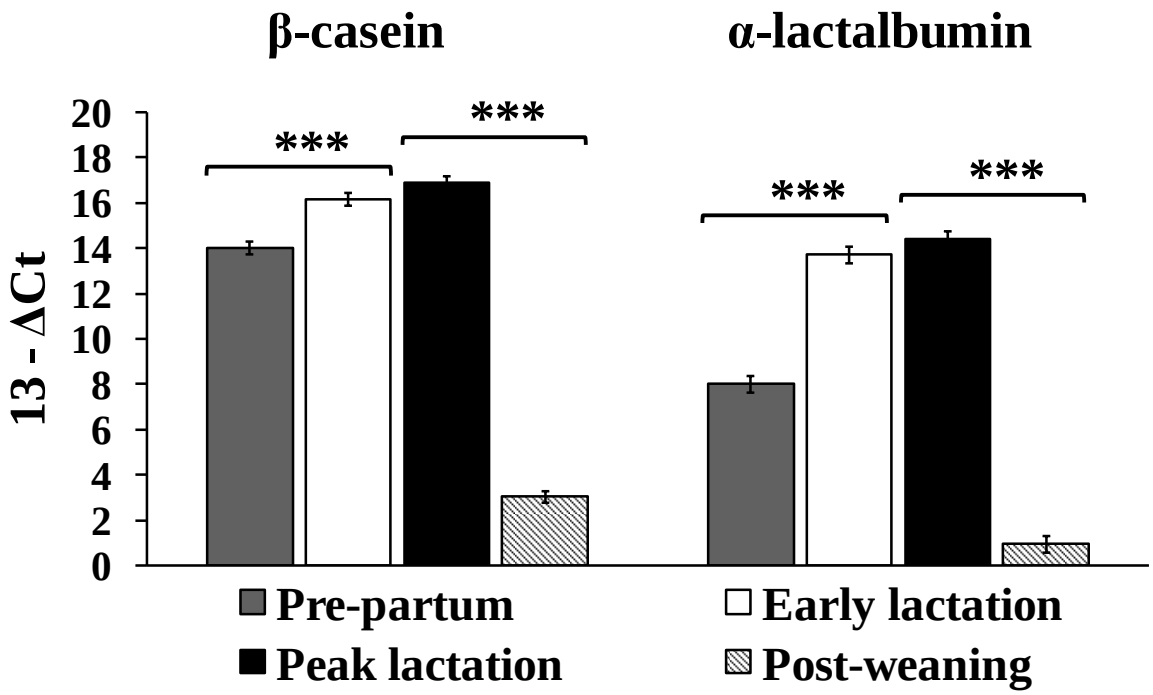


**Figure 2.4.** $ATB^{0,+}$, $y^{+}$LAT2 and CAT1, mRNA abundance in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represent 13 - ΔCt ± SE (n = 4). Data at d 2 of lactation was compared to that of d 5 of lactation (early). $^{**}P < 0.01$

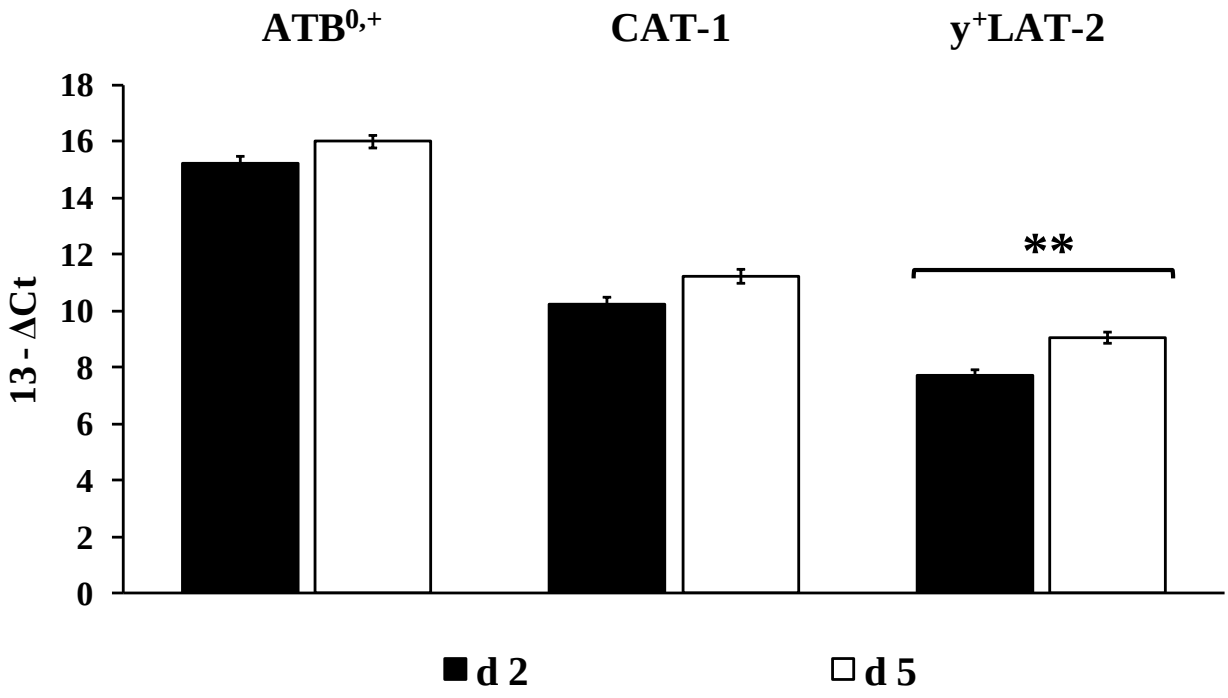
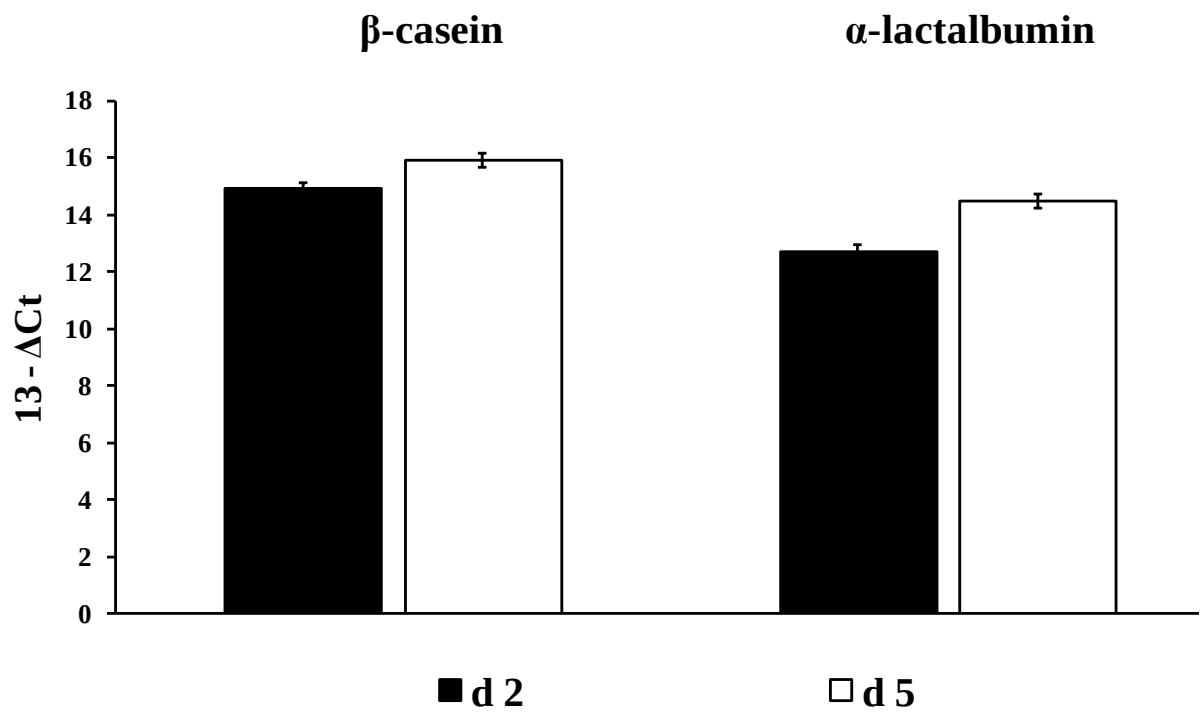


**Figure 2.5.** β-casein and α-lactalbumin mRNA abundance in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represent 13 - ΔCt ± SE (n = 4). Data at d 2 of lactation was compared to that of d 5 of lactation (early).



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# CHAPTER 3

# INCLUSION OF CRYSTALLINE AMINO ACIDS IN A REDUCED-CRUDE PROTEIN DIET MAINTAINS SOW PERFORMANCE INDEPENDENTLY OF AMINO ACID TRANSPORTER GENE EXPRESSION

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## Abstract

To test the hypothesis that reduction in dietary CP concentration coupled with crystalline amino acid (CAA) inclusion increases the efficiency of AA utilization for milk production, mammary AA arterio-venous concentration differences (A-V), AA transport efficiency (A-V/A × 100) and transcript abundance of AA transporters and milk protein-encoding genes were determined in lactating sows fed 1 of 3 diets containing 9.5 (Deficient), 13.5 (Ideal), and 17.5 % (Standard) CP but a similar indispensable and dispensable AA profile. On d 7 and 17, arterial and mammary venous blood and mammary tissue were sampled post feeding. Transcript abundance of AA transporters *SLC7A9* ($b^{0,+}$AT), *SLC7A6* ($y^{+}$LAT2), *SLC6A14* ($ATB^{0,+}$), *SLC7A1* (CAT-1), and *SLC7A2* (CAT-2b), and milk protein genes *CSN2* (β-Casein) and *LALBA* (α-Lactalbumin) were determined using RT-qPCR. Piglet ADG increased curvilinearly with increasing % CP [Q (quadratic), $P < 0.01$]; it was lower ($P < 0.05$) for Deficient compared to Ideal and Standard diets, and did not differ between Ideal and Standard diets. On d 7, Lys and

Arg A-V and transport efficiency increased curvilinearly (Q, $P < 0.05$) with increasing % CP; compared to Deficient and Standard diets, Arg A-V was higher ($P < 0.01$) and transport efficiency tended to be higher ($P = 0.09$) for the Ideal diet. On d 17, Lys A-V tended to increase linearly (L) (L, $P = 0.08$) with increasing % CP. Increasing CP linearly increased Ile and Val A-V on d 7 (L, $P = 0.05$ and $P = 0.08$, respectively) and Leu and Val on d 17 (L, $P = 0.07$ and $P = 0.04$, respectively). On d 7, plasma concentrations of BCAA:Lys, Leu:Lys and Ile:Lys decreased curvilinearly (Q, $P < 0.05$), with BCAA:Lys and Leu:Lys lower ($P < 0.01$) for Ideal compared to Standard diet. Plasma ratio of Val to Lys tended to decrease curvilinearly (Q, $P = 0.08$), and was lower ($P < 0.01$) for Ideal compared to Standard diet. Expression of genes encoding for AA transporter proteins $b^{0,+}$AT, $y^{+}$LAT2, $ATB^{0,+}$, CAT-1, CAT-2b, and for mammary synthesized proteins β-casein and α-lactalbumin, was unaffected by diet. In conclusion, decreasing the dietary CP from 19.4 (Standard diet) to 15.1 % (Ideal diet) with inclusion of CAA did not affect piglet ADG, but increased mammary transport efficiency and A-V of Lys and Arg. The increase in Lys and Arg transport efficiency was associated with a decrease in plasma concentration of BCAA to Lys ratio but unrelated to AA transporter and milk protein gene transcript abundance. These results indicate that CP reduction with CAA inclusion improves the efficiency of dietary AA utilization for litter growth, and that the mechanisms behind this response are independent of AA transporter or milk protein gene transcription.

**Introduction**

Research is increasingly focused on nutritional strategies aimed at reaching an optimal dietary AA profile for better dietary protein utilization by pigs (NRC, 1998). We recently showed that AA imbalances created by excesses or deficiencies of dietary AA reduce the

efficiency of N utilization in lactating sows, limiting milk protein synthesis and litter growth (Pérez-Laspiur et al., 2009). In growing pigs, reduction in dietary CP with concomitant inclusion of crystalline AA (CAA) increases efficiency of Lys utilization and reduces N products excreted into the environment (Otto et al., 2003). However, there is still poor mechanistic understanding of the factors that regulate dietary AA utilization efficiency at the cellular level. The intracellular availability of dietary AA is controlled by a coordinated activity of protein-carriers located in the cellular membrane that channel AA into the cells (Shennan et al., 2000, Palacín et al., 1998; Broër et al., 2008). Lysine is the dietary first limiting AA for lactating sows (NRC, 1998), and thus AA transporters involved in Lys uptake are likely to play a key role in the global efficiency of dietary protein utilization by the mammary gland. We have previously shown an increase in transcript abundance of genes encoding for Lys AA transporters in response to milk demand (Manjarín et al., 2011) and to dietary AA availability (Pérez-Laspiur et al., 2009). Herein, we hypothesize that the efficiency of AA transport by the porcine mammary gland increases in response to dietary CP reduction with CAA inclusion and that this increase is mediated via an increase mRNA abundance of mammary genes encoding for Lys transporters. The objectives of this study were 1) to test whether a reduction in dietary CP % coupled with crystalline AA (CAA) inclusion increases the efficiency of Lys utilization by the mammary gland for milk production and 2) to quantify the expression of genes encoding for specific mammary Lys transporters and dominant mammary-synthesized milk proteins.

## Material and methods

### *Animals and Tissue Collection*

All animal procedures in this study were performed with the approval of the Institutional Animal Care and Use Committee at Michigan State University (AUF#10/08-162-00). Multiparous lactating sows (Landrace x Yorkshire; n = 21) were used in a randomized incomplete block design with 3 replications. Block was defined as a farrowing cycle, with the first and second blocks consisting of 8 sows, and the third block consisting of 5 sows. Sows were individually housed in farrowing crates in a thermally controlled room ($20^0$C) throughout the study. One week prior to the expected farrowing, sows were selected and allocated to one of three dietary treatments: 1) 9.5% CP (Deficient, n = 8), 13.5% CP (Ideal, n = 6) and 17.5% CP (Standard, n = 7). All sows were fed 2.5 kg/d divided into two meals before farrowing. The day after farrowing was considered d 1 of lactation and sows were fed 1 kg at 0800 and 1600. On d 2 and 3 of lactation, sows were fed a total of 3 and 4 kg, respectively, provided in two equal meals. For the remainder of the study, a maximum of 5.5 kg/d was provided to ensure equal dry matter (DM) intake among all sows. Sow feed intake was recorded daily throughout lactation. Fresh water was freely available at all times. Sow body weight was recorded on d 2 and 18 of lactation. Litters were equalized to 8 piglets weighing approximately 15 kg in total by cross-fostering within 24 h of birth. Litter weight was recorded on d 2 and 17 of lactation, and piglets were weaned on d 21 of lactation.

***Dietary treatments and feed nutrient analysis***

Diets contained a CP concentration of 9.5 %, 13.5% and 17.5% (Deficient, Ideal and Standard, respectively) for 0.50, 0.81 and 1.01% analyzed standardized ileal digestible (SID) Lys, respectively (Table 3.1). Desired levels of CP reduction in Ideal and Deficient diets were achieved by diluting the Standard diet with cornstarch and sucrose, but keeping the soybean

meal:corn constant across diets. Crystalline AA were then added to the Ideal diet to meet the essential AA requirements and the AA:Lys for lactating sows nursing 10 piglets with a predicted average daily gain of 250 g/d based on NRC (1998) (Table 3.1). Crystalline L-Lys was then added to the Standard diet to a level that was 30% higher than that of the Ideal, i.e., 1.01% vs. 0.81% SID. The Lys level in the Standard diet was chosen to model industry feeding practices (personal communications). In order to meet the NRC (1998) AA:Lys ratio and maintain an identical dietary AA profile to that of the Ideal diet, CAA were also included in the Standard diets. Finally, L-Lys was added to the Deficient diet to meet a level of 0.50% SID Lys to achieve similar dietary SID Lys spacing between the Deficient, Ideal and Standard. As for the Standard diet, CAA were included to ensure identical AA:Lys to the Ideal diet.

In order to prevent long-term storage, diets were freshly prepared for each block, thus a total of 3 diet mixing was done. Diets were sampled from each bag and pooled per diet for each mixing. Each pooled samples were finely ground using a sample mill (Cyclotec 1093, Foss Tecator). Feed N was analyzed for each sample using a combustion-based N determinator (FP-2000, LECO Corp.) and results averaged. Amino acid concentrations in the pooled feed samples were analyzed by cation-exchange chromatography (cIEC-HPLC) coupled with post-column ninhydrin derivatization and quantitation (Agricultural Experimental Station, University of Missouri, Columbia, MO). Calculated and analyzed AA concentrations are presented in Table 3.2.

***Total mammary gland DNA quantification***

Quantification of mammary DNA concentration is as described in Chapter 1**.**

***Gene expression analysis***

*Sample collection*

Mammary parenchymal tissue was biopsied from the first and second thoracic glands of all sows 3.5 h post-feeding on d 7 (early) and d 17 (peak) of lactation, according to a method described by Kirkwood et al. (2007). During mammary tissue collection, piglets were isolated in an adjacent pen equipped with a heat lamp. Immediately following the biopsy, mammary tissue was flash frozen in liquid $N_2$ and later stored at -80$^0$C. Three hours after biopsy, piglets were returned to sows and allowed to nurse.

*RT-qPCR*

Primer sequences for reference and target genes are presented in Table 2.1 and 1.2, respectively. In order to facilitate readership throughout the remainder of the manuscript, each target gene is referred as the common name of the protein that it encodes for. Hence *SLC7A9* will be referred to as $b^{0,+}$AT, *SLC7A7* as $y^+$LAT1, *SLC7A6* as $y^+$LAT2, *SLC6A14* as $ATB^{0,+}$, *SLC7A1* as CAT-1, *SLC7A2* as CAT-2b, *CSN2* as β-Casein and *LALBA* as α-Lactalbumin. Potential reference genes were selected based on previous studies (Bionaz and Loor, 2007; Tramontana et al., 2008), but the primers used were different from those published to optimize the efficiency of the RT-qPCR reaction in our samples. RNA extraction and cDNA synthesis, primer design and optimization and RT-qPCR assay are as described in Chapter 1.

*Reference Gene Selection*

Reference gene selection is as described in Chapter 1**.**

*RT-qPCR data normalization*

Analysis of target gene expression was made by subtracting from each of the target gene Ct the arithmetic mean of the 3 selected reference genes Ct (Equation 3.1).

$$\Delta Ct_{ij} = Ct_{Cij} - [1/3 \times (Ct_{R1ij} + Ct_{R2ij} + Ct_{R3ij})] \quad [3.1]$$

where $\Delta Ct_{ij}$ is the difference between Ct of target and reference gene for the $j^{th}$ sow within the $i^{th}$ stage of lactation, and $Ct_{Cij}$ and $Ct_{Rij}$ are the cycles to threshold of target and reference gene for the $j^{th}$ sow within the $i^{th}$ stage of lactation.

A***nalysis of AA Transport Efficiency into the Mammary Gland***

Mammary vein and carotid artery catheters were prepared and surgically inserted between d 3 and 4 of lactation, as described by Trottier et al. (1995). The catheters were flushed with a sterile heparinized (20 U/mL) saline solution (0.9 %) every 12 h and maintained with a heparin block. Arterial and venous blood samples were taken simultaneously in syringes and transferred to heparinized sample tubes. The first 3 mL of fluid withdrawn was discarded to eliminate dilution from the heparin block. On d 7 and d 17 of lactation, all sows were fed 2 kg at 0800, followed by blood sampling every 30 min from 0830 to 1130, inclusive. Blood samples were centrifuged within 10 min of collection and the plasma stored at $-20^0$ C. Determination of free AA in plasma was performed by HPLC using an analytical method based on derivatization of AA with *o*-phthaldialdehyde (Wu and Meininger, 2008).

Plasma samples obtained during the 3.5-h blood collection period were pooled and each pooled sample was treated as the sampling unit. Efficiency of AA transport was calculated on d 7 and 17 as the % of circulating AA taken by mammary glands per plasma pass (Equation 3.2)

$$Y_{ij} = (A - V)/A - 100 \qquad [3.2]$$

where $Y_{ij}$ is the AA transport efficiency for the $j^{th}$ sow within the $i^{th}$ stage of lactation, and A-V is the difference between arterial and venous AA concentrations.

***Statistical analysis***

Normality of the residuals was tested using the Shapiro-Wilk test under the UNIVARIATE procedure of SAS. Piglet ADG, litter gain weight, sow feed and protein intake, DNA concentration, ΔCt, plasma AA, A-V and transport efficiency, and plasma BCAA:Lys were analyzed using a linear mixed model that included diet, stage of lactation and their interaction as fixed effects, sow and block as random effects, and sow initial body weight and parity as covariates. The statistical model is as follows (Equation 3.3):

$$Y_{ijkp} = \mu + \alpha_i + \gamma_j + \alpha\gamma_{ij} + \beta_1(x_k - \bar{x}) + \beta_2(z_k - \bar{z}) + b_k + c_p + e_{ijkp} \quad [3.3]$$

where $Y_{ijkp}$ is the variable measured for each sow (k), each stage of lactation (j), each diet (i) and each block (p), $\mu$ is the overall mean, $\alpha_i$ is the fixed effect of the $i^{th}$ level of diet, $\gamma_j$ is the fixed effect of the $j^{th}$ level of stage of lactation, $\alpha\gamma_{ij}$ *is* the fixed effect of the interaction between diet and stage of lactation, $\beta_1$ is the regression coefficient relating the covariate sow

initial body weight to the variable measured, $x_k$ is the initial body weight for each sow, x is the overall sow initial body weight mean, $\beta_2$ is the regression coefficient relating the covariate sow parity to the variable measured, $z_k$ is the parity for each sow, z is the overall parity mean, $b_k$ is the random effect of the sow, $c_p$ is the random effect of the block and $e_{ijkp}$ is the random residual effect. Relationships between dietary CP protein intake, day of lactation and their interaction and outcome variables were determined using linear (L) and quadratic (Q) contrasts. When an interaction was not significant, *P*-values of main effects (i.e., diet and stage of lactation) are reported. Multiple comparisons were accounted for with Bonferroni adjustment. Significant effects were considered at $P \leq 0.05$ and trends at $P \leq 0.1$

**Results**

Lactation performance results are presented in Table 3.3. Feed intake was not affected by dietary protein intake, and both feed and dietary protein intake increased ($P < 0.01$) from d 7 to 17 of lactation (data not shown). Sow body weight and litter size were not different between diets on d 1 or 17. Piglet and litter ADG increased curvilinearly with increasing % CP (Q, $P < 0.01$); it was lower ($P < 0.05$) for Deficient compared to Ideal and Standard diets, and not different between Ideal and Standard diets. There was no relationship between mammary DNA concentration and diet or stage of lactation.

Reference genes *MTG1*, *MRPL39* and *VAPB* had the lowest average M and therefore were selected as the most stable set of genes in porcine mammary tissue (Figure 3.1). Pairwise variation analysis between sequential normalization factors was below the 0.15 cut-off value proposed by Vandesompele et al. (2002), indicating that the optimal number of reference genes

was 3; the inclusion of a fourth gene increased (V2/3 = 0.11 and V3/4 = 0.12) the pairwise variation (Figure 3.2). Expression of genes encoding for $b^{0,+}$AT, $y^{+}$LAT2, $ATB^{0,+}$, CAT-1, CAT-2b, β-casein and α-lactalbumin were unaffected by diet and stage of lactation (Figures 3.3, 3.4, 3.5, 3.6, 3.6, 3.7, 3.8, and 3.9, respectively).

Arterial concentration of essential AA, A-V difference and transport efficiency across the mammary gland are presented in Tables 3.4, 3.5 and 3.6, respectively. Arterial concentration of non essential AA, A-V difference and transport efficiency across the mammary gland are presented in Tables 1, 2 and 3 in Appendix B. Arterial concentrations for all essential AA increased linearly ($P < 0.05$) with increasing CP concentration, except for His, Met and Trp. Arterial concentrations for non-essential AA increased linearly ($P < 0.05$) with increasing CP concentration for Asn and Tyr, decreased for Glu, Cit and Ala ($P < 0.05$), and remained unchanged for other AA. On d 7, Lys and Arg A-V and transport efficiency increased curvilinearly (Q, $P < 0.05$); compared to Deficient and Standard diets, Arg A-V was higher ($P < 0.01$) and transport efficiency tended to be higher ($P = 0.09$) for Ideal diet. On d 17, Lys A-V tended to increase linearly (L, $P = 0.08$) with increasing % CP, while there was no change in Arg A-V and transport efficiency across diets. Increasing CP linearly increased Ile and Val A-V on d 7 (L, $P = 0.05$ and $P = 0.08$, respectively) and Val and Leu on d 17 (L, $P = 0.07$ and $P = 0.04$, respectively).

Arterial BCAA to Lys ratios are presented in Table 3.7. Total BCAA:Lys, Leu:Lys and Ile:Lys plasma concentrations decreased curvilinearly (Q, $P < 0.05$) on d 7, with BCAA:Lys and Leu:Lys lower ($P < 0.01$) for Ideal compared to Standard diet. Plasma concentration of Val:Lys tended to decrease curvilinearly (Q, $P = 0.08$) on d 7, and was lower ($P < 0.01$) for Ideal

compared to Standard diet. Plasma concentration of Arg:Lys remained unchanged between diets at either d 7 or 17 of lactation.

**Discussion**

Numerous studies have focused on optimizing dietary Lys intake by the lactating sow to maximize litter growth (NRC, 1998; Dourmad et al., 1998; Touchette et al., 1998; Yang et al., 2000) but very little attention has been paid to the mechanisms that regulate the efficiency of Lys utilization for milk protein production. Such knowledge may allow the design of strategies for further optimization of dietary Lys for litter growth and reduce nitrogen losses to the environment. The first step in mammary Lys utilization involves cellular uptake, a process mediated by transporter proteins located in cellular membranes (Shennan and Peaker, 2000; Broër, 2008). Proteins known to facilitate Lys transport into mammalian epithelial cells include those of the $y^+$ system of AA transporter family specific for cationic AA (Palacín et al., 1998; Shennan and Peaker, 2000), two of which we have identified in lactating porcine mammary tissue, namely CAT-1 and CAT-2b (Pérez-Laspiur et al., 2004; 2009). Lysine may also be transported by the shared systems with BCAA, such as systems $B^{0,+}$, $b^{0,+}$ and $y^+L$; of these systems, we have shown that genes encoding for $ATB^{0,+}$ and more recently for $b^{0,+}$ AT and $y^+LAT2$, are expressed in porcine mammary tissue and regulated at a pre-translational level (Manjarín et al., 2011; Pérez-Laspiur et al., 2004; 2009). In the present study, expression of CAT-1, CAT-2b, $ATB^{0,+}$, $b^{0,+}$ and $y^+LAT2$ genes remained unchanged between diets and across stages of lactation, despite an increase in Lys and Arg transport efficiency with reduction of CP from 17.5% (Standard) to 13.5% (Ideal). Our previous studies *in vivo* (Pérez-Laspiur et al.,

2009) and that of others *in vitro* (Satsu et al., 1998) showed adaptive regulation of CAT-2b and $ATB^{0,+}$ gene expression in response to lower levels of CP intake. Reduction from 1.24% to 0.99% total Lys may not be sufficient to cause change in AA transporter gene expression. However, nor did further reduction to 0.62% total Lys (Deficient) affect AA transporter gene expression. In the study by Pérez-Laspiur et al. (2009), adaptive regulation was observed at dietary total Lys level of 0.60 %. On the other hand, the increase in transport efficiency was likely unrelated to an increase in mammary cell number, since mammary DNA concentration remained constant across diets and throughout lactation.

The response of mammary Arg and Lys extraction efficiencies paralleled the lactation performance response, whereby litter growth and piglet ADG were maximized in sows fed the Ideal diet. However, transcript abundance of the dominant milk proteins α-lactalbumin and β-casein remained unchanged across diets at both d 7 and d 17 of lactation, suggesting that changes, if any, in milk protein gene expression occurred either earlier in lactation or at a post-transcriptional level (i.e., protein translation or activity). In fact, we have previously shown that upregulation of Lys transporters and milk protein genes in porcine mammary tissue in response to milk demand takes place between pre-partum and d 5 of lactation (Manjarín et al., 2011). In addition, studies in rat muscle tissue indicated that dietary AA may regulate mRNA translation and protein synthesis via activation of mammalian target of rapamycin pathway (mTOR; O'Connor et al., 2003; Kimball and Jefferson, 2006; Hundal and Taylor, 2009). In this regard, studies in dairy cows suggest a key role of mTOR pathway in overall protein translation regulation in lactation (Hayashi et al., 2007; 2009; Burgos et al., 2010), and gene expression of mTOR pathway-related kinases have been shown in porcine mammary gland (Appendix A).

Therefore, the observed higher Lys transport efficiency and piglet ADG in the present study may be due to an upregulation of both AA transporter and milk protein expression.

Changes in Lys transport efficiency by the mammary gland paralleled changes in BCAA and Lys arterial concentrations. Feeding our ideal AA profile in a reduced-CP diet decreased the arterial BCAA:Lys and increased efficiency of Lys transport by the mammary gland on d7 of lactation. In contrast, on d17 of lactation, both the arterial concentration of BCAA:Lys and the Lys transport efficiency remained unchanged across diets. The physiologic mechanisms behind the arterial change in BCAA:Lys are unclear as dietary SID BCAA:Lys was kept constant across diets (Table 3.2). Sows fed the Ideal diet possibly had higher peripheral uptake of dietary BCAA, leading to a lower arterial BCAA:Lys. Metabolism of BCAA takes place primarily in extrahepatic tissues such as muscle and adipose tissue, as hepatocytes lack the enzyme BCAA aminotransferase involved in the first step of BCAA oxidation (Nelson and Cox, 2008). In fact, sow weight loss was numerically lower for those fed the Ideal diet compared to that of sows fed the Standard and Deficient diets, indicative of less skeletal muscle protein mobilization.

We report here a large mammary uptake of BCAA with increasing dietary CP levels. Increased mammary BCAA transport due to higher levels of protein intake has been previously shown in lactating sows (Guan et al., 2002) and cows (Bequette et al., 1996), with no increase in milk protein yield. As such, Bequette et al. (1996) hypothesized that BCAA in excess taken by the mammary gland may be oxidized in the tissue, likely decreasing the efficiency of dietary AA utilization into milk protein. This extraordinary potential for oxidative catabolism of BCAA by the mammary gland may negatively affect the efficiency of Lys and Arg transport into the mammary cells via competitive events at the AA transporter level. For instance, the AA

transporter $ATB^{0,+}$ mediates intracellular transport of both cationic and neutral AA in epithelial cells (Broër, 2008) and shows higher affinity for BCAA (Sloan and Mager, 1999). As mentioned early, we have shown here and previously (Pérez-Laspiur et al., 2004; 2009) that $ATB^{0,+}$ transcript is remarkably high in porcine mammary tissue. Despite that all diets had a similar SID BCAA:Lys, the arterial BCAA:Lys was considerably higher for both the Deficient and Standard diets compared to the Ideal diet on d 7 of lactation. Therefore, increased arterial BCAA:Lys associated with Deficient and Standard diets may have increased the competitive advantage of BCAA relative to Arg and Lys for uptake via the $ATB^{0,+}$ transporter, consequently decreasing Lys and Arg transport efficiency by the porcine mammary gland. Cationic and neutral AA also share $y^+$LAT2, an AA transporter that is also highly expressed in porcine mammary cells (Manjarín et al., 2011). In contrast to $ATB^{0,+}$, $y^+$LAT2 functions as an obligatory exchanger between cationic and large neutral AA, promoting the efflux of cationic AA from the cells (Broër, 2008). In this regard, high concentrations of Leu and Val were shown to inhibit Lys uptake and increased Lys efflux from rat mammary explants (Shennan et al., 1994; Calvert and Shennan, 1996) and porcine mammary tissue (Guan et al, 2002), respectively. Thus, in addition to the likely preferential uptake of BCAA via $ATB^{0,+}$, an increased arterial BCAA:Lys associated with Deficient and Standard diets may have facilitated Lys and Arg efflux from the mammary cells in exchange for BCAA via $y^+$LAT2 transporter, contributing to decreased Lys and Arg transport efficiency by the porcine mammary gland.

The fact that Arg transport efficiency also increased in response to feeding the Ideal diet on d 7 of lactation suggests its pivotal role in porcine mammary gland. In fact, Guan et al. (2004)

also reported a higher Arg A-V in response to lower dietary CP levels, while Nielsen et al. (2002) showed an increase in Arg mammary uptake associated to larger litter size. Arginine was not considered to limit milk protein synthesis in the sow (NRC, 1998). However, this AA is involved in numerous functions in mammary tissue directly related to milk yield (Kim and Wu, 2009). Arginine is the substrate for synthesis of vasodilator nitric oxide (NO; Wu and Morris 1998), and blood flow to the mammary gland was the main driving variable for increased mammary AA uptake in response to litter size in the study by Nielsen et al. (2002). Thus, it is possible that higher efficiency of Arg transport associated with the Ideal diet enhanced mammary blood flow, improving mammary net nutrient uptake and sow lactation performance in our study. In support of this view, dietary supplementation with arginine enhanced the production of sow's milk and piglet growth (Mateo et al., 2008). Amino acid transporter CAT-1 may offer a unique port of entry for Arg in the mammary tissue. CAT-1 is known to be highly specific for Arg and Lys transport (Sloan and Mager, 1999), and we have recently shown that CAT-1 mRNA is highly abundant in mammary gland (Manjarín et al., 2011) and uniquely localized to mammary endothelial cells (Appendix D). Additionally, Arg is also involved in the synthesis of polyamines, which are regulators of protein synthesis and lactogenesis (Meininger and Wu 2002; Wu and Morris 1998), and of proline, an indispensable AA for the young pig (Ball et al, 1986; Wu et al., 2011).

In summary, feeding a diet with less CP and containing CAA to achieve an "ideal" AA profile increased piglet ADG between d 1 and d 17 of lactation, increased mammary Lys and Arg transport efficiency and decreased plasma concentration of BCAA:Lys in the early stage of lactation, but did not increase mRNA abundance of genes encoding for Lys, Arg and BCAA transporters. These results indicate that CP reduction with CAA inclusion improves the

efficiency of dietary AA utilization for litter growth via mechanisms independent of transcription of genes encoding for milk or AA transporter proteins. We propose that such mechanism may involve competitive inhibition between cationic and BCAA at the mammary cell membrane interface.

**Table 3.1.** Ingredient composition of experimental diets (%, as fed)[1]

| Item | Deficient (9.5% CP) | Ideal (13.5% CP) | Standard (17.5% CP) |
|---|---|---|---|
| Yellow corn | 30.87 | 44.32 | 57.57 |
| Soybean meal | 14.91 | 21.25 | 27.63 |
| Cornstarch | 18.35 | 17.30 | 0 |
| Sucrose | 18.87 | 3.390 | 1.660 |
| Soybean oil | 4.770 | 4.230 | 5.510 |
| Solka floc | 7.210 | 4.470 | 2.560 |
| L-Lysine HCl | 0.169 | 0.243 | 0.316 |
| DL-Methionine | 0.009 | 0.013 | 0.017 |
| L-Threonine | 0.039 | 0.056 | 0.073 |
| L-Tryptophan | 0.002 | 0 | 0 |
| L-Valine | 0.045 | 0.065 | 0.084 |
| L-Leucine | 0.001 | 0 | 0 |
| L-Isoleucine | 0.001 | 0 | 0 |
| Calc Phos, Dical | 1.842 | 1.705 | 1.568 |
| Limestone | 1.010 | 1.055 | 1.099 |
| Vit Premix | 0.600 | 0.600 | 0.600 |
| Trace Min Premix[2] | 0.500 | 0.500 | 0.500 |
| Sow Pack[3] | 0.300 | 0.300 | 0.300 |

[1]Provided the follow vitamins per kg of diet: 4500 IU vitamin A, 458 IU vitamin $D_3$, 55 IU vitamin E, 11 mg vitamin K, 3.66 mg menadione, 0.0275 mg vitamin $B_{12}$, 3.66 mg riboflavin, 14.67 mg D-panthotenic acid, 22 mg niacin, 0.913 mg thiamine, 0.825 mg pyridoxine.

[2]Provided the following minerals per kg of diet: 335 g Ca, 5 g Fe, 5 g Zn, 5 g Cu, 150 μg Se and 75 μg I.

[3]Provided the follows amounts per kg of diet: 2755.7 IU vitamin A, 220.5 mg biotin, 385.8 mg choline, 1.65 mg folic acid.

**Table 3.2.** Calculated and analyzed (in brackets) nutrient composition of experimental diets (as fed)[1]

| Item | Deficient | Ideal | Standard |
|---|---|---|---|
| DM, % | 93.27 | 91.39 | 88.95 |
| ME, kcal/kg | 3320 | 3320 | 3320 |
| CP, % | 9.50 | 13.53 | 17.52 |
| EE, % | 6.00 | 6.00 | 7.76 |
| NDF, % | 11.50 | 10.70 | 10.70 |
| ADF, % | 1.97 | 2.82 | 3.67 |
| Ca, % | 0.80 | 0.80 | 0.80 |
| Available P, % | 0.40 | 0.40 | 0.40 |
| Na, % | 0.21 | 0.21 | 0.21 |
| Cl, % | 0.36 | 0.38 | 0.41 |
| Amino acid, total, % | | | |
| Arg | 0.66 (0.62) | 0.94 (0.96) | 1.23 (1.22) |
| Cys | 0.16 (0.15) | 0.23 (0.22) | 0.30 (0.27) |
| His | 0.27 (0.25) | 0.39 (0.39) | 0.50 (0.49) |
| Ile | 0.48 (0.43) | 0.68 (0.65) | 0.88 (0.83) |
| Leu | 0.88 (0.85) | 1.25 (1.33) | 1.63 (1.67) |
| Lys | 0.66 (0.62) | 0.94 (0.99) | 1.22 (1.24) |
| Met | 0.17 (0.15) | 0.24 (0.23) | 0.31 (0.28) |
| Met + Cys (total sulfur) | 0.33 (0.30) | 0.47 (0.45) | 0.61 (0.55) |
| Phe | 0.53 (0.49) | 0.76 (0.76) | 0.99 (0.96) |
| Phe + Tyr (total aromatic) | 0.88 (0.78) | 1.26 (1.25) | 1.64 (1.61) |
| Thr | 0.43 (0.39) | 0.61 (0.60) | 0.79 (0.74) |
| Trp | 0.12 (0.10) | 0.18 (0.16) | 0.23 (0.22) |
| Tyr | 0.35 (0.29) | 0.50 (0.49) | 0.65 (0.65) |
| Val | 0.58 (0.52) | 0.82 (0.79) | 1.07 (1.01) |
| Amino acid, SID[2] basis, % | | | |
| Arg | 0.61 (0.56) | 0.88 (0.87) | 1.14 (1.10) |
| Cys | 0.14 (0.12) | 0.20 (0.19) | 0.26 (0.23) |
| His | 0.24 (0.22) | 0.35 (0.34) | 0.45 (0.43) |
| Ile | 0.42 (0.37) | 0.60 (0.56) | 0.78 (0.72) |
| Leu | 0.79 (0.77) | 1.13 (1.21) | 1.46 (1.52) |
| Lys | 0.60 (0.50) | 0.85 (0.81) | 1.11 (1.01) |
| Met | 0.15 (0.13) | 0.22 (0.20) | 0.29 (0.25) |
| Met + Cys (total sulfur) | 0.29 (0.26) | 0.42 (0.39) | 0.55 (0.48) |
| Phe | 0.48 (0.43) | 0.68 (0.68) | 0.88 (0.86) |
| Phe + Tyr (total aromatic) | 0.79 (0.69) | 1.13 (1.11) | 1.46 (1.44) |

**Table 3.2 (cont'd)**

| | | | |
|---|---|---|---|
| Thr | 0.37 (0.32) | 0.53 (0.50) | 0.69 (0.61) |
| Trp | 0.11 (0.09) | 0.16 (0.13) | 0.21 (0.18) |
| Tyr | 0.31 (0.25) | 0.45 (0.43) | 0.58 (0.58) |
| Val | 0.51 (0.45) | 0.73 (0.68) | 0.95 (0.88) |
| Amino acid, SID[2] ratio | | | |
| Arg:Lys | 1.02 (1.12) | 1.03 (1.07) | 1.02 (1.08) |
| Cys:Lys | 0.23 (0.24) | 0.24 (0.23) | 0.23 (0.22) |
| His:Lys | 0.40 (0.44) | 0.41 (0.42) | 0.41 (0.42) |
| Ile:Lys | 0.70 (0.74) | 0.70 (0.69) | 0.70 (0.71) |
| Leu:Lys | 1.32 (1.54) | 1.33 (1.49) | 1.32 (1.50) |
| Met:Lys | 0.26 (0.26) | 0.26 (0.24) | 0.26 (0.24) |
| Total sulfur:Lys | 0.49 (0.52) | 0.49 (0.48) | 0.49 (0.47) |
| Phe:Lys | 0.80 (0.86) | 0.79 (0.83) | 0.79 (0.85) |
| Total aromatic:Lys | 1.32 (1.37) | 1.32 (1.37) | 1.32 (1.42) |
| Thr:Lys | 0.62 (0.64) | 0.62 (0.61) | 0.62 (0.60) |
| Trp:Lys | 0.18 (0.18) | 0.19 (0.16) | 0.19 (0.17) |
| Val:Lys | 0.85 (0.90) | 0.86 (0.83) | 0.86 (0.87) |

[1]Analyzed values are shown in parenthesis.

[2] Standardized ileal digestible.

**Table 3.3.** Effect of dietary CP concentration on sow and litter performance and DNA concentration in mammary gland[a]

| Item | Dietary CP, % | | | Diet | |
|---|---|---|---|---|---|
| | 9.5 | 13.5 | 17.5 | L[b] | Q[c] |
| | Deficient | Ideal | Standard | *P*-value | |
| No.[d] | 8 | 6 | 7 | | |
| Intake, kg/d | | | | | |
| Feed | 3.61 ± 0.18 | 3.88 ± 0.21 | 3.85 ± 0.19 | 0.37 | 0.56 |
| Protein | 0.3[x] 0.03 | 0.5[y] ± 0.03 | 0.7[z] ± 0.03 | < 0.001 | 0.69 |
| Sow BW, kg | | | | | |
| d 1 | 228.10 ± 7.70 | 232.40 ± 9.10 | 238.30 ± 8.30 | 0.38 | 0.94 |
| Loss, d 1 to 18 | 19.70 ± 4.50 | 15.20 ± 5.30 | 25.80 ± 4.80 | 0.35 | 0.22 |
| Litter size | | | | | |
| d 1 | 8.00 | 8.00 | 8.00 | | |
| d 18 | 8.00 | 8.00 | 8.00 | | |
| Litter wt, kg | | | | | |
| d 1 | 15.70 ± 0.69 | 15.50 ± 0.72 | 15.75 ± 0.71 | 0.93 | 0.65 |
| Gain, d 1 to 18 | 28.8[x] ± 2.30 | 36.8[y] ± 2.50 | 32.7[y] ± 2.40 | 0.07 | 0.01 |
| Pig ADG, g | | | | | |
| d 1 to 18 | 214.3[x] ± 12.60 | 281.5[y] ± 15.01 | 253.7[y] ± 13.83 | 0.03 | 0.01 |
| DNA, mg/g | | | | | |
| d 1 to 18 | 1.81 ± 0.07 | 1.81 ± 0.08 | 1.93 ± 0.08 | 0.32 | 0.54 |

[a]Data are least square means ± SE

[b]*P*-values for linear (L) contrast.

[c]*P*-values for quadratic (Q) contrast.

[d]Number of sows.

[w,x,y]Within a row, means lacking a common superscript differ ($P < 0.05$)

**Table 3.4.** Effect of dietary CP concentration on AA arterial concentrations (mmol/ L) in lactating sows on d 7 and d 17 of lactation

| | Stage of Lactation | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 7 Dietary CP, % | | | Day 17 Dietary CP, % | | | Day 7 | | Day 17 | |
| Item | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | L[j] | Q[k] | L[j] | Q[k] |
| No.[b] | 5 | 3 | 5 | 5 | 3 | 5 | | | | |
| Arg | 128.2 ± 16.4 | 175.1 ± 22.2 | 173.3 ± 16.8 | 139.9 ± 16.4 | 166.7 ± 22.2 | 189.7 ± 18.4 | 0.09 | 0.37 | 0.07 | 0.94 |
| His | 96.0 ± 6.9 | 67.7 ± 9.3 | 80.8 ± 7.0 | 93.6 ± 6.9 | 82.7 ± 9.3 | 87.3 ± 7.7 | 0.14 | 0.08 | 0.54 | 0.48 |
| Ile | 93.2[ab] ± 7.1 | 79.9[a] ± 9.8 | 112.9[b] ± 7.4 | 95.6[d] ± 7.1 | 88.6[d] ± 9.8 | 126.5[e] ± 7.7 | 0.08 | 0.08 | 0.02 | 0.08 |
| Leu | 124.8[g] ± 6.1 | 107.9[g] ± 8.2 | 171.5[h] ± 6.2 | 126.3[g] ± 6.1 | 122.2[g] ± 8.2 | 178.4[h] ± 6.8 | 0.001 | 0.002 | 0.001 | 0.01 |
| Lys | 169.3 ± 19.9 | 221.8 ± 27.5 | 226.8 ± 20.6 | 168.0[d] ± 19.9 | 213.5[de] ± 27.5 | 255.5[e] ± 21.7 | 0.06 | 0.46 | 0.01 | 0.95 |
| Met | 45.3 ± 4.6 | 43.3 ± 5.7 | 44.3 ± 4.6 | 38.2 ± 4.6 | 43.0 ± 5.7 | 47.2 ± 4.9 | 0.84 | 0.79 | 0.12 | 0.96 |
| Phe | 49.5[d] ± 5.8 | 54.3[d] ± 7.6 | 80.6[e] ± 5.9 | 52.1[g] ± 5.8 | 66.7[gh] ± 7.6 | 87.0[h] ± 6.2 | 0.001 | 0.20 | 0.001 | 0.76 |
| Thr | 108.4[a] ± 14.3 | 107.8[ab] ± 18.4 | 153.8[b] ± 14.5 | 95.3[g] ± 14.3 | 108.0[gh] ± 18.4 | 184.4[i] ± 15.5 | 0.02 | 0.24 | 0.001 | 0.13 |
| Trp | 54.4 ± 6.4 | 70.6[c] ± 9.0 | 73.6 ± 6.8 | 51.1 ± 7.3 | 78.7[d] ± 10.0 | 77.7 ± 8.0 | 0.30 | 0.94 | 0.04 | 0.25 |
| Val | 179.7[d] ± 18.3 | 192.6[d] ± 23.0 | 260.5[e] ± 18.6 | 178.4[d] ± 18.3 | 200.6[d] ± 23.0 | 278.9[e] ± 19.6 | 0.01 | 0.24 | 0.001 | 0.23 |

Within a row, means lacking a common superscript differ at [a,b,c]$P < 0.1$, [d,e,f]$P < 0.05$, and [g,h,i]$P < 0.01$.

[j]Linear.

[k]Quadratic.

**Table 3.5.** Effect of dietary CP concentration on AA arterio-venous differences (mmol / L) in porcine mammary gland at d 7 and d 17 of lactation

| | Stage of Lactation | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 7 Dietary CP, % | | | Day 17 Dietary CP, % | | | Day 7 | | Day 17 | |
| Item | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | L[k] | Q[k] | L[j] | Q[k] |
| No.[b] | 5 | 3 | 5 | 5 | 3 | 5 | | | | |
| Arg | 16.4[g] ± 5.3 | 58.4[h] ± 8.8 | 20.5[g] ± 5.4 | 26.4 ± 5.3 | 40.4 ± 7.2 | 28.9 ± 6.0 | 0.61 | 0.003 | 0.77 | 0.16 |
| His | 10.8 ± 4.2 | 10.6 ± 10.0 | 14.5 ± 4.7 | 20.5 ± 4.2 | 23.2 ± 6.7 | 14.8 ± 4.7 | 0.57 | 0.85 | 0.40 | 0.48 |
| Ile | 15.1 ± 4.3 | 21.9 ± 5.9 | 28.9 ± 4.5 | 24.8 ± 4.3 | 30.3 ± 5.9 | 33.3 ± 4.8 | 0.05 | 0.99 | 0.22 | 0.87 |
| Leu | 24.3 ± 5.1 | 28.4 ± 7.0 | 35.7 ± 5.2 | 26.8 ± 5.1 | 28.7 ± 7.0 | 44.7 ± 5.6 | 0.15 | 0.85 | 0.04 | 0.41 |
| Lys | 16.9[a] ± 4.1 | 35.4[b] ± 5.5 | 20.1[ab] ± 4.2 | 19.0 ± 4.6 | 28.0 ± 5.5 | 33.5 ± 5.3 | 0.59 | 0.03 | 0.08 | 0.80 |
| Met | 8.6 ± 2.6 | 8.9 ± 3.5 | 7.9 ± 3.0 | 9.2 ± 2.6 | 14.1 ± 3.5 | 10.4 ± 2.8 | 0.87 | 0.88 | 0.76 | 0.32 |
| Phe | 13.3 ± 2.7 | 12.4 ± 3.7 | 16.1 ± 2.8 | 13.9 ± 2.7 | 17.6 ± 3.7 | 19.6 ± 3.0 | 0.49 | 0.61 | 0.18 | 0.85 |
| Thr | 22.5 ± 3.9 | 16.2 ± 6.7 | 23.9 ± 4.3 | 22.5 ± 3.9 | 17.3 ± 5.2 | 33.5 ± 4.3 | 0.77 | 0.34 | 0.06 | 0.09 |
| Trp | 9.7 ± 4.3 | 5.05 ± 5.8 | 14.9 ± 5.9 | 6.3 ± 4.3 | 16.1 ± 5.8 | 17.1 ± 4.8 | 0.45 | 0.30 | 0.18 | 0.51 |
| Val | 25.9 ± 6.1 | 27.0 ± 8.2 | 44.3 ± 7.1 | 27.9 ± 6.1 | 39.6 ± 8.2 | 47.2 ± 6.8 | 0.08 | 0.43 | 0.07 | 0.83 |

Within a row, means lacking a common superscript differ at [a,b,c]$P < 0.1$, [d,e,f]$P < 0.05$, and [g,h,i]$P < 0.01$.

[j]Linear.

[k]Quadratic.

**Table 3.6.** Effect of dietary CP concentration on AA transport efficiency (A-V/A × 100) in porcine mammary gland at d 7 and d 17 of lactation

| | Stage of Lactation | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 7 Dietary CP, % | | | Day 17 Dietary CP, % | | | Day 7 | | Day 17 | |
| Item | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | L[j] | Q[k] | L[j] | Q[k] |
| No.[b] | 5 | 3 | 5 | 5 | 3 | 5 | | | | |
| Arg | 12.9[a] ± 3.2 | 28.8[b] ± 5.3 | 12.9[a] ± 3.2 | 19.3 ± 3.2 | 24.4 ± 4.3 | 15.8 ± 3.6 | 0.99 | 0.03 | 0.47 | 0.21 |
| His | 11.8 ± 4.6 | 15.3 ± 10.7 | 16.7 ± 5.1 | 22.1 ± 4.6 | 28.8 ± 7.3 | 16.4 ± 5.1 | 0.49 | 0.93 | 0.43 | 0.28 |
| Ile | 16.0 ± 3.4 | 27.1 ± 4.6 | 25.2 ± 3.4 | 26.0 ± 3.4 | 34.5 ± 4.6 | 25.7 ± 3.7 | 0.08 | 0.24 | 0.96 | 0.13 |
| Leu | 19.4 ± 3.6 | 26.2 ± 4.9 | 20.3 ± 3.7 | 21.3 ± 3.6 | 25.0 ± 4.9 | 24.7 ± 4.0 | 0.85 | 0.30 | 0.54 | 0.74 |
| Lys | 9.8 ± 2.1 | 16.7 ± 2.8 | 8.6 ± 2.2 | 12.0 ± 2.4 | 14.0 ± 2.8 | 14.4 ± 2.7 | 0.69 | 0.05 | 0.54 | 0.81 |
| Met | 17.6 ± 5.1 | 21.0 ± 7.0 | 16.3 ± 5.9 | 24.8 ± 5.1 | 32.3 ± 7.0 | 21.0 ± 5.7 | 0.87 | 0.64 | 0.63 | 0.28 |
| Phe | 27.3 ± 3.8 | 22.5 ± 5.2 | 19.4 ± 3.9 | 28.0 ± 3.8 | 24.8 ± 5.2 | 22.3 ± 4.3 | 0.18 | 0.90 | 0.34 | 0.95 |
| Thr | 21.1 ± 3.1 | 16.0 ± 6.2 | 16.7 ± 3.5 | 23.7 ± 3.1 | 17.0 ± 4.3 | 18.6 ± 3.5 | 0.38 | 0.69 | 0.31 | 0.43 |
| Trp | 16.1 ± 5.9 | 8.6 ± 7.9 | 18.3 ± 7.8 | 13.4 ± 5.9 | 21.0 ± 7.9 | 19.9 ± 6.6 | 0.80 | 0.35 | 0.50 | 0.61 |
| Val | 14.4 ± 2.7 | 14.1 ± 3.6 | 16.8 ± 3.1 | 16.3 ± 2.7 | 19.3 ± 3.6 | 16.3 ± 3.0 | 0.57 | 0.74 | 0.99 | 0.49 |

Within a row, means lacking a common superscript differ at [a,b,c]$P < 0.1$, [d,e,f]$P < 0.05$, and [g,h,i]$P < 0.01$.

[j]Linear.

[k]Quadratic.

**Table 3.7.** Effect of dietary CP concentration on arterial AA ratios in porcine mammary gland at d 7 and d 17 of lactation.

| | Stage of Lactation | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 7 Dietary CP, % | | | Day 17 Dietary CP, % | | | Day 7 | | Day 17 | |
| AA:Lys | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | L[j] | Q[k] | L[j] | Q[k] |
| BCAA:Lys | 0.78 ± 0.05 | 0.61[d] ± 0.06 | 0.9[e] ± 0.05 | 0.79 ± 0.05 | 0.68 ± 0.06 | 0.81 ± 0.05 | 0.12 | 0.01 | 0.78 | 0.14 |
| Ile:Lys | 0.55[a] ± 0.04 | 0.39[b] ± 0.06 | 0.55[a] ± 0.05 | 0.58 ± 0.04 | 0.44 ± 0.06 | 0.52 ± 0.05 | 0.90 | 0.03 | 0.37 | 0.12 |
| Leu:Lys | 0.74[a] ± 0.05 | 0.53[bd] ± 0.07 | 0.84[e] ± 0.05 | 0.75 ± 0.05 | 0.60 ± 0.07 | 0.75 ± 0.05 | 0.16 | 0.005 | 0.9 | 0.06 |
| Val:Lys | 1.04[a] ± 0.08 | 0.93[a] ± 0.11 | 1.33[b] ± 0.10 | 1.04 ± 0.08 | 1.01 ± 0.11 | 1.18 ± 0.10 | 0.05 | 0.09 | 0.30 | 0.50 |
| Arg:Lys | 0.76 ± 0.07 | 0.79 ± 0.10 | 0.76 ± 0.08 | 0.85 ± 0.07 | 0.80 ± 0.10 | 0.78 ± 0.08 | 0.94 | 0.82 | 0.52 | 0.89 |

Within a row, means lacking a common superscript differ at [a,b,c]$P < 0.1$, [d,e,f]$P < 0.05$, and [g,h,i]$P < 0.01$.

[j]Linear.

[k]Quadratic.

**Figure 3.1.** Average expression stability values (M) of potential reference genes *API5*, *MRPL39*, *VAPB*, *MTG1*, *RPS21*, *ACTB* and *GAPDH* plotted from least stable (left) to most stable (right).

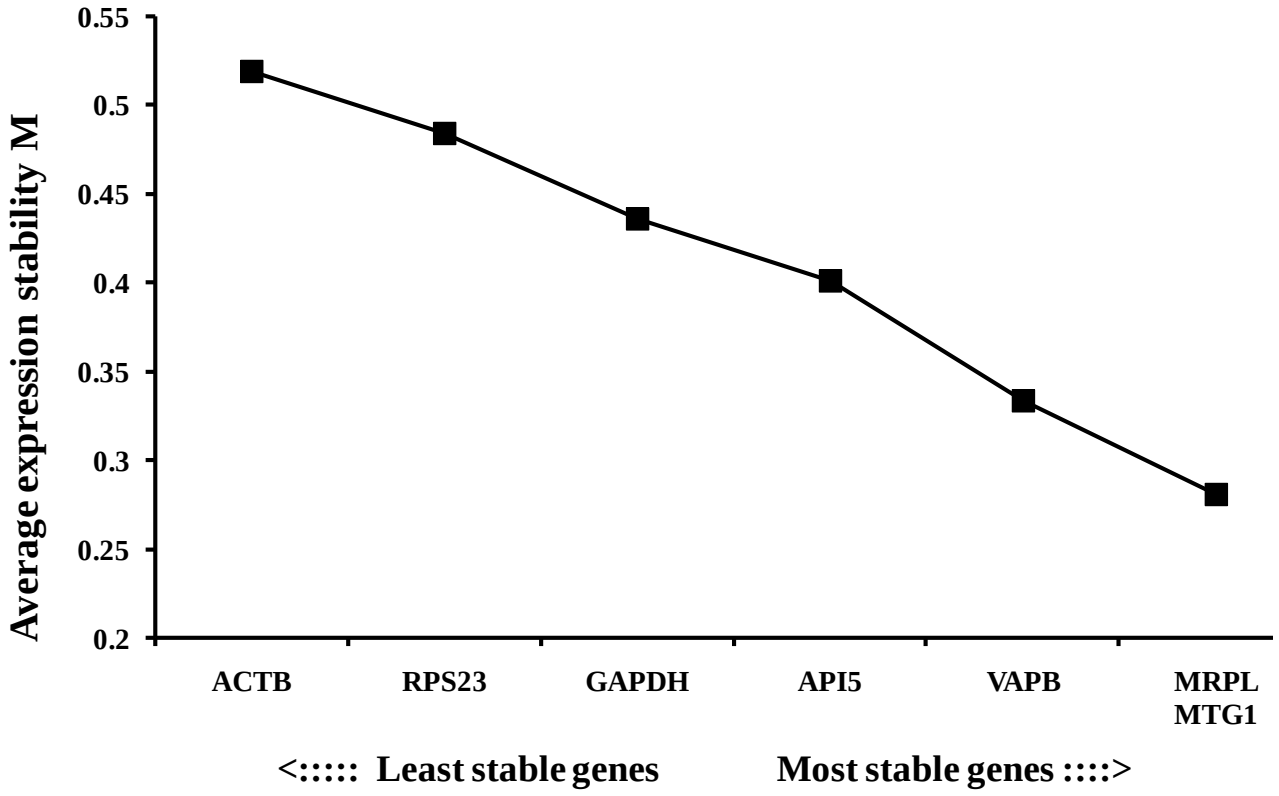


**Figure 3.2.** Pairwise variation ($V_n/V_{n+1}$) between the normalization factors $NF_n$ and $NF_{n+1}$ to determine the optimal number of reference genes for normalization.

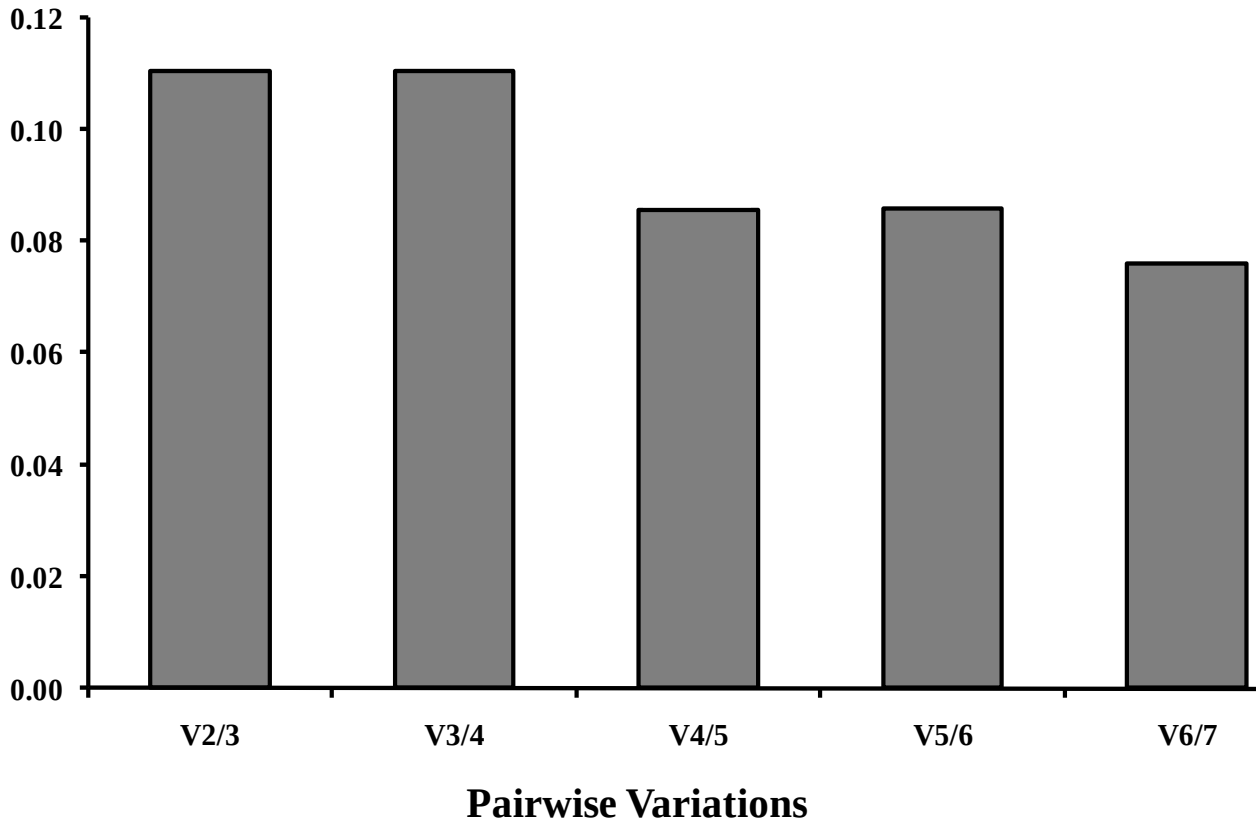


**Figure 3.3.** Messenger RNA abundance of gene encoding for amino acid transporter CAT-1 in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 14 - ΔCt ± SE (n = 4).

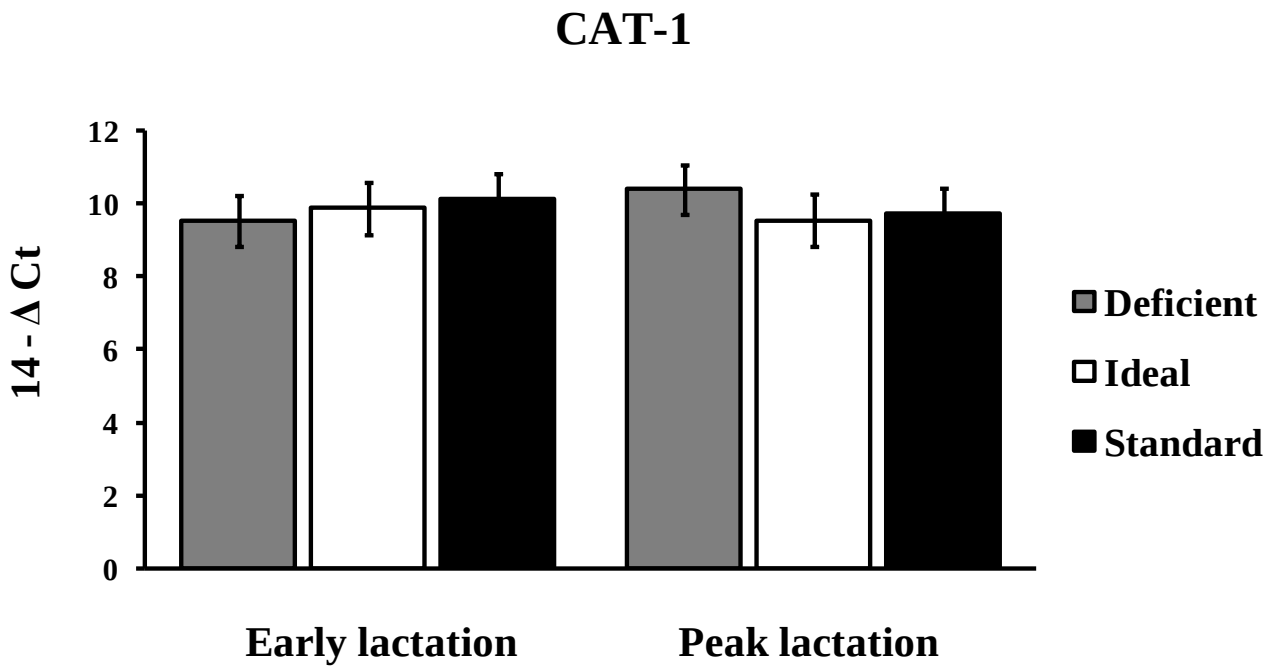


**Figure 3.4.** Messenger RNA abundance of gene encoding for amino acid transporter CAT-2b in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 14 - ΔCt ± SE (n = 4).

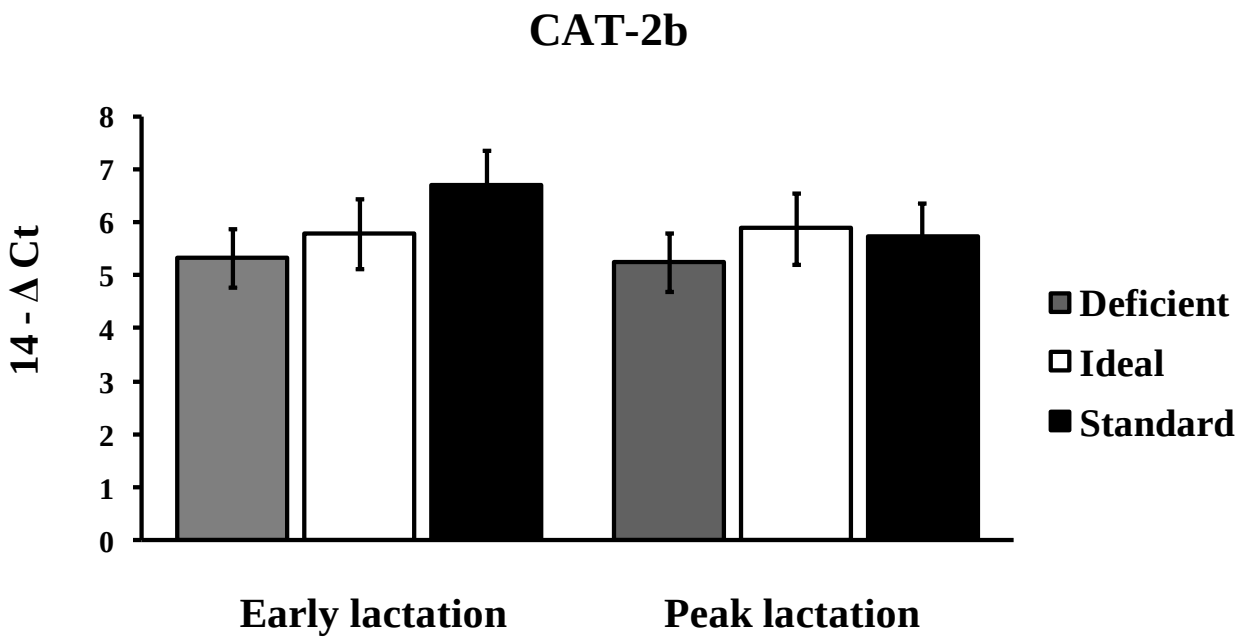


**Figure 3.5.** Messenger RNA abundance of gene encoding for amino acid transporter $b^{0,+}AT$ in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 14 - ΔCt ± SE (n = 4).

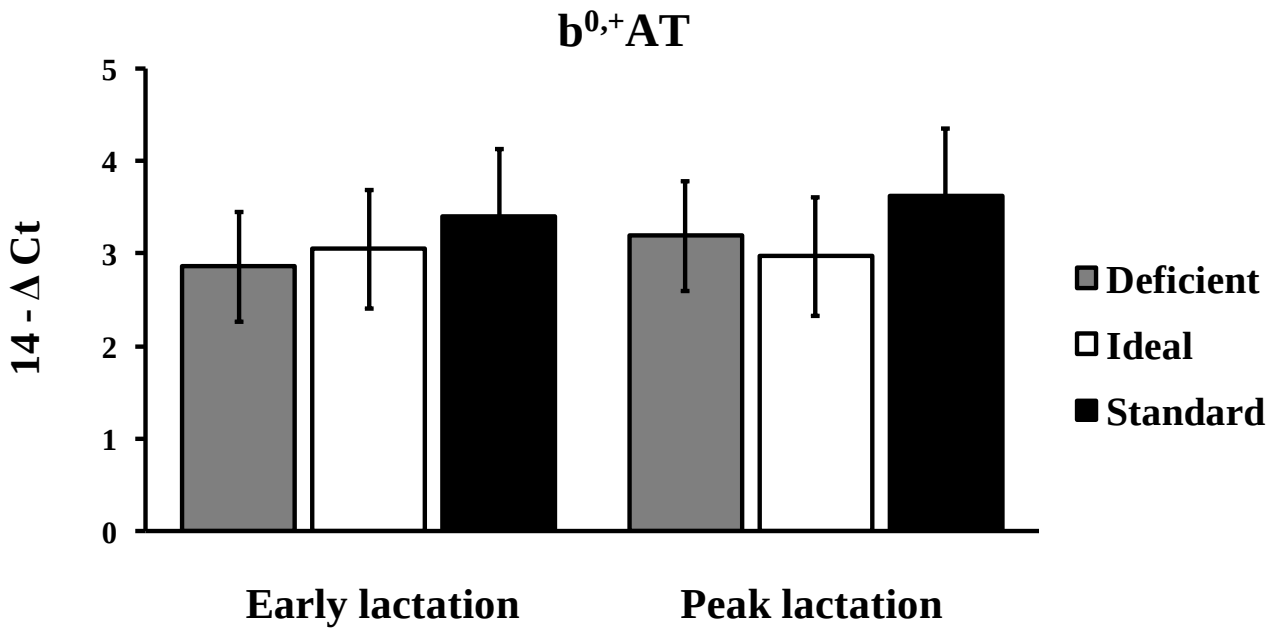


**Figure 3.6.** Messenger RNA abundance of gene encoding for amino acid transporter $ATB^{0,+}$ in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 13 - ΔCt ± SE (n = 4).

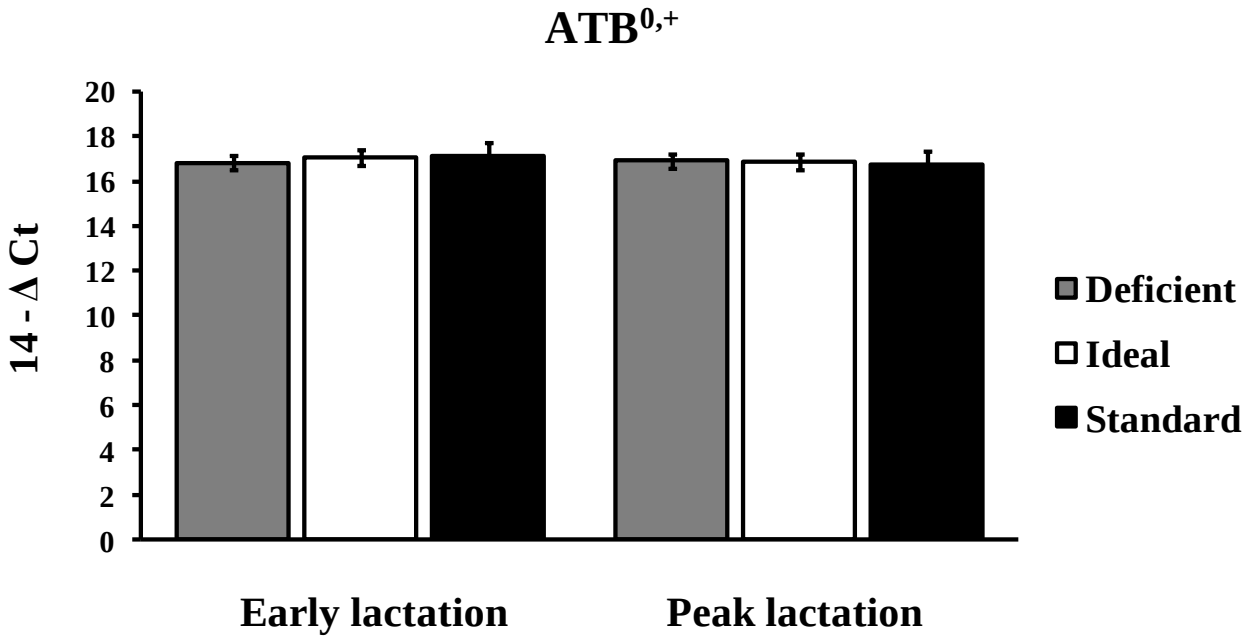


**Figure 3.7.** Messenger RNA abundance of gene encoding for amino acid transporter $y^+$LAT2 in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 13 - ΔCt ± SE (n = 4).

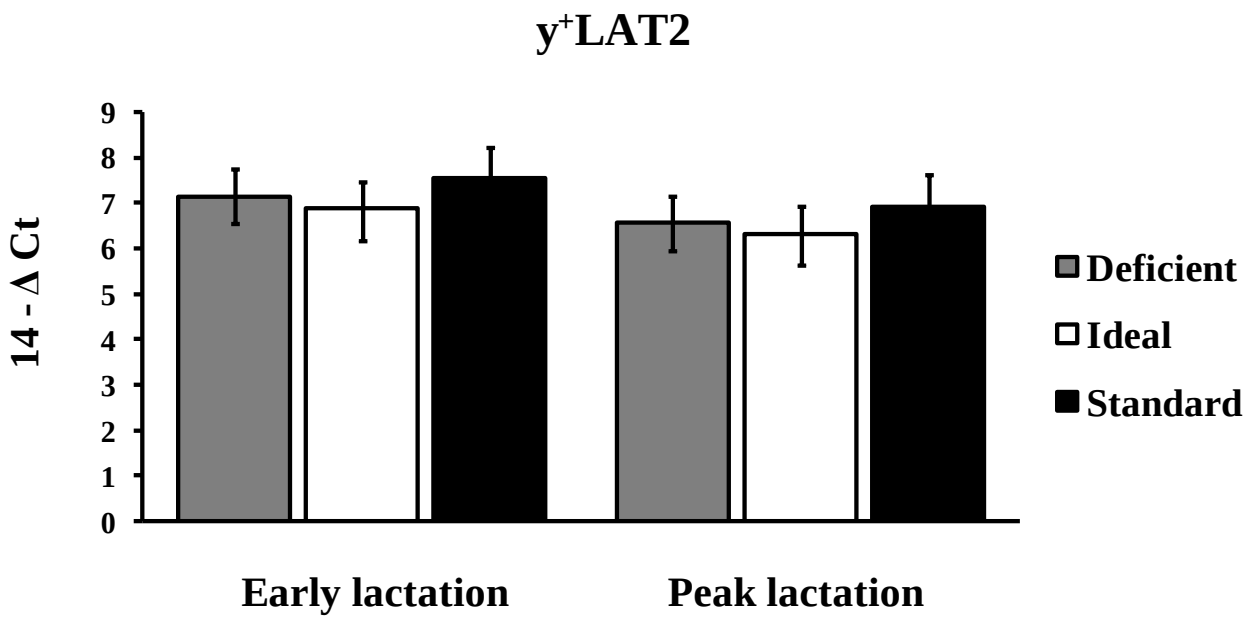


**Figure 3.8.** Messenger RNA abundance of gene encoding for milk protein gene β-casein in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 13 - ΔCt ± SE (n = 4).

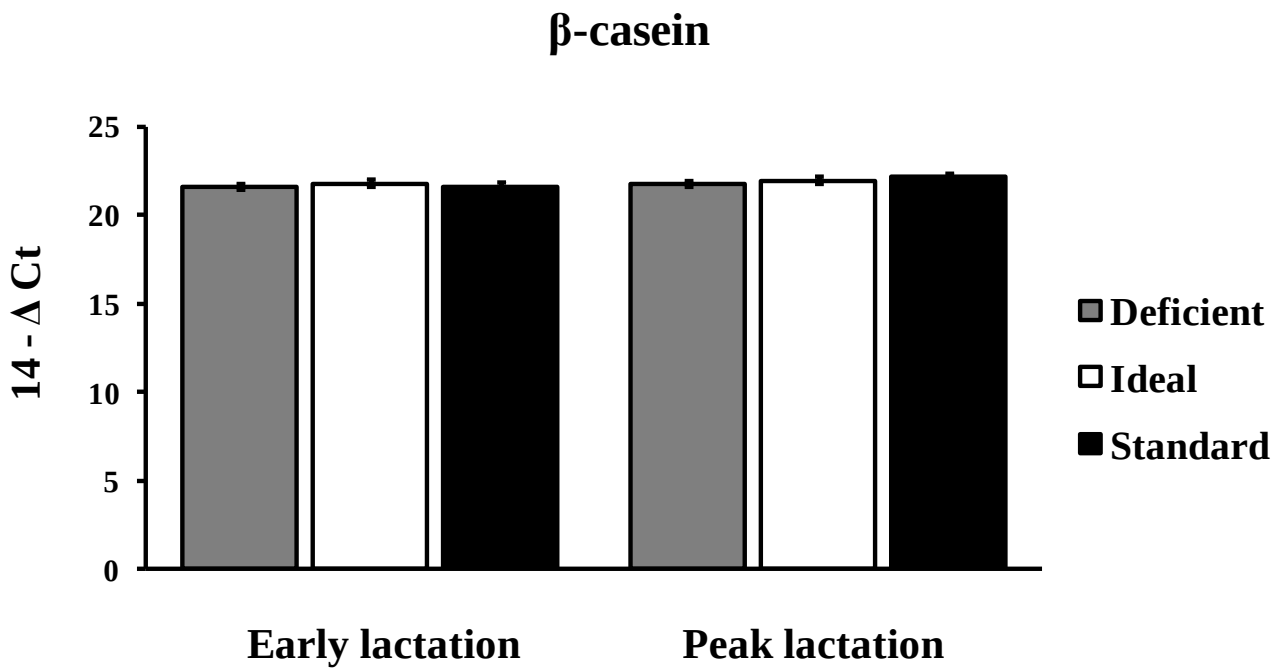
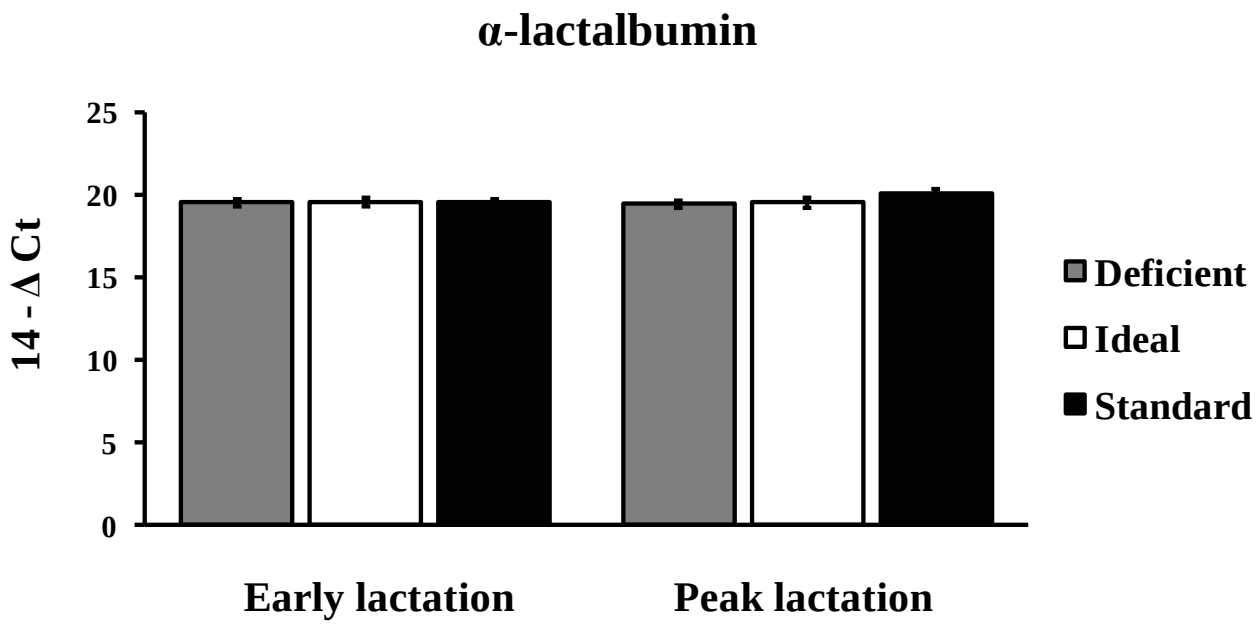


**Figure 3.9.** Messenger RNA abundance of gene encoding for milk protein gene α-lactalbumin in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents 13 - ΔCt ± SE (n = 4).



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# CHAPTER 4

## SUMMARY AND CONCLUSIONS

The first objective on the thesis outlined in Chapter 1 was to select reference genes for gene expression analysis of Lys transporters by RT-qPCR. As a result, a novel method to assess invariance of reference gene expression across different stages of mammary physiological activity was developed, and the reference genes *API5*, *GAPDH* and *MRPL39* were identified in porcine mammary tissue. The second objective of the thesis outlined in Chapter 2 was to measure the expression of genes encoding for mammary Lys transporters across different stages of mammary physiological activity, and to relate their expression to that of genes encoding for two of the most abundant milk proteins. Results showed the presence of transcripts for genes *SLC7A9*, *SLC7A7*, *SLC7A6*, *SLC6A14*, *SLC7A1* and *SLC7A2*, encoding for Lys transporters $b^{0,+}$AT, $y^{+}$LAT1, $y^{+}$LAT2, $ATB^{0,+}$, CAT-1 and CAT-2b, respectively, and for *LALBA* and *CSN2*, encoding for milk proteins α-lactalbumin and β-casein, respectively. In addition, expression of Lys transporter genes *SLC7A6*, *SLC6A14*, *SLC7A1* was correlated to *CSN2* and *LALBA*, and was maximized at early and peak of lactation. As a consequence, it was hypothesized that genes encoding for these Lys transporters would be good candidates for assessing the effect of an optimal AA profile in a reduced protein diet on the efficiency of transport of Lys across the mammary gland. Feeding a diet with 20% less CP and containing crystalline AA to achieve an optimum AA profile increased efficiency of both Lys transport across the mammary gland and apparent AA utilization for piglet growth, but did not increase mRNA abundance of genes encoding for Lys transporters or milk proteins.

The higher efficiency of mammary Lys utilization induced by the diet containing an optimum AA profile was linked to an increased Lys to BCAA arterial plasma concentration ratio, suggesting a competitive transport inhibition between these AA at the cell membrane interface. Amino acid transporters $ATB^{0,+}$ and $y^+LAT2$, both highly expressed in mammary gland, share transport of BCAA and Lys, but show higher affinity for BCAA than for lysine (Bröer, 2008). As such, an increase in Lys:BCAA may have decreased the ability of BCAA to compete against Lys for these transporters, increasing Lys uptake by the mammary cells. On the other hand, while Arg is not limiting for milk synthesis (Trottier et al, 1997), its transport across the mammary gland was the highest among all AA. In muscle, liver and brain, Arg is transported into endothelial cells of the blood vessels, where it is the substrate for synthesis of nitric oxide (Mann et al., 2003). Nitric oxide dilates blood vessels which increasese blood supply, and hence AA and other nutrients to these organs. Transport of arginine in muscle, liver and brain is mediated by AA transporter CAT-1, localized in the basolateral membrane of the endothelial cells (Closs et al, 2004; Mann et al., 2003). Results from this thesis showed that CAT-1 is localized also in endothelial cells of the porcine mammary gland, likely contributing to transport of Arg for nitric oxide synthesis.

This research demonstrates, for the first time, that lactation performance response to an optimum dietary AA balance is affected by the level of dietary protein. In this study, an optimum AA balance fed in conjunction with either excesses or deficiencies of dietary AA reduce the efficiency of mammary Lys transport. Mammary Lys extraction and litter growth rate was maximized when an optimal AA profile was fed with 4 % reduction in crude protein. Results presented in this dissertation are suggestive that changes in AA transport efficiency across mammary gland are not related to changes at gene expression level of AA transporters involved in Lys transport. This regulation may occur in part via interaction between cationic and branched-chain AA for transport across the basolateral membrane of the mammary

epithelial cell, or via an Arg-mediated increase in blood flow to the gland. Future studies investigating the regulation of AA transport efficiency in porcine mammary gland under different dietary ratios of cationic and BCAA are needed to clarify whether an interaction exists between these AA at the mammary cell membrane interface. In addition, assessment of the relationship between dietary digestible and post hepatic AA ratio is critically needed to improve the formulation of an optimal digestible AA profile for lactating sows.

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**APPENDICES**

# APPENDIX A

## TRANSCRIPT ABUNDANCE OF HORMONE RECEPTORS, GLUCOSE TRANSPORTERS, mTOR PATHWAY KINASES, IGF-1 AND MILK PROTEIN-ENCODING GENES IN MAMMARY TISSUE OF PRE-PARTURIENT, LACTATING AND POST-WEANING SOWS

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**Abstract**

The objective of this study was to test the hypothesis that transcript abundance of hormone receptors, glucose transporters, mTOR pathway related kinases, IGF-1 and milk protein-encoding genes increase in the porcine mammary gland in response to higher lactation demand. Genes included those encoding for the receptors of growth hormone (*GHR*), insulin (*INSR*), glucocorticoid (*GCR*) and prolactin (*PRLR*), for the glucose transporters GLUT1 (*SLC2A1*) and GLUT4 (*SLC2A4*), insulin-like growth factor 1 (*IGF-1*), mTOR (*FRAP1*) and p70S6 kinases (*RPS6KB1*), and the milk proteins α-lactalbumin (*LALBA*) and β-casein (*CSN2*). Mammary tissue was biopsied from 4 sows on d 110 of gestation (pre-partum), d 5 (early) and d 17 (peak) of lactation, and d 5 after weaning (post-weaning), and gene expression quantified by reverse transcription quantitative PCR (RT-qPCR). Compared to pre-partum, mRNA abundance of *GCR, SLC2A1*, *CSN2* and *LALBA* increased ($P < 0.001$), *GHR* tended to increase ($P = 0.06$) and *PRLR* decreased ($P < 0.001$) at early lactation. Compared to early lactation, mRNA abundance of *PRLR* increased ($P < 0.001$) and *GHR* decreased ($P < 0.01$) at peak lactation. Expression of *INSR* and *FRAP1* did not differ when comparing either pre-partum or peak lactation to early lactation. Compared to peak of lactation, mRNA abundance of *PRLR, CSN2* and *LALBA* decreased ($P < 0.001$), *INSR* did not change, and *IGF-1, GHR* and *SLC2A4* increased ($P < 0.001$) at post-weaning.

Across stages of mammary physiological activity, *GCR* and *SLC2A1* mRNA abundance was positively correlated to RNA:DNA (r = 0.81 and $P < 0.001$; r = 0.82 and $P < 0.001$, respectively), and to *CSN2* (r = 0.85 and $P < 0.001$; r = 0.80 and $P < 0.001$, respectively) and *LALBA* mRNA levels (r = 0.79 and $P = 0.002$; r = 0.76 and $P < 0.01$, respectively). In conclusion, *GCR*, *PRLR*, *SLC2A1* and *IGF-1* changed in porcine mammary gland from the pre-partum to peak lactation periods and thus higher milk demand, and therefore may be involved in regulation of milk synthesis in sows.

**Introduction**

Milk yield in sows is a critical factor for economic success of swine operations, as it is the main determinant of litter growth. Milk yield is dependent on lactose and protein synthetic rates, determined by intracellular glucose and AA availability. In cows, Zhao and Keating (2007) showed glucose to be channeled across mammary cells by glucose transporters in cows, whereas AA transporters mediate mammary gland AA uptake in cows and sows (Shennan and Peaker, 2000). We have previously shown that gene expression of the dominant milk proteins β-casein and α-lactalbumin is correlated to that of genes encoding for Lys AA transporters in porcine mammary gland (Manjarín et al., 2011). However, it is unknown whether expression of milk proteins is also related to expression of genes encoding for lactopoietic hormones and glucose transporters. In rats and cows, prolactin (Plaut et al., 1989), glucocorticoids (Casey and Plaut, 2007), GH (Johnson et al., 2010), insulin (Proser et al., 1987) and IGF-1 (Burgos et al., 2010) bind to mammary receptors and regulate transcription and translation of milk protein genes. Additionally, evidence in cows suggests a key role of mTOR pathway in overall milk protein translation regulation in lactation (Burgos et al., 2010). However, the relation between gene expression of lactopoietic hormone receptors, mTOR kinases, IGF-1 and glucose transporters, with that of genes encoding for β-

casein and α-lactalbumin, have not been determined in porcine mammary tissue. Such information would provide novel and critical insight on the role of glucose transporters and lactopoietic hormone receptors on milk protein synthesis regulation. Based on aforementioned notions, we hypothesized that genes encoding for GH, insulin, prolactin and glucocorticoid receptors, GLUT1 and GLUT4 glucose transporters, IGF-1, mTOR and p70S6 kinase are expressed in porcine mammary tissue, and positively correlated to mammary biosynthetic activity, and to β-casein and α-lactalbumin gene expression.

## Material and Methods

### *Animals and Tissue Collection*

Studies were performed with the approval of the Institutional Animal Care and Use Committee at Michigan State University (AUF#10/08-162-00). Four sows (all parity 5, Landrace × Yorkshire) selected on d 107 of pregnancy were moved to farrowing crates in a room maintained at $20^0$C and fed 3 kg/d divided into two meals. The diet consisted of a corn-soybean meal-based diet meeting requirement for all nutrients for lactating sows nursing 10 piglets with a predicted average daily gain of 250 g/d (NRC, 1998; Table 1). The day after farrowing was considered d 1 of lactation and sows were fed 1 kg twice a day (0800 and 1600). On d 2 and 3 of lactation, sows were fed 3 and 4 kg, respectively, in two equal meals. For the remainder of the study, sows were fed a maximum of 5.5 kg/d to ensure equal dry matter intake among all sows. Sow feed intake was recorded daily throughout lactation and fresh water was freely available at all times. Litters were equalized to 10 piglets within 48 h after farrowing and litter weight was recorded on d 2 and 17 of lactation. Piglets were weaned on d 21.

Mammary parenchymal tissue was biopsied from the first and second thoracic glands (Kirkwood et al., 2007) in the morning following a 12-h overnight feed deprivation on d 110 of gestation (pre-partum), d 5 (early) and d 17 (peak) of lactation, and d 5 after weaning (post-weaning). For mammary tissue sampling, piglets were isolated in an adjacent pen equipped with a heat lamp. Immediately following the biopsy, mammary tissue was flash-frozen and kept in liquid $N_2$ until later stored at $-80^0$C. Three hours after biopsy, piglets were returned to sows and allowed to nurse.

***Total RNA and DNA quantification.***

To assess mammary biosynthetic activity, DNA and RNA were extracted from mammary tissue using cold perchloric acid (Sigma-Aldrich, St Louis, MO) following the procedure described by Labarca and Paigen (1980) and Capuco et al. (2001), respectively. DNA concentration was quantified using bisbenzimide (Hoechst 33258, Sigma-Aldrich) and a Bio-Tek FL600 plate reader with 360/460 nm filter set (Bio-Tek Instruments, Inc., Winooski, VT), while RNA concentration was determined by spectrophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, DE). Both DNA and RNA concentrations are reported as $\mu g \times mg^{-1}$ mammary tissue.

***Gene Expression Analysis***

Primer sequences for reference and target genes are presented in Tables A.1 and A.2, respectively. In order to facilitate the readership throughout the remainder of the manuscript, each target gene is referred to as the common name of the protein that it encodes for; hence *SLC2A1* will be referred to as GLUT1, *SLC2A4* as GLUT4, *NR3C1* as glucocorticoid receptor, *PRLR* as prolactin receptor, *INSR* as insulin receptor, *GHR* as GH receptor, *FRAP1* as mTOR kinase, *RPS6KB1* as p70S6 kinase, *CSN2* as β-casein and *LALBA* as α-lactalbumin.

RNA extraction and cDNA synthesis, primer design and optimization, reference gene selection and RT-qPCR assay and normalization methods are as described in Chapter 1.

***Statistical Analysis***

Changes in ΔCt for each target gene and mammary tissue RNA and DNA concentration in response to physiological phases of mammary activity were assessed using a linear mixed model procedure (PROC MIXED) of SAS (SAS Institute Inc., Cary, North Carolina) that included the fixed effect of time and the random effect of sow (Equation A.1):

$$Y_{ij} = \mu + \alpha_i + b_j + e_{ij} \quad [A.1]$$

where $Y_{ij}$ is the response variable for the $j^{th}$ sow within the $i^{th}$ stage of lactation, $\mu$ is the overall mean, $\alpha_i$ is the fixed effect of the $i^{th}$ stage of lactation, $b_j$ is the random effect of the $j^{th}$ sow, and $e_{ij}$ is the experimental error. In experiment 1, ΔCt for each target gene were compared between early lactation and pre-partum, peak lactation and early lactation and post-weaning and peak lactation. Multiple comparisons accounted for with Bonferroni adjustment. Gene expression results reported as ΔCt values may often be confusing, as increasing ΔCt reflect decreasing mRNA abundance. Thus, subtracting ΔCt values from a constant value prior to or after statistical analysis allows for an easier interpretation of the data, i.e., increasing ΔCt reflects increasing mRNA abundance, without altering the *P*-value or the standard error. To further simplify the interpretation of results, this constant was selected to be an entire number higher than any ΔCt values among all genes, so that the values presented in the figures are all positive. Thus, mRNA abundance of individual genes is reported as 14 - ΔCt ± SEM. Differences between physiological stages of mammary activity were considered at $P \leq 0.05$.

To investigate the relationship between mRNA abundance of candidate genes, mammary biosynthetic activity and milk protein genes, correlations between mRNA abundance of genes encoding for hormone receptors, IGF-1, glucose transporters GLUT1 and GLUT4, and mTOR and p70S6 kinases, RNA:DNA and mRNA abundance of genes encoding for β-casein and α-lactalbumin were determined using the PROC CORR procedure of SAS. Likewise, correlation between genes encoding for insulin receptor and glucose transporters, and between GH receptor, IGF-1, and mTOR kinases, were also assessed. Pearson's correlation coefficient (r) was considered significant at $P \leq 0.05$.

**Results**

Lactation performance between d 2 and 17 post partum was uniform among sows, with piglet ADG of 257.8 ± 9 g and daily sow feed intake of 4.7 ± 0.32 kg. Compared to pre-partum, RNA concentration and RNA:DNA in porcine mammary gland increased ($P = 0.02$ and $P = 0.01$, respectively) at early lactation; compared to early lactation, RNA concentration remained unchanged and RNA:DNA increased at peak lactation ($P > 0.1$ and $P < 0.02$, respectively). Compared to peak of lactation, RNA concentration and RNA:DNA decreased post-weaning ($P < 0.0001$). Concentration of DNA in mammary gland remained unchanged over pre-partum to peak lactation period ($P > 0.1$), and decreased post-weaning compared to peak lactation ($P < 0.05$; Table A.3).

GeNorm analysis showed that *MTG1*, *MRPL39* and *VAPB* had the lower average expression stability parameter (M), and therefore were selected as the most stable set in porcine mammary gland (Figure 1.1). Analysis of the pairwise variation between sequential normalization factors showed that the optimal number of reference genes was 3, as their pairwise variation was below the 0.15 cut-off value proposed by Vandesompele et al. (2002),

while the inclusion of a fourth gene increased (V2/3 = 0.11 and V3/4 = 0.12) the pairwise variation (Figure 1.2).

Compared to pre-partum, glucocorticoid receptor and GLUT1 mRNA abundance increased ($P < 0.001$) at early lactation, and then remained unchanged over early lactation to post-weaning ($P > 0.1$; Figure A.1 and A.4). Compared to pre-partum, prolactin receptor mRNA abundance decreased at early lactation ($P < 0.001$) and increased at peak compared to early lactation ($P < 0.001$). Compared to peak of lactation, prolactin receptor mRNA abundance was lower post-weaning ($P < 0.0001$; Figure A.1). Compared to pre-partum, GH receptor mRNA abundance tended to increase at early lactation ($P = 0.06$) and decreased at peak compared to early lactation ($P < 0.01$). Compared to peak of lactation, GH receptor mRNA abundance was higher post-weaning ($P < 0.05$; Figure A.2). Messenger RNA abundance of GLUT4, insulin receptor, IGF-1, p70S6 and mTOR kinases remained unchanged over pre-partum to peak lactation periods ($P > 0.1$). Compared to peak of lactation, post-weaning insulin receptor, p70S6 and mTOR kinases mRNA abundance did not differ, whereas IGF-1 and GLUT4 increased ($P < 0.01$) (Figures A.2, A.3 and A.4).

Over the pre-partum to peak lactation period, mRNA abundance of glucocorticoid receptor and GLUT1 was positively correlated to RNA:DNA (r = 0.81 and $P < 0.001$; r = 0.82 and $P < 0.001$, respectively), and to β-casein (r = 0.90 and $P < 0.001$; Figure A5) and α-lactalbumin mRNA levels (r = 0.80 and $P = 0.001$; r = 0.93 and $P < 0.001$, respectively). Messenger RNA abundance of prolactin, insulin and GH receptors, IGF-1, mTOR and p70S6 kinases was not correlated with either RNA:DNA ratio or expression of milk proteins ($P > 0.1$). Growth hormone receptor mRNA abundance was not correlated to that of IGF-1 ($P > 0.1$), and neither GH receptor nor IGF-1 mRNA abundance were correlated to that of mTOR

and p70S6 kinases ($P > 0.1$). Messenger RNA abundance of insulin receptor was not correlated with that of GLUT1 and GLUT4 ($P > 0.1$).

**Discussion**

A critical step for milk production involves cellular glucose transport, as shown by a linear relationship between rates of glucose uptake by mammary cells, lactose synthesis and milk yield in dairy cows (Kim et al., 2001; Cant et al., 2002) and goats (Nielsen et al., 2001). Glucose uptake is a process mediated by transporter proteins located in the basolateral membranes of the epithelial cells. Numerous glucose transporters have been identified in mammary cells, such as $Na^+$-independent GLUT1, GLUT4, GLUT8 and GLUT12, and $Na^+$-dependent SGLT1 and SGLT2 (Zhao and Keating, 2007). Among them, GLUT1 and GLUT4 are the main insulin-sensitive transporters, cooperating in glucose uptake in insulin-responsive tissues (Zhao and Keating, 2007). In the mammary gland, GLUT1 has been detected in the basal membrane of rat (Macheda et al., 2003) and bovine (Zhao et al., 1996, 1999; Komatsu et al., 2005) epithelial cells during lactation. In rat mammary cells, GLUT1 was shown to mediate glucose uptake across cell plasma and Golgi membranes (Nemeth et al., 2000). The expression pattern of GLUT1 transporter in porcine mammary tissue in the present study was similar to that reported in cows (Zhao and Keating, 2007) and rats (Camps et al., 1994), with GLUT1 mRNA increasing with the onset of lactation. In addition, expression of GLUT1 was positively correlated to α-lactalbumin and β-casein gene, and RNA:DNA in mammary tissue. Milk protein α-lactalbumin constitutes the regulatory subunit of the enzyme lactose synthase, responsible for lactose synthesis in the mammary gland (Prager and Wilson, 1988). As such, the positive correlation between mRNA levels of GLUT1 and α-lactalbumin in porcine mammary tissue suggests a role of GLUT1 in glucose transport, presumably for lactose synthesis. Conversely, expression levels of GLUT4 were

noticeably lower than GLUT1 and remained unchanged during lactation, also consistent with previous studies in cows (Zhao et al., 1993; 1999) and rats (Camps et al., 1994; Burnol et al., 1990), who reported gene expression of GLUT4 to be very low and localized only to mammary adipocytes. Accordingly, the increase in GLUT4 mRNA levels post-weaning in the present study is likely due to a shift in adipose to epithelial cell ratio in the sow mammary gland, as previously shown in lactating rats (Camps et al., 1994; Burnol et al., 1990).

Glucose uptake by GLUT1 and GLUT4 is stimulated by insulin in response to the postprandial increase of glucose. In peripheral tissues, insulin increases both transcription and translation rates of GLUT1 and GLUT4, and promotes their redistribution to the plasma membrane from intracellular compartments (Suzuki and Tono, 1980; Cushman and Wardzala, 1980). However, the role of insulin in the mammary gland is not well understood. Insulin is critical for successful lactation (Rosen et al., 1999), as shown by the suppression of milk secretion and loss of mammary tissue DNA in rats treated with insulin inhibitors (Lau et al., 1993). However, earlier studies in pigs showed an inverse relation between insulin blood levels and milk yield and lactose synthesis (Reynolds and Rook, 1977), and administration of exogenous insulin to lactating sows decreased milk yield (Goldobin, 1976). Nonetheless, insulin appears to be involved, along with the galactopoietic hormones prolactin and glucocorticoids, in transcription regulation of milk protein genes (Rosen et al., 1999; Casey and Plaut, 2007). Receptors for insulin have been previously detected in rat (Burnol et al., 1990) and bovine mammary epithelial cells (Baumrucker and Erondu, 2000), and for the first time, in porcine mammary tissue in the present study. However, gene expression levels of insulin receptor remained constant through lactation and were not correlated to mRNA levels of milk protein genes, suggesting an indirect or permissive role in milk protein gene transcription regulation, as previously indicated by Rosen et al. (1999).

Results from the present and previous studies (Kensinger et al., 1982; Auldist et al., 1995) showed an increase in protein biosynthetic capacity per mammary cell as indicated by an RNA to DNA ratio over the pre-partum to peak lactation periods. Transcription rate is dependent on activation and synthesis of transcription factors, both events controlled by galactopoietic hormones such as prolactin and glucocorticoids during lactation (Rosen et al., 1999). Prolactin is well known as a lactogenic hormone in all mammals (Tucker, 1985), including the pig. Basal prolactin levels increase on the day before farrowing to induce lactogenesis, and then gradually decrease during lactation (Van Landeghem and Van de Wiel, 1978; Plaut et al., 1989), whereas additional surges of prolactin occur during stimulation of the mammary gland at nursing to maintain normal lactation (Knobil and Neill, 2006). Previous studies have shown that prolactin is absolutely necessary to initiate and, unlike for the dairy cow (Knight, 2001), maintain milk production in sows (Farmer, 2001), as prolactin suppression with bromocriptine (a dopamine agonist) inhibits both lactogenesis (Taverne et al., 1982) and galactopoiesis (Farmer et al., 1998). However, administration of exogenous prolactin does not increase milk production in the sow (Farmer et al., 1999; Farmer, 2001), suggesting that mammary gland receptors may be saturated with endogenous prolactin (Farmer et al., 1999). Results from the present study showed a decrease in prolactin receptor gene expression post-partum and then a rebound at peak lactation, which appears inversely related to circulating prolactin levels previously reported in the sow (Farmer et al., 1998; Plaut et al., 1989). It is well known that an increase in mRNA abundance does not always translate into increase in protein level. However, despite that prolactin receptor protein levels were not measured, results from the present study are in good agreement with previous work regarding prolactin binding activity in mammary glands of lactating sows (Plaut et al., 1989), pointing to a possible down-regulatory effect of prolactin on its own receptor, as previously demonstrated in rat mammary glands (Barash et al., 1983). Therefore, it is possible that the

galactopoietic effect of prolactin on sow mammary tissue is more related to the prolactin receptor density or affinity than to the circulating prolactin levels. Such a key role of prolactin receptors in milk yield is supported by the finding that mammary prolactin receptors of Meishan-derived sows, which produced more milk than Large White sows, had a greater affinity for the hormone than those from Large White sows (Farmer et al., 2000). However, results from the present study showed no correlation between gene expression of prolactin receptors and mRNA abundance of milk proteins, despite the fact that prolactin has been shown to regulate milk protein synthesis by increasing transcription of β-casein and α-lactalbumin (Rosen et al., 1999). Consequently, prolactin may play a permissive rather than a regulatory role in milk protein synthesis, as previously acknowledged by Farmer (2001).

Conversely, mRNA abundance of glucocorticoid receptor increased with the onset of lactation, and was positively correlated to α-lactalbumin and β-casein genes, and RNA:DNA in mammary tissue, indicative of a potential regulatory role in sow milk protein synthesis. In fact, numerous studies have shown that glucocorticoids play a major role in mammary secretory activation and milk synthesis, regulation of milk protein gene expression, and maintenance of secretory cell differentiation and lactation (reviewed by Casey and Plaut, 2007). Glucocorticoid receptors are localized within the cellular cytoplasm, and upon activation exert a synergistic action with prolactin-induced transcription factors to increase milk protein transcription rate (Akers, 2006; Doppler et al., 2000, 2001). As such, transcriptional regulation of glucocorticoid receptors may play a key role in adapting the porcine mammary gland to higher lactation demand, possibly by regulating transcription of mammary genes encoding for milk proteins in the lactating sow.

Knockout studies (Casey and Plaut, 2007; Lau et al., 1993) and hormone inhibitor treatments (Farmer et al., 1998; Powell and Keisler, 1995) indicate that the presence of

prolactin, insulin and glucocorticoids is necessary to reach maximum milk yield. However, administration of these hormones to lactating sows does not increase milk production or litter growth. Furthermore, treatment with exogenous glucocorticoids at supraphisiological levels inhibits galactopoiesis in both sows (Norby et al., 2001) and dairy cows (Shamay et al., 2000). Conversely, administration of GH to dairy cows results in an increase in milk yield (Bauman & Vernon, 1993; Bauman, 1999), but fails to improve sow or litter performance in lactating sows (Farmer et al., 1992, 1996; Toner et al., 1996), the reason for which remains unknown. Growth hormone is secreted in a pulsatile manner by the anterior pituitary gland and upon binding to its membrane receptor in target tissues, stimulates cell growth and changes in protein, carbohydrate, and fat metabolism (Bauman, 1999). Several studies have shown the positive effects of GH on bovine lactation to be related to an increase in proliferation and activity of bovine mammary epithelial cells. The decline in milk production after peak lactation is attributable to a reduction in the number of secretory cells, while administration of GH during mid-lactation increased mammary cell proliferation (Capuco et al., 2001). Furthermore, response to GH treatment in dairy cows was negligible when hormone was administered prior to peak lactation (Chilliard, 1989; McBride et al., 1988). Administration of GH to lactating sows also increased DNA concentration in mammary tissue (Farmer et al., 1997). However, and in contrast to bovine lactation, data obtained in the present study showed that DNA concentration in porcine mammary tissue remained constant, or even increased through lactation (Kim et al., 1999; Kensinger et al., 1982). Sow milk yield peaks at 2 to 3 weeks post partum, very shortly before weaning. Therefore, in the absence of decreasing mammary cell number, administration of exogenous GH is likely to have no beneficial effects on milk synthesis. Results from the present study showed a temporally regulated expression of gene encoding for GH receptor over the pre-partum to post-weaning period, suggesting a potential role in milk protein synthesis in the sow. Expression of GH

receptor has been detected also in bovine mammary tissue (Plath-Gabler et al., 2001) and bovine mammary cell lines (Johnson et al., 2010; Zhou et al., 2008), and treatment with GH increased β-casein gene expression in dairy cows (Yang et al., 2005), indicative of a direct effect of the hormone at the mammary tissue level. However, the molecular mechanisms mediating such effects remain unknown. It has been suggested that GH increased milk production indirectly, via endocrine and paracrine secretion of IGF-1 from liver and mammary stromal cells (Akers et al., 2000; Allan et al., 2002). Indeed, previous work has shown an increased IGF-1 receptor abundance during lactogenesis in bovine mammary tissue (Dehoff et al., 1988), while treatment with IGF-1 stimulated β-casein synthesis in cultured mammary cells from lactating cows (Hanigan et al., 1992). Results from the present study indicate that the gene encoding for IGF-1 is expressed at the transcription level in porcine mammary tissue. However, neither GH receptor nor IGF-1 expression levels were positively correlated to mRNA abundance of β-casein and α-lactalbumin or RNA:DNA during lactation.

It has been recently shown that the galactopoietic effect of GH in bovine mammary tissue is partially due to an increase in mRNA translation initiation and elongation, possibly mediated via the mammalian target of rapamycin (mTOR) pathway (Hayashi et al., 2007; 2009; Burgos et al., 2010). The mTOR pathway regulates both cell growth and cell cycle progression in the organism through its ability to integrate signals from nutrients (e.g., AA) and growth factors (IGF-1 and GH) to increase protein translation, via activation of translation factors such as p70S6 kinase (Ma and Blenis, 2009). In this regard, Toerien and Cant (2007) reported an increase in p70S6 kinase in the mammary gland of lactating cows compared to dry animals, whereas stimulation of mammary protein synthesis by growth factors was associated with activation of several mTOR downstream translation factors (Burgos et al., 2010). To our knowledge, the present work is the first to report mTOR and p70S6 kinases gene expression in the porcine mammary gland, although neither showed

changes at the mRNA level over pre-partum to post-weaning periods. Accordingly, it is possible that activation of both kinases during lactation depends on changes at transcription and phosphorylation level, as previously shown in bovine mammary gland (Hayashi et al., 2007; 2009; Burgos et al., 2010).

In summary, results from the present study showed an increase in mRNA abundance of glucocorticoid receptor and GLUT1 transporter genes with the onset of lactation, and a positive relationship between genes encoding for glucocorticoid receptor and GLUT1 and those encoding for α-lactalbumin and β-casein proteins, and RNA:DNA in mammary tissue. As such, transcriptional regulation of GLUT1 and glucocorticoid receptors may play a key role in adapting the porcine mammary gland to higher lactation demand, possibly by increasing glucose uptake and milk protein synthesis, and thus are potential molecular targets for improving sow milk production during lactation. Genes encoding for prolactin, GH and insulin receptors, IGF-1, GLUT4, and mTOR and p70S6 kinases were all expressed in porcine mammary cells. However, despite that mRNA abundance of prolactin and GH receptors, GLUT4 transporter and IGF-1 genes changed over lactation, it was unrelated to expression of milk protein genes. It is possible that variation in protein abundance and activity, rather than changes at gene expression levels, determine the role of these receptors, transporters and kinases during lactation.

**Table A.1.** Primer information for reverse transcription quantitative PCR (RT-qPCR) assays

| Accesion Number[1] | Gene | Protein | Primer[2] | Primer (5'-3') | E[3] (%) |
|---|---|---|---|---|---|
| CV872150.1 | API5 | Apoptosis inhibitor 5 | F. 502<br>R. 568 | CTGGAGTGGTGGCAATAATCTCT<br>CCAAGGGAGCTCAGGTTTAGC | 99.4 |
| AY610067.1 | *MRPL39* | Mitochondrial ribosomal protein L39 | F. 540<br>R. 601 | TCGCTGGAGCTTTCTGCTATG<br>TGTTGGCATCCACTCATCAAG | 103.5 |
| NM_001123213.1 | *VAPB* | Vesicle-associated membrane protein-associated protein B/C | F. 1012<br>R.1072 | TGGCGCTGGTGGTTTTG<br>CCTACAAGGCGATCTTCCCTATG | 101.9 |
| DQ452569.1 | *ACTB* | β-Actin | F. 746<br>R. 803 | TGCGGGACATCAAGGAGAA<br>GCCATCTCCTGCTCGAAGTC | 111.8 |
| AF017079.1 | *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | F. 376<br>R. 429 | CGTCCCTGAGACACGATGGT<br>CCCGATGCGGCCAAAT | 97.6 |
| XM_001927465.1 | *RPS23* | Ribosomal protein 23 | F. 52<br>R.115 | CCACCGACGGGACCATAA<br>CAGGGCTGTGCCCAAATG | 99.6 |
| XM_001927648.1 | *MTG1* | Mitochondrial GTPase 1 | F:463<br>R:525 | GGCAAGTCCTCGCTCATCAA<br>CTTGGTGGCTTTTCCTTTCCT | 102.7 |

[1]Accesion number corresponds to the complementary DNA (cDNA) or the expressed sequence tag (EST) sequence deposited in the National Center for Biotechnology and Information database from which the primers were designed.

[2]Direction (F = forward; R = reverse) and hybridization position for each primer (5′–3′) within the nucleotide sequence from which the primers were designed.

[3]Primer pair efficiency (E) was calculated as follow: $E = -1+10^{(-1/slope)} \times 100$. The $R^2$ values for all standard curves for reference and candidate genes were > 0.98, indicating excellent linear relationships between quantities of serially diluted cDNA and Ct when RT-qPCR was performed.

**Table A.2** Primer information for reverse transcription quantitative PCR (RT-qPCR) assays.

| Accesion Number[1] | Gene | Protein | Primer[2] | Primer (5'-3') | E[3] (%) |
|---|---|---|---|---|---|
| AF102858 | *INSR* | Insulin Receptor | F. 110<br>R. 172 | TTCTTCGAACCCCGAGTACCT<br>CCGGCACGTACACAGAACAT | 97.7 |
| NM_001001868 | *PRLR* | Prolactin Receptor | F. 796<br>R. 854 | TGCATCCTTCCGCCAGTT<br>TCCAACAGATGGGTGTCAAATC | 100.9 |
| NM_001008481.1 | *NR3C1* | Glucocorticoid receptor | F. 2155<br>R. 2219 | ACTTACACCTGGATGACCAAATGA<br>GGCGAACACCATGAGAAACA | 88.7 |
| NM_214254.2 | *GHR* | Growth hormone receptor | F. 1617<br>R. 1674 | TGCCAAAAAGTGCATCGCTAT<br>TGGCGCTACACGTGATTCAA | 107.9 |
| X17058 | *SLC2A1* | GLUT1 | F. 1514<br>R. 1575 | CTGCGGCTCCAGGATTTTT<br>TGTTGCTTGTCTGAATGGACTGA | 88.2 |
| M_001128433.1 | *SLC2A4* | GLUT4 | F. 1938<br>R. 2043 | ACTCCTCCCTCTCTGGCACTT<br>CCCCCTTTCACCCAGAGTCT | 108.3 |
| NM_214256.1 | *IGF-1* | Insulin-like growth factor 1 | F. 24<br>R. 110 | ATCCTCTTCGCATCTCTTCTACTTG<br>GCCCCACAGAGGGTCTCA | 103.9 |
| EU288086.1 | *FRAP1* | mTOR kinase | F. 75<br>R. 135 | TTGTTGCCCCCTATTGTGAAG<br>CCTTTCGAGATGGCAATGGA | 103.1 |
| XM_003131672.1 | *RPS6KB1* | p70S6 kinase | F. 352<br>R. 416 | GGAAACAAGTGGAATAGAGCAGATG<br>TTGGAAGTGGTGCAGAAGCTT | 105.1 |
| NM_214434.1 | *CSN2* | β-casein | F. 333<br>R. 398 | CTGTGGTGGTGCCTCTTCTTC<br>GAACAATGGTCTCCTTAGCTTTGG | 96.4 |
| NM_214360.1 | *LALBA* | α-lactalbumin | F. 433<br>R. 495 | CCCGCTGTCTTGCTGCTT<br>AGGTAGCCTTAGGGAAGAGGAGTT | 94.5 |

[1]Accesion number corresponds to the complementary DNA (cDNA) or the expressed sequence tag (EST) sequence deposited in the National Center for Biotechnology and Information database from which the primers were designed.

[2]Direction (F = forward; R = reverse) and hybridization position for each primer (5′–3′) within the nucleotide sequence from which the primers were designed.

**Table A.2 (cont'd)**

[3]Primer pair efficiency (E) was calculated as follow: $E = -1+10^{(-1/slope)} \times 100$. The $R^2$ values for all standard curves for reference and candidate genes were > 0.98, indicating excellent linear relationships between quantities of serially diluted cDNA and Ct when RT-qPCR was performed.

**Table A.3.** Concentration of RNA and DNA in sow mammary gland, expressed as mg × $g^{-1}$ tissue. Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation and d 5 after weaning (post-weaning) and peak lactation.

| Item | Pre-partum | Early lactation | Peak lactation | Post-weaning | SE | *P*-value[a] |
|---|---|---|---|---|---|---|
| RNA | $3.4^{w}$ | $5.7^{x}$ | $7.04^{x}$ | $1.01^{y}$ | 0.52 | < 0.0001 |
| DNA | $2.22^{w}$ | $2.2^{w}$ | $2.05^{wx}$ | $1.66^{x}$ | 0.14 | 0.04 |
| RNA:DNA | $1.5^{w}$ | $2.6^{x}$ | $3.4^{y}$ | $0.63^{z}$ | 0.22 | < 0.0001 |

[a]*P*- value for main effect of stage of lactation (Equation 1.2)
[w,x,y]Within a row, means lacking a common superscript differ ($P < 0.05$)

**Figure A.1.** Messenger RNA abundance of glucocorticoid and prolactin receptors in pig mammary cells quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represent 14 - ΔCt ± SE. Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (post-weaning) and peak lactation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

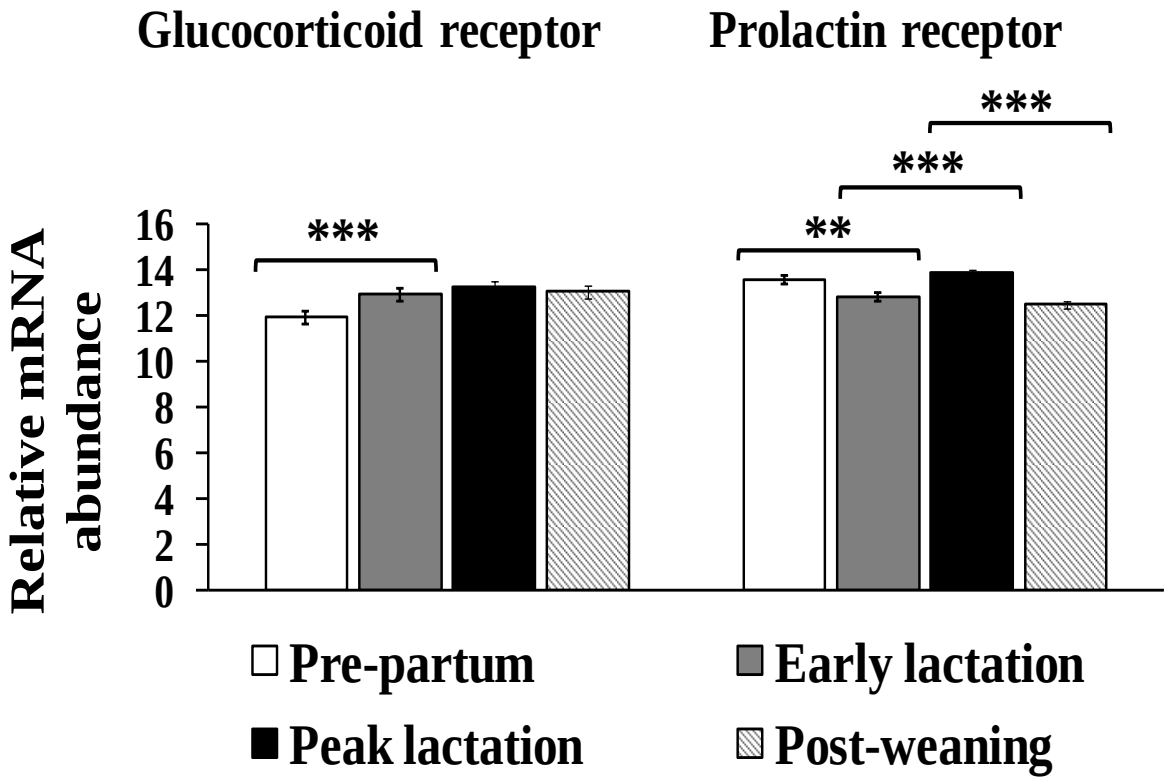


**Figure A.2.** Messenger RNA abundance of growth hormone receptor (GHR) and insulin-like growth factor 1 (IGF-1) in pig mammary cells quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represent 14 - ΔCt ± SE. Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (post-weaning) and peak lactation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

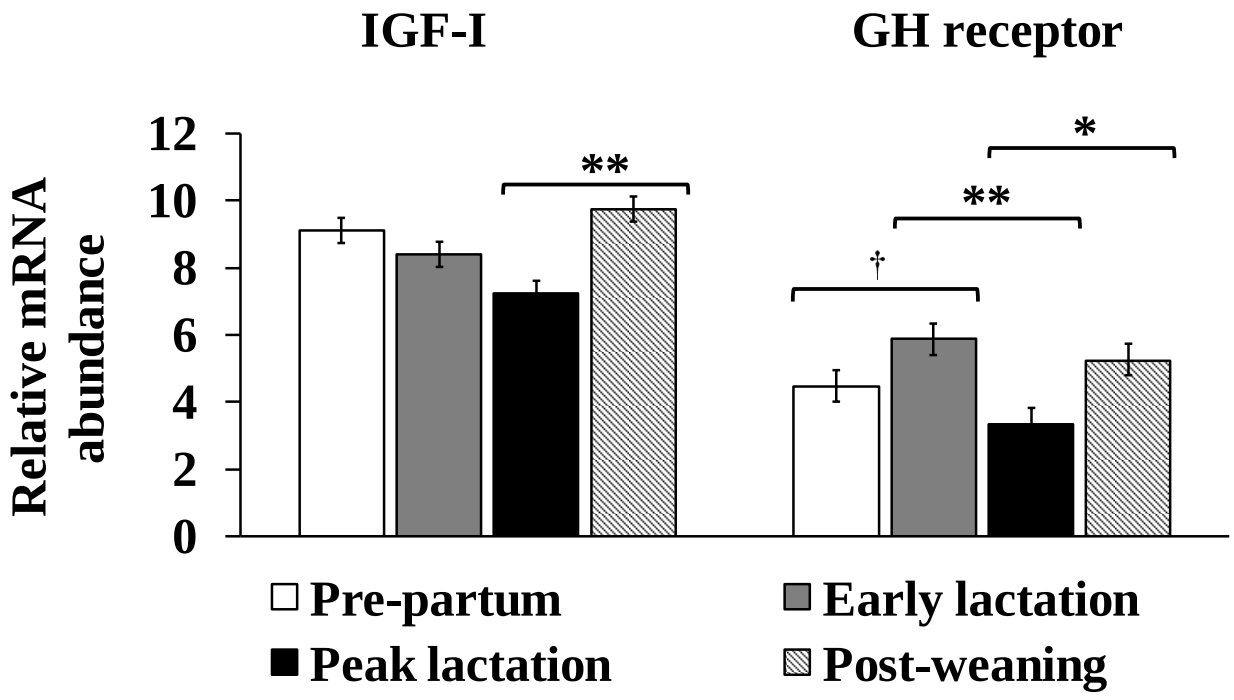


**Figure A.3.** Messenger RNA abundance of mTOR and p70S6 kinases in pig mammary cells quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represent 14 - ΔCt ± SE. Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (post-weaning) and peak lactation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

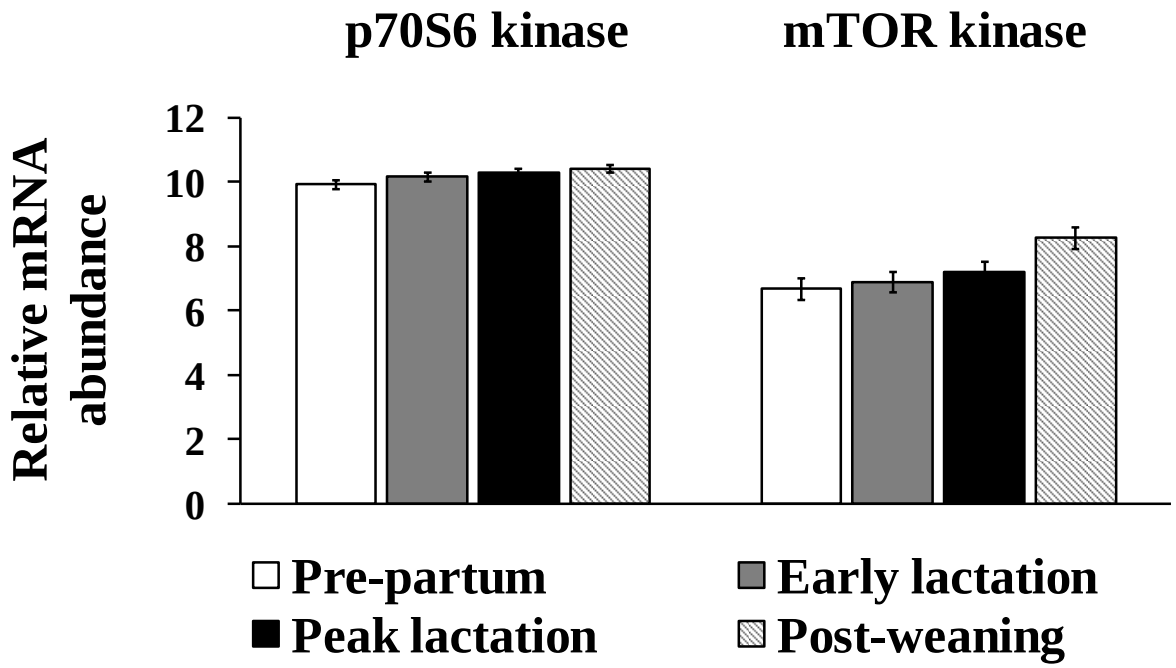
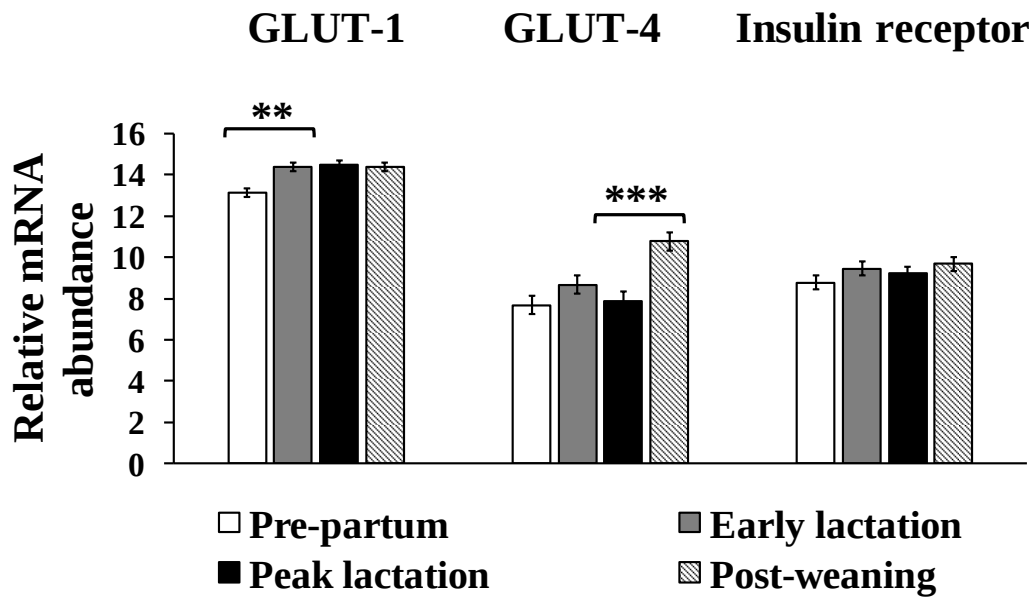


**Figure A.4.** Messenger RNA abundance of glucose transporters GLUT1 and GLUT4, and insulin receptors, in pig mammary cells quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represent 14 - ΔCt ± SE. Data was compared between d 5 of lactation (early) and d 110 of gestation (pre-partum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (post-weaning) and peak lactation. $^{**}P < 0.01$; $^{***}P < 0.001$



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## APPENDIX B

## TABLES NON ESSENTIAL AA

**Table B.1.** Effect of dietary CP concentration on AA arterial concentrations (mmol / L) in lactating sows at d 7 and d 17 of lactation

| Item | Stage of Lactation | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 7 Dietary CP, % | | | Day 17 Dietary CP, % | | | Day 7 | | Day 17 | |
| | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | L[j] | Q[k] | L[j] | Q[k] |
| No.[b] | 5 | 3 | 5 | 5 | 3 | 5 | | | | |
| Ala | 575.7[d] ± 37.6 | 487.0[e] ± 50.4 | 306.4[f] ± 38.3 | 637.92[a] ± 37.6 | 558.8[b] ± 50.4 | 391.0[c] ± 42.2 | 0.001 | 0.45 | 0.001 | 0.47 |
| Asn | 62.2 ± 12.1 | 99.7 ± 15.3 | 91.7 ± 12.2 | 62.1[d] ± 12.1 | 93.6[de] ± 15.3 | 103.2[e] ± 13.0 | 0.05 | 0.16 | 0.02 | 0.49 |
| Asp | 27.8 ± 4.8 | 22.8 ± 6.4 | 26.2 ± 4.9 | 25.2 ± 4.8 | 25.0 ± 6.4 | 27.7 ± 5.3 | 0.80 | 0.54 | 0.70 | 0.83 |
| Cit | 68.2 ± 8.5 | 72.4 ± 10.9 | 50.1 ± 8.6 | 76.3 ± 8.5 | 62.2 ± 10.9 | 58.7 ± 9.2 | 0.09 | 0.26 | 0.12 | 0.65 |
| Gln | 515.3 ± 40.7 | 562.5 ± 54.3 | 423.3 ± 41.5 | 419.0 ± 40.7 | 447.7 ± 54.3 | 441.8 ± 44.9 | 0.10 | 0.14 | 0.68 | 0.77 |
| Glu | 271.0[g] ± 32.8 | 206.8[gh] ± 43.8 | 148.4[h] ± 33.6 | 239[a] ± 32.8 | 171.3[ab] ± 43.8 | 145.8[b] ± 34.2 | 0.01 | 0.95 | 0.04 | 0.65 |
| Gly | 583.7 ± 58.0 | 470.7 ± 78.1 | 539.1 ± 59.2 | 655.5 ± 58.0 | 526.4 ± 78.1 | 581.3 ± 64.8 | 0.58 | 0.33 | 0.40 | 0.32 |
| Ser | 103.1 ± 6.7 | 95.5 ± 8.9 | 104.7 ± 6.9 | 107.5 ± 6.7 | 113.2 ± 8.9 | 118.3 ± 7.3 | 0.84 | 0.39 | 0.22 | 0.97 |
| Tyr | 58.9[g] ± 7.7 | 73.4[gh] ± 10.6 | 99.8[h] ± 8.0 | 52.5[g] ± 7.7 | 87.5[gh] ± 10.6 | 109.28[h] ± 8.3 | 0.01 | 0.64 | 0.001 | 0.61 |

Within a row, means lacking a common superscript differ at [a,b,c]$P < 0.1$, [d,e,f]$P < 0.05$, and [g,h,i]$P < 0.01$.

[j]Linear.

[k]Quadratic.

**Table B.2.** Effect of dietary CP concentration on AA arterio-venous differences (mmol / L) in porcine mammary gland at d 7 and d 17 of lactation.

| Item | Stage of Lactation | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 7 Dietary CP, % | | | Day 17 Dietary CP, % | | | Day 7 | | Day 17 | |
| | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | L[j] | Q[k] | L[j] | Q[k] |
| No.[b] | 5 | 3 | 5 | 5 | 3 | 5 | | | | |
| Ala | 39.8 ± 17.2 | 34.3 ± 20.8 | 37.7 ± 18.1 | 76.8 ± 17.3 | 51.7 ± 20.8 | 43.9 ± 17.3 | 0.94 | 0.87 | 0.22 | 0.74 |
| Asn | 8.7 ± 5.9 | 29.2 ± 7.9 | 18.0 ± 6.0 | 15.1 ± 5.9 | 18.9 ± 7.9 | 25.0 ± 6.6 | 0.28 | 0.11 | 0.28 | 0.90 |
| Asp | 3.6 ± 4.5 | 11.7 ± 6.0 | 7.0 ± 4.6 | 5.5 ± 4.5 | 3.2 ± 6.0 | 10.9 ± 5.0 | 0.61 | 0.38 | 0.44 | 0.50 |
| Cit | 2.8 ± 3.8 | 5.0 ± 6.3 | 2.1 ± 3.8 | 3.8 ± 3.8 | 19.9 ± 5.1 | 2.5 ± 4.2 | 0.90 | 0.72 | 0.82 | 0.02 |
| Gln | 65.4 ± 17.9 | 77.4 ± 24.6 | 73.3 ± 18.5 | 58.1 ± 17.9 | 83.2 ± 24.6 | 91.2 ± 19.7 | 0.76 | 0.79 | 0.24 | 0.77 |
| Glu | 65.8 ± 10.3 | 80.7 ± 13.6 | 48.2 ± 10.4 | 78.0 ± 10.3 | 63.2 ± 13.6 | 50.6 ± 11.3 | 0.20 | 0.14 | 0.08 | 0.94 |
| Gly | 15.6 ± 23.0 | 34.1 ± 30.9 | 25.3 ± 23.5 | 44.7 ± 23.0 | 24.3 ± 30.9 | 16.2 ± 25.9 | 0.77 | 0.71 | 0.43 | 0.87 |
| Ser | 28.9 ± 4.7 | 39.6 ± 6.3 | 34.3 ± 4.8 | 34.3 ± 4.7 | 41.5 ± 6.3 | 39.9 ± 5.3 | 0.43 | 0.31 | 0.44 | 0.57 |
| Tyr | 9.8 ± 2.9 | 11.5 ± 4.0 | 14.0 ± 3.4 | 16.3 ± 2.9 | 21.5 ± 4.0 | 17.3 ± 3.3 | 0.38 | 0.94 | 0.82 | 0.34 |

Within a row, means lacking a common superscript differ at [a,b,c]$P < 0.1$, [d,e,f]$P < 0.05$, and [g,h,i]$P < 0.01$.

[j]Linear.

[k]Quadratic.

**Table B.3.** Effect of dietary CP concentration on AA transport efficiency (A-V/A × 100) in porcine mammary gland at d 7 and d 17 of lactation.

| Item | Stage of Lactation | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 7 Dietary CP, % | | | Day 17 Dietary CP, % | | | Day 7 | | Day 17 | |
| | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | 9.50 Deficient | 13.50 Ideal | 17.50 Standard | L[j] | Q[k] | L[j] | Q[k] |
| No.[b] | 5 | 3 | 5 | 5 | 3 | 5 | | | | |
| Ala | 6.8 ± 2.7 | 7.0 ± 3.3 | 13.5 ± 2.8 | 11.6 ± 2.7 | 9.4 ± 3.3 | 10.7 ± 2.7 | 0.14 | 0.45 | 0.83 | 0.66 |
| Asn | 15.9 ± 6.5 | 32.2 ± 8.8 | 18.5 ± 6.7 | 26.0 ± 6.5 | 20.8 ± 8.8 | 23.1 ± 7.4 | 0.78 | 0.18 | 0.78 | 0.72 |
| Asp | 8.4 ± 14.5 | 34.5 ± 19.4 | 24.7 ± 14.8 | 20.0 ± 14.5 | 11.5 ± 19.4 | 39.5 ± 16.2 | 0.44 | 0.44 | 0.39 | 0.44 |
| Cit | 3.7 ± 5.3 | 6.0 ± 8.9 | 2.4 ± 5.5 | 5.5 ± 5.3 | 25.1 ± 7.2 | 4.2 ± 6.0 | 0.86 | 0.78 | 0.87 | 0.04 |
| Gln | 12.9 ± 3.9 | 13.5 ± 5.3 | 17.4 ± 4.0 | 12.6 ± 3.9 | 18.8 ± 5.3 | 21.0 ± 4.3 | 0.44 | 0.80 | 0.18 | 0.76 |
| Glu | 27.6 ± 7.3 | 39.6 ± 9.3 | 34.8 ± 7.4 | 37.5 ± 7.3 | 36.4 ± 9.3 | 37.9 ± 7.8 | 0.37 | 0.37 | 0.97 | 0.89 |
| Gly | 2.5 ± 3.8 | 6.8 ± 5.0 | 4.1 ± 3.8 | 7.2 ± 3.8 | 6.8 ± 5.0 | 3.1 ± 4.2 | 0.77 | 0.57 | 0.48 | 0.79 |
| Ser | 28.6 ± 4.2 | 41.5 ± 5.8 | 32.0 ± 4.4 | 32.2 ± 4.2 | 37.5 ± 5.8 | 33.9 ± 4.8 | 0.59 | 0.13 | 0.80 | 0.52 |
| Tyr | 18.0 ± 3.5 | 17.6 ± 4.8 | 12.4 ± 4.1 | 33.0[g] ± 3.5 | 25.5[gh] ± 4.8 | 15.0[h] ± 3.9 | 0.37 | 0.63 | 0.01 | 0.73 |

Within a row, means lacking a common superscript differ at [a,b,c]$P < 0.1$, [d,e,f]$P < 0.05$, and [g,h,i]$P < 0.01$.

[j]Linear.

[k]Quadratic.

# APPENDIX C

## ANALYSIS OF REFERENCE GENE EXPRESSION AND RNA AND DNA CONCENTRATION IN PORCINE MAMMARY GLAND DURING LACTATION

Test for changes of tissue RNA and DNA concentrations was performed using a linear mixed model in SAS (Figure 1.6). The model included stage of lactation as a fixed effect and sow as random effect. The statistical model is as follows (Equation C.1):

$$Z_{ij} = \mu + \alpha_i + b_j + e_{ij} \quad [C.1]$$

where $Z_{ij}$ is the tissue RNA or DNA concentration for each sow (j) at each stage of lactation (i), $\mu$ is the overall tissue RNA or DNA concentration mean, $\alpha_i$ is the fixed effect of the $i^{th}$ level of stage of lactation, $b_j$ is the random effect of the sow, and $e_{ij}$ is the experimental error.

Test for invariance expression of reference genes without accounting for tissue RNA and DNA concentration (Figures 1.7, 1.8, and 1.9) was performed using a linear mixed model in SAS The model included stage of lactation as a fixed effect and sow as random effect. The statistical model is as follows (Equation C.2):

$$Y_{ij} = \mu + \alpha_i + b_j + e_{ij} \quad [C.2]$$

where $Y_{ij}$ is the value of potential reference genes corrected by the standard curve and log base 2 transformed for each sow (j) at each stage of lactation (i), $\mu$ is the overall mean, $\alpha_i$ is

the fixed effect of the $i^{th}$ level of stage of lactation, $b_j$ is the random effect of the sow, and $e_{ij}$ is the experimental error.

# APPENDIX D

## AMINO ACID TRANSPORTER CAT-1 IS LOCALIZED TO ENDOTHELIAL CELLS IN THE MAMMARY GLAND OF LACTATING SOWS.

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## Material and Methods

### *Animals and Tissue Collection*

Studies were performed with the approval of the Institutional Animal Care and Use Committee at Michigan State University (AUF#10/08-162-00). Two sows (parity 5 and 7, Landrace × Yorkshire) were fed a corn-soybean meal-based diet meeting all nutrient requirements for lactating sows nursing piglets with a predicted average daily gain of 250 g/d (NRC, 1998; Table 1). Litters were equalized to 10 piglets within 48 h and piglets were weaned on d 21. In the first sow mammary parenchymal tissue was biopsied from the first and second thoracic glands (Kirkwood et al., 2007) in the morning following a 12-h overnight fast on d 110 of gestation (pre-partum), d 5 (early) and d 17 (peak) of lactation. Immediately following the biopsy, mammary tissue was flash-frozen in liquid $N_2$ and stored at -80$^0$C until later protein analysis. In the second sow, mammary parenchymal tissue from one of the first thoracic glands and skeletal muscle from the quadriceps was collected following euthanasia on d 21 of lactation. Tissue was fixed in formalin and 24 h later embedded in paraffin for immunohistochemical staining. Muscle tissue was used as a control for immunochemical staining of CAT-1.

***Protein Expression Analysis***

*Protein Isolation*

Mammary parenchymal tissue from the first sow (200 mg) was homogenized in 2 mL of containing 10mM Tris (pH 7.5; Invitrogen, Carlsbad, CA), 100 mM NaCl (J.T. Baker; Phillipsburg, NJ), 1 mM EDTA (J.T. Baker), 1 mM EGTA (ICN Biomedicals; Aurora, Ohio), 1% Igepal CA-630 (Sigma-Aldrich; St. Louis; MO), 0.4% Deoxycholic acid sodium salt monohydrate (ICN Biomedicals), and 60 mM Octyl β-D-glucopyranoside (Sigma-Aldrich). The homogenate was centrifuged for 30 min at 20,000 × g. Supernatant was collected and protein content was measured using a Qubit protein assay kit (Quant-iT, Invitrogen, Carlsbad, CA). The remainder of the supernatent was diluted to a working stock containing 10ng/µL of protein and stored at -20$^0$C stored until the blot was performed.

*Western Blot Analysis*

CAT-1 abundance in porcine mammary parenchymal tissue from the first sow was determined by western blotting using an anti-CAT-1 antibody produced in rabbit (Sigma-Aldrich; #AV43838). Protein (36 µg) was diluted (1:4) in Laemmli sample buffer (BIO-RAD; Hercules, CA) mixed with β-mercaptoethanol (Sigma-Aldrich), heated in boiling water for 5 min, and centrifuged at 400 × g for 15 sec. Samples were loaded into a 10% Tris-glycine gel (Mini-Protean TGX Precast Gel, BIO-RAD) and then run at 200V. Protein was electrotransferred to a nitrocellulose membrane at 100V for 1 hour. The membranes were stained with Ponceau S staining solution (Sigma-Aldrich) to evaluate loading of samples. The membranes were then rinsed with double-distilled $H_2O$ and blocked with SuperBlock (BIO-RAD) for 1 h. The

membrane was then washed in BupH buffer (Thermo-Scientific; Rockford, IL) before applying the primary antibody against CAT-1. This antibody was diluted 1:500 in a solution with 10 % blocking buffer and 0.1% Tween-20 (Sigma-Aldrich), and incubated overnight at $4^0$C. The secondary antibody was goat antirabbit conjugated with horseradish peroxidase (SuperSignal West Pico kit, Thermo-Scientific; Rockford, IL), diluted 1:50,000 in a solution with 10 % blocking buffer and 0.1% Tween-20 (Sigma-Aldrich), and incubated for 1 h at room temperature.

*Immunohistochemistry*

Specimens were processed, embedded in paraffin and sectioned on a rotary microtome at 4 -5 µs. Sections were placed on slides coated with 3-Aminopropyltriethoxysilane and dried at 56°C overnight. The slides were subsequently deparaffinized in Xylene and hydrated through descending grades of ethanol to distilled water; then placed in tris buffered saline pH 7.5 for 5 minutes to allow for pH adjustment. Following TBS, slides were antigen retrieved using 0.03% Protease E (Sigma-St. Louis, MO) for 10 minutes at 37degrees. Followed by running tap and distilled water rinses. Endogenous Peroxidase was blocked utilizing 3% Hydrogen Peroxide / Methanol bath for 30 minutes followed by running tap and distilled water rinses. Following pretreatment standard avidin-biotin complex staining steps were performed at room temperature on the DAKO Autostainer. Slides are rinsed for two minutes between each staining step; after blocking for non-specific protein with Normal Goat Serum (Vector Labs – Burlingame, CA) for 30 minutes; sections were incubated with Avidin / Biotin blocking system for 15 minutes (Avidin – Vector Labs / d-Biotin – Sigma Chemical – St. Louis, MO). Following subsequent rinsing in Tris Buffered Saline + Tween 20 (Scytek – Logan, UT) slides were incubated in the following primary antibodies: Polyclonal Rabbit Anti CT1 1:500 60 minutes. Upon completion

of primary incubations, slides were incubated for 30 minutes with the corresponding Biotinylated secondary Goat Anti- Rabbit IgG (H+L) (Vector) at a concentration of 11µg/ml; followed by the application of R.T.U. Vectastain Elite ABC Reagent (Vector) for 30 minutes. The reaction is developed with Nova Red (Vector) peroxidase substrate for 15 minutes; followed by distilled water rinses. Samples were counterstained with Gill 2 Hematoxylin (ThermoFisher – Pittsburgh, PA) for 1 ½ minutes, differentiated in 1% Aqueous Glacial Acetic Acid, rinsed in running tap water; followed by dehydration through ascending grades of ethanol, clearing in Xylene, and coverslipped with synthetic permanent mounting media.

**Results**

Western Blot analysis showed CAT-1 protein expression in porcine mammary tissue at all stages of mammary physiological activity (Figure D.1). Immunohistochemical staining revealed labeling of CAT-1 in the basolateral membrane of endothelial cells of mammary tissue (Figure D.2a) and muscle tissue (Control; Figure D.2b)

**Figure D.1**. Western blot analysis of CAT-1 in porcine mammary parenchymal tissue at d 110 of gestation (pre-partum), d 5 (early) and d 17 (peak) of lactation.

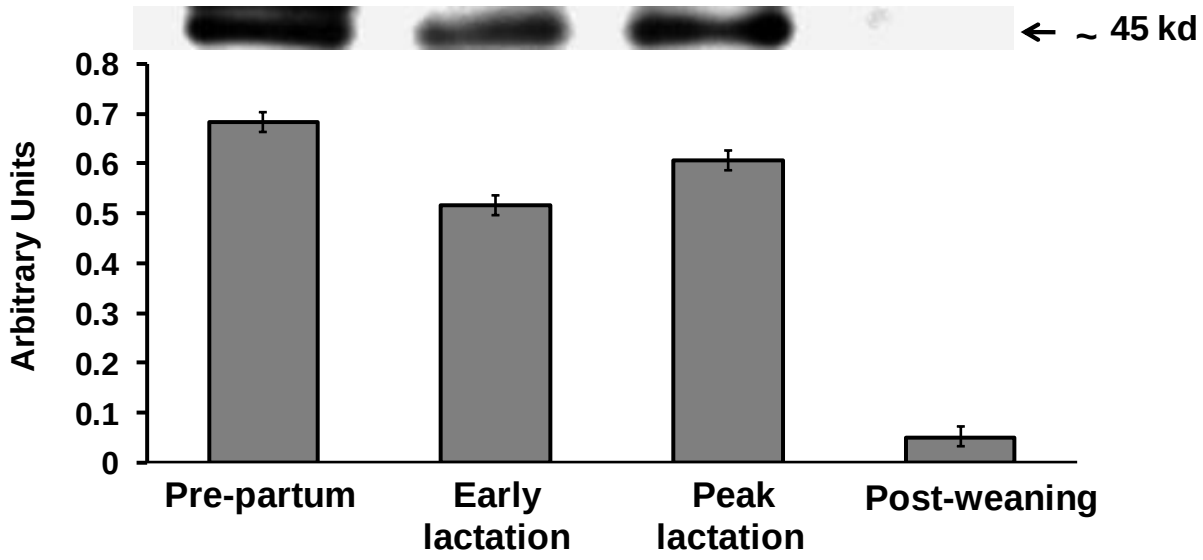

**Figure D.2a**. Cellular localization of CAT-1 in porcine mammary parenchymal tissue at d 21 of lactation. CAT-1 is only localized in the basolateral membrane of endothelial cells.

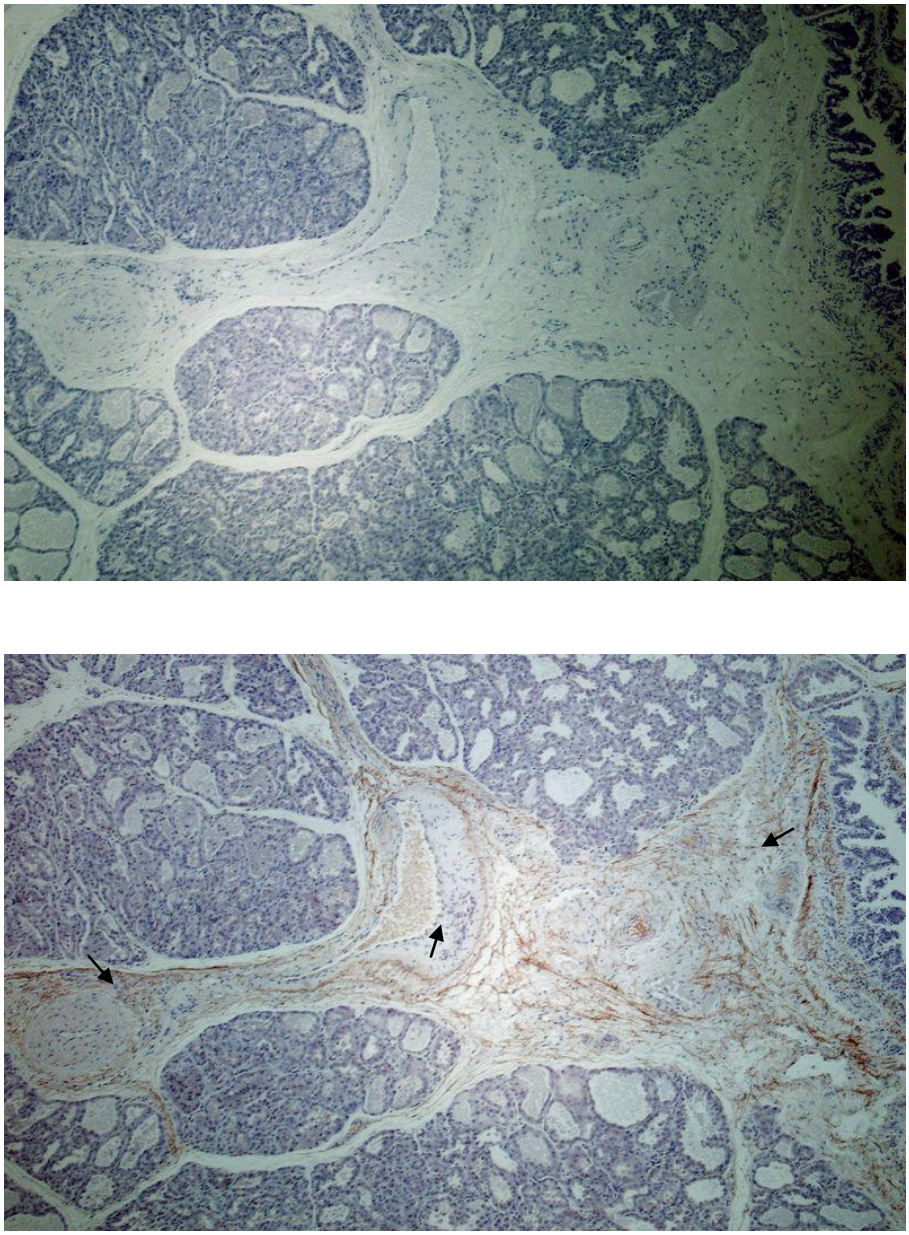

**Figure D.2b.** Cellular localization of CAT1 in porcine skeletal muscle tissue at d 21 of lactation. CAT-1 is only localized in the basolateral membrane of endothelial cells.