RESEARCHREPORT

ANIMAL SCIENCE — Nutritional Efficiency Consortium

Title: Regulation of Amino Acid Transport Efficiency by the Porcine Mammary Gland - revised
NPB #08-113

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Industry Summary: Modern swine production systems require highly productive animals. Thus, continued development of nutritional strategies to improve milk production and litter weight gain is critical. However, very little is known about the physiological mechanisms that control milk protein synthesis in pigs. Furthermore, with increasing concerns over environmental nitrogen losses, research has focused on strategies to improve efficiency of dietary protein utilization in growing pigs, with the breeding herd lagging behind. Indeed, the focus of amino acid nutrition for the lactating sow has been on litter growth maximization rather than on minimizing nitrogen excretion. However, lactating sows consume approximately 20 kg of crude protein (CP) over a 21-d lactation period, with substantial nitrogen losses in the urine. Hence, there is significant contribution of dietary nitrogen losses to the environment from the breeding herd as well. Understanding of the factors governing the efficiency of utilization of dietary protein and amino acid for milk synthesis is critical in order to minimize nitrogen excretion from the breeding herd while maintaining or even improving lactation performances. The objectives of this study were to 1) quantify the expression of genes that encode for milk and Lys transporter proteins and assess whether these genes are correlated; 2) measure the efficiency of Lys utilization for milk production in response to feeding an optimum amino acid balance in a reduced crude protein diet; 3) quantify the expression of genes that encode for milk and Lys transporter proteins in response to feeding an optimum amino acid balance in a reduced CP diet; 4) assess whether change in Lys utilization efficiency for milk production is related to changes in the expression of genes encoding for Lys transporter proteins.

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For objective 1, the expression of 5 genes that encode for amino acid transporter proteins and of 2 that encode for mammary synthesized milk proteins were measured in sow mammary tissue across different stages of mammary physiological activity and milk demand. Results of objective 1 indicated that there was a high correlation between transcript abundance for 2 of the 5 amino acid transporter genes selected and the transcript abundance of genes encoding for milk proteins. Therefore, a select number of amino acid transporter genes were identified as potential molecular targets for improvement of sow milk production during lactation.

For objectives 2 to 4, 24 sows were used. The rate of uptake of amino acids by the mammary gland (that is, the percentage of AA that the mammary gland uses per blood pass) was quantified, along with the expression of genes that encode for transporter proteins responsible for amino acid uptake. Sows were fed 1 of 3 diets that contained 9.5 (Deficient), 13.5 (Ideal), and 17.5 % (Standard) CP but had similar indispensable and dispensable amino acid profile formulated to target an optimum profile. The results of objectives 2 to 4 demonstrated that decreasing the dietary CP from 19.4 (Standard diet) to 15.1 % (Ideal diet) with inclusion of crystalline amino acids 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 branched chain amino acids to Lys ratio but was unrelated to the expression of genes encoding for amino acid transporter and milk proteins. These results indicate that CP reduction with crystalline amino acid 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.

The study demonstrated that feeding an optimum balance of amino acids achieved through crystalline amino acid inclusion improves the efficiency of Lys utilization (mammary extraction efficiency) and maintains lactation performance if provided in a reduced CP diet. These findings will allow the development of effective nutritional tools that can be immediately implemented to positively impact both lactation performance and the environment. Second, the study demonstrated that there are two Lys transporter genes that are related to genes encoding for mammary synthesized milk proteins, which in the longer term may be targeted to further understand how amino acid transporters regulate milk production. Finally, the results of the study demonstrated that changes in expression of genes encoding for Lys transporter proteins were not related to Lys extraction efficiency, indicating that other or additional mechanisms regulate Lys transport; for instance, in this study, increased Lys extraction efficiency by the mammary gland was related to lower circulating levels of the branched-chain amino acids, pointing to competitive inhibition mechanism between amino acids for uptake into the mammary gland. **The later finding is highly significant to the swine industry**: for the first time, it is shown that improvement in Lys utilization for milk production may be achieved by decreasing the levels of non-limiting amino acids, such as the branched-chain amino acids, in order to decrease the competition between Lys and those amino acids for mammary uptake. This latter finding does not only provide an important initial

understanding of the biological basis behind the so called 'ideal protein' or 'optimum amino acid balance', but provides an alley for future technology development at a mechanistic level, including, but not limited to, modulators of nutrient repartitioning.

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Scientific 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 proteins-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 *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 increased 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

To address concerns over environmental N losses, research has focused on strategies to improve efficiency of dietary protein utilization in growing pigs rather than the breeding herd. The focus of AA nutrition for the lactating sow has been on litter growth maximization rather than on minimizing nitrogen excretion. However, lactating sows consume approximately 20 kg of crude protein (CP) over a 21-d lactation period, of which 35% of the absorbed protein is excreted in the urine. Hence, there is substantial contribution of dietary N losses to the environment from the breeding herd as well but a clear dearth of understanding of the factors governing the efficiency of utilization of dietary protein for milk synthesis remains. We recently reported 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). As part of a National Pork Board-funded project, we have shown that reduction in dietary CP with concomitant inclusion of crystalline amino acids (CAA) increases the efficiency of Lys utilization by the growing pig 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 fed a corn-soybean meal based diet (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. The research question of this application was to determine whether the efficiency of AA transport by the porcine mammary gland increases in response to dietary CP reduction coupled with CAA inclusion in an optimum balance, and whether an increase in efficiency is mediated via an increase the expression of mammary genes encoding for Lys transporters. **The question is highly relevant to swine producers. First, demonstration that an optimum balance of AA achieved through CAA inclusion and CP reduction, improves the efficiency of Lys utilization, will allow the development of effective nutritional tools that can be immediately implemented to positively impact both lactation performance and the environment. Second, investigating whether improvement of Lys utilization efficiency is related to changes in expression of genes encoding for Lys transporter proteins will provide an alley for future technology development at a mechanistic level, including, but not limited to, gene targeting and modulators of nutrient repartitioning.**

Objectives

(1) Quantify the expression of genes that encode for milk and Lys transporter proteins and assess whether these genes are correlated.

(2) Determine if crystalline AA inclusion in a reduced-CP diet modulates the expression of porcine mammary cell genes encoding for specific AA transporter proteins.

(3) Determine if crystalline AA inclusion in a reduced-CP diet increases the extraction efficiency (mammary AA/ AA intake) of limiting AA by the lactating mammary gland.

(4) Determine if changes in AA transporter gene expression is directly related to the changes in *in viv*o mammary AA uptake and extraction efficiency (mammary AA/ AA intake).

Materials & 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 = 24) 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 (20^0C) 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). The number of sows allocated to each diet represents the final number at the end of the study because 3 sows had to be removed from the study: one sow died from spleen rupture, and two sows had elevated fever. All sows were fed a ration of 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 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 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 Tables 1 and 2.

Table 1. Ingredient composition of experimental diets (%, as fed)

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

Table 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)

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.

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 the 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.

RNA extraction and cDNA 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 quality and quantity using the Agilent Bioanalyzer 2100 with the RNA 6000 Nano Labchip (Agilent Technologies, Palo Alto, CA). The *RNA* integrity *number* (*RIN*) values ranged from 8.7 to 10 for all samples. 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 cDNA concentration was quantified by spectrophotometry (NanoDrop 1000) and then diluted to a working stock of 10ng/µL and stored at -20^0C.

Primer design. In order to facilitate readership throughout the remainder of the manuscript, each target gene is referred to by 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 in order 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 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 in this study. Evaluation of primer-dimer formation was based on 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 DNAase treatment (PerfectPure RNADNase, Gaithersburg, MD) in order to eliminate genomics 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 $(10^{-1/slope} - 1) \times 100$, 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.

Reference Gene Selection. A relative standard curve (Larionov et al., 2005) was used as the RT-qPCR method to measure relative mRNA abundance of potential reference genes. Relative mRNA amounts from the standard curve were entered directly into geNorm software to select the most stable reference genes within the analyzed set, as described by Vandesompele et al. (2002). Briefly, expression stability value (M) for each gene 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 (Vn/Vn+1) between 2 sequential normalization factors (NFn and NFn+1). Such normalization factors (NFn) are based on the geometric mean of the expression levels of n and n+1 best reference genes (Vandesompele et al., 2002).

Reverse Transcription Quantitative PCR Assay. Reverse transcription quantitative PCR reactions were performed in MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems). To each well were added 3µL (30 ng) 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). Plates were sealed, centrifuged at 400 × *g* for 1 min and loaded into the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The amplification program included 2 initial steps (50°C for 2 min and 95°C for 10 min) followed for 40 cycles (step 3; 95°C for 15 s and 60°C for 1 min) and a dissociation curve (step 4; 95°C for 15 s, 60°C for 1 min, 95°C for 15 s). Data were analyzed with the 7000 RQ Sequence Detection Systems Software (version 2.2.1, Applied Biosystems).

RT-qPCR data normalization. Normalization of target gene expression values was made according to the following formula:

$$\Delta Ct_{ijk} = Ct_{Cijk} - \left[\frac{1}{3}\left(Ct_{R1ijk} + Ct_{R2ijk} + Ct_{R3ijk}\right)\right] \quad [1]$$

where ΔCt_{ij} is the normalized target gene expression for the j^{th} sow within the i^{th} stage of lactation and the k^{th} diet, and Ct_{Cijk} and Ct_{Rijk} are the Ct values for target and reference genes, respectively. 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 simpler interpretation of the data, whereby increasing ΔCt values correspond to 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. Consequently all values presented in the figures are positive.

Analysis 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 4)

$$Y_{ij} = \frac{A-V}{A} - 100 \qquad [2]$$

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.

Total mammary gland DNA quantification

Total DNA was extracted from mammary tissue using cold perchloric acid (Sigma-Aldrich, St Louis, MO) following the procedure described by Labarca and Paigen (1980). Following extraction, DNA was quantified by spectrophotometry 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), and reported as $\mu g \cdot mg^{-1}$ mammary tissue.

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:

$$Y_{ij} = \mu + \alpha_i + \gamma_i + \alpha_i \times \gamma_i + \beta_1(x_{ij} - \bar{x}) + \beta_2(y_{ij} - \bar{y}) + b_j + c_j + e_{ij} \quad [3]$$

where Y_{ij} is the variable measured (j) within each stage of lactation (i), μ is the overall mean, α_i is the fixed effect of the i^{th} level of diet, γ_i is the fixed effect of the i^{th} level of stage of lactation, $\alpha_i \times \gamma_i$ *is* the fixed effect of the i^{th} level of interaction between diet and stage of lactation, β_i is the regression coefficient relating the covariate sow initial body weight to the variable measured, x_{ij} is the initial body weight for the j^{th} sow within the i^{th} stage of lactation, x is the overall sow initial body weight mean, β_2 is the regression coefficient relating the covariate sow parity to the variable measured, y_{ij} is the parity for the j^{th} sow within the i^{th} stage of lactation, y is the overall parity mean, b_j is the random effect of the sow, c_j is the random effect of the block and e_{ij} is the experimental error. 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

Results common to all 3 objectives. Lactation performance results are presented in Table 3. Feed intake was not affected by dietary protein intake, and both feed and daily dietary protein intake increased ($P < 0.01$) from d 7 to 17 of lactation (data not shown). Sow BW 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.

Table 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 ($P < 0.05$).

Results for objective 1: Quantify the expression of genes that encode for milk and Lys transporter proteins and assess whether these genes are correlate. 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.

Results for objective 2: Determine if crystalline AA inclusion in a reduced-CP diet modulate the expression of porcine mammary cell genes encoding for specific AA transporter proteins. 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 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. Expression of genes encoding for α-lactalbumin, β-casein and CAT-1, CAT-2b, $b^{0,+}AT$, $ATB^{0,+}$, $y^{+}LAT2$ were unaffected by diet (Figures 1a, b, c, d, e, f, g, and h, respectively).

Figure 1a

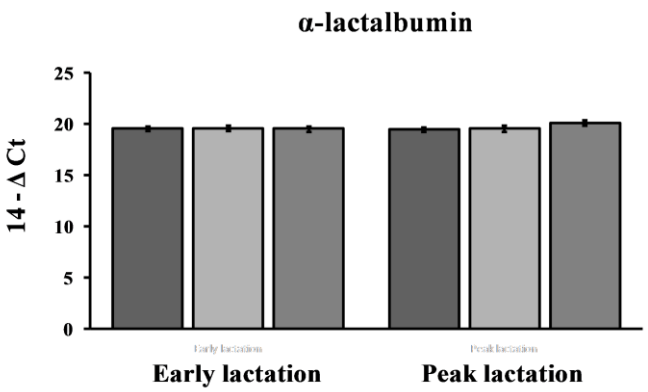


Figure 1b

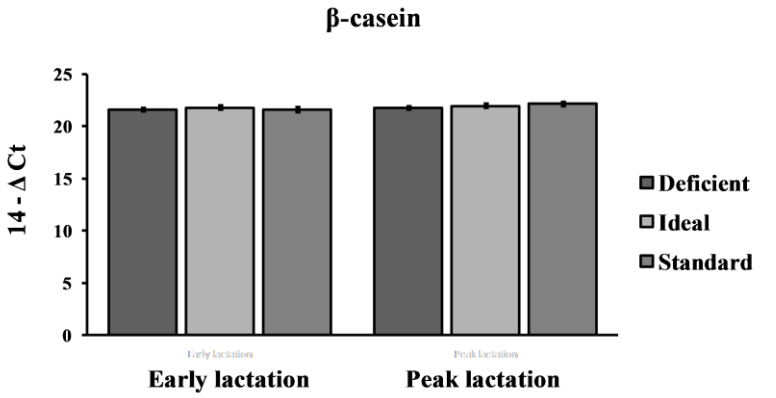


Figure 1c

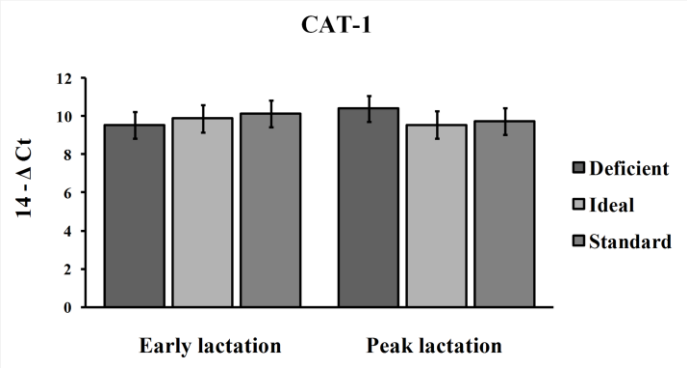


Figure 1d

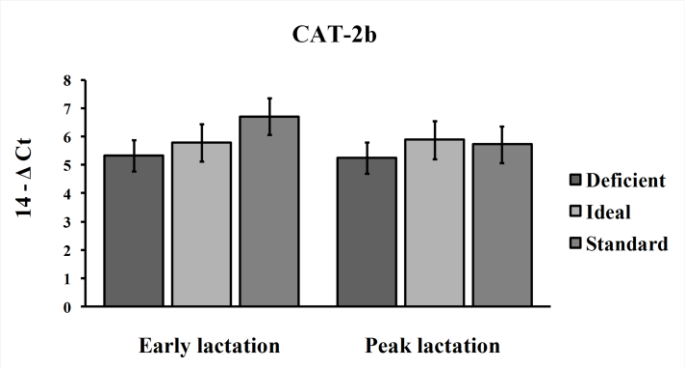


Figure 1e

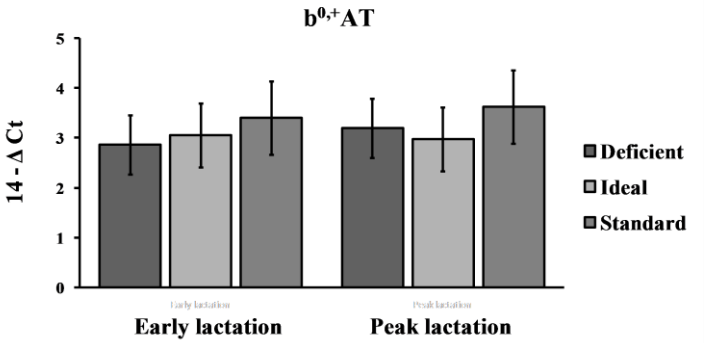


Figure 1f

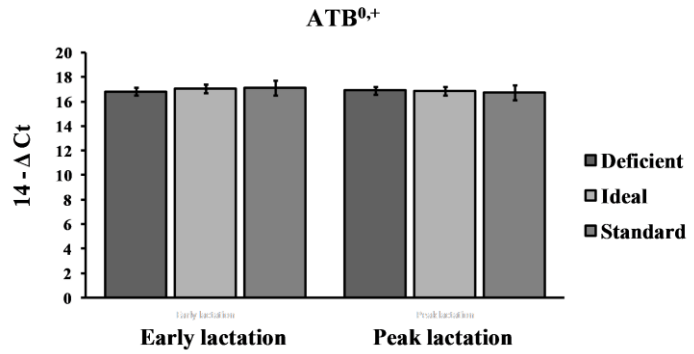
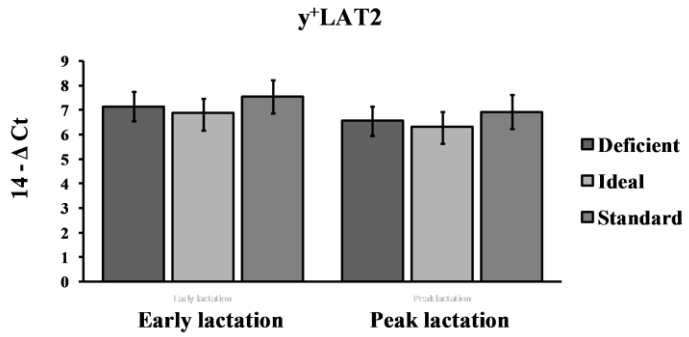


Figure 1g



Results for objective 3: Determine if crystalline AA inclusion in a reduced-CP diet increases the extraction efficiency (mammary AA/ AA intake) of limiting AA by the lactating mammary gland. Arterial concentration of essential AA, A-V difference and transport efficiency across the mammary gland are presented in Tables 4, 5 and 6, respectively. Arterial concentration of non essential AA, A-V difference and transport efficiency across the mammary gland are presented in Tables 3, 4 and 5 in Appendix. Arterial concentrations for all essential AA increased linearly ($P < 0.05$) with increasing CP concentration, except for His, Met and Trp. Arterial concentrations of Asn and Tyr increased linearly ($P < 0.05$) with increasing CP concentration decreased for Glu, Cit and Ala ($P < 0.05$), and remained unchanged for other non-essential 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 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 decreased 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.

Tables 4 to 7 are presented at the end of this document.

Results for objective 4: Determine if changes in AA transporter gene expression is directly related to the in vivo changes in mammary AA uptake and extraction efficiency (mammary AA/ AA intake). There was no relationship between AA uptake and extraction efficiency and expression of genes at the transcriptional level encoding for the Lys transporter proteins selected.

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 protein $ATB^{0,+}$ and more recently proteins $b^{0,+}$ 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) affected 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 where 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 (Manjarín et al., unpublished data). 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 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 (Manjarín et al., unpublished data). 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.

Results from this NPB-funded project were disseminated and acknowledged according to the NPB contract, so far, via the following:

Peer-reviewed literature

Manjarin, R., Zamora, V., Wu, G., Steibel, J.P., Kirkwood, R.N., Taylor, N.P., Liesman, J., Trifilo, K., and **N.L. Trottier**. Inclusion of crystalline amino acids in a reduced-crude protein diet maintains sow performance independently of amino acid transporter gene expression. J. Anim. Sci. *Accepted on 09/12/2011 with revisions.*

Manjarin, R., Steibel, J.P., Kirkwood, R.N., Taylor, N.P., and **N. L. Trottier**. 2011. Transcript abundance of hormone receptors, glucose transporters, mTOR pathway related kinases and ligand, and milk protein-encoding genes in mammary tissue of peri-parturient, lactating and post-weaned sows. J. Anim. Sci. doi: 10.2527/jas.2011-4179.

Manjarin, R., **Trottier, N.L**., Weber, P.S., Taylor, N.P., and J.P. Steibel. 2011. Simple analytical and experimental procedure for selection of reference genes for qPCR normalization data. J. Dairy Sci. 94: 4950-4961.

Manjarin, R., Steibel, J.P., Zamora, V., Am-in, N., Kirkwood, R.N., Ernst, C.W., Weber, P.S., Taylor, N.P., **Trottier, N.L**. 2011. Transcript abundance of amino acid transporters, β-casein and α-lactalbumin in mammary tissue of peri-parturient, lactating and post-weaned sows. J. Dairy Sci. 94: 3467-3476.

Invited talks (and papers published in conference proceedings)

Manjarin, R., Taylor, N.P., **Trottier, N.L.,** Weber, P.S., and J. P. Steibel. 2011. Simple analytical and experimental procedure for selection of reference genes for qPCR normalization data. **Presented at the 5th international qPCR Symposium & Industrial Exhibition & Application Workshop, Technical University of Munich, Physiology-Weihenstephan, Germany. 28th March – 1st April**.

Trottier, N.L. and Manjarin, R. 2010. Linking our understanding of mammary gland metabolism to amino acid nutrition. Midwest Swine Nutrition Conference Proceedings: 58-63. **Presented at the Midwest Swine Nutrition Conference, 10th Year Anniversary, Indianapolis, Indiana, September 9.**

Manjarin, R., Steibel, J.P., Zamora, V., Am-in, N., Kirkwood, R.N., Ernst, C.W., Weber, P.S., Taylor, N.P., **Trottier, N.L.** 2010. Transcript abundance of amino acid transporters, β-casein and α-lactalbumin in mammary tissue of peri-parturient, lactating and post-weaned sows. **Presented at the 5th Annual Animal**

Science Graduate Research Forum, September 10. **Department of Animal Science, Michigan State University.**

Abstracts in Conference Proceedings

Manjarin, R., Zamora, V., Wu, G., Steibel, J.P., Kirkwood, R.N., Taylor, N.P., Liesman, J., Trifilo, K., and **N.L. Trottier**. 2011. Crystalline amino acid inclusion in a reduced-crude protein diet increases mammary arginine and lysine apparent utilization and maintains sow performance independently of amino acid transporter gene expression. J. Anim. Sci. 89(E-Suppl. 2):80. **Presented at the American Society of Animal Science Midwest Meetings, Des Moines, IA, March 2011.**

Manjarin, R., Steibel, J.P., Kirkwood, R.N., Taylor, N.P., and **N. L. Trottier**. 2011. Transcript abundance of hormone receptors, glucose transporters, mTOR pathway related kinases and ligand, and milk protein-encoding genes in mammary tissue of peri-parturient, lactating and post-weaned sows. J. Anim. Sci. 89(E-Suppl. 2):141. **Presented at the American Society of Animal Science Midwest Meetings, Des Moines, IA, March 2011.**

Manjarin R., Steibel, J.P., Zamora, V., Am-in, N., Kirkwood, R., Ernst, C., Weber, P., Taylor, N.P., **Trottier, N.L**. 2010. Amino acid transporter mRNA abundance in porcine mammary tissue during pregnancy, lactation and post-weaning periods. J. Dairy. Sci. 93(E-Suppl. 1):672. **Presented at the American Society of Animal Science Midwest Meetings, Des Moines, IA, March 2010.**

Note: R. Manjarin was the Doctorate student that received partial funding from the NPB for his assistantship and conducted the research presented in this final report. Dr. Manjarin is now a post-doctoral fellow at University of California Davis under the supervision of Dr. Russ Hovey in the area of sow lactation physiology. Funding from the NPB allowed the generation of novel data, advancement in the area of sow nutrition and lactation physiology, environment and the training of many undergraduate students. Finally, Dr. Manjarin was recently awarded the 2012 ASAS Midwest Yound Scholar recognition in great part due to his work conducted under NPB funding.

Table 4. Effect of dietary CP concentration on AA arterial concentrations (μmol/ L) in lactating sows on d 7 and d 17 of lactation

	Stage of Lactation						*P*-value			
	Day 7			Day 17						
	Dietary CP, %			Dietary CP, %			Day 7		Day 17	
	9.50	13.50	17.50	9.50	13.50	17.50				
Item	Deficient	Ideal	Standard	Deficient	Ideal	Standard	L[e]	Q[f]	L[e]	Q[f]
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]$P < 0.01$. [e]Linear. [f]Quadratic.

Table 5. Effect of dietary CP concentration on AA arterio-venous differences (μmol / L) in porcine mammary gland at d 7 and d 17 of lactation

	Stage of Lactation						*P*-value			
	Day 7			Day 17						
	Dietary CP, %			Dietary CP, %			Day 7		Day 17	
	9.50	13.50	17.50	9.50	13.50	17.50				
Item	Deficient	Ideal	Standard	Deficient	Ideal	Standard	L[e]	Q[f]	L[e]	Q[f]
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]$P < 0.01$. [e]Linear. [f]Quadratic.

Table 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			Day 17						
	Dietary CP, %			Dietary CP, %			Day 7		Day 17	
	9.50	13.50	17.50	9.50	13.50	17.50				
Item	Deficient	Ideal	Standard	Deficient	Ideal	Standard	L[e]	Q[f]	L[e]	Q[f]
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]$P < 0.01$. [e]Linear. [f]Quadratic.

Table 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			Day 17						
	Dietary CP, %			Dietary CP, %			Day 7		Day 17	
AA:Lys	9.50	13.50	17.50	9.50	13.50	17.50				
	Deficient	Ideal	Standard	Deficient	Ideal	Standard	L[c]	Q[b]	L[c]	Q[b]
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]$P < 0.01$. [e]Linear. [f]Quadratic.