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ESPRESSION OF AMINO ACID TRANSPORTERS IN PORCINE MAMMARY TISSUE DURING LACTATION

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EXPRESSION OF AMINO ACID TRANSPORTERS IN PORCINE MAMMARY GLAND DURING LACTATION

By

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ABSTRACT

EXPRESSION OF AMINO ACID TRANSPORTERS IN PORCINE MAMMARY GLAND DURING LACTATION

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Efficient synthesis of milk protein is crucial to sustain normal growth of the nursing neonate and is dependent upon the intracellular availability of amino acids in mammary epithelial cells in very specific proportions. Thus, mammary cells must be equipped to recognize extracellular amino acids and regulate amino acid entry into the intracellular space in a coordinated fashion in order to optimize their utilization for milk protein synthesis. The extracellular availability of amino acids also is directly dependent upon dietary provision of amino acids. It is well recognized that a diet providing proteins composed of amino acids in the proportions in which they are required improves efficiency of utilization. There is an obvious gap of mechanistic understanding, however, between the processes involved in mammary use of extracellular amino acids and amino acid nutrition. Optimizing milk production and dietary protein utilization remains an overarching goal of swine production. The overall goal of dissertation research described herein was to determine the impact of nutritional and production (milk demand) factors on mammary amino acid utilization. The studies described in this dissertation are the first in lactating pigs and focus on amino acid transporters CAT-1, CAT-2B, ASCT1, and $B^{0,+}$. These transporters are distinguished for

transporting amino acids that are indispensable in the diet of sows during lactation.

The main hypothesis of this dissertation was that under conditions of protein nutritional stress (deficiency or excess), the mammary gland adaptively regulates amino acid transport by increasing (during deficiency) and decreasing (during excess) the level of expression of CAT-1, CAT-2B, ASCT1, and $B^{0,+}$ in porcine mammary gland, respectively. To test this hypothesis changes in transcript and protein abundance of amino acid transporters were determined in porcine mammary gland at two stages of lactation (milk demand).

Fundamental to the described studies was the development of a novel mammary biopsy technique that allowed for tissue collection from the same animal at different stages of lactation without affecting lactation performance. Results of the described studies provided (1) direct evidence for the presence of CAT-1 and CAT-2B, $B^{0,+}$, and ASCT1 mRNA and CAT-1 and ASCT1 protein in porcine mammary tissue; (2) evidence of a linear decrease in CAT-2B but not CAT-1 mRNA abundance with increasing maternal dietary protein intake from deficient to excess was determined; and (3) evidence of increasing ASCT1 and $B^{0,+}$ mRNA abundance in porcine mammary gland with milk demand was shown.

The observed differential regulation of these amino acid transporters in response to amino acid availability explain, in part, how the mammary gland responds to dietary protein intake and stage of lactation and the respective outcome in lactation performance. These contributions improve our understanding on protein nutrition of the lactating sow and enhance our ability to develop mechanistic models to ultimately maximize milk production.

In memory of

Jeannie L. Burton

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KEY TO ABBREVIATIONS

AA = amino acid

AV = arterio-venous

BCAA = branched chain amino acids

EAA = essential amino acid

MT = mammary tissue

NEAA = nonessential amino acid

PCR = polymerase chain reaction

Q-RT-PCR = quantitative real-time polymerase chain reaction

CHAPTER ONE

INTRODUCTION

The postnatal survival and optimum growth of neonates in mammalian species depends solely on the care and nutrition the mother provides through lactation. During this stage of development, maternal milk is the primary source of nutrients to guarantee the survival of the progeny and hence, the species.

Sow's milk yield and composition is the most important factor limiting growth of nursing pigs (Hodge, 1974; Harrell et al., 1993; Pluske and Dong, 1998). Milk yield increases with advancement of lactation and plateaus as early as day 15 and up to day 18 of lactation (Jones and Stahly, 1999). Conversely, up to 25% of nursing pigs are currently weaned at a very young age, i.e., between days 12 to 14. The early weaning practice provides health and economic benefits over conventional weaning, which is usually performed around 21 days of age (Hohenshell et al., 2000), but does not allow the nursing pig to benefit from the maximum milk production period. In addition, sow milk yield during the early phase of lactation is lower than desired to maximize the growth potential of her nursing pigs (Hartmann et al., 1997). For instance, artificially-reared piglets weaned shortly after birth and provided *ad libitum* access to liquid milk diet have the potential to duplicate their weight gain by 3 weeks of age (Harrell et al., 1993). Increasing piglet growth rate during lactation leads to higher weaning weights and decreased time to reach market weight (Slade and Miller, 1999). Selection pressure for prolific sows coupled with lower incidence of

piglet death has set milk production as one of the most important limiting factors behind nursing pig growth (Boyd and Kensinger, 1998).

Research efforts on malnutrition and its effect on lactation performance and milk composition are increasing worldwide, particularly in developing countries. Maternal nutrient intake, especially in suboptimal quantities affects milk composition and consequently, growth and development of neonates (Forsum and Lönnerdal, 1980; Noblet and Etienne, 1986; King et al., 1993; Motil et al., 1995; Zubieta and Lönnerdal, 2006).

Milk composition varies at different stages of lactation (Klobasa et al., 1987; Wu and Knabe, 1994). Shortly after parturition, the mammary gland secretes colostrum in small quantities. Colostrum is composed of high concentrations of immunoglobulins and growth factors. Protein percentage in sow's colostrum can be as high as 15 % (Oftedal, 1984). Post colostrogenesis, mature milk composition is relatively stable throughout the remainder of lactation (Klobasa et al., 1987; Csapó et al., 1996). Sow's mature milk is composed of 7.6 % fat, 5.5 % protein, 5.3 % carbohydrates and 18.7 % total solids (Oftedal, 1984; Csapó et al., 1996).

Studies in rat and other species have demonstrated that depressed maternal protein intake can diminish the total protein, nitrogen and non-protein nitrogen content of milk (Ronayne de Ferrer and Sambucetti, 1993; Sturman et al., 1986; Forsum and Lönnerdal, 1980; Motil et al., 1995). These changes in milk protein and nitrogen content are attributable mainly to changes in the casein-to-whey ratio, which decreases with increasing deficiency in dietary protein intake. It

has been hypothesized that nutritional stress impairs the functional integrity of epithelial cells, thus diminishing their synthetic capacity (Ronayne de Ferrer and Sambucetti, 1993). It has also been suggested that sow's milk is deficient in protein content for optimal lean body mass deposition (Williams, 1976). Sow's milk has a relatively low protein to energy ratio ranging from 9.2 to 10.4 g of protein per MJ GE, consequently, fat deposition is favored over protein deposition (Pluske et al., 1995). However, attempts to alter the protein profile of replacement milk to achieve higher protein to energy ratios and therefore, increase growth rate of neonates, have yielded controversial results (Sherry et al., 1978).

Milk protein secretion results from a series of processes that can be divided into four main steps: 1) amino acids removal from blood by mammary epithelial cells; 2) expression of milk protein-encoding genes in the mammary epithelial cells for synthesis of new proteins (Boisgard et al., 2001); 3) transport of plasma-borne proteins directly into mammary epithelial cells by transcytosis (Boisgard et al., 2001); and 4) transport and post-translational modification of newly synthesized and plasma borne proteins within the mammary epithelial cells. Of all organs, the lactating mammary gland has the highest demand for amino acids to meet the requirements of both milk protein synthesis and tissue growth (Boyd and Kensinger, 1998). In lactating sows, more than 95% of the essential dietary amino acid requirement is attributable to the need of the udder for protein synthesis (NRC, 1998). During lactation, 31 to 45% of whole-body protein flux is attributed to milk protein synthesis compared to 1 to 6% in dry,

non-pregnant animals (Champredon et al., 1990). In the dairy cow and goat, whole body protein synthesis is increased during lactation, a large proportion being attributable to mammary metabolism (Bequette and Backwell, 1997).

Of the indispensable amino acids utilized by the porcine mammary gland, lysine, threonine and valine in practical diets fed to sows have the potential to limit milk protein synthesis. To date, our understanding of the limitation of these amino acids is confined to empirical studies demonstrating their high requirements for milk protein synthesis relative to their low concentrations in feeds fed to lactating sows (Pettigrew, 1993). Uptake of certain indispensable amino acids by mammary epithelial cells is rate limiting for milk protein synthesis *in vitro* (Mephan, 1982; Maas et al; 1997).

Of the proteins synthesized by the mammary gland, up to 60% are excreted in the milk (Champredon et al., 1990; Oddy et al., 1988). Mammary protein secretion rate varies from 60 to 600 g per day in lactating humans and sows, corresponding to as much as 676 and 240 mg milk protein per g of weight gain in nursing infants and neonatal pigs, respectively. Amino acids extracted by the mammary epithelial cells are used mainly for the synthesis of caseins, the most abundant proteins in mammalian milks (Aimutis et al., 1982). Caseins are phosphoproteins, mammary derived and constitute over 40% of the proteins found in porcine milk; the remaining proteins include immunoglobulins and other blood borne proteins, collectively called whey proteins. Caseins and whey proteins average 2.74 and 2.22 %, respectively, in sow's milk (Oftedal, 1984). Whey proteins account for 85% of total protein in colostrum and 60% in mature

milk. The whey proteins, except for α-lactalbumin, are synthesized ex-situ and brought into the mammary gland to be deposited in milk.

Apart from their role as substrates for casein proteins synthesis, amino acids are used for the synthesis of constitutive proteins needed to support the metabolic functions of mammary cells. For instance, total protein synthesis in the dairy cow and goat lactating mammary gland is 1.3 to 2.5 fold the rate of milk protein secretion (Bequette and Backwell, 1997; Bequette et al., 1996). Other proteins in milk include enzymes such as lipoprotein lipase, lysozyme, plasmin, alkaline phosphatase, lactoferrin, and lactoperoxidase that facilitate digestion of the major milk components once milk is secreted and also act as immunomodulators (Hartmann and Holmes, 1989).

The genes that encode the proteins found in milk are not exclusive to the mammary gland and/or lactation. Caseins are encoded by a cluster of single-copy genes in a locus of around 350 kb (Rijnkels et al, 1997). The αs1- casein and β- casein genes are closely related genes while the κ-casein gene is not evolutionary related to these genes; the expression of these genes is nonetheless coordinated. In contrast, the principal whey proteins are encoded by a relatively small, single-copy, genes.

Expression of milk-specific genes occurs in the lobulo-alveolar epithelium of the lactating mammary gland and is regulated by nutrient availability and hormonal stimuli transmitted through a set of transcription factors that bind to enhancer elements on these genes (Rosen et al., 1998). Interestingly, the transcription factors that regulate the promoter regions of milk protein genes are

not specific to the mammary gland, nor restricted to lactation. Nevertheless, the unique combination and orchestrated efforts of these transcription factors and hormones result in mammary- and lactation-specific expression of milk protein genes. Of the processes involved in milk protein synthesis, the rate of amino acid uptake by mammary epithelial cells and the rate of intracellular protein synthesis may represent the two major limiting processes.

1. Intracellular milk protein synthesis

1.1. Role of transcription factors

Milk protein gene expression is controlled by promoter regions containing complex DNA elements referred to as composite response elements or CoREs. The CoREs are cluster of binding sites for transcription factors that act as positive or negative regulators of gene expression. Transcription factors act cooperatively to either enhance or repress expression of these genes. This type of gene regulation permits different levels of transcription depending on the combination of these positive and negative regulators of transcription. Some of the most studied transcription factors related to these CoREs are Stat5, CAAT enhancer binding proteins (C/EBPs), Nuclear factor (NFI), glucocorticoid receptor (GR), Yin Yang 1 (YY1), and octamer binding transcription factor 1 (Oct-1).

Stat5 is not mammary specific (Kazansky et al., 1995; Liu et al., 1995) and its expression in the mammary gland is not lactation-specific either. Stat5 is the primary transcription factor responsible for prolactin signaling in the mammary gland. Then again, other hormones not related to lactation, cytokines, and growth factors can activate Stat5. Two Stat5 genes (Stat5a and Stat5b) have been identified, with their protein products exhibiting 93% similarity at the amino acid level. In addition, both these genes encode different Stat5 isoforms that arise by alternative splicing. These alternative spliced isoforms cannot activate transcription and thus may act as transcription inhibitors instead (Jolivet et al., 1996).

The β-casein gene promoter contains four C/EBPs binding sites required for hormonal regulation. The C/EBP genes, like Stat5, also encode multiple protein isoforms by using alternative translational activation sites. Some of these isoforms can act as negative transcription factors inhibiting transcription. The ratio of the different C/EBPs therefore determines the resulting activation or repression of the milk protein genes.

NFI plays a critical role in the expression of WAP and several other whey proteins. However, NFI is not only expressed during the lactation period, and also plays a major role during involution of the mammary gland. Several NFI isoforms have been identified, and similarly to Stat5 and C/EBPs, some of these are activators while others are repressors of transcription.

Glucocorticoid response elements in milk protein genes are responsible for the cooperative effects of hydrocortisone and prolactin on expression at least in mammary epithelial cell lines (Lechner et al., 1997). However, the action of GR on regulation of milk protein gene expression has not been extensively studied apart from its cooperative role with Stat5.

Recently, another ubiquitous transcription factor, Oct-1, was shown to be present in mammary epithelial cells and to bind to a promoter region of the β-casein gene (Dong and Zhao, 2007). Oct-1 has a critical role in the hormonal activation of expression of the β-casein gene as demonstrated by a 70% reduced hormonal induction when the Oct-1 binding site is mutated.

The β-casein promoter region also contains a negative regulatory region that represses transcription in the absence of the lactogenic hormones in

mammary epithelial cells (Meier and Groner, 1994). Mutation of this region leads to hyper-transcription of these genes. YY1 has been shown to interact directly with this inhibitory region repressing transcription of the milk protein genes (Raught et al., 1994). Furthermore, C/EBPs and YY1 associate through protein-protein interactions to this negative regulatory site. Interestingly, the effects of YY1 in transcription repression are counteracted by Stat5 and vice versa (Raught et al., 1994). This suggests that the ratio of YY1 to Stat5 is important in the induction or repression of milk protein gene expression.

1.2. Role of lactogenic hormones and metabolites

Milk protein synthesis during lactation requires a major increase in amino acid partitioning towards the mammary gland (Bequette et al., 1994), which in turns is under complex homeorhetic control. Recently, relationships between circulating concentrations of lactogenic hormones and extraction by the porcine mammary gland on mammary amino acid uptake were investigated (Farmer et al., 2008). Amino acid AV difference was shown to be positively correlated to prolactin AV difference and to insulin arterial plasma concentration. This is supported by the fact that both prolactin and insulin are required for the full expression of milk protein genes (Nagaiah et al., 1981; Worthington-Roberts, 1997; Kozakai et al., 2002).

In both animals and humans, the synthesis of milk proteins and mammary enzymes is induced primarily by prolactin and further stimulated by insulin and cortisol (Nagaiah et al., 1981; Worthington-Roberts, 1997). Prolactin stimulates

β-casein gene expression in epithelial cell lines of mouse mammary gland after stimulation with dexamethasone (Kozakai et al., 2002). The effect of prolactin on milk protein gene expression is mostly mediated by activation of Stat5a and Stat5b by tyrosine phosphorylation through the JAK/STAT signal transduction pathway (Darnell, 1997). Circulating prolactin binds to the extracellular component of the prolactin receptor initiating the JAK/STAT signal transduction cascade. Specifically, prolactin induces dimerization of the receptor which, by means of transphosphorylation, activates JAK2 (Rui et al., 1994). The activated JAK2 tyrosine kinase in turn phosphorylates the prolactin receptor where proteins containing SH2 domains can dock (Pezet et al., 1997). The Stat5s contain such domains and consequently are phosphorylated, form a heterodimer, and translocate to the nucleus where they can activate transcription of the milk protein genes. In the absence of Stat5a or Stat5b, mice are unable to lactate (Liu et al., 1997) but β-casein gene expression is not dramatically altered (Liu et al., 1997) suggesting that the activation of one of the Stat5 alone is enough to induce β-casein gene expression.

Insulin and hydrocortisone alone cause little induction of β-casein gene expression in mammary epithelial cells, while addition of prolactin results in a marked increase in induction of this gene. In fact, pretreatment with glucocorticoids is crucial for induction of this gene by prolactin (Doppler et al., 1990). It has been hypothesized that glucocorticoids act indirectly by altering the levels of different C/EBPs isoforms in a way that lactogenic hormones can then prevent the repression in transcription caused by the C/EBPs transcription

factors directly at the promoter level (Schmitt-Ney et al., 1991). In contrast, the glucocorticoid receptor associates with Stat5 through protein:protein interactions and can act directly and synergistically to regulate β-casein gene expression (Stöcklin et al., 1996). Although the role of insulin on lactation in intact animals is well established (Lau et al., 1993), the molecular mechanism by which insulin cooperates with prolactin and glucocorticoids to induce milk protein gene expression is not well defined. What is clear is that the effect of insulin on milk protein gene expression occurs at the level of transcription and this effect is only minimally mediated by IGF-I and IGF-II (Prosser et al., 1987). One potent mechanism by which insulin could mediate induction of milk protein gene transcription is through its well established role in nutrient uptake and metabolism. For example, through induction of glucose uptake and the glucose metabolic pathway, insulin can activate transcription of several genes in the liver like GLUT2 (a glucose transporter) and insulin itself, and other enzymes (Vallet et al., 1997). Using primary cultures of mammary epithelial cells, a glucose-dependent increase in transcription of β-casein and α-lactalbumin that occurs at normal circulating glucose levels was demonstrated (Rosen et al., 1999).

1.3. Role of the extracellular matrix

The role of the extracellular matrix in mammary gland development and maintenance of differentiated lobulo-alveolar ducts is well established (Parry et al., 1987). However, our interest lies in the role of the extracellular matrix on milk protein gene expression during lactation. Studies using primary epithelial cells and mammary epithelial cell lines have revealed that, in addition to the presence of lactogenic hormones, interaction with the extracellular matrix is required for high levels of milk protein gene expression. This effect occurs via induction of cell morphological changes required for expression and induction of a signal that, in the presence of lactogenic hormones, induces milk protein gene expression. Specifically, laminin and β1-integrins are responsible for producing this signal (Edwards et al., 1998; Streuli et al., 1991). The induction of milk protein gene expression occurs via interaction with several promoter regions in these genes collectively called extracellular matrix-dependent enhancers (Schmidhauser et al., 1992). Most importantly, signaling by the prolactin receptor is not initiated in primary mammary epithelial cells in the absence of extracellular matrix (Edwards et al., 1998).

The importance of the cell:stratum interaction between the extracellular matrix and mammary epithelial cells on milk protein gene expression cannot be ignored when studying *in vitro* milk protein gene expression as cell and tissue culture systems may not simulate the intact mammary gland environment.

2. Intracellular Amino Acid Availability: Role of Amino Acid Transport Processes

Control of milk protein synthesis ultimately occurs within epithelial cells and it is here that amino acid supply may be limiting to milk protein synthesis. The intracellular availability of amino acids is determined in part by the activity of a range of amino acid transport processes (Baumrucker, 1985). In spite of their central importance to milk synthesis, there is relatively little known about mammary transport systems and their control in agriculturally valuable animals. Most of the available knowledge comes from rodent species (Shennan, 1998).

2.1. Current knowledge on amino acid uptake by the mammary gland

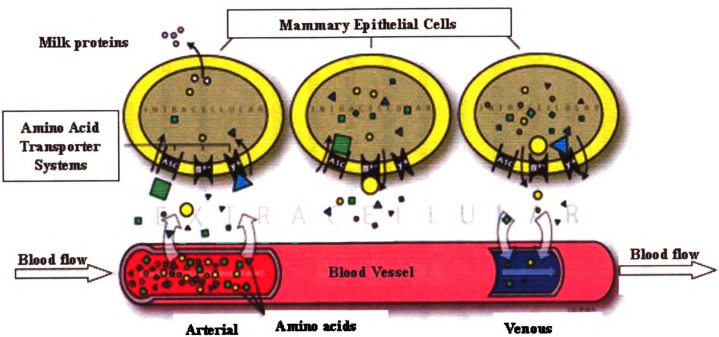
Arteriovenous (AV) exchange of amino acids across the lactating udder has been investigated in pigs (Linzell et al., 1969; Spincer et al., 1969; Trottier et al., 1995a and 1995b; Guan et al., 2002; Nielsen et al., 2002), cows (Guinard et al., 1994; Metcalf et al., 1994; Cant and McBride, 1995; Metcalf et al., 1996) and goats (Mepham and Linzell, 1966; Mepham, 1982; Bequette et al., 1997; Bequette et al., 2000) as a mean to indirectly assess the rate of transport of amino acids into mammary glands for milk production. This technique is based on the knowledge that amino acids are carried either as free entities or bound with albumin to cells through arterial supply and removed by cells via transport systems located on the apical surface of cell membranes. Amino acids not removed by cells appear in the venous circulation. Thus, the difference between arterial and venous amino acid concentration (AV difference) represents the

concentration of a particular amino acid removed by the cell. In all species studied, lactation is accompanied by a significant increase in AV difference for all amino acids compared to the non-lactating state. However, regulatory mechanisms facilitating this uptake are unknown. A simplified amino acid removal scheme across the circulatory system and mammary epithelial cells is presented in Figure 1.1

A number of AV tracer kinetic models have been used to address whether amino acid transport processes place a limitation on milk protein synthesis by the mammary gland *in vivo*, and whether transport activity defines limiting amino acids for milk protein synthesis. In lactating pigs, lactating goats (Bequette et al., 2000) and dairy cows (Schwab et al., 1992; Guinard et al., 1994), AV differences across the mammary gland for the majority of amino acids parallel milk protein secretion rate. It is not known if the parallelism observed between amino acid AV difference and milk protein secretion rate is directly related to transporter functions. Despite the reported abundant variety of amino acid transporter systems in numerous tissues and animal species, very few have been studied in mammary tissue. New experimental approaches are essential to address whether these transport processes place a limitation on milk protein synthesis by the porcine mammary glands during the developmental phases of lactation.

Maternal protein nutrition alters mammary uptake of amino acids in lactating sows (Guan et al., 2002; Trottier et al., 1997; Guan et al., 2004). Changes in amino acid extraction rate by the mammary gland in response to dietary and/or arterial amino acid availability indicate that mammary amino acid

Figure 1.1. Simplified representation of amino acid movement between the circulatory system, the extracellular space and mammary epithelial cells. Amino acids are carried via the arterial system (red) and diffuse into the extracellular space via blood flow (indicated by horizontal arrow) and concentration gradient. Amino acids are also transported via transporter systems from the intracellular space to the extracelular space, and from the extracellular space to the venous compartment (blue).


Mammary Epithelial Cells
Milk proteins
Amino Acid Transporter Systems
Blood flow
Blood Vessel
Blood flow
Arterial
Amino acids
Venous

uptake is a regulated process (Trottier et al., 1997; Bequette et al., 2000; Guan et al., 2004). For instance, when histidine was severely limiting in systemic supply, the goat mammary gland was able to increase extraction efficiency of histidine from 17 to 74% to avoid a dramatic decrease in milk protein synthesis (Bequette et al., 2000). To accomplish this, rates of inward and outward transport of histidine across the mammary gland were altered in favor of its net uptake (Bequette et al., 2000). Similar response was observed when feeding diets deficient in lysine (Guan et al., 2002). However, while lysine extraction increased, the mammary gland was unable to transfer sufficient quantities of lysine to maintain protein synthesis and net mammary protein balance presumably due to the fact that there is no endogenous synthesis of lysine compared to some for histidine. It is unknown whether the increase in extraction efficiency for lysine as reported by Guan et al. (2002) or histidine as reported by Bequette et al. (2000) was consequent to an increase in amino acid transporter capacity.

Milk production increases from approximately 6.5 to 8 kg per day (litter size of 7 to 8 pigs) from day 9 to 17 of lactation (23% increase) (Nielsen et al., 2002), respectively, which corresponds to a milk protein secretion rate of approximately 325 to 400 g per day, respectively. Several reports show that amino acid uptake is limited by stage of lactation (Nielsen et al., 2002; Guan et al., 2002) and presumably by milk demand. During peak lactation, AV difference for the majority of amino acids across the porcine udder increased by approximately 25% relative to day 9 of lactation. The increase in AV difference

corresponded to an increase in extraction rate of approximately 40% for the majority of amino acids to up to 74% for arginine (Nielsen et al., 2002). Similarly, in a recent study, Guan and coworkers (2004) found that the total AV difference of dietary essential amino acids across the porcine udder increased from 287 to 377 μmol/L from day 10 to 18 of lactation, corresponding to a 31% increase. Similarly, Nielsen and coworkers (2002) reported that the increase in milk production with progression of lactation was associated with increased plasma amino acid AV difference rather than blood flow across the porcine mammary gland. It was also demonstrated that changes in plasma amino acid AV difference paralleled changes in milk production level and net milk protein secretion (Guan et al. 2002; Trottier, 1997; Nielsen et al., 2002). In both studies (Nielsen et al., 2002 and Guan et al., 2002), amino acid AV difference and extraction rate also decreased past peak milk production (i.e., from day 18 to 24 of lactation) by approximately 15 and 25%, respectively, for the majority of the essential amino acids. This decrease in AV and extraction rate paralleled a 10% decrease in milk production (Nielsen et al., 2002). Schawb and coworkers (1992) demonstrated a reduced responsiveness of the udder to amino acid availability for milk protein synthesis with advancement of lactation. Taken together, stage of lactation may dictate milk protein synthesis in part via regulatory control of amino acid transfer processes across the mammary gland.

To date, two main restrictions to the net quantitative uptake of amino acid across the capillary wall into the extracellular mammary space have been suggested: 1) the rate at which blood flow transports amino acids to the arterial

capillary; and 2) blood concentrations of amino acids. However, these factors are probably not important in defining differences in mammary amino acid uptake and milk protein secretion with increasing days of lactation based on the notion that blood flow remains relatively constant throughout lactation (Nielsen et al. 2002) and that arterial concentrations of indispensable amino acids either vary inversely with milk production (in particular of threonine and valine) or do not change as in the case for lysine.

2.2. Amino acid transport systems and proteins

Uptake of amino acids by mammary gland epithelial cells is thought to be mediated by several transport systems with unique kinetic and regulatory properties (Shennan et al., 1997). Several transport systems with different substrate specificities for amino acids have been identified and characterized in a variety of mammalian cell systems. Amino acid transfer processes across mammary tissue have been studied using *in vitro* approaches in mice (Van Winkle et al., 1985; Verma and Kansal, 1995), rats (Kilberg et al., 1981; Shennan et al., 1994; Shennan et al., 2002; Howard et al., 2004; Lopez et al., 2006) and pigs (Hurley et al., 2000; Jackson et al., 2000) and have enabled kinetic definitions of amino acid transport and, with these, assignments of amino acids to specific systems sharing similar transport kinetic properties. Transport systems are characterized by the type of amino acid that they transport and by the thermodynamic properties of the transport. Specific transport systems carry different types of amino acids and different transport systems show overlapping

specificities for amino acids (Palacín et al., 1998). Transport systems are either ubiquitous or tissue-specific. Tissue-specific transport systems are usually isoforms of ubiquitous transport systems (Palacín et al., 1998). Of main interest for sow lactation is the study of the transporter systems y^+, ASC, and $B^{0,+}$ in mammary tissue because these are specific for amino acids that are essential in the diet of sows and thus can limit milk protein synthesis. These systems have been isolated and their cDNAs cloned from various non-mammary tissues as well as human and ovine mammary tissue (Table 1.1). Amino acid transport systems that have been kinetically defined in mammary tissue are presented and discussed below. Of all the studies reviewed, only two (Hurley et al., 2000; Jackson et al., 2000) were found that pertain to amino acid transport systems in porcine mammary tissue, highlighting an important gap in knowledge.

2.3. Transport characteristics of amino acid transport systems of interest

The uptake of cationic amino acids (lysine, arginine, ornithine, and histidine) is mediated by five transporter systems, namely y^+, y^+L, b^+, $b^{0,+}$, and $B^{0,+}$ (Hyde et al., 1993; Palacin et al., 1998). The y^+ system and possibly the $B^{0,+}$ system likely make the major contributions to lysine uptake in sow mammary tissue (Shennan et al., 1997). These systems differ in their affinity for cationic amino acids, their dependence on sodium for transport of amino acids, and their capacity to additionally transport zwitterionic amino acids (Palacin et al., 1998). System y^+, also referred to as the CAT (cationic amino acid transporter) family

TABLE 1.1

Amino acid transporter systems and proteins names, amino acid affinity, and known species and tissues distribution.

Amino acid transporter system	Amino acid transporter protein	Affinity for amino acids	Known tissue distribution	Main species studied	References
Y^+	CAT-1	lysine, arginine, ornithine, histidine	*mammary gland,* testis, bone marrow, brain, stomach, spleen, kidney, lung, ovary, uterus, large and small intestine, thymus, heart, skeletal muscle, skin	Rodent, Ovine	Kakuda et al., 1993; Kim et al., 1991; Kiaei et al., (unpubl.)
Y^+	CAT-2A	lysine, arginine, ornithine, histidine	*mammary gland* , liver, skeletal muscle, skin, ovary, stomach	Rodent, Human	Pérez Laspiur et al., 2004; Closs et al., 1993 & 1997; MacLeod and Kakuda, 1996
Y^+	CAT-2B	lysine, arginine, ornithine, histidine	*mammary gland* , liver, lung, brain, activated macrophages, splenocytes, ovary, skeletal muscle, testis, thymus	Rodent, Human	Pérez Laspiur et al., 2004; Closs et al., 1997; Kakuda et al., 1993; Ito and Groudine, 1997
$B^{0,+}$	$B^{0,+}$	isoleucine, leucine, valine, methionine, lysine, arginine, alanine, glycine	*mammary gland*, lung, trachea, salivary gland, stomach, pituitary gland	Human	Sloan and Mager, 1999; Pérez Laspiur et al., 2004
ASC	ASCT1	alanine, serine, cysteine, threonine	*mammary gland* , hippocampus, skeletal muscle, pancreas, brain, heart, placenta, lung, kidney, liver	Human	Shafqat et al., 1993; Arriza et al., 1993; Pérez Laspiur et al., 2004

transport system, is a high affinity, sodium-independent transporter, and is specific for cationic amino acids. It is also capable of carrying zwitterionic amino acids across cell membranes, though with low affinity and in a sodium-dependent manner (Closs, 1996). The y^+ transporter system is of particular interest because lysine is the first dietary limiting amino acid for milk protein synthesis in lactating pigs (Shennan et al., 1997), and growing evidence shows that arginine may be a dietary essential amino acid for lactation in the pig (Pérez Laspiur and Trottier, 2001; O'Quinn et al., 2002). Thus, it is likely that the y^+ system is predominantly responsible for lysine and arginine transport into mammary tissue for milk protein synthesis.

The nature of cationic amino acid transport system in porcine mammary tissue is controversial, and the transporter for lysine has not been identified (Hurley et al., 2000). Lysine was originally proposed by Baumrucker (1985) to be primarily transported via the classical y^+ system in mammary tissue. Now, however, lysine is believed to be transported in porcine mammary tissue via a Na^+-independent system that differs from the classical y^+ system (Hurley et al., 2000). This belief is based on two findings: 1) lysine uptake in porcine mammary tissue occurs via a Na^+-independent transport mechanism with a *K*m of approximately 1.4 mM (Hurley et al., 2000), which is 3 to 10-fold higher than the reported *K*m for y^+ systems in any other tissues (Closs et al, 1997); and 2) lysine uptake in porcine mammary tissue is not specific for lysine, because lysine uptake can be inhibited 50% by supra-physiological concentrations of L-leucine, L-alanine, and L-methionine (Calvert and Shennan, 1996; Hurley et al., 2000). In

support of this argument, arginine was shown to be transported via two systems in the mouse mammary gland, one specific for cationic amino acids (i.e., the classical y^+ system) and the other capable of interacting with both cationic and neutral amino acids, such as leucine (Sharma and Kansal, 1999). In fact, the *K*m for arginine uptake in mouse mammary tissue via the y^+ system was reported to be 0.76 mM (Sharma and Kansal, 1999), which, as argued for lysine, is approximately 10-fold higher than the reported *K*m for arginine uptake in other tissues (Closs et al., 1997). Admittedly, one cannot rule out that the *K*m for lysine uptake via the y^+ system in porcine mammary tissue may be much higher than that found in other tissues, given the uniqueness of the mammary gland in protein synthetic capacity. On the other hand, the fact that lysine uptake is only partly inhibited by supra-physiological concentrations of neutral amino acids but strongly inhibited by physiological concentration of arginine (Hurley et al., 2000) suggests that system y^+ contribution to lysine uptake in sow mammary tissue is of physiological importance, supporting the original argument of Baumrucker (1985). Calvert and Shennan (1996) and Sharma and Kansal (1999) proposed that the mammary cationic amino acid transport system that interacts with neutral amino acids may be a tissue specific variant of system y^+ or y^+L, respectively. With the notion that lysine uptake by lactating rat mammary tissue (Shennan and McNeillie, 1994) and porcine mammary tissue (Hurley et al., 2000) is not dependent upon Na^+, the contribution to cationic and neutral amino acid interactive uptake by system $B^{0,+}$ is unlikely. Both y^+ and $B^{0,+}$ transport system family members remain to be cloned from bovine or porcine mammary tissue.

The branched-chain amino acids (BCAA) isoleucine, leucine and valine are retained by the porcine (Trottier et al., 1997; Nielsen et al., 2002) and bovine (Clark et al., 1980; Hanigan et al., 1991) mammary gland in excess of their output in milk. The branched chain amino acids, in particular valine, seem to play an important role in the process of milk protein synthesis and overall mammary metabolism (Richert et al., 1996; Richert et al., 1997; Guan et al., 2002). To date, there is very little understanding of their importance relative to metabolic functions other than protein synthesis in the mammary gland. The BCAA are potentially taken up via at least four systems, including the ASC and $B^{0,+}$ systems (Palacin et al., 1998). These systems transport zwitterionic amino acids in a sodium-dependent manner. Jackson and coworkers (2000) previously found that only about half of the uptake of valine by lactating porcine mammary tissue occurred via a sodium-independent mechanism, presumably the L system. Valine is largely taken up by a Na^+-dependent system (Jackson et al., 2000). Two Na^+-dependent amino acid transport systems with substrate specificity for BCAA have been identified in mammary tissues of some species, system $B^{0,+}$ (Sloan and Mager, 1999), and system ASC (Arriza et al., 1993; Shennan, 1998).

System ASC has a preference for linear dipolar amino acids (alanine, serine, and cysteine) but has highest affinity for amino acids containing distal hydroxyl group, such as threonine (Arriza et al., 1993). It is discernible from system A (neutral amino acid transporter) in that it does not recognize *N*-methylated amino acids and is pH insensitive (Makowske and Christensen, 1982; Vadgama and Christensen, 1984; and Utsunomiya-Tate et al., 1996). System

ASC has been shown to be a major component of Na^+-dependent transport of amino acids in a number of tissues and species, including rabbit intestine and reticulocytes, pigeon erythrocytes, and human fibroblasts (White, 1985). Cellular uptake of BCAA in sow mammary tissue was characterized to describe the kinetic properties and substrate specificity of the valine uptake system (Jackson et al., 2000). The valine transport system in porcine mammary tissue has a *K*m of 0.64 mM and a V_{max} of 1.84 mmol/kg cell. The characterization of the level of expression of system ASC is of interest because of its specificity for BCAA, and to the ASCT1 specificity for threonine (Neville et al., 1980), the second limiting amino acid for milk protein synthesis in lactating sows (Pettigrew, 1993).

System $B^{0,+}$, characterized in mouse blastocysts, has broad specificity with high affinity for both cationic and neutral amino acids that is Na^+ and Cl^--dependent (Van Winkle et al., 1985), but has highest affinity for hydrophobic amino acids (the BCAA isoleucine, leucine, and valine, the sulfur amino acids methionine and cysteine, and the aromatic amino acids phenylalanine and tryptophan) (Sloan and Mager, 1999). System $B^{0,+}$ is well characterized for its inhibition by bicyclic amino acids such as 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid. Because system $B^{0,+}$ is a broad specificity transporter, characterization of its expression level in porcine mammary tissue will provide further insight into its physiological relevance to amino acid uptake regulation in that system.

While the changes in AV difference reported in the literature may be mediated via changes in amino acid transporter affinity and kinetics (i.e., *K*m and

Vmax), the focus of this dissertation is to begin to test the hypothesis that AV differences and milk protein secretion differences related to dietary protein intake and across day of lactation are related to the level of expression of mammary amino acid transporters.

2.4. Molecular and kinetic characteristics of transporter systems and proteins of interest

2.4.1. CAT family system

CAT-1 was the first of the CAT family of transporters studied (Palacin et al., 1998). Cloning of this transporter resulted from studies demonstrating induction of cationic amino acid transport by the murine ecotropic leukemia virus (ecoR) (Kim et al., 1991), which was cloned and called CAT-1. The murine CAT-1 is a 622 amino acid glycoprotein with a predicted molecular mass of 67 kDa (Albritton et al., 1989). It has the substrate specificity and kinetic properties consistent with the high-affinity, low-capacity, system y^+ properties (White, 1985). The human gene homologous to the murine CAT-1 was cloned and mapped to chromosome 13q12-13 (Yoshimoto et al., 1992; Albritton et al., 1992). This gene consists of 10 introns and 11 exons, which code for an mRNA transcript of 9kb with an open reading frame of 1.8kb (reviewed by Malandro and Kilberg, 1996).

Isolation and cloning of CAT-2A, the second CAT transporter to be studied, occurred from investigations of genes involved in T-cell function during immune responses (MacLeod et al., 1990). The full-length cDNA (called Tea) predicted a 50-kDa protein of 658 amino acids (Reizer et al., 1993). This low

affinity transporter was subsequently isolated from murine liver, where it appears to be abundantly expressed (Closs et al., 1993a). Cloning of the third member of the CAT family, CAT-2B, was performed by Closs and coworkers (1993b) using mouse lymphocytes and macrophages. CAT-2A and CAT-2B are encoded by one gene in mouse and are known to arise through alternative splicing, which appears to confer tissue expression characteristics of these transporters (Closs et al., 1993b).

The fourth member of the CAT family is called CAT-3 and was isolated from mouse and rat brain tissue by Hosokawa and coworkers (1997) and Ito and Groudine (1997). The CAT-3 transporter was shown by these investigators to be brain-specific, and the mouse and rat counterparts exhibit 95% identity in amino acid sequence. Vékony and coworkers (2001) cloned the homo sapiens CAT- 3 transporter and reported that it is expressed in the thymus, lymphoma cells, uterus, testis, mammary gland, ovary, stomach, as well as the brain in humans. The human cationic amino acid transporter (formerly CAT-3) is now called CAT-4 and maintains high sequence homology to the other members of the cationic transporters (Sebastio et al., 1997). However, the literature on this transporter is scarce (Palacín et al., 1998) and will not be discussed further.

The members of the cationic amino acid transporters have good homology between them at the DNA sequence level. The amino acid sequences of murine CAT-2A and CAT-2B maintain ~60% homology with CAT-1 (Closs et al., 1993a; Reizer et al., 1993). The deduced amino acid sequences for CAT-2A and CAT-2B differ in 19 amino acid residues in humans (Closs et al., 1997), and on a

stretch of 42 amino acid residues in mouse (Albritton et al., 1992; Kavanaugh et al., 1994). It has been proposed that all the members of this cationic amino acid transporter group are isoforms resulting from mutually exclusive alternate splicing of a primary transcript (MacLeod and coworkers quoted in MacLeod and Kaduka, 1996). There is also evidence that the different isoforms result from transcriptional initiation at distinct promoters (Finley et al., 1995). Posttranscriptional regulation of CAT-1 gene expression with highly regulated and rapid turnover of the CAT-1 transcript modifies the expression of CAT-1 in various tissues (Aulak et al., 1999; Closs, 2002). CAT-1 and CAT-2A/2B expression has been reported in epithelial cells of several tissues and in various cell lines (Palacin et al., 1998; Closs et al., 1993a; Closs, 1996; Closs et al., 1997; Burger-Kentischer et al., 1998; Cariappa et al., 2002). Tissue distribution of CAT-1 is relatively ubiquitous in humans, consistent with system y^+ expression properties in several other species, except in hepatic tissue (Closs et al., 1997; Burger- Kentischer et al., 1998). CAT-2A is expressed abundantly in hepatic tissue (Closs et al., 1993a).

2.4.2. ASC System

Two members of the ASC family have been isolated: ASCT1 from human hippocampus (Shafqat et al., 1993; Arriza et al., 1993) and ASCT2 from mouse testis (Utsunomiya-Tate et al., 1996). It is proposed herein to study system ASCT1 due to its specificity for threonine, the second limiting amino acid after lysine for milk protein synthesis in lactating pigs (Pettigrew, 1993). Arriza and

coworkers (1993) and Shafqat and coworkers (1993) cloned the ASCT1 transporter cDNA and reported that it codes for a 532-amino acid protein. They also reported that ASCT1 has an open reading frame of 1572 bp which encodes 524 amino acid residues distributed over 8 exons. ASCT1 also functions as a receptor for the baboon endogenous retrovirus in certain conditions (Marin et al., 2000).

ASCT2 was cloned by Fairman and coworkers (1995) and encodes for a 553-amino acid protein. The difference between the ASCT1 and ASCT2 isoforms is substrate specificity, where ASCT2 recognizes as substrate a broader set of amino acids (Bussolati et al. 1992). The system ASC transporters show 40 to 44% identity to the human glutamate transporter, and can also transport glutamate with low affinity at neutral pH (Vadgama and Christensen, 1984).

2.4.3. $B^{0,+}$ System

System $B^{0,+}$ was first cloned by Sloan and Mager (1999) and called $hATB^{0+}$. The human cDNA for this transporter is 4.5 kb in length and encodes a 642-amino acid protein. It has a high sequence similarity to the glycine and proline transporters. Tissue distribution of this transporter has been reported for humans, with the highest expression in the lung, trachea, and salivary gland, and lower levels of expression in the mammary gland, stomach, and pituitary gland (Sloan and Mager, 1999).

There is no published information demonstrating the molecular presence of amino acid transporters in porcine mammary tissue. Indeed, only human $B^{0,+}$

(Sloan and Mager, 1999), CAT-3 (Vékony et al., 2001), GLT-1 (Berger and Hediger, 2006), the ovine CAT-1 (Kiaei et al., unpublished), and the rat LAT1, LAT2 (Shennan et al., 2002), and SNAT2 (Lopez et al., 2006) transport proteins have been studied in the context of mammary tissue expression. In this dissertation, the presence of these transporters in porcine mammary tissue during lactation will be investigated.

2.5. Regulation of amino acid transporter expression

2.5.1. Amino acid availability

Expression of various amino acid transporters is affected by amino acid availability (Shennan et al., 1994; Taketani et al., 1998; Fernandez et al., 2001; Christie et al., 2001; Closs, 2002; Kimball and Jefferson, 2006). In general, excess amino acid availability promotes anabolism and global protein synthesis (Fafournoux et al., 2000). This can occur by a global increase in mRNA transcription, stability and translation. Conversely, when amino acids are in short supply, global protein synthesis is depressed to the point of entering a catabolic state. In this state of proteolysis, a select group of genes are transcribed and translated at an increase rate via a process known as adaptive regulation. These groups of selected genes are related to the biosynthesis and transport of amino acids into cells (Barbosa-Tessmann et al., 2000). During adaptive regulation, amino acid transporter expression, stability and activity are increased to ensure the uptake of amino acids for the sustainability and survival of the cell in a nutrient deprived environment. An example of these genes are the transporters

SNAT2 (system A; Lopez et al., 2006); CAT-1 (system y^+; Aulak et al., 1999), ASCT1 (system ASC; Howard et al., 2004) and $B^{0,+}$ (system $B^{0,+}$; Satsu et al., 1998) among others.

Amino acid availability stimulates uptake of amino acids via the system y^+ through *trans* stimulation, with CAT-1 exhibiting a greater rate of *trans* stimulation than CAT-2 (Closs et al., 1997). On the other hand, through adaptive regulation, amino acid starvation increases CAT-1 mRNA and protein levels. Indeed, mRNA levels for CAT-1 increased 3.8 to 18 folds in C6 glioma and NRK kidney cells following a 2-h amino acid starvation (Aulak et al.; 1999). An increase in translation via an internal ribosomal entry sequence within the 5' untranslated region of the CAT-1 transcript has also been demonstrated (Fernandez et al., 2001). Yaman and coworkers (2002) also demonstrated that during amino acid starvation, CAT-1 mRNA stability increased with corresponding increase in CAT-1 protein expression. Yet, long-term protein malnutrition or dietary protein deficiency causes down regulation of these amino acid transporters (Hyde et al., 2003).

2.5.2. Lactogenic hormones and metabolites

Hormonal availability also plays a role in the expression of several amino acid transporters. Increased uptake of amino acids by mammary tissue explants has been reported in the presence of lactogenic hormones such as prolactin, insulin, and also in the presence of glucocorticoids and glucose (Sharma and Kansal, 2000; Simmons et al., 1996). In the same manner, hepatic treatment with

insulin and glucocorticoids increased CAT-1 expression through increase in mRNA transcription and stability (Liu and Hatzoglou, 1998). Another important piece of evidence related to regulation of amino acid transporter expression is the finding that glucose limitation can cause an increase in CAT-1's mRNA and protein levels, with a consequent increase in arginine uptake (Fernandez et al., 2002) similar to the effect of amino acid starvation. The amino acid transporter $B^{0,+}$ capacity of transport seems to increase in blastocysts during implantation by the presence of estrogen (Kilberg et al., 1993).

2.5.3. Inflammation

The importance of CAT-2 regulation by external factors can be clearly appreciated in cytokine-activated cells in which nitric oxide (NO) production is increased. CAT-2 seems to be required for sustained NO production, and its expression increased when cells or tissues are activated in order to increase their NO supply (Nicholson et al., 2001). This is accomplished by increasing the expression of CAT-2B and, in some cases, an increase in CAT-1. Kakuda and coworkers (1998) reported that food deprivation and surgical trauma increases CAT-2A transcripts and that activated macrophages show an increase in CAT-2 transcript and protein, but a decrease in CAT-1 transcripts. Similar results have been reported subsequently (Kakuda et al., 1999; Nelin et al., 2001). In some cases where cells are challenged with lipopolysaccharide and interferon gamma, all CATs transcripts (CAT-1, CAT-2A and CAT-2B) increase (Baydoun et al., 1999) and this increase appears to be regulated by the p38 mitogen-activated

protein kinases. On the other hand, polyamines like spermine inhibit NO production via depressed arginine uptake in activated macrophages by downregulating CAT-2B mRNA abundance (Mossner et al., 2001).

MAIN HYPOTHESIS AND OBJECTIVES

In spite of their central importance to milk synthesis, there is relatively little known about mammary transport systems and their control in agriculturally valuable animals. It is not known if the parallelism observed between amino acid AV difference and milk protein secretion rate is directly related to transporter functions. Although there is abundant variety of amino acid transporter systems in numerous tissues and animal species, very few have been studied in porcine mammary tissue. Furthermore, the extent to which these transport processes control the entry of amino acids into porcine mammary tissue for milk protein synthesis has not been studied. New experimental approaches are essential to address whether these transport processes place a limitation on milk protein synthesis by the porcine mammary glands during the developmental phases of lactation.

Increases in amino acid transport into mammary epithelial cells during lactation may be mediated via two main mechanisms: (i) increased tissue sensitivity for amino acid transport via increased affinity of transporters for amino acids; and (or) (ii) a change in capacity for transport via an increase in mammary expression of key amino acid transporter systems. Acknowledging that amino acid transport into mammary epithelial cells may be mediated via multiple

complex mechanisms, it is proposed that the change observed in milk protein secretion rate with lactation demand is regulated, in part, by an alteration in capacity for amino acid transport across mammary epithelial cells.

The studies described herein will begin to address whether or not maternal protein nutrition and stage of lactation exerts regulatory control of the amino acid transfer processes across the mammary gland. The hypothesis of the present study is that under conditions of protein nutritional stress, the mammary gland adaptively regulates amino acid transport. To test this hypothesis gene expression of amino acid transporters (i.e., CAT-1, CAT-2A, CAT-2B, ASCT1, and $B^{0,+}$) will be determined at two stages of lactopoesis. The main objective is to examine changes occurring in the expression of amino acid transporters specific for the transfer of lysine, threonine, and valine across mammary tissue during lactation as these amino acids are limiting in diets of lactating sows. To achieve this, mammary parenchyma mRNA and protein abundance for the various transporters will be measured as an indicator of expression. Additional objectives of this thesis include 1) the development of a biopsy technique in mammary gland that permits collection of mammary tissue at different stages in lactation from the same animal, thus decreasing the number of animals used in the study and biological variation in the analysis; 2) investigate the effect of protein nutritional stress in lactation performance, specially milk protein yield and piglet growth rate; and 3) determine the effect of protein nutritional stress and milk demand on mammary amino acid transporter expression. These objectives are addressed in the following chapters.

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CHAPTER TWO

Kirkwood[b], R. N., Pérez Laspiur[a], J., Ames[b], N. K., Moore[c], J. B., Cegielski[d], A., and N. L. Trottier[e]. 2007. Mammary gland biopsy does not affect lactation performance in sows. Can. J. Anim. Sci. 87: 281-284.

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CHAPTER TWO

MAMMARY GLAND BIOPSY DOES NOT AFFECT LACTATION PERFORMANCE IN SOWS

Abstract

To determine morphological and molecular characteristics of porcine mammary tissue *in vivo*, mammary tissue was collected from 18 sows at 3 to 6 days of lactation and 17 to 19 days of lactation using a biopsy technique. The success of the technique was determined by monitoring lactation performance, as evidenced by sow rectal temperature, voluntary feed intake, milk somatic cell count, and piglet average daily gain. Up to 1.7 g of mammary tissue was collected at each biopsy without decreasing sow feed intake or piglet growth.

Keywords: Biopsy, mammary gland, lactation, sow

Introduction

Investigating developmental changes in pig mammary tissue composition and metabolism during lactation has historically been achieved by partial or total mammary tissue collection following the serial slaughter of sows (e.g., Kim et al. 1999). Serial slaughter is costly and wasteful of animals, in particular because it requires a relatively large number of sows to account for animal variation. Furthermore, post-mortem decay of cellular components can occur in only

minutes (Wilusz et al. 2001), so time of tissue collection after euthanasia, compared to tissue collection in vivo, may alter the integrity of these components. A mammary needle biopsy approach has been used in different animal species, including lactating goats (Cvek et al. 1998) and sows (Magnusson 1999, Thiel et al. 2005). However, experimental requirements may arise necessitating the collection of relatively large tissue samples. Cerveza et al. (2000) reported the collection of relatively large (1.5 g) sow mammary gland samples in early and late lactation, but few details of the procedure were provided. A mammary gland biopsy procedure was adequately described by Richert et al. (1998), but only single samples of mammary tissue were obtained. Serial biopsy samples to monitor changes in mammary composition or metabolism may be necessary, but subsequent biopsies may be impacted by inflammatory responses to the first biopsy affecting sow lactation performance. In none of the earlier studies were potential adverse effects on sow lactation performance investigated. Therefore, our primary goal was to obtain mammary tissue from the same sow at two stages of lactation in sufficient quantity to perform RNA and protein abundance assays, morphometric analysis and immunohistochemistry. Our secondary goal was to determine any effects on the welfare of the sow and litter as evidenced by piglet growth.

Materials and Methods

The All-University Committee on Animal Use and Care of Michigan State University approved all procedures performed in this study. At 110 day of gestation, 18 multiparous sows (12 Landrace x Yorkshire and 6 Yorkshire) were moved to individual crates within an environmentally controlled farrowing room. Room temperature was maintained at 20°C and photoperiod controlled at 10 h of light and 14 h of darkness. Litters were equalized to 8 piglets per sow by cross-fostering within 36 h of birth.

Sows were fed corn-soybean meal-based diets formulated to meet all nutrient requirements according to NRC (1998) for lactating sows housed in a thermoneutral environment (20°C) and nursing 8 piglets with a predicted average daily gain of 225 g d^{-1}. Throughout the study, sows were fed twice daily at 0800 and 1600. Prior to farrowing, sows received a total of 2.5 kg d^{-1}. After farrowing, on days 1, 2, and 3 of lactation sows were fed a total of 2, 4, and 6 kg, respectively. Thereafter, sows were fed to-appetite. Fresh water was freely available throughout the experimental period.

Sow feed intake was recorded daily from 1 to 20 days of lactation. Orts were collected before the morning meal, weighed and discarded. Sows were weighed on 1 and 17 days of lactation and piglets were weighed individually on days 1, 4, and 18 of lactation. Milk samples were collected manually at 3 and 17 days of lactation from the first and second thoracic mammary glands on each side of the udder (4 glands total) and pooled within sow. Milk letdown was

stimulated by intramuscular injection of 10 IU oxytocin (Vet Tek, Phoenix Scientific, St.Joseph, MO). Milk samples were preserved with 8 mg of 2-bromo-2-nitropropane-1,3-diol (bronopol) and 0.3 mg of natamycin (Broad Spectrum Microtabs II, D&F Control Systems, Inc., Son Ramon, CA) and kept refrigerated for determination of somatic cell counts (Dairy Herd Improvement Association, East Lansing, MI, USA).

Mammary gland tissue was collected between 3 and 6 days and again between 17 and 19 days of lactation from the first and second thoracic mammary glands, respectively. On the day of biopsy sows were fed 2 kg of their morning meal and then from 60 min after feeding, piglets were isolated for 45 min. Isolated piglets were kept in a pen adjacent to the sow equipped with a heat lamp and ample bedding. Following the 45 min isolation period, piglets were returned to sows and allowed to nurse. Piglets suckling the glands to be biopsied were identified on their back using a permanent marker. Immediately upon cessation of milk letdown, sows were rapidly anesthetized by intramuscular injection 1.0 mL per 34 kg BW of TKX [(250 mg tiletamine and 250 mg zolazapam (Telozol®; Fort Dodge Animal Health, Fort Dodge, IA), dissolved in 2.5 mL ketamine (VetaKet®; Lloyd Laboratories, Shenandoah, IA) and 2.5 mL xylazine-100 (AnaSed®; Lloyd Laboratories)].

Piglets were removed to their isolation pen, and the sow remained in her farrowing crate. While under anesthesia, sows were positioned in lateral recumbency to expose one entire lateral side of the udder. One mammary gland was prepared for biopsy using Povidone-iodine scrub, USP, 7.5 % (Novaplus,

Novation, Irving, TX) followed by rinsing with 70% ethanol and cleaning with Povidone-iodine solution, USP, 10% (Novaplus, Novation) (Figure 2.1). Following subcutaneous and intramammary injection of 1.0 mL of 2% lidocaine (Lidoject®; Butler Animal Health supply, Dublin, OH) (Figure 2.2), a 2-cm incision was made vertical to the *plica lateralis*, aligned with the nipple and approximately 5 cm dorsal to the perimeter of the nipple areola. Hemorrhage was minimal but, if evident, was controlled by pressure with gauze. Two pieces of mammary tissue (~400 to 850 mg each) were exteriorized by gently grasping a small piece of mammary tissue with forceps and using this to elevate a much larger piece of tissue, which was then excised with a scalpel in a circular motion (Figure 2.3). Mammary tissue was rapidly flash-frozen in liquid nitrogen and stored at -80°C to determine the relative transcript abundance of key genes of interest or incubated in 10% buffered formalin for histological preparation and *in situ* hybridization. The incision was closed with simple interrupted sutures using size 0 nylon (CP-1 ethilon suture, Ethicon, Inc., Piscataway, NJ) (Figure 2.4). Time from incision to closing was approximately 5 min. Sows received an intramuscular injection of Banamine® (1.1 mg/kg BW) (flunixin meglumine, Schering-Plough Animal Health, Omaha, NE) immediately after biopsy and again at 24 and 48 h later. Following full recovery from anesthesia, sows were fed the reminder of their morning meal and piglets were returned to the sow and allowed to suckle normally. Rectal temperature was recorded after biopsy and again at 24 and 48 h to monitor health status of the sow. Sutures were removed 7 days after biopsy.

Analysis of variance was conducted to determine differences in average daily gain for piglets suckling the mammary glands subjected to biopsy compared to piglets suckling the intact glands, and to determine differences in sow daily feed intake, rectal temperature and milk somatic cell count one day prior to biopsy (pre) compared to two days following biopsy (post). Sources of variation in the model included effect of sow, stage of lactation, and mammary gland treatment (biopsy vs intact). Statistical analysis was performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Repeated measures analysis was used for repeated measures over days of lactation based on different covariance structures (Littell et al. 1998). The best fitting repeated measures covariance structure was determined using the Akaike information criterion. Separation of means was performed by least squares estimates. Least square means were considered different at $P<0.05$. Pre- and post-test power calculations were performed as previously described by Stroup (1999, 2002).

Results and Discussion

Up to 1.7 g of mammary tissue was obtained from live lactating sows at both early and late stages of lactation without apparent effect on sow welfare as indicated by sow feed intake, rectal temperature, and milk somatic cell count (Table 2.1). One sow developed a local infection at the biopsy site and was treated with a topical triple antibiotic containing neomycin, polymyxin B sulfates, and bacitracin zinc (Fougera, Altana Inc, Melville, NY) for 5 days until infection

subsided. No local or systemic infections were observed for the remaining 17 sows. Average sow rectal temperature was lower ($P<0.001$) on the two days following biopsy compared to that of the day of biopsy, reflecting the efficacy of the Banamine. Sow feed intake was not different on the day following biopsy than that on the day prior to biopsy. On average, sows gained 3.3 kg of body weight during the 18-day lactation period. Average daily gain from days 4 to 18 of lactation of the piglets suckling the glands subjected to biopsy versus that of piglets suckling intact glands did not differ (244.23 ± 12.72 $g \cdot day^{-1}$ vs 237.13 ± 9.76 $g \cdot day^{-1}$, respectively, $P=0.54$). Somatic cell counts from biopsied glands in early lactation period (3 – 6 days) were not different during the late lactation period (17 - 19 days) when compared to that of milk from non-biopsied glands. Sows were weaned at 20 day of lactation and returned to the breeding herd.

Using mammary mRNA transcript abundance data for two amino acid transporters (Chapter 4), the power to detect differences with stage of lactation was calculated using serial slaughtering design and biopsy techniques. Power to detect stage of lactation effects on mRNA transcript abundance using 18 animals in a serial slaughtering design was 0.64 compared to 0.98 when using a biopsy approach. A similar power (0.93) could be achieved with a serial slaughtering design by doubling the number of animals, i.e., using 36 sows.

The data presented indicates that mammary tissue can be collected from the same sow at different stages of lactation without adversely affecting lactation performance. Voluntary feed intake, rectal temperature, or apparent sow behavior were not compromised using the biopsy technique approach described

herein, suggesting that welfare of the sow was not impaired. The present biopsy technique describes a practical method for collection of mammary tissue in sufficient quantities for quantification of RNA and protein abundance, morphometric analysis, and immuno-histochemistry (Pérez Laspiur et al. 2004; Chapters 3, 4, and 5). Therefore, when total collection of parenchymal tissue is not required, this mammary biopsy technique allows for larger amount of mammary tissue compared to that of the needle biopsy technique and offers an alternative to serial slaughtering thus decreasing the number of animals needed to detect differences with stage of lactation.

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TABLE 2.1

Sow feed intake, rectal temperature and milk somatic cell count pre and post mammary biopsy[1]

Item	Time relative to biopsy		P-value
	Before	After	
Sow feed intake (kg d^{-1})	5.13 ± 0.32	5.02 ± 0.33	0.79
Sow rectal temperature (°C)	39.2 ± 0.07	38.7 ± 0.07	<0.001
Milk somatic cell count (1000 mL^{-1})	3677 ± 781	4969 ± 918	0.15

[1]Data are least square means ± standard error of the mean.

Figure 2.1. Mammary biopsy technique: One mammary gland (thoracic) was prepared for biopsy with a combination of povidone-iodine scrub and 70 % ethanol.

Figure 2.2. Mammary biopsy technique: Lidocaine was injected sub-cutaneously and intramuscularly 1 min prior to incision.

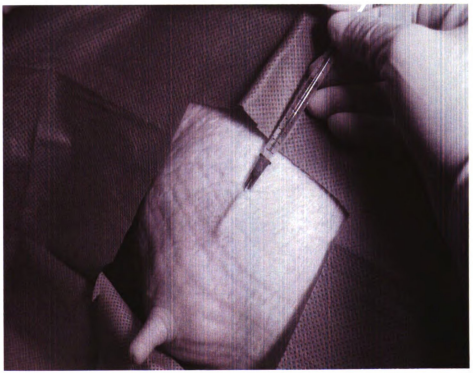


Figure 2.3. Mammary biopsy technique: Mammary tissue was exteriorized with forceps and excised with a scalpel in a circular motion.

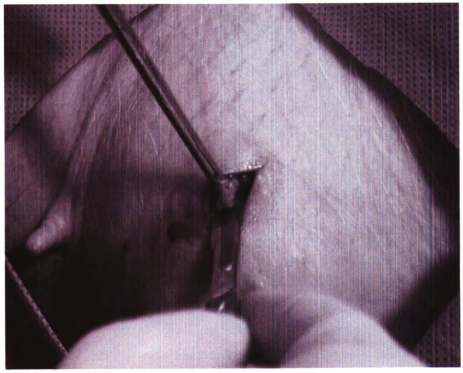
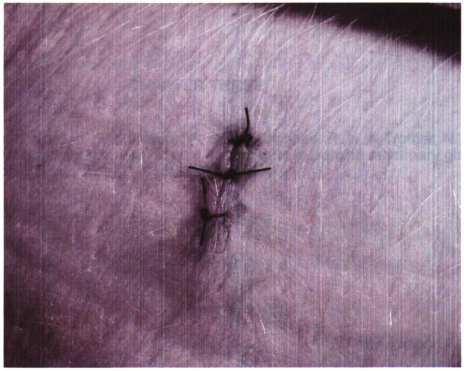


Figure 2.4. Mammary biopsy technique: The incision was closed using simple interrupted sutures. Three to four sutures were sufficient to close the incision and ensuring space for drainage.



CHAPTER THREE

Pérez Laspiur, J., Burton, J. L., Weber, P. S. D., Kirkwood, R. N. & Trottier, N. L. (2004) Short Communication: Amino acid transporters in porcine mammary gland during lactation. J. Dairy Sci. 87: 3235-3237.

CHAPTER THREE

AMINO ACID TRANSPORTERS IN PORCINE MAMMARY GLAND DURING LACTATION

Abstract

The objective of this study was to determine if mRNA transcripts for the amino acid (AA) transporter proteins CAT-1, CAT-2B, $B^{0,+}$ and ASCT1 are present in porcine mammary tissue (MT). Transcript abundance on d 7 and 17 of lactation was determined by northern blot analysis and absolute quantification performed by real-time polymerase chain reaction. Porcine MT expresses CAT-1, CAT-2B, $B^{0,+}$ and ASCT1 during lactation. Preliminary findings indicate that $B^{0,+}$ mRNA abundance tended to decrease on d 17 compared to that of d 7.

Keywords: Amino acids transporters, Mammary gland, Lactation, Porcine

Introduction

Despite the central role of AA transporters in provision of AA necessary for synthesis of proteins for cell structure, enzymes and signaling mechanisms (Meyer, 2001), little is known about regulation of AA transport and (or) the factors that affect their activity in various tissues. Expression of AA transporter transcripts and proteins known to mediate AA transport in other tissues have not

been studied in porcine MT. Such knowledge is critical to enhance our understanding of the factors controlling milk protein synthesis in lactating sows. Kinetic studies using porcine MT explants have provided indirect evidence that AA transport in MT is specific and carrier-mediated (Jackson et al., 2000; Hurley et al., 2000). This led us to hypothesize that AA transporter proteins CAT-1, CAT-2A and CAT-2B (system y^+), $B^{0,+}$ (system $B^{0,+}$), and ASCT1 (system ASC) are present in porcine MT. The objectives were to determine the presence of RNA transcripts for these transporters and quantify their abundance during early (d 7) and peak (d 17) lactation.

Materials and Methods

Mammary biopsies from randomly selected lactating sows (n=3) were performed at two stages of lactation and tissue rapidly flash-frozen in liquid nitrogen and stored at -80°C. Liver from a pre-pubertal gilt was collected following euthanasia and handled in same manner as MT. Liver was collected as a negative control for CAT-1 and CAT-2B, and as a positive control for CAT-2A. Total RNA was isolated from liver and MT using TRIzol Reagent (Invitrogen Life Technologies) following manufacturer's instructions as described in Weber et al. (2001).

Human CAT-1, CAT-2A, and CAT-2B cDNA probes were donated by Dr. E. Closs (Johannes Gutenberg University, Germany). A 400bp CAT-2B specific cDNA probe was developed from the region where human CAT-2A and CAT-2B

differ (Closs et al., 1997). To confirm its identity to the human CAT-2B (GenBank Accession number U76369), the DNA fragment was sequenced using a dye-terminator fluorescent cycle sequencing technique and an ABI® 3100 Genetic Analyzer (PerkinElmer Applied Biosystems, Foster City, CA). The sequenced fragment was then used to synthesize a ^{32}P-labeled CAT-2B cDNA probe. Porcine $B^{0,+}$ and ASCT1 cDNA probes were developed by polymerase chain reaction (PCR) using pooled cDNA from porcine MT as a template. PCR primers were designed from the published human $B^{0,+}$ (GenBank Accession number AF151978) and human ASCT1 (GenBank Accession numbers L14595 and L19444) cDNA sequences. PCR was carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA) using Taq DNA polymerase as recommended by manufacturer (Invitrogen, Life Technologies, USA). Resulting PCR amplification products were visualized as single bands of correct size using agarose gel electrophoresis (1.8% gel), gel purified (Wizard® PCR Preps DNA Purification System, Promega, Madison, WI), ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI), and the recombinant plasmids transformed into JM109 competent cells (Promega, Madison, WI). Prior to radioactive labeling for Northern blot hybridizations, the cloned inserts were excised from purified plasmid DNA using EcoRI, gel purified (Wizard kit, Promega, Madison, WI), and visualized as single bright bands on 1.8% agarose checking gels stained with ethidium bromide. The 1058 and 373 bp cDNA probes for $B^{0,+}$ and ASCT1, respectively, were DNA sequenced in both directions to confirm identities using a dye-terminator fluorescent cycle sequencing technique and an ABI® 3100

Genetic Analyzer (PerkinElmer Applied Biosystems, Foster City, CA), and the sequence information deposited in GenBank (Accession numbers: AY375264 and AY375265) (Appendix B).

The CAT-1, CAT-2A, CAT-2B, $B^{0,+}$, and ASCT1 probes were validated by Northern blot analysis using duplicate aliquots of porcine liver and MT collected as described in Weber et al. (2001). Hybridizations were carried out for 18 to 24 h at 42°C using a ^{32}P-labeled cDNA probes (NEN Life Science Products, Inc., Boston, MA) generated by the random prime method (Feinberg and Vogelstein, 1983.). The blot was probed first with β-actin cDNA to verify equality of RNA loading across lanes, followed by CAT-1, CAT-2A, CAT-2B, $B^{0,+}$, and ASCT1 cDNA with complete stripping of the blot between hybridizations. Nylon membranes (Amersham Biosciences, NJ) were then washed and exposed to BioMax MS film (Fisher Scientific, Pittsburgh, PA) for 24 to 240 h at -80°C with an intensifying screen (Fisher Scientific, Pittsburgh, PA).

Results and Discussion

Figure 3.1 shows Northern blot validations of CAT-1, CAT-2A, CAT-2B, $B^{0,+}$ and ASCT1 cDNA probes. Human CAT-1 probe hybridized to a predominant transcript of expected size (~5.2 kb) in MT. CAT-1 also hybridized to a predominant transcript in liver tissue, which is in contrast to most species studied to date, but in agreement with Liu and Hatzoglou (1998) who also demonstrated the presence of CAT-1 in rat liver. CAT-1 transcript presence in MT has also

been demonstrated in sheep (Kiaei et al., unpublished; GenBank Accession number AF212146) and humans (Vékony et al., 2001). CAT-2A hybridized to a predominant transcript of expected size (~7.9 kb) in porcine liver but not MT, consistent with other studies (Closs et al., 1993) demonstrating to date the specificity of CAT-2A for hepatic tissue only. In contrast to CAT-2A, the 400 bp CAT-2B probe hybridized to a single transcript in both liver and MT.

Porcine $B^{0,+}$ probe hybridized to a predominant transcript in porcine MT, but not in liver. To date, $B^{0,+}$ transcript presence in MT had been reported in humans only (Sloan and Mager, 1999). Porcine ASCT1 probe hybridized to a predominant transcript in both porcine liver and MT. While ASCT1 expression has been demonstrated in a variety of tissues (Arriza et al., 1993), its expression in MT had not been reported to date. In summary, results demonstrate the presence of CAT-1, CAT-2B, $B^{0,+}$, and ASCT1 transcripts in lactating porcine MT.

Transcript abundance on d 7 and d 17 of lactation of AA transporters CAT-1, CAT-2B, $B^{0,+}$, and ASCT1 was determined by Northern blot analysis using the validated probes as described above. Figure 3.2A suggests that $B^{0,+}$ transcript abundance was lower on d 17 compared to d 7. No apparent change in transcript abundance was observed for CAT-1, CAT-2B, and ASCT1 (data not shown). Absolute quantification of $B^{0,+}$ and β-actin mRNA abundance on d 7 and 17 was also determined by real-time PCR. Porcine $B^{0,+}$ and β-actin cDNA probes developed for northern blot analysis were used as a standard template. Mammary tissue RNA from d 7 and 17 was converted into first-strand cDNA and quantitative real-time RT-PCR was conducted as previously described

(Coussens et al., 2003; Madsen et al., 2004) with 50 ηg of template cDNA and gene specific primers designed using Primer Express (PerkinElmer Applied Biosystems, Foster City, CA). The $B^{0,+}$ forward primer was 5' GGTGGTCCATTTTGGTCCATAT 3' and reverse primer was 5' GTGATCGTTTCAATCGAAGCAA 3'. β-actin was used as the control gene in this system with forward primer 5' CTCCTTCCTGGGCATGGA 3' and reverse primer 5' CGCACTTCATGATCGAGTTGA 3'. Three and five-point standard curves for $B^{0,+}$ and β-actin were run on each plate and determined to be linear and parallel to each other, indicating similar reaction efficiency. All samples were run in triplicates. Three wells per plate had all reagents added except cDNA template to serve as negative controls. All analyses were conducted using an ABI® 7700 DNA sequence detection system (PerkinElmer Applied Biosystems, Foster City, CA). For each test amplification reaction, mean $B^{0,+}$ expressions were normalized against β-actin (within sow and sample day) to account for differences between individual samples in RNA loading, cDNA synthesis efficiency, and amplification efficiency. Differences between means were detected using the MIXED procedure of SAS (1998). Figure 3.2B demonstrates that $B^{0,+}$ transcript abundance tended ($P = 0.08$) to decrease from early to peak lactation in porcine MT.

Considering that the protein synthetic capacity and AA uptake by porcine MT are higher during peak lactation compared to that of early lactation (Kim et al., 1999; Nielsen et al., 2002), an increase in transcript abundance of AA transporter proteins was expected. However, data presented herein on transcript

abundance suggest that CAT-1, CAT-2B and ASCT1 do not change with day of lactation and that $B^{0,+}$ decreases from early to peak lactation. With the notion that mammary tissue is heterogeneous and composed of both stromal and epithelial cells, the possibility that expression of AA transporters differed between cell types cannot be refuted. We recognize that these results are limited due to the small sample size, and that further research with larger sample size is critical to determine the relationship between AA transporters mRNA abundance and day of lactation. Nevertheless, as a whole, the data are novel and represent a gateway for the study of AA utilization and transport regulation in porcine MT at a molecular level.

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Figure 3.1. Northern blots of human CAT-1, CAT-2A, CAT-2B and porcine $B^{0,+}$ and ASCT1 in porcine mammary (lanes 1 & 2) or liver tissue (lanes 3 & 4). Blots were exposed to X-ray film for 120h (CAT-1), 96h (CAT-2A), 122h (CAT-2B), 48h ($B^{0,+}$), 24h (ASCT1) or 240h (ß-actin).

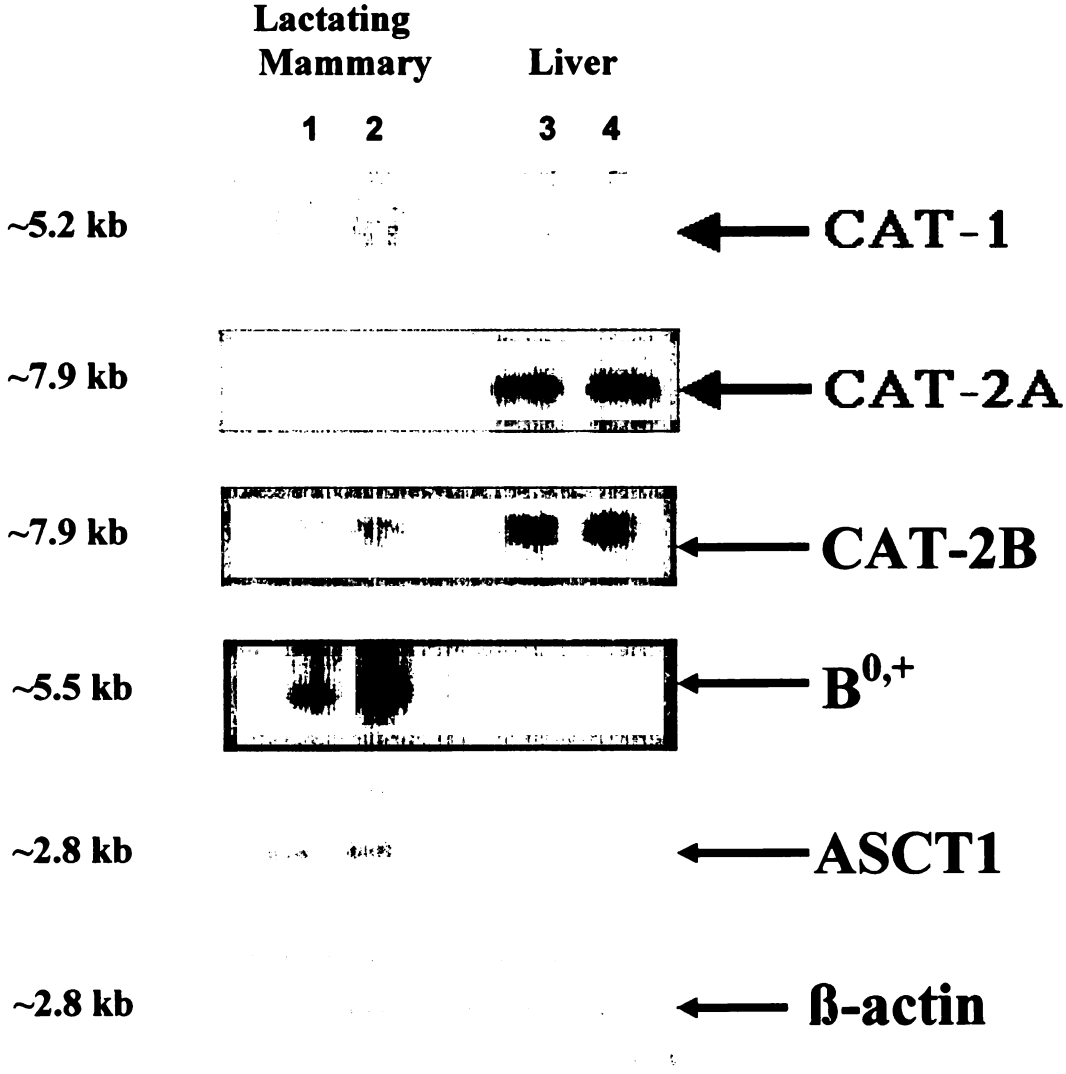
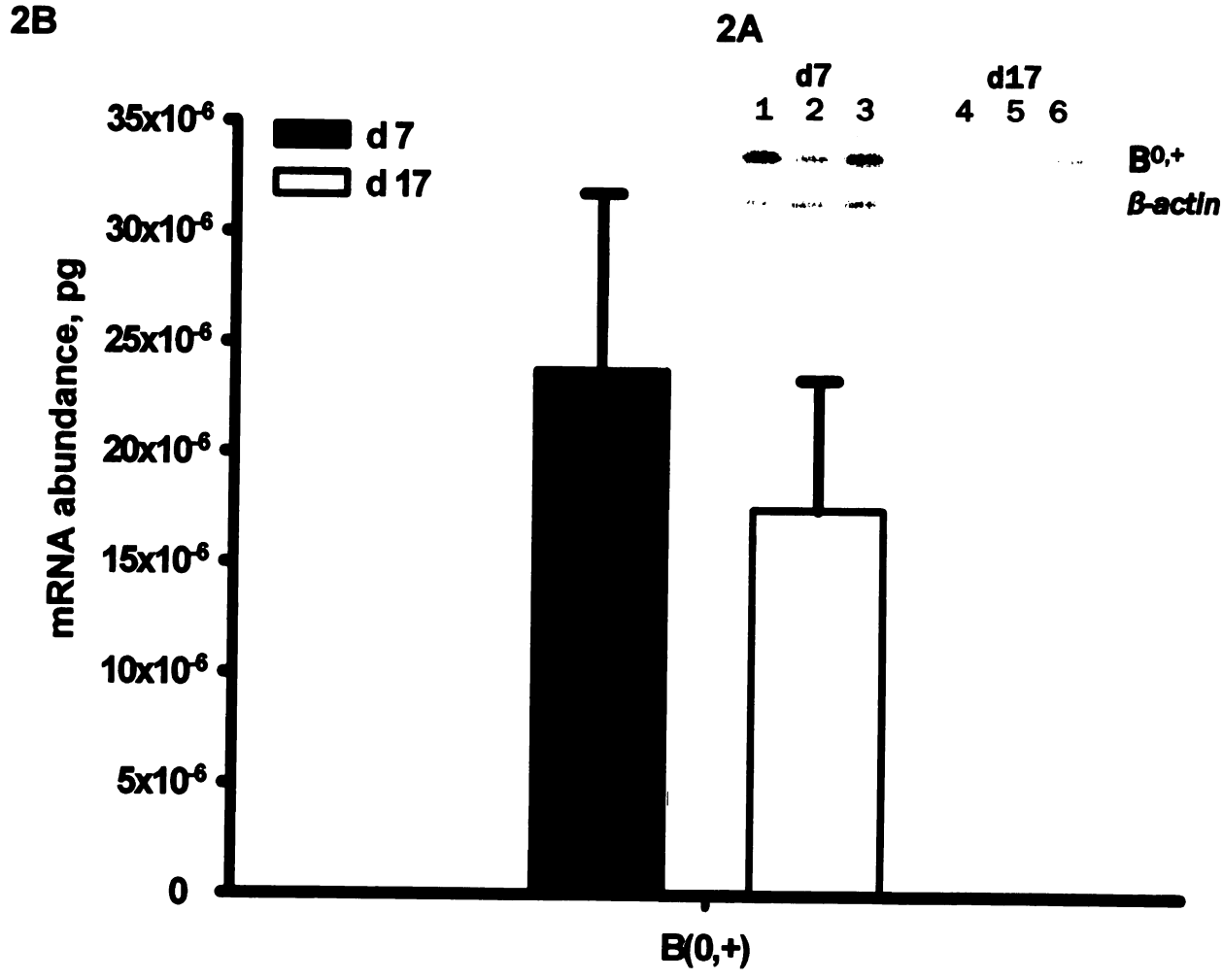

Lactating Mammary
Liver
1 2
3 4
~5.2 kb
CAT-1
~7.9 kb
CAT-2A
~7.9 kb
CAT-2B
~5.5 kb
B0,+
~2.8 kb
ASCT1
~2.8 kb
ß-actin

Figure 3.2. Northern blots of $B^{0,+}$ at early (d 7) and peak (d 17) lactation in MT of 3 multiparous lactating sows (A). Lanes 1 and 3, sow 1; lanes 2 and 5, sow 2; lanes 3 and 6, sow 3. Blots were exposed to X-ray film for 13h ($pB^{0,+}$ ~5.3 Kb) or 24h (pß-actin ~2.8 Kb). Porcine $B^{0,+}$ transcript abundance in 50ηg cDNA as a function of day of lactation by using RT-PCR analysis (B). Bars represent means ± standard errors from 3 multiparous tended to be lower than on day 7 for (P = 0.08).


2B
2A
d7
d17
1 2 3
4 5 6
B0,+
β-actin
35x10-6
30x10-6
25x10-6
20x10-6
15x10-6
10x10-6
5x10-6
0
d 7
d 17
mRNA abundance, pg
B(0,+)

CHAPTER FOUR

DIETARY PROTEIN INTAKE AND STAGE OF LACTATION ALTER AMINO ACID TRANSPORTER EXPRESSION IN PORCINE MAMMARY TISSUE

Abstract

To test the hypothesis that under conditions of restricted or surfeit amino acid (AA) availability, the mammary gland undergoes adaptations aimed at optimizing AA uptake, changes in AA transporter mRNA and protein abundance were investigated at two distinct stages of lactopoesis. Eighteen multiparous lactating sows were used in a 2×3 randomized incomplete block design consisting of two stages of lactation and three dietary treatments. Diets were formulated to restrict (Deficient), meet (Adequate) or exceed (Excess) the quantity of valine, lysine, threonine, methionine, tyrosine and tryptophan required by lactating sows. Mammary tissue was biopsied at Early and Peak lactation. Compared to Adequate, sows fed Excess diet had lower piglet growth rate ($P<0.05$). Compared to Adequate, sows fed Deficient and Excess diets had lower milk protein and casein yield ($P<0.05$). Compared to sows fed the Adequate diet, feeding the Excess diet decreased CAT-2B mRNA abundance ($P<0.05$). Dietary protein intake had no effect on CAT-1, ASCT1 and $B^{0,+}$ mRNA abundance. Abundance for ASCT1 and $B^{0,+}$ mRNA was higher at Peak compared to that at Early stage of lactation ($P<0.01$). Lactation demand and dietary protein intake did not alter CAT-1 and ASCT1 protein levels. In conclusion, dietary protein intake modulated CAT-2B expression but not CAT-1,

ASCT1 or $B^{0,+}$. These results suggest that changes in AA extraction rates by the mammary gland in response to sow protein intake may be mediated by other mechanisms, including changes in other amino acid transporters with overlapping specificity or by changes in activity of these AA transporters.

Keywords: Amino acid transporter, mammary gland, lactation, protein intake

Milk yield and composition are crucial factors limiting neonatal growth (Grantham-McGregor et al., 2000). During the early phase of lactation, milk protein yield is insufficient to maximize growth of piglets (Hartmann et al., 1997). The same is true in humans, in particular those living in poor communities and developing countries (Cisse et al., 2002; Kaseb et al., 2002). Despite the crucial importance of efficient milk protein secretion to sustain normal rate of growth of the nursing neonate (Raiha et al., 1986), current understanding of mammary protein synthetic processes is lacking, in particular those involved in the transport of AA across the blood-mammary gland barrier.

The porcine mammary gland has the highest demand for AA compared to that of other organs (Shennan and Peaker, 2000) and thus represents a unique model of AA supply and demand at very high rates of AA utilization for protein synthesis (Baracos, 2006). Dietary protein intake below that of requirement depresses milk protein yield and subsequent neonatal pig growth (Guan et al., 2002). Likewise, excessive intake of protein by the dam depresses piglet growth, but does so to an even greater extent than that of dietary protein deficiency (King

et al., 1993; Yang et al., 2000). These changes are accompanied by what seems to be some adaptive mechanisms whereby the mammary gland modulates the extraction rates of circulating AA in response to dietary AA imbalances, on one hand in an attempt to meet the need of milk protein demand, but on the other hand to limit the unnecessary uptake of certain AA (Guan et al., 2004), suggesting that AA transport is a regulated step of AA utilization for mammary protein synthesis.

It is well documented that AA are transported across cells by a large number of transport systems with distinct molecular and functional characteristics (Palacín et al., 1998). Several AA transport systems have been characterized in mammary glands of humans, rats, mice, and pigs, but only a few of their molecular entities have been identified. Of the molecular entities, only the human $B^{0,+}$ (Sloan and Mager, 1999), CAT-3 (Vékony et al., 2001) and GLT-1 (Berger and Hediger, 2006), and the rat LAT1, LAT2 (Shennan et al., 2002) and SNAT2 (Lopez et al., 2006) transport proteins have been cloned from mammary tissue. Relative to porcine mammary tissue, transcripts encoding for transport proteins CAT-1, CAT-2B, ASCT1 and $B^{0,+}$ have been identified (Pérez Laspiur et al., 2004). Amino acid transporters act as nutrient sensors in many tissues and respond to AA availability by initiating signaling cascades that promote cell survival under different environments (Hyde et al., 1993). For instance, several AA transporters, including the ASCT1 and some belonging to the CAT family may be adaptively regulated with increased uptake activity and expression in conditions of limited AA availability (Hyde et al., 1993; Hatzoglou et al., 2004;

Howard et al., 2004). In contrast, under conditions of surfeit AA, uptake of lysine (Hurley et al., 2000), valine (Jackson et al., 2000), alanine (Sharma and Kansal, 1999), and glycine (Rehan et al., 2000), among others (Tovar et al., 2000) is depressed.

In vitro, AA availability alters transcription, translation and activity of AA transporters (Hyde et al., 2003; Hatzoglou et al., 2004). The CAT-1 gene is one of the few nutrient sensitive genes that are up-regulated under nutrient deficient environments and down-regulated under nutrient rich environments (Fernandez et al., 2001; Bruhat et al., 2002; Yaman et al., 2002; Hyde et al., 2003; Fernandez et al., 2003). However, changes in mRNA abundance of AA transporters do not necessarily equate to changes in protein levels (Hatzoglou et al., 2004). In addition, while we have previously demonstrated the presence of CAT-1, CAT-2B, ASCT1 and $B^{0,+}$ transcripts in whole mammary tissue (Pérez Laspiur et al., 2004), it is possible that these transcripts may be primarily expressed in stromal cells in addition to epithelial cells. In other tissues studied to date, CAT-1 was shown to be expressed in epithelial cells (Koga et al., 2003; Verri et al., 2005). Whether or not these AA transporter proteins are also expressed in mammary epithelial cells where caseins, the most abundant proteins in milk, are synthesized remains to be ascertained.

Determining whether or not adaptive regulation of AA transporters in response to AA availability occurs in the mammary gland of lactating sows would provide some initial and fundamental understanding of the mechanisms defining the efficiency of dietary AA utilization in response to dietary AA imbalances. In

addition, with the notion that milk demand constitutes a driving force for milk protein synthesis (Daly and Hartmann, 1995; Spinka et al., 1997) and that AA influx increases with increasing milk demand (Nielsen et al. 2002, Guan et al. 2004), investigating if these changes are associated with modulating AA transporter abundance would offer additional insight into differential regulation amongst AA.

To test the hypothesis that under conditions of restricted or surfeit AA availability, the mammary gland differentially expresses AA transporters aimed at optimizing AA uptake, changes in AA transporter mRNA and protein expression were investigated at two distinct stages of lactopoesis.

Materials and Methods

The All-University Committee on Animal Use and Care of Michigan State University approved all procedures performed in this study (AUF#11/02-161-00).

Dietary treatments and feed nutrient analysis

Diets were formulated to contain 12 % (Deficient), 18 % (Adequate), and 24% (Excess) protein, for a targeted protein intake respectively meeting 77, 116, and 157 % of lysine required by lactating sows to support nursing pigs with a predicted growth rate of 225 g/d. (NRC, 1998) (Table 1). Both Adequate and Deficient protein diets were mixed by diluting the Excess protein diet with

cornstarch in order to equalize the soybean meal to corn ratio and thus the AA profile relative to lysine across all diets. The Deficient diet restricted the quantity of valine, lysine, threonine, methionine, tyrosine and tryptophan required by 25, 23, 22, 15, 15, and 13%, respectively. The Excess diet exceeded the quantity of all AA by over 157%. Solka floc was included in the Adequate and Deficient protein diets to maintain an identical level of NDF. Corn oil was used to reduce dustiness, improve palatability and maintain isocaloricity across diets. Feed samples were analyzed for DM, CP, starch, and AA concentrations. Samples were finely ground using a sample mill (Cyclotec 1093, Foss Tecator). Feed N was analyzed using a combustion based N/protein determinator (FP-2000, LECO Corp.). Starch content of diets was determined enzymatically after samples were gelatinized with NaOH as described by Karkalas (1985). Amino acid concentrations in feed samples were analyzed by reverse-phase HPLC (Alliance 2690, Waters Corp.) after hydrolysis in 6*N* HCL at 110°C for 24 hrs (Guay and Trottier, 2006).

Animals and experimental design

Eighteen multiparous lactating sows (12 Landrace × Yorkshire and 6 Yorkshire) were used in a randomized incomplete block design with two replications. Block was defined as a farrowing cycle, the first block or replicate consisted of 7 sows and the second block or replicate consisted of 11 sows. Sows were individually housed in farrowing crates in a thermally controlled room

(20°C) during the length of the study. Sows were randomly allocated to one of three dietary treatments (Deficient, Adequate, or Excess) one week prior to the expected farrowing date and fed 2.5 kg divided into two meals per day. Litters were equalized to 8 piglets by cross-fostering within 36 h of birth. The day after farrowing was considered day one of lactation and sows received two 1-kg meals (0800 and 1600 h). On day 2 and 3 of lactation sows were fed 4 and 6 kg, respectively, in two meals. Sows fed the Excess diet were provided ad libitum access to feed thereafter, while sows fed the Adequate and Deficient diets were pair-fed to sows fed the Excess diet. Sow feed intake was recorded daily throughout the lactation period. Orts were collected before the morning meal and weighed. Sow body weight was recorded on d 1 and 17 of lactation. Piglets were weighed individually on d 1, 4, and 18 of lactation. Milk yield was estimated based on litter average daily gain (ADG in g/d) and litter size (No. of piglets in litter) according to Noblet and Etienne (1989) where milk yield (g/d) = 2.42 x ADG + 78.2 x Bwi + 26 x No. of piglets in litter, where Bwi is the initial whole litter body weight in kg.

Mammary tissue collection

Mammary tissue was collected between d 3 and 6 (Early) and d 17 and 19 (Peak) of lactation from the first and second thoracic mammary glands, respectively following the biopsy procedure of Kirkwood and coworkers (2007). Sows were fed 2 kg of their morning meal and piglets were removed from the

sows 1 hr post-feeding. Piglets remained isolated for 45 min in an adjacent pen equipped with a heat lamp and ample bedding. Following the 45-min isolation period, piglets were returned to sows and allowed to nurse. Immediately following completion of the nursing bout and while sows still remained in lateral recumbence, sows were sedated and mammary tissue collected as described in Kirkwood and coworkers (2007). Mammary tissue was rapidly flash-frozen in liquid N_2 and stored at -80°C. For immunohistochemistry analysis, collected tissue (~ 0.5 g) was immediately transferred in 10% buffered formalin and incubated for 16 hr at 4°C. The tissue was then rinsed in phosphate buffered saline and stored in 70% ethanol at 4°C until analysis.

Blood collection and analysis

Blood samples were collected at Early and Peak lactation immediately following mammary tissue collection while sows were at rest under residual sedation. Blood (7.0 mL) was collected via an ear vein using a butterfly needle attached to an extension and BD Vacutainer™ glass blood tubes containing EDTA (2.1 % EDTA/mL blood) (Becton, Dickinson and Company). Samples were immediately centrifuged and plasma stored at -20°C until later analysis of free AA and glucose concentrations. Determination of free AA in plasma was performed by reverse-phase HPLC (Pico Tag, Waters) as previously described (Guay and Trottier, 2006). Plasma glucose concentration was determined using a glucose oxidase method (Wako Diagnostics, Wako Chemicals) and absorbance was

determined with a micro-plate reader (Spectramax 340, Molecular Devices Corporation).

Milk collection and analysis

Approximately 30 mL of milk was manually collected from the first and second thoracic mammary glands on each side of the udder (4 glands total) on the day prior to biopsy following i.m. administration of 10 I.U. of oxytocin (Vet Tek, Phoenix Scientific) and pooled within sow. Approximately 15 mL of milk was kept refrigerated and analyzed for true protein, fat, lactose and solids by energy absorption analysis (Bentley 2000, Bentley Instruments), and for somatic cell counts by flow cytometry method (Somacount 500, Bentley Instruments). The remaining 15-mL milk aliquot was frozen and stored at -20°C for later analysis of free AA, total N, total casein and for casein-N determination. Milk samples were thawed on ice and whole milk was defatted by centrifugation for 30 min (1500 x g at 4°C) followed by removal of the fat layer by low vacuum suction. A 1-mL defatted milk aliquot was used for determination of free AA by HPLC as described for plasma after centrifugation at 1500 x g for 15 min at 4°C. Total N in defatted milk was determined using a combustion based N/protein determinator (FP-2000, LECO Corp.) and EDTA (Sigma) as a calibration standard. Milk protein concentration was determined from total milk N using a factor of 6.38. For casein determination, casein was precipitated out of a weighed aliquot of fluid milk at room temperature after adjusting the pH to 4.6 using 1*N* HCl. Following

centrifugation (1500 x g for 15 min at 4°C), the precipitated casein pellets were washed twice with distilled water, solubilized at pH 7.0, freeze-dried, and stored at -20°C. Casein concentration was determined by dividing the freeze-dried pellet weight by the defatted liquid milk aliquot weight. Casein-N in defatted milk was determined as described above for milk N.

RNA isolation

Total RNA was isolated from mammary tissue using TRIzol Reagent (Invitrogen Life Technologies) following manufacturer's instructions as described in Weber and coworkers (2001). RNA isolated from mammary tissue was treated with RQ1 RNase-free DNase (Promega Corp.) for 15 min at 37°C to remove any contaminating genomic DNA. RNA was then purified further by phenol/chloroform (1:1) extraction and ethanol precipitation. Pellets of RNA were suspended in RNase-free water, and quantified using a spectrophotometer (DU-650 Beckman) and the 260 and 280 nm readings, and evaluated for RNA integrity on 1.2% agarose gels (Weber et al., 2001; Madsen et al., 2002).

cDNA synthesis

RNA samples (1 ug) were converted to cDNA using Superscript II reverse transcriptase (Invitrogen Life Technologies) as recommended by the manufacturer and as previously described by Coussens and coworkers (2003)

and Madsen and coworkers (2004). Remaining RNA was degraded by RNase H and the resulting cDNA extracted with phenol/chloroform (1:1). Following precipitation with ethanol, final cDNA pellets were suspended in 20 µL of RNase-free water for quantitative real-time polymerase chain reaction (Q-RT-PCR) analysis of abundance of the various AA transporters. Concentration of cDNA was measured using a nano-spectrophotometer (Nano Drop ND-1000, NanoDrop Technologies Inc.).

Transcript abundance

Absolute quantification of transcript expression of the AA transporters CAT-1, CAT-2B, ASCT1, and $B^{0,+}$ was determined in mammary tissue using Q-RT-PCR. Primers for Q-RT-PCR assessment of CAT-1, CAT-2B, ASCT1, and $B^{0,+}$ mRNA abundance were designed using Primer Express (PE ABI Inc.) (Table 2). Standards for each AA transporter were developed using these primers and our cloned AA transporters cDNAs as templates (Pérez Laspiur et al., 2004). Standard curves for Q-RT-PCR are shown in Figures 4.5 to 4.8. Quantitative RT-PCR was conducted in 96-well optical plates (Perkin Elmer Corp.) using triplicate 2-uL (25ng) of starting cDNA for each reaction. To run the Q-RT-PCR assays, cDNA templates were combined with SYBR green dye mix (Perkin Elmer Corp.) and Q-RT-PCR conducted as recommended by the manufacturer. Triplicate five-point standard curves for each test gene were run on every plate. Three wells per plate had all reagents added except cDNA template to serve as negative

controls. All analyses were conducted using a DNA sequence detection system (PE 7700 Perkin Elmer Corp.). Abundance data of the cDNAs for triplicate samples were recorded automatically by the instrument software as cycles to threshold (C_t), based on threshold lines adjusted to intersect PCR amplification lines in the linear portion of the amplification standard curves. For each test amplification reaction, mean CAT-1, CAT-2B, ASCT1, and $B^{0,+}$ expressions were normalized against starting cDNA concentration to account for differences between individual samples in RNA loading and cDNA synthesis efficiency. B-actin was not used as a housekeeping gene as its abundance was shown to change with stage of lactation (data not shown).

Protein isolation and quantification

Total protein was isolated from mammary tissue using the procedure described by Krotova et al. (2003) with modifications. Briefly, mammary tissue (30 - 45 mg) was pulverized in liquid N2 with a mortar and pestel and transferred to a 15-mL glass tube containing 1 mL chilled lysis buffer (10mM Tris, pH 7.5; 100mM NaCl; 1mM EDTA; 1mM EGTA; 1% Nonidet P-40; 0.4% deoxycholate; 60mM octylglucoside) per 10 mg of tissue and homogenized at medium speed with a tissue homogenizer (PT 10-35 Brinkmann Instruments). Samples were then centrifuged at 20,000 x g for 30 min and the supernatant collected. Samples were centrifuged a second time at 13,000 x g for 10 min and the supernatant collected and pooled with the first supernatant. Protein concentration was

determined using a modified Lowry assay (RC DC Protein Assay, Bio-Rad Laboratories).

Antibodies

A rabbit polyclonal anti-CAT-1 antibody and pre-immune serum was donated by Dr. E. R. Block (Malcom Randall VA Medical Center, Gainesville, Florida). This antibody recognizes the fourth extracellular domain of the human CAT-1 peptide (T-Y-F-G-V-S-A-A-L-T-L-M-M-P-Y-F-C-L-D-K-D-T-P-L-P-D-A-F-K-H-V-G-W-G) as described by Krotova et al. (2003). For detection of ASCT1, a commercially available rabbit polyclonal anti-human ASCT1 antibody (Chemicon International) recognizing a 12-AA peptide sequence (M-E-K-S-N-E-T-N-G-Y-L-D) corresponding to the NH_2 terminus was used for western blot analysis. The secondary antibody used for detection of both CAT-1 and ASCT1 was a commercially available donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Biosciences UK).

Western blot analysis

Protein samples (~100 – 150 ug) were denatured and separated by one dimensional SDS-PAGE on a 10% Tris-HCl Ready Gel (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blotted overnight at 4°C with either the anti-CAT-1 antibody at a 1:3,000

dilution or without a primary antibody. All membranes were incubated for 1 hr at room temperature with our HRP-conjugated secondary antibody at a 1:2,500 dilution. Following stripping, membranes were blotted overnight at 4°C with either anti-ASCT1 antibody at a 1:5,000 dilution or without a primary antibody. Expressed CAT-1 and ASCT1 were detected using an ultra sensitive HRP detection system (femtoLucent™ PLUS-HRP Reagent Kit, Geno Technology). The density of the band was quantified using a scanning densitometer (Bio-Rad GS-700 Imaging Densitometer, Bio-Rad Laboratories). Uniformity of protein loading was confirmed by staining of the blots with Ponceau S staining (Sigma) as described previously (Schedin et al., 2004).

Immunohistochemistry analysis

Mammary tissue from three lactating sows at Peak lactation (day 17) was biopsied as described previously (Kirkwood et al., 2007). Immunohistochemistry analysis was performed using a modified vector protocol (Vectastain Universal Elite ABC Kit, Vector Laboratories). Unstained slides for antigen retrieval were prepared by the Histology Laboratory, Division of Human Pathology, Department of Physiology at Michigan State University. Tissues were processed on an automated tissue processor (Thermo Electron Excelsior) and embedded in paraffin on a Miles Tissue Tek II embedding station (Sakura Finetek U.S.A. Inc). Paraffin blocks were sectioned on a Reichert Jung 2030 Rotary microtome (Leica) and placed on adhesive slides coated with 3-aminoethylalkylsilane

solution (Sigma Chemical). Slides were then dried overnight in a slide drying oven not exceeding 60°C. Slides were deparaffinized in xylene 100% and tissue hydrated through descending grades of ethanol to distilled water. Antigen retrieval protocol was performed per manufacturer's instructions (Citra Antigen Retrieval, Biogenex Laboratories). Slides were then incubated with 3% H_2O_2 in methanol with the purpose of total quenching of endogenous peroxidase activity. Slides were then rinsed, washed in phosphate buffered saline and air-dried. To prevent non-specific binding, slides were incubated for 35 min with diluted goat normal blocking serum. Sections were incubated for 60 min with CAT-1 antiserum at 1:300 dilution in normal goat serum or no primary antibody in normal goat serum as a negative control. For ASCT1 detection, sections were incubated overnight at 4°C with ASCT1 at 1:200 dilution in normal goat serum or no primary antibody in normal goat serum as a negative control. Slides were washed and incubated for 30 min with biotinylated goat anti-rabbit IgG antibody (HRP-conjugated) at 1:200 and then washed in phosphate saline buffer and ABC reagent prepared as instructed by manufacturer. Slides were then rinsed, washed and incubated for 30 min with ABC reagent. Slides were mounted and stained with diaminobenzidine tetrahydrochloride (DAB) peroxidase and staining observed under a microscope (Leica Microskopie and Systeme GmbH). Morphological analysis was performed to identify and distinguish epithelial and stromal cells.

Statistical analyses

Differences in lactation performance, plasma AA concentrations, milk composition and yield, RNA and protein levels as affected by dietary protein intake and stage of lactation were determined by analysis of variance. Sources of variation in the full model included sow, diet, stage of lactation, breed, parity, and replicate as classification variables, and the two-way and three-way interactions of interest. Sow nested within diet was included as a random effect. For all AA transporter genes, a reduced model was used that included stage of lactation, diet, and the stage of lactation x diet interaction. When the effect of replicate, diet x replicate, day x replicate, replicate x breed, and replicate x parity were significant they were included in the model. Sow nested within diet was included as a random effect. Statistical analysis was performed using the MIXED procedure of SAS (1998). Repeated measures analysis was used for repeated measures over days of lactation based on different covariance structures (Litell et al., 1998). The best fitting repeated measures covariance structure was determined using the Akaike information criterion (AIC). Relationships between dietary protein intake and response variables were determined by linear and quadratic orthogonal contrasts. Separation of means was performed by the least squares estimates. Differences were considered significant at $P<0.05$. Suggestive differences between treatments were considered at $P\leq0.10$. A square root transformation was used to normalize distributions of mRNA abundance for CAT-1, ASCT1, and $B^{0,+}$. A logarithmic transformation was used to normalize mRNA abundance of CAT-2B. Data are presented as backtransformed means on the original scale of measurement. Abundance of

CAT-1 and ASCT1 in porcine mammary tissue at Peak lactation was expressed relative to abundance at Early lactation per individual sow. Effects of Deficient or Excess dietary protein level on CAT-1 protein abundance in porcine mammary tissue was expressed relative to Adequate dietary protein level per individual sow.

Results

Lactation performance

Effect of protein intake and stage of lactation on sow lactation performance, and milk composition and yield are presented in Tables 3 and 4 respectively. Pair-feeding Adequate and Deficient diet-fed sows to sows fed the Excess diet resulted in similar DM intake across dietary treatments. Sows in the Deficient, Adequate, and Excess diets consumed 86, 135, and 195 % of their lysine requirement, respectively (NRC, 1998), which was marginally higher than the targeted lysine intakes, but close to the targeted differences in protein intake between diets. Due to the starch concentration of the diets, starch intake was 3000, 2422, and 2006 g/d in sows fed Deficient, Adequate, and Excess diets, respectively ($P<0.001$). Sows consuming the Deficient diet lost 2.13 kg over the 21-d lactation period, while sows in the Adequate and Excess diets gained 2.50 and 9.75 kg, respectively (Linear, $P=0.09$). Compared to Adequate fed sows, piglet daily weight gain was ($P<0.05$) lower for sows fed Excess diet.

Relationship between piglet daily weight gain and sow protein intake was curvilinear, with increasing gain from Deficient to Adequate and decreasing from Adequate to Excess (quadratic, $P<0.05$). Dietary sow feed intake and protein intake did not differ with stage of lactation, while starch intake was higher ($P<0.001$) at Peak lactation compared to Early lactation.

Milk composition and yield

Effect of protein intake on milk composition and yield are presented in Table 3 and Figures 4.1 and 4.2. Compared to Adequate fed sows, % milk CP was lower in sows fed Deficient ($P<0.05$) but not different for sows fed Excess. Percentage of milk lactose and total solids were not affected by protein intake. Fat % increased linearly ($P<0.05$) with increasing protein intake, but was not different for either Deficient or Excess fed sows when compared to Adequate fed sows. The interaction between dietary protein intake and stage of lactation on milk true protein and casein percentage was significant ($P<0.05$) and results are presented in Figures 4.1 and 4.2, respectively. Milk protein percentage was higher ($P<0.05$) for sows fed the Deficient diet compared to sows fed the Adequate diet at Early lactation, while it was lower for sows fed the Deficient diet compared to sows fed the Adequate diet at Peak lactation ($P<0.05$). Milk casein percentage was lower for sows fed the Deficient and Excess diet compared to sows fed the Adequate diet ($P<0.05$) and was lower at Peak lactation compared to Early lactation only for sows fed the Deficient diet ($P<0.05$).

Milk yield tended to decrease in sows fed the Excess diet (P=0.07) compared to sows fed the Adequate diet. Compared to Adequate fed sows, milk CP yield was lower (P<0.05) for sows fed Deficient diet. Compared to Adequate fed sows, milk casein yield was lower (P<0.05) for both sows fed either Deficient or Excess diets. Relationship between true protein (P=0.08), CP (P< 0.05), and casein yield (g/d) (P<0.01) was quadratic, with increasing values with increasing protein intake from Deficient to Adequate and decreasing values with increasing protein intake from Adequate to Excess. Fat yield did not differ between dietary treatments. Yield of lactose and solids did not differ with increasing dietary protein intake (P>0.10).

The effect of stage of lactation on milk composition and yield are presented in Table 4. Milk yield was higher (P<0.001) at Peak lactation compared to that of Early lactation. On a percentage basis, true protein, CP, fat and casein were lower (P<0.05) and lactose was higher (P<0.001) at Peak lactation compared to those in Early lactation. Casein-N % did not change with stage of lactation but the contribution of casein-N to total N was higher (P<0.01) at Peak lactation than that of Early lactation. Total solids percentage did not differ with stage of lactation. Yield of lactose and total solids were higher (P<0.001) while yield of true protein tended to be higher (P<0.10) at Peak lactation compared to Early lactation. Casein, CP, and fat yield did not differ with stage of lactation.

Plasma free AA and glucose concentration

Plasma glucose and AA concentrations as affected by protein intake are presented in Table 5. With the exception of arginine and histidine where no changes were observed, total plasma essential amino acid (EAA) concentration increased linearly ($P<0.001$) with increasing protein intake, while total and the majority of plasma nonessential amino acid (NEAA) concentrations decreased linearly ($P<0.05$) with increasing protein intake. Plasma glucose concentration did not differ with protein or starch intake. Glucose and AA concentrations as affected by stage of lactation are presented in Table 6. Stage of lactation had no effect on total AA concentrations or on the majority of plasma EAA and NEAA concentrations. The interaction between dietary protein intake and stage of lactation on plasma glucose was significant ($P<0.05$).

Milk free AA concentration

Milk free AA concentrations as affected by protein intake are presented in Table 7. Total free EAA concentration in milk increased linearly ($P<0.01$) with increasing protein intake. Total free NEAA and the majority of NEAA concentration in milk did not differ between dietary treatments ($P<0.10$; Table 7).

Amino acid transporter mRNA abundance

The effect of dietary protein intake and stage of lactation on mRNA abundance is presented in Figures 4.5 to 4.9. The interaction between diet and

stage of lactation was not significant for ASCT1 (P>0.10) and CAT-1 (P>0.10) but tended to be significant for CAT-2B (P=0.09) and $B^{0,+}$ (P=0.08).

Protein intake. In Early lactation, CAT-2B mRNA abundance was lower (P<0.01) in sows fed the Excess diet when compared to Adequate fed sows but did not differ for sows fed the Deficient diet (Figure 4.5). Overall, in early lactation, CAT-2B mRNA abundance decreased linearly with increasing dietary protein intake (P<0.01). At Peak lactation, when compared to Adequate fed sows, CAT-2B mRNA abundance did not differ for either sows fed Deficient nor fed Excess. Overall, at Peak lactation, CAT-2B mRNA abundance tended to decrease linearly with increasing protein intake (P=0.12). During both Early and Late stages of lactation, when compared to sows fed Adequate, mRNA abundance of CAT-1 (Figure 4.6), $B^{0,+}$ (Figure 4.7) and ASCT-1 (Figure 4.8) did not differ for sows fed either Deficient or Excess diets. Overall, during both stages of lactation, there was no linear or quadratic relationships between dietary protein intake and mRNA abundance for CAT-1, $B^{0,+}$, and ASCT-1.

Stage of lactation. Compared to Early lactation, CAT-1 mRNA abundance tended to be higher at Peak lactation only for sows consuming the Excess diet (P<0.10; Figure 4.6), and $B^{0,+}$ mRNA abundance was higher only for sows consuming the Adequate diet (P<0.01; Figure 4.7). Dietary protein intake had no effect on ASCT1 mRNA abundance (P>0.10; Figure 4.8). Abundance for ASCT1

was higher at Peak compared to that at Early stage of lactation (P<0.01; Figure 4.10).

Amino acid transporter protein abundance

The CAT-1 antibody used in this experiment recognized a single band of expected size for CAT-1 protein (Figure 4.11) demonstrating that this protein is expressed in porcine mammary tissue during lactation. The ASCT1 antibody used in this experiment also recognized a single band of expected size for ASCT1 (Figure 4.12) demonstrating that this protein is expressed in porcine mammary tissue during lactation. No bands were detected when membranes were blotted with secondary antibody alone, this observations were used as negative controls.

Relative abundance of the CAT-1 protein in mammary tissue during Early and Peak lactation and at different levels of dietary protein intake is presented in Figures 4.13 and 4.14, respectively. Relative abundance of CAT-1 protein in lactating mammary tissue did not differ with protein intake (P>0.10) or stage of lactation (P>0.10). Relative abundance of the ASCT1 protein in mammary tissue during Early and Peak lactation is presented in Figure 4.15. Relative abundance of the ASCT1 protein in response to dietary protein intake could not be used due to a significant replicate by diet interaction. Relative abundance of the ASCT1 protein in lactating mammary tissue did not respond to changes in milk demand (P>0.10).

Amino acid transporter cellular localization

Immunohistochemical analysis of lactating porcine mammary tissue at Peak lactation revealed that CAT-1 (Figure 4.15) and ASCT1 (Figure 4.16) expression occurs mostly in epithelial cells with negligible staining in stromal cells and none in fat globules. CAT-1 expression, however, showed abundant localization in the perinuclear area of epithelial cells besides the expected staining in the cell membrane.

Discussion

P rotein intake. Feeding sows both under conditions of restricted and surfeit protein levels depressed piglet growth rate. This is in agreement with others reporting a curvilinear relationship between protein intake and piglet growth rate (Stahly et al., 1992, King et al., 1993; Johnston et al., 1999; Yang et al., 2000; Guan et al., 2002). In this study, the depression in growth rate was accompanied by a decrease in total protein and casein yield, indicating that protein intake below or in excess of requirements depress neonatal growth in part via change in mammary protein and/or casein synthesis. Values for true milk protein were lower than expected while values for total protein and for casein-N and casein-N as a percentage of total N are in agreement with others (Guan et al., 2002), indicating that true milk protein analysis underestimated true

protein yield. The strong depressive effect of excess protein intake on piglet growth rate may also have resulted from a combined decrease in both milk protein and lactose yield. In parallel, plasma concentrations for the majority of EAA increased linearly with increasing protein intake, indicating that extracellular availability of AA to the mammary gland in sows fed protein in excess of requirements was not a limiting factor to mammary protein synthesis.

Excessive protein intake decreases AA arterio-venous difference across the porcine mammary gland *in vivo* regardless of the linear increase in plasma AA availability (Guan et al., 2004), suggesting that the mammary gland is unable to utilize excess AA. Others have also demonstrated, using porcine mammary tissue explants, that excess availability of several EAA and NEAA depresses uptake of limiting AA for milk synthesis, namely lysine and valine. Hurley and coworkers (2000) reported a lysine uptake inhibition in porcine mammary tissue explants in the presence of high concentration of arginine, methionine, leucine, alanine, and ornithine in the medium. Similarly *in vivo*, Guan and coworkers (2004) reported a decreased AV difference for lysine while AV difference for leucine increased with excess intake of protein. Jackson and coworkers (2000) demonstrated an inhibition of valine uptake by porcine mammary tissue explants when methionine, leucine, alanine, and glutamine were present in the medium. In our study, plasma concentrations of alanine and glutamine in plasma of sows fed Excess protein were lower compared to sows fed Adequate protein suggesting that alanine and glutamine may not interact with lysine and valine uptake. Although the ratio of all AA to lysine were constant across all diets, the ratio of

leucine to lysine increased from 1.30 to 1.43 in sows consuming Excess protein intake compared to Adequate (data not shown). Likewise, the ratio of valine to lysine and isoleucine to lysine increased from 2.13 to 2.43 and 0.77 to 0.88, respectively. It is unlikely that the depressed lysine and valine uptake in the studies by Hurley and coworkers (2000) and Jackson and coworkers (2000), and the lower AV difference of AA in the study by Guan and coworkers (2004), was due to saturation of these transporters as the estimated Michaelis Constant (*K*m) for the transport of lysine and valine is 10-fold and 2-fold higher, respectively, than their plasma concentrations of sows consuming excess dietary protein intake. Inhibition or a depressed AA uptake can occur via interaction among AA and transporter proteins or by a change in the expression of amino acid transporter proteins. Regulation of AA transporter proteins expression by AA availability is well documented (Shennan et al., 1994; Taketani et al., 1998; Fernandez et al., 2001; Christie et al., 2001; Closs, 2002; Kimball and Jefferson, 2006). Furthermore, the evidence for a role of AA transporters in regulating the intracellular AA pool size by changes in transporter expression is growing (Hyde et al., 2003). We had hypothesized that AA transporter gene expression plays a role in modulating milk protein synthesis in the porcine mammary gland, presumably via changes in intracellular AA availability.

Four AA transporters belonging to three major transport systems were studied herein, two of the y^+ transport system, namely CAT-1 and CAT-2B, one of the ASC system, namely ASCT1, and one which constitutes in itself the $B^{0,+}$ system. Expression and/or activity of these AA transporters, among others, may

be crucial for the uptake of AA across the mammary gland. The y^+ transport system is Na^+-independent and specific for cationic AAs, namely arginine, histidine and lysine. Lysine is the first limiting AA for milk protein synthesis and neonatal pig growth (Kirchgessner et al., 1993; Knabe et al., 1996; Richert et al., 1997; O'Quinn et al., 2002). Arginine is taken up by the mammary gland in excess of that excreted in milk suggesting a requirement for mammary tissue metabolism that extends far above that required for milk protein synthesis and neonatal growth (Trottier et al., 1997). Under conditions of restricted protein or lysine intake, mammary extraction of lysine and arginine increased (Pérez Laspiur et al., 2006) and mammary lysine retention increased (Guan et al., 2002), respectively. In lactating goats fed diets limiting in histidine, mammary extraction rate of histidine increased from 17 to 74% to avoid a dramatic decrease in milk protein (Bequette et al., 2000). Adaptive regulation of CAT-1 by AA availability *in vitro* is well documented (Fernandez et al., 2001; Bruhat et al., 2002; Yaman et al., 2002; Hyde et al., 2003; Fernandez et al., 2003). Under nutrient deficient environments, global protein synthesis is depressed, while the synthesis of crucial proteins required for cell survival, including AA transporters, is increased (Hyde et al., 2003). Amino acid starvation is sensed by an AA response element sequence in the first exon of the CAT-1 gene (Bruhat et al., 2002) and this response element, when induced, stimulates the CAT-1 gene promoter (Fernandez et al, 2003). In addition to increased transcription of the CAT-1 gene, AA starvation increases CAT-1 mRNA stability via an additional nutrient sensor, an AU-rich element, located at the 3'-end of the transcript

(Yaman et al., 2002). *In vitro*, adaptive regulation of CAT-1 occurs not only by deficiency or excess of the CATs AA substrates but also via depletion or excess of any single EAA (Fernandez et al., 2003). In addition, mRNA abundance of CAT-1 is increased 3 to 18-fold by AA starvation in *in vitro* studies (Hyatt et al., 1997; Aulak et al., 1999).

We therefore hypothesized that under dietary protein deficiency, CATs mRNA and protein abundance would increase, and under protein excess, CATs mRNA and protein abundance would decrease. However, feeding a protein Deficient diet and subsequent decreased plasma AA concentration, did not upregulate CAT-1 mRNA abundance. Similarly, in the rat small intestine *in vivo*, restricted luminal availability of AAs failed to alter mRNA abundance of CAT-1 in this tissue (Howard et al., 2004). It is possible that the previously observed changes in CAT-1 gene expression with varying AA availability *in vitro* require complete starvation of AA. Unlike starvation, protein deficiency may not be sensed by the animal as an immediate life-threatening situation and therefore the response of these AA transporters to decreased AA availability may be attenuated. In addition, animals respond through changes in dietary protein availability by mobilizing body protein and thus provide supplemental endogenous AA. However, our dietary treatments must have created a real deficiency of AA whereby endogenous sources of AA were not sufficient since milk protein yield in particular and piglet growth rate were depressed in the Deficient group. In contrast to CAT-1, CAT-2B mRNA mammary abundance overall did respond to dietary protein intake with decreasing abundance with

increasing protein intake. In early phase of lactopoesis however, a critical period during which the lactogenic process establishes milk production capacity for the later stages of lactation (Ostrom, 1990), CAT-2B responded *per se* only to the excess in AA availability. CAT-2B has been studied *in vitro* in other cell types and shown to be inducible by infection and cellular stress to increase arginine uptake for NO synthesis (Stevens et al., 1996; Irie, et al., 1997; Kakuda et al., 1998).

Although modulation of the CAT-1 gene has been well documented *in vitro*, very few studies have been conducted and/or reported similar changes *in vivo*. Despite that CAT-1 is the major contributor to system y^+ transport in most cells (Closs, 2002), in this study dietary protein intake and thus AA availability did not alter CAT-1 protein levels either. It is possible that the lysine and/or arginine deficit resulting from intake of the Deficient diet was not sufficient to generate a starvation response at a cellular level similar to that observed with total AA depletion *in vitro*. In fact, arginine concentration, albeit considerably lower than that of the Adequate and Excess protein diets, was not below the NRC (1998) recommended dietary arginine concentration. CAT-1 may be more responsive to arginine deficiency than that of lysine. While AA transport activity was not measured in this experiment, it is possible that dietary protein and stage of lactation modulate AA transport across the mammary gland via changes in CAT-1 activity. Alternatively, it has been shown that posttranslational CAT-1 activity can be altered via its interactions with cytoskeletal proteins like fodrin (Zharikov and Block, 2000) and also by internalization of the transporter away from the plasma membrane rendering it inactive (Hatzoglou et al., 2004). Other

transporters are also compartmentalized away form the plasma membrane in order to decrease or totally devoid their function (Piroli et al., 2004; Watson and Pessin, 2001).

Mammary tissue is a heterogeneous mixture of cells with 80% of the total cell population consisting of epithelial cells (data not shown). Morphological identification of mammary epithelial cells, fat lobules, connective tissue and lobular ducts was performed by visual and comparative analysis guided by previous publications (Pitelka and Hamamoto, 1977; Wooding, 1977; Caruolo, 1980; Keenan and Dylewski, 1985; Akers et al., 2006). As demonstrated by immunohistochemistry, AA transporter CAT-1 and ASCT1 are localized in epithelial cells of mammary tissue and in the perinuclear area of epithelial cells. The CAT-1 antibody used in our study was raised against the 4^{th} putative extra cellular domain of the human sequence (Krotova et al., 2003). The perinuclear staining observed in this study and others may be due to a weak recognition of our antibody for non-solubilized non-reduced CAT-1; therefore, the perinuclear binding observed may be non-specific binding. On the other hand, it is possible that CAT-1 perinuclear staining indicates internalization of the CAT-1 transporter. Hence, if the CAT-1 transcript can indeed sense AA availability directly, the lack of response of CAT-1 to extracellular AA availability may be explained by internalization of the transporter. Others (Woodward et al., 1994) have also shown abundant perinuclear staining in addition to staining in plasma membrane clusters in porcine arterial endothelial cells and human fibroblasts using antibodies against the same CAT-1 epitope but of the murine peptide. In a review

on plasma membrane transporters for cationic AA, Closs and coworkers (2004) stated that subcellular localization of CAT-1 depends on the cell type. For instance, CAT-1 localizes to the plasma membrane of kidney and glioblastoma cells (Kizhatil and Albritton, 2002; Wolf et al., 2002) as well as in association with caveoli (McDonald et al., 1997), fodrin (Zharikov and Block, 2000), or actin cytoskeleton (Zharikov et al., 2001) of porcine artery endothelial cells.

In lactating mouse mammary tissue, *in vitro* starvation increased uptake of AA via system ASC by an increase in V_{max} suggesting an increase in transporter protein expression (Verma and Kansal, 1995). Thus, it was expected herein that ASCT1 would respond to AA deficiency through increase in mRNA abundance. System ASC, in addition to its capacity to transport linear dipolar NEAAs, such as alanine, serine, and cysteine, has highest affinity for AA containing a distal hydroxyl group, such as threonine (Kilberg et al., 1981). Although the ASCT1 transporter functions in a Na^+-dependent manner, predominantly as a mediator of neutral AA exchange rather than net uptake (Arriza et al., 1993), it can effectively induce net transport of a particular AA using as driving force the concentration gradient of another AA (Zerangue and Kavanaugh, 1996). In other words, high intracellular concentration of alanine has the potential to drive the uptake of threonine into the mammary gland. In this study, protein deficiency or excess did not modulate ASCT1 mRNA abundance. In contrast, Howard and coworkers (2004) reported an increase in ASCT1 mRNA abundance in rat small intestine by dietary removal of luminal AA. Surprisingly, in our study, plasma concentration of ASCT1 substrates, alanine and serine, decreased linearly with increasing dietary

protein intake, while threonine concentration in plasma increased. This may suggest that ASCT1 has a role as an AA exchanger translocating threonine from the mammary gland into plasma, while translocating alanine and serine from plasma into the mammary gland during Excess protein intakes. It is noteworthy to mention, however, that without kinetically defined movements of these AA, one should be cautious in interpreting the results as such. Nonetheless, the changes observed in plasma alanine and serine profile in parallel with changes in plasma threonine do indicate that threonine utilization by the mammary gland decreased in sows fed the Excess protein diet. Its adaptable role as an AA exchanger may explain why ASCT1 mRNA failed to respond to protein deficiency or excess.

It is possible that uptake of cationic AA and BCAA in porcine mammary gland may also be mediated by other transport systems such as system $B^{0,+}$. System $B^{0,+}$ transports a wide range of AA, including the normal substrates for CATs and ASCT1 (Van Winkle et al., 1985), with highest affinity for hydrophobic AA (Sloan and Mager, 1999). To our knowledge, this study is the first to investigate mRNA abundance responses of $B^{0,+}$ to dietary protein intake in an *in vivo* system. While $B^{0,+}$ substrates availability varied greatly with changes in dietary protein intake, $B^{0,+}$ mRNA abundance remained unaffected. In some cases *in vitro*, system $B^{0,+}$ exhibited adaptive regulation in the same manner as reported for CAT-1 (Taylor et al., 1996; Satsu et al., 1998). On the contrary, regulation of $B^{0,+}$ transport activity occurs in enterocytes under hormonal stimulation *in vitro* resembling that of feeding. Ray et al. (2005) reported a 250% increase in enterocyte glutamine uptake by system $B^{0,+}$ following treatment with

growth hormone. Several AA at ultraphysiological high concentrations can induce growth hormone release. For instance, arginine infusion to pigs and humans increased plasma growth hormone concentration (Rakoff et al., 1973; Atinmo et al., 1978). In our study, it is unlikely that plasma arginine concentration of sows consuming the Excess diet was sufficiently high to increase plasma growth hormone concentration. In fact, there were little changes in plasma arginine concentrations across the range of dietary protein intake. The lack in response of ASCT1 and $B^{0,+}$ mRNA abundance to deficiency and excess of AA availability indicate that changes in mRNA abundance of these AA transporters may not limit the inward or outward movements of AA across the porcine mammary gland.

Stage of lactation. Milk protein yield increased with advancement of lactation, while dietary protein intake and plasma concentrations for the majority of AA (and thus presumably AA availability to the mammary gland) remained unchanged, indicating that plasma AA retrieval by the mammary gland increased to meet milk protein demand by the growing progeny. Other studies have demonstrated that for the cationic AA lysine and arginine, arterio-venous differences across the mammary gland remain unchanged with advancement of lactation while that of other AA increase (Nielsen et al., 2002). In parallel to these observations, increasing day of lactation from 4 to 18 in this study increased overall transporter ASCT1 mRNA and $B^{0,+}$ mRNA abundance but did not affect the cationic specific AA transporters CAT-1 and CAT-2B abundance. In mammary glands of dairy cows, other nutrient transporters, including the glucose

transporter GLUT-8 (Zhao et al., 2004) are up-regulated with advancement of lactation, possibly to facilitate increase glucose extraction by the mammary gland.

Because the synthetic capacity of the porcine mammary gland is higher at peak lactation compared to early lactation (Kim et al., 1999), a higher demand for AA and energy is likely. The increase in synthetic capacity at peak lactation is sustained by increases in cell numbers (DNA concentration) and cell volume. Thus, one should acknowledge that the observed increase in ASCT1 and $B^{0,+}$ mRNA abundance may be due to an increase in mammary cell numbers. If so, our interpretation for the lack of CAT-1 and CAT-2B mRNA abundance response to lactopoesis may not be correct.

Similarly to CAT-1, ASCT1 is subject to *trans*-stimulation by its substrates, meaning that the rate of transport of ASC AA substrates is higher when the extracellular concentration of those AA is increased (Christensen, 1990). Although there is direct evidence for regulation of the ASCT1 gene by AA availability *in vivo* (Howard et al., 2004) and *in vitro* (Sakai et al., 2003), AA availability in this study did not alter mRNA abundance of the ASCT1 gene in porcine mammary tissue. On the other hand, ASCT1 mRNA was higher at Peak lactation (peak milk demand) compared to that of Early lactation. As for CAT-1, we hypothesized that ASCT1 may be adaptively regulated at the translational level and that the increase in mRNA abundance with milk demand would translate in higher ASCT1 protein abundance.

Neither AA availability nor milk demand altered the expression of ASCT1 transporter protein. Hence, the changes observed in mRNA abundance of ASCT1 with stage of lactation did not equate to changes in ASCT1 protein levels.

In this study, milk protein and casein yield, and neonatal pig growth were impacted by dietary protein intake with a corresponding impact on AA availability to the mammary gland. Of the four AA transporters studied herein, only CAT-2B responded to changes in AA availability at the mRNA abundance level, suggestive of CAT-2B involvement in promoting and restricting arginine and lysine transport into mammary tissue for protein synthesis. CAT-2B may play a crucial role in regulating milk protein synthesis in response to changes in protein concentration and dietary AA imbalance of sow diets.

On the other hand, increasing lactation demand increased ASCT1 and $B^{0,+}$ mRNA abundance, while the CATs remained unchanged, suggestive of a different regulatory pathway for stimulation of neutral AA transport across the mammary gland *in vivo*. Changes in AA uptake in response to AA availability and milk demand may occur via two other mechanisms: (1) changes in abundance of other amino acid transporters and/or (2) changes in kinetic properties (i.e., changes in *K*m, not in V_{max}) of the CAT and ASC transporter systems. Future studies on the regulation of amino acid availability to the mammary gland during lactation are necessary to investigate changes in kinetic and expression of other AA transporters such as those of systems A and L which also transport neutral AA in several tissues (Palacín et al., 1998).

Acknowledging that other AA transporters may be regulated by changes in AA availability and lactation demand, results nonetheless indicate that the AA transporters studied herein are differentially regulated by diet and stage of lactopoeisis, exemplifying the complexity and the dearth of knowledge about the regulation of these important genes in porcine mammary tissue. Furthermore, it is unknown if mRNA transcription rate and stability were altered by AA availability and stage of lactation. Nor if the observed changes in mRNA abundance translated into changes in transporter protein levels for CAT-2B and $B^{0,+}$. The possibility that the expression of these AA transporters may be regulated post-transcriptionally in mammary tissue, as shown in other tissues (Palacín et al., 1998), cannot be ruled out.

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TABLE 4.1

Ingredient and nutrient composition of experimental diets on as fed basis (%)

Item	Dietary treatments		
	12% CP	18% CP	24% CP
Ingredients			
Corn, dent yellow	23.96	36.34	48.22
Soybean meal, solv	22.75	34.50	45.78
Corn oil	0.60	1.45	2.30
Dicalcium phosphate	2.05	1.45	0.90
Limestone	0.60	0.87	1.10
Trace mineral[1]	0.50	0.50	0.50
Vitamin premix[2]	0.60	0.60	0.60
Salt, NaCl	0.30	0.30	0.30
Sowpac[3]	0.30	0.30	0.30
Solka floc	5.40	2.65	0.00
Cornstarch	42.94	21.04	0.77
Chemical analysis			
CP, %	12.08	18.19	24.05
Starch, %	58.44	50.65	36.29
Calculated analysis	12.08	18.19	24.05
DE, kcal/kg	3409	3454	3499
ME, kcal/kg	3305	3300	3298
Ca, %	0.75	0.75	0.75
P, %	0.61	0.60	0.60
NDF, %	10.73	10.73	10.72
Fat, %	1.37	1.34	2.57
Lysine, %	0.62	0.94	1.25

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

[2] Provided the following per kilogram of diet: 4,583 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-pantothenic acid, 22 mg niacin, 0.913 mg thiamine, 0.825 mg pyridoxine.

[3] Provided the following per kg premix: 918583 IU vitamin A, 73487 mcg biotin, 128602 mg choline, 551 mg folic acid.

TABLE 4.2

Primer sequences for real time PCR (5'→3')

cDNA	GenBank Accession #	Primers			
		Forward	Reverse	Tms	nt
CAT-1	NM_003045	TCATCATCACCTT CTGCATTGTG	CACAGCGCCCC TTTGG	60/58	63
CAT-2B	U76369	CTTTGATGGCCTT TCTGTTTGAC	GGCCATGAGTG TGCCAATG	60/59	69
$B^{0,+}$	AF151978	GGTGGTCCATTT TGGTCCATAT	GTGATCGTTTC AATCGAAGCAA	59/59	68
ASCT1	L14595 and L19444	GAAAGGCGAGCA GGAACTTG	GGCGATGTCTC CTCCTCAGA	59/59	61

TABLE 4.3

Effect of protein intake on sow lactation performance, milk yield and composition[1]

Item	Crude protein concentration			SEM[2]	Statistics[3]	
	12% (Deficient)	18% (Adequate)	24% (Excess)		L	Q
No. observations[4]	6	6	6			
Sow feed intake (kg/d)	5.080	5.152	5.517	0.306	0.33	0.70
Protein intake (g/d)	565^{c}	911^{b}	1315^{a}	46.7	<0.0001	0.63
Starch intake (g/d)	3000^{a}	2423^{b}	2006^{c}	99.5	<0.0001	0.51
Sow weight change (kg)	-2.13^{b}	2.50^{ab}	9.75^{a}	4.51	0.09	0.81
Piglet weight gain (g/d)	224^{ab}	254^{a}	220^{b}	15	0.51	0.05
Milk composition (%)						
True protein	4.46	4.58	4.62	0.17	0.50	0.86
Crude protein[5]	5.73^{b}	6.49^{a}	6.50^{a}	0.22	0.03	0.20
Fat	6.96^{b}	7.20^{ab}	8.19^{a}	0.43	0.04	0.48
Lactose	5.33	5.20	5.08	0.13	0.17	0.98
Solids	10.57	10.61	10.52	0.17	0.84	0.76
Casein	3.74^{bc}	4.54^{a}	4.38^{act}	0.27	0.11	0.18
Casein-N	12.65†	11.89	12.30	0.31	0.42	0.16
Casein-N, % of total N	52.42^{ab}	54.57^{a}	51.55^{b}	1.34	0.65	0.14

TABLE 4.3 continuation

Milk yield (kg/d)	12.4	13.6	12.3[†]	0.65	0.90	0.16
True protein, (g/d)	594	660	570[†]	33	0.59	0.08
Crude protein,(g/d)	751[a]	914[b]	782[ab]	55	0.69	0.05
Fat, g/d	978	1088	1041	96	0.61	0.51
Lactose,g/d	696	742	612[†]	53	0.25	0.20
Solids,g/d	1400	1513	1273[†]	90	0.30	0.13
Casein,g/d	492[b]	695[a]	480[b]	48	0.86	<0.01

[1] Data shown are least squares means for a lactation period of 18 days.
[2] Standard error of the mean.
[3] P-value of linear (L) and quadratic (Q) orthogonal contrasts.
[4] Number of observations per treatment.
[5] % N x 6.38.
[a,b,c] Means with different superscripts differ at P<0.05.
[†] Means tend to differ from Adequate at P<0.1 for milk composition and yield data.

TABLE 4.4

Effect of stage of lactation on sow lactation performance and milk composition[1]

Item	Stage of lactation		SEM[2]	Statistics[3]
	Early	Peak		P-value
No. Observations[4]	18	18		
Sow feed intake (kg/d)	5.381	5.576	0.379	0.43
Protein intake (g/d)	945	992	58	0.50
Starch intake (g/d)	1756	2899	157	<0.001
Milk composition (%)				
True protein	5.17	4.28	0.14	<0.001
Crude protein[5]	7.03	5.46	0.14	<0.001
Fat	8.90	6.72	0.49	0.03
Lactose	4.59	5.51	0.13	<0.001
Solids	10.44	10.63	0.14	0.41
Casein	4.61	3.83	0.18	<0.001
Casein N	12.06	12.49	0.23	0.18
Casein N / Total N	51.51	54.19	0.84	0.003
Milk yield (kg/d)	11.4	15.1	0.43	<0.001
True protein (g/d)	578	638	24	0.06
Crude protein (g/d)	802	830	36	0.38
Fat (g/d)	1030	1041	80	0.93
Lactose (g/d)	532	834	36	<0.001
Solids (g/d)	1176	1615	57	<0.001
Casein (g/d)	535	576	36	0.38

[1] Data are least squares means for a lactation period of 18 days.
[2] Standard error of the mean.
[3] P-value of ANOVA.
[4] Number of observations per treatment.
[5] % N x 6.38.

TABLE 4.5

Effect of protein intake on plasma glucose and amino acid concentration[1]

Item	Crude protein concentration			SEM[2]	Statistics[3]	
	12% (Deficient)	18% (Adequate)	24% (Excess)		L	Q
No. Observations[4]	6	6	6			
Glucose, mg/dL	67.31	75.25	76.82	5.10	0.19	0.62
Amino Acid, µmol/L						
Essential						
Total	782.11	1033.89	1400.05	63.30	<0.001	0.61
Arg	87.29	116.42	123.84	15.60	0.11	0.58
His	85.53	98.11	106.65	12.19	0.24	0.89
Ile	65.31	82.70	124.86	9.37	<0.001	0.30
Leu	98.81	138.47	203.24	15.48	<0.001	0.52
Lys	77.01	106.79	142.48	16.78	0.01	0.89
Met	16.00	17.51	27.53	3.03	0.02	0.27
Phe	39.14	49.70	77.62	5.94	<0.001	0.25
Thr	116.85	142.66	182.08	14.53	<0.01	0.71
Trp	36.71	54.13	66.45	5.33	<0.01	0.70
Val	159.83	227.40	345.67	21.94	<0.001	0.36

TABLE 4.5 continuation

Nonessential						
Total	3215	2751	2588	190	0.04	0.51
Ala	637.38	399.28	279.36	41.49	<0.001	0.26
Asn	22.70	21.90	18.85	2.39	0.27	0.71
Asp	8.60	8.83	10.19	1.01	0.28	0.66
Cit	59.73	63.02	75.00	6.74	0.13	0.61
Cys	8.89	9.57	8.48	1.47	0.85	0.63
Gln	1424.75	1327.64	1231.15	103.45	0.20	0.99
Glu	278.17	188.10	161.72	26.01	<0.01	0.33
Gly	87.63	99.40	107.18	10.19	0.19	0.88
Orn	41.87	55.59	78.79	7.69	<0.01	0.62
Pro	387.73	373.89	427.80	41.04	0.50	0.51
Ser	49.37	46.56	37.50	2.68	<0.01	0.36
Tau	63.99	61.27	63.60	4.64	0.95	0.66
Tyr	81.84	109.75	136.04	13.39	0.01	0.96

[1] Data shown are least square means for a lactation period of 18 days.
[2] Standard error of the mean.
[3] P-value of linear (L) and quadratic (Q) orthogonal contrasts.
[4] Number of observations per treatment.

TABLE 4.6

Effect of stage of lactation on plasma glucose and amino acid concentration[1]

Item	Stage of lactation		SEM[2]	Statistics[3]
	Early	Peak		P-value
No. Observations[4]	18	18		
Glucose, mg/dL	79.86	66.40	3.69	<0.01
Amino Acid, µmol/L				
Essential				
Total	1028.45	1115.58	63.41	0.24
Arg	101.86	116.50	10.47	0.18
His	78.16	115.36	7.93	<0.001
Ile	84.68	97.24	6.04	0.03
Leu	142.22	151.47	10.10	0.32
Lys	117.11	100.41	11.20	0.14
Met	25.81	14.89	2.08	<0.01
Phe	52.06	58.91	4.16	0.16
Thr	146.58	147.81	11.94	0.95
Trp	50.46	54.40	3.59	0.29
Val	229.76	258.84	15.28	0.10
Nonessential				
Total	2924.40	2778.29	142.76	0.42
Ala	447.98	429.37	28.82	0.18
Asn	22.09	20.21	1.61	0.25
Asp	10.10	8.32	0.70	0.03
Cit	56.98	74.85	4.98	0.02
Cys	11.02	6.95	1.14	0.05
Gln	1406.22	1249.48	76.22	0.14
Glu	227.83	190.83	16.66	0.02

TABLE 4.6 continuation

Gly	100.02	96.12	7.12	0.63
Orn	56.83	60.67	4.91	0.34
Pro	397.94	395.01	30.91	0.95
Ser	49.82	39.13	2.13	<0.01
Tau	70.07	55.85	3.44	<0.01
Tyr	103.62	114.80	9.81	0.36

[1] Data are least squares means for a lactation period of 18 days.
[2] Standard error of the mean.
[3] P-value of ANOVA.
[4] Number of observations per treatment.

TABLE 4.7

Effect of protein intake on milk free amino acid concentration[1]

Item	Crude protein concentration			SEM[2]	Statistics[3]	
	12% (Deficient)	18% (Adequate)	24% (Excess)		L	Q
No. Observations	6	6	6			
Amino Acid, µmol/L						
Total	4750.43	4276.15	4666.63	363.59	0.87	0.35
Essential						
Total	1708.17	1923.61	2254.75	126.86	0.01	0.72
Arg	165.89	284.05	192.86	54.20	0.73	0.14
His	126.25	109.94	120.31	15.20	0.78	0.49
Ile	7.99	8.72	6.53	1.65	0.54	0.49
Leu	53.31	77.16	67.95	12.72	0.41	0.30
Lys	77.78	75.43	84.61	10.08	0.63	0.65
Met	19.33	18.02	19.22	3.62	0.98	0.78
Phe	52.31	57.54	58.62	5.92	0.46	0.78
Tau	1128.10	1128.58	1562.31	78.16	0.001	0.04
Thr	69.94	72.56	73.41	15.17	0.87	0.96
Trp	37.40	40.20	42.39	5.82	0.55	0.97
Val	23.95	43.43	35.44	6.46	0.23	0.11

TABLE 4.7 continuation

Nonessential						
Total	2949.66	2368.87	2406.92	262.55	0.16	0.35
Ala	241.87	177.99	151.81	22.95	0.02	0.51
Asn	496.31	382.58	321.24	59.75	0.05	0.73
Asp	208.31	257.42	274.84	24.93	0.08	0.61
Cys	138.75	137.69	136.56	18.94	0.93	0.99
Gln	662.39	503.40	426.13	82.19	0.06	0.69
Glu	588.56	333.44	482.92	74.54	0.33	0.05
Gly	165.05	156.96	166.27	22.29	0.97	0.76
Orn	21.99	20.49	19.71	1.17	0.18	0.81
Pro	89.99	103.82	109.47	16.80	0.42	0.85
Ser	133.23	92.43	103.33	21.57	0.34	0.35
Tyr	168.48	216.61	221.26	30.30	0.24	0.57

[1] Data are least squares means for a lactation period of 18 days.
[2] Standard error of the mean.
[3] P-value of linear (L) and quadratic (Q) orthogonal contrasts.
[4] Number of observations per treatment.

Figure 4.1. Effect of dietary protein intake and stage of lactation on milk true protein percentage. Bars with different letters differ at $P<0.05$ across stages of lactation.

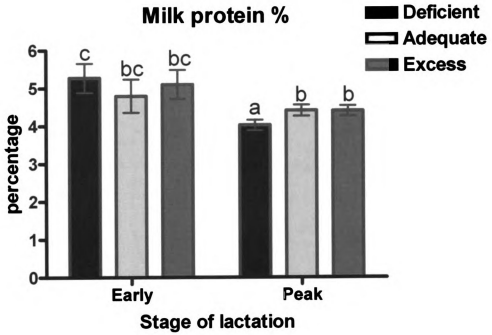

Milk protein %
Deficient
Adequate
Excess
percentage
6
5
4
3
2
1
0
c
bc
bc
a
b
b
Early
Peak
Stage of lactation

Figure 4.2. Effect of dietary protein intake and stage of lactation on milk casein percentage. Bars with different letters differ at $P<0.05$ across stages of lactation.

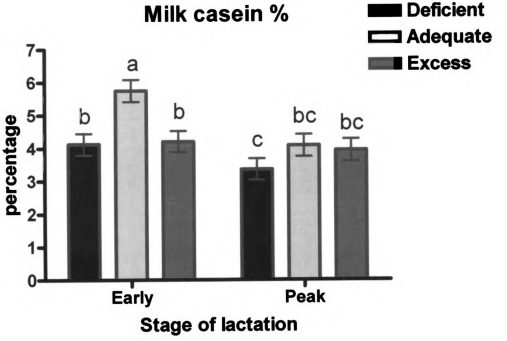

Milk casein %
Deficient
Adequate
Excess
percentage
7
6
5
4
3
2
1
0
a
b
b
c
bc
bc
Early
Peak
Stage of lactation

Figure 4.3. Effect of dietary protein intake and stage of lactation on plasma glucose concentration. Bars with different letters differ at $P<0.05$ across stages of lactation.

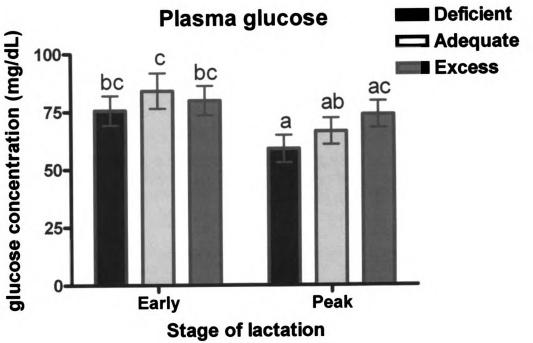

Plasma glucose
Deficient
Adequate
Excess
glucose concentration (mg/dL)
100
75
50
25
0
bc
c
bc
a
ab
ac
Early
Peak
Stage of lactation

Figure 4.4. Effect of protein intake on piglet average daily gain (g/d) and milk casein and true protein yield (g/d). The relationship was curvilinear between protein intake, piglet average daily gain (quadratic, $P<0.05$) and yields of milk casein (quadratic, $P<0.01$) and true protein (quadratic, $P=0.08$).

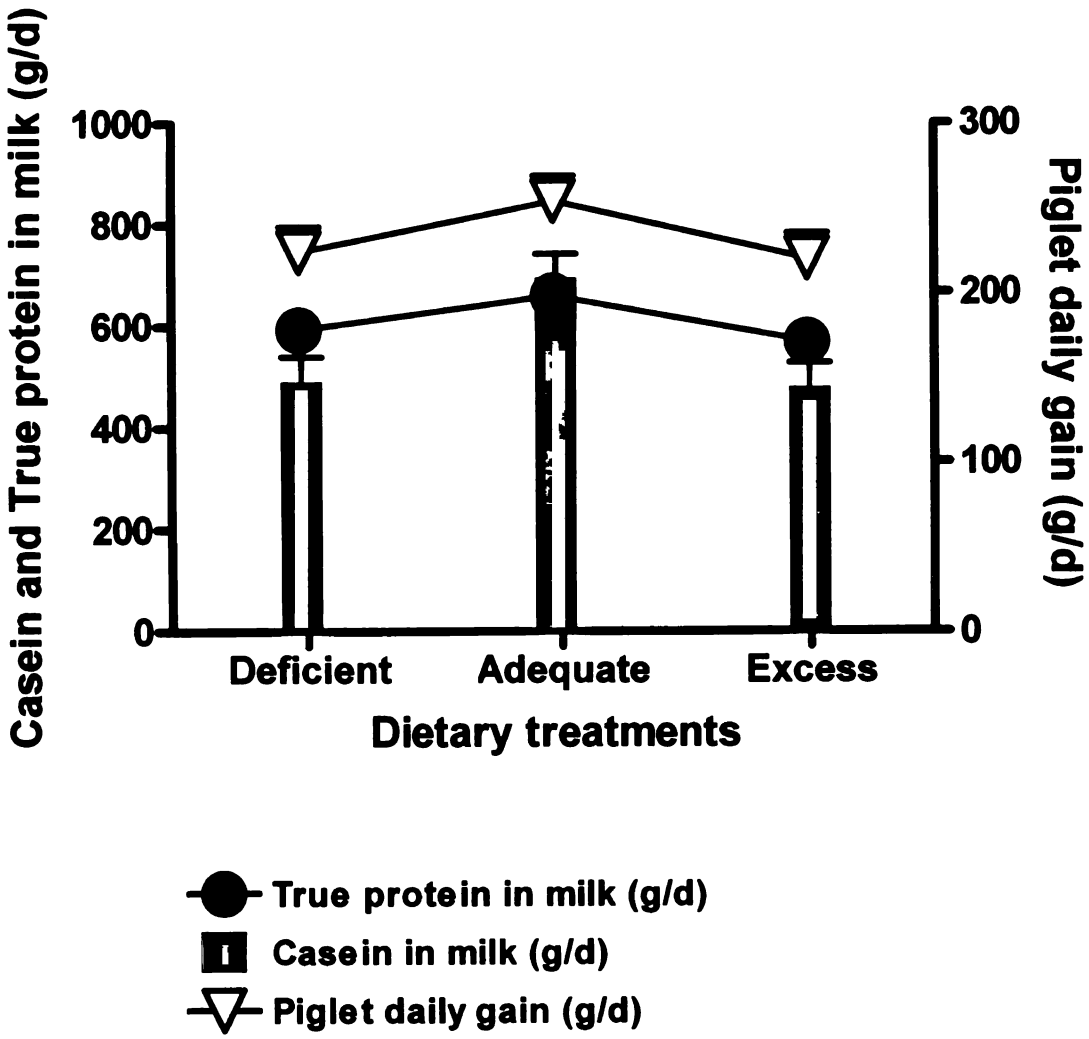
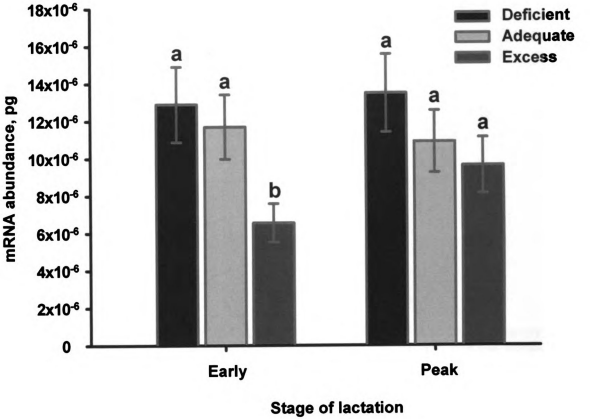

Casein and True protein in milk (g/d)
1000
800
600
400
200
0
300
200
100
0
Piglet daily gain (g/d)
Deficient
Adequate
Excess
Dietary treatments
True protein in milk (g/d)
Casein in milk (g/d)
Piglet daily gain (g/d)

Figure 4.5. Effect of dietary protein intake and stage of lactation on CAT-2B mRNA transcript abundance. Relationship between protein intake and mRNA abundance in Early lactation was linear ($P<0.01$). Relationship between protein intake and mRNA abundance at Peak lactation tended to be linear ($P=0.12$). Bars with different letters a vs b differ at $P<0.01$ across stages of lactation.

CAT-2B


18x10-6
16x10-6
14x10-6
12x10-6
10x10-6
8x10-6
6x10-6
4x10-6
2x10-6
0
mRNA abundance, pg
Deficient
Adequate
Excess
a
a
b
a
a
a
Early
Peak
Stage of lactation

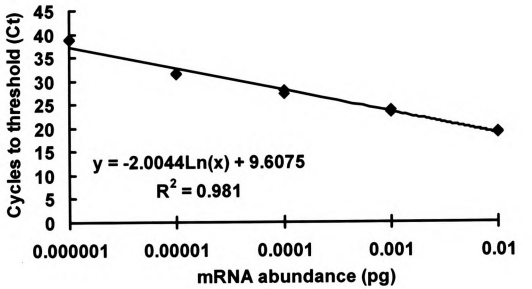
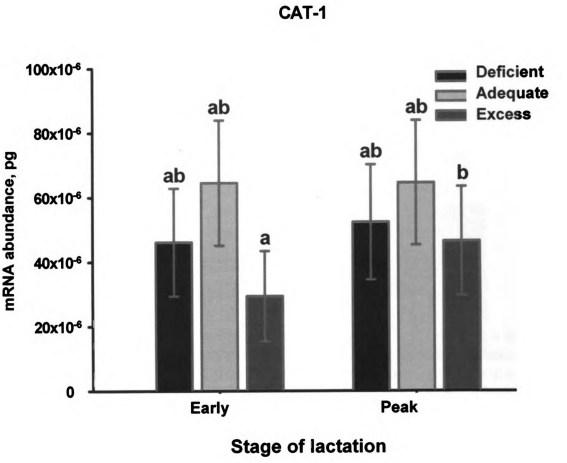

45
40
35
30
25
20
15
10
5
0
Cycles to threshold (Ct)
y = -2.0044Ln(x) + 9.6075
R2 = 0.981
0.000001
0.00001
0.0001
0.001
0.01
mRNA abundance (pg)

Figure 4.6. Effect of dietary protein intake and stage of lactation on CAT-1 mRNA transcript abundance. There was no relationship (linear or quadratic) between protein intake and mRNA abundance. Bars with different letters tended to differ at $P<0.10$ across stages of lactation.


CAT-1
100x10-6
80x10-6
60x10-6
40x10-6
20x10-6
0
mRNA abundance, pg
Deficient
Adequate
Excess
ab
ab
a
ab
ab
b
Early
Peak
Stage of lactation

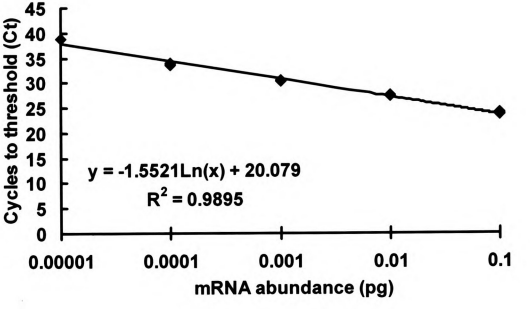
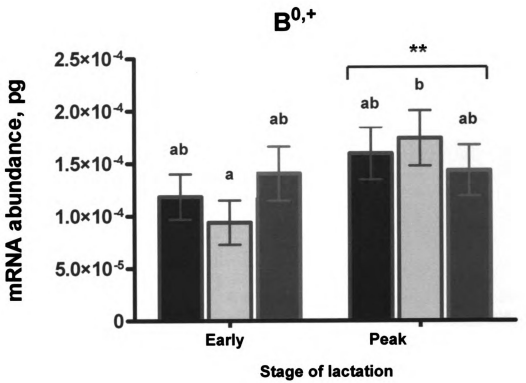

45
40
35
30
25
20
15
10
5
0
Cycles to threshold (Ct)
y = -1.5521Ln(x) + 20.079
R2 = 0.9895
0.00001
0.0001
0.001
0.01
0.1
mRNA abundance (pg)

Figure 4.7. Effect of dietary protein intake and stage of lactation on $B^{0,+}$ mRNA abundance. There was no relationship (linear or quadratic) between protein intake and mRNA abundance. Bars with different letters differ at P<0.01 across stages of lactation. Diet x day interaction tended to differ at P=0.09. Overall (across diets), $B^{0,+}$ mRNA abundance was higher at Peak compared to Early stage (**P < 0.01).


B0,+
**
mRNA abundance, pg
2.5×10-4
2.0×10-4
1.5×10-4
1.0×10-4
5.0×10-5
0
ab
a
ab
ab
b
ab
Early
Peak
Stage of lactation

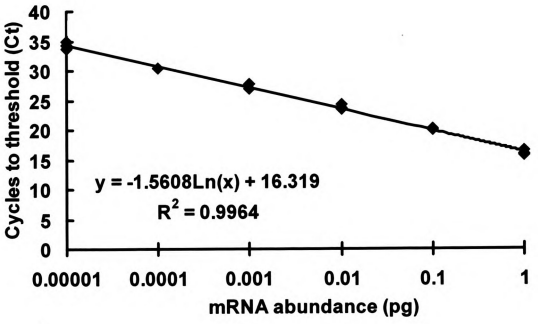
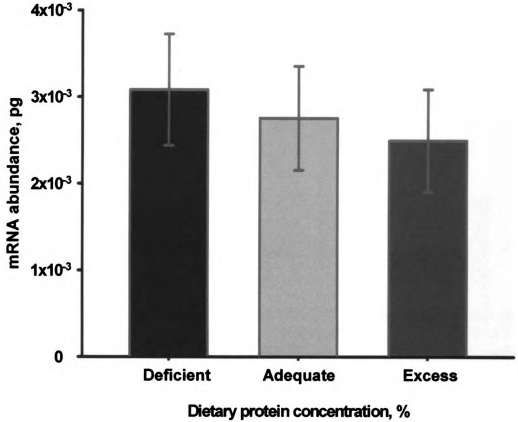

Cycles to threshold (Ct)
40
35
30
25
20
15
10
5
0
y = -1.5608Ln(x) + 16.319
R2 = 0.9964
0.00001
0.0001
0.001
0.01
0.1
1
mRNA abundance (pg)

Figure 4.8. Effect of dietary protein intake on ASCT1 mRNA transcript abundance. There was no relationship (linear or quadratic) between dietary protein intake and ASCT1 mRNA abundance.

ASCT1


4x10-3
3x10-3
2x10-3
1x10-3
0
mRNA abundance, pg
Deficient
Adequate
Excess
Dietary protein concentration, %

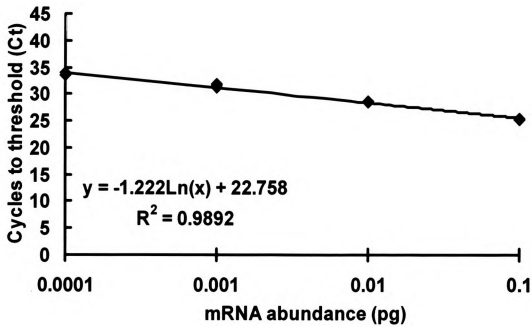

45
40
35
30
25
20
15
10
5
0
Cycles to threshold (Ct)
y = -1.222Ln(x) + 22.758
R2 = 0.9892
0.0001
0.001
0.01
0.1
mRNA abundance (pg)

Figure 4.9. Effect of stage of lactation on ASCT1 mRNA transcript abundance. ASCT1 mRNA abundance was higher at Peak lactation compared to Early lactation (***P<0.001).

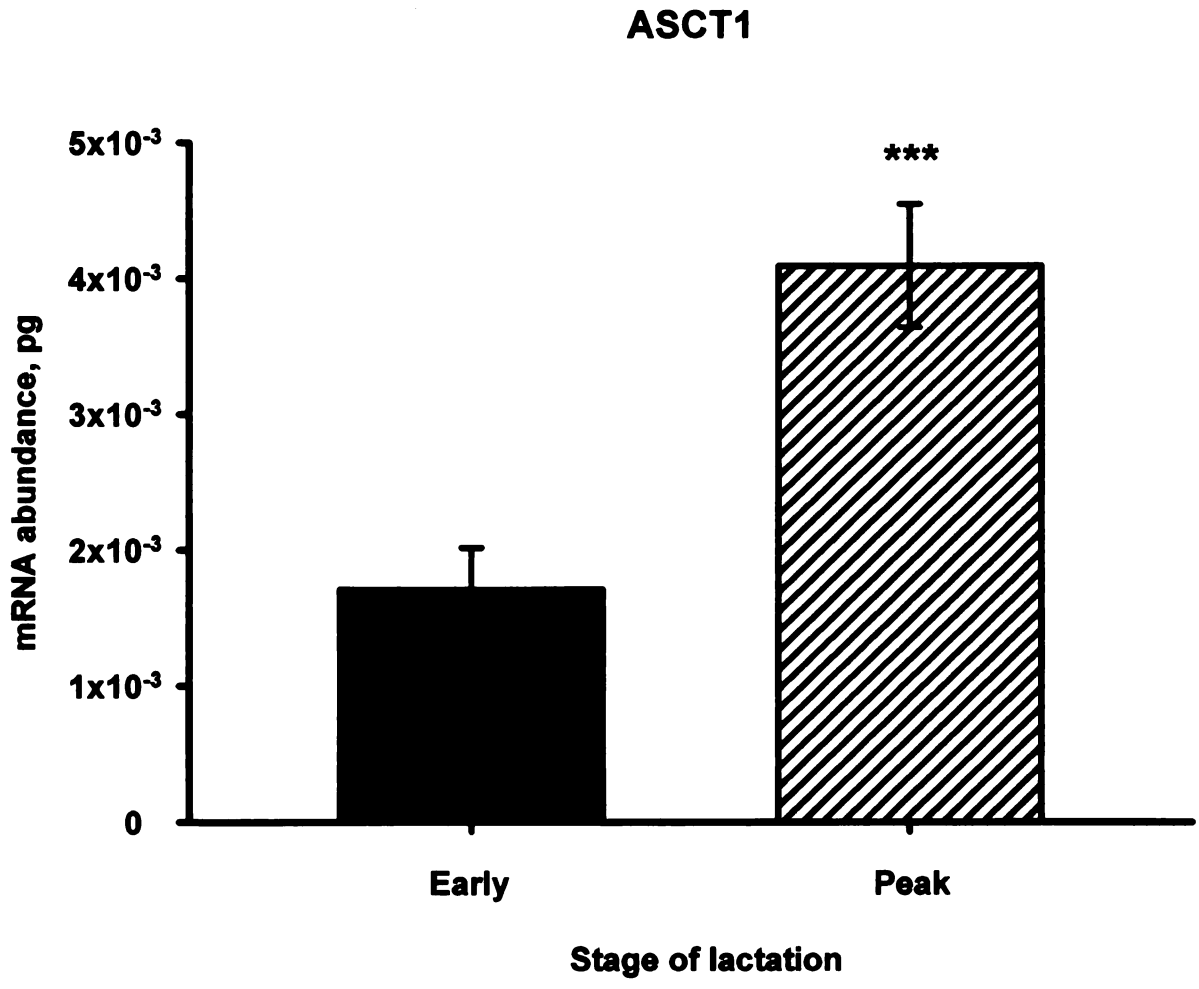

ASCT1
5x10-3
4x10-3
3x10-3
2x10-3
1x10-3
0
mRNA abundance, pg
***
Early
Peak
Stage of lactation

Figure 4.10. Representative western blot demonstrating the presence of CAT-1 amino acid transporter in porcine mammary tissue during lactation at three levels of dietary protein intake. Uniformity of loading was confirmed by Ponceus S staining and scanning of the membranes. Representative band is shown.

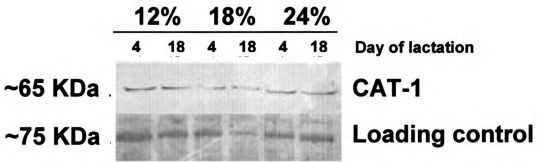

12%
18%
24%
4
18
4
18
4
18
Day of lactation
~65 KDa
CAT-1
~75 KDa
Loading control

Figure 4.11. Representative western blot demonstrating the presence of ASCT1 amino acid transporter in porcine mammary tissue during lactation at three levels of dietary protein intake. Uniformity of loading was confirmed by Ponceus S staining and scanning of the membranes. Representative band is shown.

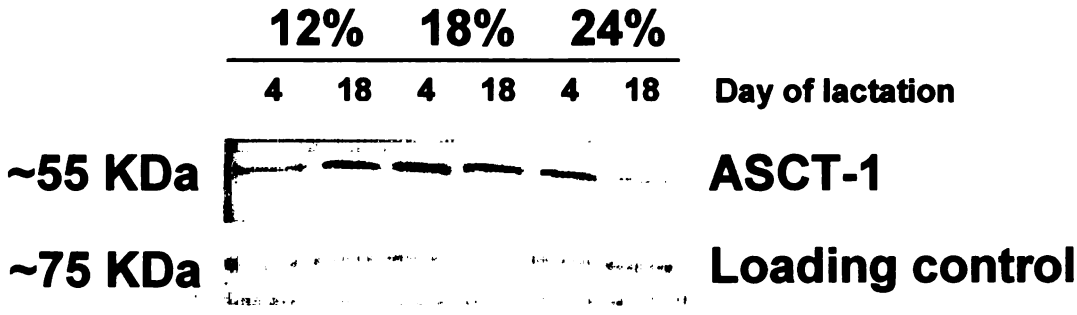

12%
18%
24%
4
18
4
18
4
18
Day of lactation
~55 KDa
ASCT-1
~75 KDa
Loading control

Figure 4.12. Relative abundance of CAT-1 protein in porcine mammary tissue at Early and Peak stage of lactation. CAT-1 protein levels did not different with stage of lactation in porcine mammary tissue ($P>0.10$).

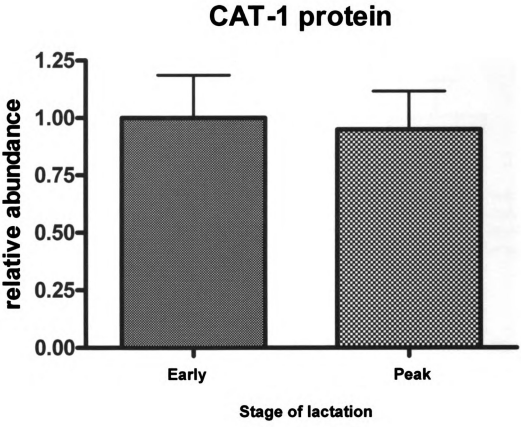

CAT-1 protein
relative abundance
1.25
1.00
0.75
0.50
0.25
0.00
Early
Peak
Stage of lactation

Figure 4.13. Relative abundance of CAT-1 protein in porcine mammary tissue from sows fed a Deficient (12%), Adequate (18%), or Excess (24%) protein diets. CAT-1 protein levels were not altered with dietary protein intake in porcine mammary tissue during lactation ($P>0.10$).

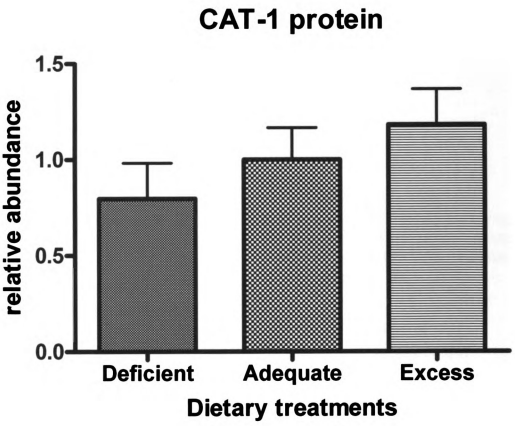

CAT-1 protein
relative abundance
1.5
1.0
0.5
0.0
Deficient
Adequate
Excess
Dietary treatments

Figure 4.14. Relative abundance of ASCT1 protein in porcine mammary tissue at Early and Peak stage of lactation. ASCT1 protein levels did not different with stage of lactation in porcine mammary tissue ($P>0.10$).

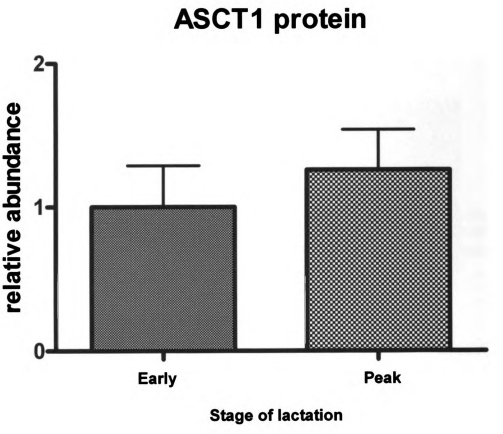

ASCT1 protein
relative abundance
2
1
0
Early
Peak
Stage of lactation

Figure 4.15. Immunohistochemistry staining of porcine mammary tissue at Peak lactation with CAT-1 antiserum (a) or negative control (b). CAT-1 localizes to membrane and perinuclear areas in epithelial cells (1) but not stromal (2) cells.

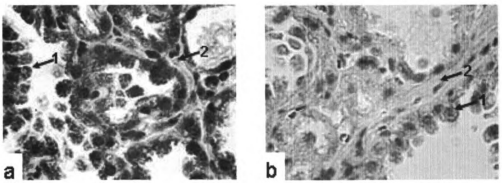
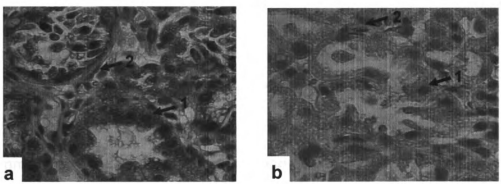

1
2
a
2
1
b

Figure 4.16. Immunohistochemistry staining of porcine mammary tissue at Peak lactation with ASCT1 (a) or negative control (b). ASCT1 localizes to membrane and perinuclear areas in epithelial cells (1) but not stromal (2) cells.


a
2
1
b
2
1

SUMMARY AND CONCLUSIONS

During lactation, intake of protein in adequate quantity and a balanced amino acid profile by the mother is crucial to provide amino acids essential for the synthesis of milk proteins. Conversely, excessive intake of dietary protein may be detrimental to milk protein production as revealed by depressed neonatal growth. Dietary amino acids are transported via the circulatory system to organs and enter tissues via specific transporters. The transport processes are seemingly regulated by mechanisms that are still largely undefined. In the present study, a porcine model was used to begin addressing whether protein nutrition and milk demand modulate amino acid transport in mammary tissue. To test the hypothesis that under conditions of protein nutritional stress the mammary gland adaptively regulates amino acid transport, gene expression of amino acid transporters (i.e., CAT-1, CAT-2A, CAT-2B, ASCT1, and $B^{0,+}$) were determined at two stages of lactopoiesis. The specific objectives of this dissertation were to 1) develop a non-invasive mammary biopsy technique allowing repeated collection of large quantities of mammary tissue from nursing sows; 2) determine the presence of amino acid transporter transcripts and proteins, respectively, in mammary tissue of nursing sows; 3) investigate the effect of nutritional protein stress (i.e. deficiency and excess) in nursing sows on their milk protein and casein yields, and their piglet growth rate; and 4) determine the level of amino acid transporter transcript and protein expression in mammary

tissue of nursing sows in response to dietary protein deficiency and excess at two levels of milk demand.

Transcripts for CAT-1, CAT-2B, ASCT1, and $B^{0,+}$ were detected while that of CAT-2A was not detected in lactating porcine mammary gland under the experimental conditions presented in this thesis and within the limits of the technique used (i.e., Northern blots). The presence of CAT-1, CAT-2B, ASCT1, and $B^{0,+}$ mRNA was furthered confirmed using Q-RT-PCR. Proteins at least for CAT-1 and ASCT1 were detected in mammary tissue of nursing sows.

Dietary protein deficiency and protein excess decreased milk protein and casein yields, and nursing pig growth rate while neither dietary protein deficiency nor excess affected CAT-1, ASCT1 or $B^{0,+}$ mRNA abundance. In contrast, increasing dietary protein intake from deficient to excess conditions decreased mRNA abundance of CAT-2B, and more so for sows fed excessive levels of protein under lower milk demand status or early stage of lactation. Similar, protein excess diet was more detrimental than protein deficiency on piglet growtg rate. Changes in CAT-2B mRNA abundance in response to protein deficiency and excess indicate that mammary cells adaptively down-regulated CAT-2B, in response to conditions of protein excess. Conversely, there was no evidence for adaptive regulation both at the mRNA abundance for $B^{0,+}$ and at mRNA and protein abundance for CAT-1 and ASCT1.

Increasing milk demand had no effect on amino acid transporters CAT-1 and CAT-2B mRNA abundance, but increased ASCT1 and $B^{0,+}$ mRNA abundance. An increase in ASCT1 and $B^{0,+}$ transcript abundance with increase

milk demand may be a result of the well recognized increase in cell number (DNA concentration) in porcine mammary tissue with advancement of lactation. In this case, the lack of response observed for CAT-1 and CAT-2B mRNA abundance, and ASCT1 and CAT-1 protein abundance, to an increase in milk demand may be translated into masked decreases in abundance of these transporters or changes in the relative number of epithelial cells versus stromal cells. It is unknown if the increase in mammary cell numbers with advancement of lactation is a result of proportional increases in both epithelial and stromal cells equally. It remains to be investigated if protein levels of CAT-2B and $B^{0,+}$ are altered with increasing milk demand.

Transporter CAT-2B may be of physiological importance in terms of modulating the entry of the nutritionally first limiting amino acid for lactation, and may be responsible in part for the improved efficiency of lysine utilization in the face of reduced extracellular lysine availability. More prominently so, CAT-2B may play a larger role behind the reduced efficiency of lysine utilization upon excess extracellular exposure to lysine, or to other amino acids in particular arginine and the large neutral amino acids. For instance in this study, sows fed excessive quantities of protein were not only exposed to high concentrations of arginine, but to even higher levels of large neutral amino acids, in particular leucine and valine. These amino acids either specifically share transport with lysine (arginine) or utilize with lower affinity the y^+ system, and have been well shown in various *in vitro* systems to reduce lysine uptake.

At least in regards to the amino acid transporters studied in this thesis, the response to either protein nutritional stress or milk demand was not uniform across the selected transporters. These results indicate that amino acid transporters may be differentially regulated in response to protein nutritional stress. Differentially regulating expression of amino acid transporters in response to amino acid availability would serve as a biological advantage to mammary cells in order to adapt and survive in extreme scenarios and utilize available amino acids and nutrients with precise efficiency. Various amino acid transport systems with different specificities and affinities for amino acids have been identified and these can be expressed and active in cells at different stages of development as well as at different states of the cell. A mosaic of amino acid transporters can then be expressed at a certain time in a cell and it is the combination of these transport systems that capacitate cells to utilize extracellular amino acids effectively for their survival and growth. For example, a hallmark of mammary tumor cells is an overexpression of the System A transporter. This transporter is overexpressed in tumor cells to a degree that these cells have a biological advantage over normal cells in terms of amino acid uptake and therefore thrive and survive at better rates than non-tumor cells. Thus, differential expression of this amino acid transporter make tumor cells more fit to survive than non-tumor cells. In a way, the ability of expressing the right assortment of amino acid transporters at the right time, thus, being able to sense amino acid availability and responding accordingly, would make mammary cells more efficient in synthesizing milk proteins with the available nutrients and,

ultimately, in providing their offspring with the best milk protein quantity and quality possible ensuring survival of their young, hence, the species.

The mere fact that the transporters CAT-1, ASCT1 and $B^{0,+}$ studied herein did not respond with changes in mRNA abundance in some cases or in protein abundance, at least in two of the transporters with available antibodies, does not necessarily minimize or eliminate their theorized role in modulating amino acid transport; it remains to be determined whether these transporters modulate amino acid uptake through changes in affinity. Overall, the results of this study highlight the hidden complexity between amino acid interaction and lactation performance outcome, yet also shed some lights on one potential mechanism responsible for defining the efficiency of dietary protein utilization for milk protein synthesis.

While this thesis was limited to the study of four transporter genes, it is recognized that many other genes important to milk protein synthesis and mammary epithelial and stromal cell functions may be influenced by the treatments imposed on the sows in these studies. Increased knowledge of the molecular basis regulating amino acid availability for milk protein synthesis will improve our understanding of porcine protein nutrition during lactation, specifically regarding lactation performance responses to amino acid interaction in diets of lactating sows. This knowledge ultimately contributes to expanding and enhancing our models of amino acid utilization for increasing milk production efficiently in lactating sows.

APPENDICES

APPENDIX A

AMINO ACID EXTRACTION BY PORCINE MAMMARY GLAND: A PILOT STUDY TO TEST FEED INTAKE RESPONSE TO DIETARY PROTEIN INTAKE

Abstract

Six multiparous sows were used to test the hypothesis that amino acid (AA) extraction rate by the porcine mammary gland decreases with increasing availability of dietary AA. Sows were fed graded concentrations of protein consisting of 12, 18, and 24% protein to provide Deficient, Adequate, and Excess protein and AA, respectively. Sows were fitted with mammary vein and carotid artery catheters between d 3 and 5 of lactation. Arterial and venous blood samples were collected on d 10, 14, and 18 of lactation. For each blood sampling day, blood samples were collected every 30 min for a total of 3 h, and samples pooled per sow. Piglet average daily gain (ADG) tended to decrease linearly ($P=0.12$) with increasing dietary crude protein concentration. Mammary extraction rates of arginine and lysine decreased linearly ($P=0.06$) with increasing dietary crude protein concentration. Leucine, threonine, and phenylalanine extraction rates tended to increase with increasing protein intake from 12 to 18% and to decrease with increasing protein intake from 18 to 24% (quadratic, $P<0.10$). Mammary gland AA extraction rates decreased with increasing dietary protein intake, indicating that AA uptake processes are regulated in part by AA availability.

Keywords: amino acid uptake, dietary protein, mammary gland, lactation

Introduction

Milk protein intake is crucial to neonatal development and growth (Millar, 1970). Free AA in blood are the major precursors for milk protein synthesis (Mepham, 1982) and their arterial concentration appears to be the most limiting factor affecting their uptake by the mammary gland (Bequette et al., 2000). Uptake of essential AA by mammary epithelial cells *in vitro* is rate limiting for milk protein synthesis (Gomez et al., 1995). However, the regulatory mechanisms behind arterial AA uptake by the mammary gland are unknown. Both deficiency and excess of dietary protein intake by lactating sows result in depression of piglet ADG compared to that of sows fed adequate amount of protein (Stahly et al., 1992, King et al., 1993; Yang et al., 2000; Guan et al., 2002). Depression in piglet ADG is correlated to lower milk protein yield (Guan et al., 2002). In lactating goats and sows, reduced availability of any one of the essential AA in plasma decreases mammary AA uptake and milk protein synthesis (Bequette et al., 2000; Guan et al., 2002). In lactating sows, AA arterio-venous (AV) difference and extraction rates across the mammary gland is also a function of AA arterial concentration (Trottier et al., 1997). Mammary AA AV difference responds curvilinearly to dietary protein intake, with protein deficiency leading to low AV difference but high extraction rate and protein excess leading to low AV difference but with low extraction rate (Guan et al., 2004). This suggests that AA uptake and transport processes can be modulated by AA availability, possibly as an attempt by the mammary tissue to maintain optimum intracellular

concentrations of AA for protein synthesis (Bequette et al., 1996). In the study by Guan et al. (2004), dietary AA profiles across diets were adjusted to be the same using AA in crystalline form. The objective of this study was to determine AA extraction rate response by the porcine mammary gland to dietary protein intake independent from crystalline AA inclusion or maintenance of AA profile across diets. A second objective was to test the feasibility of using corn and soybean meal as the sole ingredients to vary dietary crude protein concentration. It was hypothesized that increasing dietary total protein concentration from deficient to excess would decrease extraction rates of AA by the porcine mammary gland as observed in the study by Guan et al. (2004).

Materials and Methods

Dietary Treatments

Experimental diets were formulated to vary in CP concentrations by modifying the ratio of corn to soybean meal. An Adequate diet (18 % protein) was formulated to meet the protein and AA requirements for high producing sows and all other nutrients according to the NRC (1998). A Deficient (12 % protein) and an Excess (24 % protein) diets were formulated to provide CP and AA intake below and above those of NRC (1998), respectively. All three dietary treatments were formulated to be isocaloric with a metabolizable energy (ME) of 3.4 Mcal/kg. Diet ingredient and nutrient composition are shown in Table A1.

Animals

Six multiparous (parity 2 or 3) sows (Landrace x Yorkshire) were selected one week prior to parturition and allocated to dietary treatments. Sows were provided ad libitum access to feed throughout the lactation period and feed intakes were recorded daily from parturition to weaning on d 18. Litters were randomly cross-fostered within 48 hours after birth to ensure litter size of 11 pigs and uniform suckling demand across all sows. Live body weight, ultrasonic backfat depth and loin area were recorded post-farrowing (d 0) and at weaning (d 18). Pigs were individually weighed at birth (d 0), and on d 7, 14 and 18.

Sample collection

Cannulation of the anterior main mammary vein and the carotid artery were performed between d 3 and 5 of lactation to allow for recovery after parturition and proper ingestion of colostrums by piglets, as described by Trottier et al. (1995). Sows were allowed to recover for 5 to 6 days prior to blood and milk sample collection. Blood samples were collected on d 10, 14 and 18 of lactation. A total of seven arterial and mammary venous blood samples were obtained from each sow on each sampling day at 30 min intervals. Plasma was separated, pooled per sampling day and sow, and stored at -20°C. Amino acids in plasma samples were quantified by reverse-phase HPLC (Pico Tag, Waters) as previously described (Guan et al., 2002).

Amino acid extraction rates

Amino acid extraction rates were calculated as described previously (Guan et al., 2002) where mammary AA extraction rate = AV difference across the mammary gland / plasma arterial AA concentration.

Statistical Analysis

Differences in lactation performance and AA extraction rates were determined by analysis of variance. Sources of variation in the model included sow, diet and day of lactation as classification variables and the two-way interactions of interest, diet by day of lactation. Sow nested within diet was included as a random effect. Statistical analysis was performed using the MIXED procedure of SAS (1998). Repeated measures analysis was used for repeated measures over days of lactation based on different covariance structures (Litell et al., 1998). The best fitting repeated measures covariance structure was determined using the Akaike information criterion (AIC). Relationships between dietary protein intake and response variables were determined by linear and quadratic orthogonal contrasts. Differences were considered significant at. $P<0.05$. Tendency for differences between treatments was considered at $P\leq0.10$

Results and Discussion

Sow and litter lactation performances are shown in Table A2. The combined voluntary feed intake response by the sows during the 18-d lactation period with dietary protein concentration did not create significant differences between protein intake. Consequently, the low dietary energy intake per se may have limited litter growth rate.

Amino acid extraction rates by the mammary gland are shown in Table A3. Mammary extraction rates represent the amount of AA being taken up by the mammary gland relative to AA availability. Mammary extraction rates of lysine and arginine tended to decrease with increasing dietary protein concentration (linear, $P=0.06$), suggesting protein excess limited milk protein synthesis as piglet ADG also decreased linearly ($P=0.12$). Extraction rates of leucine, threonine, and phenylalanine also were lowest ($P<0.10$) in sows fed the Excess diet.

Because energy intake may have been limiting, it is possible that AA extraction rates decreased in response to a decrease in energy substrates availability. In the study by Guan et al. (2004), sows were offered similar dietary protein concentrations with no impact on voluntary dry matter intake, yet AA extraction rates decreased with feeding excess protein. Nonetheless, AA extraction rate responses to changes in AA availability in this pilot study resemble that of Guan et al. (2004), who suggested that AA transporter processes may be regulated in part by AA availability. Amino acid uptake by the mammary gland may be regulated by AA transporters that sense AA availability

and regulate cellular uptake of AAs (Hyde et al., 2003). The majority of amino acid transporters in the porcine mammary gland have not been characterized to date. However, of the diverse group of AA transport systems characterized so far in other tissues and species, three systems appear to play a major role in transport of all essential AA. An AA transport system specific for cationic AA, namely y^+, has been characterized in a number of tissues (Palacín et al., 1998), including the porcine mammary gland (Hurley et al., 2000). In this study, lysine and arginine extraction were notably affected by dietary protein availability, suggesting that system y^+ transporter may be involved in mediating this response. So far, there is no reported evidence for the molecular presence of system y^+ transporters in the mammary gland. To a lesser extent, threonine extraction rate was also depressed with deficiency and excess protein intake. System ASC transports alanine, serine, and cysteine but has highest affinity for threonine (Kilberg et al., 1981), the second limiting AA for milk protein synthesis in lactating sows (Pettigrew, 1993). The molecular presence of system ASC in mammary gland is unknown. Extraction rates of the branched chain AA, leucine, valine and isoleucine, were depressed with increasing protein intake. System $B^{0,+}$ transports a wide range of AAs with the highest affinity for hydrophobic AAs, such as the branched-chain AAs valine, leucine and isoleucine (Van Winkle et al., 1985; Sloan and Mager, 1999). The branched chain AAs play important roles in the process of milk protein synthesis and overall mammary metabolism (Trottier et al., 1997; Nielsen et al., 2002) and therefore a depressed extraction rate of these AAs may also limit milk production.

Implication

Amino acid availability to the mammary gland modulates AA uptake and thus possibly milk protein synthesis. Depressed postnatal growth of piglets under conditions of deficiency and excesses of sow dietary protein intake may be related to changes in AA extraction by the mammary gland and thus milk protein yield. Preliminary results generated by this study imply that AA uptake by the mammary gland may be regulated by AA availability. This regulation may occur in part via AA transporter systems Future studies investigating the expression and regulation of AA transporters in porcine mammary gland during lactation are essential to our understanding of AA utilization and milk protein synthesis. Results of this study reinforces the importance of maintaining the AA profile equal across dietary treatments in order to insure similar voluntary feed intake across dietary treatments and thus expected changes in dietary crude protein intake. Accordingly, to test AA transporter gene expression response to AA availability, corn to soybean meal ratio was maintained and variation in crude protein concentrations was achieved with dilution with corn starch.

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TABLE A1

Ingredient and nutrient composition of experimental diets on as fed basis (%)

	Dietary treatments		
Item	Deficient	Adequate	Excess
Ingredients			
Corn, dent yellow	80.16	64.16	47.16
Soybean meal,	13.00	29.00	46.00
Corn oil	2.30	2.30	2.30
Dical. phos.	1.97	1.97	1.97
Limestone	0.87	0.87	0.87
MSU vit premix[1]	0.60	0.60	0.60
MSU min premix[2]	0.50	0.50	0.50
Salt, NaCl	0.30	0.30	0.30
Sow pack[3]	0.30	0.30	0.30
Calculated analysis			
CP, %	12.35	18.03	24.06
ME, Kcal/kg	3348	3310	3270
CFat, %	3.32	2.94	2.53
CFiber, %	9.42	10.02	10.65
Ca, %	0.81	0.86	0.91
P, %	0.67	0.73	0.80
Ca:P	1.20	1.17	1.14

[1] Provided the following per kilogram of diet: 4,583 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-pantothenic acid, 22 mg niacin, 0.913 mg thiamine, 0.825 mg pyridoxine.

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

[3] Provided the following per kg premix: 918583 IU vitamin A, 73487 mcg biotin, 128602 mg choline, 551 mg folic acid.

TABLE A2

Protein intake on sow lactation performance and piglet average daily gain[1]

	Dietary treatments[3]				Statistics[4]	
Item	Deficient	Adequate	Excess	SEM[2]	L	Q
Weight change, kg[5]	-23.36	-14.29	-18.37	8.71	0.71	0.58
Average feed intake, kg/d	5.641	4.467	3.883	0.849	0.23	0.80
Average protein intake, g/d	732.1	787.1	871.1	144.0	0.55	0.94
Backfat change, mm	0.75	-6.86	-4.19	2.78	0.30	0.23
Loin eye area change, cm^2	-4.77	-1.00	-3.00	3.19	0.72	0.51
Piglet ADG, g	259.7	238.3	201.3	19.2	0.12	0.76

[1] Data shown are least squares means for a lactation period of 18 days with n=2 per dietary treatment.
[2] Standard error of the mean.
[3] Dietary treatments: Deficient (12% protein), Adequate (18% protein) and Excess (24% protein).
[4] P-value of linear (L) and quadratic (Q) orthogonal contrasts.
[5] Lactation period (d 18 – d 1).

TABLE A3

Extraction rate (%)by the mammary gland[1]

	Dietary treatments[3]				Statistics[4]	
Amino acid	Deficient	Adequate	Excess	SEM[2]	L	Q
Arginine	25.66	21.10	15.90	2.27	0.06	0.92
Histidine	12.62	14.92	12.82	2.30	0.95	0.49
Isoleucine	35.04	38.74	26.83	4.22	0.26	0.23
Leucine	37.19	40.58	31.45	2.03	0.14	0.09
Lysine	59.13	41.10	21.54	9.10	0.06	0.95
Phenylalanine	27.96	34.53	25.14	2.41	0.47	0.07
Threonine	21.31	22.99	13.93	2.98	0.18	0.24
Tryptophan	9.87	7.96	6.28	3.43	0.51	0.98
Valine	25.95	24.39	17.07	2.97	0.13	0.49

[1] Data shown are least square means for a lactation period of 18 days with n=2 per dietary treatment.
[2] Pooled standard error of the mean.
[3] Dietary treatments: Deficient (12% protein), Adequate (18% protein) and Excess (24% protein).
[4] P-value of linear (L) and quadratic (Q) orthogonal contrasts.

APPENDIX B

Sus scrofa Amino Acid Transporter $B^{0,+}$ and ASCT1 mRNA, partial sequences

Porcine $B^{0,+}$ and ASCT1 cDNA probes were developed by polymerase chain reaction (PCR) using pooled cDNA from porcine MT as a template. PCR primers were designed from the published human $B^{0,+}$ (GenBank Accession number AF151978) and human ASCT1 (GenBank Accession numbers L14595 and L19444) cDNA sequences. PCR was carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA) using Taq DNA polymerase as recommended by manufacturer (Invitrogen, Life Technologies, USA). Resulting PCR amplification products were visualized as single bands of correct size using agarose gel electrophoresis (1.8% gel), gel purified (Wizard® PCR Preps DNA Purification System, Promega, Madison, WI), ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI), and the recombinant plasmids transformed into JM109 competent cells (Promega, Madison, WI) and stored at -80°C. The 1058 and 373 bp cDNA probes for $B^{0,+}$ and ASCT1, respectively, were DNA sequenced in both directions to confirm identities using a dye-terminator fluorescent cycle sequencing technique and an ABI® 3100 Genetic Analyzer (PerkinElmer Applied Biosystems, Foster City, CA), and the sequence information deposited in GenBank (Accession numbers: AY375264 and AY375265). Sequence alignment and homology information to the human $B^{0,+}$ (GenBank Accession number AF151978) and human ASCT1 (GenBank Accession number L14595) cDNA sequences is provided.

The porcine partial cDNA sequence for amino acid transporter $B^{0,+}$ (*italics*) has a 93% homology to the human $B^{0,+}$ cDNA sequence (GenBankAccession numbers AY375265 and AF151978, respectively).

```
433  AGGTGTGGGAATTACAATGGTCCTGATCTCCATTTTTGTGACAATCTATTACAATGTCAT  492
     ||||||||||||||||||||||||| |||||||||||||||||| ||||||||||
1    AGGTGTGGGAATTACAATGGTCCTGATATCCATTTTTGTGACAATCTACTACAATGTCAT  60

493  AATTGCCTATAGTCTTTACTACATGTTTGCTTCTTTTCAAAGTGAACTACCATGGAAAAA  552
     |||||||||||||||||||||||||||||||||||||||| |||||||||| ||||
61   AATTGCCTATAGTCTTTACTACATGTTTGCTTCTTTTCAAAGTAAACTACCATGGGAAAA  120

553  TTGTTCTTCGTGGTCAGATAAAAACTGTAGCAGATCACCAATAGTAACTCACTGTAATGT  612
     |  ||||   ||| ||||| ||||||||||||||| || |||||||| || ||||||||
121  TKKTTCTWWTTGGGCAGATGAAAACTGTAGCAGATCGCCTATAGTAACACATTGTAATGT  180

613  GAGTA  617
     |||||
181  GAGTA  185
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641   ATCATCCAAATGAATAAAAGCTGGGTAGACATCAACAATTTTACCTGCATCAACGGCAGT  700
      ||| |||||||||||||||||||||||   ||||||| |||||||||||| || ||
200   ATCTTCCAAATGAATAAAAGCTGGGTAGATGCCAACAATCTTACCTGCATCAATGGAAGC  259

701   GAAATTTATCAGCCAGGGCAGCTTCCCAGTGAACAATATTGGAATAAAGTGGCGCTCCAA  760
      ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
260   GAAGTTTATCAGCCAGGGCAGCTTCCCAGTGAACAATATTGGAATAAAGTGGCGCTCCAA  319

761   CGGTCAAGTGGAATGAATGAGACTGGAGTAATTGTTTGGTATTTAGCACTTTGTCTTCTT  820
      ||||| ||||||||| |||||||||||||| ||||| |||||||||||||||||||||||
320   CGGTCGAGTGGAATGGATGAGACTGGAGTCATTGTGTGGTATTTAGCACTTTGTCTTCTT  379

821   CTGGCTTGGCTCATAGTTGGAGCAGCACTATTTAAAGGAATCAAATCGTCTGGCAAGGTG  880
      ||||| |||||||||||||||||||| |||||||||||||| || ||||||||||||
380   CTGGCCTGGCTCATAGTTGGAGCAGCACTGTTTAAAGGAATCAAGTCTTCTGGCAAGGTG  439

881   GTATATTTTACAGCTCTTTTCCCCTATGTGGTCCTACTCATCCTGTTAGTACGAGGTGCA  940
      |||||||||||||| |||||||||||| || |||||||||||||  | ||||| |||
440   GTATATTTTACAGCTATTTTCCCCTATGTTGTTCTACTCATCCTGTTGATCCGAGGAGCA  499

941   ACTCTGGAGGGTGCTTCAAAAGGCATTTCATACTATATTGGAGCCCAGTCAAATTTTACA 1000
      |||||||| ||||| |||||||||||||||||||||||||| ||||||||||||||||
500   ACTCTGGATGGTGCATCAAAAGGCATTTCATACTATATTGGAGCACAGTCAAATTTTACA  559

1001  AAACTTAAGGAAGCTGAGGTATGGAAAGATGCTGCCACTCAGATATTTTACTCCCTTTCA 1060
      ||||||| |||||||||||| |||||||||||||||| |||||||||||||||||||||
560   AAACTTAGGGAAGCTGAGGTTTGGAAAGATGCTGCCACACAGATATTTTACTCCCTTTCT  619

1061  GTGGCTTGGGGTGGCTTAGTTGCTCTATCATCTTACAATAAGTTCAAAAACAACTGCTTC 1120
      |||||||||||||| |||||||||||||||||||||| |||||||| ||||| |||| |
```

```
620   GTGGCTTGGGGTGGTTTAGTTGCTCTATCATCTTACAACAAGTTCAATAACAATTGCTAC  679

1121  TCTGATGCCATTGTGGTTTGTTTGACAAACTGTCTCACTAGCGTGTTTGCTGGATTTGCT 1180
      || |||||||||| |||||||||||||||| || ||||| ||||| ||||||||||||
680   TCAGATGCCATTGTAGTTTGTTTGACAAACTGCCTTACTAGTGTGTTCGCTGGATTTGCT  739

1181  ATTTTTTCTATATTGGGACACATGGCCCATATATCTGGAAAGGAAGTTTCTCAAGTTGTA 1240
      |||||||||||||||||||||||| | |||||||||||||||||| ||||||||||||
740   ATTTTTTCTATATTGGGACACATGGCTCGTATATCTGGAAAGGAAGTCTCTCAAGTTGTA  799

1241  AAATCAGGTTTTGATTTGGCATTCATTGCCTATCCAGAGGCTCTAGCCCAACTCCCAGGT  300
      |||||||||||| |||||||| |||||||| |||||||| ||||||||||||||||||||
800   AAATCAGGTTTCGATTTGGCGTTCATTGCTTATCCAGAAGCTCTAGCCCAACTCCCAGGT  859

1301  GGTCCATTTTGGTCCATATTATTTTTTTTCATGCTTTTAACTTTGGGTCTCGATTCTCAG 1360
      || |||||||||||||||||||||| |||||||||| |||||||||| || ||||||
860   GGGCCATTTTGGTCCATATTATTTTTCTTCATGCTTTTGACTTTGGGTCTGGACTCTCAG  919

1361  TTTGCTTCGATTGAAACGATCACAACAACAATTCAAGATTTATTTCCCAAAGTGATGAAG 1420
      |||||||| |||||||||| |||||||| ||||||||||||||||||||||||||||||
920   TTTGCTTCCATTGAAACGATTACAACAACGATTCAAGATTTATTTCCCAAAGTGATGAAG  979

1421  AAAATGAGGGTTCCCATAACTTTGGGCTGCTGCTTGGTTTTGTTTCTCCTTGGTCTCGTC 1480
      ||||||||||||||| ||||||||| ||||||||||||||||||||||||||||||||
980   AAAATGAGGGTTCCCACAACTTTGGGTTGCTGCTTGGTTTTGTTTCTCCTTGGTCTCGTC 1039

1481  TGTGTGACTCAGGCTGGAAT  1500
      |||| ||||||||||||||
1040  TGTG-GACTCAGGCTGGAAT  1058
```

The porcine partial cDNA sequence for amino acid transporter ASCT1 (*italics*) has a 92% homology to the human ASCT1 cDNA sequence (GenBankAccession numbers AY375264 and L14595, respectively).

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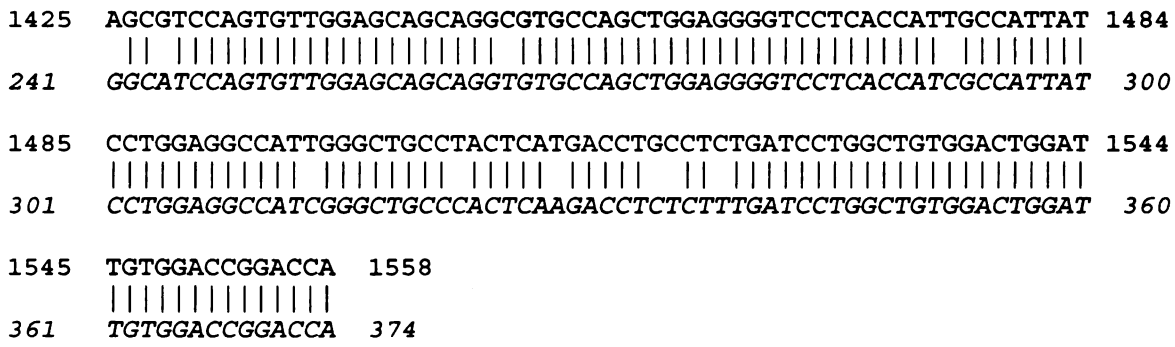
1185  CCCATTTGCGACAGCATTTGCTACCTGCTCCAGCTCAGCGACCCTTCCCTCTATGATGAA 1244
      |||||||||||||||||||| |||||||||||||||| |||||||||| ||||||||
1     CCCATTTGCGACAGCATTTGCCACCTGCTCCAGCTCAGCAACCCTTCCCTCGATGATGAA   60

1245  GTGCATTGAAGAGAACAATGGTGTGGACAAGAGGATCAGCAGGTTTATTCTCCCCATCGG 1304
      |||||||||||| |||||||||| |||||||||||||||||||| ||||| ||||||||
61    GTGCATTGAAGATAACAATGGTGTAGACAAGAGGATCAGCAGGTTCATTCTTCCCATCGG  120

1305  GGCCACCGTGAACATGGACGGAGCAGCCATCTTCCAGTGTGTGGCCGCGGTGTTCATTGC 1364
      |||||||||||||||||||||||| ||||||||||||| |||||||| ||||||||||
121   GGCCACCGTGAACATGGACGGAGCTGCCATCTTCCAGTGCGTGGCCGCCGTGTTCATTGC  180

1365  GCAACTCAACAACATAGAGCTCAACGCAGGACAGATTTTCACCATTCTAGTGACTGCCAC 1424
       || ||||||||  | ||||| || |||||||||||||||||||| |||||||| |||||
181   CCAGCTCAACAATGTGGAGCTGAAAGCAGGACAGATTTTCACCATCCTAGTGACAGCCAC  240
```

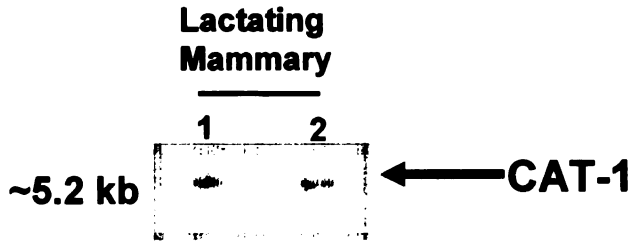

1425 AGCGTCCAGTGTTGGAGCAGCAGGCGTGCCAGCTGGAGGGGTCCTCACCATTGCCATTAT 1484
241 GGCATCCAGTGTTGGAGCAGCAGGTGTGCCAGCTGGAGGGGTCCTCACCATCGCCATTAT 300
1485 CCTGGAGGCCATTGGGCTGCCTACTCATGACCTGCCTCTGATCCTGGCTGTGGACTGGAT 1544
301 CCTGGAGGCCATCGGGCTGCCCACTCAAGACCTCTCTTTGATCCTGGCTGTGGACTGGAT 360
1545 TGTGGACCGGACCA 1558
361 TGTGGACCGGACCA 374

APPENDIX C

Porcine Poly A+ RNA Northern Blot Analysis of CAT-1

As shown in Figure 3.1 (Chapter 3), hCAT-1 hybridized to porcine mammary tissue during lactation. While the band was of expected size, it required intensification. Thus, a second northern blot using poly A^+ RNA was performed (Figure C1). Following total mammary RNA isolation and assessment of RNA purity and quality as described in Chapter 3, purification of poly A^+ RNA was performed using the Oligotex method (Qiagen Inc.; Valencia, CA). Duplicate 5 µg-samples of this Poly A^+ RNA were size separated on a 1.2% 0.2M formaldehyde agarose gel. RNA was then transferred to a nylon membrane and hybridized with the CAT-1 probe as described in Chapter 3.

Figure C1. Northern blot analysis of CAT-1 mRNA abundance in poly A^+ RNA from lactating porcine mammary tissue, demonstrating that the human CAT-1 cDNA probe hybridized well to a single transcript of correct size (5.2 kb). Lanes 1 and 2 contained 5µg of mRNA from mammary tissue of one sow. The hCAT-1 cDNA was donated by Dr. Closs. Blot was exposed to X-ray film for 168h.


Lactating
Mammary
1
2
~5.2 kb
CAT-1

APPENDIX D

Representative Standard Curves for Candidate Genes

Standard curves for Q-RT-PCR were generated using cDNA templates and primers as described in Chapters 3 and 4. Representative standard curves for the genes of interest are reported in Figures D1 to D5 below.

Figure D1. Representative standard curve generated by Q-RT-PCR for porcine CAT-1.

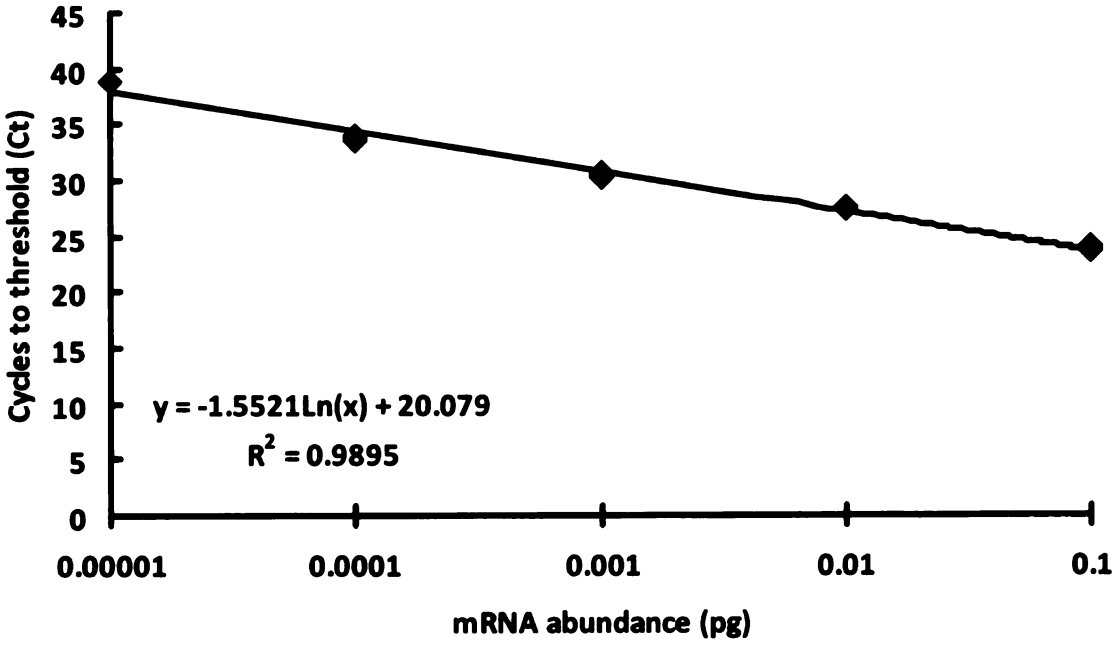

45
40
35
30
25
20
15
10
5
0
Cycles to threshold (Ct)
y = -1.5521Ln(x) + 20.079
R2 = 0.9895
0.00001
0.0001
0.001
0.01
0.1
mRNA abundance (pg)

Figure D2. Representative standard curve generated by Q-RT-PCR for porcine CAT-2B.

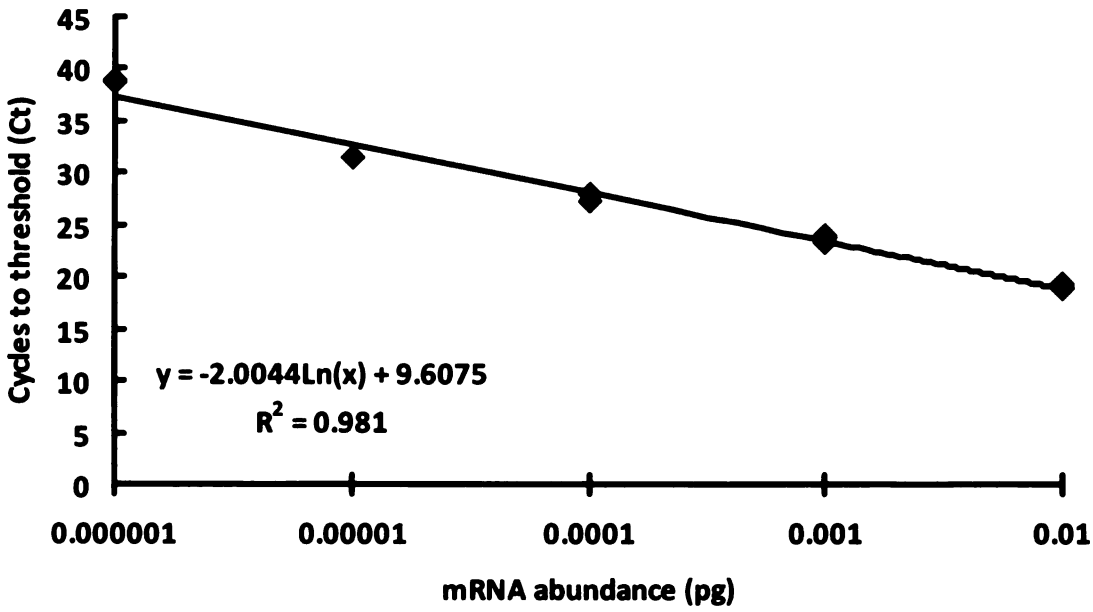

45
40
35
30
25
20
15
10
5
0
Cycles to threshold (Ct)
y = -2.0044Ln(x) + 9.6075
R2 = 0.981
0.000001
0.00001
0.0001
0.001
0.01
mRNA abundance (pg)

Figure D3. Representative standard curve generated by Q-RT-PCR for porcine $B^{0,+}$.

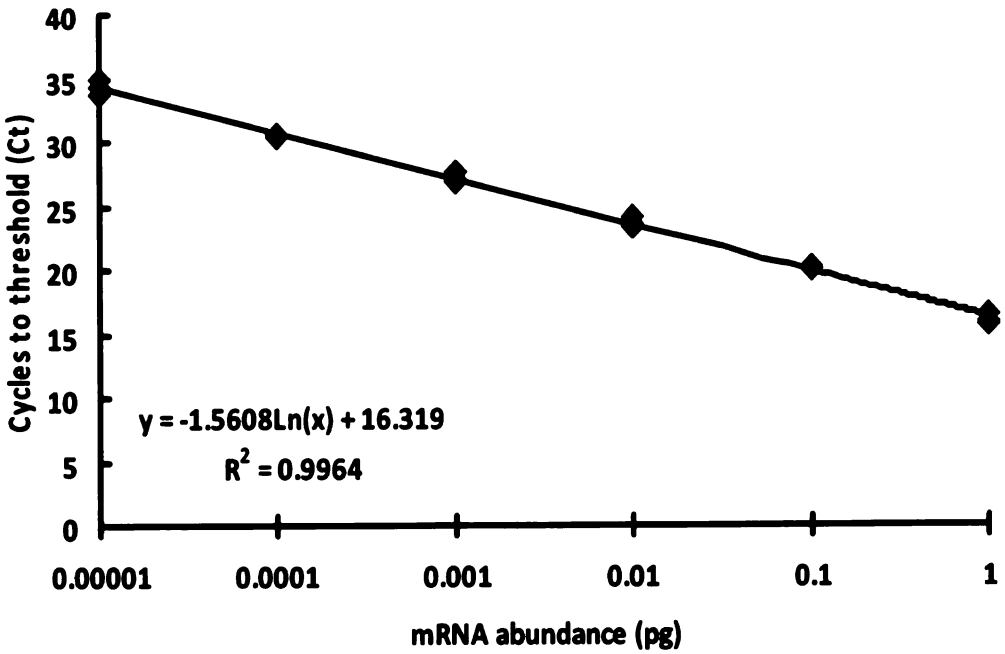

40
35
30
25
20
15
10
5
0
Cycles to threshold (Ct)
y = -1.5608Ln(x) + 16.319
R2 = 0.9964
0.00001
0.0001
0.001
0.01
0.1
1
mRNA abundance (pg)

Figure D4. Representative standard curve generated by Q-RT-PCR for porcine ASCT1.

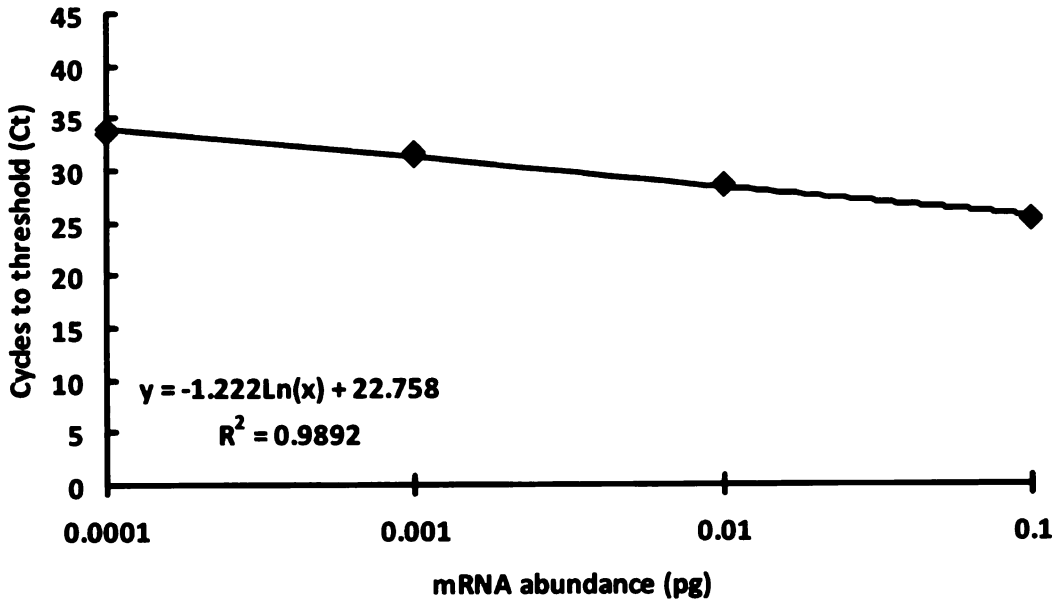
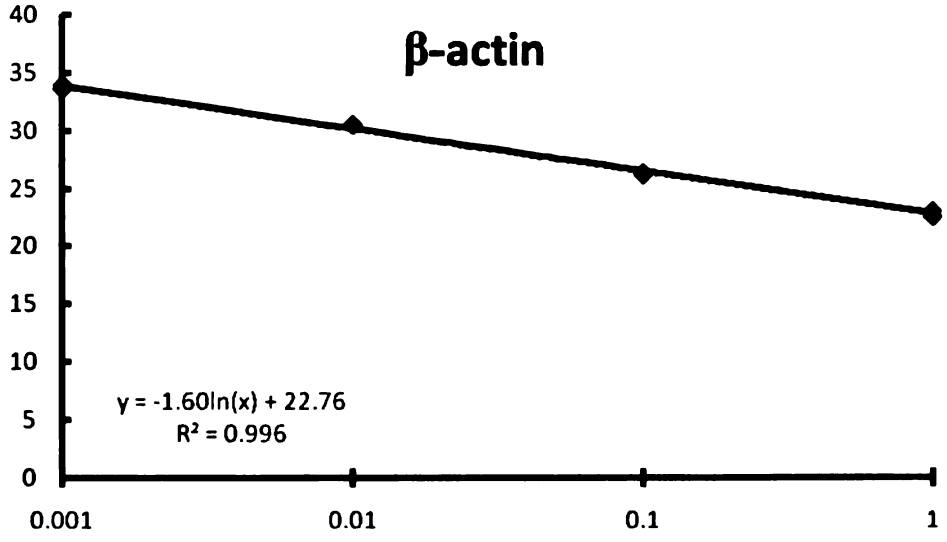

45
40
35
30
25
20
15
10
5
0
Cycles to threshold (Ct)
y = -1.222Ln(x) + 22.758
R2 = 0.9892
0.0001
0.001
0.01
0.1
mRNA abundance (pg)

Figure D5. Representative standard curve generated by Q-RT-PCR for porcine β-actin.


β-actin
40
35
30
25
20
15
10
5
0
y = -1.60ln(x) + 22.76
R² = 0.996
0.001
0.01
0.1
1

APPENDIX E

β-actin Transcript Abundance in Porcine Mammary Tissue During Lactation

Transcript abundance of a gene of interest can be quantified by real-time PCR using two main methods, relative and absolute quantification. Relative abundance of a gene of interest refers to its expression compared to a control gene. Control genes for relative transcript abundance can be endogenous or external standards added to the samples. Endogenously expressed genes or housekeeping genes are most often used and encode for proteins that are essential for maintenance of cell function (e.g., cytoskeletal, glycolytic, and ribosomal proteins) (Eisenberg and Levanon, 2003). These housekeeping genes are therefore presumed to be expressed in every experimental sample. In addition, similar expression of these genes is assumed in different tissues and cell types since their protein products are required for cell viability. In previous studies of relative RNA abundance in mammary tissue throughout stages of lactation, several housekeeping genes have been commonly used as control genes in real-time PCR assays. These are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and cyclophilin (Komatsu et al., 2005; Zhao et al., 2004; Kozakai et al., 2002; Bonnet et al., 2002).

Two well-known housekeeping genes were evaluated during the development and validation of the real-time assays performed in the experiments described in this dissertation. Due to the nature of dietary treatments imposed,

glycolytic enzymes, such as GAPDH, were expected to be differentially expressed. A pilot study using three animals was conducted to determine whether GAPDH transcript abundance varied with stage of lactation. Relative abundance of GAPDH varied significantly ($P<0.05$) with stage of lactation (data not shown). When analyzing β-actin suitability as a normalizing gene to correct for qualitative and quantitative differences in transcript abundance caused by variations in cDNA synthesis efficiency and sample loading, transcript abundance was found to change as well (data not shown).

Others have also found β-actin and GAPDH to be unsuitable normalizing genes (Glare et al., 2002; Chang et al., 1998; Foss et al., 1998; Yamada et al., 1997; Marten et al., 1994). Consequently, transcript abundance analysis was modified. Absolute quantification of genes of interest was performed using specific standard curves for all genes including β-actin. For experiments in Chapter 4, diluted cDNA was quantified using a nano-spectrophotometer (Nano Drop ND-1000, NanoDrop Technologies, Inc., Rockland, DE) and these values were then used to correct for differences in efficiency and loading of cDNA. When samples from the experiments described in Chapter 4 ($n = 18$) were normalized with cDNA concentration, β-actin transcript abundance was indeed different at Early versus Peak lactation (Figure E1).

Using β-actin and GAPDH expression as housekeeping genes to normalize transcript abundance of genes of interests in the mammary gland at

different stages of lactation should be avoided. The assumption that the housekeeping gene expression does not change with stage of lactation is erroneous as the mammary gland is in constant proliferation during lactation. Not only the number of cells in porcine mammary tissue increases with advancement of lactation, the size of these cells increase as well (Kim et al., 1999). Taken together, caution is necessary when studying previous experiments investigating gene expression in mammary tissue across stages of lactation by relative abundance using GAPDH and β-actin as normalizing genes.

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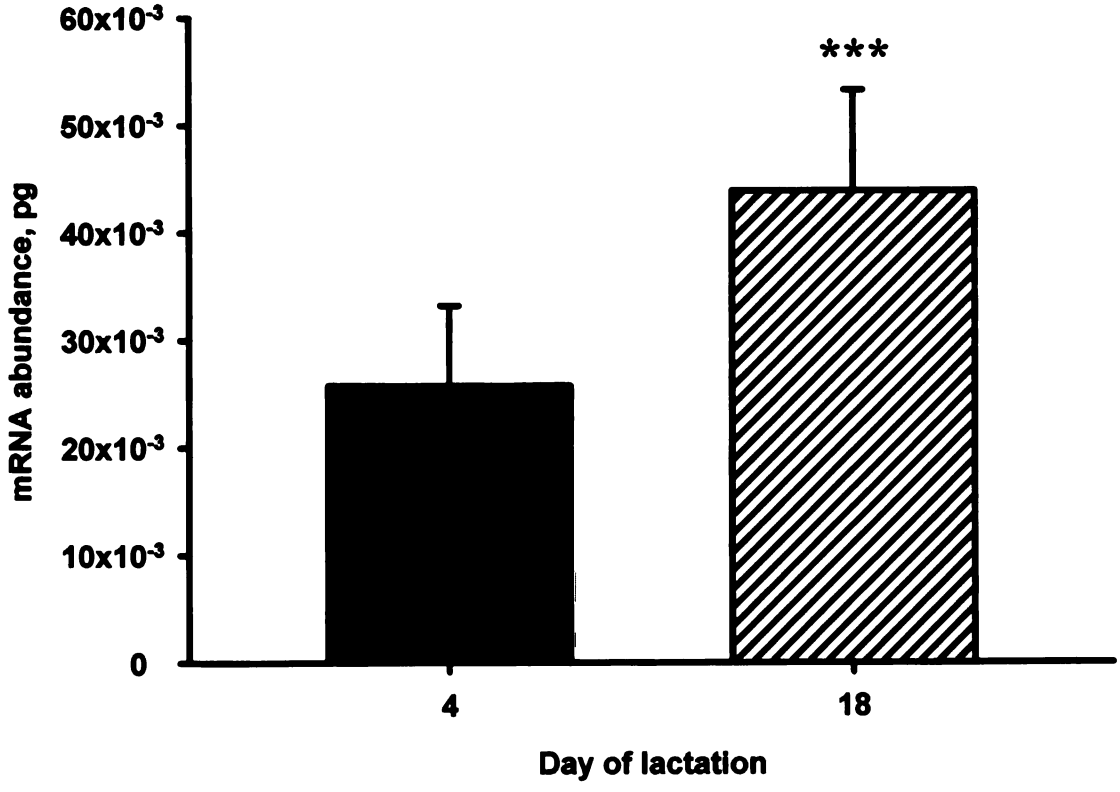
Figure E1. β-actin transcript abundance in porcine mammary tissue (n=18) at Early and Peak lactation. β-actin transcript abundance was higher at Peak lactation compared to Early lactation (***P < 0.001).


60x10-3
50x10-3
40x10-3
30x10-3
20x10-3
10x10-3
0
mRNA abundance, pg
***
4
18
Day of lactation

APPENDIX F

Contribution of Epithelial Cells to Porcine Mammary Tissue at Peak Lactation

With the notion that mammary tissue is heterogeneous and composed of both stromal and epithelial cells, the possibility that expression of amino acid transporters differed between cell types in the experiments described in this dissertation cannot be refuted. Relative contribution of epithelial and stromal cells in porcine mammary tissue during lactation was therefore estimated. Two thoracic mammary glands were collected and sliced in four main regions as shown in Figure F1. Each region was then divided in small sections (~0.5g) and incubated separately in 10% formalin for 16 hrs at 4°C. Sections were then rinsed in phosphate buffered saline and stored in 70% ethanol at 4°C. Sections of mammary tissue were embedded in paraffin following the procedure described by Weber-Hall and Dale (2000). Embedded samples were sent to Dr. R. M. Akers at Virginia Polytechnic Institute and State University for histological assessment of relative density of epithelial cells across the sampling regions as described previously (Berry et al., 2003). Briefly, 5mm sections were cut and placed on positively charged microscope slides (Fisher Scientific, Pittsburgh, PA). Slides were first de-paraffinized in two changes of xylene (5 min each) and subsequently rehydrated through a graded series (100%, 95%, and 70%) of ethanol followed by incubation in water. Sections were then stained by incubation in Ehrlich's Hematoxylin and Eosin (Sigma, St. Louis, MO) for 10 min. Following staining, slides were washed in water for 5 to 10 min, dehydrated (2

min each in 70%, 95% and 100% ethanol), incubated in two changes of xylene (5 min each), and cover slipped using Permount mounting media (Fisher Scientific, Pittsburgh, PA).

Mammary epithelial cells, stromal cells, and fat globules are identified in Figure F2 as described in Chapter 5. Table F1 shows the relative distribution of epithelial and stromal cells in porcine mammary tissue at peak lactation.

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Figure F1. Longitudinal cut of one porcine mammary gland depicting the four regions used for histological examination.

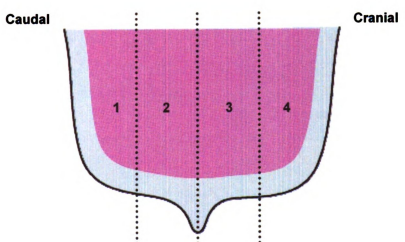
Caudal
Cranial
1
2
3
4

Figure F2. Photographic representation of cell type distribution in porcine mammary tissue on day 18 of lactation at a 100X magnification. a: mammary epithelial cells; b: stromal cells; c:, and fat globules.

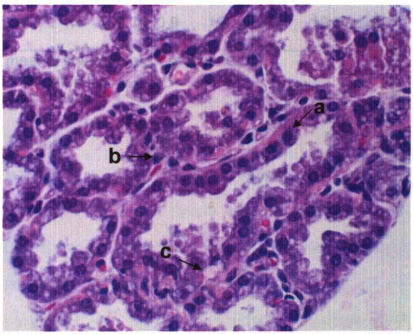
a
b
c

TABLE F1

Relative distribution of epithelial and stromal cells in porcine mammary tissue on day 18 of lactation[1], %

Cell type	Region[2]				P-value[3]
	1	2	3	4	
Epithelial	81.6 ± 1.4	80.3 ± 0.9	80.2 ± 0.7	80.5 ± 0.9	> 0.1
Stromal	18.4 ± 1.4	19.7 ± 0.9	19.8 ± 0.7	19.5 ± 0.9	> 0.1

[1]Values are mean ± standard error of the mean.
[2]As illustrated in Figure 2.
[3]t-tests of paired means.

APPENDIX G

Dietary Protein Intake Does Not Alter Protein Abundance of ASCT1 in Porcine Mammary Gland during Lactation.

System ASC is a ubiquitous AA transport system that provides cells with AAs required for cellular metabolism and nutrition (Christensen, 1990). ASC transporter mediates Na+-dependent exchange of small neutral AAs such as alanine, serine, cystine, threonine, and in some situations proline (Pinilla-Tenas et al., 2003). Extraction rates of these amino acids by the porcine mammary gland *in vivo* were higher at peak lactation compared to early lactation (Nielsen et al., 2002). In addition, similarly to CAT-1, ASCT1 is subject to *trans*-stimulation by its substrates. This means that the rate of transport of ASC substrates is higher when the extracellular concentration of those AAs is increased (Christensen, 1990). Although there is direct evidence for regulation of the ASCT1 gene by AA availability (Sakai et al., 2003; Howard et al., 2004), AA availability did not alter mRNA abundance of the ASCT1 gene in porcine mammary tissue, however, it was higher at Peak lactation (peak milk demand) compared to Early lactation (Chapter 4).

The objective was to test the hypothesis that ASCT1 is subject to adaptive regulation in the porcine mammary gland during lactation and responds to milk demand. Western blot analysis was used to determine the relative abundance of ASCT1 protein levels in mammary tissue.

The experimental design, animals, dietary treatments, tissue collection and protein isolation, and western blotting procedures are described in Chapter 5. Relative abundance of the ASCT1 protein in mammary tissue during Early and Peak lactation are presented in Chapter 5. Relative abundance of ASCT1 protein in response to different levels of dietary protein intake is presented in Figure G1. Relative abundance of the ASCT1 protein in lactating mammary tissue did not differ with AA availability ($P>0.10$). However, there was an interactive effect of dietary treatment x replicate where ASCT1 protein levels were lower and higher with Excess protein intake in replicates 1 and 2, respectively, compared to Adequate protein intake (Figure G2). The interactive effect of dietary treatment and replicate could not be explained, and consequently, the data was not included within the main body of the thesis.

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Pinilla-Tenas, J., Barber, A. & Lostao, M. P. (2003) Transport of praline and hydroxyproline by the neutral amino-acid exchanger ASCT1. J. Membr. Biol. 195: 27-32.

Sakai, K., Shimizu, H., Koike, T., Furuya, S. & Watanabe, M. (2003) Neutral amino acid transporter ASCT1 is preferentially expressed in L-Ser-synthetic/storing glial cells in the mouse brain with transient expression in developing capillaries. J. Neurosci. 23: 550-560.

Figure G1. Relative abundance of ASCT1 protein in porcine mammary during lactation at three levels of dietary protein intake (n=6). ASCT1 protein levels did not change with dietary protein intake ($P>0.10$).

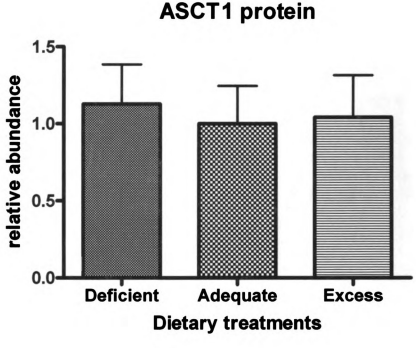
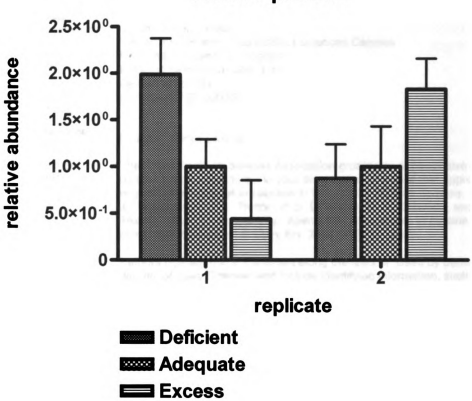

ASCT1 protein
relative abundance
1.5
1.0
0.5
0.0
Deficient
Adequate
Excess
Dietary treatments

Figure G2. The effect of dietary protein intake on ASCT1 relative abundance was different within replicates. Relative abundance of ASCT1 in porcine mammary tissue by experimental replicate. Excess dietary protein intake (24%) decreased ASCT1 protein abundance compared to Adequate protein intake on sows in replicate 1, while on sows in replicate 2, Excess dietary protein intake increased ASCT1 abundance when compared to the Adequate protein diet ($P<0.01$).


ASCT1 protein
relative abundance
2.5×10⁰
2.0×10⁰
1.5×10⁰
1.0×10⁰
5.0×10⁻¹
0
1
2
replicate
Deficient
Adequate
Excess

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February 26, 2007

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