REGULATION OF PORCINE CONCEPTUS SURVIVAL AND GROWTH BY L-ARGININE

A Dissertation

by

XILONG LI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Nutrition

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Approved by:

Co-Chairs of Committee,	Guoyao Wu
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ABSTRACT

Regulation of Porcine Conceptus Survival and Growth

by L-arginine. (December 2011)

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Co-Chairs of Advisory Committee: Dr. Guoyao Wu
Dr. Fuller W. Bazer

This study was conducted to test the hypothesis that dietary supplementation with L-arginine during early pregnancy will ameliorate embryonic loss in pigs. Gilts were bred at the second estrus, and housed individually in pens and fed twice daily 1 kg of a corn- and soybean meal-based diet supplemented with 0.0%, 0.4%, or 0.8% L-arginine (w/w) between d 0 and 25 of gestation (Experiment 1) or between d 14 and 25 of gestation (Experiments 2 and 3). At d 25 (Experiment 1 and 2) or d 60 (Experiment 3) of gestation, gilts were hysterectomized to obtain uteri and conceptuses. Total RNA and protein were extracted from the frozen tissues. Quantitative RT-PCR, western blotting, and microarray analyses were performed to determine the changes of gene expression at mRNA and protein levels.

Dietary supplementation with 0.8% L-arginine between d 0 and 25 of gestation decreased uterine weight, total number of fetuses, number of corpora lutea (CL), total fetal weight, total volume of allantoic and amniotic fluids, concentrations of progesterone in maternal plasma and allantoic fluid, compared to the control group.

However, dietary supplementation with 0.4% or 0.8% L-arginine between d 14 and 25 of gestation increased total volume of amniotic fluid, total amounts of arginine in allantoic and amniotic fluids, total amounts of fructose and most amino acids in amniotic fluid, placental growth, and the number of viable fetuses per litter by 2. Dietary supplementation with 0.4% or 0.8% L-arginine between d 14 and 25 of gestation increased the total number of fetuses and number of live fetuses, rate of embryonic survival, and volumes of allantoic and amniotic fluids in gilts with 15 to 18 CL on d 60 of gestation compared with the control group. The abundance of placental protein and expression of mRNA related to the genes for arginine transport and metabolism, including cationic amino acid transporter 1, endothelial nitric oxide synthase (NOS3), phosphorylated-NOS3, ornithine decarboxylase, and guanosine triphosphate cyclohydrolase-I was increased by dietary supplementation with 0.8% L-arginine between d 0 and 25 of gestation. The abundance of total and phosphorylated mechanistic target of rapamycin was also enhanced by dietary 0.8% L-arginine supplementation between d 0 and 25 of gestation. Microarray analysis revealed that supplementation with 0.8% arginine between d 14 and 25 of gestation affected placental expression of 575 genes.

Findings from the current study not only advance basic knowledge of mammalian reproductive biology, but also have important implications for developing practical means to enhance fertility in female pigs.

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Teamwork was essential for all of the pig surgeries in this project. The surgeries were done early in the morning most times. They started 5:00 AM several times to avoid time conflicts with other investigators using the surgery facility. At least one-third of the

surgeries were done on weekends and holidays. I wish to express my deepest appreciation to all the professors, postdoctoral fellows, graduate students, and visiting scholars who participated in the pig surgeries. The crew of the pig surgery teams included Dr. Fuller W. Bazer, Dr. Guoyao Wu, Dr. Robert C. Burghardt, Dr. Gregory A. Johnson, Dr. Qinglei Li, Dr. Junjun Wang, Dr. Zhaolai Dai, Dr. Kang Yao, Dr. Pengbin Xi, Dr. David W. Erikson, Dr. Bryan G. White, Dr. Carmen D. Tekwe, Ms. Fang Chen, Mr. Reza Rezaei, Mr. James W. Frank, Mr. Jian Lei, Mr. Sudath Dahanayaka, Mr. Xiaoqiu Wang, Mr. Gregory Fritz, and Mr. Merrick Gearing. I thank them for their wonderful cooperation and kind help.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Profitability of the pig industry critically depends on reproductive efficiency of sows, including the number of live piglets weaned per sow per year. However, prenatal mortality is a big challenge that must be overcome in order to improve the reproductive efficiency of modern high-prolific sows. Prenatal mortality is estimated to be 30-50% in pigs (Pope 1994). As a result, gilts produce an average of 9.62 piglets born per litter in the United States, which is much lower than the potential of 14 or more piglets per litter based on the total number of oocytes ovulated (USDA 2009). More than 75% of prenatal loss occurs during the first 25 d of gestation, but another peak in fetal death occurs between d 40 to 60 of gestation (Pope 1994). Many factors contribute to embryonic/fetal loss, including ovulation rate, fertilization rate, disease (e.g., virus infection), chromosomal abnormalities, non-uniform development of conceptuses, and intra-uterine crowding or uterine capacity (Pope 1994; Wu et al. 2010). Of those causes, failure of development of conceptuses (embryo and extra-embryonic membranes) during the peri-implantation period is the main cause for embryonic loss (before d 30) and inadequate uterine capacity is the major reason for fetal deaths after d 30 (Wu et al. 2010; Bazer et al. 2009).

This dissertation follows the style and format of Amino Acids.

Many efforts have been made to improve embryonic/fetal survival in pigs and other mammalian species. Animal breeding can improve litter size, but the efficiency is very low. Litter size in the U.S. swine industry increased at a rate of only 0.052 pigs/year between 1980 and 2000 (Johnson 2000). The reason for low efficiency of genetic selection is low heritability for litter size. Heritability estimates for litter size born is 0.1, and even less for live-born pigs in a litter (~0.07) (Rothschild 1996). Another approach has been to increase ovulation rate through superovulation; however, an increase in litter size was not realized because high ovulation rates in sows have the potential to cause excessive intra-uterine crowding of conceptuses which increases fetal mortality (Town et al. 2005).

Arginine is a conditionally essential amino acid for mammals, including pigs (Wu et al. 2009). In addition to being a building block for proteins, it is the precursor for synthesis of many biologically active molecules, including nitric oxide (NO), ornithine, polyamines (putrescine, spermine and spermadine), creatine, and agamatine (Wu and Morris 1998). Of those, NO and polyamines are most important as they stimulate cell proliferation, cell migration, cellular remodeling, angiogenesis, and dialation of blood vessels to increase blood flow.

Although several studies have been conducted to determine effects of arginine supplementation on reproductive performance in pigs (Mateo et al. 2007; Zeng et al. 2008; Campbell 2009; Berard et al. 2009), there are many issues to be addressed. There is limited knowledge about arginine metabolism in the uterus and placenta. There is no clear answer as to whether effects are mediated directly by arginine or by its metabolites,

such as NO and polyamines. Moreover, we do not understand how arginine regulates gene expression in the uterus and placenta. Importantly, few studies have focused on early pregnancy which is the most critical stage for embryonic/fetal survival. There is a need to answer questions such as whether arginine supplementation is safe during early pregnancy, if it is effective in improving embryonic survival, and the duration of supplementation required to effectively improve embryonic survival during early pregnancy.

Implantation and placentation in pigs

Following fertilization, the zygotes develop and cleave into 2- and 4-cell stage embryos in the oviduct. After entering into the uterus on about d 3 of gestation, embryos continue to cleave, develop to the blastocyst stage by d 7 or 8 of gestation and hatch from the zona pellucida. After pig blastocysts hatch from the zona pellucida on d 7 or 8 of gestation, they migrate within the uterus to achieve equal spacing among themselves. Then, blastocysts undergo dramatic changes in morphology from expanded spherical blastocysts to tubular, and filamentous forms between d 10 and 12 of pregnancy. The diameter of spherical blastocysts is only 5 to 10 mm by d 10 of gestation. However, when reaching a spherical diameter of 10 mm at about d 11 of gestation, it takes only 3 or 4 h for blastocysts to elongate to tubular and then filamentous conceptuses that are 150 to 200 mm in length; and by d 15 they approach 1,000 mm in length (Geisert and Yelich 1997). Interestingly, this dramatic morphological change occurs initially through cellular remodeling rather than cellular hyperplasia, but the final phase of elongation between d 12 and 15 involves both cellular hyperplasia and cellular remodeling (Geisert

et al. 1982). Pig conceptuses initiate attachment of trophectoderm to uterine luminal epithelium (LE) on d 13 of pregnancy and implantation is accomplished by about d 18 of gestation in advance of placentation and formation of a true epitheliochorial placenta. Unique characteristics of domestic animals, including pigs, are the prolonged pre-implantation period for elongation of conceptus trophectoderm, followed by orientation of the blastocyst, apposition between trophectoderm and uterine LE, and then adhesion of trophectoderm to uterine LE (Bazer et al. 2009).

This prolonged preimplantation period for blastocyst/conceptus elongation allows for establishment of maximum surface area of contact between trophectoderm and uterine LE for absorption of products secreted by or transported by maternal uterine epithelia into the uterine lumen (histotroph) that are essential for survival and growth of the conceptus which has superficial and noninvasive attachment between trophectoderm and uterine LE in pigs. Although early trophectoderm elongation depends mainly on histotrophic nutrition from uterine LE, superficial glandular epithelium (sGE), and GE (Spencer and Bazer 2004), rapid growth of blood vessels in the yolk sac (d 16-21) and allantois (d 21 to term) of the placenta prepares the conceptus for hematotrophic exchange of nutrients and gases between maternal and fetal-placental blood in addition to nutrients supplied by histotroph via areolae of the chorioallantoic membranes from the post-implantation period of pregnancy to the end of gestation to support growth and development of the conceptus (Linton et al. 2008). Importantly, maternal recognition of pregnancy starts at d 11 of gestation when the blastocyst begins its dramatic morphological changes and also initiates secretion of estrogen which is the signal from

trophectoderm for maternal recognition of pregnancy in pigs (Bazer and Thatcher 1977). To support these dramatic events in conceptus development, many genes for nutrient transport, cellular remodeling, angiogenesis, relaxation of vascular tissues, cell proliferation and migration are involved (Bazer et al. 2010). Early embryonic losses result from a failure of conceptus development and implantation during the peri-implantation period of pregnancy (Bazer et al. 2009).

Arginine metabolism

Synthesis

Circulating arginine comes from the exogenous sources in the diet and from endogenous synthesis. Dietary arginine requirements vary with species, nutritional status and developmental stage (Wu and Morris 1998). *De no*vo arginine synthesis and whole-body protein turnover are two main endogenous sources. Whole-body protein turnover accounts for 85-95% of total endogenous arginine flux in adult animals and humans. However, *de no*vo synthesis of arginine provides 30% of endogenous arginine in neonatal pigs (Wu and Morris 1998). The high rate of *de novo* synthesis may be a strategy for the neonate to compensate for arginine deficiency in milk (Davis et al. 1993b; Wu and Knabe 1994).

De novo synthesis of arginine from citrulline was first reported for the mammalian kidney in the early 1940s (Borsook H and Dubnoff 1941; Cohen and Hayano 1946). However, the mechanism of conversion was not identified until one-decade later (Ratner and Petrack 1953). During this *de novo* synthesis process, citrulline first condenses with aspartic acid to produce argininosuccinate by argininosuccinate synthase (ASS), and

argininosuccinate is cleaved by argininosuccinate lyase (ASL) to form arginine and fumaric acid (Wu and Morris 1998). In addition to the kidney, brain can also produce net arginine at a low level in adult animals (Ratner et al. 1960). Although both ASS and ASL activities are high in the mammalian liver, there is no net synthesis or release of arginine by the liver due to very high hepatic arginase activity which rapidly degrades arginine (Wu and Morris 1998).

Although citrulline, an immediate precursor of arginine, is synthesized from ornithine by ornithine carbamoyltransferase (OCT), the activity of OCT is very low in the kidney (Raijman 1974), suggesting that the kidney must take up citrulline from blood to synthesize arginine. The liver does not normally contribute citrulline to the circulation (Drotman and Freedland 1972) and there is net release of citrulline into the circulation only when there are much higher than physiological levels of substrate (e.g., ornithine and NH_4Cl) are provided (Drotman and Freedland 1972). However, Windmueller and Spaeth (1981) reported that in adult rats, the small intestine is the primary organ to release significant amounts of citrulline into the blood, which is taken up by the kidneys to synthesize arginine. These authors further estimated that 83% of the citrulline taken up by the kidney was released from the organ as arginine. In support of this conclusion, arginine becomes a nutritionally essential amino acid after massive resection of the small intestine in adult rats (Wakabayashi et al. 1994). All of these studies indicate that the intestinal-renal axis is the main pathway for *de novo* synthesis of arginine in adult animals.

The substrates for citrulline synthesis in the small intestine were not known until the 1990s. Glutamine, which was found to be taken up extensively from arterial blood and diet, was considered to be a precursor of citrulline in the intestine (Windmueller and Spaeth 1981). With improved methodology for amino acid analysis, biochemical evidence for citrulline production from glutamine was first reported for porcine enterocytes (Wu et al. 1994). During intensive studies to explain the paradoxical change in intestinal synthesis of citrulline from glutamine in postnatal pigs (Wu et al. 1995; Wu and Knabe 1995), Wu (1997) discovered that proline is a major substrate for citrulline production by enterocytes. This finding has been confirmed in studies involving rats (Wu 1997), humans (Tomlinson et al. 2011), and sheep (Wu et al. 2008). Endogenous synthesis of arginine from proline plus glutamine provides approximately 60% of the total arginine required by neonatal and postnatal pigs (Wu et al. 2004).

Studies with porcine enterocytes established the enzymological basis for arginine synthesis from glutamine and proline (Wu et al. 1994; Wu 1997). Glutamine is converted into glutamate by phosphate-activated glutaminase. Pyrroline-5-carboxylate (P5C) is formed from glutamate by P5C synthase. P5C can also be synthesized from proline by proline oxidase. P5C is converted into ornithine by ornithine aminotransferase (OAT). Citrulline is produced from ornithine and carbamoyl phosphate by carbamoyl phosphate synthase I (CPS I). Interestingly, all of the enzymes involved in arginine synthesis exist in the enterocyte of neonates (Wu and Knabe 1995). Importantly, arginase activity is nearly absent from neonatal enterocytes, thereby maximizing the release of arginine from the small intestine (Wu and Knabe 1995).

Transport

Arginine is essential for cell survival and growth. However, cells cannot take up a significant quantity of extracellular arginine by simple diffusion. Specific transporters are needed for transporting arginine across the cell membrane. As a cationic amino acid, arginine shares the same transporters with lysine, ornithine, and histidine. The system y+ is the first transport system identified as a cationic amino acid transporter (CAT; Christensen and Antonioli 1969). This transport system is selective for cationic amino acids and it is Na^+-independent. System y+ was thought to be the only transport system for cationic amino acids until other novel systems were discovered two decades later (Van Winkle et al. 1985, 1988; Devés et al. 1992, 1993). Specifically, system $b^{o,+}$, $B^{o,+}$ was discovered for mouse blastocysts (Van Winkle et al. 1985, 1988). In contrast to CAT that is highly selective to cationic amino acids, systems $b^{o,+}$ and $B^{o,+}$ can transport neutral amino acids in addition to cationic amino acids (Van Winkle et al. 1985, 1988). These two systems are distinguished by their dependence on Na^+. System $B^{o,+}$ is Na^+-dependent, while system $b^{o,+}$ is Na^+-independent (Van Winkle et al. 1985, 1988). Subsequently, the fourth transport system named y+L was discovered in the course of studies involving human erythrocytes (Devés et al. 1992, 1993). This system has high affinity for both neutral and cationic amino acids. Transport of cationic amino acids through this system is Na^+-independent; however, its apparent affinity for neutral amino acids decreases dramatically when Na^+ in the medium is replaced with K^+ (Devés et al. 1992).

The most important transporter for arginine uptake in most cell types is system y+, which has high-affinity for arginine and is Na^+-independent. Recombinant DNA technology provided the means to identify the proteins involved in the transport of cationic amino acids. Interestingly, it was found that the membrane receptor for ecotropic murine leukemia viruses (ecoR) induced cationic amino acid transport (Kim et al. 1991; Wang et al. 1991). Because both transport properties and the expression pattern of ecoR is the same as system y+, this virus receptor was named mouse cationic amino acid transporter (mCAT). Three different genes of CAT (SLC7A1, SLC7A2, and SLC7A3) were identified, which encode four homologous proteins CAT-1, CAT-2A plus CAT-2B, and CAT-3, respectively (Devés and Boyd 1998).

The placenta plays a critical role in the delivery of amino acids from mother to fetus, and, therefore, fetal growth. In the human placenta, there are two cell layers between the maternal and fetal circulation: syncytiotrophoblast and the fetal capillary endothelium. The endothelium is considered to transport amino acids through pores within the interendothelial cleft (Leach and Firth 1992). However, the polarized plasma membranes of syncytiotrophoblast represent a significant barrier to transport of amino acids. Two plasma membranes, the microvillous plasma membrane (MVM; maternal facing) and basal plasma membrane (BM; fetal facing), exist in the syncytiotrophoblast. The concentrations of most amino acids in plasma of the fetus are higher than those in the mother (Philipps et al. 1978), suggesting the need for transporters of amino acids across the placenta. More than 15 amino acids transport systems exist in the human placenta (Jansson 2001).

There are four possible cationic amino acid systems (y^+, y^+L, b^{0+}, and B^{0+}) that can transport L-arginine in animal cells (Devés and Boyd 1998), but only two of them are present in the syncytiotrophoblast: a high affinity, low capacity system y^+ system and a lower affinity, higher capacity y+L system (Ayuk et al. 2000). It is well established that y^+ and y^+L systems are present in the MVM for arginine transport, but the y+ system is the main transport system for transport of cationic amino acids in most of the cell types studied (Ayuk et al. 2000). The transport systems in BM are different from those in MVM. In particular, the y^+L system represents the principal transport pathway in BM (Ayuk et al. 2000). However, there is growing evidence for the existence of system y^+ in the BM (Speake et al. 2003). Total L-arginine transporter activity is higher in BM from preeclamptic placentae compared with those from control placentae (Speake et al. 2003), which is predominantly due to increased activity of system y^+ (Ayuk et al. 2002). Moreover, low concentrations of L-arginine can up-regulate expression of system y^+ in endothelial cells (Bogle et al. 1996). The presence of system y^+ in the BM suggests its importance in regulating arginine metabolism in intra-uterine growth restriction (IUGR).

Different genes of CAT have different spatial and temporal patterns of expression in the ovine conceptus (Gao et al. 2009). The abundance of SLC7A1 mRNA is high in uterine LE and sGE on d 16 of the estrous cycle and on d 16 to 20 of pregnancy. SLC7A2 mRNA is most abundant in uterine LE and mid- to deep-glandular epithelia on d 14–20 of gestation. However, the abundance of SLC7A3 was not affected by day of the estrous cycle or by pregnancy status. In contrast to expression in the uterus, SLC7A1, SLC7A2, and SLC7A3 mRNAs were weak in the trophectoderm and

endoderm of conceptuses from d 13 to 18 of pregnancy. Expression of the CAT gene is induced by P4 and further stimulated by interferon tau (IFNT) in sheep. Long-term treatment of ewes with P4 stimulated SLC7A1 in LE and GE, and IFNT tended to increase SLC7A1 abundance in LE. SLC7A2 mRNA abundance increased by short-term treatment with P4 and IFNT, but SLC7A1 expression was not affected.

Catabolism

There are multiple pathways in cells for arginine catabolism. Arginine serves as the precursor for synthesis of many biological molecules, including ornithine, polyamines (putrescine, spermine and spermidine), proline, glutamine, creatine, agamatine, and nitric oxide (NO), as well as protein (Wu and Morris 1998).

Arginine-ornithine pathway

The classic pathway of arginine catabolism is initiated by arginase to produce ornithine. Ornithine is subsequently converted to polyamines, proline, glutamate, and glutamine. Arginase exists as two distinct isoenzymes in mammals. Type I arginase is a cytosolic enzyme mainly expressed in the liver. Type II arginase is expressed in mitochondria of extra-hepatic tissues including kidney, brain, small intestine, mammary gland and macrophages (Wu and Morris 1998). Although hepatic cells have a limited ability to extract circulating arginine (Castillo et al. 1996), type I arginase is a component of the urea cycle to catalyze urea production from hepatic arginine which is important for detoxifying waste nitrogen. All enzymes of the urea cycle are expressed in the small intestine of weaned pigs. This may serve as a first line of defense against the toxicity of ammonia which is generated from amino acid metabolism in both enterocytes and

luminal bacteria in the small intestine (Wu 1995). Arginase isoforms also were detected in endothelial cells of various mammalian species (Morris 2009). Li et al. (2001) first reported that over-expression of arginase I or arginase II in endothelial cells reduces NO synthesis from arginine. Subsequently, elevated arginase activity in endothelium inhibits NO production in blood vessels (Lim et al. 2007; Zhang et al. 2001). These results suggest that arginase may compete with NOS for arginine in the vasculature to regulate blood flow.

Ornithine produced from arginine is an important precursor for synthesis of polyamines, proline, and glutamine (Wu and Morris 1998). Ornithine is converted into putrescine by ODC1. Spermidine is synthesized from putrescine by adding an aminopropylic group from decarboxylated S-adenosyl-L-methionine (SAM), and this reaction is catalyzed by spermidine synthase. With the presence of spermine synthase, spermidine is converted into spermine by adding another aminopropylic group from decarboxylated SAM (Wu and Morris 1998). Of these enzymes, ODC1 is the rate-controlling enzyme for the polyamine biosynthetic pathway. However, arginase-deficient cells cannot proliferate unless ornithine or polyamines are added in serum-free medium (Holtta and Pohjanpelto 1982). This suggests that arginase regulates the availability of ornithine for polyamine synthesis. In both the placenta (Kwon et al. 2003; Wu et al. 2005) and small intestine (Wu et al. 2000), ornithine is synthesized from proline via proline oxidase.

Ornithine aminotransferase catalyzes the formation of P5C from ornithine. P5C is the common substrate for the production of proline and glutamate via P5C reductase and

P5C dehydrogenase, respectively (Wu and Morris 1998). Endogenous synthesis of proline is important for protein synthesis in growing animals (Wu et al. 2011). Proline production accounts for 54% of arginine catabolism in enterocytes of post-weaning pigs (Wu et al. 1996). In many cell types, glutamine synthetase catalyzes ATP-dependent synthesis of glutamine from glutamate. The importance of OAT in ornithine metabolism is epitomized by gyrate atrophy of the choroid and retina in adult patients with OAT deficiency. These patients have 10- to 20- times higher concentrations of ornithine in their plasma due to substantially lower OAT levels (Simmell and Takki 1973). Similar results were reported for OAT-knockout adult mice fed a standard diet (Wang et al. 1995).

Synthesis of proline and glutamine from arginine is also important in the lactating mammary gland which takes up more arginine, but less proline and glutamine from blood than their outputs in milk (Mepham and Linzell 1966; Trottier et al. 1997). This means extra proline and glutamine in milk is synthesized from arginine in the mammary gland. The enzymes for proline and glutamine synthesis from arginine include arginase, OAT, P5C reductase and P5C dehydrogenase (Wu and Morris 1998). Activities of these enzymes increase substantially with advancing stages of lactation (Mezl and Knox 1977).

Arginine-NO pathway

The pathway of NO production from arginine is most exciting for arginine catabolism. Arginine is the precursor of NO, which is catalyzed by NO synthase (NOS) (Bredt and Snyder 1994). There are three isoforms of NOS: neuronal NOS (nNOS; also known as

NOS1), inducible NOS (iNOS; also known as NOS2), and endothelial NOS (eNOS; also known as NOS3). NOS1 and NOS3 are expressed constitutively in a cell-specific manner and produce low levels of NO. While NOS2 is induced by certain immunological stimuli (including LPS and inflammatory cytokines) to generate large amounts of NO which is a major endothelial cell-dependent relaxing factor (Ignarro 1987).

Nicotinamide adenine dinucleotide phosphate hydrogen, calmodulin, flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin (BH_4) are essential cofactors for enzymatic activities of all isoforms of NOS (Wu and Morris 1998). In addition, NOS1 and NOS3, but not NOS2, require calcium for generation of NO (Bredt and Snyder 1994). Nearly all cell types can recycle citrulline into arginine via ASS and ASL, and this intracellular arginine-citrulline cycle helps sustain sufficient concentrations of arginine to support NO production (Wu and Brosnan 1992). In cells and blood, NO is rapidly oxidized via many nonenzymatic reactions to nitrite and nitrate, with nitrate being the major product. For example, NO is readily oxidized to nitrite via autoxidation or reacts with superoxide anion to yield peroxynitrite (an oxidant). NO and nitrite can also be oxidized by oxyhemoglobin or oxymyoglobin to form nitrate. Nitrite and nitrate are excreted by the kidneys. The half-life of NO in physiological solutions is extremely short (~5 s), but it can be transported as a glutathione adduct to conserve its biological activity (Wu et al. 2004). Determination of nitrite and nitrate provides a valid indicator of NO synthesis by cells. Synthesis of both NOS and citrulline from arginine in

enterocytes of suckling piglets decreases with development. However, they both increase markedly in enterocytes of post-weaning pigs (Wu et al. 1996).

Arginine-agmatine pathway

Another pathway of arginine catabolism is agmatine synthesis. Arginine decarboxylase (ADC) decarboxylates arginine to produce agmatine. This pathway has long been recognized in plants and bacteria. It was thought to be absent from mammals until it was discovered in the brain of animals in 1994 (Li et al. 1994). Expression of the ADC gene in mammalian cells was confirmed by cloning and characterization of human ADC (Zhu et al. 2004). ADC is also present in liver, kidney, adrenal gland, and macrophages (Wu and Morris 1998). However, ADC activity is absent from porcine enterocytes (Wu et al. 1996). ADC has 48% amino acid sequence homology with ODC1, but has no ODC1 activity (Zhu et al. 2004). Interestingly, agmatine irreversibly inhibits neuronal NOS and down-regulates inducible NOS (Halaris and Plietz 2007).

Arginine-creatine pathway

Arginine is one of three amino acid precursors for creatine synthesis. The amidino group from arginine is transferred to the amino group of glycine for synthesis of guanidinoacetic acid (GAA) by L-arginine:glycine amidinotransferase (AGAT). GAA can then be methylated by the methyl donor SAM which is produced from methionine (Wyss and Kaddurah-Daouk 2000). AGAT is the first rate-limiting enzyme for creatine synthesis. Although hepatocytes can readily convert GAA into creatine, creatine cannot be produced directly from arginine, glycine, and methionine in the liver (da Silva et al. 2009). In the rat, AGAT is predominantly expressed in the kidney, whereas high GAMT

activity occurs in the liver (da Silva et al. 2009). This suggests inter-organ cooperation for creatine synthesis. *De novo* synthesis is the major source of creatine in neonatal animals (Lamarre et al. 2010; Brosnan et al. 2009). Approximately 20% of whole-body arginine utilization in young pigs is accounted for by creatine production (Wu et al. 2004). This is an important reason why arginine is an essential amino acid for neonates.

Function of arginine and its metabolites

Multiple catabolic fates and unique chemical features enable arginine to have versatile functions in cardiovascular, neurological, endocrine, and immunological systems (Wu and Meininger 2000; Barbul 1990; Calabrese et al. 2007; Schmidt et al. 1992). Moreover, arginine was discovered to regulate the mechanistic target of rapamycin (MTOR) cell signaling pathway which plays fundamental roles in protein synthesis, cell proliferation and modulation of cytoskeletal structure (Kim et al. 2011; Yao et al. 2008). Since the report that dietary supplementation with 0.83% arginine enhances litter size in gilts (Mateo et al. 2007), there has been growing interest in the role of arginine in embryonic and fetal survival and growth.

Arginine and cardiovascular functions

The cardiovascular system is an essential transport network to supply oxygen and nutrients to tissues and remove metabolic by-products. Cardiovascular disease is the first leading cause for death in the U.S (Lloyd-Jones et al. 2010). Discovery of NO as an endothelium-derived vascular smooth muscle cell relaxing factor fundamentally changed classical views about the role of endogenous gases in cell physiology (Ignarro et al. 1987). Results of a study involving anesthetized rabbits indicated that N-monomethyl-L-

arginine (L-NMMA), a specific inhibitor of NO synthesis from L-arginine, induced a dose-dependent increase in blood pressure. However, the effect of L-NMMA was reversed by infused L-arginine (Rees et al. 1989). This suggests that NO is a key regulator of cardiovascular function. In a study with conscious Long-Evans rats, Gardiner et al. (1990) found that administration of L-NMMA increased blood pressure in internal carotid, mesenteric, renal, and hindquarters vascular beds indicating that NO can regulate regional blood flow. Moreover, the aortic ring of NOS3-knockout mice had no relaxation reaction to acetylcholine, and these mutant mice develop hypertension (Huang et al. 1995). Results of these studies indicate that NO plays an important role in maintenance of vascular tone and hemodynamics. NO, synthesized from arginine, binds the heme group of soluble guanylate cyclase (sGC), thereby activating this enzyme for generation of cyclic guanosine monophosphate (cGMP) from guanosine-5'-triphosphate (GTP) (Bredt and Snyder 1994). The cGMP activates cGMP-dependent protein kinases and the phosphorylation of target proteins that elicit a series of physiological responses (e.g., relaxation of vascular smooth muscle cells, vasodilation, and mitochondrial biogenesis). In addition, NO inhibits the release of endothelin-1 (a vasoconstrictor) by EC, and prevents leukocyte adhesion to the endothelium and platelet aggregation (Huang 2000).

Arginine may regulate the synthesis of carbon monoxide (CO) and hydrogen sulfide (H_2S) from glycine and cysteine, respectively (Li et al. 2009). These gases have important biological functions in the cardiovascular system (Maines 1997; Yang et al. 2008). NO stimulates H_2S production in vascular tissues (Zhao et al. 2003), whereas H_2S

inhibits the arginine–NO pathway in aorta and EC (Geng et al. 2007). Additionally, endothelial NO has a permissive role in CO- and perhaps H_2S-induced vascular dilation (Barkoudah et al. 2004). Thus, there may be cross-talk between various gaseous signaling pathways, and physiological levels of NO regulate vascular tone and hemodynamics in synergy with other gaseous vasoactive factors.

There is compelling evidence that arginine is a useful nutrient to prevent and treat cardiovascular disorders. For example, acute infusion of L-arginine rapidly reduced both systolic and diastolic blood pressures in patients with hypertension (Nakaki et al. 1990). Blood pressure also decreased significantly in response to 4 weeks of dietary supplementation with 12 g L-arginine daily (Ast et al. 2010). Paradoxically, although intracellular concentrations of arginine are several hundred times higher than the *K*m of NOS (~5 μM). exogenous L-arginine administration still increases NOS activity even when levels of L-arginine are excessive. This was termed the arginine paradox (Kurz and Harrison 1997). Several theories have been proposed for this paradox, but no one can explain it perfectly (Wu and Meininger 2000). However, the discovery of increased BH_4 availability in response to exogenous L-arginine administration shed light on this mystery (Shi et al. 2004). BH_4 is the essential cofactor of all NOS isoforms. Arginine increases NOS activity and NO production by increasing BH_4 availability. In addition to hypertentive patients, arginine supplementation is also beneficial for patients with cardiovascular disorders, including coronary artery disease, peripheral arterial disease, ischemia/reperfusion, and heart failure which are also associated with impaired NO synthesis and endothelial dysfunction (Wu and Meininger 2000).

Arginine function in reproduction

Among the products of arginine catabolism, NO and polyamines are most important in reproduction as they stimulate cell proliferation, cell migration, cellular remodeling, angiogenesis and dilation of blood vessels to increase blood flow. Notably, NOS gene knockout mice have proved to be an excellent model system to evaluate the functions of NO on reproductive process in females. Results indicate that NO is essential for ovulation, embryonic development, and implantation (Maul et al. 2003). There is no significant influence of NOS2 deficiency on length of the estrous cycle or ovulation rate. However, cycle length was significantly increased and ovulation rate was markedly decreased in NOS3 knockout mice (Jablonka-Shariff et al. 1999). The number of implanted blastocysts was also significantly lower in NOS3 knockout than wild-type mice (Pallares et al. 2008). Remodeling of the uterine vascular wall is essential for increasing uterine blood flow which is required for successful pregnancy outcomes (Osol and Cipolla 1993). Knockout of the NOS3 gene decreased the remodeling capacity of the uterine artery during pregnancy in mice (van der Heijden et al. 2005). This may be a major cause for the decline in fetal and neonatal survival in NOS3 knockout mice. Although there were no effects on implantation rates and early development of implantation sites, viable embryos at mid-gestation and litter size at term were significantly reduced in NOS3 knockout mice. This impairment was associated with reduced cellularity and abnormally thickened walls of decidual arteries in the absence of NOS3 gene expression (Burnett et al. 2002). Results of all of these studies suggest that NO is essential for successful pregnancy outcomes.

As noted previously, the key function for ornithine catabolism is the synthesis of polyamines (putrescine, spermidine, and spermine) which are crucial for cell growth, migration, and proliferation, as well as angiogenesis (Wu, 2009). Results of several studies have confirmed that ODC1 and polyamines are essential for healthy pregnancy. ODC1 activity increases sharply between d 6 and 8 of gestation which is just after implantation (d 4 to 5 of gestation) in mice (Fozard et al. 1980). Similar changes occur for uterine levels of putrescine and spermidine. However, when adding 2% DL-α-difluoromethylomithine (α-DFMO), an irreversible inhibitor of ODC1, to the drinking water from d 5 to 8 of gestation, ODC1 activity and concentrations of uterine putrescine and spermidine decreased significantly compared with mice not treated with α-DFMO in drinking water (Fozard et al. 1980). Meanwhile, all of the mice treated with α-DFMO showed pregnancy loss by d 18 of gestation. It was determined that embryonic development failed to progress beyond d 6 to 7 of gestation in these mice. However, decidualization of the uterine stroma did occur normally after implantation. This indicated that ODC1 activity and polyamines are important for embryogenesis after implantation (Fozard et al. 1980). This effect of α-DFMO has been confirmed in rats and rabbits (Fozard et al. 1980). Polyamines are also essential for blastocyst implantation as implantation of blastocysts is significantly inhibited by the ODC1 inhibitor, α-DFMO (Zhao et al. 2008). The benefit of polyamines in pregnancy may relate to its regulation of synthesis of steroid hormones as well as embryonic, placental and fetal growth and development. The activity of ovarian ODC1 increases immediately after ovulation and is required to enhance secretion of progesterone by the corpora lutea which is essential for

implantation of blastocysts (Bastida et al. 2002). Ovarian growth and the formation of Graafian follicles were also inhibited by blocking ODC1 activity in immature female mice (Bastida et al. 2005). The decrease in concentrations of progesterone and estradiol at diestrus caused by α-DFMO treatment was associated with its inhibitory effects on expression of the genes for steroidogenic factor 1, cytochrome cholesterol side chain cleavage enzyme, and steroidogenic acute regulatory protein in the ovary (Bastida et al. 2005).

Maternal nutrition plays a critical role in fetal growth and development. The versatile functions of arginine make it an ideal nutrient for intervention to overcome undesirable reproductive problems such as IUGR and preeclampsia (Wu et al. 2004; Wu et al., 2009). A major problem in human medicine and animal production is IUGR, which is defined as impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy (Wu et al. 2006). Approximately 5% of human infants born in the U.S. suffer from IUGR (Marsal 2002). After birth, IUGR offspring often have many severe disorders and need special care. Therefore, health costs for managing IUGR infants are particularly high and there is also a considerable negative psychological impact on parents. Moreover, such offspring have high risks for diabetes, obesity and cardiovascular disease in adulthood (Valsamakis et al. 2006). A major factor for IUGR is impaired vascular development in the mother and the fetus, resulting in insufficient delivery of nutrients and oxygen required for fetal growth. At present, the pathogenesis of this disease has not been clarified. Many experiments have been conducted to study the effects of arginine supplementation on fetal development in pregnant women and

animals. Daily oral administration of 3 g L-arginine to women with pregnancies complicated by IUGR resulted in enhanced fetal weights and birth weights (Sieroszewski et al. 2004). Intravenous administration of L-arginine (20 g/day) to women with IUGR fetuses also increased birth weights (Xiao and Li 2005).

Preeclampsia is another severe reproductive disease in women. It affects about 5 to 8% of all pregnancies (Lain and Roberts 2001). Preeclamptic women usually have endothelial dysfunction and hypertention (Roberts 1999). Concentrations of arginine in plasma are markedly reduced in patients with preeclampsia (D'Aniello et al. 2001). However, asymmetric dimethylarginine, a naturally occurring inhibitor of NO in plasma, increased in patients with preeclampsia (Sandrim et al. 2010). Infusion of L-arginine to pregnant women is associated with increased NO production and decreased blood pressure (Facchinetti et al. 1999). Long-term dietary supplementation with low doses of L-arginine decreased blood pressure through increased synthesis and/or bioavailability of NO in women with preeclampsia (Rytlewski et al. 2005). These studies suggest that arginine is a functional nutrient that can be used to treat and/or prevent preeclampsia.

Embryonic/fetal loss is a major problem in pig reproduction (Pope 1994). However, there are few effective ways to reduce high embryonic/fetal loss in pigs (Johnson 2000). Interestingly, several lines of experimental evidence suggest that arginine supplementation may be effective in enhancing embryonic/fetal survival in pigs. First, dietary supplementation with 1.0% arginine-HCl between d 30 and 114 of gestation increased the number of live-born piglets by 2 and litter birth-weight by 24% (Mateo et al. 2007). Similar results were obtained when 1% arginine-HCl was supplemented to

gilts and multiparous sows between d 22 and 114 of gestation (Gao et al. 2011). Second, dietary supplementation with 1% arginine to gilts or sows between d 14 and 28 of gestation increased the number of live-born piglets by approximately 1 at birth (Ramaekers et al. 2006; Campbell 2009). Third, supplementation with 1% arginine between d 14 and 28 of gestation increased the number of fetuses per litter by 3 on d 70 of gestation in gilts (Berard et al. 2009). Similarly, dietary supplementation with arginine during early- or mid-gestation increased embryonic survival and litter size in rats (Zeng et al. 2008). Furthermore, dietary arginine supplementation reduced embryonic and fetal deaths in mice infected with type 2 porcine circovirus (Ren et al. 2011). Taken together, these results strongly support an important role for arginine in improving embryonic/fetal survival in mammals.

Summary and objectives

Embryonic/fetal loss is a major problem in the swine industry. There has been little progress to improve litter size in pigs using genetic and animal breeding approaches. Failure of conceptus development and implantation during the peri-implantation period of pregnancy is regarded as major causes for embryonic losses in pigs. Arginine is a physiologically versatile amino acid. It is a nitrogenous precursor for synthesis of ornithine, polyamines (putrescine, spermine and spermidine), proline, glutamine, creatine, agamatine, and NO. These biological molecules play key roles in stimulating cell proliferation, cell migration, cellular remodeling, angiogenesis and dilation of blood vessels, as well as stimulation of various cell signaling pathways. More importantly,

arginine and its metabolites have important roles in reproduction, including enhancement of embryonic and fetal survival.

Our central hypothesis is that dietary supplementation with L-arginine during early pregnancy will ameliorate embryonic loss in pigs. The overall objectives of this dissertation research were to: (1) determine the effects of dietary arginine supplementation during early pregnancy on survival and growth of the porcine conceptus; and (2) elucidate the underlying molecular mechanisms associated with increased conceptus survival and development. Findings from the current study not only advance basic knowledge of mammalian reproductive biology, but also have important implications for developing practical means to enhance fertility in female swine.

CHAPTER II

DIETARY SUPPLEMENTATION WITH 0.8% L-ARGININE BETWEEN DAYS 0 AND 25 OF GESTATION REDUCES LITTER SIZE IN GILTS*

This study determined the effects of L-arginine supplementation during early pregnancy on embryonic/fetal survival and growth in gilts. Gilts were housed individually in pens and fed twice daily 1 kg of a corn- and soybean meal-based diet supplemented with 0.0%, 0.4%, or 0.8% L-arginine (w/w) between d 0 and 25 of gestation (10 gilts/treatment). The diets were made isonitrogenous by addition of appropriate amounts of L-alanine. At d 25 of gestation, gilts were fed L-alanine or L-arginine, and hysterectomized 30 min later to obtain uteri and conceptuses (embryos and associated fetal membranes and fluids). Dietary supplementation with 0.4% or 0.8% L-arginine enhanced ($P < 0.05$) its concentrations in maternal plasma (64% and 98%, respectively) as well as the vascularity of the allantoic membrane, compared with the control group. Reproductive performance [number of corpora lutea (CL) and fetuses, placental and fetal weights, and embryonic mortality] did not differ between the 0.4% Arg and control groups. However, supplementation with 0.8% L-arginine decreased ($P < 0.05$) uterine weight (-20%), total number of fetuses (-24%), CL number (-17%), total fetal weight

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(-34%), total volume of allantoic and amniotic fluids (-34 and -42%, respectively), concentrations of progesterone in maternal plasma (-33%), as well as total amounts of progesterone (-35%), estrone (-40%), and estrone sulfate (-37%) in allantoic fluid, compared to the control group. These results indicate that dietary supplementation with 0.8% L-arginine between d 0 and 25 of gestation, while increasing placental vascularity, adversely affects the reproductive performance of gilts.

Introduction

Embryonic loss is a significant problem for both pigs and humans (Bazer et al. 2010). For example, 30-50% of porcine embryos do not survive to term, with most of the losses occurring before d 25 of gestation. After hatching from the zona pellucida, the blastocysts migrate along the length of the uterus and undergo a dramatic change in morphology (Bazer et al. 2010). At d 11 or 12 of gestation, the conceptus elongates rapidly from a 10-mm spherical to 100-150 mm filamentous form within 3 to 4 h (Geisert and Yelich 1997). Thereafter, the conceptuses begin to attach to the uterine wall, and attachment is completed approximately by d 18 of gestation. Although early trophoblast elongation depends mainly on histotrophic nutrients secreted from uterine glands (Spencer and Bazer 2004), blood vessels in the yolk sac and allantois of the placental membranes start to grow rapidly to prepare for hematotrophic nutrition, that is the transfer of nutrients and gases between the maternal and fetal vascular systems (Linton et al. 2008). Thus, the early events of pregnancy are associated with rapid changes in expression of genes for nutrient transport, cellular remodeling, angiogenesis and relaxation of vascular tissues, as well as cell proliferation and migration (Bazer et al.

2009). Failure of conceptuses to undergo implantation and/or cell death during the peri-implantation period results in early embryonic loss.

Arginine is a conditionally essential amino acid for mammals, including pigs (Wu et al. 2009). It is the nitrogenous precursor of nitric oxide (NO), which is a vasodilator and a cell signaling molecule (Li et al. 2009). Furthermore, NO is essential for ovulation, embryonic development, and implantation (Maul et al. 2003). In addition to NO, other products of arginine catabolism (e.g., proline and polyamines) are crucial for cell growth, migration and proliferation, as well as angiogenesis (Wu 2009). Interestingly, supplementing 1.2% L-arginine to rats for 7 d immediately after breeding substantially reduced embryonic mortality by 30% (Zeng et al. 2008). Similarly, supplementing 0.83% L-arginine to gilts between d 30 and 114 of gestation enhanced the number of live-born piglets by 2 per litter (Mateo et al. 2007), which represents an important breakthrough in swine nutrition and production (USDA 2009). At present, little is known about effects of arginine supplementation on embryonic survival or growth and development of conceptuses in pigs.

The present study was conducted to test the hypothesis that dietary supplementation with L-arginine during the first 25 d of pregnancy would ameliorate embryonic loss in pigs. Our results indicated that supplementation with 0.8% L-arginine between d 0 and 25 of gestation reduced the number of corpora lutea (CL), concentrations of progesterone in maternal blood and the conceptus, as well as litter size. These unexpected findings are novel and important, because they question the long-standing view that augmenting total daily feed intake of gilts (e.g., from 2 to 4 kg/d and,

therefore, doubling arginine intake) reduces embryonic/fetal survival due to increased intake of dietary energy (Bazer et al. 1968; Dyck and Strain 1983; Virolainen et al. 2004).

Materials and methods

Chemicals

L-arginine and L-alanine were provided by Ajinomoto Co., Inc. (Tokyo, Japan). Amino acids for HPLC analysis were purchased from Sigma Chemicals (St. Louis, MO). The RIA kits for progesterone (DSL-3400), estrone (DSL-8700), and estrone sulfate (DSL-5400) were obtained from Diagnostic Systems Laboratories (Webster, TX). Anticoagulant vascutainer tubes were procured from BD (Franklin Lakes, NJ).

Animals and diets

This study was conducted at the Veterinary Medical Park of Texas A&M University and approved by the Texas A&M University Laboratory Animal Care and Use Committee. During the entire experimental period, all pigs had free access to drinking water.

Gilts (F1 crosses of Yorkshire X Landrace sows and Duroc X Hampshire boars) had free access to a sorghum grain- and soybean meal-based diet for finishing swine (Table 2.1) until 8 wk before breeding at 8 mo of age. During the 8-wk period before breeding, gilts were fed 2.7 kg/d of the same diet to meet the National Research Council (NRC)-recommended requirements of nutrients for pre-breeding gilts (National Research Council 1998), and all gilts consumed 100% of the feed provided daily.

Table 2.1 Composition of the diet for pre-breeding gilts[1,2]

Ingredients	%
Sorghum grain	72.35
Wheat middlings	10.0
Porcine meat and bone meal	3.5
Soybean meal (47.5% crude protein)	7.55
Soybean hulls	5.0
Ground limestone	0.58
Salt mix	0.50
Monocalcium phosphate	0.34
Choline chloride	0.05
Trace mineral premix[3]	0.08
Vitamin premix[4]	0.05

[1]All values are expressed on an as-fed basis.

[2]Providing 12.8 MJ metabolizable energy per kg diet and the following macronutrients (%): dry matter, 90.0; crude protein, 14.0; fat, 2.50; fiber, 3.73; L-lysine, 0.63; L-methionine, 0.21; L-cysteine, 0.22; L-tryptophan, 0.16; L-threonine, 0.48; L-isoleucine, 0.72; L-arginine, 0.83; calcium, 0.65; phosphorus, 0.56; sodium, 0.21; chlorine, 0.35; potassium, 0.61; sulfur, 0.17; and magnesium, 0.22.

[3]Providing the following microminerals (mg/kg diet): manganese: 64.9; iron, 215; copper, 21.0; cobalt, 0.16; zinc, 153; iodine, 0.49; and selenium, 0.38.

[4]Providing the following vitamins (mg/kg diet): retinyl acetate, 1.76; cholecalciferol, 0.01; D-α-tocopheryl acetate, 36.3; menadione sodium bisulfate, 2.22; choline, 1,190; riboflavin, 4.81; niacin, 71.7; pantothenic acid, 25.5; vitamin B-12, 0.023; biotin, 0.17; vitamin B-6, 5.35; and thiamine, 6.01.

Table 2.2 Composition of the basal diet for gestating gilts[1]

Ingredients	%
Corn grain	80.1
Soybean meal (48.5% crude protein)	10.0
Alfalfa meal	5.0
Monocalcium phosphate	1.9
Potassium chloride	0.75
Ground limestone	1.0
Soybean oil	0.50
Salt mix	0.35
Vitamin premix[2]	0.30
Mineral premix[3]	0.10
Chemical composition	
Dry matter	89.5
Metabolizable energy, *MJ/kg*	12.9
Crude protein[4]	12.0
Fiber	3.62
Lysine	0.57
Calcium	0.82
Potassium	0.94
Magnesium	0.15
Sulfur	0.19
Sodium	0.16
Chlorine	0.61
Total phosphorus	0.70
Available phosphorus	0.47

[1]All values are expressed on an as-fed basis.

[2]Providing the following (mg/kg of the basal diet): retinyl acetate, 8.07; cholecalciferol, 0.05; D-α-tocopheryl acetate, 63.6; menadione sodium bisulfate, 1.76, choline, 1,106; riboflavin, 8.57; niacin, 65.2; pantothenic acid, 34.6; vitamin B-12, 0.04; biotin, 0.23; vitamin B-6, 7.93; and thiamine, 4.51.

[3]Providing the following (mg/kg of the basal diet): manganese: 45.2; iron, 228; copper, 22.2; cobalt, 0.15; zinc, 176; iodine, 0.61; selenium, 0.39.

[4]Providing the following (% of the basal diet): alanine, 0.74; arginine, 0.70; aspartate plus asparagine, 1.29; cysteine, 0.20; glutamate plus glutamine, 2.24; glycine, 0.55; histidine, 0.30; isoleucine, 0.52; leucine, 1.09; lysine, 0.57; methionine, 0.22; phenylalanine, 0.54; proline, 1.11; serine, 0.53; threonine, 0.49; tryptophan, 0.14; tyrosine, 0.42; and valine, 0.61.

Gilts were checked daily for estrus with boars in the morning and bred at onset of the second estrus and 12 h later. Three fertile boars were used randomly for breeding to minimize boar effects. At the time of breeding (d 0 of gestation), the body weight (BW) of gilts was 112.6 ± 3.6 kg (mean ± SEM). Immediately after breeding, gilts were assigned randomly to one of three treatment groups (0.0%, 0.4% and 0.8% L-arginine), and penned individually. There were 10 gilts per treatment. Between d 0 and 24 of gestation, gilts were fed twice daily (0700 h and 1800 h) 1 kg of a corn- and soybean meal-based diet (Table 2.2) supplemented with 0.0% (Control), 0.4%, or 0.8% L-arginine (w/w). All gilts consumed 100% of the feed provided daily. The basal diet (2 kg/gilt per day) met the NRC-recommended requirements of nutrients for gestating gilts (National Research Council 1998). The three diets were made isonitrogenous by addition of appropriate amounts of L-alanine and cornstarch: (a) 32.8 g L-alanine/2 kg diet for the control group; (b) 16.4 g L-alanine + 8 g L-arginine + 8.4 g cornstarch per 2 kg diet for the 0.4% L-arginine group; and (c) 16.0 g L-arginine + 16.8 g cornstarch per 2 kg diet for the 0.8% L-arginine group. L-Arginine or L-alanine was added to the basal diet as top dressing. L-Alanine, rather than a mixture of amino acids, was used as the isonitrogenous control, because it is rapidly catabolized by pigs (Wang et al. 2008), is not a substrate for arginine synthesis (Wu et al. 2009), and does not affect any of the measured variables of reproductive performance on d 25 of gestation [(a) CL number; (b) uterine, placental, and embryonic/fetal weights; (c) the total number of fetuses, embryonic survival, and the number of live fetuses; and (d) volumes of amniotic and allantoic fluids, compared with non-supplemented gilts (our unpublished observations).

At d 25 of gestation, 22 h after the last meal and 30 min after consumption of L-arginine (4 or 8 g) or L-alanine (isonitrogenous amounts), gilts were prepared for anesthesia (approximately 15 min) and then hysterectomized to obtain uteri and conceptuses (embryos and associated fetal membranes and fluids), as described previously (Wu et al. 2005). After the abdomen of the gilt was opened, uterine venous and arterial blood samples (10 mL) were collected separately into EDTA-coated vacutainer tubes. Plasma was obtained after centrifugation (10,000 g for 5 min) and stored at -80^{o}C until analyzed for metabolites and hormones. Umbilical or fetal blood samples could not be obtained at d 25 of gestation due to the small size of blood vessels. After the uterus was obtained and weighed, the numbers of CL, total embryos, and live embryos were counted. Additionally, crown-rump length and weight of each fetus, as well as placental weight of each conceptus were recorded. Finally, the volumes of allantoic fluid (ALF) and amniotic fluid (AMF) for each fetus were measured. Allantoic fluid (10 mL) and all amniotic fluid were stored at -80^{o}C for analyses for amino acids and hormones.

Analysis of metabolites and hormones

Amino acids in uterine arterial and venous samples as well as amniotic and allantoic fluids were analyzed by HPLC methods involving precolumn derivatization with o-phthaldialdehyde (Wu and Knabe 1994). Ammonia, urea and glucose were determined using enzymatic methods (Wu et al. 2005). Progesterone, estrone, and estrone sulfate were determined using RIA kits according to the instructions of the manufacturer. The minimum detection limit was 0.1 ng/mL, 1.2 pg/mL, and 0.01 ng/mL for progesterone,

estrone, and estrone sulfate, respectively. The intra-assay coefficients of variation were 6.3%, 4.7%, and 4.2% for progesterone, estrone, and estrone sulfate assays, respectively. The inter-assay coefficients of variation were 9.2%, 5.4%, and 10.7% for progesterone, estrone, and estrone sulfate assays, respectively.

Statistical analysis

Data were analyzed using General Linear Model procedures of SPSS [Statistical Package for the Social Sciences] (Version 12.0, Chicago, IL) for a randomized complete block design. Gilt was considered as the experimental unit. Differences among treatment means were determined by the Student-Newman-Keuls multiple comparison test. Data on the embryonic survival rate was analyzed using the Chi-Square test of SPSS. Probability values < 0.05 were considered statistically significant.

Results

Reproductive performance of gilts

Four gilts (1, 1, and 2 gilts in the 0.0%, 0.4% and 0.8% L-arginine groups, respectively) were not pregnant at the time of hysterectomy. None of the measurements of reproductive performance in gilts differed between the control and 0.4% L-arginine groups (Table 2.3). However, compared with the control group, dietary supplementation with 0.8% L-arginine reduced ($P < 0.05$) uterine weight, the total number of fetuses, the number of live fetuses, CL number, total fetal weight, and volumes of ALF and AMF. Embryonic survival and total placental weight did not differ between the control and 0.8% arginine groups. However, embryonic survival was lower ($P < 0.05$) in gilts supplemented with 0.8% L-arginine than in gilts supplemented with 0.4% L-arginine.

Maternal plasma metabolites and hormones

Concentrations of aspartate, glutamate, alanine, and tyrosine were lower ($P < 0.05$), but concentrations of arginine, ornithine and proline were higher ($P < 0.05$) in plasma from gilts supplemented with 0.8% L-arginine, compared with control gilts (Table 2.4). No

Table 2.3 Reproductive performance of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) from d 0 through d 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
BW at breeding, *kg*	113.9	110.1	113.7	3.6	0.696
BW at d 25 of gestation, *kg*	120.9	115.0	116.4	3.2	0.492
BW gain, *kg/25 d*	7.0	4.9	2.7	2.7	0.556
Uterine weight, *kg*	3.11[a]	2.94[ab]	2.48[b]	0.1	0.041
Total fetus, *n*	13.1[a]	12.6[a]	10.0[b]	0.4	0.003
Live fetus, *n*	12.7[a]	12.3[a]	9.6[b]	0.5	0.007
CL, *n*	15.2[a]	13.7[ab]	12.6[b]	0.4	0.011
Embryonic survival rate, %	83.1[ab]	90.8[a]	75.6[b]	2.3	0.022
Total viable fetal weight, *g*	8.3[a]	8.9[a]	5.5[b]	0.8	0.020
Total placental weight, *g*	174.9	147.1	136.3	22.1	0.255
Fetal length, *cm*	2.11	2.11	2.01	0.01	0.190
Total ALF volume, *L*	1.28[a]	1.26[a]	0.85[b]	0.07	0.008
Total AMF volume, *mL*	2.70[a]	3.37[a]	1.56[b]	0.29	0.029

*Values are means with pooled SEM, n = 9 gilts (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg group). Means in a row with superscripts without a common letter differ, $P < 0.05$.

differences in concentrations of lysine (Table 2.4) and other amino acids (Table 2.5) in maternal plasma were detected between the control and 0.8% L-arginine groups. Compared with the control group, dietary supplementation with 0.4% L-arginine enhanced ($P < 0.05$) concentrations of arginine, alanine, ornithine, and proline in

maternal plasma (Table 2.4) but had no effect on other amino acids (Table 2.5). Concentrations of amino acids, other than alanine and arginine, in maternal plasma did not differ between the 0.4% and 0.8% L-arginine groups. Concentrations of ammonia, urea and progesterone were lower ($P < 0.05$) in maternal plasma of gilts supplemented with both 0.4% and 0.8% L-arginine, when compared with control gilts (Table 2.4). However, concentrations of estrone and estrone sulfate (Table 2.4) as well as glucose and free fatty acids (Table 2.5) in maternal plasma did not differ among the three treatment groups of gilts.

Table 2.4 Concentrations of free amino acids, ammonia, urea and hormones in uterine arterial plasma of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine from d 0 through d 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Ala, *μmol/L*	751[a]	490[b]	258[c]	74	<0.001
Arg, *μmol/L*	123[c]	210[b]	251[a]	12	<0.001
Asp, *μmol/L*	14[a]	12[ab]	10[b]	1	0.008
Glu, *μmol/L*	194[a]	167[ab]	148[b]	7	0.023
Lys, *μmol/L*	139	156	132	13	0.486
Orn, *μmol/L*	64[b]	97[a]	93[a]	9	0.028
Pro, *μmol/L*	220[b]	295[a]	310[a]	16	<0.001
Ammonia, *μmol/L*	165[a]	128[b]	124[b]	5	<0.001
Urea, *μmol/L*	2112[a]	1594[b]	1426[b]	82	<0.001
Progesterone, *μg/L*	20.1[a]	12.6[b]	13.5[b]	1.19	0.013
Estrone, *ng/L*	130	118	100	8	0.331
Estrone sulfate, *μg/L*	8.27	8.93	7.36	0.61	0.589

*Values are means with pooled SEM, n = 9 gilts (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg group). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 2.5 Concentrations of free amino acids, glucose, and non-esterified fatty acids (NEFA) in uterine arterial plasma of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) from d 0 through d 25 of gestation*

Amino Acid	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol/L		
β-Ala	17.1	15.2	11.8	1.51	0.073
Asn	36.4	37.4	32.5	2.7	0.396
Cit	54.3	61.0	54.1	5.0	0.538
Gln	406.2	387.7	330.5	15.4	0.118
Gly	692.0	692.7	612.0	26.6	0.386
His	60.3	62.9	61.3	3.8	0.881
Ile	73.0	73.2	75.8	6.1	0.937
Leu	140.3	144.8	145.0	11.4	0.948
Met	27.8	29.7	26.6	1.9	0.512
Phe	45.5	50.6	48.0	3.4	0.570
Ser	83.2	82.3	74.6	5.7	0.525
Taurine	120.6	94.4	108.6	13.2	0.376
Thr	99.8	102.6	87.3	8.7	0.434
Trp	35.5	35.2	34.8	2.5	0.981
Tyr	54.3	52.8	44.0	3.41	0.101
Val	164.0	172.7	169.4	14.29	0.910
Glucose	4054	4129	3953	153	0.902
NEFA	127	126	158	17	0.714

*Values are means with pooled SEM, n = 9 gilts (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg group).

Table 2.6 Concentrations of free amino acids in allantoic fluid of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 0 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol/L		
Asp	8	8	10	0.8	0.524
Glu	49	39	48	3.9	0.576
Asn	54	56	54	2.9	0.972
Ser	405	381	407	16	0.778
Gln	526	527	498	26	0.886
His	61	64	66	2.8	0.811
Gly	452	391	434	23	0.566
Thr	141	142	136	7.7	0.962
Cit	9.4	9.6	9.6	0.8	0.996
Arg	100	141	135	10	0.229
β-Ala	22	20	24	1.6	0.693
Tau	322	309	347	23	0.801
Ala	145	131	157	7.9	0.436
Tyr	29	31	27	1.8	0.781
Trp	9	8	12	1.0	0.353
Met	10	10	11	0.6	0.760
Val	50	50	54	2.6	0.796
Phe	20	21	22	1.2	0.778
Ile	14	14	14	0.9	0.903
Leu	32	31	35	1.8	0.604
Orn	102	109	110	4.6	0.755
Lys	239	252	221	13	0.656
Pro	168	198	214	11	0.213

* Values are means and pooled SEM, n = 9 gilts (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg group).

Table 2.7 Concentrations of free amino acids in amniotic fluid of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 0 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol		
Asp	21	24	24	2.2	0.788
Glu	132	159	152	12	0.664
Asn	66	70	77	3.1	0.361
Ser	346	397	380	17	0.478
Gln	869	941	978	32	0.376
His	53	58	59	2.4	0.540
Gly	324	300	357	16	0.392
Thr	159	146	156	9.4	0.843
Cit	8	8	7	0.3	0.550
Arg	93	94	118	7.9	0.364
β-Ala	12	11	13	0.8	0.379
Tau	157	135	184	11	0.184
Ala	271	295	304	12	0.563
Tyr	61	68	60	4.5	0.726
Trp	11	13	15	1.0	0.239
Met	39	44	42	2.2	0.623
Val	140	143	153	7.8	0.785
Phe	51	53	59	3.0	0.601
Ile	35	35	47	2.5	0.096
Leu	114	110	128	5.8	0.417
Orn	63	72	72	3.0	0.392
Lys	110	121	120	7.1	0.795
Pro	205	214	192	8.5	0.590

* Values are means and pooled SEM, n = 9 gilts (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg group).

Table 2.8 Total amounts of free amino acids and hormones in fetal fluids of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) from d 0 through d 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*-Value
		Allantoic fluid, *μmol*			
Arg	132[b]	189[a]	128[b]	10	0.034
Asn	70[a]	71[a]	43[b]	5	0.019
Gln	670[a]	681[a]	401[b]	48	0.025
His	79[a]	82[a]	54[b]	5	0.042
Lys	314[a]	312[a]	179[b]	25	0.035
Orn	131[a]	134[a]	92[b]	8	0.046
Pro	211	253	254	23	0.685
Ser	519[a]	488[a]	330[b]	32	0.038
Tyr	36[a]	39[a]	24[b]	3	0.012
		Allantoic fluid, *μg*			
Progesterone	2.29[a]	2.24[a]	1.50[b]	0.127	0.016
Estrone	0.63[a]	0.62[a]	0.38[b]	0.035	0.002
Estrone sulfate	56[a]	59[a]	35[b]	4	0.013
		Amniotic fluid, *nmol*			
Arg	205[b]	310[a]	212[b]	18	<0.001
Asn	192[a]	227[a]	117[b]	20	0.043
Gln	2300[b]	3680[a]	1967[b]	305	0.026
His	121[b]	224[a]	103[b]	15	<0.001
Lys	303[a]	338[a]	201[b]	21	0.019
Orn	148[b]	229[a]	125[b]	15	<0.001
Pro	454[b]	613[a]	410[b]	34	0.017
Ser	1028[a]	1352[a]	550[b]	89	0.018
Tyr	129[b]	256[a]	108[b]	16	<0.001

*Values are means with pooled SEM, n = 9 gilts (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg group). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 2.9 Total amounts of free amino acids, glucose, fructose, and non-esterified fatty acids (NEFA) in allantoic fluid and amniotic fluid of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) from d 0 through d 25 of gestation*

Amino Acid	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		Allantoic fluid, *μmol*			
Ala	186	168	135	11	0.076
β-Ala	29	26	18	2	0.131
Asp	10	11	10	1	0.944
Cit	13	12	10	1	0.194
Glu	61	53	48	5	0.309
Gly	587	498	435	40	0.215
Ile	17	16	13	1	0.124
Leu	41	38	34	3	0.072
Met	13	13	10	1	0.145
Phe	26	25	20	2	0.085
Taurine	418	404	339	38	0.281
Thr	184	178	140	16	0.136
Trp	11	12	10	1	0.710
Val	64	62	47	4	0.096
Glucose	1875[b]	2496[a]	1622[b]	144	0.032
Fructose,*mg*	1479[a]	1250[ab]	882[b]	91	0.023
NEFA	47[a]	47[a]	26[b]	4.1	0.044
		Amniotic fluid, *nmol*			
Ala	764	821	659	74	0.133
β-Ala	34	40	20	5	0.223
Asp	59	64	48	7	0.205
Cit	23	25	17	3	0.108
Glu	366	423	315	50	0.120
Gly	974	985	787	96	0.226
Ile	90	107	76	12	0.172
Leu	301	348	259	33	0.096
Met	89	125	77	15	0.103
Phe	131	150	116	17	0.128
Taurine	420	467	354	56	0.384
Thr	434	502	383	50	0.118
Trp	30	36	27	4	0.177
Val	335	387	296	34	0.082
Glucose	2277[b]	3459[a]	1502[c]	186	0.004
Fructose,*mg*	2.61[ab]	3.56[a]	1.62[b]	0.31	0.035
NEFA	0.22[a]	0.22[a]	0.11[b]	0.01	0.001

*Values are means with pooled SEM, n = 9 gilts (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg group). Means in a row with superscripts without a common letter differ, $P < 0.05$.

ALF and AMF metabolites and hormones

The concentration of amino acids in ALF and AMF did not differ among the three treatment groups of gilts (Table 2.6; 2.7). Total amounts of asparagine, glutamine, histidine, lysine, ornithine, serine, threonine, tyrosine (Table 2.8), glucose, fructose, and free fatty acids (Table 2.9) in ALF or AMF were lower ($P < 0.05$) in gilts supplemented with 0.8% L-arginine, compared with values for ALF and AMF from conceptuses of gilts in the control and 0.4% L-arginine groups. Total amounts of arginine and alanine (Table 2.8), as well as other amino acids (Table 2.9) in ALF and AMF did not differ among the three treatment groups of gilts. Total amounts of ammonia, urea and hormones (progesterone, estrone and estrone sulfate) in ALF and AMF did not differ between the control and 0.4% L-arginine groups, but values for these two treatment groups were greater ($P < 0.05$) than those for the 0.8% L-arginine groups (Table 2.8).

Placental vascularity

Visual examination of the conceptuses indicated the presence of small and pale blood vessels on the allantoic membranes of conceptuses from control gilts at d 25 of gestation (Fig. 2.1). Of particular note, blood vessels of the allantoic membranes of conceptuses from gilts supplemented with 0.4% or 0.8% L-arginine were more extensive and larger, compared with those for conceptuses from control gilts (Fig. 2.1). Placental vascularity did not appear to differ between conceptuses from gilts receiving 0.4% and 0.8% L-arginine.

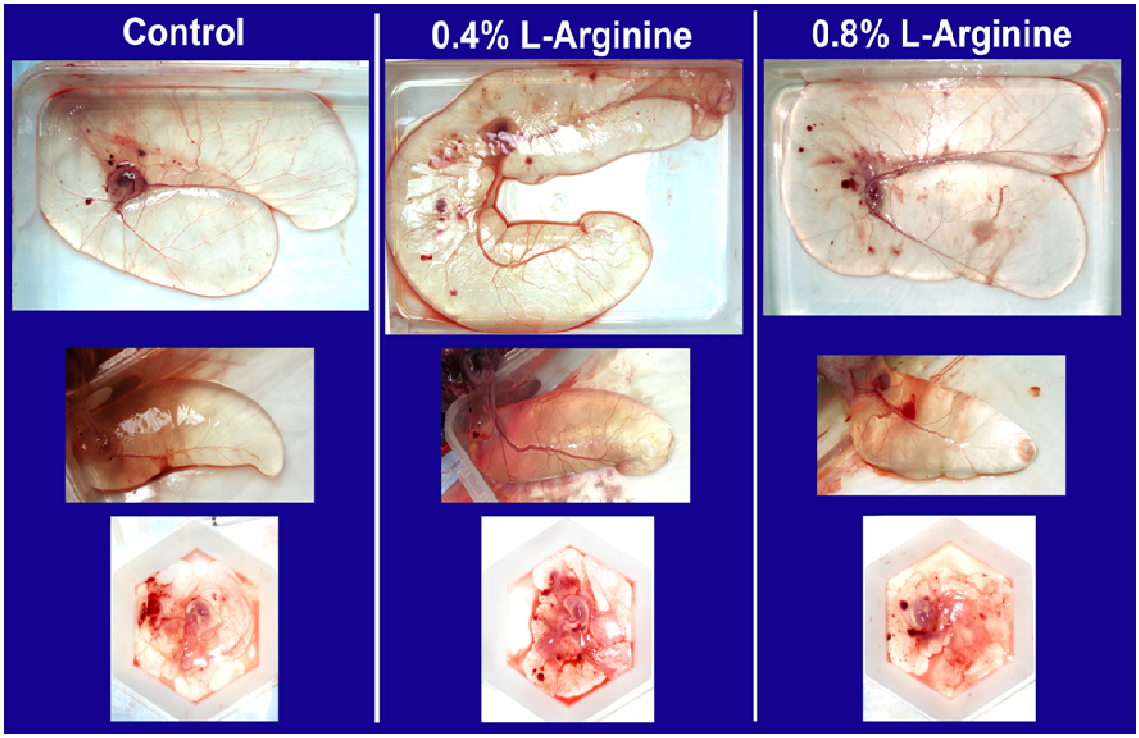

Fig. 2.1 Representative conceptuses of gilts fed diets supplemented with 0 (Control), 0.4 or 0.8% L-arginine from d 0 through d 25 of gestation. At d 25, blood vessels on allantoic membranes in the control group were small and pale in comparison with more extensive and larger vessels in allantoic membranes of gilts supplemented with 0.4% or 0.8% L-arginine.

Discussion

Dietary supplementation with 0.83% L-arginine between d 30 and 114 of gestation (Mateo et al. 2007) or 1% L-arginine between d 14 and 28 of gestation (Ramaekers et al. 2006; Berard et al. 2009) has been reported to enhance fetal survival and growth in gilts. Surprisingly, results of the present study indicated that supplementation with 0.8% L-arginine reduced CL and embryo numbers as well as uterine weight and conceptus development (i.e., reduced volumes of both AFL and AMF) in gilts at d 25 of gestation. Additionally, increasing the supplemental dose of L-arginine from 0.4% to 0.8% reduced

embryonic survival rate at d 25 of gestation by 17% (Table 2.3). To our knowledge, this is the first report of an adverse effect of dietary L-arginine supplementation during early gestation on pregnancy outcome in mammals. Indeed, our results led to the discovery that 0.8% L-arginine supplementation immediately after breeding severely reduced CL numbers, resulting in impaired production of progesterone in early pregnant gilts.

Nutrition, particularly the balance of amino acids in the diet, affects tissue protein synthesis (Suryawan et al. 2009; Elango et al. 2009; Deng et al. 2009) and pregnancy outcomes (Fiorotto et al. 1995) in mammals. Arginine supplementation (up to 0.8% of the diet) had no effect on intestinal absorption of basic amino acids in pregnant gilts as concentrations of lysine (Table 2.4) or histidine (Table 2.5) in maternal plasma did not differ among gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine. However, our studies indicate that the period of gestation in which L-arginine is supplemented to gilts is critical because 0.8% L-arginine was detrimental to embryonic survival between d 0 and 25. This phenomenon is consistent with previous reports indicating that a high plane of feeding between d 0 and 10 of gestation, but not d 11-20, decreased embryonic survival and progesterone levels at d 30 in pigs (Dyck and Strain 1983). Similarly, embryonic survival on d 28 was greater in gilts fed 1.8 kg of a diet daily between d 0 and 15 after mating, compared with 2.5-kg diet/d (Pharazyn 1992). Furthermore, embryonic survival and concentrations of progesterone in blood were greater in gilts fed 2.1 kg/d of a commercial ration during the first 34 d of pregnancy, in comparison with gilts fed daily 4.3 kg of the diet during that period (Virolainen et al. 2004). Previous researchers concluded that the adverse effects of increased feed intake (e.g., augmenting total feed

intake from 2 of 4 kg/d) on embryonic survival resulted from increased intake of dietary energy (Bazer et al. 1968; Dyck and Strain 1983; Virolainen et al. 2004). In our study, total intake of feed and metabolizable energy (2.0 kg/d and 26 MJ/d, respectively; currently recommended by NRC) did not differ among the treatment groups. However, doubling L-arginine intake by gilts from 14 to 30 g/d through supplementing 0.8% L-arginine to the basal diet (which occurred without increasing feed intake) was sufficient to decrease concentrations of progesterone in maternal plasma (Table 2.8) and embryonic survival (Table 2.3). Thus, the unexpected observations from the current work question the long-standing view, based on assumptions, that elevated energy intake was solely responsible for high embryonic/fetal mortality in gilts with high feed intake (Bazer et al. 1968; Dyck and Strain 1983; Virolainen et al. 2004).

Another novel and important finding of this study is that dietary L-arginine supplementation during early gestation reduced CL number in gilts (Table 2.3), thereby decreasing concentrations of progesterone in maternal blood and the conceptus (Table 2.8). In the ovary, follicular development and discharge of mature oocytes with the formation of CL depends on cell signaling via mitogen activated protein kinases 3 and 1 [also known as extracellular-regulated protein kinases 1 and 2 (ERK1/2)] (Duggavathi and Murphy 2009) and liver receptor homolog 1 [Lrh1] (Duggavathi et al. 2008). Recent evidence shows that Lrh1 is essential for ovulation in mice through a mechanism involving expression of the *NOS3* gene (Duggavathi et al. 2008). Based on available data, we suggest that increased production of NO through arginine supplementation may impair ERK1/2 signaling and Lrh1 function in the porcine ovary, therefore reducing the

number of follicles that ovulated and, therefore, the number of CL and concentrations of progesterone in maternal plasma. Regression of CL rarely occurs in pregnant pigs under normal feeding conditions (e.g., 2 kg daily of a typical corn- and soybean meal-based diet) because CL are the main source for progesterone required for establishment and maintenance of pregnancy (Bazer et al. 2009). Prostaglandin F_{2a} ($PGF_{2\alpha}$) is a luteolytic agent for CL regression when it is secreted from the uterine epithelia into the uterine circulation (endocrine secretion) rather than into the uterine lumen (exocrine secretion) (Henderson and McNatty 1975; Bazer and Thatcher 1977). Although $PGF_{2\alpha}$ production is known to be modulated by estrogen secreted by the conceptus (Bazer and Thatcher 1977), recent studies have shown that NO can stimulate this biochemical event by up-regulating expression of cyclooxygenase II, a key enzyme for prostaglandin synthesis (Roberto et al. 2008; Salvemini et al. 1993). Thus, dietary L-arginine supplementation between d 0 and 25 of gestation, which promotes systemic NO synthesis in animals (Wu et al. 2009), may lead to CL regression through a $PGF_{2\alpha}$-dependent pathway. Additionally, because ovulation usually takes place at about 44 h after onset of estrus (Bazer et al. 2010), initiation of L-arginine supplementation within 24 h after onset of estrus may inhibit or interfere with ovulation, thereby decreasing the numbers of CL in gilts. Future studies are warranted to test these novel hypotheses.

Progesterone plays an important role in up-regulating expression of amino acid transporters in the uterine endometrium and conceptus (Wu et al. 2009; Johnson et al. 2009). Thus, total amounts of neutral amino acids (asparagine, glutamine, serine, threonine and tyrosine) and basic amino acids (histidine, lysine and ornithine) were

lower in ALF and AMF of gilts supplemented with 0.8% L-arginine, compared with values for conceptuses from control gilts (Table 2.8). Note that the reduced availability of these amino acids in fetal fluids occurred independent of their concentrations in maternal plasma (Table 2.4). Indeed, concentrations of asparagine, glutamine, serine, threonine, tyrosine, histidine, and lysine in maternal plasma did not differ between the control and L-arginine-supplemented gilts, whereas concentrations of ornithine in maternal plasma were higher in L-arginine-supplemented gilts (Table 2.4). Thus, lowered production of progesterone due to reduced CL numbers not only impairs embryonic/fetal survival, but also reduces the availability of total amino acids for uterine and conceptus growth. This is consistent with by our finding that uterine weight and total viable fetal weight were 20% and 34% lower, respectively, in gilts supplemented with 0.8% L-arginine, compared with values for control gilts (Table 2.3).

Based on our current knowledge about hormonal regulation of conceptus development (Bazer et al. 2010), we suggest that a reduction in concentrations of progesterone in maternal plasma and total amounts of progesterone, estrone and estrone sulfate in ALF may mediate, at least in part, adverse effects of 0.8% L-arginine supplementation on embryonic survival in gilts. Consistent with previous reports (Chen et al. 1995; Horne et al. 1983), a reduction in the amounts of estrone and estrone sulfate in ALF was associated with fewer live embryos in gilts (Table 2.8). Interestingly, circulating levels of progesterone are also reduced in gilts receiving a high plane of feeding in association with compromised embryonic survival (Jindal et al. 1996). Importantly, daily administration of progesterone has been reported to be effective in

ameliorating embryonic and fetal mortality in gilts and sows induced by high feeding levels (Jindal et al. 1996). Thus, it can be surmised that supplementation of both L-arginine and progesterone during early gestation may have the desired benefit of enhancing vascular development and angiogenesis in the placenta (Fig. 2.1) while preventing embryonic/fetal loss and impairment of uterine growth in gilts. Additional work is required to test this novel idea using molecular, genomic and proteomic techniques (Wang et al. 2009; Palii et al. 2009).

In summary, dietary supplementation with 0.4% L-arginine between d 0 and 25 of gestation had no beneficial effect on the reproductive performance of gilts. However, supplementation with 0.8% L-arginine during this period of pregnancy, while increasing placental vascularity, decreased litter size in gilts. This finding did not support the original hypothesis of the present study, but led to an important discovery that 0.8% L-arginine supplementation immediately after breeding reduced numbers of CL and their production of progesterone, therefore impairing conceptus survival and growth in gilts.

CHAPTER III

DIETARY SUPPLEMENTATION WITH L-ARGININE BETWEEN DAYS 14 AND 25 OF GESTATION ENHANCES REPRODUCTIVE PERFORMANCE OF GILTS

This study determined effects of dietary L-arginine supplementation between d 14 and 25 of gestation on embryonic survival and growth in gilts. Gilts were checked daily for estrus with boars in the morning and bred at onset of the second estrus and 12 h later (the time of breeding = d 0 of gestation). Between d 14 and 25 of gestation, 15 gilts per treatment were housed individually and fed twice daily 1 kg of a corn- and soybean meal-based diet supplemented with 0.0%, 0.4%, or 0.8% L-arginine. All diets were made isonitrogenous by addition of L-alanine. At d 25 of gestation, gilts were hysterectomized to obtain conceptuses. Compared with control gilts, dietary supplementation with 0.4% or 0.8% L-arginine increased ($P \leq 0.05$) concentrations of arginine in maternal plasma, total volume of amniotic fluid, total amounts of arginine in allantoic and amniotic fluids, total amounts of fructose and most amino acids in amniotic fluid, placental growth, and the number of viable fetuses per litter by 2. The numbers of total fetuses or corpora lutea, total fetal weight, total volume of allantoic fluid, maternal circulating levels of progesterone and estrogen, or total amounts of the hormones in allantoic fluid did not differ among the three treatment groups. Reproductive performance of gilts did not differ between the 0.4% and 0.8% L-arginine groups. These

novel results indicate that dietary supplementation with 0.4% or 0.8% L-arginine between d 14 and 25 of gestation can enhance embryonic/fetal survival in gilts.

Introduction

Arginine serves as the physiological precursor for synthesis of many biological molecules, including ornithine, polyamines (putrescine, spermine and spermidine), proline, glutamine, creatine, agamatine, and nitric oxide (NO), as well as proteins (Wu and Morris 1998). Arginine and its metabolites have versatile functions in cardiovascular, neurological, immunological and endocrine systems (Wu and Meininger 2000; Barbul 1990; Calabrese et al. 2007; Schmidt et al. 1992). Notably, results of recent studies led to the discovery that arginine can activate the mechanistic target of rapamycin (MTOR) cell signaling pathway (Yao et al. 2008; Kim et al. 2011), which plays crucial roles in protein synthesis, cell growth, and cytoskeletal remodeling (Bazer et al. 2011a). More importantly, NO and polyamines, two metabolites of arginine catabolism, may regulate conceptus survival and growth by promoting cell proliferation and migration, angiogenesis, and dilation of blood vessels to increase blood flow.

There is evidence that the number of live-born piglets is enhanced by 2 per litter in gilts receiving dietary supplementation with 0.83% L-arginine between d 30 and 114 of gestation (Mateo et al. 2007). Moreover, embryonic mortality in rats was reduced by 30% in response to dietary supplementation with 1.2% L-arginine for 7 d immediately after breeding (Zeng et al. 2008). These studies represent an important breakthrough for developing strategies to reduce embryonic loss, a major problem in reproduction of mammals, including pigs and humans. Early pregnancy (before d 25 of gestation) is the

period when 75% of embryonic losses occur (Pope 1994), so this is a critical window to control embryonic mortality in pigs (Wu et al. 2010).

It was proposed that dietary supplementation with L-arginine during the first 25 d of pregnancy would ameliorate embryonic loss in pigs (Li et al. 2010). Unexpectedly, the results from the first study (see Chapter Π) revealed that supplementing the diet of gilts with 0.8% L-arginine between d 0 and 25 of gestation reduced reproductive performance as indicated by reductions in embryonic survival, number of corpora lutea (CL), and concentrations of progesterone in maternal plasma, as compared with the control group. The adverse effects of 0.8% dietary L-arginine supplementation immediately after breeding suggest that excessive intake of dietary L-arginine interferes with CL formation or promote CL regression by increasing NO synthesis (Roberto et al. 2008; Salvemini et al. 1993). This, in turn, reduces concentrations of progesterone in maternal plasma and the conceptus. Interestingly, there are reports that dietary supplementation with L-arginine for 2 wk beginning on d 14 of gestation increases litter size at birth in pigs (Ramaekers et al. 2006; Berard et al. 2009). These results suggest that initiation of arginine supplementation after CL formation may capitalize on the benefits of arginine on conceptus survival and development without adverse effects on CL number and progesterone production. However, experimental results in support of this proposition are lacking. The present study tested the hypothesis that dietary supplementation with arginine between d 14 and 25 of gestation increases survival and development of conceptuses on d 25 of gestation.

Materials and methods

Chemicals

L-arginine and L-alanine were provided by Ajinomoto Co., Inc. (Tokyo, Japan). The RIA kits for progesterone (DSL-3400), estradiol (DLS-4400), estrone (DSL-8700), and estrone sulfate (DSL-5400) were obtained from Diagnostic Systems Laboratories (Webster, TX). Anticoagulant vacutainer tubes were procured from BD (Franklin Lakes, NJ). Amino acid standards for HPLC analysis were purchased from Sigma Chemicals (St. Louis, MO).

Animals and diets

The experimental design was similar to that described in Chapter II with some modifications. Briefly, following breeding during the second period of estrus, 45 gilts (F1 crosses of Yorkshire X Landrace sows and Duroc X Hampshire boars) were assigned randomly to three treatment groups (0.0, 0.4, and 0.8% L-arginine) and penned individually. Fifteen gilts were used for each treatment group. Between d 0 and 13 of gestation, gilts were fed twice daily (0700 and 1800 h) 1 kg of a corn and soybean meal-based diet (2 kg diet/d). The basal diet met NRC (1998) recommended nutrient requirements for gestating gilts. Starting on d 14 of gestation, gilts were fed twice daily (0700 and 1800 h) 1 kg of a corn- and soybean meal-based diet supplemented with 0.0% (control), 0.4%, or 0.8% L-arginine (wt/wt) as described in Chapter II.

Hysterectomy and tissue collection

At d 25 of gestation, gilts were hysterectomized to obtain uteri and conceptuses after 10 mL samples of uterine venous and arterial blood were collected for analysis of

metabolites and hormones (Li et al. 2010). Uterine weight, CL number, total number of fetuses, total number of viable fetuses, fetal weight and length, placental weight, and volumes of allantoic (ALF) and amniotic fluids (AMF) were measured and recorded. ALF (10 mL) and all AMF from each fetus were collected for assays of metabolites and hormones (Wu et al. 1998a). No fetal blood samples could be obtained on d 25 of gestation due to the very small size of umbilical vessels (Li et al. 2010). Portions of each placenta from the gilts were snap-frozen in liquid nitrogen. Endometrium was separated from myometrium using curved scissors, and snap-frozen in liquid nitrogen (Wu et al. 1998b). All snap frozen samples were stored at -80^{o}C until analyzed.

Homogenization of placenta and endometrium

Frozen placenta (~200 mg) and endometrium (~100 mg) from eight gilts in each treatment group were homogenized with a glass homogenizer in 1 mL of ice-cold 1.5 M $HClO_4$. The homogenate was transferred into 15 mL BD Falcon™ conical tubes. The homogenizer was rinsed twice each with 1 mL of 1.5 M $HClO_4$. The combined homogenized solution was neutralized with 1.5 mL of 2 M K_2CO_3. The solution was centrifuged at 10, 000 g for 2 min, and the supernatant fluid was used for HPLC analysis of amino acids.

Analysis of amino acids, fructose, and hormones

Amino acids in plasma from uterine arterial plasma and amniotic and allantoic fluids as well as placental and endometrial extracts were analyzed by HPLC methods (Li et al. 2010). Fructose was determined in duplicate as described by Roe (1934) with modifications. Briefly, 100 μL samples were deproteinized with 200 μL of 4.7%

trichloroacetic acid. The solution was centrifuged at 10,000 g for 1 min, and the supernatant fluid was used for fructose assay. The reagents were added into a clear 96-well microplate in the order of 40 μL of sample or fructose standards (STD), 40 μL of 1 mg/mL resorcinol, and 120 μL of 30% HCl. A microplate was covered by a clear film followed by a gentle vortex (30 sec) and incubation at 80 ^{o}C for 8 min. After the microplate cooled in running tap water, absorbance was measured at 490 nm. Fructose concentrations in samples were calculated on the basis of the fructose standard curve.

Progesterone, estradiol, estrone, and estrone sulfate were determined using RIA kits according to the instructions of the manufacturer. The minimum detection limit was 0.1 ng/mL, 4.7 pg/mL, 1.2 pg/mL, and 0.01 ng/mL for progesterone, estradiol, estrone, and estrone sulfate, respectively. The intra-assay coefficients of variation were 6.3%, 4.6%, 4.7%, and 4.2% for progesterone, estrone, and estrone sulfate assays, respectively. The inter-assay coefficients of variation were 9.2%, 8.5%, 5.4%, and 10.7% for progesterone, estradiol, estrone, and estrone sulfate assays, respectively.

Calculations and statistical analysis

The total amount of a substance in allantoic or amniotic fluid was calculated as concentration times the total volume of the fluid. Data, expressed as means ± SEM, were analyzed using General Linear Model procedures of SPSS [Statistical Package for the Social Sciences] (Version 15.0, Chicago, IL) for a randomized complete design. Gilt was considered as the experimental unit. Differences among treatment means were determined by the Duncan multiple comparison test. Data on embryonic survival were

analyzed using the Chi-Square test of SPSS. Probability values ≤ 0.05 were considered statistically significant.

Results

Reproductive performance of gilts

One gilt from the control group and one gilt from the 0.8% L-arginine group were not pregnant at the time of hysterectomy (Table 3.1). There were no differences among the three groups of gilts in maternal body-weight gain, total uterine weight, total number of fetuses, number of CL, total fetal weight, fetal length, or volume of ALF (Table 3.1). However, compared with the control group, dietary supplementation with 0.4% or 0.8% L-arginine increased placental weight by 21% and 34% ($P < 0.01$), respectively and the number of live fetuses per litter by 2 ($P = 0.05$), while reducing ($P < 0.01$) embryonic mortality by 14% and 15%, respectively (Table 3.1). The total volume of AMF was greater ($P < 0.01$) for conceptuses from gilts supplemented with either 0.4% or 0.8% L-arginine (Table 3.1). Reproductive performance of gilts did not differ between the 0.4% and 0.8% L-arginine groups.

Concentrations of amino acids, glucose, and free fatty acids in maternal plasma

Gilts in the 0.4% and 0.8% L-arginine groups had higher ($P < 0.05$) concentrations of arginine and ornithine in maternal plasma, compared with the control group (Table 3.2). Concentrations of aspartate, glutamate, and alanine were lower ($P < 0.05$) in plasma from gilts supplemented with 0.8% L-arginine compared with control gilts, but the values did not differ between the 0.4% arginine and control groups (Table 3.2).

Concentrations of other amino acids, glucose, and free fatty acids in maternal plasma did not differ among the three treatment groups of gilts (Tables 3.2 and 3.3).

Table 3.1 Reproductive performance of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Number of gilts, *n*	14	15	14		
BW at breeding, *kg*	119.9	113.3	110.9	4.8	0.762
BW at d 25 of gestation, *kg*	121.3	114.4	112.1	4.8	0.745
BW gain, *kg/25 d*	1.1	1.1	1.2	1.0	0.998
Uterine weight, *kg*	2.46	2.66	2.48	0.07	0.389
Total fetuses, *n*	11.3	12.8	12.4	0.4	0.228
Live fetuses, *n*	10.5[b]	12.7[a]	12.2[a]	0.4	0.050
CL, *n*	13.9	14.4	13.6	0.3	0.586
Embryonic mortality, %	24.7[a]	11.2[b]	10.1[b]	1.9	0.001
Weight of viable fetuses, *g*	5.58	6.27	5.83	0.18	0.252
Total placental weight, *g*	93.0[b]	124.6[a]	112.5[a]	4.3	0.004
Fetal length, *cm*	1.84	1.82	1.81	0.02	0.872
Total ALF volume, *L*	0.95	1.04	0.99	0.04	0.631
Total AMF volume, *mL*	2.52[b]	4.06[a]	3.42[a]	0.16	0.001

*Values are means with pooled SEM; means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Concentrations of amino acids in allantoic and amniotic fluids

Concentrations of arginine in ALF were greater ($P < 0.05$) in conceptuses from gilts supplemented with 0.4 and 0.8% arginine, compared with control gilts (Table 3.4). No difference was detected for concentrations of arginine in AMF among the three treatment groups (Table 3.5). Total arginine in ALF (Table 3.6) and AMF (Table 3.7)

increased ($P < 0.01$) in response to dietary supplementation with 0.4% or 0.8% L-arginine. In ALF, total amounts of other amino acids were not affected by arginine supplementation; but total amounts of most amino acids in AMF were greater for conceptuses from gilts supplemented with 0.4% and 0.8% arginine (Table 3.7).

Concentrations of amino acids in placentae and endometria

Concentrations of arginine were greater ($P < 0.05$), but concentrations of alanine were lower ($P < 0.01$) in gilts supplemented with 0.8% L-arginine compared with control gilts and the gilts supplemented with 0.4% arginine (Table 3.8). Similar results were obtained for concentrations of alanine in endometrial samples (Table 3.9). Concentrations of other amino acids in placentae and endometria did not differ among the treatment groups of gilts.

Table 3.2 Concentrations of free amino acids in uterine artery of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acid	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol/L		
Asp	18[a]	17[a]	11[b]	1.1	0.018
Glu	274[a]	246[a]	172[b]	15	0.008
Arg	161[b]	313[a]	336[a]	24	0.002
Ala	1114[a]	999[a]	437[b]	105	0.013
Orn	79[b]	123[a]	119[a]	7.5	0.026
Lys	217	202	229	9.9	0.558

* Values are means and pooled SEM. n = 10; means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 3.3 Concentrations of other free amino acids, glucose, and non-esterified fatty acids (NEFA) in uterine arterial plasma of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol/L		
Asn	82	70	71	2.9	0.215
Ser	130	122	112	4.9	0.366
Gln	544	512	456	24	0.322
His	90	95	93	3.5	0.878
Gly	734	643	676	54	0.798
Thr	190	169	167	8.1	0.457
Cit	71	76	62	3.6	0.291
β-Ala	25	21	16	1.8	0.114
Tau	75	79	70	5.5	0.082
Tyr	107	95	93	3.0	0.075
Trp	62	55	56	2.6	0.531
Met	44	40	39	1.7	0.539
Val	311	283	278	11	0.424
Phe	80	71	73	2.5	0.308
Ile	124	111	113	4.6	0.499
Leu	200	186	179	6.5	0.427
Pro	252	234	247	8.4	0.663
Cys	205	236	240	16	0.623
OH-Pro	19	19	18	1.0	0.940
Glucose	4177	4444	4050	112	0.359
NEFA	146	133	142	7.7	0.777

* Values are means and pooled SEM, n = 10.

Table 3.4 Concentrations of free amino acids in allantoic fluid of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol/L			
Asp	8	8	7	0.5	0.735
Glu	64	62	47	4.3	0.209
Asn	89	84	78	4.7	0.647
Ser	526	544	461	27	0.451
Gln	699	677	597	43	0.613
His	94	89	92	6.1	0.935
Gly	460	401	414	26	0.645
Thr	206	196	204	13	0.945
Cit	10.3	10.1	9.5	0.8	0.916
Arg	104[b]	154[a]	191[a]	12	0.006
β-Ala	33	28	28	1.4	0.319
Tau	432	396	392	29	0.835
Ala	196	217	187	12	0.564
Tyr	79	74	68	3.1	0.369
Trp	12	14	13	0.8	0.842
Met	15	15	16	0.9	0.934
Val	88	86	87	5.6	0.991
Phe	34	34	37	2.8	0.911
Ile	24	23	24	1.6	0.977
Leu	50	48	47	3.7	0.966
Orn	141	136	145	7.4	0.904
Lys	299	291	321	19	0.801
Pro	248	271	234	13	0.546
Cys	42	39	44	2.7	0.802
OH-Pro	63	70	59	2.6	0.242

* Values are means and pooled SEM, n = 10. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table 3.5 Concentrations of free amino acids in amniotic fluid of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol/L		
Asp	25[a]	24[a]	16[b]	1.6	0.026
Glu	266[a]	218[a]	139[b]	15	0.001
Asn	104	98	101	3.8	0.789
Ser	513	537	499	21	0.781
Gln	1235	1121	977	48	0.093
His	68	74	78	3.7	0.481
Gly	392	446	430	17	0.442
Thr	214	231	230	11	0.778
Cit	22	21	21	0.9	0.873
Arg	118	114	120	5.5	0.910
β-Ala	27	26	26	1.2	0.882
Tau	204	249	221	12	0.255
Ala	361	381	336	13	0.404
Tyr	121	117	116	3.5	0.864
Trp	18	17	18	0.7	0.587
Met	57	55	55	1.5	0.859
Val	227	220	219	9.0	0.938
Phe	86	80	84	4.5	0.869
Ile	63	60	62	2.4	0.882
Leu	157	151	160	5.5	0.824
Orn	78	87	74	3.6	0.283
Lys	174	168	168	7.7	0.942
Pro	199	214	196	9.2	0.728
Cys	26	26	27	1.3	0.945
OH-Pro	39	44	38	1.3	0.144

* Values are means and pooled SEM, n = 10. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table 3.6 Total amounts of free amino acids in allantoic fluid of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol			
Asp	7	8	7	0.5	0.803
Glu	59	61	44	5.2	0.394
Asn	78	82	75	6.2	0.898
Ser	467	524	437	36	0.629
Gln	614	661	585	54	0.854
His	79	85	87	6.5	0.868
Gly	400	390	394	29	0.991
Thr	171	192	195	15	0.804
Cit	8.8	9.0	9.2	0.8	0.980
Arg	106[b]	168[a]	195[a]	14	0.008
β-Ala	28	27	27	1.5	0.929
Tau	351	361	372	24	0.940
Ala	195	209	189	12	0.805
Tyr	69	71	66	4.7	0.891
Trp	11	13	12	1.0	0.567
Met	13	14	15	1.1	0.815
Val	74	84	84	6.6	0.810
Phe	29	33	36	3.0	0.653
Ile	20	23	23	2.0	0.801
Leu	42	46	45	3.8	0.876
Orn	118	131	135	7.8	0.659
Lys	249	284	302	22	0.600
Pro	221	262	225	18	0.619
Cys	36	40	41	3.3	0.867
OH-Pro	62	62	60	4.6	0.974

* Values are means and pooled SEM, n = 10. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table 3.7 Total amounts of free amino acids in amniotic fluid of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol		
Asp	76	84	53	6.0	0.061
Glu	674[a]	815[a]	471[b]	50	0.007
Asn	263	346	333	20	0.192
Ser	1280[b]	2090[a]	1642[ab]	124	0.033
Gln	3294[b]	4136[a]	3227[b]	187	0.032
His	163[b]	285[a]	274[a]	20	0.026
Gly	1065[b]	1849[a]	1660[a]	136	0.050
Thr	497[b]	889[a]	846[a]	66	0.021
Cit	58	72	68	6.0	0.152
Arg	303[b]	492[a]	425[a]	28	0.010
β-Ala	68	96	87	6.9	0.245
Tau	538[b]	967[a]	715[ab]	68	0.022
Ala	1043[b]	1436[a]	1059[b]	72	0.026
Tyr	294[b]	431[a]	363[ab]	21	0.015
Trp	46[b]	60[a]	66[a]	3.1	0.016
Met	159	186	193	14	0.242
Val	533[b]	815[a]	833[a]	49	0.012
Phe	221	295	284	19	0.250
Ile	147[b]	214[a]	209[a]	12	0.035
Leu	401[b]	564[a]	521[a]	31	0.012
Orn	183[b]	342[a]	216[a]	22	0.001
Lys	445[b]	654[a]	514[ab]	38	0.049
Pro	532[c]	857[a]	664[b]	65	0.001
Cys	75[b]	126[a]	97[ab]	7.4	0.010
OH-Pro	98[b]	176[a]	136[ab]	12	0.020

* Values are means and pooled SEM, n = 10. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table 3.8 Concentrations of free amino acids in placentae of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		nmol/mg tissue			
Asp	0.46	0.31	0.36	0.030	0.126
Glu	0.82	0.70	0.69	0.120	0.596
Asn	0.20	0.14	0.17	0.011	0.094
Ser	0.57	0.48	0.46	0.048	0.432
Gln	2.44	1.90	1.85	0.124	0.596
His	0.24	0.20	0.15	0.024	0.328
Gly	0.96	0.86	0.85	0.053	0.649
Thr	0.43	0.36	0.38	0.032	0.671
Cit	0.025	0.019	0.021	0.008	0.104
Arg	0.29[b]	0.30[b]	0.36[a]	0.020	0.034
Tau	1.01	0.90	0.91	0.063	0.755
Ala	0.59[a]	0.54[a]	0.41[b]	0.026	0.001
Tyr	0.17	0.20	0.16	0.016	0.867
Trp	0.05	0.06	0.05	0.005	0.633
Met	0.08	0.08	0.07	0.006	0.438
Val	0.33	0.31	0.32	0.019	0.515
Phe	0.15	0.14	0.13	0.013	0.770
Ile	0.12	0.12	0.10	0.008	0.331
Leu	0.24	0.23	0.19	0.037	0.902
Orn	0.13	0.11	0.11	0.006	0.193
Lys	0.38	0.41	0.37	0.034	0.338

* Values are means with pooled SEM, n = 8. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table 3.9 Concentrations of free amino acids in endometria of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine between d 14 and 25 of gestation [*]

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		nmol/mg tissue			
Asp	0.74	0.59	0.64	0.117	0.720
Glu	2.05	1.80	1.83	0.075	0.370
Asn	0.064	0.069	0.063	0.004	0.588
Ser	0.29	0.32	0.23	0.018	0.076
Gln	0.86	0.80	0.87	0.048	0.804
His	0.19	0.19	0.14	0.013	0.302
Gly	1.88	1.78	1.76	0.086	0.829
Thr	0.24	0.23	0.23	0.008	0.657
Cit	0.068	0.072	0.064	0.004	0.572
Arg	0.27	0.29	0.30	0.024	0.129
Tau	2.93	2.79	2.53	0.159	0.612
Ala	1.35[a]	1.59[a]	0.74[b]	0.128	0.012
Tyr	0.16	0.14	0.15	0.008	0.538
Trp	0.026	0.024	0.024	0.002	0.187
Met	0.067	0.064	0.085	0.009	0.109
Val	0.31	0.31	0.29	0.010	0.617
Phe	0.10	0.092	0.084	0.005	0.208
Ile	0.19	0.16	0.12	0.011	0.067
Leu	0.33	0.31	0.24	0.018	0.147
Orn	0.082	0.10	0.084	0.008	0.373
Lys	0.25	0.22	0.23	0.009	0.728

[*] Values are means with pooled SEM, n = 8; Means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table 3.10 Concentrations of hormones in uterine artery and total amounts of the hormones in allantoic fluid in gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Hormones	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Uterine vein					
Progesterone, *ng/ml*	16.3	17.9	17.9	1.14	0.817
Estradiol, *pg/ml*	102.2	108.5	86.9	8.08	0.550
Estrone, *pg/ml*	126.7	112.5	89.4	7.70	0.142
Estrone sulfate, *ng/ml*	9.22	9.07	6.19	0.74	0.174
Allantoic fluid					
Progesterone, *μg*	1.13	1.22	1.14	0.10	0.923
Estradiol, *ng*	670.5	630.8	607.8	42.7	0.842
Estrone, *ng*	614.6	590.1	581.7	58.2	0.974
Estrone sulfate, *μg*	60.6	58.7	63.7	4.57	0.909

*Values are means with pooled SEM, n = 14 gilts.

Hormones in maternal plasma and ALF

Progesterone was more abundant ($P < 0.01$) than estradiol, estrone and estrone sulfate in maternal plasma (Table 3.10). In contrast, concentrations of estrone sulfate in ALF were approximately 50-fold greater ($P < 0.01$) than concentrations of progesterone. Concentrations and total amounts of progesterone, estradiol, estrone, and estrone sulfate in maternal uterine vein and ALF did not differ among the three treatment groups of gilts (Table 3.10).

Fructose, glucose, and free fatty acids in ALF and AMF

Concentrations of fructose in ALF were similar to those in AMF (Table 3.11). Concentrations of fructose, glucose, and free fatty acids in ALF and AMF did not differ among the treatment groups of gilts (Table 3.11). Likewise, dietary supplementation

Table 3.11 Concentrations and total amounts of fructose, glucose, and non-esterified fatty acids (NEFA) in fetal fluids of gilts fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*-Value
Concentration in ALF					
Fructose, *mg/mL*	1.13	1.13	1.09	0.04	0.884
Glucose, *μmol/L*	1961	2076	1707	93	0.254
NEFA, *μmol/L*	39	38	37	0.5	0.262
Concentration in AMF					
Fructose, *mg/mL*	0.76	0.88	0.82	0.03	0.277
Glucose, *μmol/L*	644	664	731	49	0.803
NEFA, *μmol/L*	35	36	41	1.3	0.092
Total amount in ALF					
Fructose, *mg*	1045	1178	1099	63	0.682
Glucose, *μmol*	1863	2159	1690	96	0.129
NEFA, *μmol*	37^{b}	40^{a}	36^{b}	0.6	0.018
Total amount in AMF					
Fructose, *mg*	1.93^{b}	3.38^{a}	2.84^{a}	0.18	0.001
Glucose, *μmol*	1.62^{b}	2.70^{a}	2.50^{a}	0.19	0.034
NEFA, *μmol*	0.09^{b}	0.15^{a}	0.14^{a}	0.01	0.001

*Values are means with pooled SEM, n = 10 gilts; means in a row with superscripts without a common letter differ, $P \leq 0.05$.

with arginine did not affect total amounts of fructose in ALF (Table 3.11). However, total amounts of fructose, glucose and free fatty acids were greater ($P < 0.05$) in AMF of gilts receiving dietary supplementation with 0.4% or 0.8% L-arginine, compared with the control group. Neither concentrations nor total amounts of fructose, glucose, and free fatty acids in ALF and AMF differed between the 0.4% and 0.8% L-arginine groups, with exception for free fatty acids which have a higher total amount in ALF with 0.4%

L-arginine supplementation with compared to control and 0.8% L-arginine group (Table 3.11).

Discussion

Embryonic loss is a major problem in pig reproduction (Pope 1994). Unfortunately, there are few effective ways to reduce such a high loss of embryos in gestating swine (Johnson 2000). Interestingly, dietary supplementation with 1.0% arginine-HCl between d 30 and 114 of gestation increased the number of live-born piglets per litter by 2 (Mateo et al. 2007). Although there are two critical windows for fetal death after d 30 of gestation, more than 75% of prenatal loss occurs during the first 25 d of gestation (Pope 1994). Threrefore dietary L-arginine supplementation may have positive effects on fertility when it is initiated during early pregnancy because it is the most critical window of opportunity to control embryonic mortality in pigs. However, results of our previous study (Chapter II) indicated that the number of live fetuses, CL number, and concentrations of progesterone in maternal plasma were decreased by supplementing 0.8% L-arginine to the diet of gilts between d 0 and 25 of gestation. In contrast, results of this study with arginine supplementation beginning on d 14 of gestation indicated that dietary supplementation with 0.4% or 0.8% L-arginine did not affect number of CL, but did increase the number of live fetuses and decrease embryonic mortality at d 25 of gestation as compared to control gilts (Table 3.1). The only difference in experimental design between the present and previous study as the day when L-arginine supplementation was initiated (d 14 in the present study vs d 0 in the previous study). A decrease in CL number in response to L-arginine supplementation immediately after

breeding may have resulted from the interference with growth and ovulation of ovarian follicles and formation of CL (Smith et al. 1994). Maternal recognition of pregnancy starts on d 11 of pregnancy in swine when blastocysts begin their dramatic morphological changes (Bazer and Thatcher 1977). Supplementing the diet with 0.8% L-arginine before d 14 of gestation may impede this process, resulting in decreased embryonic survival. However, when dietary L-arginine supplementation starts at d 14 of gestation, CL formation and maternal recognition of pregnancy are not affected by L-arginine supplementation.

Ramaekers et al. (2006) reported that dietary supplementation with 1% arginine from d 14 to 28 increased the number of live-born piglets at birth by approximately 1 per sow. The results from the present study indicate that enhanced embryonic survival before d 25 of gestation is a major factor contributing to increased litter size at term. It is known that some fetal loss can occur between d 25 of gestation and parturition in gilts and sows with large litters early in gestation (Wu et al. 2006). Daily supplementation with arginine beyond d 25 of gestation until term will help prevent fetal loss and increase litter size to a greater extent (approximately one more live-born piglet per litter), when compared with dietary arginine supplementation between d 14 and 25 of gestation. Enhanced growth (Table 3.1) and vascularization of the placenta (Chapter II) likely promotes embryonic survival in arginine-supplemented gilts and positively impacts fetal survival and growth in subsequent stages of gestation. Note that total fetal weight on d 25 of gestation was not affected by arginine, likely because the embryos receive nutrients primarily from uterine secretions during early pregnancy (Bazer et al. 2010).

Another important finding from this study is that CL number was not affected by dietary supplementation with arginine between d 14 and 25 of gestation. Similarly, no difference was detected in concentrations of progesterone in maternal plasma among the three groups of gilts. The CL are the only source of progesterone in pigs and it is essential for normal pregnancy (Bazer et al. 2010). Estrogen, another important hormone in pregnancy, was not affected by L-arginine supplementation. Thus, an increase in embryonic survival in arginine-supplemented gilts is likely mediated by factors other than progesterone and estrogen. However, based on results of the previous study (Li et al. 2010), it is clear that adequate amounts of progesterone are necessary for arginine to enhance embryonic survival during early gestation.

The total volume of AMF increased in gilts receiving diets supplemented with 0.4% or 0.8% L-arginine, compared with the control group. The mechanisms responsible for enhanced transport of water, arginine, most other amino acids, sugars, and ions across placentae and the amniotic membrane are unclear. Results of this study suggest an important role for arginine in regulating these physiological processes. Total amounts of fructose in AMF also increased in the 0.4% or 0.8% L-arginine groups as compared to the control group. It is possible that arginine stimulates the conversion of glucose to fructose in placentae and subsequent transport of fructose across the fetal side of the placenta into the amniotic fluid. This observation is novel and important, as fructose is now known to be a substrate for the synthesis of glycoproteins in porcine placentae (Appendix Table A-2) and to stimulate MTOR cell signaling in porcine trophectoderm cells (Bazer 2011b). Fructose does not move from the fetal system to maternal

circulation (Alexander et al. 1955) and an increase in availability of this sugar in conceptuses of arginine-supplemented gilts warrants further investigation.

Consistent with the previous study (Chapter II), concentrations of aspartate, glutamate, and alanine were lower, but concentrations of arginine and ornithine were higher in plasma of gilts supplemented with 0.8% L-arginine compared with control gilts. Moreover, gilts in the 0.4% L-arginine group had higher circulating levels of arginine than control gilts. It is possible that rates of synthesis of aspartate and glutamate were greater, or rates of degradation of these amino acids were lower in the whole body of control gilts (receiving isonitrogenous amounts of alanine) than in gilts supplemented with 0.8% arginine. The findings that embryonic survival was greater in gilts supplemented with 0.4% arginine than control gilts even though concentrations of aspartate or glutamate in maternal plasma did not differ between the control and 0.4% arginine groups indicate that modest changes in aspartate and glutamate availability did not affect litter size in gilts. Thus, dietary supplementation with 0.4% L-arginine is sufficient to enhance reproductive performance in gilts as is 0.8% L-arginine. This new knowledge is very important for developing a cost-effective strategy to enhance swine production worldwide.

In summary, supplementing the diet of gilts with 0.4% or 0.8% L-arginine between d 14 and 25 of gestation increased concentrations of arginine in maternal plasma, total amounts of arginine in ALF and AMF, and the number of live fetuses per litter by 2 on d 25 as compared to control gilts. Arginine supplementation also increased the volume of AMF as well as total amounts of fructose and most amino acids in AMF possibly due to

enhanced transport of ions, water, sugar and amino acids across placentae and into the amniotic fluid. These findings will aid in developing cost-effective strategies to enhance litter size in swine and also have important implications for improving embryonic survival in other mammals.

CHAPTER IV

EFFECTS OF DIETARY SUPPLEMENTATION WITH L-ARGININE BETWEEN DAYS 14 AND 25 OF GESTATION ON REPRODUCTIVE PERFORMANCE OF GILTS AT DAY 60 OF GESTATION

This experiment was conducted to determine the effects of dietary L-arginine supplementation between d 14 and 25 of pregnancy on reproductive performance of gilts at d 60 of gestation. Sixty gilts were assigned randomly into three treatment groups (0.0, 0.4, and 0.8% L-arginine) after breeding during their second period of estrus. Gilts were fed daily 2 kg of a corn- and soybean meal-based diets supplemented with 0.0% (control), 0.4%, or 0.8% L-arginine between d 14 and 25 of gestation. Then gilts were returned to the 2 kg/d basal diet between d 26 and 59 of gestation without arginine supplementation. At d 60 of gestation, gilts were hysterectomized to obtain uteri and conceptuses. The results from all gilts with corpus luteum (CL) numbers ranging from 9 to 14 indicated no differences among the three treatment groups of gilts: (1) concentrations of arginine in maternal plasma; (2) concentrations and total amounts of amino acids in allantoic and amniotic fluids; (3) maternal weight gains and uterine weight; (4) total number of fetuses, number of live fetuses, and number of CL; (5) rate of embryonic mortality; (6) total fetal and placental weights; and (7) volumes of allantoic and amniotic fluids. However, analysis of data from gilts with CL numbers ranging from 15 to 18 revealed that dietary supplementation with 0.4% or 0.8% L-arginine increased

the total number of fetuses and the number of live fetuses, rate of embryonic survival, and volumes of allantoic and amniotic fluids compared with the control group. Thus, arginine supplementation can increase litter size in gilts which have relatively high ovulation rates and adequate uterine capacity on d 60 of gestation.

Introduction

Dietary supplementation with arginine is a new strategy to decrease prenatal embryonic or fetal losses in pigs. However, the timing of arginine supplementation is critical for it to be effective. The number of live-born fetuses increased significantly in gilts fed a diet supplemented with 0.83% arginine between d 30 and 114 of gestation (Mateo et al. 2007). However, the numbers of total and live embryos were decreased by dietary supplementation 0.8% L-arginine between d 0 and 25 of gestation (See Chapter II). Interestingly, when initiated on d 14 of gestation, L-arginine supplementation increased the number of live fetuses per litter and embryonic survival rate on d 25 of gestation, compared with the control group (see Chapter III).

Although 70% of embryonic and fetal deaths occur before d 25 of gestation, another 20% of fetal deaths may occur between d 40 to 60 of gestation (Pope 1994). Starting at d 30 of pregnancy, placental capacity to support fetal development becomes a limiting factor for fetal survival and growth (Wilson 2002). An increase in ovulation rate can increase the number of embryos that survive during early pregnancy, but it is not proportional to the number of live-born piglets (Freking et al. 2007; van der Waaij et al. 2010). The embryos are either dead or suffer from intra-uterine growth retardation if they cannot obtain enough nutrients from dams due to limited uterine capacity. High

numbers of embryos in early pregnancy have a negative effect on the survival and development of fetuses during mid- and late-pregnancy (Town et al. 2004). This suggests that the increase in number of embryos at d 25 of gestation in gilts receiving dietary L-arginine supplementation between d 14 and 25 of gestation (Chapter III) may not be maintained to a later stage of pregnancy or term. Likewise, it is unknown whether arginine supplementation during early gestation may have a programming effect on placentae or fetuses to support conceptus survival and growth at advanced stages of pregnancy.

This study was conducted to determine the effects of dietary arginine supplementation between d 14 and 25 of pregnancy on reproductive performance of gilts at d 60 of gestation.

Materials and methods

Animals and diets

The experimental design was similar with that described for the study in Chapter III, except that gilts received no arginine supplementation after d 25 of gestation. Briefly, 60 gilts were assigned randomly to one of three treatment groups (0.0, 0.4, and 0.8% L-arginine) after breeding during their second period of estrus. Throughout the trial, gilts were fed twice daily (0700 and 1800 h) 1 kg of a corn and soybean meal-based diet (2 kg/d). Between d 14 and 25 of gestation, gilts were fed the basal diet supplemented with 0.0, 0.4, and 0.8% L-arginine. After d 25 of gestation, arginine supplementation was terminated. At d 60 of gestation, gilts were hysterectomized to obtain uteri and conceptuses after obtaining 10 mL of uterine venous and arterial blood samples for

amino acid assays. Uterine weight, CL number, total number of fetuses, total number of viable fetuses, fetal weights and lengths, placental weights, and volumes of ALF and AMF were measured and recorded. The ALF (10 mL) and AMF (10 mL) from each fetus were analyzed for amino acids.

Analysis of amino acids

Amino acids in uterine arterial samples as well as ALF and AMF were analyzed by HPLC methods as described in Chapter II.

Statistical analysis

Data were analyzed using the General Linear Model procedures of SPSS [Statistical Package for the Social Sciences] (Version 15.0, Chicago, IL) for a randomized complete block design. Gilt was considered as the experimental unit. Differences among treatment means were determined using Duncan's multiple comparison test. Data on embryonic survival were analyzed using the Chi-Square test of SPSS. Probability values < 0.05 were considered statistically significant.

Results

Reproductive performance of gilts on d 60 of gestation

Two gilts from the control group, three gilts from the 0.4% L-arginine groups, and two gilts from the 0.8% L-arginine group were not pregnant at the time of hysterectomy. The number of CL for all gilts was 14.2 ± 0.36 (mean ± SEM, n = 53) with a range from 9 to 18. Thus, we considered CL number of 9 to 14 (12.4 ± 0.33; mean ± SEM, n = 31) and 15 to 18 (16.0 ± 0.20; mean ± SEM, n = 22) to be below and above average, respectively. On d 60 of gestation, there were no differences among the three treatme-

Table 4.1 Reproductive performance of gilts with 9 to 14 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Number of gilts, *n*	11	10	10		
BW at breeding, *kg*	117	119	121	2.36	0.755
BW at d 60 of gestation, *kg*	128	129	138	3.58	0.502
BW gain, *kg/60 d*	11	10	16	1.79	0.344
Uterine weight, *kg*	10.1	10.8	9.7	0.48	0.726
Total no. fetuses, *n*	11.3	11.1	10.7	0.29	0.700
Live fetuses, *n*	11.3	10.5	10.6	0.26	0.423
No. CL	12.6	12.3	12.4	0.31	0.911
Embryonic mortality, %	9.9	13.1	14.5	1.79	0.593
Total viable fetal weight, *kg*	1.30	1.25	1.28	0.04	0.864
Total placental weight, *kg*	1.98	1.61	1.78	0.12	0.473
Fetal Length, *cm*	13.4	13.4	13.7	0.11	0.562
Total ALF volume, *L*	2.30	2.49	2.81	0.32	0.819
Total AMF volume, *L*	1.45	1.30	1.35	0.06	0.562

*Values are means with pooled SEM.

nt groups of gilts with 9 to 14 CL in any measured variable of reproductive performance, including body weight gain, uterine weight, number of total and number of live fetuses, CL number, rate of embryonic mortality, total fetal and placental weights, and volumes of ALF and AMF (Table 4.1). However, the analysis of data from gilts with 15 to 18 CL revealed a different picture regarding effects of arginine supplementation on reproductive performance. Specifically, for gilts with 15 to and 18 CL, dietary supplementation with 0.4% or 0.8% L-arginine increased ($P < 0.05$) the number of total fetuses and number of live fetuses, rate of embryonic survival, and ALF and AMF

Table 4.2 Reproductive performances of gilts with 15 to 18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Number of gilts, *n*	7	7	8		
BW at breeding, *kg*	121	120	119	2.46	0.937
BW at d 60 of gestation, *kg*	145	142	135	3.09	0.381
BW gain, *kg/60 d*	24	22	16	1.86	0.181
Uterine weight, *kg*	10.1	11.1	11.3	0.54	0.612
Total no. fetuses, *n*	11.4[b]	13.0[a]	13.6[a]	0.34	0.018
Live fetuses, *n*	10.6[b]	12.3[a]	12.5[a]	0.29	0.007
No. CL	15.9	16.0	16.0	0.20	0.952
Embryonic mortality, %	33.2[a]	23.0[b]	22.7[b]	2.20	0.031
Total viable fetal weight, *kg*	1.22	1.42	1.39	0.05	0.177
Total placental weight, *kg*	1.80	1.80	1.86	0.11	0.973
Fetal Length, *cm*	13.0	13.1	13.1	0.14	0.988
Total ALF volume, *L*	2.36[b]	3.93[a]	3.95[a]	0.34	0.013
Total AMF volume, *L*	1.17[b]	1.53[a]	1.80[a]	0.08	0.002

*Values are means with pooled SEM. Means in a row with superscripts without a common letter differ, $P < 0.05$.

volumes, compared with the control group (Table 4.2). The ratio of fetal to placental weights did not differ among the three groups of gilts (0.111 to 0.115). Likewise, dietary supplementation with 0.4% and 0.8% arginine did not affect the weights of fetal organs, including brain, heart, kidney, leg muscle, liver, lung, Intestine, spleen, and stomach, regardless of CL numbers (Appendix Table A-3).

Table 4.3 Concentrations of free amino acids, glucose, and non-esterified fatty acids (NEFA) in uterine artery of gilts with 9 to 14 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol/L			
Asp	12	12	11	1.1	0.906
Glu	166	169	173	8.2	0.950
Asn	37	45	37	2.5	0.249
Ser	97	113	120	4.3	0.099
Gln	550	610	584	19	0.454
His	95	99	95	6.1	0.959
Gly	829	929	931	38	0.500
Thr	141	143	117	9.9	0.551
Cit	52	62	58	2.8	0.307
Arg	147	154	150	5.9	0.894
β-Ala	28	32	25	3.0	0.693
Tau	59	55	54	2.3	0.739
Ala	405	462	502	24	0.301
Tyr	80	90	72	3.8	0.173
Trp	54	59	68	4.6	0.495
Met	39	39	47	1.8	0.184
Val	217	200	213	6.4	0.547
Phe	57	58	60	2.6	0.937
Ile	85	78	80	2.9	0.655
Leu	181	176	175	3.9	0.831
Orn	57	78	84	5.3	0.079
Lys	161	185	148	11	0.365
Glucose	4476	5020	4480	205	0.480
NEFA	170	162	181	26	0.962

* Values are means and pooled SEM; n = 11 (Control) or n = 10 gilts (0.4% and 0.8% L-Arg groups).

Table 4.4 Concentrations of free amino acids, glucose, and non-esterified fatty acids (NEFA) in uterine artery of gilts with 15 to 18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol/L			
Asp	18	14	12	1.1	0.147
Glu	197	163	157	8.7	0.143
Asn	46[a]	43[a]	35[b]	1.8	0.049
Ser	114	129	114	7.8	0.703
Gln	503	423	503	34	0.587
His	70	63	83	3.6	0.089
Gly	892	972	841	63	0.734
Thr	104	101	138	8.4	0.183
Cit	72	86	63	6.4	0.370
Arg	126	138	149	6.4	0.447
β-Ala	19	16	15	1.7	0.675
Tau	55	67	63	3.4	0.385
Ala	490	486	477	11	0.931
Tyr	84	70	75	3.1	0.170
Trp	54	58	49	2.4	0.441
Met	44	45	39	3.4	0.821
Val	174[b]	181[b]	211[a]	5.8	0.024
Phe	59	57	51	1.8	0.323
Ile	71	75	89	3.5	0.121
Leu	161	159	166	2.9	0.679
Orn	92	93	73	4.4	0.153
Lys	88	106	103	8.4	0.084
Glucose	4212	4260	4653	226	0.730
NEFA	84	91	117	8.1	0.195

* Values are means and pooled SEM; n = 7 (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Concentrations of amino acids, glucose, and fatty acids in maternal uterine plasma on d 60 of gestation

Concentrations of all measured amino acids, glucose, and fatty acids did not differ on d 60 of gestation among the treatment groups for gilts with 9 to 14 CL (Table 4.3). Similar results were obtained for gilts with 15 to 18 CL, except that concentrations of asparagine were lower ($P < 0.05$) and concentrations of valine were higher ($P < 0.05$) in gilts supplemented with 0.8% arginine, compared with the control group (Table 4.4).

Concentrations and total amounts of amino acids in ALF on d 60 of gestation

For gilts with 9 to 14 CL, concentrations of glutamine, phenylalanine, and serine were higher ($P < 0.05$) in ALF in response to supplementation with 0.8% arginine, compared with the control and 0.4% arginine groups (Table 4.5). In these gilts, concentrations of other amino acids in ALF did not differ among the three treatment groups of gilts. Dietary supplementation with 0.4% or 0.8% arginine did not affect concentration of any amino acid in ALF of gilts with 15 to 18 CL (Table 4.6).

For gilts with 9 to 14 CL, total amounts of glutamine, phenylalanine, and serine in ALF were higher ($P < 0.05$) in response to supplementation with 0.8% arginine, compared with the control and 0.4% arginine groups (Table 4.7). Total amounts of glycine, taurine and tryptophan in ALF were greater ($P < 0.05$) in gilts supplemented with 0.8% arginine as compared to the control group. Total amounts of other amino acids in ALF did not differ due to treatment. In contrast, for gilts with 15 to 18 CL, total amounts of all measured amino acids, except for threonine, β-alanine, methionine,

Table 4.5 Concentrations of free amino acids in ALF of gilts with 9 to14 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol/L		
Asp	44	49	40	3.6	0.583
Glu	1207	1080	1312	124	0.751
Asn	36	56	46	4.0	0.121
Ser	61[b]	68[b]	91[a]	4.0	0.006
Gln	288[b]	380[b]	528[a]	28	0.002
His	48	52	57	4.0	0.719
Gly	261	375	345	25	0.173
Thr	94	125	121	9.1	0.349
Cit	30	36	36	3.5	0.797
Arg	589	645	633	45	0.891
β-Ala	115	130	115	9.4	0.768
Tau	328	270	362	22	0.197
Ala	89	104	78	7.6	0.359
Tyr	19	20	12	2.3	0.380
Trp	19	24	22	1.2	0.217
Met	5.6	8.7	4.3	0.9	0.110
Val	32	35	26	2.5	0.332
Phe	8.3[b]	11[b]	17[a]	1.2	0.022
Ile	29	30	30	1.3	0.941
Leu	63	74	45	5.3	0.079
Orn	811	1023	877	64	0.379
Lys	591	625	612	19	0.779

* Values are means and pooled SEM; n = 11 (Control) or n = 10 gilts (0.4% and 0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 4.6 Concentrations of free amino acids in ALF of gilts with 15 to 18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol/L			
Asp	54	66	55	6.6	0.722
Glu	608	556	757	55	0.408
Asn	37	49	58	4.4	0.172
Ser	75	83	81	4.5	0.753
Gln	303	345	373	21	0.427
His	46	52	41	3.9	0.561
Gly	314	261	299	19	0.510
Thr	122	113	123	12	0.940
Cit	47	40	46	5.0	0.828
Arg	487	437	514	43	0.782
β-Ala	97	66	90	8.5	0.266
Tau	241	264	337	25	0.347
Ala	110	96	115	9.4	0.733
Tyr	20	19	25	2.2	0.522
Trp	14	22	18	1.8	0.196
Met	18	14	11	1.9	0.398
Val	40	49	50	4.9	0.687
Phe	19	25	20	3.0	0.685
Ile	36	36	35	1.7	0.982
Leu	46	41	33	3.1	0.333
Orn	603	646	669	62	0.926
Lys	480	385	342	33	0.278

* Values are means and pooled SEM; n = 7 (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg groups).

Table 4.7 Total amounts of free amino acids in ALF of gilts with 9 to 14 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol			
Asp	102	122	112	9.1	0.696
Glu	2776	2690	3687	330	0.408
Asn	82	138	130	10.4	0.060
Ser	140[b]	169[b]	255[a]	13	0.001
Gln	661[b]	945[b]	1484[a]	84	0.001
His	111	129	160	11	0.199
Gly	600[b]	934[a]	969[a]	84	0.050
Thr	216	310	339	24	0.103
Cit	70	89	101	8.8	0.423
Arg	1355	1605	1778	119	0.424
β-Ala	265	323	323	24	0.559
Tau	754[ab]	672[b]	1016[a]	59	0.040
Ala	204	259	220	18	0.432
Tyr	44	49	35	5.4	0.562
Trp	43[b]	59[a]	62[a]	3.4	0.044
Met	13	22	12	2.2	0.125
Val	73	88	73	6.1	0.521
Phe	19[b]	27[b]	48[a]	3.5	0.003
Ile	67	75	84	3.4	0.177
Leu	157	183	127	12	0.155
Orn	1864	2548	2465	161	0.178
Lys	1360[b]	1556[ab]	1720[a]	54	0.035

* Values are means and pooled SEM; n = 11 (Control) or n = 10 gilts (0.4% and 0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 4.8 Total amounts of free amino acids in ALF of gilts with 15 to18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acids	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol			
Asp	113[b]	258[a]	216[a]	26	0.035
Glu	1562[b]	2318[a]	2685[a]	150	0.002
Asn	87[b]	193[a]	228[a]	20	0.004
Ser	177[b]	328[a]	318[a]	24	0.004
Gln	715[b]	1356[a]	1473[a]	111	0.004
His	109[b]	206[a]	177[a]	16	0.039
Gly	742[b]	1026[a]	1182[a]	70	0.046
Thr	287	371	392	25	0.186
Cit	98[b]	180[a]	185[a]	17	0.038
Arg	1150[b]	1900[a]	2029[a]	174	0.016
β-Ala	229	259	356	30	0.283
Tau	568[c]	898[b]	1332[a]	90	0.001
Ala	258[b]	377[a]	389[a]	24	0.031
Tyr	39[b]	67[a]	88[a]	6.6	0.006
Trp	30[b]	86[a]	72[a]	9.2	0.010
Met	43	56	44	5.7	0.587
Val	102[b]	191[a]	199[a]	17	0.026
Phe	44[b]	98[a]	81[a]	5.5	0.032
Ile	84[b]	140[a]	137[a]	8.5	0.002
Leu	116	159	130	8.4	0.071
Orn	1423[b]	2333[a]	2069[a]	131	0.003
Lys	1132	1512	1349	89	0.144

* Values are means and pooled SEM; n = 7 (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

phenylalanine, leucine, and lysine, were greater ($P < 0.05$) in the 0.4% and 0.8% arginine groups, compared with the control group (Table 4.8). In gilts with above average number of CL, total amounts of all amino acids in ALF except for taurine did not differ between the 0.4% and 0.8% arginine groups.

Concentrations and total amounts of amino acids in AMF on d 60 of gestation

For gilts with 9 to 14 CL, concentrations of threonine and citrulline in AMF were higher ($P < 0.05$) in the 0.8% arginine than the control group, but total amounts of other amino acids did not differ due to treatment (Table 4.11). Compared with the control group, dietary supplementation with 0.8% arginine increased ($P < 0.05$) concentrations of glutamate, glycine, arginine, and taurine in AMF, but had no effect on concentrations of other amino acids (Table 4.10).

For gilts with 9 to 14 CL, total amounts of threonine and citrulline in AMF were greater ($P < 0.05$) in response to supplementation with 0.8% arginine, compared with the control group, but total amounts of other amino acids in AMF were not affected by dietary supplementation with 0.4% or 0.8% arginine (Table 4.11). For gilts with 15 to 18 CL, total amounts of all amino acids, except for methionine, were greater ($P < 0.05$) in AMF in the 0.8% arginine than in the control group (Table 4.12). In these gilts, total amounts of asparagine, glutamine, glycine, threonine, arginine, β-alanine, taurine, alanine, tryptophan, phenylalanine, leucine, and lysine in AMF were greater ($P < 0.05$) in the 0.8% arginine than the 0.4% arginine group, but total amounts of other amino acids did not differ between the two groups. Compared with the control group, dietary supplementation with 0.4% arginine increased ($P < 0.05$) total amounts of glutamate,

serine, glycine, citrulline, arginine, β-alanine, taurine, alanine, valine, leucine, ornithine, and lysine, but had no effect on total amounts of other amino acids (Table 4.12).

Table 4.9 Concentrations of free amino acids in AMF of gilts with 9 to 14 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acid	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol/L		
Asp	14	13	11	1.1	0.626
Glu	364	371	352	24	0.947
Asn	36	32	33	1.5	0.666
Ser	128	150	146	8.6	0.556
Gln	976	966	1051	52	0.792
His	47	42	44	3.3	0.830
Gly	309	359	386	14	0.109
Thr	59[b]	61[b]	86[a]	4.1	0.024
Cit	33[b]	51[a]	54[a]	3.1	0.014
Arg	111	121	124	3.7	0.374
β-Ala	11	15	9.3	1.1	0.068
Tau	78	65	87	4.9	0.196
Ala	644	756	744	25	0.136
Tyr	29	35	22	3.5	0.276
Trp	9	8.7	7.8	1.0	0.885
Met	29	28	26	1.2	0.630
Val	89	113	94	5.7	0.162
Phe	13	14	11	0.9	0.524
Ile	19	29	21	2.8	0.266
Leu	75	78	56	5.9	0.303
Orn	46	51	50	2.6	0.693
Lys	227	227	212	8.8	0.750

* Values are means and pooled SEM; n = 11 (Control) or n = 10 gilts (0.4% and 0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 4.10 Concentrations of free amino acids in AMF of gilts with 15-18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acid	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol/L			
Asp	11	11	15	1.2	0.426
Glu	235[b]	320[a]	355[a]	19	0.019
Asn	38	33	38	1.8	0.454
Ser	121	148	144	6.9	0.205
Gln	943	849	1087	43	0.086
His	37	37	35	1.9	0.933
Gly	279[b]	309[b]	428[a]	19	0.001
Thr	81[ab]	63[b]	98[a]	5.4	0.034
Cit	41[b]	56[a]	50[ab]	2.7	0.035
Arg	103[b]	114[ab]	143[a]	6.5	0.042
β-Ala	11	14	14	1.1	0.554
Tau	56[b]	78[a]	84[a]	4.3	0.015
Ala	714	681	754	25	0.558
Tyr	30	28	36	2.7	0.493
Trp	10	7.7	13	0.9	0.151
Met	37	43	39	5.4	0.884
Val	100	128	106	7.7	0.279
Phe	12	9.9	13	0.8	0.316
Ile	24	23	34	2.9	0.292
Leu	53	50	54	1.6	0.492
Orn	58	54	46	2.2	0.110
Lys	221	223	235	7.7	0.789

* Values are means and pooled SEM. n = 7 (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 4.11 Total amounts of free amino acids in AMF of gilts with 9 to 14 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acid	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			µmol		
Asp	19	17	15	1.5	0.509
Glu	520	482	475	32	0.857
Asn	51	42	44	2.2	0.246
Ser	183	195	197	12	0.879
Gln	1395	1256	1418	71	0.592
His	67	54	59	4.5	0.530
Gly	442	467	520	18	0.257
Thr	84[b]	79[b]	116[a]	5.5	0.026
Cit	48[b]	63[a]	67[a]	3.0	0.014
Arg	159	158	167	4.8	0.730
β-Ala	16	20	12	1.4	0.113
Tau	111	85	117	6.9	0.105
Ala	921	983	1004	32	0.581
Tyr	41	46	30	4.6	0.346
Trp	13	11	10	1.3	0.777
Met	42	36	35	1.7	0.275
Val	127	147	127	7.2	0.442
Phe	18	18	15	1.2	0.553
Ile	27	38	28	3.6	0.397
Leu	108	101	76	8.0	0.290
Orn	66	67	67	3.5	0.987
Lys	324	295	286	12	0.470

* Values are means and pooled SEM; n = 11 (Control) or n = 10 gilts (0.4% and 0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 4.12 Total amounts of free amino acids in AMF of gilts with 15 to 18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Amino Acid	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
		µmol			
Asp	13[b]	17[b]	27[a]	2.0	0.022
Glu	300[b]	533[a]	639[a]	45	0.002
Asn	45[b]	51[b]	68[a]	3.3	0.010
Ser	142[b]	227[a]	259[a]	15	0.001
Gln	1103[b]	1298[b]	1957[a]	100	0.001
His	43[b]	57[ab]	64[a]	3.4	0.045
Gly	326[c]	473[b]	771[a]	46	0.001
Thr	94[b]	96[b]	176[a]	11	0.001
Cit	47[b]	86[a]	91[a]	5.9	0.001
Arg	121[c]	175[b]	257[a]	15	0.001
β-Ala	13[b]	23[a]	28[a]	1.7	0.001
Tau	66[c]	119[b]	150[a]	9.3	0.001
Ala	835[c]	1042[b]	1356[a]	59	0.001
Tyr	35[b]	43[ab]	65[a]	5.4	0.048
Trp	12[b]	12[b]	23[a]	1.7	0.014
Met	43	66	71	8.6	0.393
Val	117[b]	196[a]	191[a]	14	0.017
Phe	15[b]	15[b]	23[a]	1.2	0.042
Ile	28[b]	35[b]	61[a]	5.7	0.045
Leu	62[c]	76[b]	97[a]	3.8	0.001
Orn	68[b]	83[a]	84[a]	3.1	0.010
Lys	259[c]	342[b]	423[a]	19	0.001

*Values are means and pooled SEM; n = 7 (Control and 0.4% L-Arg groups) or n = 8 gilts (0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 4.13 Concentrations and total amounts of fructose, glucose, and non-esterified fatty acids (NEFA) in fetal fluid of gilts with 9 to 14 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) from d 14 through d 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Concentration in ALF					
Fructose, *mg/mL*	1.07	0.83	0.97	0.06	0.216
Glucose, *µmol/L*	310	286	298	34	0.961
NEFA, *µmol*	9.9	12	8.9	1.51	0.811
Concentration in AMF					
Fructose, *mg/mL*	1.17	1.37	1.47	0.07	0.207
Glucose, *µmol/L*	1365	1522	1290	81	0.493
NEFA, *µmol*	15	15	12	1.47	0.608
Total amount in ALF					
Fructose, *mg*	2.44	2.06	2.73	0.14	0.154
Glucose, *µmol*	769	787	837	93	0.965
NEFA, *µmol*	23	29	25	3.76	0.833
Total amount in AMF					
Fructose, *mg*	1.70	1.78	1.99	0.09	0.446
Glucose, *µmol*	1980	1979	1741	110	0.611
NEFA, *µmol*	22	20	16	2.04	0.492

* Values are means and pooled SEM; n = 11 (Control) or n = 10 (0.4% and 0.8% L-Arg groups).

Table 4.14 Concentrations and total amounts of fructose, glucose, and non-esterified fatty acids (NEFA) in fetal fluid of gilts with 15 to 18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) from d 14 through d 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Concentration in ALF					
Fructose, *mg/mL*	1.05	1.53	1.24	0.11	0.194
Glucose, *μmol/L*	279	222	213	24	0.535
NEFA, *μmol*	7.0	5.1	6.8	0.85	0.743
Concentration in AMF					
Fructose, *mg/mL*	1.69[a]	1.60[a]	1.26[b]	0.06	0.011
Glucose, *μmol/L*	1120	1245	1372	61	0.249
NEFA, *μmol*	12	10	8.1	0.92	0.237
Total amount in ALF					
Fructose, *mg*	2.48[b]	5.20[a]	4.89[a]	0.44	0.016
Glucose, *μmol*	659[b]	873[a]	841[a]	33	0.009
NEFA, *μmol*	15[b]	20[b]	29[a]	2.13	0.014
Total amount in AMF					
Fructose, *mg*	1.98	2.44	2.26	0.08	0.064
Glucose, *μmol*	1311[c]	1905[b]	2470[a]	134	0.001
NEFA, *μmol*	14	15	15	1.28	0.900

*Values are means and pooled SEM; n = 7 (Control and 0.4% L-Arg groups) or n = 8 (0.8% L-Arg groups). Means in a row with superscripts without a common letter differ, $P < 0.05$.

Fructose, glucose, and free fatty acids in ALF and AMF on day 60 of gestation

The concentration and total amounts of fructose, glucose, and free fatty acids were not different among three treatment groups in gilts with 9 to 14 CL (Table 4.13). However, total amounts of fructose and glucose in ALF were greater in 0.4% L-arginine group or 0.8% L-arginine group with compared to control group in gilts with 15 to 18 CL (Table

4.14). Total amounts of free fatty acids in ALF and total amounts of glucose in AMF were higher in the 0.8% L-arginine group compared with control group, but not different from the 0.4% L-arginine group in gilts with 15 to 18 CL (Table 4.14).

Discussion

The number of live fetuses and the rate of embryonic survival at d 25 of gestation were enhanced by 0.4% or 0.8% L-arginine supplementation between d 14 and 25 of gestation (Chapter III). This benefit of improved embryonic/fetal survival in response to short-term arginine supplementation was carried forward to d 60 of gestation for gilts with 15 to 18 CL (Table 4.2). Interestingly, re-examination of the data in Chapter II revealed that in response to dietary supplementation with 0.4% and 0.8% arginine, gilts with high CL numbers (15 to 19) had similar rates of embryonic survival to gilts with 10 to 14 CL (Appendix Table A-4; A-5). Of particular note, results of our recent study indicated that supplementing 0.4% and 0.8% arginine to the diet of gilts between d 14 and 25 of gestation increased the number of live-born piglets by 0.58 and 0.91, respectively, compared with control gilts (Jeffrey L. Vallet, Xilong Li, Fuller W. Bazer, and Guoyao Wu, unpublished observations). Similarly, studies involving gilts and multiparous sows showed that dietary supplementation with 1% arginine between 14 and 28 of gestation enhanced the number of live-born piglets at term by approximately 1 per litter as compared to the control (Ramaekers et al. 2006). Because arginine is expensive in today's market and its supplementation for a prolonged period of time is not expected to result in an appreciable economic return to swine producers, identifying a short, but

effective window of arginine supplementation, holds great promise to cost-effectively enhance litter size in swine (Wu et al. 2006).

Uterine capacity is a major limiting factor for the survival and development of fetuses in modern highly prolific swine after d 30 of gestation (Wilson 2002). Both the structure and function of the placenta plays a key role in regulating the transfer of nutrients and oxygen from mother to fetus (Reynolds et al. 2006). Placental efficiency, defined by some investigators as the ratio of fetal to placental weights (Molteni et al., 1978; Kurz et al., 1999), is often used to evaluate uterine capacity. However, this simplistic definition does not take into account fetal survival and development, and should be revised accordingly. For example, we found that neither fetal nor placental weights differed between control and arginine-supplemented gilts with above average numbers of CL numbers, but arginine supplementation increased the number of live fetuses on d 60 of gestation by 2 per litter (Table 4.2). Higher placental efficiency is correlated with a higher number of live fetuses per litter, which is due, in part, to enhanced vascular development of the placenta (Vonnahme and Ford 2004). This is well demonstrated by the marked difference between highly prolific Chinese Meishan pigs and western breeds of pigs. Compared to western breeds (e.g., Large White), Meishan gilts have similar ovulation rates, but produce three to five more live piglets per litter at birth despite having smaller placentae (Bazer et al. 1988; Christenson, 1993; Galvin et al., 1993; Lee et al., 1995).

A novel and important finding of this study is that gilts respond differentially to dietary arginine supplementation, depending on CL numbers. Specifically, dietary

supplementation with 0.4% or 0.8% L-arginine increased the number of live fetuses and embryonic survival in gilts with 15 to 18 CL (Table 4.2), which is 2.6 to 5.6 greater than the average number of CL (12.4) for gilts that did not exhibit improved reproductive performance (Table 4.1). Results of our previous study (Chapter II) suggest that progesterone plays a permissive role in the action of arginine to enhance embryonic/fetal survival. Because body weights of gilts did not differ between the control and arginine-supplemented groups, more CL may result in greater concentrations of progesterone in maternal circulation and conceptus. It is tempting to speculate that expression of some progesterone-inducible genes may be necessary for arginine to enhance embryonic/fetal survival in mammals. Alternatively, arginine or its metabolic products (e.g., creatine, NO, and polyamines) may improve the uterine environment in gilts with high CL numbers. Further research is warranted to test this novel hypothesis.

Results of this study indicate a programming effect of arginine supplementation during early pregnancy on the availability of amino acids in the conceptus. Specifically, although arginine supplementation was discontinued after d 25 of gestation, there were marked differences on d 60 of gestation in total amounts of several key amino acids (e.g., glutamine, serine, glycine, and arginine) in ALF and AMF that are related to one-carbon-unit metabolism as well as the synthesis of glucose and polyamines. These pathways play crucial roles in protein synthesis, cell proliferation, and development of the conceptus (Wu et al. 2008). This may provide a nutritional basis to explain improved embryonic survival at mid-gestation despite short-term supplementation with arginine between d 14 and 25 of gestation. Interestingly, in arginine-supplemented gilts with 9 to

14 CL, increases in total amounts of some amino acids were observed on d 60 of gestation. Whether this change plays a role in reducing fetal deaths between d 60 of gestation and parturition is not known. Such a possibility would provide an additional mechanism for improving litter size at birth.

Because feed intake and concentrations of amino acids in plasma did not differ between control and arginine-supplemented gilts, possible mechanisms responsible for increased amounts of water, ions, and amino acids in ALF and AMF would be: a) altered metabolism of nutrients in the conceptus; and b) enhanced transport of nutrients across the placenta, amniotic membrane, and allantoic membrane. In this regard, it is noteworthy that we recently discovered that osteopontin rapidly stimulates ion transport across the porcine placenta (Johnson et al. 2011). We hypothesize that arginine up-regulates expression of this integrin-binding protein in the placenta and endometrium, thereby enhancing the transfer of nutrients from mother to fetus. Future experiments are necessary to test this novel hypothesis.

In summary, reproductive performance of gilts with 15 to 18 CL at d 60 of gestation was improved by dietary arginine supplementation between d 14 and 25 of gestation. In contrast, no changes in fetal growth and survival were observed in gilts with 9 to 14 CL. Arginine supplementation during early gestation can improve uterine capacity and possibly have a programming effect on placental transport of nutrients from mother to fetus. These novel findings provide a much-needed basis for design of future experiments to optimize beneficial effects of arginine on improving embryonic survival and development in swine and other mammals.

CHAPTER V

MECHANISM OF ARGININE FUNCTION IN PIG REPRODUCTION

This study was conducted to determine the effects of arginine supplementation on the expression of key genes related to arginine transport, nitric oxide and polyamine synthesis, mechanistic target of rapamycin (MTOR) activation, and other possible cell signaling pathways. Placentae and endometria were collected and snap-frozen in liquid nitrogen. Total RNA and protein were extracted from the frozen tissues. Quantitative RT-PCR, western blotting, and microarray analyses were performed to determine the changes of gene expression at the mRNA and protein levels. Results indicated that placental the abundance of proteins encoded for by genes related to arginine transport and metabolism, including cationic amino acid transporter 1, endothelial nitric oxide synthase (NOS3), phosphorylated-NOS3, ornithine decarboxylase, and guanosine triphosphate cyclohydrolase-I were increased by dietary supplementation with 0.8% L-arginine between d 0 and 25 of gestation. The abundance of total and phosphorylated-MTOR, but not eukaryotic translation initiation factor 4E binding protein 1 and ribosomal protein S6 kinase 1, were enhanced by dietary 0.8% L-arginine supplementation between d 0 and 25 of gestation. Interestingly, the mRNA and protein levels of the genes related to MTOR signaling and syntheses of NO and polyamines were not affected by dietary supplementation with 0.8% L-arginine between d 14 and 25 of gestation. Microarray analysis revealed that supplementation with 0.8% arginine between d 14 and 25 of gestation affected placental expression of 575 genes, with 146

genes being up-regulated and 429 genes being down-regulated. These differentially expressed genes play important roles in nutrient metabolism, as well as insulin, TGFB, and Notch signaling pathways.

Introduction

There is growing interest in the role of arginine nutrition in enhancing litter size in livestock species. However, few studies have been conducted to explore the underlying mechanisms. Thus, there is limited understanding of regulatory functions of arginine in the placenta. Results of recent studies indicated that L-arginine is not only a building block for proteins, but also has roles in cell signaling (Wu et al. 2009).

Specific transporters are needed for transporting arginine across cell membranes. There are four possible systems (y^+, y^+L, b^{0+}, and B^{0+}) for transport of L-arginine by animal cells. The most important transporter for arginine uptake in most cell types is system y+, which is a high-affinity Na^+-independent system. Three different Solute Carrier Family 7 (Cationic Amino Acid Transporter, Y+ System) Member genes (SLC7A1, SLC7A2, SLC7A3) which encode for four homologous proteins SLC7A1, SLC7A2, SLC7B2 and SLC7A3 (Devés and Boyd 1998). Arginine supplementation may increase arginine availability by increasing expression of its transporters in placental cells.

Arginine metabolites such as nitric oxide (NO) and polyamines may be key mediators for the function of L-arginine in reproduction. NO is essential for ovulation, embryonic development, and implantation (Maul et al. 2003). NO is synthesized from L-arginine by NO synthase (NOS) (Bredt and Snyder 1994). There are three isoforms of NOS:

neuronal NOS (nNOS; also known as NOS1), inducible NOS (iNOS; also known as NOS2), and endothelial NOS (eNOS; also known as NOS3). NOS1 and NOS3 are constitutively expressed in a cell-specific manner and produce low levels of NO (Ignarro 1987). In contrast, NOS2 is induced by certain immunological stimuli, including LPS and inflammatory cytokines, to generate large amounts of NO (Li et al. 2007). Tetrahydrobiopterin (BH_4) is an essential cofactor for all isoforms of NOS. Increasing extracellular levels of arginine enhances NO production through increasing the availability of BH_4 whose *de novo* synthesis from guanosine triphosphate (GTP) requires GTP cyclohydrolase-I (GCH1) as the first and rate-controlling enzyme (Shi et al. 2004) The key function for ornithine (another metabolite of arginine) is the synthesis of polyamines (putrescine, spermidine, and spermine) via ornithine decarboxylase (ODC1). Polyamines are crucial for cell growth, migration, and proliferation, as well as angiogenesis (Wu 2009).

The mechanistic target of rapamycin (MTOR) cell signaling pathway in skeletal muscle (Yao et al. 2008) and small intestine (Rhoads et al. 2006) is known to be activated by physiological levels of arginine. MTOR is a highly conserved serine/threonine kinase of the phosphatidylinositol kinase-related kinase family (Inoki and Guan 2006). The MTOR signaling pathway plays a central role in regulating cell growth by sensing and responding to environmental cues, including nutrients (Wullschleger et al. 2006). Two distinct MTOR complexes, MTORC1 and MTORC2, have been identified in eukaryotic cells (Wullschleger et al. 2006). MTORC1 plays a key role in cell proliferation and mRNA translation for protein synthesis, whereas MTORC2

is associated with cell migration and cytoskeletal reorganization which is not inhibited by rapamycin (Bazer et al. 2009). MTOR phosphorylates eukaryotic initiation factor 4E binding protein 1 (elF4EBP1) and ribosomal protein S6 kinase (RPS6K) (Carrera 2004). Most recently, L-arginine was reported to stimulate proliferation of ovine and porcine trophectoderm cells through the MTOR-RPS6K-RPS6 signaling cascade (Kim et al. 2011; Kong et al. 2011).

This study was conducted to determine the effects of arginine supplementation on: 1) expression of key genes related to arginine transport, synthesis of NO and polyamines, and MTOR cell signaling based on RT-PCR and western blotting techniques; and 2) global gene expression based on microarray analysis.

Materials and methods

Tissue collection

At the time of sample collection in Experiments 1 and 2, each placenta from a live fetus was snap-frozen in liquid nitrogen. Endometrium and myometrium were separated using curved scissors and placed immediately in liquid nitrogen. All snap-frozen samples were stored at -80^{o}C until analyzed. Eight placentae from each gilt in each group were selected randomly for extraction of total RNA and protein.

Western blotting

Frozen placentae and endometria (approximately 200 mg) were homogenized at 4^{o}C with a model PRO200 homogenizer (PRO Scientific, Oxford, CT) in 0.6 mL of lysis buffer (1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 20 mM Tris, 150 mM NaCl, 5mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM β-

glycerophosphate) containing 1X protease inhibitor cocktail (Set I, Calbiochem, La Jolla, CA). The lysates were centrifuged at 16,000 g and 4°C for 15 min. The supernatant fluid was transferred into a new tube for protein assay and western blot analyses. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA) with bovine serum albumin as a standard. Proteins were denatured in 4X NuPAGE LDS Sample Buffer (Invitrogen) containing 10% mercaptoethanol. Denatured protein (60 μg for phosphoproteins and 40 μg for other proteins) were loaded into 4-12% NuPAGE® Novex Bis-Tris Pre-Cast Gels (Invitrogen). Electrophoresis was conducted at 16 W constantly for 1.5 h in NuPAGE® MOPS SDS Running Buffer (Invitrogen). Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) in Transfer Buffer (25 mM Tris, 192 mM glycine, and 5% methanol) at 12 V overnight, using the Bio-Rad Transblot apparatus (Hercules, CA). Membranes were blocked in 5% nonfat dry milk (5% BSA for phosphoproteins) which was dissolved in the Tris-buffered saline-Tween solution (TBST; 20 mM Tris, 150 mM NaCl, pH 7.6, and 0.1% Tween-20) for 3 h at room temperature. The membranes were incubated with primary antibodies (Table 5.1) overnight at 4°C with gentle rocking. After being washed three times with TBST, the membranes were incubated at room temperature for 1 h with a secondary antibody (0.8 mg/mL Donkey Anti-Mouse IgG or Donkey Anti-Rabbit IgG, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:50,000. Finally, the membranes were washed four to six times with TTBS, followed by development using Supersignal West Dura Extended Duration Substrate according to the manufacturer's instructions (Thermo Fisher Scientific Inc., USA). Western blots were quantified by

measuring the intensity of target protein bands using a ChemiDoc XRS system and Quantity One software (Bio-Rad, Hercules, CA). Images for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein were used to normalize the abundance of the target proteins.

Table 5.1 Information on antibodies used for western blotting

Protein	Company	Catalog No.	Dilution	Size (kDa)
CAT1	Sigma	AV43838	1:1000	67
NOS3	BD Transduction Laboratories	610297	1:1000	140
P-NOS3 (Ser1177)	Cell Signaling Technology	9571S	1:500	140
ODC1	Atlas Antibodies	HPA001536	1:500	51
GCH1	Produced by Drs. C.J. Meininger and G. Wu, Texas A&M University		1:500	29
MTOR	Cell Signaling Technology	2983	1:1000	289
P- MTOR (Ser2448)	Cell Signaling Technology	2974	1:500	289
RPS6K	Cell Signaling Technology	9202	1:500	70
P-RPS6K (Thr389)	Cell Signaling Technology	9206	1:1000	70
EIF4EBP1	Cell Signaling Technology	9452	1:1000	15-20
P- EIF4EBP1 (Thr70)	Cell Signaling Technology	9455	1:1000	15-20
GAPDH	Cell Signaling Technology	2118	1:1000	37

Total RNA isolation

Total RNA was isolated from the frozen placentae according to the manual of the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The quality of total RNA was determined by 1% agarose electrophoresis. The quantity of the total RNA was measured by NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA).

Microarray analysis

Total RNA (400 ng) was reverse-transcribed to cDNA. T7 RNA polymerase-driven RNA synthesis was used for the preparation and labeling of cRNA with Cy3 or Cy5 dye. The labeled cRNA probes were purified with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Purified cRNA was quantified with the NanoDrop 1000, and 825 ng for each were hybridized on a 44 K Agilent porcine gene expression microarray (Agilent, Santa Clara, CA). This array includes 43,803 probes which were prepared using gene sources from RefSeq, UniGene, and TIGR. The slide format was printed using the Agilent's 60-mer SurePrint technology. The hybridized slides were washed according the manual of a commercial kit (Agilent Technology, Palo Alto, CA), followed by scanning with a Genepix 4100A scanner (Molecular Devices Corporation, Sunnyvale, CA) with the tolerance of saturation setting of 0.005%. A locally weighted linear regression (LOWESS) method was applied to normalize the data by the median of the signal intensity and local background values. SAS 9.1.3 program (SAS Institute Inc. Cary, NC) with mixed model was used to analyze the normalized data (Chiang et al. 2008). Statistical significance to detect differentially expressed genes was determined by the approximate t-test for least-square means, where $P < 0.05$ was considered to be

statistically different. The false discovery rate (Q value) was calculated for each *P*-value using the R program (Chiang et al. 2008). Genes were annotated by basic local alignment search tool (BLAST) in the database of the national center for biotechnology information (NCBI) and the institute for genomic research (TIGR). The database for annotation, visualization and integrated discovery (DAVID) version 6.7 was used to generate specific functional annotations of biological processes for the differentially expressed genes (Dennis et al. 2003).

Quantitative real-time PCR

Total RNA (1 μg) from each sample was used for cDNA synthesis with a random hexamer primer of a Thermoscript RT PCR system kit (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. The cDNAs were quantified by quantitative RT-PCR using the ABI Prism 7900HT system with SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA). The primers for each gene were designed by using the Oligo6 program (Table 5.2). The cycling conditions of quantitative RT-PCR amplification were: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and optimal annealing temperature for 1 min (Table 5.2). The porcine tubulin α gene used as the housekeeping gene. Dissociation curves were performed at the end of amplification for validating data quality. All samples were run in triplicate and the average critical threshold cycle (Ct) was used for calculating relative mRNA levels of target genes by fold-change and statistical significance (Fu et al. 2010).

Table 5.2 Sequence and optimal annealing temperatures for primers used in the quantitative RT-PCR

Accession No.	Gene	Primer sequence	Product Length (bp)	Annealing Temp. (^{o}C)
TC290976	Antigen	Forward: 5'- ACTAACTTGAAGTAGCGTGG -3'	75	48
		Reverse:5'- TTTCTGTGTCTGGGACTGTT-3'		
AY610027	ARV1	Forward: 5'- CTCTGCGTCTTCTGTTTGCT-3'	109	53
		Reverse:5'- CCCATTCCTTGGCATATCTG-3'		
TC274873	AMPK	Forward: 5'- CAACATTTTCCACCCTTTCG-3'	94	53.7
		Reverse:5'- GGGCTGCTTTCCAGATTACC-3'		
EW039857	CALCR	Forward: 5'- TCCAGCCTTGTTATCGTCTC-3'	79	53
		Reverse:5'- GTGATTTGGATGCAGCTTTG-3'		
EW109654	CASC5	Forward: 5'- CTGTGGTCCTATGAATGTTA -3'	289	48
		Reverse:5'- AACTGTCCTTTCCAGGTTAC-3'		
NM_001001861	CXCL2	Forward: 5'- CACTGTGACCAAACGGAA -3'	120	53
		Reverse:5'- GTTGGCACTGCTCTTGTTT-3'		
TC295311	Cytc	Forward: 5'- CCATTTCGGTGACATTACTG-3'	294	51.4
		Reverse:5'- TCTCTCATTCCGTAGGTTCT-3'		
TC249250	DRP	Forward: 5'- CAAGTGTGAAAGGTGAAGC-3'	220	49
		Reverse: 5'-GCTATTCTGTGGTCTGCCT-3'		
TC277265	E2F3	Forward: 5'- GCTTTGCGACAAGTGCCTAC-3'	113	51.6
		Reverse:5'- GGCAGCTAACCAGATGAGAT-3'		
EW299999	ElF	Forward: 5'- GGAAGACACCACAGAAAGT-3'	110	48.9
		Reverse:5'- CTTCTCTTAGCCTCTTAGC-3'		
TC246681	EndoRT	Forward: 5'- CAACATGGATGGACCTAGAA-3'	146	50
		Reverse:5'- TGTCTGTGAATCAGCATCTG-3'		
NM_214003	IGFBP2	Forward: 5'- GTGGATGGGAACGTGAACTT-3'	111	56.8
		Reverse:5'- GTGCTGCTCCGTGACTTTCT-3'		
TC274812	Mdase	Forward: 5'- CAGTCATAAGCGTGGTGGAA-3'	94	55
		Reverse:5'- CGTGACTTTCTCCAGCATCC-3'		
TC254633	NAS	Forward: 5'- CCTCTACCTGACTTCCATTT-3'	98	48
		Reverse: 5'- CATGTTCACAGATACCACGA-3'		
TC267605	PFKFB1	Forward: 5'- GCCTAAGATGACTCAAGAGA-3'	187	53.3
		Reverse:5'- CGTGGAGATGTAGGTCTTT-3'		

Table 5.2 Continued

Accession No.	Gene	Primer sequence	Product Length (bp)	Annealing Temp. (°C)
AK233690	PGM1	Forward: 5'- GATTGCTTTGTACGAGACCC-3'	118	54
		Reverse: 5'- CTCACGGATGTGGTCAGAAC-3'		
TC279371	PI3K	Forward: 5'- TGAAGGCACCGAAGTTGTCC-3'	149	58
		Reverse: 5'- TGAAGCCCTGTGTCGTCTGG-3'		
NM_213963	PPARGC	Forward: 5'- AACCCACAGAGACCCGAAAC-3'	82	53
		Reverse:5'- AAATGTTGCGACTGCGATTG-3'		
AK231515	Presenilin	Forward: 5'- AAGGAGCACAGCGGACTCT-3'	299	57
		Reverse:5'- TGGGTACTGAACGGGTGTTT-3'		
TC275071	RAG	Forward: 5'- ATGCCAGATCCTTAACCCAC-3'	82	53
		Reverse:5'- GCAGCAGAAATGAATCCAAC-3'		
BI341657	RasGEF	Forward: 5'- CTCCCATCTACAGCGAGGAA-3'	104	56
		Reverse: 5'- GAGCGTGGTCCTGAGGGTCT-3'		
TC243513	RHBG	Forward: 5'- GTGCCTACTTTGGGTTGGTC-3'	103	56
		Reverse:5'- ATGGCAAAGAGGTCCGAATG-3'		
TC257543	RU2S	Forward: 5'- CACTTCTGGAACCCTGCACT-3'	103	53
		Reverse:5'- TGATCCCACTGATTCAAGGC-3'		
NM_001001863	TNNT3	Forward: 5'- CCTGTACCARCTGGAGATTG-3'	78	51
		Reverse: 5'- CTGAGGTTGATGATGTCGTA-3'		
DQ225365	Tubulin α	Forward: 5'-GCAGTGTTTGTAGACCTG GA-3'	139	55
		Reverse:5'-CAATGGTGTAGTGACCTCGG-3'		
TC293083	unknown	Forward: 5'- TAACCTATCAAATGGCAGTT-3'	144	48
		Reverse:5'- CAGAAACAGGACTTTGGGA-3'		
NM_214295	NOS3	Forward: 5'- GCCTACAGGACCCAAGATG-3'	100	57
		Reverse: 5'- TGATGAAGCAGGGTACAGGG-3'		
EU534186	ODC1	Forward: 5'- GGCGCTGAGGTCGGTTTC-3'	166	57
		Reverse:5'- TGCCTGGCTCCGCTATGATT-3'		
XM_001924171	GTPCH	Forward: 5'- CATGCAGTTCTTCACCAA -3'	98	52
		Reverse: 5'- CCTTCACAATCACCATCTCA-3'		
EU288086	MTOR	Forward: 5'- GTCTCTATCAAGTTGCTGGC-3'	126	53
		Reverse: 5'- CTTTCGAGATGGCAATGGAA-3'		
NM_001012613	SCF7A1	Forward: 5'- ACTCGACTCTCGTGGACCTT-3'	134	54
		Reverse:5' GGTCAGTTGACTTTCTGCCT-3'		

Statistical analysis

Data were analyzed using the General Linear Model procedures of SPSS (Version 15.0, Chicago, IL) for a randomized complete block design. Gilt was considered as the experimental unit. Differences among treatment means were determined by using the Duncan multiple comparison test. Probability values < 0.05 were considered statistically significant.

Results

Expression of genes related to arginine transport and metabolism

Dietary supplementation with 0.8% L-arginine between d 0 and 25 of gestation increased (P<0.05) the abundance of SLCA1, NOS3, pNOS3, ODC1 and GCH1 placental proteins (Fig. 5.1). However, expression of these genes at the mRNA and protein levels in placentae (Table 5.3; 5.4) and endometria was not affected when dietary L-arginine supplementation was provided between d 14 and 25 of gestation (Table 5.5). Dietary supplementation with 0.4% L-arginine either between d 0 and 25 of gestation or between d 14 and 25 of gestation had no effect on expression of these genes in the placentae (Fig. 5.1; Table 5.3).

Expression of proteins related to the MTOR signaling pathway

Dietary supplementation with 0.8% L-arginine between d 0 and 25 of gestation increased ($P < 0.05$) the abundance of total and p-MTOR (Fig. 5.2), but did not affect abundance of total or p-EIF4EBP1 and RPS6K (Fig. 5.2). Compared with the control group, dietary supplementation with 0.4% arginine either between d 0 and 25 of gestation or between d

14 and 25 of gestation had no effect on the abundance of placental MTOR protein (Fig. 5.2; Table 5.3).

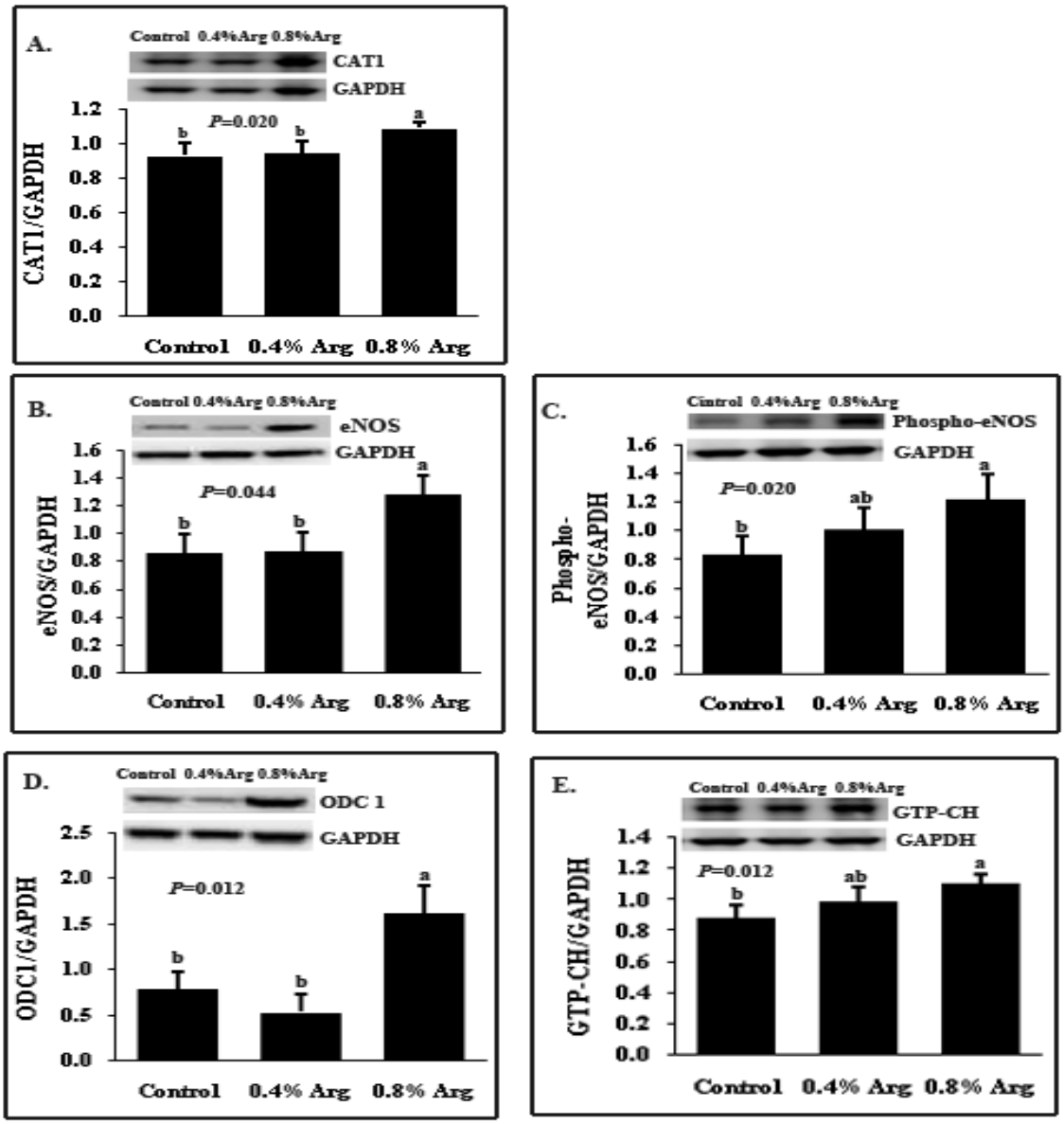

Fig. 5.1 Relative abundance of proteins related to arginine transport and metabolism in placentae of gilts fed diets supplemented with 0.0, 0.4, or 0.8% L-arginine (Arg) between d 0 and 25 of gestation. Protein levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to normalize the abundance of target proteins. (A.) CAT1; (B.) NOS3; (C.) p-NOS3; (D.) ODC1; E.) GCH1.

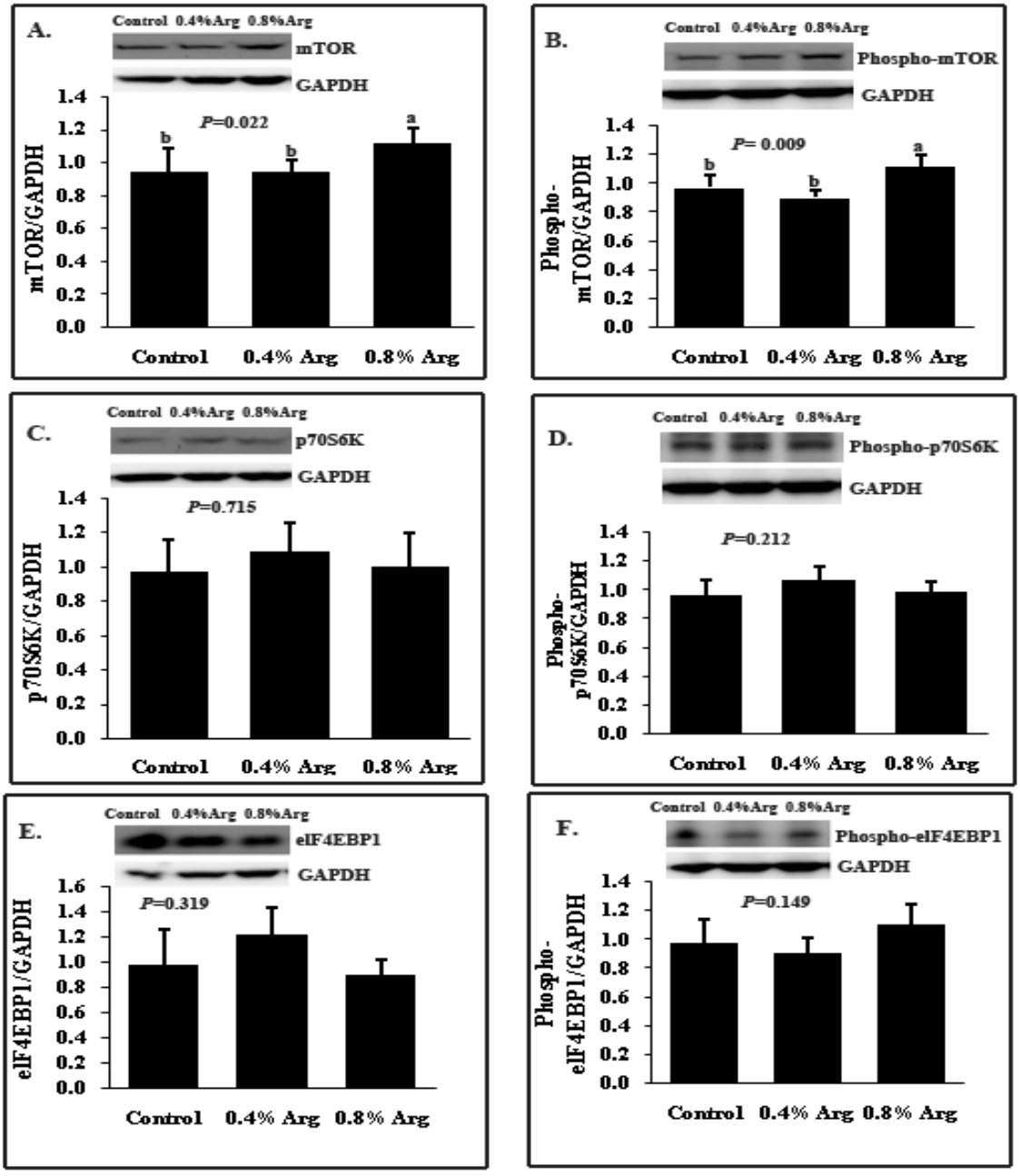

Fig. 5.2 Relative abundance of proteins related to MTOR cell signaling pathway in placentae of gilts fed diets supplemented with 0.0, 0.4, or 0.8% L-arginine (Arg) between d 0 and 25 of gestation. Protein levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to normalize the abundance of target proteins. (A.) MTOR; (B.) p-MTOR; (C.) RPS6K; (D.) p-RPS6K; (E.) EIF4EBP1; (F.) p-EIF4EBP1.

Table 5.3 Relative abundance of proteins related to arginine transport, metabolism, and MTOR cell signaling pathway in placentae of gilts fed diets supplemented with 0.0%, 0.4% or 0.8% L-arginine (Arg) between d 14 and 25 of pregnancy*

Protein	Control	0.4%Arg	0.8%Arg	SEM	P-Value
CAT1	1.155	0.908	0.938	0.052	0.114
NOS3	0.894	1.075	0.968	0.167	0.746
P-NOS3	1.041	1.026	0.954	0.062	0.581
ODC1	0.913	1.126	0.835	0.301	0.780
GCH1	1.014	1.036	0.954	0.032	0.214
MTOR	0.931	1.091	1.020	0.055	0.153
P-MTOR	1.018	1.015	0.983	0.020	0.414
RPS6K	1.358	0.869	0.928	0.200	0.205
P-RPS6K	1.015	1.063	0.902	0.055	0.144
EIF4EBP1	1.291	0.954	0.866	0.108	0.343
P-EIF4EBP1	1.073	1.119	0.799	0.088	0.265

*Pooled SEM; n=8; protein levels were determined by western blotting; protein levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to normalize the abundance of target proteins.

Table 5.4 Relative levels of mRNA for select genes in placentae of gilts supplemented with 0.8% L-arginine in the diet, compared with the control group*

Gene	Fold change	*P*-value
NOS3	0.76	0.383
GCH1	0.77	0.131
MTOR	1.04	0.520
ODC1	0.83	0.044
SCF7A1	0.94	0.550

*n = 8; $P < 0.05$ was considered significant.

Table 5.5 Relative abundance of proteins in endometria of gilts fed diets supplemented with 0.0%, 0.4% or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Protein	Control	0.4% Arg	0.8% Arg	SEM	*P*-Value
MTOR	0.978	1.053	0.979	0.021	0.275
RPS6K	1.060	0.953	1.008	0.175	0.972
EIF4EBP1	1.103	0.901	1.006	0.142	0.857
P-MTOR	0.978	1.006	1.019	0.035	0.897
P-RPS6K	1.026	0.963	1.008	0.053	0.889
P-EIF4EBP1	0.976	0.910	1.106	0.067	0.936
NOS3	1.073	1.003	0.903	0.073	0.652
P-NOS3	0.995	1.034	0.936	0.088	0.909
ODC1	1.208	0.875	0.891	0.084	0.196

*Pooled SEM; n=8; protein levels were determined by western blotting; protein levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to normalize the abundance of target proteins

Genes related to arginine function based on microarray analysis

Microarray results from 22 expressed sequence tags (ESTs) were verified using quantitative RT-PCR (Table 5.6). One hundred and forty six ESTs were up-regulated and 429 ESTs were down-regulated by dietary supplementation with 0.8% arginine between d 14 and 25 of gestation (Table 5.7; 5.8). Functional analysis by the DAVID program revealed alterations in placental expression of genes in response to dietary supplementation with 0.8% L-arginine. These genes are known to play important roles in fatty acids biosynthesis, as well as insulin, transforming growth factor beta, and notch signaling pathways (Table 5.9).

Table 5.6 Verification of microarray data using quantitative RT-PCR

Accession No.	Gene name	Microarray *P* Value	Microarray Fold change	RT-PCR *P* Value	RT-PCR Fold change
EW039857	CALCR	0.038	3.23	0.408	0.57
TC246681	EndoRT	0.024	2.87	0.157	1.36
AK231515	Presenilin	0.006	2.31	0.021	1.77
AK233690	PGM1	0.033	2.30	0.624	0.96
TC275071	RAG	0.006	1.76	0.068	1.36
TC267605	PFKFB1	0.025	1.55	0.162	1.39
TC279371	PI3K	0.027	1.45	0.595	0.96
TC254633	NAS	0.045	1.39	0.556	1.11
EW109654	CASC5	0.002	1.35	0.164	1.21
EW299999	elF	0.015	1.17	0.910	1.02
TC277265	E2F3	0.043	1.16	0.171	0.90
TC249250	DRP	0.051	1.14	0.786	1.03
TC295311	Cytc	0.016	0.84	0.238	1.33
AY610027	ARV1	0.008	0.80	0.273	1.09
NM_213963	PPARGC	0.032	0.72	0.088	0.70
TC274812	Mdase	0.000	0.71	0.612	0.96
TC274873	AMPK	0.022	0.66	0.638	0.94
NM_001001861	CXCL2	0.013	0.49	0.016	0.43
NM_214003	IGFBP2	0.002	0.47	0.094	0.51
TC243513	RHBG	0.006	0.45	0.016	0.63
TC290976	Antigen	0.006	0.35	0.839	1.04
TC257543	RU2S	0.015	0.23	0.044	0.38

Table 5.7 Genes for which expression in the porcine placentae was up-regulated by dietary supplementation with 0.8% L-arginine between d 14 and 25 of gestation in comparison with the control group

EST	Accession No.	Gene Name	*P*-Value	Fold Change
BX918610	NM_001001863	Troponin T type 3	0.004	4.61
TC292911*	XM_003129590	Leucine-rich repeat-containing protein 51-like	0.001	4.49
AK231515	EU287432	Presenilin 2	0.006	2.31
TC275071*	NM_000536	RAG-2		
TC278497*	NM_018941	Ceroid-lipofuscinosis, neuronal 8	0.010	2.23
TC289044*	XM_001929300	Sus scrofa leucine-rich repeat-containing protein 18-like	0.020	2.10
TC275071*	AB091391	Recombination activating protein 2	0.006	1.76
TC275071*	AB091391	Recombination activating protein 2	0.006	1.76
TC274023*	NM_001097446	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F	0.005	1.70
TC274023*	NM_001097446	APOBEC3F	0.005	1.70
BX666795	XM_001924347	Similar to SLCO3A1 protein	0.003	1.67
BX666795	XM_001924347	SLCO3A1 protein	0.003	1.67
TC278155*	NM_214378	RH	0.019	1.66
EW660666	NM_001045886	Phenazine biosynthesis-like protein domain containing	0.013	1.54
TC246855*	AY208121	Myostatin	0.022	1.54
AY610045	XM_001924474	Similar to androgen-induced 1	0.018	1.42

Table 5.7 Continued

EST	Accession No.	Gene Name	*P*-Value	Fold Change
TC261962*	EW422073	HBE1	0.035	1.31
AK234630	XM_001927389	FK506-binding protein	0.009	1.28
AJ584674	NM_213757	ST3GAL4	0.000	1.27
AK239509	AB529869	PECR mRNA for peroxisomal trans-2-enoyl-CoA reductase	0.027	1.25
BX667232	XM_001925672	Similar to pecanex-like protein 1	0.030	1.23
CN155716	EU617320	Small calcium-binding mitochondrial carrier 1	0.038	1.23
EV880225	DQ629170	RPS6	0.017	1.22
CK467702	NM_001035277	CDH13	0.013	1.22
CD572284	AJ009912	PLP	0.006	1.21
DN125568	GQ184633	CDC2	0.048	1.18

*Sequence can be accessed on http://compbio.dfci.harvard.edu/cgi-bin/tgi.

Table 5.8 Genes for which expression in porcine placentae was down-regulated by dietary supplementation with 0.8% L-arginine between d 14 and 25 of gestation in comparison with the control group

EST	Accession No.	Gene Name	*P*-value	Fold Change
BI341657	XM_001926447	RasGEF domain family, member 1A	0.013	0.18
TC273367*	XM_003129699	Probable dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1,3-glucosyltransferase-like	0.010	0.20
TC257543*	XM_001927988	RU2S	0.015	0.23
DN100844	FJ263680	Acetyl-coenzyme A carboxylase alpha	0.003	0.27
NP321728	AF274712	Pig endogenous retrovirus group Beta3 polymerase	0.014	0.29
BI360386	XM_003133904	Oncostatin-M-specific receptor subunit beta-like	0.009	0.31
TC238637*	NM_214376	Amphiregulin	0.045	0.31
CF178669	AJ427478	ASIP	0.023	0.33
CX061534	XM_003130350	Torsin-1A-interacting protein 1-like	0.007	0.40
TC301037*	XM_003357826	Serine/threonine-protein kinase DCLK1-like	0.012	0.42
TC243513*	NM_213996	Rh family, B glycoprotein	0.006	0.45
DN106254	NM_001098597	OSTN	0.025	0.47
AY577905	NM_001001861	Chemokine (C-X-C motif) ligand 2	0.013	0.49
TC278652*	NM_214003	Insulin-like growth factor binding protein 2	0.002	0.49

Table 5.8 Continued

EST	Accession No.	Gene Name	*P*-value	Fold Change
BP443132	XM_864245.3	CYP2C33	0.037	0.50
TC280345*	XM_003122165	Golgin A1	0.018	0.51
AY198323	NM_214257	DPP4	0.030	0.51
DN106254	NM_001098597	OSTN	0.048	0.51
TC290654*	NM_001105290	Bmp7	0.030	0.55
AK235882	NM_214048	Arginase 1	0.014	0.55
CO989438	XM_001928917	Potassium large conductance calcium-activated channel, subfamily M, beta member 4	0.017	0.56
DQ836054	NM_001097442	DAB1	0.021	0.57
TC270858*	AF228059	Decay-accelerating factor CD55	0.026	0.58
CV878027	XM_001926796	SAMD4A	0.018	0.58
TC290589*	XM_003132094	Upstream binding protein 1	0.005	0.58
CA513725	XM_003129205	Heat shock 70kDa protein 4-like	0.016	0.58
EV881857	XM_003132080	Sodium bicarbonate cotransporter 3-like	0.009	0.59
TC266622*	XM_003127574	Methylenetetrahydrofolate reductase (NAD(P)H), transcript variant 1	0.018	0.60
TC286353*	NM_001243919	CUE domain containing 1	0.007	0.60
TC250322*	NM_001037965	Inhibitor of DNA binding 2	0.007	0.61
CN159399	NM_001128506	Charged multivesicular body protein 4b-like	0.012	0.61
AK230591	NM_001128488	Antizyme inhibitor 1	0.016	0.62
AK234300	XM_003125957	RIB43A-like with coiled-coils protein 2-like	0.005	0.627

Table 5.8 Continued

EST	Accession No.	Gene Name	*P*-value	Fold Change
TC247541*	XM_003134192	Pericentriolar material 1	0.015	0.64
CF181641	XM_003128338	Dystonin, transcript variant 2	0.015	0.64
AK233736	XM_001927836	Similar to Down syndrome critical region gene 1-like 1 protein	0.033	0.65
DQ866834	DQ279926	RXRalpha	0.047	0.65
AB271924	NM_001099924	FGFR2	0.019	0.68
AY850382	NM_001011505	KLF13	0.006	0.68
AB116561	NM_213772	IFNAR1	0.012	0.69
TC248589*	NM_001077215	ROD1	0.025	0.70
AY610204	NM_214296	RND3	0.039	0.70
BP142559	XM_001926474	AKAP13	0.016	0.70
TC257240*	XM_001925375	Similar to PR domain containing 1	0.042	0.71
AY284842	AY284842	GPAT	0.016	0.71
AK235700	NM_001078670	Interferon regulatory factor 9	0.024	0.71
AK235466	DQ105589S2	CDS2	0.013	0.71
EU095967	NM_001105286	TRAF6	0.023	0.71
BP444119	NM_214224	HPD	0.007	0.72
AY159788	NM_214266	PRKAA2	0.025	0.72
AK235681	NM_213963	PPARGC-1	0.032	0.72
DQ853415	NM_001078666	PSEN2	0.034	0.72
AK240475	XM_001927539	Similar to general transcription factor IIH	0.006	0.73
BP446317	NM_001097440	BIN1	0.036	0.73

Table 5.8 Continued

EST	Accession No.	Gene Name	*P*-value	Fold Change
CK461960	NM_001162401	LPAR2	0.048	0.73
AB271924	NM_001099924	FGFR2	0.048	0.73
BI184146	XM_001927725	PTGFRN	0.002	0.74
CV875504	XM_001926134	Similar to chloride channel 3	0.040	0.74
EU009401	NM_001098605	PNPLA2	0.014	0.74
TC261381	NM_213973	HSP90	0.036	0.75
AK233668	NM_213830	FBP	0.029	0.75
AY609622	AY609622	Similar to small nuclear RNA activating complex	0.037	0.76
AB254406	NM_001101814	NR1H3	0.028	0.77
DN120475	XM_001927228	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	0.013	0.77
AY644721	NM_001009581	PAP7	0.037	0.78
AJ955195	XM_001929149	Similar to transmembrane protein 77	0.036	0.79
AK237448	XM_001928092	Similar to Rab-1C	0.033	0.79
AK234427	XM_001928746	Similar to adenosine deaminase-like protein	0.046	0.79
TC278200*	XM_001925656	Similar to procollagen	0.038	0.79
AK235686	XM_001925381	Similar to insulin-degrading enzyme	0.016	0.80
DN100853	AF339885	Mannose-6-phosphate/insulin-like growth factor II receptor	0.038	0.81

*Sequence can be accessed on http://compbio.dfci.harvard.edu/cgi-bin

Table 5.9 Pathway analysis for genes using functional annotation of the DAVID program

Gene Name	Species	Database	Pathway
5,10-methylenetetrahydrofolate reductase (NADPH)	Homo sapiens	KEGG_PATHWAY	hsa00670:One carbon pool by folate
			hsa00680:Methane metabolism,
Acetyl-Coenzyme A carboxylase alpha	Homo sapiens	KEGG_PATHWAY	hsa00061:Fatty acid biosynthesis
			hsa00620:Pyruvate metabolism
			hsa00640:Propanoate metabolism
			hsa04910:Insulin signaling pathway
Arginase, liver	Sus scrofa	KEGG_PATHWAY	ssc00330:Arginine and proline metabolism
Asparagine-linked glycosylation 8, alpha-1,3-glucosyltransferase homolog (S. cerevisiae)	Homo sapiens	KEGG_PATHWAY	hsa00510:N-Glycan biosynthesis
Chemokine (C-X-C motif) ligand 2	Sus scrofa	KEGG_PATHWAY	ssc04062:Chemokine signaling pathway
Chromatin modifying protein 4B; similar to LOC616164 protein	Bos taurus	KEGG_PATHWAY	bta04144:Endocytosis
Inhibitor of DNA binding 2	Sus scrofa	KEGG_PATHWAY	ssc04350:TGF-beta signaling pathway
Oncostatin M receptor	Homo sapiens	KEGG_PATHWAY	hsa04060:Cytokine-cytokine receptor interaction
			hsa04630:Jak-STAT signaling pathway
Potassium large conductance calcium-activated channel, subfamily M, beta member 4	Sus scrofa	KEGG_PATHWAY	ssc04270:Vascular smooth muscle contraction
Presenilin 2	Sus scrofa	KEGG_PATHWAY	ssc04330:Notch signaling pathway
			ssc05010:Alzheimer's disease
Recombination activating gene 2	Sus scrofa	KEGG_PATHWAY	ssc05340:Primary immunodeficiency
Acetyl-Coenzyme A carboxylase alpha	Homo sapiens	BIOCARTA	h_leptinPathway:Reversal of Insulin Resistance by Leptin
5,10-methylenetetrahydrofolate reductase (NADPH)	Homo sapiens	PANTHER_PATHWAY	P02743:Formyltetrahydroformate biosynthesis
doublecortin-like kinase 1	Homo sapiens	PANTHER_PATHWAY	P00031:Inflammation mediated by chemokine and cytokine signaling pathway
5,10-methylenetetrahydrofolate reductase (NADPH)	Homo sapiens	REACTOME_PATHWAY	REACT_11193:Metabolism of vitamins and cofactors
Acetyl-Coenzyme A carboxylase alpha	Homo sapiens	REACTOME_PATHWAY	REACT_1505:Integration of energy metabolism
			REACT_602:Metabolism of lipids and lipoproteins
Pericentriolar material 1	Homo sapiens	REACTOME_PATHWAY	REACT_152:Cell Cycle, Mitotic

Discussion

The placenta plays a critical role in transporting amino acids from mother to fetus, thereby having an enormous impact on fetal survival, growth, and development. The pig has true epitheliochorial placentation, meaning that the placenta is only superficially attached to the uterine luminal epithelium. Such a placental structure increases the efficiency of gas and nutrient exchanges between fetus and mother. Consistent with the increased availability of arginine in the conceptus of arginine-supplemented gilts (Chapter II), long-term (between d 0 and 25 of gestation) dietary supplementation with 0.8% L-arginine increased expression of arginine transporter SLCA1 in the placenta. However, the expression of SLCA1 was not affected by short-term supplementation with 0.8% arginine (d 14-25) or low dose of arginine (0.4%). Similar results were obtained for NOS3, ODC1, GCH1, MTOR, S6K1 and 4EBP1. These results indicate that arginine regulation of expression of arginine transporters and MTOR cell signaling pathways depend on dose and timing of arginine supplementation. To our knowledge, this is the first study of effects of dietary arginine supplementation on *in vivo* expression of placental genes in any animal species.

NO and polyamines are crucial for cell growth, migration, and proliferation, as well as angiogenesis (Wu 2009). They are regarded as major mediators for arginine function in the cell. NO and polyamines play key roles in pregnancy, including ovulation, implantation and fetal development (Maul et al. 2003). Interestingly, we found that long-term (between d 0 and 25 of gestation) dietary supplementation with 0.8% L-arginine increased expression of genes for NOS3, p-NOS3, and ODC1. Additionally, dietary

supplementation with 0.8% arginine enhanced expression of GCH1 in porcine placentae, which is the first and rate-controlling enzyme in the *de novo* synthesis of BH_4 (essential cofactor for all NOS isoforms) (Shi et al. 2004). This is in keeping with the previous report that dietary L-arginine supplementation stimulates endothelial NO synthesis by increasing BH_4 availability in both normal and diabetic rats (Kohli et al. 2004). Results of *in vitro* studies have also demonstrated that increasing extracellular L-arginine concentration dose-dependently enhanced GCH1 expression and BH_4 availability for NO production in cultured endothelial cells (Wu et al. 2004).

The MTOR signaling pathway plays a central role in regulating cell growth (Wullschleger et al. 2006). This pathway can be regulated by the availability of amino acids (Martin and Sutherland 2001) with elF4EBP and p70S6K being two important genes downstream of MTOR (Carrera 2004). elF4EBP normally binds elF4E (a eukaryotic translation initiation factor) to inactivate mRNA translation. However, phosphorylation of 4EBP1 by MTOR releases elF4E from its binding with elF4E to allow initiation of mRNAtranslation (Gingras et al. 2004). Upon activation by MTOR, p70S6K phosphorylates p70S6 to facilitate ribosome biogenesis and translation elongation (Gingras et al. 2004). A novel observation of this study is that long-term (between d 0 and 25 of gestation) dietary supplementation with 0.8% L-arginine increased expression of total and p-MTOR in porcine placentae. However, expression of total and p-elF4EBP and p70S6K was not affected by dietary 0.8% L-arginine supplementation between d 0 and 25 of gestation. This suggests that long-term L-arginine supplementation can activate MTOR, but its effect may not increase

phosphorylation of elF4EBP and S6K in the placenta. It is not clear how MTOR activation is disassociated with phosphorylation of its two downstream target proteins. However, the experimental conditions for the study (e.g., reduced levels of progesterone in maternal plasma and allantoic fluid) do not favor optimal survival or growth of fetuses. It is possible that the action of arginine on the placenta depends on adequate progesterone signaling or receptivity of the organ to physiological levels of arginine. Nonetheless, L-arginine could increase the abundance of p-MTOR, p-p70S6K, p-p70S6, and p-elF4EBP1 proteins in explant cultures of sheep conceptuses (Kim et al. 2011) and in ovine trophectoderm cells (Kim et al. 2011).

Because dietary supplementation with arginine between d 14 and 25 of gestation did not affect placental expression of genes at either the mRNA or protein levels that are known to regulate protein synthesis and cell growth, we used microarray technology to identify novel genes that may impact placental growth and development. Importantly, such an approach identified differentially expressed genes in the placenta of arginine-supplemented gilts. Of particular interest, these genes are related to fatty acids biosynthesis, as well as insulin, TGF-B, and Notch signaling pathways. Specifically, increased expression of type-3 troponin may beneficially enhance the growth of the placenta and alter its structure, as reported for myogenesis (Wong and Ordahl, 1996), to allow for efficient transfer of oxygen and nutrients from mother to fetus. Additionally, expression of leucine-rich repeat-containing proteins in the placenta of arginine-supplemented gilts may facilitate gene transcription, as reported for other cell types (Warfel et al. 2011), to enhance receptivity of the organs to arginine or its metabolites in

placental cells. In coordination with these changes, down-regulation of expression of IGF-2 binding protein can enhance the availability of IGF-2 to promote placental cell growth and differentiation via PI3 and MAP kinase signaling pathways (Kim et al. 2008). Moreover, reduced expression of heat shock protein 70 in placentae of arginine-supplemented gilts is consistent with an important role for arginine to reduce oxidative stress in animal cells (Jobgen et al. 2009) and improve their survival (Tan et al. 2010).

In summary, long-term dietary supplementation with 0.8% L-arginine increased the abundance of proteins in the porcine placenta that are related to arginine transport and metabolism. Relative abundances of total and p-MTOR were also enhanced by long-term supplementation with 0.8% arginine. In addition to the MTOR pathway, arginine may also affect other cell signaling pathways that promote placental growth and development.

CHAPTER VI

SUMMARY AND DIRECTION OF FUTURE RESEARCH

Arginine is the nitrogenous substrate for synthesis of both nitric oxide and polyamines in animals, including pigs. Although increasing concentration of arginine in plasma within the physiological range has been reported to enhance fetal survival and growth in swine, little is known about effects of the effects of arginine on conceptus survival, growth, or development during early gestation. Four series of experiments were conducted to fill in this gap of knowledge. Dietary supplementation with 0.4% L-arginine between d 0 and 25 of gestation had no beneficial effect on the reproductive performance of gilts. However, supplementation with 0.8% L-arginine during this period of pregnancy, while increasing placental vascularity, decreased litter size in gilts. This unexpected finding did not support the original hypothesis of the present study, but led to an important discovery that 0.8% L-arginine supplementation immediately after breeding reduced the number of corpora lutea (CL) and their production of progesterone, thereby impairing conceptus survival and growth in gilts. In contrast, supplementing the diet of gilts with 0.4% or 0.8% L-arginine between d 14 and 25 of gestation resulted in increased concentrations of arginine in maternal plasma, total amounts of arginine in allantoic fluid (ALF) and amniotic fluid (AMF), and the number of live fetuses per litter by 2 on d 25 of gestation as compared to the control gilts. Arginine supplementation between d 14 and 25 of gestation also increased the volume of AMF as well as total amounts of fructose and most amino acids in AMF possibly due to enhanced transport of ions,

water, sugar and amino acids across placentae and into the amniotic fluid. Reproductive performance of gilts with 15 to 18 CL at d 60 of gestation was improved by dietary arginine supplementation between d 14 and 25 of gestation. Interestingly, on d 60 of gestation, when the basal diet was supplemented with 0.4% or 0.8% arginine between d 14 and 25 of gestation, no changes in fetal growth and survival were observed in gilts with 9 to 14 CL. Collectively, the results indicate that arginine supplementation between d 14 and 25 of gestation can improve uterine capacity and possibly have a programming effect on placental transport of nutrients from mother to fetus. Long-term dietary supplementation with 0.8% L-arginine during early pregnancy increased the abundance of proteins in the porcine placenta that are related to arginine transport and metabolism. Relative abundances of total and phosphorylated mechanistic target of rapamycin (MTOR) were also enhanced by the long-term supplementation with 0.8% arginine. In addition to the MTOR pathway, arginine may also affect other cell signaling pathways that can promote placental growth and development.

These novel findings from this dissertation will aid in developing cost-effective strategies to enhance litter size in swine and also have important implications for improving embryonic survival in other mammals. These results also provide a much-needed basis for design of future experiments to optimize beneficial effects of arginine on improving embryonic survival and development in swine and other mammals. Findings from the current study not only advance basic knowledge of mammalian reproductive biology, but also have important implications for developing practical means to enhance fertility in female swine.

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APPENDIX

Table A-1 Oxidation of glucose and fructose in pig placentae*

	5 mM Glucose		5 mM Fructose	
Variable	Areolae	Inter-areolae	Areolae	Inter-Areolae
CO_2 production, nmol per mg tissue	1.73±0.36[a]	0.24±0.03[b]	0.053±0.008[c]	0.034±0.007[d]

*d 60 of pregnancy; n = 5. Chorioallantois tissue (100 mg) was incubated at 37°C in 1 ml of a culture medium containing physiological concentrations of amino acids (Kong et al. 2011) and 5 mM D-[U-^{14}C]glucose or 5 mM D-[U-^{14}C]fructose for 2 h. The specific activity of D-[U-^{14}C]glucose and D-[U-^{14}C]fructose in the incubation medium was 990 and 48 dpm/nmol, respectively. At the end of the 2-h incubation, ^{14}CO2 was collected in 0.2 ml of Soluene for measurement of radioactivity by a liquid scintillation counter (Wu 1997). In glucose oxidation, the average dmp for the blanks was 1234, and the average dmp for the samples was 7693 and 4288 for areolae and inter-areolae, respectively. In fructose oxidation, the average dmp for the blanks was 91, and the average dmp for the samples was 288 and 143 for areolae and inter-areolae, respectively.

[a-d] Means with different superscripts differed ($P < 0.01$), as analyzed by one-way ANOVA.

Table A-2 Incorporation of radiolabled fructose into proteins in chorioallantois of pig placentae *

Variable	Blank[a]	0.1 mM Fructose
DPM in protein	87	676[†]

* d 60 of pregnancy; n = 6. Chorioallantois tissue (200 mg) was incubated for 6 h at 37°C in 1 ml of an oxygenated (95% O_2/5% CO_2) culture medium containing physiological concentrations of amino acids (Kong et al. 2011) and 0.1 mM D-[U-^{14}C]fructose. The specific activity of D-[U-^{14}C]fructose in the medium was 474 dpm/nmol. At the end of 6-h incubation, the placenta was washed with 5 ml of Krebs buffer and then homogenized in 2 ml of 10% trichloroacetic acid. The trichloroacetic acid-insoluble fraction (protein) was solubilized in 0.5 ml of 1 M NaOH and then analyzed for ^{14}C radioactivity.

[a]No chorioallantois tissue in the medium.

[†]Differed from the blank ($P < 0.001$), as analyzed by the Independent-Samples T-Test.

Table A-3 Weight of organs of fetuses from all gilts with 9-18 CL at d 60 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) from d 14 through d 25 of gestation

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
			g		
Number of gilts, *n*	11	13	13		
Brain	3.03	2.94	2.95	0.04	0.659
Heart	0.88	0.85	0.91	0.02	0.570
Kidney	1.43	1.33	1.49	0.03	0.129
Leg Muscle	4.86	4.52	4.80	0.11	0.386
Liver	5.35	4.85	5.26	0.13	0.241
Lung	4.08	3.97	4.33	0.08	0.156
Intestine	2.18	1.88	2.08	0.07	0.158
Spleen	0.08	0.09	0.08	0.003	0.735
Stomach	0.44	0.44	0.45	0.006	0.551

* Values are means with pooled SEM.

Table A-4 Reproductive performance of gilts with 10 to 14 CL at d 25 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation[*]

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Number of gilts, *n*	10	8	10		
BW at breeding, *kg*	117.2	117.5	102.1	4.9	0.349
BW at d 25 of gestation, *kg*	119.4	120.4	103.4	4.9	0.284
BW gain, *kg/25 d*	2.0	2.9	1.3	1.3	0.903
Uterine weight, *kg*	2.32	2.44	2.46	0.08	0.736
Total fetuses, *n*	10.9	11.0	11.3	0.3	0.848
Live fetuses, *n*	9.9	11.0	11.1	0.3	0.197
CL, *n*	13.2	12.3	12.6	0.2	0.174
Embryonic mortality, *%*	25.3[a]	9.5[b]	11.5[b]	2.6	0.012
Weight of viable fetuses, *g*	5.33	5.73	5.73	0.22	0.701
Total placental weight, *g*	89.3[b]	123.5[a]	112.3[a]	4.9	0.009
Fetal length, *cm*	1.82	1.80	1.84	0.02	0.825
Total ALF volume, *L*	0.88	0.93	0.99	0.05	0.608
Total AMF volume, *mL*	2.19[b]	3.90[a]	3.36[a]	0.22	0.001

[*]Values are means with pooled SEM; means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table A-5 Reproductive performance of gilts with 15 to 19 CL at d 25 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation*

Variable	Control	0.4% Arg	0.8% Arg	SEM	*P*- Value
Number of gilts, *n*	4	7	4		
BW at breeding, *kg*	147.6	108.4	132.8	12	0.519
BW at d 25 of gestation, *kg*	141.2	107.5	133.8	11	0.517
BW gain, *kg/25 d*	-2.1	-1.0	1.0	1.7	0.815
Uterine weight, *kg*	2.95[a]	2.91[a]	2.54[b]	0.07	0.037
Total fetuses, *n*	12.7	14.9	15.3	0.6	0.325
Live fetuses, *n*	12.7	14.7	15.0	0.6	0.415
CL, *n*	16.3	16.9	16.0	0.4	0.664
Embryonic mortality, *%*	22.4	13.1	6.6	2.6	0.093
Weight of viable fetuses, *g*	6.32	6.81	6.07	0.21	0.366
Total placental weight, *g*	98.3	125.9	106.3	8.7	0.428
Fetal length, *cm*	1.90	1.84	1.75	0.03	0.161
Total ALF volume, *L*	1.20	1.15	0.98	0.05	0.199
Total AMF volume, *mL*	3.59	4.17	3.50	0.16	0.150

*Values are means with pooled SEM; means in a row with superscripts without a common letter differ, $P \leq 0.05$.

Table A-6 Adhesion force between the chorioallantoic membrane and epithelium of endometrium in gilts at d 25 of gestation fed diets supplemented with 0, 0.4 or 0.8% L-arginine (Arg) between d 14 and 25 of gestation *

Variable	Control	0.4% Arg	0.4% Arg	SEM	*P*-value
Adhesion force	6.8[b]	8.7[a]	7.5[ab]	0.29	0.025

* n = 10. Measured by the force to seperate the chorioallantoic membrane and the epithelium of endometrium (lowest force = 1; highest force = 10).

[a, b] Means with different superscripts differed ($P < 0.05$), as analyzed by one-way ANOVA.

Table A-7 Genes for which expression was up-regulated in porcine placentae by dietary supplementation with 0.8% arginine between d 14 and 25 of gestation in comparison with the control group*

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
TNNT3	NM_001001863	TNNT3	0.004	4.61
TC292911	TC292911	Unknown	0.001	4.49
CX058159	CX058159	Unknown	0.024	4.11
EW039857	NM_001742	Homo sapiens calcitonin receptor (CALCR) on chromosome 7	0.038	3.23
TC246681	XM_001788623	Bos taurus similar to endonuclease reverse transcriptase (LOC100140677) mRNA	0.024	2.87
CF362298	NM_001075988	Bos taurus transmembrane emp24 protein transport domain containing 6 (TMED6)	0.047	2.84
TC267763	TC267763	Unknown	0.049	2.60
DY425109	DY425109	Unknown	0.039	2.59
TC301630	NG_011688	Homo sapiens growth hormone receptor (GHR) on chromosome 5	0.050	2.59
AK231515	EU287432	Sus scrofa presenilin 2 mRNA complete cds	0.006	2.31
PGM1	NM_001076903	Bos taurus phosphoglucomutase 1 (PGM1)	0.034	2.30
TC278497	NM_018941	Homo sapiens ceroid-lipofuscinosis neuronal 8 (epilepsy progressive with mental retardation) (CLN8)	0.010	2.23
CK462699	CK462699	Unknown	0.028	2.15
TC289044	Q98459	Unknown	0.021	2.10
TC252173	Q8P941	Unknown	0.029	2.07
TC255795	Q9TT95	Unknown	0.041	2.00
BG896072	BG896072	Unknown	0.037	1.97
TC293083	TC293083	Unknown	0.011	1.89
TC290408	P60837	Unknown	0.043	1.87
PGM1	NM_001076903	Bos taurus phosphoglucomutase 1 (PGM1)	0.030	1.86
BX923086	NM_006377	Homo sapiens unc-13 homolog B (C. elegans) (UNC13B)	0.048	1.80
TC275071	AB091391	Sus scrofa RAG-2 gene for recombination activating protein 2	0.006	1.76
TC274023	NM_001097446	Sus scrofa apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3F (APOBEC3F)	0.005	1.70
BX666795	XM_001924347	Sus scrofa similar to SLCO3A1 protein (LOC100156054)	0.003	1.67
RH	NM_214378	RH	0.019	1.66
DN106369	NM_001109960	Canis lupus familiaris glycophorin A (MNS blood group) (GYPA)	0.022	1.64
TC279102	XR_021586	Pan troglodytes leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)	0.036	1.61
TC267605	NM_001143721	Sus scrofa 6-phosphofructo-2-kinase/fructose-2 6-biphosphatase 1 (PFKFB1)	0.025	1.55
EW660666	NM_001045886	Bos taurus phenazine biosynthesis-like protein domain containing (PBLD)	0.013	1.54
TC246855	AY208121	sus scrofa myostatin gene	0.022	1.54
TC295643	Q4TB76	Unknown	0.045	1.51
TC266252	Q4ZMW3	Unknown	0.019	1.50
AJ947838	NM_178177	Homo sapiens nicotinamide nucleotide adenylyltransferase 3 (NMNAT3)	0.022	1.50

Table A-7 Continued

Gene ID	Accession No.	GeneName	*P*-value	Fold Change
DN116615	Q74P05	Reverse transcriptase	0.047	1.49
BX674383	XM_001136904	Pan troglodytes hypothetical LOC465780 transcript variant 2 (LOC465780)	0.013	1.46
TC279371	XM_001496778	Equus caballus phosphoinositide-3-kinase regulatory subunit 4 (PIK3R4	0.027	1.45
DN102244	XM_511600	Pan troglodytes mannose receptor C type 2 (MRC2)	0.034	1.43
CK466870	Q2XYG1	Unknown	0.003	1.42
AY610045	XM_001924474	Sus scrofa similar to androgen-induced 1 (LOC100151943)	0.018	1.42
TC248086	Q00994	Unknown	0.040	1.41
TC276293	NM_001077619	homo sapiens UBX domain protein 2B (UBXN2B)	0.024	1.40
DY428406	NG_016762	Homo sapiens pyruvate dehydrogenase kinase isozyme 3 (PDK3) on chromosome X	0.042	1.40
AK236663	XM_873525	Bos taurus myoferlin transcript variant 12 (FER1L3)	0.031	1.39
DN110652	NM_174525	Beta-crystallin A4	0.041	1.39
BM484590	NM_174492	Bos taurus tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta polypeptide (YWHAH)	0.025	1.37
EW109654	NM_170589	Homo sapiens cancer susceptibility candidate 5 (CASC5) transcript variant 1	0.002	1.35
CF361829	A9YMB8	NADH dehydrogenase subunit 2	0.012	1.34
TC270961	Unknown	Unknown	0.024	1.34
LOC733663	Unknown	Unknown	0.037	1.34
CYP3A39	NM_214422	CYP3A39	0.027	1.34
GUCY2C	NM_214105	GUCY2C	0.025	1.33
AJ947745	NG_007956	cytochrome P450 family 20 subfamily A polypeptide 1 (CYP20A1)	0.022	1.33
AY609525	NM_001101198	Chromobox protein homolog 3	0.015	1.32
EW203657	A2AIM8	Talin 1	0.002	1.32
CJ019155	XM_001489617	Equus caballus dedicator of cytokinesis 11 (DOCK11)	0.006	1.32
AK233854	NM_001099022	Bos taurus Era G-protein-like 1 (E. coli) (ERAL1)	0.010	1.32
TC271032	Unknown	Unknown	0.036	1.31
HBE1	EW422073	HBE1	0.035	1.31
TC267213	Unknown	Unknown	0.026	1.30
TC255027	Unknown	Unknown	0.007	1.30
TC302833	NM_001165887	Homo sapiens zinc finger protein 268 (ZNF268) transcript variant 9	0.021	1.30
EW225983	Unknown	Unknown	0.034	1.28
TC263030	Unknown	Unknown	0.044	1.28
TC272293	Unknown	Unknown	0.019	1.28
AK234630	XM_001927389	sus scrofa FK506-binding protein (LOC100152728)	0.009	1.27
TC259073	XM_001488075	Equus caballus kelch-like 13 (Drosophila) (KLHL13)	0.021	1.27
BW980922	XM_001113023	Macaca mulatta dUTP pyrophosphatase isoform 2 transcript variant 4 (DUT)	0.021	1.27
ST3GAL-IV	NM_213757	Sus scrofa ST3 beta-galactoside alpha-2 3-sialyltransferase 4 (ST3GAL4) ST3GAL-IV	0.000	1.27

Table A-7 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
TC269098	Unknown	Unknown	0.008	1.27
AW429646	Unknown	Unknown	0.046	1.27
TC260428	Unknown	Unknown	0.019	1.26
AK236998	XM_847200	Canis familiaris similar to Nonhistone chromosomal protein HMG-17 (High-mobility group nucleosome binding domain 2) (LOC609853)	0.048	1.26
AK239509	AB529869	Sus scrofa PECR mRNA for peroxisomal trans-2-enoyl-CoA reductase	0.027	1.25
TC259570	Unknown	Unknown	0.017	1.24
TC246828	Unknown	Unknown	0.017	1.24
TC287179	NM_014857	Homo sapiens RAB GTPase activating protein 1-like (RABGAP1L) transcript variant 1 mRNA	0.001	1.24
TC258132	Unknown	Unknown	0.047	1.24
EW484397	NM_001103101	Bos taurus zinc finger protein 502 (ZNF502)	0.025	1.24
TC293624	AM229312	Porcine endogenous retrovirus C complete proviral genome clone PERV-C(1312)	0.032	1.24
TC277299	XM_534651	Canis familiaris similar to M-phase phosphoprotein 9 (LOC477453)	0.037	1.24
BX667232	XM_001925672	Sus scrofa similar to pecanex-like protein 1 (LOC100154536)	0.030	1.23
CN155716	EU617320	Sus scrofa small calcium-binding mitochondrial carrier 1 (SCAMC-1) mRNA	0.038	1.23
EW131859	XM_001498163	Equus caballus similar to ribosomal protein L9 (LOC100055158)	0.001	1.23
AK230973	XM_532879	Canis familiaris similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 transcript variant 1 (LOC475671)	0.047	1.23
AK237164	XM_534550	Canis familiaris similar to CG1218-PA transcript variant 1 (LOC477355)	0.013	1.23
EV880225	DQ629170	Sus scrofa RPS6 (RPS6) mRNA	0.017	1.22
CK467702	NM_001035277	cadherin 13 H-cadherin (heart) (CDH13)	0.013	1.22
AK232944	XM_535692	Canis familiaris similar to CG18769-PB isoform B (LOC478513)	0.018	1.22
TC245796	Unknown	Unknown	0.014	1.22
TC276127	Unknown	Unknown	0.010	1.22
TC292002	XM_001150978	Pan troglodytes similar to LUC7L2 protein transcript variant 3 (LOC739990)	0.049	1.22
TC278978	Unknown	Unknown	0.033	1.22
CK453467	XM_001924194	Sus scrofa similar to WD repeat and HMG-box DNA-binding protein 1 (Acidic nucleoplasmic DNA-binding protein 1) (And-1) (LOC100152808)	0.029	1.21
CD572284	AJ009912	Sus scrofa plp gene	0.006	1.21
BX670823	XM_001494501	Equus caballus similar to replication protein A3 14kDa (LOC100063929)	0.001	1.21
TC270552	XM_001503315	Equus caballus pumilio homolog 2 (Drosophila) (PUM2)	0.050	1.21
TC292269	Unknown	Unknown	0.047	1.21
CF366738	Unknown	Unknown	0.029	1.21
TC272391	Unknown	Unknown	0.033	1.21
CETN3	DN132970	CETN3	0.002	1.20

Table A-7 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
TC287511	NM_145647	Homo sapiens WD repeat domain 67 (WDR67) transcript variant 1	0.015	1.20
BG695764	NM_006122	Homo sapiens mannosidase alpha class 2A member 2 (MAN2A2)	0.011	1.20
TC298854	XM_580298	Bos taurus similar to trinucleotide repeat containing 6C (TNRC6C)	0.033	1.20
AK235662	NG_012170	Homo sapiens RAB23 member RAS oncogene family (RAB23) on chromosome 6	0.043	1.20
BX675945	XM_542777	Canis familiaris similar to SH3 domain-binding protein 5 (SH3 domain-binding protein that preferentially associates with BTK) (LOC485657)	0.033	1.20
TC258084	NM_006690	Homo sapiens matrix metallopeptidase 24 (membrane-inserted) (MMP24)	0.045	1.19
DY437500	NM_015203	Homo sapiens regulation of nuclear pre-mRNA domain containing 2 (RPRD2)	0.026	1.19
TC255075	Unknown	Unknown	0.009	1.19
TC284683	Unknown	Unknown	0.043	1.18
TC287282	Unknown	Unknown	0.033	1.18
EV898729	XR_045439	Sus scrofa misc_RNA (LOC100152987) miscRNA	0.017	1.18
HMGB2	NM_214063	HMGB2	0.045	1.18
DN125568	GQ184633	Sus scrofa cell division cycle 2 variant 1 (CDC2) mRNA	0.048	1.18
TC280036	Unknown	Unknown	0.020	1.17
TC276408	XR_042873	Bos taurus misc_RNA (LOC534434) miscRNA	0.041	1.17
EW299999	XM_001498308	Equus caballus similar to eukaryotic translation elongation factor 1 beta 2 (LOC100068470)	0.015	1.17
GADD45A	NM_001044599	GADD45A	0.044	1.17
BX672323	XM_001927571	Sus scrofa similar to PC4 and SFRS1-interacting protein (Lens epithelium-derived growth factor) (LOC100157597)	0.019	1.17
CN162044	NM_024947	Homo sapiens polyhomeotic homolog 3 (Drosophila) (PHC3)	0.029	1.17
TC277265	XM_001915541	Equus caballus similar to Transcription factor E2F3 (E2F-3) (LOC100052248)	0.043	1.16
AK233465	BC102499	Bos taurus LSM8 homolog U6 small nuclear RNA associated (S. cerevisiae) mRNA (cDNA clone MGC:127377 IMAGE:7953297)	0.048	1.16
AY609929	XM_001927909	Sus scrofa similar to DEK oncogene transcript variant 1 (LOC100156871)	0.040	1.16
TC248286	Unknown	Unknown	0.007	1.16
CD572531	XM_001929144	Sus scrofa similar to transmembrane 6 superfamily member 1 (LOC100155238)	0.048	1.16
TC251927	XM_001926317	Sus scrofa similar to TBCC domain containing 1 (LOC100154090)	0.013	1.16
TC274000	Unknown	Unknown	0.037	1.16
EW046833	NR_002211	Homo sapiens Meis homeobox 3 pseudogene 1 (MEIS3P1) non-coding RNA	0.029	1.16
EW304485	XM_531801	Canis familiaris similar to protein phosphatase 1B isoform 2 transcript variant 1 (LOC474573)	0.029	1.15
AY610084	GQ369460	Sus scrofa clone 1 F-box protein 7 (FBXO7) mRNA	0.046	1.15
AY609917	XM_001488642	Equus caballus similar to CCHC-type zinc finger nucleic acid binding protein transcript variant 1 (LOC100050146)	0.049	1.15
RPS29	NM_001001633	RPS29	0.031	1.15

Table A-7 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
DB800055	XM_001490197	Equus caballus cytidine and dCMP deaminase domain containing 1 (CDADC1)	0.040	1.15
ANG2	NM_213808	ANG2	0.046	1.14
CK467169	XM_001926109	Sus scrofa similar to SNX25 protein (LOC100157280)	0.018	1.14
TC258796	XM_001928025	Sus scrofa Calcineurin A protein transcript variant 2 (LOC396603)	0.049	1.14
AK235945	XM_001928672	Sus scrofa similar to FRA10AC1 protein transcript variant 1 (LOC100152110)	0.027	1.14
LOC414417	NM_001001635	Sus scrofa translation factor sui1-like protein (LOC414417)	0.040	1.14
AK230600	NM_001101220	Bos taurus dynein light chain Tctex-type 3 (DYNLT3)	0.026	1.13
EW593120	NM_001137619	Sus scrofa ribosomal protein S3A (RPS3A) mRNA	0.018	1.13
SCYE1	NM_001114283	Sus scrofa aminoacyl tRNA synthetase complex-interacting multifunctional protein 1 (AIMP1)	0.026	1.12
TC281417	XM_876757	Bos taurus similar to Heterogeneous nuclear ribonucleoprotein H (hnRNP H) transcript variant 26 (HNRPH1)	0.044	1.12
TC285283	XM_001493049	Equus caballus similar to pinin desmosome associated protein (LOC100060952)	0.020	1.12
TC248717	XM_001926594	Sus scrofa similar to ring finger protein 20 (LOC100154259)	0.040	1.12
TCTP	NM_214373	TCTP	0.027	1.12
AJ659363	XM_001925659	Sus scrofa similar to MGC165949 protein (LOC100158143)	0.036	1.11
AY609728	BC112734	Bos taurus mitochondrial ribosomal protein L30 mRNA (cDNA clone MGC:137661 IMAGE:8165094)	0.034	1.09

* Determined by microarray

Table A-8 Genes for which expression was down-regulated in porcine placentae by dietary supplementation with 0.8% arginine between d 14 and 25 of gestation in comparison with the control group*

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
AJ964783	O48246	Cytochrome b	0.001	0.15
BI341657	XM_001926447	Sus scrofa similar to RasGEF domain family member 1A (LOC100156683)	0.013	0.18
TC273367	NG_008926	Homo sapiens asparagine-linked glycosylation 8 alpha-1 3-glucosyltransferase homolog (S. cerevisiae) (ALG8) on chromosome 11	0.010	0.20
TC257543	XM_001927988	Sus scrofa similar to RU2S (LOC100153025)	0.015	0.23
DN100844	FJ263680	Sus scrofa clone CH242-27L18 acetyl-coenzyme A carboxylase alpha (ACACA) gene	0.003	0.27
NP321728	AF274712	Sus scrofa pig endogenous retrovirus group Beta3 polymerase gene	0.014	0.29
TC267851	Unknown	Unknown	0.004	0.30
AK235514	XM_001151324	Pan troglodytes hypothetical protein LOC745470 (LOC745470)	0.037	0.30
BI360386	XM_001083849	Macaca mulatta similar to oncostatin M receptor transcript variant 2 (LOC693569)	0.009	0.31
AREG	NM_214376	Sus scrofa amphiregulin (AREG)	0.045	0.31
CF178669	AJ427478	Sus scrofa ASIP gene for agouti signalling protein and AHCY gene for S-adenosylhomocysteine hydrolase	0.023	0.33
TC257832	NM_001143983	Homo sapiens chordin-like 1 (CHRDL1) transcript variant 4	0.027	0.33
TC290976	A7AUI6	41-2 protein antigen	0.006	0.35
CX061534	NM_015602	Homo sapiens torsin A interacting protein 1 (TOR1AIP1)	0.007	0.40
CN158380	Q9QF03	Envelope glycoprotein V3 region	0.024	0.41
TC301037	BC152456	Homo sapiens doublecortin-like kinase 1 mRNA (cDNA clone MGC:176710 IMAGE:8862589)	0.012	0.41
DY414270	NM_003828	Homo sapiens myotubularin related protein 1 (MTMR1) on chromosome X	0.010	0.42
TC251548	XM_600715	Bos taurus similar to kelch-like 18 (LOC522434)	0.046	0.42
AK232310	NM_001009778	Ovis aries aldehyde dehydrogenase 1 family member A1 (ALDH1A1)	0.041	0.43
TC269985	A0QK03	UDP-glucose 6-dehydrogenase	0.029	0.44

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
BP997825	XM_864249	Bos taurus similar to Zinc finger ZZ-type and EF-hand domain-containing protein 1 transcript variant 1 (ZZEF1)	0.045	0.44
RHBG	NM_213996	Sus scrofa Rh family B glycoprotein (RHBG)	0.006	0.45
EW614253	Unknown	Unknown	0.038	0.45
IGFBP2	NM_214003	IGFBP2	0.002	0.47
OSTN	NM_001098597	OSTN	0.025	0.47
CXCL2	NM_001001861	CXCL2	0.013	0.49
TC278652	NM_214003	Sus scrofa insulin-like growth factor binding protein 2 (IGFBP2)	0.002	0.49
CYP2C33	NM_214414	Sus scrofa cytochrome P450 2C33 (CYP2C33)	0.037	0.50
AK238519	XM_874285	Bos taurus similar to phosphofructokinase platelet transcript variant 12 (PFKP)	0.025	0.50
BX674271	XM_001489186	Equus caballus calpain 6 (CAPN6)	0.038	0.50
TC280345	XM_864245.3	Bos taurus similar to golgin 97 transcript variant 2 (GOLGA1)	0.018	0.50
DPPIV	NM_214257	Sus scrofa dipeptidyl-peptidase 4 (DPP4)	0.030	0.51
EV918706	Unknown	Unknown	0.006	0.51
OSTN	NM_001098597	OSTN	0.048	0.51
OSTN	NM_001098597	OSTN	0.045	0.53
OSTN	NM_001098597	OSTN	0.043	0.54
BX924685	XM_613086	Bos taurus myotubularin 1 (MTM1)	0.038	0.54
CH242-235B3.1	NM_001105290	Sus scrofa bone morphogenetic protein 7 (Bmp7)	0.030	0.55
ARG1	NM_214048	Sus scrofa arginase liver (ARG1)	0.014	0.55
TC282384	NM_207334	Homo sapiens family with sequence similarity 43 member B (FAM43B)	0.046	0.56
CO989438	XM_001156630	Pan troglodytes calcium-activated potassium channel beta 4 subunit (KCNMB4)	0.017	0.56
TC301414	Unknown	Unknown	0.036	0.56

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
DAB1	NM_001097442	DAB1	0.021	0.57
BP441060	BC151769	Bos taurus MARVEL domain containing 2 mRNA (cDNA clone IMAGE:7942171)	0.040	0.57
TC288678	Unknown	Unknown	0.022	0.57
TC293066	Unknown	Unknown	0.026	0.57
TC270858	AF228059	Sus scrofa decay-accelerating factor CD55 mRNA complete cds	0.026	0.58
CV878027	XM_001926796	Sus scrofa similar to SAMD4A protein (LOC100156574)	0.018	0.58
TC290589	BC047235	Homo sapiens upstream binding protein 1 (LBP-1a)	0.005	0.58
CA513725	XM_533297	Canis familiaris similar to Heat shock 70 kDa protein 4L (Osmotic stress protein 94) (Heat shock 70-related protein APG-1) transcript variant 1 (LOC476089)	0.016	0.58
EV881857	XM_001493559	Equus caballus solute carrier family 4 sodium bicarbonate cotransporter member 7 (SLC4A7)	0.009	0.59
TC289774	Unknown	Unknown	0.013	0.59
DB803904	XM_524602	Pan troglodytes leucine zipper protein 1 transcript variant 3 (LUZP1)	0.047	0.60
TC266622	Unknown	Unknown	0.018	0.60
TC286353	NM_001099064	Bos taurus CUE domain containing 1 (CUEDC1)	0.007	0.60
ID2	NM_001037965	ID2	0.007	0.61
DY419449	XM_001492190	Equus caballus similar to TPR repeat-containing protein C1orf34 homolog (LOC100062643)	0.025	0.61
CN159399	NM_001128506	Bos taurus chromatin modifying protein 4B (CHMP4B)	0.012	0.61
AK230591	XM_853643	Canis familiaris similar to ornithine decarboxylase antizyme inhibitor transcript variant 10 (LOC475058)	0.016	0.62
AK234050	NM_001075748	Bos taurus CCCTC-binding factor (zinc finger protein) (CTCF)	0.047	0.62
AK234300	NM_001038191	Bos taurus RIB43A domain with coiled-coils 2 (RIBC2)	0.005	0.63
AJ955807	Unknown	Unknown	0.006	0.63
DN132980	Unknown	Unknown	0.008	0.63

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
AK232193	XM_532475	Canis familiaris similar to islet cell autoantigen 1 isoform 1 transcript variant 2 (LOC475242)	0.040	0.63
TC247541	XM_001489418	Equus caballus pericentriolar material 1 transcript variant 1 (PCM1)	0.015	0.64
CF181641	XM_001252266	Bos taurus similar to dystonin transcript variant 1 (DST)	0.015	0.64
TC261918	XM_001916323	Equus caballus AHNAK nucleoprotein (AHNAK)	0.006	0.64
TC286370	XM_001927561	Sus scrofa hypothetical protein LOC100155972 (LOC100155972)	0.029	0.64
AK232477	NM_001077833	Bos taurus protein kinase C zeta (PRKCZ)	0.047	0.64
CRP	NM_213844	CRP	0.028	0.65
AK233736	XM_001927836	Sus scrofa similar to Down syndrome critical region gene 1-like 1 protein (LOC100153773)	0.033	0.65
AK232606	XM_001102892	Macaca mulatta similar to Protein C14orf133 homolog transcript variant 2 (LOC706315)	0.046	0.65
RXRA	DQ279926	Sus scrofa retinoid X receptor alpha transcript variant 1 (RXRalpha)	0.047	0.65
EW635567	XM_543735	Canis familiaris similar to solute carrier family 2 (facilitated glucose transporter) member 13 (LOC486609)	0.022	0.65
TC294557	XR_025023	Pan troglodytes similar to PAR-6 beta (LOC458334)	0.012	0.65
CV867559	AJ009912	Sus scrofa plp gene	0.043	0.65
TC274128	Unknown	Unknown	0.010	0.66
TC246408	Unknown	Unknown	0.029	0.66
TC300920	XM_001493065	Equus caballus similar to zinc finger protein 783 (LOC100060969)	0.013	0.66
EV918706	AJ560639	Homo sapiens mRNA for aminopeptidase O (APO gene)	0.046	0.67
PPARGC-1	NM_213963	PPARGC-1	0.034	0.67
TC258272	NM_001162429	Bos taurus similar to programmed cell death 6 interacting protein transcript variant 1 (PDCD6IP)	0.033	0.67
TC239249	NM_001128934	Homo sapiens synaptopodin 2 (SYNPO2) transcript variant 3	0.006	0.67
EW196031	NM_052885	Homo sapiens solute carrier family 2 (facilitated glucose transporter) member 13 (SLC2A13)	0.050	0.67
TC278516	Unknown	Unknown	0.042	0.68

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
HSPA5	X92446	S.scrofa mRNA for grp78 protein	0.019	0.68
FGFR2	NM_001099924	Sus scrofa fibroblast growth factor receptor 2 (FGFR2)	0.019	0.68
KLF13	NM_001011505	KLF13	0.006	0.68
LOC100037960	NM_001097457	Sus scrofa major facilitator superfamily domain containing 6 (MFSD6)	0.044	0.68
TC291433	NM_015691	Homo sapiens WWC family member 3 (WWC3)	0.021	0.69
AK236939	BC128144	Homo sapiens component of oligomeric golgi complex 4 mRNA (cDNA clone IMAGE:40112536)	0.008	0.69
IFNAR1	NM_213772	IFNAR1	0.012	0.69
AK235751	NM_001172415	Homo sapiens BCL2-associated athanogene (BAG1) transcript variant 1	0.035	0.69
TC273652	NM_001098104	Bos taurus par-6 partitioning defective 6 homolog beta (C. elegans) (PARD6B)	0.044	0.69
CK454680	XM_001927223	Sus scrofa similar to PAB-dependent poly(A)-specific ribonuclease subunit 3 (hPan3) (LOC100157385)	0.015	0.69
CK458354	XM_537163	Canis familiaris similar to niban protein isoform 2 (LOC480041)	0.049	0.69
CK467413	NM_001033348	Mus musculus Ral GTPase activating protein alpha subunit 2 (catalytic) (Ralgapa2)	0.030	0.69
TC278165	Unknown	Unknown	0.030	0.70
HADHA	NM_213962	HADHA	0.037	0.70
TC272775	NM_001003022	Canis lupus familiaris glucocorticoid receptor DNA binding factor 1 (GRLF1)	0.000	0.70
ROD1	NM_001077215	ROD1	0.025	0.70
RND3	NM_214296	RND3	0.039	0.70
AKAP13	XM_001926474	Sus scrofa A kinase (PRKA) anchor protein 13 (AKAP13)	0.016	0.70
DT324917	XM_001924319	Sus scrofa similar to DnaJ homolog subfamily B member 12 (LOC100156234)	0.026	0.70
AY609497	BC010370	Homo sapiens tumor suppressor candidate 3 mRNA (cDNA clone MGC:13453 IMAGE:4334284)	0.015	0.70
TC268146	Unknown	Unknown	0.006	0.70
AK234157	NM_001024571	Bos taurus chromosome 12 open reading frame 41 ortholog (C5H12orf41)	0.007	0.70

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
AK236119	NM_001135021	Homo sapiens ELMO/CED-12 domain containing 3 (ELMOD3) transcript variant 2	0.005	0.70
AK236625	XM_001088315	Macaca mulatta checkpoint suppressor 1 transcript variant 5 (CHES1)	0.040	0.70
BP434590	NM_024776	Homo sapiens NKF3 kinase family member (SGK269)	0.003	0.70
PPARGC-1	NM_213963	PPARGC-1	0.037	0.71
TC257240	XM_001925375	Sus scrofa similar to PR domain containing 1 with ZNF domain transcript variant 2 (LOC100154284)	0.042	0.71
AK234388	NM_001162886	Sus scrofa v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1)	0.039	0.71
SM22A	NM_001110134	Equus caballus transgelin (TAGLN) mRNA	0.035	0.71
AK235923	XM_510991	Pan troglodytes hypothetical LOC454118 transcript variant 5 (LOC454118)	0.021	0.71
EW263623	XM_001139429	Pan troglodytes similar to CREB transcript variant 2 (LOC459901)	0.011	0.71
GPAT	AY284842	GPAT	0.016	0.71
TC277497	AB120429	kinesin-family protein KIF1Bbeta3 {Rattus norvegicus}	0.049	0.71
LOC780415	NM_001078670	Sus scrofa interferon regulatory factor 9 (LOC780415)	0.024	0.71
AK235466	DQ105589S2	Sus scrofa CDP-diacylglycerol synthase 2 (CDS2) mRNA partial cds	0.013	0.71
TRAF6	NM_001105286	TRAF6	0.023	0.71
CV877363	XM_001500207	Equus caballus similar to DEAH (Asp-Glu-Ala-His) box polypeptide 38 (LOC100054293)	0.039	0.71
TC274812	Unknown	Unknown	0.000	0.71
LOC448984	Unknown	Unknown	0.040	0.71
TC241377	Unknown	Unknown	0.034	0.71
AK240289	XM_001498786	Equus caballus CTTNBP2 N-terminal like (CTTNBP2NL)	0.007	0.71
AK232343	AK232343	Unknown	0.025	0.71
HPD	NM_214224	HPD	0.007	0.71
AK234841	NM_016075	Homo sapiens vacuolar protein sorting 36 homolog (S. cerevisiae) (VPS36)	0.048	0.71
AK239867	BC063709	Homo sapiens hypothetical protein LOC126917 mRNA (cDNA clone IMAGE:4801936) partial cds	0.028	0.71
PRKAA2	NM_214266	Sus scrofa protein kinase AMP-activated alpha 2 catalytic subunit (PRKAA2)	0.025	0.71

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
BX923341	NM_001098924	Bos taurus ubiquitin specific peptidase 10 (USP10)	0.008	0.72
TC299183	X58430	Homo sapiens Hox1.8 gene for homeobox protein	0.008	0.72
TC247962	Unknown	Unknown	0.015	0.72
PPARGC-1	NM_213963	Sus scrofa peroxisome proliferator activated receptor gamma coactivator 1 (PPARGC-1) mRNA [NM_213963]	0.032	0.72
PPARGC-1	NM_213963	Sus scrofa peroxisome proliferator activated receptor gamma coactivator 1 (PPARGC-1) mRNA [NM_213963]	0.036	0.72
EV929901	XM_870793	Bos taurus similar to Protein FAM101B (LOC618459)	0.017	0.72
CV872688	XM_001926594	Sus scrofa similar to ring finger protein 20 (LOC100154259)	0.044	0.72
AK233041	NM_001133129	Pongo abelii zinc finger with KRAB and SCAN domains 1 (ZKSCAN1)	0.011	0.72
AK238640	NM_006466	Homo sapiens polymerase (RNA) III (DNA directed) polypeptide F 39 kDa (POLR3F)	0.006	0.72
PSEN2	NM_001078666	PSEN2	0.034	0.72
ATP6V1H	NM_214240	ATP6V1H	0.040	0.72
AK235914	XM_001168855	Pan troglodytes TMEM9 domain family member B transcript variant 2 (TMEM9B)	0.032	0.72
AK240543	XM_001928943	Sus scrofa similar to Ubiquitin domain containing 1 (LOC100156395)	0.025	0.73
CJ012713	XM_001926430	Sus scrofa similar to mast cell proteinase-3 (LOC100155263)	0.039	0.73
EV978656	NG_011790	Homo sapiens ATP-binding cassette sub-family A (ABC1) member 3 (ABCA3) on chromosome 16	0.027	0.73
TC273277	Unknown	Unknown	0.036	0.73
DR083551	NM_001075289	Bos taurus activating transcription factor 1 (ATF1)	0.013	0.73
AK240475	XM_001927539	Sus scrofa similar to general transcription factor IIH polypeptide 3 34kDa (LOC100152121)	0.006	0.73
EW069422	XM_528043	Pan troglodytes Smad ubiquitination regulatory factor 1 (SMURF1)	0.030	0.73
TC283069	XM_001156044	Pan troglodytes sideroflexin 1 transcript variant 1 (SFXN1)	0.027	0.73
BX665101	Unknown	Unknown	0.035	0.73
TC288457	XM_869579	Bos taurus similar to EMI domain containing 2 (EMID2)	0.036	0.73
BIN1	NM_001097440	BIN1	0.036	0.73

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
TC283021	Unknown	Unknown	0.043	0.73
AK232443	XM_001501049	Equus caballus similar to Cullin-5 (CUL-5) (Vasopressin-activated calcium-mobilizing receptor) (VACM-1) (LOC100061726)	0.014	0.73
LOC396848	NM_176636	Bos taurus myosin light chain kinase (MYLK)	0.009	0.73
CJ038329	P47843	Solute carrier family 2 facilitated glucose transporter member 3	0.028	0.73
DN101455	XM_001788616	Bos taurus similar to SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily c member 1 (LOC522045)	0.016	0.73
LOC100038015	NM_001162401	Sus scrofa lysophosphatidic acid receptor 2 (LPAR2)	0.048	0.73
E4	NM_213947	E4	0.026	0.73
FGFR2	NM_001099924	FGFR2	0.048	0.73
TC266079	Unknown	Unknown	0.026	0.73
AK236120	XM_001929348	Sus scrofa similar to RIKEN cDNA 2810048G17 (LOC100157433)	0.014	0.73
CV876228	XM_001093735	Macaca mulatta similar to chromatin modifying protein 4C (LOC702310)	0.023	0.73
BI184146	XM_001927725	Sus scrofa prostaglandin F2 receptor negative regulator (PTGFRN)	0.002	0.73
ROD1	ROD1	ROD1	0.018	0.74
CV875504	XM_001926134	Sus scrofa similar to chloride channel 3 (LOC100156049)	0.040	0.74
BW961052	XM_001928697	Sus scrofa similar to coatomer protein complex subunit alpha transcript variant 1 (LOC100157296)	0.016	0.74
AK234049	XM_001112035	Macaca mulatta similar to SP140 nuclear body protein isoform 1 transcript variant 2 (LOC710925)	0.003	0.74
TC260069	Unknown	Unknown	0.033	0.74
CB285502	XM_001162866	Pan troglodytes PR domain containing 4 transcript variant 2 (PRDM4)	0.045	0.74
DN106070	NM_001105626	Bos taurus tweety homolog 2 (Drosophila) (TTYH2)	0.029	0.74
BX919286	XM_001493063	Equus caballus similar to tetratricopeptide repeat domain 3 (LOC100060967)	0.046	0.74
PNPLA2	NM_001098605	PNPLA2	0.014	0.74
TC289012	Unknown	Unknown	0.026	0.74

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
AK232121	Q9ASK4	Putative LRR receptor-like protein kinase	0.037	0.74
TC278409	Unknown	Unknown	0.014	0.74
AK230999	BC010355	Homo sapiens zinc finger protein 638 mRNA (cDNA clone IMAGE:4248516) with apparent retained intron	0.007	0.74
CJ014614	AF480462	Homo sapiens mixed lineage kinase-related kinase MRK-beta mRNA	0.022	0.75
TC268607	Unknown	Unknown	0.026	0.75
AK234388	NM_001162886	Sus scrofa v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1)	0.013	0.75
CN157587	NM_014611	Homo sapiens MDN1 midasin homolog (yeast) (MDN1)	0.003	0.75
AK231618	NM_152305	Homo sapiens KTEL (Lys-Tyr-Glu-Leu) containing 1 (KTELC1) transcript variant 1	0.014	0.75
CF361296	Unknown	Unknown	0.021	0.75
BP165217	NM_145160	Homo sapiens mitogen-activated protein kinase kinase 5 (MAP2K5)	0.040	0.75
EW259004	NM_024091	Homo sapiens FAST kinase domains 3 (FASTKD3)	0.022	0.75
TC257345	Unknown	Unknown	0.033	0.75
HSP90	NM_213973	HSP90	0.036	0.75
AJ940394	XM_001925507	Sus scrofa similar to Protein strawberry notch homolog 1 (Monocyte protein 3) (MOP-3) (LOC100158131)	0.023	0.75
FBP	NM_213830	FBP	0.029	0.75
AK240409	BC109100	Homo sapiens zinc finger protein 75D mRNA (cDNA clone MGC:126327 IMAGE:40034784)	0.011	0.75
EW046701	BC157843	Homo sapiens protein phosphatase 1H (PP2C domain containing) mRNA (cDNA clone MGC:189738 IMAGE:9057062)	0.026	0.75
TC270146	AB209365	Homo sapiens mRNA for diacylglycerol kinase epsilon variant protein	0.023	0.76
TC294387	Unknown	Unknown	0.006	0.76
DN107931	BC143266	Homo sapiens uveal autoantigen with coiled-coil domains and ankyrin repeats mRNA (cDNA clone MGC:176785 IMAGE:9051768)	0.048	0.76
TC266056	XM_001927219	Sus scrofa similar to single-strand selective monofunctional uracil DNA glycosylase (LOC100153912)	0.011	0.76
EW214587	NM_001037806	Homo sapiens NCK-associated protein 5-like (NCKAP5L)	0.021	0.76

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
AY609622	AY609622	Sus scrofa similar to small nuclear RNA activating complex polypeptide 3 50kDa (LOC100156386)	0.037	0.76
EW656850	Unknown	Unknown	0.042	0.76
AK234698	NM_005723	Homo sapiens tetraspanin 5 (TSPAN5)	0.019	0.76
AK231358	XM_001496368	Equus caballus chromodomain helicase DNA binding protein 4 (CHD4)	0.034	0.76
TC250538	NM_014997	Homo sapiens kelch domain containing 10 (KLHDC10)	0.005	0.76
DT327635	NM_015175	Homo sapiens neurobeachin-like 2 (NBEAL2)	0.017	0.76
TC299692	NM_001025107	Homo sapiens adenosine deaminase RNA-specific (ADAR)	0.046	0.76
DY420532	NM_017902	Homo sapiens hypoxia inducible factor 1 alpha subunit inhibitor (HIF1AN)	0.047	0.76
BX918062	BC000030	Homo sapiens Wolf-Hirschhorn syndrome candidate 1-like 1 mRNA (cDNA clone IMAGE:3505788)	0.044	0.76
CN157824	NM_052855	Homo sapiens ankyrin repeat domain 40 (ANKRD40)	0.022	0.77
INSIG1	INSIG1	INSIG1	0.017	0.77
TC256419	XM_589799	Bos taurus similar to zinc finger and BTB domain containing 38 transcript variant 1 (ZBTB38)	0.020	0.77
TC261725	NM_015459	Homo sapiens atlastin GTPase 3 (ATL3)	0.034	0.77
EW054519	NM_014016	Homo sapiens SAC1 suppressor of actin mutations 1-like (yeast) (SACM1L)	0.030	0.77
TC290580	Unknown	Unknown	0.047	0.77
TC297603	Unknown	Unknown	0.048	0.77
BW977049	NM_022750	Homo sapiens poly (ADP-ribose) polymerase family member 12 (PARP12)	0.027	0.77
NR1H3	NM_001101814	NR1H3	0.028	0.77
TC265732	Unknown	Unknown	0.049	0.77
TC278658	Unknown	Unknown	0.034	0.77
TC275612	Unknown	Unknown	0.006	0.77
AK236642	BC011558	Homo sapiens nuclear factor (erythroid-derived 2)-like 2 mRNA (cDNA clone MGC:20033 IMAGE:4548874)	0.027	0.77

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
AK236072	XM_001150552	Pan troglodytes BTB (POZ) domain containing 7 (BTBD7)	0.010	0.77
TC277856	Unknown	Unknown	0.011	0.77
AK231141	XM_857278	Canis familiaris similar to helicase with zinc finger domain transcript variant 7 (LOC490907)	0.038	0.77
YWHAZ	XM_001927228	Sus scrofa tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ)	0.013	0.77
CX060615	XM_001926596	Sus scrofa similar to HBS1-like protein (LOC100155814)	0.041	0.77
TC273432	NM_001075242	Bos taurus KIAA1737 (KIAA1737)	0.049	0.77
AK233400	BT026260	Bos taurus Wiskott-Aldrich syndrome-like (WASL)	0.028	0.77
BX926031	NR_002323	Homo sapiens taurine upregulated 1 (non-protein coding) (TUG1)	0.001	0.77
AJ963284	Unknown	Unknown	0.000	0.77
DY426447	NM_001081544	Bos taurus leucine zipper down-regulated in cancer 1 (LDOC1)	0.044	0.77
BI399717	Unknown	Unknown	0.030	0.77
AJ655057	NM_003274	Homo sapiens trafficking protein particle complex 10 (TRAPPC10)	0.033	0.78
TC288314	Unknown	Unknown	0.029	0.78
CJ038028	XM_862420	Canis familiaris similar to filamin A interacting protein 1 transcript variant 4 (LOC481882)	0.003	0.78
DY419617	NM_001076046	Bos taurus small nuclear ribonucleoprotein 70kDa (U1) (SNRNP70)	0.044	0.78
AK233061	XM_001926938	Sus scrofa similar to Uncharacterized protein C20orf4 homolog (LOC100152525)	0.047	0.78
TC300581	XM_001928761	Sus scrofa similar to EP300 interacting inhibitor of differentiation 1 (LOC100155122)	0.021	0.78
DN103217	XM_847823	Canis familiaris similar to bruno-like 4 RNA binding protein transcript variant 1 (LOC610838)	0.025	0.78
AK236656	NM_001105501	Bos taurus ubiquitin family domain containing 1 (UBFD1)	0.011	0.78
TC298889	Unknown	Unknown	0.026	0.78
EW078178	XM_863956	Bos taurus similar to zinc finger protein 403 transcript variant 2 (GGNBP2) mRNA	0.024	0.78
TC239883	Unknown	Unknown	0.036	0.78
CJ022253	Unknown	Unknown	0.030	0.78
TC272845	Unknown	Unknown	0.040	0.78

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
PAP7	NM_001009581	PAP7	0.037	0.78
TC283651	Unknown	Unknown	0.008	0.78
TC272853	XM_001916241	Equus caballus SEC31 homolog A (S. cerevisiae) (SEC31A)	0.042	0.78
AK234063	XM_875085	Bos taurus similar to Dynactin subunit 4 (Dynactin subunit p62) transcript variant 3 (DCTN4)	0.043	0.79
DB801941	XM_001167842	Pan troglodytes adaptor-related protein complex 3 sigma 2 subunit transcript variant 1 (AP3S2)	0.024	0.79
AJ949764	Unknown	Unknown	0.020	0.79
EW044715	Unknown	Unknown	0.021	0.79
BX926252	Unknown	Unknown	0.031	0.79
AJ955195	XM_001929149	Sus scrofa similar to transmembrane protein 77 (LOC100158051)	0.036	0.79
TC257321	NM_014817	Homo sapiens TLR4 interactor with leucine rich repeats (TRIL)	0.045	0.79
CJ029886	XM_002708672	Oryctolagus cuniculus integrator complex subunit 4 (LOC100350308)	0.023	0.79
TC289051	Unknown	Unknown	0.035	0.79
AK237448	XM_001928092	Sus scrofa similar to Ras-related protein Rab-35 (Rab-1C) (GTP-binding protein RAY) (LOC100151805)	0.033	0.79
TC252571	BC025865	Mus musculus vacuolar protein sorting 37C (yeast) (Vps37c) mRNA	0.015	0.79
AK234427	XM_001928746	Sus scrofa similar to adenosine deaminase-like protein (predicted) (LOC100155388)	0.046	0.79
TC273185	NM_024139	Rattus norvegicus calcium binding protein p22 (Chp)	0.040	0.79
NOG	XM_001104355	Macaca mulatta similar to Noggin precursor (NOG)	0.036	0.79
AJ962580	XM_587548	Bos taurus similar to Inactive phospholipase C-like protein 2 (Phospholipase C epsilon 2) (Phospholipase C-L2) (PLC-L(2)) (PLC-L2) (PLCL2)	0.046	0.79
TC275311	Unknown	Unknown	0.031	0.79
TC266450	NM_001034841	Homo sapiens inositol 1 4 5-triphosphate receptor interacting protein-like 2 (ITPRIPL2) transcript variant 2 non-coding RNA	0.035	0.79
AJ943355	XM_001148863	Pan troglodytes haloacid dehalogenase-like hydrolase domain containing 2 transcript variant 5 (HDHD2)	0.020	0.79

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
CK464272	NM_001167741	Homo sapiens metaxin 3 (MTX3) transcript variant 1	0.027	0.79
AK231096	BC149255	Bos taurus signal-regulatory protein alpha mRNA (cDNA clone IMAGE:8115519)	0.047	0.79
TC278200	XM_001925656	Sus scrofa similar to procollagen type IV alpha 1 (LOC100151842)	0.038	0.79
AK236606	XM_001151850	Pan troglodytes A-kinase anchor protein 11 transcript variant 4 (AKAP11)	0.013	0.79
TC299921	Unknown	Unknown	0.026	0.79
EW027983	NM_025107	Homo sapiens myc target 1 (MYCT1)	0.015	0.79
TC284254	XM_851809	Canis familiaris similar to serine/threonine-protein kinase PRP4K transcript variant 15 (LOC488199)	0.033	0.79
AK237711	XM_001488261	Equus caballus similar to PHD finger protein 6 (LOC100054177)	0.012	0.79
EW589095	Unknown	Unknown	0.047	0.79
AK233313	XM_545906	Canis familiaris similar to SH3 domain and tetratricopeptide repeats 1 (LOC488788)	0.023	0.79
TC300859	AB007872	Homo sapiens zinc finger protein 264 (ZNF264)	0.019	0.80
TC245799	Unknown	Unknown	0.007	0.80
TC255319	XM_001495384	Equus caballus similar to SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily c member 1 (LOC100064492)	0.028	0.80
EW420742	XM_542033	Canis familiaris similar to immediate early response 2 (LOC484917)	0.025	0.80
AK237711	XM_001488261	Equus caballus similar to PHD finger protein 6 (LOC100054177)	0.009	0.80
AK235686	XM_001925381	Sus scrofa similar to insulin-degrading enzyme (LOC100155309)	0.016	0.80
CV874785	XM_001788601	Bos taurus similar to FIP1-like 1 transcript variant 2 (LOC100138550)	0.029	0.80
AK232495	XM_537970	Canis familiaris similar to Gamma-taxilin (Lipopolysaccharide specific response protein 5) (LOC480853)	0.048	0.80
DT325774	XM_001109176	Macaca mulatta similar to solute carrier family 39 (zinc transporter) member 9 transcript variant 2 (LOC712430)	0.034	0.80
AK237044	XM_001499279	Equus caballus similar to ubiquitin-conjugating enzyme E2Z (LOC100069535)	0.034	0.80
TC285668	Unknown	Unknown	0.036	0.80

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
BX676607	XM_001928537	Sus scrofa similar to Platelet receptor Gi24 (LOC100154373)	0.049	0.80
EW369833	XM_606371	Bos taurus par-3 partitioning defective 3 homolog (C. elegans) (PARD3)	0.042	0.80
BP434962	XM_001928205	Sus scrofa similar to T-complex protein 1 subunit alpha (TCP-1-alpha) (CCT-alpha) (LOC100153485)	0.044	0.80
AK238011	NM_001105646	Bos taurus aldehyde dehydrogenase 4 family member A1 (ALDH4A1) nuclear gene encoding mitochondrial protein	0.046	0.80
AK234809	NM_001075556	Bos taurus small nuclear ribonucleoprotein 35kDa (U11/U12) (SNRNP35)	0.012	0.80
AY610027	XM_001926484	Sus scrofa similar to ARV1 homolog (S. cerevisiae) (LOC100151849)	0.008	0.80
AJ944053	XM_507955	Pan troglodytes phosphoinositide-3-kinase adaptor protein 1 (PIK3AP1)	0.012	0.81
TC294130	Unknown	Unknown	0.032	0.81
TC281094	Unknown	Unknown	0.018	0.81
TC246475	Unknown	Unknown	0.033	0.81
DN100853	AF339885	Sus scrofa mannose-6-phosphate/insulin-like growth factor II receptor (m6p/igf2r) mRNA partial cds	0.038	0.81
CN161695	XM_545559	Canis familiaris similar to Integrin alpha-V precursor (Vitronectin receptor alpha subunit) (CD51 antigen) transcript variant 1 (LOC488437)	0.020	0.81
TC270371	Unknown	Unknown	0.006	0.81
ODC	ODC	ODC	0.037	0.81
TC268131	XM_001150696	Pan troglodytes HMG-BOX transcription factor BBX transcript variant 13 (BBX)	0.042	0.81
DN125536	XM_596205	Bos taurus similar to LEM domain containing 2 transcript variant 1 (LEMD2)	0.029	0.81
AY609497	XM_001488031	Equus caballus similar to tumor suppressor candidate 3 transcript variant 1 (LOC100049974)	0.035	0.81
TC273861	Unknown	Unknown	0.036	0.81
TC268537	AF070556	Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta polypeptide (YWHAQ)	0.001	0.81
BX671510	NM_001075838	Bos taurus myosin ID (MYO1D)	0.009	0.81
DN134244	XM_001106950	Macaca mulatta A kinase (PRKA) anchor protein 2 (AKAP2)	0.022	0.81

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
EW533203	XM_001139700	Pan troglodytes similar to APOLD1 protein (LOC738701)	0.013	0.81
TC289943	Unknown	Unknown	0.045	0.81
AK232486	NM_001159481	Bos taurus pyruvate dehydrogenase kinase isozyme 2 (PDK2) transcript variant 1	0.042	0.81
TC272320	BC107228	Mus musculus carcinoembryonic antigen-related cell adhesion molecule 19 mRNA (cDNA clone MGC:130156 IMAGE:40051962)	0.026	0.81
TAF1B	TAF1B	TAF1B	0.045	0.81
DB802589	Unknown	Unknown	0.017	0.81
CF177025	NM_001105048	Bos taurus immunoglobulin superfamily member 1 (IGSF1)	0.034	0.82
TC275305	XM_856269	Canis familiaris similar to RNA-binding protein 6 (RNA binding motif protein 6) (RNA-binding protein DEF-3) (Lung cancer antigen NY-LU-12) (Protein G16) transcript variant 2 (LOC608064)	0.027	0.82
DR066002	BC103112	Bos taurus CD36 molecule (thrombospondin receptor) mRNA (cDNA clone MGC:128284 IMAGE:7985341) complete cds	0.044	0.82
AK232065	NM_020921	Homo sapiens ninein (GSK3B interacting protein) (NIN) transcript variant 2	0.027	0.82
EW120221	NM_001014942	Bos taurus retinoic acid receptor alpha (RARA)	0.022	0.82
TC249497	Unknown	Unknown	0.027	0.82
TC296294	NM_022740	Homo sapiens homeodomain interacting protein kinase 2 (HIPK2) transcript variant 1	0.029	0.82
TC282743	Unknown	Unknown	0.022	0.82
DY405668	AB188402	Sus scrofa CD3Z for CD3 zeta chain CD3 eta chain partial cds alternative splicing	0.024	0.82
CN157435	XM_001918217	Equus caballus cation channel sperm associated 2 (CATSPER2)	0.042	0.82
CF362786	Unknown	Unknown	0.037	0.82
DV224594	Unknown	Unknown	0.045	0.82
TC274382	NM_001101064	Bos taurus TSPY-like 4 (TSPYL4)	0.022	0.82
CK451297	XM_001914696	Equus caballus bromodomain containing 4 (BRD4)	0.018	0.82
TC242066	BC105323	Bos taurus Rho GTPase activating protein 1 mRNA (cDNA clone IMAGE:7945456)	0.030	0.82
EW387350	XM_001928378	Sus scrofa hypothetical protein LOC100155837 (LOC100155837)	0.021	0.82

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
AK231743	NM_001011685	Bos taurus 5 10-methylenetetrahydrofolate reductase (NADPH) (MTHFR)	0.032	0.82
ODC	AB529865	Sus scrofa ODC1 mRNA for ornithine decarboxylase 1	0.038	0.82
EW475595	XM_001929082	Sus scrofa similar to tetraspanin 15 (LOC100157123)	0.047	0.82
CN158962	NM_001105420	Bos taurus cytoplasmic polyadenylation element binding protein 4 (CPEB4)	0.044	0.82
AK239900	NM_001105370	Bos taurus similar to leucine-rich-domain inter-acting protein 1; LeR inter-acting protein 1; LEAP1 (LOC515042)	0.031	0.83
TC302515	AJ504726	Sus scrofa mut gene for methylmalonyl-CoA mutase exons 1-7	0.012	0.83
TC258946	NM_001102136	Bos taurus nucleoredoxin (NXN)	0.024	0.83
EW575812	XM_001487868	Equus caballus translocated promoter region (to activated MET oncogene) transcript variant 1 (TPR)	0.042	0.83
LOC733605	NM_001044554	Sus scrofa NADH dehydrogenase 1 beta subcomplex 6 (LOC733605)	0.039	0.83
DN108591	XM_001499231	Equus caballus PHD finger protein 20-like 1 (PHF20L1)	0.035	0.83
EW525979	XM_001928262	Sus scrofa similar to B-cell CLL/lymphoma 10 (LOC100153411)	0.016	0.83
TC256227	Unknown	Unknown	0.040	0.83
TC258553	NM_001106592	Rattus norvegicus zinc finger protein 282 (Znf282)	0.042	0.83
TC264353	XM_524602	Pan troglodytes leucine zipper protein 1 transcript variant 3 (LUZP1)	0.013	0.83
EW141994	GU144288	Sus scrofa breed Meishan CDP-diacylglycerol-inositol 3-phosphatidyltransferase (CDIPT) mRNA	0.029	0.83
BX922324	XM_001097043	Macaca mulatta similar to neighbor of BRCA1 gene 1 (LOC708558)	0.037	0.83
TC252375	NG_011605	Homo sapiens optic atrophy 1 (autosomal dominant) (OPA1) on chromosome 3	0.016	0.83
AK233485	Unknown	Unknown	0.023	0.83
TC275758	NM_021705	Homo sapiens nuclear transcription factor Y alpha (NFYA) transcript variant 2	0.029	0.83
CV874400	XR_013993	Macaca mulatta similar to carboxypeptidase D precursor (LOC712407)	0.042	0.83
BP464264	NM_001033763	Bos taurus DnaJ (Hsp40) homolog subfamily B member 1 (DNAJB1)	0.042	0.83
EW622475	Unknown	Unknown	0.027	0.83
NLN	NM_214359	NLN	0.025	0.83

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
BQ599146	XM_001929267	Sus scrofa similar to ubiquinol-cytochrome c reductase binding protein (LOC100157621)	0.035	0.83
TC250527	Unknown	Unknown	0.017	0.83
LOC733594	Unknown	Unknown	0.031	0.83
TC280160	NM_001080267	Bos taurus hypothetical protein LOC512679 (KIAA1549)	0.034	0.83
AJ655828	XM_001088983	Macaca mulatta similar to cytoplasmic polyadenylation element binding protein 3 (LOC698133)	0.047	0.83
AK236892	XM_001087186	Macaca mulatta similar to TGF-beta induced apoptosis protein 12 transcript variant 5 (LOC694549)	0.020	0.84
AK234787	NM_001083760	Bos taurus solute carrier family 30 (zinc transporter) member 7 (SLC30A7)	0.034	0.84
TC285101	Unknown	Unknown	0.009	0.84
TC244961	Q8BU92	RAS protein activator like 2	0.050	0.84
EW417533	Unknown	Unknown	0.033	0.84
BX923434	XR_024055	Pan troglodytes similar to KIAA0178 (SMC1L1)	0.035	0.84
DN101466	XM_585116	Bos taurus similar to axin interaction partner and dorsalization antagonist transcript variant 1 (LOC508353)	0.047	0.84
TC284856	NM_001106613	Rattus norvegicus protein phosphatase 4 regulatory subunit 2 (Ppp4r2)	0.018	0.84
AK233507	XM_541506	Canis familiaris similar to Nucleobindin 1 precursor (CALNUC) transcript variant 1 (LOC484391)	0.012	0.84
TC289103	Unknown	Unknown	0.041	0.84
TC293356	XM_001148120	Pan troglodytes limb region 1 protein (LMBR1)	0.024	0.84
LOC733610	XM_001488738	Equus caballus similar to Transmembrane protein 59 transcript variant 1 (LOC100050223)	0.044	0.84
AK234479	NM_001045939	Bos taurus glutaryl-Coenzyme A dehydrogenase (GCDH) nuclear gene encoding mitochondrial protein	0.027	0.84
CJ029361	XM_001929357	Sus scrofa similar to transcription termination factor RNA polymerase II (LOC100156166) mRNA	0.016	0.84
TC295311	Unknown	Unknown	0.016	0.84
TC289215	XM_001103594	Macaca mulatta similar to hepatic leukemia factor (LOC706623)	0.013	0.84

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
TC256781	XM_510504	Pan troglodytes coronin actin binding protein 2B (CORO2B)	0.021	0.85
TC291534	Unknown	Unknown	0.028	0.85
AK232325	XM_509243	Pan troglodytes PTPRF interacting protein alpha 2 (PPFIA2) mRNA	0.032	0.85
AK235920	XM_001926875	Sus scrofa similar to FYVE and coiled-coil domain containing 1 (LOC100154150)	0.048	0.85
AK233030	NM_004820	Homo sapiens cytochrome P450 family 7 subfamily B polypeptide 1 (CYP7B1)	0.027	0.85
DN100970	Unknown	Unknown	0.049	0.85
BW979660	NM_004428	Homo sapiens ephrin-A1 (EFNA1) transcript variant 1	0.031	0.85
TC262793	BC144616	Homo sapiens family with sequence similarity 63 member B mRNA	0.008	0.85
DV224971	NM_015027	Homo sapiens pyridoxal-dependent decarboxylase domain containing 1 (PDXDC1)	0.043	0.85
TC276349	Unknown	Unknown	0.004	0.85
TC283027	NG_009369	Homo sapiens gap junction protein alpha 5 40kDa (GJA5) on chromosome 1	0.022	0.85
AK235477	XM_001501249	Equus caballus trinucleotide repeat containing 6A (TNRC6A)	0.010	0.85
BM190617	NM_024776	Homo sapiens NKF3 kinase family member (SGK269)	0.048	0.85
TC261566	XM_001147305	Pan troglodytes required for meiotic nuclear division 5 homolog B transcript variant 8 (RMND5B)	0.042	0.85
AK233115	NM_001076539	Bos taurus zinc finger protein 574 (ZNF574)	0.046	0.85
TC287870	NM_001077984	Bos taurus RNA polymerase II associated protein 1 (RPAP1)	0.013	0.86
CK453045	XM_518711	Pan troglodytes karyopherin alpha 5 (importin alpha 6) (KPNA5)	0.016	0.86
P2RY2	P2RY2	P2RY2	0.045	0.86
BX664868	NM_001046448	Bos taurus tetraspanin 14 (TSPAN14)	0.049	0.86
BP440893	NM_001046478	Bos taurus rhomboid domain containing 2 (RHBDD2)	0.047	0.86
AK237910	NG_011499	Homo sapiens JAZF zinc finger 1 (JAZF1) on chromosome 7	0.026	0.86
TC253559	Unknown	Unknown	0.036	0.86
AJ684796	BC120080	Bos taurus calmodulin 3 (phosphorylase kinase delta) mRNA (cDNA clone MGC:140648 IMAGE:8272819)	0.023	0.86

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
VWF	AF052036	VWF	0.023	0.86
AK234934	NM_001131045	Sus scrofa solute carrier family 39 (zinc transporter) member 7 (SLC39A7)	0.007	0.86
AK235860	NM_001166044	Sus scrofa cyclin-dependent kinase 9 (CDK9)	0.044	0.86
EW618205	Unknown	Unknown	0.045	0.86
CX064989	Unknown	Unknown	0.037	0.86
DY413627	XP_001926148	similar to KIAA1398 protein	0.024	0.86
NCOA1	NM_001025228	NCOA1	0.022	0.86
CV874500	Unknown	Unknown	0.036	0.87
TC276672	AK127343	Homo sapiens cDNA FLJ45415 fis clone BRHIP3033734 highly similar to Protein FAM53B	0.029	0.87
TC261431	Unknown	Unknown	0.039	0.87
MITF	GU097381	Sus scrofa microphtalmia-associated transcription factor isoform A (MITF) mRNA complete cds alternatively spliced	0.008	0.87
AK233061	XM_001926938	Sus scrofa similar to Uncharacterized protein C20orf4 homolog (LOC100152525)	0.010	0.87
TC252427	X68453	S.scrofa mRNA for tubulin-tyrosine ligase	0.016	0.87
TC259850	XM_001503832	Equus caballus similar to coiled-coil domain containing 28B (LOC100070339)	0.010	0.87
AK237279	NM_001034374	Bos taurus microtubule-associated protein RP/EB family member 2 (MAPRE2)	0.025	0.88
DN107664	XM_596055	Bos taurus similar to AT rich interactive domain 2 (ARID RFX-like) (ARID2)	0.018	0.88
TC239752	AF097750	Gallus gallus chromatin assembly factor 1 p48 subunit mRNA	0.044	0.88
TC277649	NM_001015627	Bos taurus resistance to inhibitors of cholinesterase 8 homolog A (C. elegans) (RIC8A)	0.043	0.88
AY609407	XM_001926939	Sus scrofa similar to syndecan 2 (LOC100152754)	0.024	0.88
CF359413	NM_001132379	Pongo abelii Der1-like domain family member 1 (DERL1)	0.032	0.88
TC270932	XM_001253150	Bos taurus prothymosin alpha (PTMA)	0.025	0.88
TC290163	AY550038	Sus scrofa ribosomal protein S28 (RPS28)	0.028	0.88
TC263689	O77783	Bos taurus exostoses (multiple) 2 (EXT2)	0.034	0.88

Table A-8 Continued

Gene ID	Accession No.	Gene Name	*P*-value	Fold Change
CN030549	XM_591968	Bos taurus similar to aurora borealis transcript variant 1 (LOC514162)	0.021	0.89
EW570014	XM_001925192	Sus scrofa similar to Transmembrane 9 superfamily member 3 precursor (SM-11044-binding protein) (EP70-P-iso) (LOC100157588)	0.036	0.89
TC272557	NM_001075824	Bos taurus tripartite motif-containing 32 (TRIM32)	0.033	0.89
TC248797	Unknown	Unknown	0.025	0.90
BP171980	XM_001254303	Bos taurus similar to ubiquitin specific protease 48 (USP48)	0.016	0.90
AK235128	XM_001104624	Macaca mulatta v-akt murine thymoma viral oncogene homolog 3 (AKT3)	0.041	0.90
TC297181	AF196186	Homo sapiens atypical PKC isotype-specific interacting protein short variant mRNA complete cds	0.041	0.91
DN117823	Q34177	NADH-ubiquinone oxidoreductase chain 5	0.037	0.92
IFNGR2	NM_001111258	IFNGR2	0.041	0.93

* Determined by microarray

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Research Interests

My research interest is in the fields of amino acid metabolism and female reproductive biology. I am especially interested in doing research that will increase understanding of mechanisms critical to implantation, uterine receptivity to implantation and placentation, uterine biology and pregnancy. I am also interested in diseases and pathologies that affect reproduction and successful outcomes of pregnancy.

Publications in Refereed Scientific Journals

Li X, Rezaei R, Li P, Wu G (2011) Composition of amino acids in feed ingredients for animal diets. Amino Acids 40:1159-1168

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Abstracts

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