ARGININE SUPPLEMENTATION STRATEGIES DURING GESTATION: IMPACTS ON DAMS AND OFFSPRING

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MASTER OF SCIENCE

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ABSTRACT

We hypothesize rumen-protected arginine supplementation during gestation will mitigate deleterious offspring effects caused by undernutrition. Experiment 1: non-pregnant ewes were supplemented with rumen-protected arginine at varying doses to assess effects on circulating amino acids and carotid hemodynamics. Arginine concentrations post-supplementation were greater in ewes supplemented with 180 vs. 90 mg/kg BW, and vascular resistance indices were lesser with 180 mg/kg BW; therefore, 180 mg/kg BW was used in experiment 2. Experiment 2: nutrient-restricted pregnant ewes were supplemented with rumen-protected arginine and maternal and offspring growth and physiological responses were measured. Arginine supplementation to nutrient restricted ewes improved offspring development compared to restricted ewes without supplementation. Circulating amino acids in offspring were efficiently metabolized, which may contribute to improved growth and development. There was no change in carotid hemodynamics in supplemented pregnant ewes. Further research should determine how arginine improves development, as this dietary supplement could rescue at-risk pregnancies.

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LIST OF ABBREVIATIONS

AA .... amino acids

ADF .... acid detergent fiber

ADG .... average daily gain

AGAT .... arginine:glycine amidinotransferase

AOAC .... Association of Official Agricultural Chemists

$b^{0,+}$ .... Sodium independent amino acid intestinal transporter

$B^{0,+}$ .... Sodium dependent amino acid intestinal transporter

B-mode .... grayscale imaging mode

BCS .... body condition score

BH_4 .... tetrahydrobiopterin

BW .... body weight

°C .... degrees Celsius

Ca .... calcium

cGMP .... cyclic guanosine monophosphate

cm .... centimeters

CON .... control

CP .... crude protein

CSA .... cross-sectional area

d .... day

D-mode .... spectral Doppler mode

DM .... dry matter

DNA .... deoxyribonucleic acid

eNOS ....endothelial nitric oxide synthase

EDV .... end diastolic velocity

FAD ....flavin adenine dinucleotide

FGR ....fetal growth restriction

FMN .... flavin mononucleotide

g ....grams

GI .... gastrointestinal

h ....hour(s)

iNOS ....inducible nitric oxide synthase

IU ....international units

IUGR ....intrauterine growth restriction

IV ....intravenous

kg ....kilograms

K_m ....Michaelis-Menten constant

mM..millimolar

ME .... metabolizable energy

mg .... milligrams

MHz ....megahertz

mL ....milliliters

MnV ....mean velocity

N .... nitrogen

Na^+ ....Sodium

NaCl ....sodium chloride

NADH .... nicotinamide adenine dinucleotide

NADPH .... nicotinamide adenine dinucleotide phosphate

NDF .... neutral detergent fiber

NH_4^+ .... ammonium

nNOS .... neuronal nitric oxide synthase

NO .... nitric oxide

NOS .... nitric oxide synthase

NRC .... national research council

O_2 .... oxygen

OKG .... ornithine α-ketoglutarate

P .... Phosphorous

PBS .... phosphate buffered saline

PEPT1 .... peptide transporter 1

PI .... pulsatility index

PSV .... peak systolic velocity

RES .... restricted

RES-ARG .... restricted with arginine

RI .... resistance index

RP-ARG .... rumen-protected arginine

SAS .... statistical analysis system

Se .... selenium

Trt .... treatment

UPLC .... ultra-performance liquid chromatography

USDA .... United States Department of Agriculture

vs. .... versus

y^+ .... arginine intestinal transporter

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Introduction

Fetal growth restriction (**FGR**) in utero, or intrauterine growth restriction (**IUGR**), can be defined as any impairment to growth and development of the fetus. A major cause of FGR/IUGR is inappropriate maternal nutrition (Wu et al., 2006; Caton and Hess, 2010). Poor maternal nutrition during gestation may permanently defect the structure and physiology of vascular development of the fetus. If undernutrition continues into late pregnancy, fetal amino acid metabolism may shift to the placenta for energy production, causing amino acids normally destined for the fetus to be catabolized by the placenta for energy (Barker et al, 1993). We believe that arginine supplementation may be a solution to "rescue" at risk offspring from compromised maternal nutrition during pregnancy.

Arginine is the most abundant carrier of protein nitrogen (**N**) in animals, and has been at the center of amino acid research for many years (Flynn et al., 2002). Arginine is classified as a semi-essential amino acid, meaning that the body is able to synthesize it, but at certain physiological states the body isn't able to synthesize enough to meet requirements. Times of critical arginine demand and likely elevated conditional requirements include growth and catabolic states, along with health challenges involving trauma, burn injury, massive small-bowel resection, and renal failure (Cynober, 1994; Flynn et al., 2002).

Supplementation of amino acids to ruminants is more complex than to monogastrics due to the microbial population present in the rumen. To alleviate this challenge, past researchers have used jugular (Recabarren et al., 1996) or abomasal (Davenport et al., 1990) infusion technologies to deliver arginine to ruminants for absorption, however these infusion techniques

are not practical for everyday production purposes. Rumen-protection technologies have been utilized to make dietary amino acid supplements protected from microbial degradation and available for absorption in the small intestine in ruminants (Oke et al., 1986). These rumen-protected supplements can be fed along with a normal ration, so it is much more practical for producers to implement. By using these rumen-protected amino acids, a producer can be more confident that their ruminant animal is receiving the amino acid they are administering them. However, it is essential that a producer knows that they are administering the most effective dose, both for the physiological benefit of the animal, and for the economic benefit of the producer. One of the objectives of this study will be to determine the most effective dose at which to administer a dietary rumen-protected arginine supplement to gestating ewes.

Once the most effective dose is established, we seek to examine whether arginine supplementation is affecting related amino acid balance in both the dam and her offspring, despite the fact that the offspring aren't receiving the arginine supplement directly. Our main objective in supplementation is to test whether dams receiving arginine during gestation will benefit their subsequent offspring despite effects of inappropriate maternal nutrition during pregnancy. We believe these effects may, at least partially, be achieved through enhanced blood flow during gestation induced by arginine supplementation.

Fetal growth restriction and maternal nutrition

Inappropriate maternal nutrition is a relevant concern in livestock production. For example, in the Western United States, grazing ewes can often receive less than 50% of their nutrient requirements during times of summer drought or winter dormancy (Wu et al., 2006). Nutrient restrictions of this magnitude are documented to cause IUGR (Meyer et al., 2010;

Swanson et al., 2008). Not surprisingly, maternal condition during gestation is impacted by inappropriate nutrition. Meyer et al. (2010) found that ewes fed a restricted diet (60% of requirements) lost weight from day (**d**) 39 to 95 of gestation, while ewes on adequate nutrient diets (100% of requirements) gained weight. From d 96 through term of gestation restricted ewes gained markedly less weight than ewes receiving adequate nutrients. These changes were also reflected in ewe body condition score (**BCS**); restricted nutrient ewes lost condition from d 39 to 95 of gestation while ewes receiving adequate nutrition gained condition.

In addition to a loss of ewe body weight during pregnancy, ewes can demonstrate reduced lactation performance which will impact future neonatal and postnatal growth (Wu et al., 2006). In a similar study to the aforementioned, ewes restricted in nutrients yielded less colostrum than ewes fed adequate nutrients throughout pregnancy; these results continued into early lactation when those restricted ewes continued to produce less milk than adequately fed ewes (Meyer et al., 2011a). This means that in addition to impaired neonatal development, offspring will be receiving inadequate postnatal nutrition; this contributes to the overall challenge of postnatal healthy life and survival.

Implications of IUGR in offspring will often manifest as low birth weights and pre-weaning deaths (Wu et al., 2006). Undernutrition during the last one-third of gestation is particularly detrimental to fetal development, as this is the window in which most fetal growth occurs in ruminants (Caton and Hess, 2010). Most pre-weaning deaths of offspring in the United States occur within the first days of postnatal life (Wu et al., 2006). The offspring that survive the first few days of life are more prone to have neurological, respiratory, intestinal, and circulatory disorders. All of these effects result in a longer period of time required to adapt to postnatal life (Wu et al., 2006; Caton and Hess, 2010; Reynolds and Caton, 2012). In the sheep

industry lamb loss is a major issue, making the ability of offspring to adapt to postnatal life rapidly all the more important; most lamb deaths occur before reaching the age of being marked, docked, or branded (USDA 2012). In the aforementioned study, Meyer et al. (2010) found that offspring from ewes receiving restricted nutrients during gestation had 8.1% lower birth weights than offspring from ewes receiving adequate nutrients. Offspring from ewes receiving restricted nutrients continued to weigh less at various early stages of life, which resulted in decreased ADG as compared to those offspring from adequately fed dams. Restricted offspring also had lower heart girth measurements than offspring from ewes receiving adequate nutrients. A combination of all of these traits could seriously impair the ability for offspring to develop normally and have a healthy life.

Arginine metabolism, absorption, and interactions

Arginine metabolism

Metabolism of arginine is initiated when dietary protein is broken down to its components by proteolytic enzymes. Pancreatic enzymes trypsin and carboxypeptidase B are proteolytic enzymes that release arginine and lysine (Grimble, 2007). We would expect that dietary arginine supplementation could potentially up-regulate activity of these pancreatic enzymes.

Arginine synthesis is often characterized as the “intestinal-renal axis”, because this process takes place first in the intestine and eventually in the kidney (Morris, 2007). Citrulline is synthesized in the enterocyte and enters the bloodstream to travel to the kidney, where arginosuccinate will convert the citrulline to arginine (Cynober et al., 1995; Wu et al., 2009). Unlike the liver, the kidney is practically devoid of arginase activity so arginine synthesis can

actually result in net arginine accumulation (Flynn et al., 2002). This conversion of citrulline to arginine takes place in many cell types other than enteroctyes, including adipocytes, endothelial cells, macrophages, neurons, and myocytes. However, the kidney appears to be a primary driver of arginine synthesis from citrulline (Wu et al., 2009).

Arginine synthesis in the liver due to the urea cycle does occur; however, due to large amounts of arginase in the liver there is no net accumulation (Cynober et al., 1995). Arginine serves as an allosteric activator to N-acetylglutamate synthetase, which would synthesize N-acetylglutamate. This N-acetylglutamate is an activator of carbamoyl phosphate synthetase, which is a key enzyme in the urea cycle (Cynober et al., 1995; Cynober, 2002). Therefore, greater amounts of arginine will allosterically increase ureagenesis in the liver. This may partially explain why higher protein diets often stimulate increased ureagenesis, and indicates that supplemental dietary arginine would probably induce a similar effect.

Arginase enzymes are the primary drivers of arginine degradation. Arginases convert arginine to ornithine, which causes downstream synthesis of proline, glutamate, glutamine, and polyamines. Arginases can be type I or type II; type I arginases are primarily used by the liver for the urea cycle, while type II arginases are located in the kidney, brain, small intestine, endothelial cells, mammary gland, and macrophages (Flynn et al., 2002). Another key product of arginine catabolism is creatine. Creatine synthesis occurs by arginine:glycine amidinotransferase (AGAT) transferring the guanidine group from arginine to the amino group of glycine to yield guanidinoacetate. Guanidinoacetate is later methylated to eventually become creatine (Wu et al., 2013).

Arginases compete for arginine with another family of enzymes: nitric oxide synthases (**NOS**; Wu et al., 2009). Arginine provides the N atom for nitric oxide (**NO**) in its reaction with

these NOS enzymes (Morris, 2007). The NOS enzymes include three families: eNOS, which is present in endothelial cells, nNOS, which is present in neuronal tissue, and iNOS, which are inducible by cytokines under certain immune conditions. Collectively, the NOS enzymes require arginine, O_2, tetrahydrobiopterin (**BH_4**), nicotinamide adenine dinucleotide phosphate (**NADPH**), calmodulin, flavin mononucleotide (**FMN**), and flavin adenine dinucleotide (**FAD**) to produce NO; BH_4 is the rate-limiting cofactor (Cynober et al., 1995; Wu et al., 2009). The iNOS family produces the greatest amount of NO, which is used for immune function (Flynn et al., 2002). The NO produced by iNOS has been used to fight tumor cells through glyceraldehyde-3-phosphate dehydrogenase, aconitase, nicotinamide adenine dinucleotide (**NADH**)-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase inhibition to impair cellular respiration (Cynober et al., 1995). Increased interest has centered on eNOS production of NO, and its role in vasodilation to regulate blood flow and vascular tone (Martin et al., 2001). This NO released by eNOS activates guanylyl cyclase, which increases cellular cGMP levels to induce smooth muscle relaxation (Cynober, 1994).

A key question often posed in arginine supplementation is ‘how much is too much’, at what point are the NOS enzymes reaching saturation? Considering the K_m of NOS is 5 µm and under normal conditions endothelial cellular arginine concentrations are somewhere around 100 times greater than this, it seems that these enzymes are probably almost always saturated with substrate. However, it appears that NOS is co-localized with arginine transporters in the plasma membrane. This suggests that some arginine is going to its transporter to enter the intracellular arginine pool for later use, however arginine could rather be preferentially sent to NOS to be used for NO production (Cynober, 2002).

Arginine can also be degraded by arginine decarboxylase to agmatine; this reaction takes place in the brain, liver, kidney, adrenal gland, macrophages, and the small intestine. Agmatine will then be converted to putrescine, a polyamine, by agmatinase (Flynn et al., 2002). Polyamines serve many functions, including protecting cells from oxidative damage, regulating gene expression, signal transduction, ion channel function, DNA and protein synthesis, and apoptosis. Polyamines are essential for cell proliferation, differentiation, and function (Flynn et al., 2002). These functions make them especially important during periods of growth and development, including fetal development during gestation. Polyamines are also required in blastocyst implantation, and are therefore important in achieving pregnancy (Wu et al., 2013).

Arginine absorption and transport

Absorption and transport of arginine will be indicative of its further metabolism and interaction with amino acids. Intestinal absorption of arginine in mammals takes place in the ileum and jejunum by both transporters and physical diffusion components. Physical diffusion takes place at high arginine concentrations, and can represent up to 45% total arginine uptake (Cynober et al., 1995). At the brush border of the intestine there are several arginine transporters present, including $B^{0,+}$ (Na^+-dependent) and $b^{0,+}$ (Na^+-independent). These transport systems are primarily found in fibroblasts and endothelial cells, and are shared with lysine, ornithine, and cysteine (Cynober et al., 1995). In addition, peptide transporter 1 (**PEPT1**) would be present at the brush border to transport arginine present in peptide forms.

Finally, y^+ is a primary arginine transport system present at both the brush border and in the basolateral membrane. This is a high affinity, pH-insensitive, Na^+-independent basic amino acid transporter with a steric constraint that will not permit methylated forms of amino acids to

bind (Cynober et al., 1995). The y^+ transporter is stimulated by intracellular arginine accumulation (Morris, 2007). This transporter is shared with lysine, histidine, and ornithine, which can cause concern during arginine supplementation (Flynn et al., 2002; Wu et al., 2009). Note that this transporter is not used by citrulline, and therefore citrulline does not compete with arginine for transport. Most of the dietary arginine taken up by the intestine will be released as citrulline, or metabolized in the liver (Cynober et al., 1995).

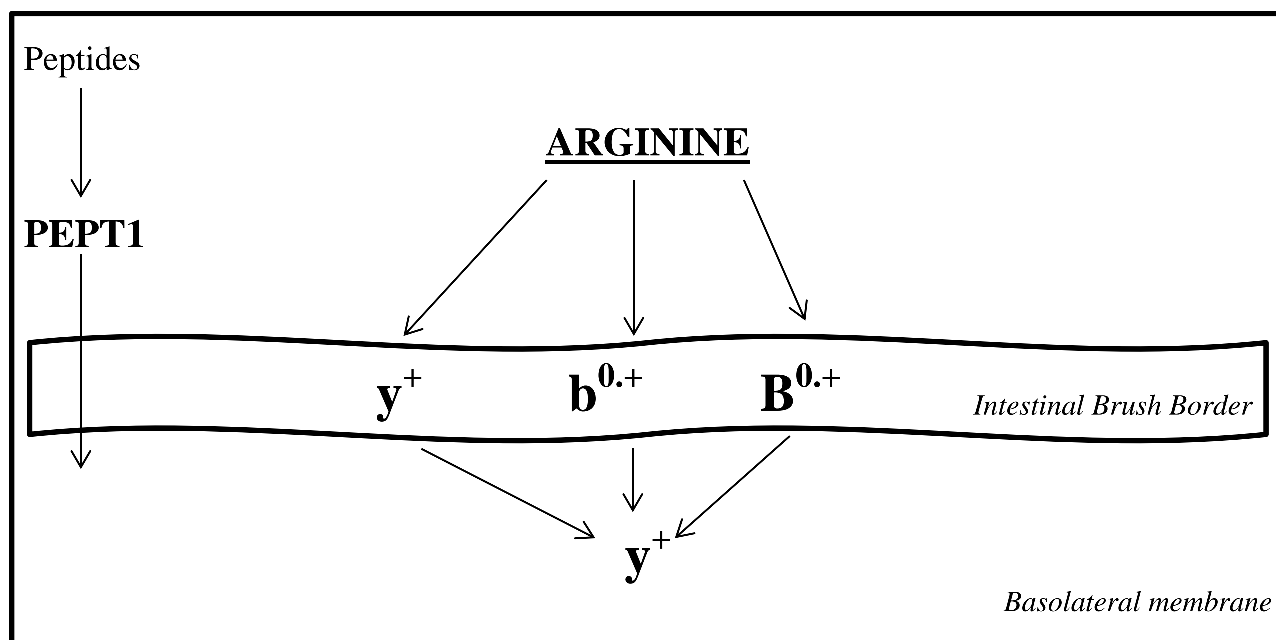

Figure 1.1. Intestinal absorption and transport of arginine. Arginine can be absorbed by more general transporters, including $b^{0,+}$ and $B^{0,+}$, or by more specific transporters like y^+. The y^+ transporter absorbs arginine at both the brush border and the basolateral membrane. Adapted from Cynober et al., 1995.

Interaction between arginine and other amino acids

Citrulline. The relationship between arginine and citrulline is complex and intriguing. Arginine can be both metabolized to and synthesized from citrulline in enterocytes and the kidneys, as mentioned previously. Low protein diets will cause animals to down-regulate arginine flux to the liver in order to spare the arginine from degradation in ureagenesis. Instead,

the arginine will be metabolized into citrulline in the small intestine, which will be sent to the kidney for arginine synthesis. This is an arginine-sparing effect because citrulline is not metabolized in the gut and will not compete for arginine transporters (Cynober, 2002). Very little citrulline goes to the liver for metabolism; most will be transported to the kidney. For these reasons, citrulline has been implicated as a better precursor for arginine than ornithine (Cynober et al., 1995). Citrulline supplementation has been shown to increase plasma arginine, and bioavailability between the three amino acids (citrulline, arginine, and ornithine) is greatest in citrulline, followed by ornithine and arginine, respectively (Cynober, 2007). Based on this information, we would hypothesize that citrulline levels would increase with arginine supplementation. With no competition for intestinal absorption transporters, citrulline would readily be synthesized from the increased amounts of arginine in the small intestine. If protein supply was not reduced, we believe less citrulline would eventually be used for arginine synthesis in the kidney because the body would not require arginine synthesis due to its adequate or increased arginine status.

Ornithine. Like citrulline, ornithine is also synthesized from arginine in the gut. This conversion is performed by arginase enzymes (Cynober, 2002). Ornithine can be further metabolized to putrescine, which can form spermidine and spermine downstream, all of which are polyamines (Flynn et al., 2002). Ornithine can also generate glutamine via ornithine aminotransferase, citrulline via ornithine carbamoyl transferase, and proline. Most of the ornithine generated from arginine will enter the portal bloodstream to travel to the liver and participate in ureagenesis; only a small fraction will eventually synthesize polyamines (Cynober et al., 1995). Unlike arginine, ornithine has a nitrogen-sparing effect; when ornithine is combined with $2NH_4^+$ it synthesizes arginine, whereas arginine generates urea to utilize N (Cynober, 2007).

In its α-ketoglutarate salt form, OKG, ornithine works to regulate N balance during malnutrition (Cynober, 1994). Ornithine may also work with arginine to improve immune response to inflammation. Activated macrophages synthesize NO and release arginase around inflammatory site upon their lysis. Arginine would convert to ornithine via these arginases, and ornithine would be transported to lymphocytes for polyamine synthesis. Also, ornithine could generate proline for collagen synthesis (Cynober et al., 1995). Considering the relationship between ornithine and arginine, we would anticipate that arginine supplementation would increase ornithine due to up regulated arginase activity. We would also expect polyamine synthesis to increase, which would normally be beneficial to the animal. Because supplemental arginine would most likely increase ureagenesis activity, we would expect the majority of the increased ornithine levels to be shuttled to the liver to participate in the urea cycle.

Lysine. In contrast to citrulline and ornithine, lysine is not synthesized from arginine but is thought to antagonize arginine transport and mobilization for metabolism. Antagonism in transport is due to competition for use of the y^+ transport system and its potential saturation. Antagonism in arginine mobilization for metabolism is hypothesized to be due to inhibition of trypsin and carboxypeptidase B, either at the intestinal action level or the pancreatic synthesis level, to decrease arginine availability for further metabolism (Jones et al., 1967). The results in experiments testing this antagonism are mixed. In young pigs, reduced growth was seen accompanying lysine supplementation, but this was thought to be due to a shift causing amino acid imbalance as opposed to simply arginine antagonism (Edmonds and Baker, 1987). However, in tissue culture lysine has been successful in suppressing negative immune consequences of arginine and herpes simplex virus (Griffith et al., 1981). The chick is the animal model in which most lysine-arginine research has been conducted, because chicks cannot

synthesize arginine to correct the antagonism caused by lysine. Adding excess L-lysine to the diet of chicks increases the requirement for arginine. If this requirement is not met, chicks will exhibit decreased growth rate, symptoms of arginine deficiency, and decreased plasma arginine concentrations. All of these symptoms are able to be reversed with arginine supplementation (Jones, 1964; Jones et al., 1967). Studies examining transporter saturation in the chick have found that transporter competition is not very significant (Jones et al., 1967). Plasma-to-muscle ratios of lysine:arginine were not altered by high dietary lysine concentrations. In addition, though plasma concentrations of lysine were high, urinary arginine losses did not change. Studies in the chick that tested pancreatic enzyme availability to metabolize arginine have been inconclusive and not very repeatable. Therefore, performance differences caused by lysine supplementation were believed to be due to decreased tissue arginine concentrations and a delayed response in kidney arginase activity (Jones et al., 1967). All of this data could indicate that lysine-arginine antagonism is more easily seen in vitro than in vivo. Performance changes in vivo due to lysine supplementation are more likely due to amino acid balance shifts than a specific arginine antagonism effect. Also, since the chick is one of the few animals incapable of producing any arginine, most animals would be able to “override” antagonism effects induced by lysine by mobilizing citrulline to synthesize more arginine. We believe that arginine supplementation would, therefore, not impact lysine concentrations present in the blood stream due to antagonism in mammals.

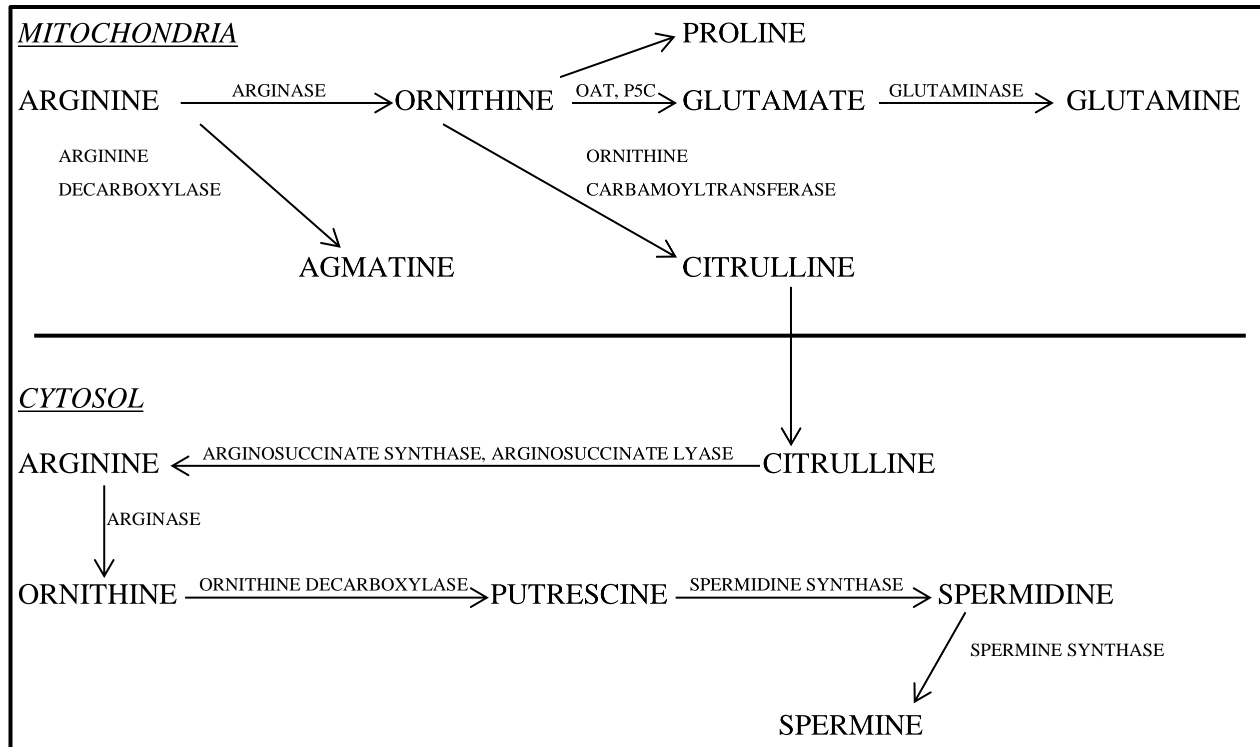

Figure 1.2. Relationships between arginine and related amino acids and metabolites. Adapted from Flynn et al., 2002. Nested within this figure is the urea cycle (Citrulline → Arginosuccinate → Arginine → Ornithine); urea is synthesized in the arginase-catalyzed reaction of arginine → ornithine. Note that studies have shown that agmatine may also have a direct pathway to polyamine synthesis via an intermediate between arginine and putrescine (Wang et al., 2014).

The role of dietary arginine in maternal and fetal tissue development during gestation

Nitric oxide and polyamines play important roles in fetal growth and development: nitric oxide as a vasodilator (Martin et al., 2001) and polyamines to regulate DNA and protein synthesis, cell proliferation and differentiation, regulation of gene expression, and signal transduction (Kwon et al., 2003). Placental angiogenesis and vascular growth are stimulated by NO. This could serve to provide more blood to the placenta, which would carry increased nutrients to support fetal development during gestation. Other reproductive processes that require NO include ovulation and embryonic development and implantation (Wu et al., 2013).

Polyamines are also required in blastocyst implantation (Wu et al., 2013). All of this suggests the importance of arginine during fetal conception and development. Encouraging the importance of arginine to young animals, data further suggests that arginine requirements of young piglets are greater than current NRC recommendations reflect (Kim et al., 2004). This poses the question: is this difference in requirement only present in piglets or in more young species, including ruminants?

Supplementation of arginine directly to young offspring has been shown to have beneficial effects in monogastrics. Yao et al. (2011) found that dietary arginine supplementation to weaned piglets enhanced daily BW gain, feed efficiency, and relative small intestinal weights. Maternal supplementation during pregnancy has been another documented method of supplementing arginine to offspring in utero. Mateo et al. (2007) provided arginine as a dietary supplement to pregnant sows from d 30 of gestation through parturition. Their findings demonstrated ability for arginine to increase both litter size and live litter birth weights in piglets. In a similar study, Gao et al. (2012) found that dietary arginine supplementation to pregnant sows increased total number of piglets per litter, number of live born piglets, litter birth weight, and placental weights. This data strongly suggests that dietary arginine supplementation has both neonatal and postnatal beneficial effects on offspring. However, as previously mentioned, dietary supplementation to monogastrics does not present the same challenges as to ruminants

Often in ruminants, arginine and other amino acids are provided as infusions. Several studies have been done involving intravenous injection of arginine to ewes on either control (100% NRC nutrient requirements) or restricted (less than 100% NRC nutrient requirements; often between 50 and 60% requirements) diets. Lassala et al. (2010) found that injection of arginine from d 60 to term of gestation increased birth weights of lambs from restricted ewes by

21% as compared to saline-injected restricted ewes. Satterfield et al. (2013) found that after injecting arginine vs. saline to ewes receiving 100% NRC requirements or 50% NRC requirements, weights of lambs were unaffected by arginine treatment. However, Satterfield injected arginine for only 25 days (d 100 to 125 of gestation) during pregnancy.

Based on previous research from our lab, we hypothesize that rumen-protected arginine supplementation will provide a more steady-state metabolism than injection of arginine. Injection of arginine induces a short-term spike in bioavailability, while steady dietary supplementation over time seems to be more consistently bioavailable in the serum (A. M. Meyer, C. B. Saevre, D. V. Dhuyvetter, R. E. Musser, and J. S. Caton, unpublished data). For this reason, we are seeking to establish a proven supplementation protocol using rumen-protected arginine instead of arginine injections. We hypothesize that we will see similar or enhanced results as compared to previous studies using arginine infusions.

The effects of arginine on hemodynamic responses during gestation

It has been long known that blood circulation plays vital roles in fetal development during gestation. Blood flow to the placenta undergoes massive shifts throughout stages of gestation (Rosenfeld et al., 1974). In 1977, Rosenfeld measured blood flow to several organs in ewes throughout gestation, including the brain, pancreas, spleen, heart, liver, kidneys, adrenals, uterus, and mammary gland. In these ewes heart rate increased by 20% as gestation progressed, while cardiac output increased by a marked 75%. Throughout gestation, blood flow to the brain, liver, and kidneys decreased, while blood flow to the uterus increased as a percentage of total cardiac output (Rosenfeld et al., 1977). These data help to illustrate the important role blood flow and its distribution has during gestation.

In compromised pregnancies, such as those caused by inappropriate maternal nutrition, these trends in blood flow may shift inappropriately. The placenta is the major organ for nutrient exchange between mother and fetus, and blood flow mediates much of these exchanges. The blood flow via uterine vessels feeds the maternal portion of the placenta, while blood flow via umbilical vessels feeds the fetal portion of the placenta; both uterine and umbilical blood flows increase throughout progression of pregnancy (Reynolds et al., 2006). However, pregnancies with risk of reduced fetal and placental growth often present reduced rates of placental blood flow and reduced fetal nutrient uptake. These compromised pregnancies may be susceptible to nutritional therapies to counteract these blood flow effects. For example, ewes in a compromised pregnancy model that were fed high dietary selenium displayed an increase in placental vascularity (Reynolds et al., 2006).

Arginine may be a nutritional therapeutic to increase blood flow to possibly counteract defects in compromised pregnancies. Altered eNOS expression can alter placental vascular development, and low levels of placental eNOS have been associated with IUGR pregnancies. In addition, NO may interact with vascular endothelial growth factor (**VEGF**: an indicator of vascularity) to further impact fetal growth (Reynolds et al., 2006).

In a 4 × 4 Latin square experiment, Meyer et al. (2011b) supplemented steers with one of two dietary doses of rumen-protected arginine (180 or 360 mg/kg), an injectable arginine supplement, or no supplementation at all. Hemodynamics were measured via Doppler ultrasonography at the carotid artery. Their findings showed that steers receiving 180 mg/kg BW of rumen-protected arginine had greater distal tissue perfusion than those steers receiving no supplement at all, or those supplemented with injectable arginine (Meyer et al., 2011b). In addition, steers receiving 180 mg/kg BW of rumen-protected arginine had decreased vascular

resistance compared to those steers receiving no supplement at all, or those supplemented with 360 mg/kg BW of rumen-protected arginine (Meyer et al., 2011b). These data suggest that rumen-protected arginine supplementation may enhance blood flow. However, further studies need to be performed in pregnant animals to test the effects during gestation. We hypothesize that arginine supplementation to pregnant animals will induce similar effects, and improve vascular hemodynamics throughout gestation.

The effects of arginine on intestinal characteristics in young animals

Intestinal health is often examined as an indicator of increased growth, as the intestine is the primary organ of nutrient absorption and initiation of nutrient assimilation. Several studies have evaluated the effect of arginine supplementation on intestinal characteristics in young animals. Zhan et al. (2008) fed arginine supplement to early weaned piglets, and found that supplementation increased villus height in the small intestine. In addition, VEGF showed increased immunoreactive expression in the duodenum, jejunum, and ileum, suggesting increased vascularity to these portions of the organ. This study found dose dependent effects: the lower dose of arginine supplementation induced an increase in VEGF expression as compared to control, but the higher dose did not. This further illustrates the imperative nature of administering the most efficient dose to illicit desired effects.

In a similar study, Yao et al. (2011) also found that arginine supplementation up until 21 d of age, weaned piglets had increased villus height and VEGF protein levels in the duodenum, ileum, and jejunum. These data support a potential role for arginine supplementation in increased intestinal health. We will examine intestinal health by studying jejunal characteristics of offspring from arginine-supplemented dams. We hypothesize that these offspring will express

characteristics that reflect better intestinal health and therefore potential for enhanced nutrient absorption and assimilation.

Conclusions

Arginine should be examined as a potential therapeutic for at-risk pregnancies due to maternal undernutrition through strategic supplementation that will be provided in concentrations that will be transported and utilized by the body without negatively impacting related amino acid metabolism. If this is achieved, arginine can potentially increase blood flow to the fetus during gestation to increase nutrient availability for development. Also, maternal supplementation of arginine during gestation could improve gut health of the offspring, and further aid in nutrient assimilation. Based on these priorities and previous research in our lab, we believe that a dietary rumen-protected arginine supplement at a specified dose will accomplish these goals most efficiently in pregnant ewes, and result in healthier, more viable lambs

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CHAPTER 2. DOSE TITRATION OF RUMEN-PROTECTED ARGININE IN EWE LAMBS: EFFECTS ON CAROTID ARTERIAL HEMODYNAMICS AND CIRCULATING SERUM AMINO ACIDS

Abstract

Our hypothesis was that rumen-protected arginine supplementation would impact concentrations of arginine-related serum amino acids in non-pregnant ewes. This would increase concentrations of amino acids resulting from the metabolism of arginine (citrulline, ornithine), and decrease levels of amino acids competing for transporters with arginine (lysine). We further hypothesized that arginine supplementation would increase blood flow by inducing vasodilation via nitric oxide production. To test these hypotheses, non-pregnant primiparous Rambouillet ewe lambs (n = 60) were penned individually in a temperature-controlled facility for a dose titration study to determine the most effective dose of rumen-protected arginine to positively influence both circulating amino acids and blood flow. Ewes were randomly assigned to one of four treatments: a control group receiving no supplement (**CON**), and groups receiving 90 mg/kg BW (**90**), 180 mg/kg BW (**180**), or 360 mg/kg BW of rumen-protected arginine supplement (**360**). Supplements were administered for 15 d, and fully consumed before delivery of a total pelleted diet. Blood samples were attained at 0600 on d 5, 8, 12, and 15 of the treatment period. In addition, Doppler ultrasound was used to assess carotid arterial blood flow on d 5, 8, 12, and 15 of the supplementation period. After 15 d of supplementation, ewes receiving 180 mg/kg BW of rumen-protected arginine had greater serum ornithine ($P = 0.05$) and arginine ($P = 0.05$), and tended to have greater aspartate ($P = 0.08$) than ewes receiving 90 mg/kg BW, and 180 were similar to 360 fed ewes ($P \geq 0.55$). All ewes receiving arginine supplement (90, 180, and 360)

had greater distal tissue perfusion than CON ewes ($P = 0.02$). Ewes receiving 90 or 180 mg/kg BW of arginine supplement showed the least resistance of blood flow ($P = 0.01$). Based on these serum amino acid data and blood flow analyses, we accept our hypothesis that arginine supplementation will impact serum amino acid concentrations and blood flow. In addition, we determine 180 mg/kg BW to be the most effective dose at which to administer this rumen-protected arginine supplement.

Introduction

Using strategic arginine supplementation to improve various aspects of livestock production has been a promising area of research for many years. Much of this research has been done in monogastrics, where provision of arginine has shown positive impacts on early embryonic survival in rats (Zeng et al., 2008), and increased fetal survival and litter sizes in swine (Mateo et al., 2007).

Due to amino acid breakdown by microbes in the rumen, dietary amino acid supplementation in ruminants requires direct intravenous injection, postruminal infusion, or use of a protection agent to permit the amino acid to be absorbed for metabolism in the small intestine. Lassala et al. (2010) found that arginine injection in pregnant ewes resulted in enhanced lamb birth weights from nutrient restricted dams. In a further experiment, Lassala et al. (2011) found that arginine improved fetal survival in dams carrying multiple fetuses. Because dietary supplementation of arginine is more practical in livestock production than intravenous injections and postruminal infusions, our research strategy focused on effects of feeding rumen-protected arginine supplement.

Arginine supplementation has been shown to increase circulating arginine and ornithine concentrations, both in monogastrics (Southern and Baker, 1982) and via protection agents in ruminants (Meyer et al., 2011a). Ornithine often follows similar circulation patterns to arginine because arginases commonly break down arginine into ornithine, which can be used either in the urea cycle or to synthesize polyamines. In contrast, arginine supplementation has been hypothesized to decrease circulating lysine and/or histidine due to competition for a common y^+ transporter at the brush border and basolateral membrane (Cynober et al., 1995; Flynn et al., 2002). However, results regarding this antagonism effect between arginine, lysine, and histidine are mixed. This antagonism is more pronounced in poultry, due to their inability to synthesize arginine to compensate for reduced transport caused by lysine supplementation (Jones et al., 1967). In contrast, young pigs fed dietary arginine in excess did not see a change in circulating lysine or histidine (Southern and Baker, 1982). However, an antagonism effect between arginine and lysine has been reported using rumen-protected arginine supplementation in steers, most notably in supplementation of greater concentrations (Meyer et al., 2011a). We hypothesized that due to arginine supplementation, we would note an increase in circulating ornithine and citrulline, in addition to a decrease in circulating lysine.

In addition to its amino acid interactions, arginine serves as a substrate for NOS enzymes, which in turn synthesize NO (Wu et al., 2009; Wu et al. 2013). Nitric oxide is a known vasodilator, which increases blood flow by regulating vascular tone (Martin et al., 2001). Because the carotid artery is one of the first major vessels leaving the aorta, the carotid artery can be used as an indicator for systemic blood flow. Rumen-protected arginine supplementation has been shown to influence carotid arterial hemodynamics by increasing distal tissue perfusion and

decreasing resistance in the vessel (Meyer et al. 2011b). We hypothesized that arginine supplementation would increase blood flow due to increased NO production.

Materials and methods

Protocols described herein were approved by the North Dakota State University Institutional Animal Care and Use Committee. Nulliparous Rambouillet-cross ewe lambs (n = 60; 51 ± 1.4 kg initial BW) from NDSU Hettinger Research Extension Center were housed individually in a temperature-regulated facility (12 to 21°C; Animal Nutrition and Physiology Center; Fargo, ND) with free access to water. Facility lighting was timed to mimic normal daylight patterns.

Experimental design and treatments

All ewes were designated a complete pelleted diet (Table 2.1) fed twice daily (0630 and 1830) to meet NRC (1985, 2007) recommendations. Dietary intake for each individual ewe was calculated based on BW and metabolizable energy (**ME**) requirements. Diets were analyzed for dry matter (**DM**), ash, and crude protein (**CP**) following the Association of Official Agricultural Chemists (**AOAC**; 1990), and neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) using an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY).

Table 2.1. Ingredient and nutrient composition of pelleted diet fed to ewes[1]

Item	%
Ingredient	
Alfalfa meal, dehydrated	34.0
Beet pulp, dehydrated	27.0
Wheat middlings	25.0
Ground corn	8.4
Soybean meal	5.0
Trace mineral premix[2]	0.6
Nutrient composition	
DM	89.9
CP	15.5
NDF	37.2
ADF	21.5

[1]Diets administered to ewes daily at 0630 and 1830.
[2]Premix: 18 to 21% Ca, 9% P, 10 to 11% NaCl, 49.3 mg/kg Se, 700,000 IU/kg Vitamin A, 200,000 IU/kg Vitamin D, 400 IU/kg Vitamin E

In addition to this pelleted diets ewes were randomly assigned either 0, 90, 180, or 360 mg rumen-protected arginine (**RP-ARG;** Kemin Industries, Des Moines, IA) per kg BW (based on initial BW); Arginine was mixed with 50 g of fine ground corn and fed twice daily prior to offering the pelleted diet. Control ewes (0 mg/kg RP-ARG) were also provided 100 g of fine ground corn daily, without the added rumen protected arginine. This resulted in four treatment groups consisting of 0 mg/kg (**CON**, n = 15), 90 mg/kg (**90**, n = 15), 180 mg/kg (**180**, n = 15), and **360** mg/kg (360, n = 15) BW RP-ARG in addition to a complete pelleted diet. A 7 d adaptation period to the pelleted diet was implemented before beginning rumen-protected arginine treatments; treatment period continued for a total of 15 d. Ewe BW and BCS was measured at initiation (d 0) and end (d 15) of 15 d treatment period. Body condition score was measured on a 1 to 5 scale (1 = emaciated and 5 = obese) by two independent evaluators.

Blood sample collection and analysis

Blood samples were attained at 0600 on d 5, 8, 12, and 15 of the treatment period. Collections were obtained via right jugular venipuncture so as to avoid puncturing the left carotid

artery, which was used for Doppler ultrasonography. Blood (10 mL) was collected with Corvac serum separator vacuum tubes with thrombin (Tyco Healthcare, Mansfield, MA), which were placed on ice and held a minimum of 45 minutes. Whole blood was centrifuged at 1,500 × g for 30 min at 4°C, after which supernatant was pipetted into 2-mL screw-cap vials and stored at -20°C.

Serum AA concentrations were evaluated using ultra performance liquid chromatography (**UPLC**) methods similar to Meyer et al., 2011a. Amino acids separation utilized a reverse-phase chromatography column (MassTrak AAA 2.1 x 150 mm Column, Waters Corporation, Milford, MA).

Doppler ultrasonography

Carotid arterial hemodynamics were evaluated using duplex B-mode and D-mode programs of the color Doppler ultrasound instrument (model SSD-3500; Aloka America, Wallingford, CT) with an attached 5.0-MHz finger transducer probe (Aloka UST-672). Ultrasound assessments began with a baseline measurement on d 0, continued by d 5, 8, 12, and 15 of the treatment period. All scans were obtained without use of anesthesia, 10 cm below the mandible on the left carotid artery. Prior to scanning the affected area was cleaned and sheared of wool; Aquasonic transmission gel (Parker Laboratories, Fairfield, NJ) was utilized as needed. Using B-mode, a cross-sectional view of the carotid artery was visualized, and arterial pulsatility was confirmed using a duplex view of B-mode and D-mode. This duplex mode also allowed for obtaining final hemodynamic measurements by using B-mode for visualization while simultaneously using D-mode for recording pulsatile waves. As described in Lemley et al. (2012) three comparable cardiac cycle waveforms from three separate ultrasonography

assessments were averaged per ewe within a treatment day (9 measurements per day). Hemodynamic measurements were calculated using peak systolic velocity (**PSV**), end-diastolic velocity (**EDV**), mean velocity (**MnV**), and cross-sectional area of the vessel (**CSA**). These parameters were used to calculate pulsatility index (**PI**; PI = [PSV (cm/s) – EDV (cm/s)]/MnV (cm/s)), an indicator of tissue blood perfusion, and resistance index (**RI**; RI = [PSV (cm/s) – EDV (cm/s)]/PSV (cm/s)), an indicator of vascular resistance.

Statistical analysis

There were four treatments (n = 15 per treatment) and four sampling periods (after baseline hemodynamic measurements). Hemodynamic data were calculated as percent change from baseline and then normalized to the control treatment by setting it to zero. No treatment × sampling period interactions were present, so main effect least square means are reported (n = 60). Contrasts were used to address specific questions: CON vs. RP-ARG (CON vs. 90 + 180 + 360) to test for an overall effect of RP-ARG, linear to test for linear effect of increasing level of RP-ARG, and quadratic to test for a quadratic effect of increasing level of RP-ARG. For linear and quadratic contrasts unequal spacing coefficients were generated and used for contrast statements. For the standard error of the mean (SEM) n = 60.

Ewe performance data were analyzed as a completely random design using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NY) with ewe serving as the experimental unit. These contrasts addressed whether overall RP-ARG supplementation had an effect on ewe performance (CON vs. 90 + 180 + 360), and whether 180 was the optimal dose of RP-ARG supplementation (180 vs. 90, 180 vs. 360). When specific contrast P - values ≤ 0.05 they were considered different.

Amino acid data were analyzed similar to performance data using contrasts to address specific questions. These contrasts addressed whether overall RP-ARG supplementation had an effect on circulating serum amino acids (CON vs. 90 + 180 + 360), and whether 180 was the optimal dose of RP-ARG supplementation (180 vs. 90, 180 vs. 360).

Results and discussion

Ewe performance data

There were no significant differences in BW either before or after the supplementation period ($P \geq 0.12$; Table 2.2). This data is similar to Peine et al. (2013) where no difference in ewe BW was noted due to arginine supplementation. Ewes receiving 360 mg/kg BW of rumen-protected arginine were initially more heavily conditioned (greater BCS) than 180 ewes ($P = 0.001$), and this difference followed throughout supplementation ($P = 0.02$). Ewes receiving no supplement were initially less conditioned than 360 ewes ($P = 0.02$), but were statistically not different at the end of supplementation ($P = 0.08$). This small change in condition is probably not biologically relevant in this particular study.

Table 2.2. Body weight and BCS measurements in nulliparous ewes fed varying doses of rumen-protected arginine

	Treatments[1]						Contrasts[2]		
Item	0	90	180	360	SEM	*P* - value	0 vs. 90, 180, 360	180 vs. 90	180 vs. 360
Initial BW, kg	50.7	47.4	48.7	52.7	1.87	0.22	0.62	0.63	0.14
Final BW, kg	53.3	49.3	50.3	55.2	1.92	0.12	0.44	0.70	0.08
BW change, kg	2.7	1.9	1.7	2.6	0.44	0.28	0.21	0.70	0.15
Initial BCS	2.65	2.70	2.57	2.87	0.063	0.01	0.40	0.14	0.001
Final BCS	2.67	2.68	2.60	2.85	0.072	0.10	0.59	0.42	0.02

[1] Treatments were administered to ewes daily for 14 d mixed in a 50 g fine ground corn carrier. Treatments were 0, 90, 180, and 360 mg of RP-ARG/kg BW daily and are coded 0 (0 mg/RP-ARG/kg BW), 90 (90 mg/RP-ARG/kg BW), 180 (180 mg/RP-ARG/kg BW), and 360 (360 mg/RP-ARG/kg BW). Control ewes (0) were fed the 50 g of fine ground corn carrier without additions of RP-ARG.

[2]There were four treatments (n = 15/treatment). Contrasts were used to address specific questions: 0 (0 mg/RP-ARG/kg BW) vs. RP-ARG (CON vs. RP-ARG90 + RP-ARG180 + RP-ARG360) to test for an overall effect of RP-ARG, 180 vs. 90, and 180 vs. 360 to test for the optimal rumen-protected arginine supplementation dose. For the standard error of the mean (SEM), n = 60.

Circulating amino acids

Baseline levels of circulating serum amino acids (Table 2.3) showed that the only amino acids of interest with inherent differences among treatment were glutamate and lysine. Before initiating supplementation, circulating levels of glutamate and lysine were greater in 180 ewes than in 90 ewes ($P = 0.04$ and $P = 0.02$, respectively).

Upon completion of the supplementation period, circulating serum levels of arginine were greater in 180 vs. 90 ewes ($P = 0.05$; Table 2.4). This trend was also seen in circulating ornithine ($P = 0.05$) and glutamate ($P = 0.02$), both of which are downstream of arginine metabolism. These results are similar to findings by Southern and Baker (1982), in which dietary arginine supplementation increased circulating arginine and ornithine levels. Meyer et al. (2011a) also found that increasing doses of rumen-protected arginine supplementation resulted in increased serum arginine and ornithine.

Though there were initial treatment differences in circulating glutamate levels, the increase throughout supplementation was greater in 180 ewes than in 360 ewes ($P = 0.05$; Table 2.5). Aspartate followed a similar pattern, with a tendency to have greater circulating levels in 180 vs. 90 ewes at the completion of supplementation ($P = 0.08$), and a tendency to have a greater increase throughout supplementation in 180 vs. 360 ewes ($P = 0.07$). Both of these AA play roles in the urea cycle, therefore these changes may suggest a threshold for urea cycle activity induced with supplementation at 180 mg/kg BW. Previous literature has noted increases in closely-related glutamine with increasing dose of rumen-protected arginine supplementation, but no change in serum glutamate levels (Meyer et al., 2011a).

Circulating lysine levels were greater ($P = 0.04$) in 180 vs. 90 ewes and tended to be greater ($P = 0.06$) in 180 vs. 360 ewes upon completion of supplementation. Throughout

supplementation lysine tended to increase in 180 ewes and decrease in 360 ewes (P = 0.06), suggesting a possible threshold in doses to induce antagonism in transport. This same relationship between doses was reported by Meyer et al. (2011a), in which steers receiving 180 mg/kg BW rumen-protected arginine had greater serum lysine levels than those receiving supplement dosed at 360 mg/kg BW. Similarly, we found circulating levels of histidine increased (P = 0.02) in 180 ewes and decreased in 360 ewes throughout supplementation. When comparing all arginine supplemented treatments to control, we did not find any evidence for antagonism in transport due to arginine supplementation; lysine levels did not change throughout supplementation in non-supplemented ewes vs. supplemented ewes (0 vs. 90, 180, and 360; P = 0.72) and neither did circulating histidine levels (0 vs. 90, 180, and 360; P = 0.58). Southern and Baker (1982) found similar data in pigs fed dietary arginine. They noted no change in plasma lysine or histidine due to arginine supplementation. This is probably due to the species' ability to synthesize arginine to overcome these effects.

Based on these amino acid data, it appears that a dose of 180 mg/kg BW is the most appropriate dose tested in this study. This is reflected in the increased serum arginine and ornithine concentrations at the completion of the supplementation period, along with the efficiency in lysine transport that is demonstrated with 180 mg/kg BW dosage, and decreased with 360 mg/kg BW dosage.

Table 2.3. Baseline serum amino acid levels in nulliparous ewes supplemented varying doses of rumen-protected arginine

	Baseline AA levels by Treatment, µmole per L[1]						Contrasts[2]		
	0	90	180	360	SEM	*P* - value	0 vs. 90, 180, 360	180 vs. 90	180 vs. 360
Arginine	164.0	173.1	180.6	175.6	5.87	0.25	0.07	0.37	0.55
Urea Cycle AA[3]									
Ornithine	45.3	39.4	45.0	46.5	2.12	0.11	0.46	0.07	0.70
Citrulline	149.9	153.1	143.9	148.1	7.82	0.87	0.86	0.41	0.71
Aspartate	10.9	10.4	12.1	12.5	0.69	0.13	0.32	0.10	0.65
Arginine metabolism AA[3]									
Ornithine	45.3	39.4	45.0	46.5	2.12	0.11	0.46	0.07	0.70
Citrulline	149.9	153.1	143.9	148.1	7.82	0.87	0.86	0.41	0.71
Proline	89.0	82.9	91.2	93.9	3.70	0.20	0.94	0.12	0.62
Glutamate	54.9	44.1	52.2	54.6	2.69	0.02	0.14	0.04	0.53
Glutamine	472.7	464.2	446.3	486.0	14.33	0.27	0.66	0.38	0.06
Polyamine AA[3]									
Ornithine	45.3	39.4	45.0	46.5	2.12	0.11	0.46	0.07	0.70
Methionine	17.3	17.3	18.5	19.3	0.92	0.33	0.30	0.38	0.52
Cationic transporter AA[3]									
Lysine	98.2	91.6	115.7	114.7	6.98	0.04	0.26	0.02	0.92
Ornithine	45.3	39.4	45.0	46.5	2.12	0.11	0.46	0.07	0.70
Histidine	78.9	77.1	77.3	83.3	2.69	0.33	0.91	0.97	0.12

[1] Treatments were administered to ewes daily for 14 d mixed in a 50 g fine ground corn carrier. Treatments were 0, 90, 180, and 360 mg of RP-ARG/kg BW daily and are coded 0 (0 mg/RP-ARG/kg BW), 90 (90 mg/RP-ARG/kg BW), 180 (180 mg/RP-ARG/kg BW), and 360 (360 mg/RP-ARG/kg BW). Control ewes (0) were fed the 50 g of fine ground corn carrier without additions of RP-ARG.

[2]There were four treatments (n = 15/treatment); sampling occurred at d 0 prior to implementation of supplementation. Contrasts were used to address specific questions: 0 (0 mg/RP-ARG/kg BW) vs. RP-ARG (CON vs. 90 + 180 + 360) to test for an overall effect of RP-ARG, 180 vs. 90, and 180 vs. 360 to test for the optimal rumen-protected arginine supplementation dose. For the standard error of the mean (SEM), n = 60.

[3]All amino acids absent already-stated arginine.

Table 2.4. Final serum amino acid levels in nulliparous ewes supplemented varying doses of rumen-protected arginine

	Final AA levels by Treatment, µmole per L[1]						Contrasts[2]		
	0	90	180	360	SEM	*P* - value	0 vs. 90, 180, 360	180 vs. 90	180 vs. 360
Arginine	160.3	158.5	178.3	176.1	7.10	0.11	0.20	0.05	0.83
Urea Cycle AA[3]									
Ornithine	48.8	45.5	53.2	48.6	2.69	0.26	0.93	0.05	0.23
Citrulline	134.5	137.5	138.6	155.0	8.41	0.32	0.35	0.93	0.17
Aspartate	10.3	10.7	13.0	11.1	0.90	0.18	0.23	0.08	0.16
Arginine metabolism AA[3]									
Ornithine	48.8	45.5	53.2	48.6	2.69	0.26	0.93	0.05	0.23
Citrulline	134.5	137.5	138.6	155.0	8.41	0.32	0.35	0.93	0.17
Proline	95.1	94.3	99.3	91.8	3.48	0.50	0.99	0.31	0.13
Glutamate	63.7	59.0	75.1	64.5	4.61	0.10	0.64	0.02	0.11
Glutamine	300.1	295.5	293.8	309.0	8.89	0.63	0.95	0.89	0.23
Polyamine AA[3]									
Ornithine	48.8	45.5	53.2	48.6	2.69	0.26	0.93	0.05	0.23
Methionine	18.1	17.3	19.2	17.6	1.13	0.65	0.94	0.24	0.31
Cationic transporter AA[3]									
Lysine	102.9	100.1	124.1	102.6	7.87	0.12	0.51	0.04	0.06
Ornithine	48.8	45.5	53.2	48.6	2.69	0.26	0.93	0.05	0.23
Histidine	80.2	78.7	80.7	76.7	2.71	0.72	0.64	0.60	0.30

[1] Treatments were administered to ewes daily for 14 d mixed in a 50 g fine ground corn carrier. Treatments were 0, 90, 180, and 360 mg of RP-ARG/kg BW daily and are coded 0 (0 mg/RP-ARG/kg BW), 90 (90 mg/RP-ARG/kg BW), 180 (180 mg/RP-ARG/kg BW), and 360 (360 mg/RP-ARG/kg BW). Control ewes (0) were fed the 50 g of fine ground corn carrier without additions of RP-ARG.

[2]There were four treatments (n = 15/treatment), sampling occurred at d 15 on the last day of supplementation period. Contrasts were used to address specific questions: 0 (0 mg/RP-ARG/kg BW) vs. RP-ARG (CON vs. 90 + 180 + 360) to test for an overall effect of RP-ARG, 180 vs. 90, and 180 vs. 360 to test for the optimal rumen-protected arginine supplementation dose. For the standard error of the mean (SEM), n = 60.

[3] All amino acids absent already-stated arginine.

Table 2.5. Change in serum amino acid levels in nulliparous ewes supplemented varying doses of rumen-protected arginine

	Change in AA levels by Treatment, µmole per L[1] (Final – Baseline)						Contrasts[2]		
	0	90	180	360	SEM	*P* - value	0 vs. 90, 180, 360	180 vs. 90	180 vs. 360
Arginine	-3.69	-14.6	-2.4	0.4	6.22	0.35	0.80	0.17	0.75
Urea Cycle AA[3]									
Ornithine	3.5	6.1	8.2	2.4	2.73	0.45	0.51	0.59	0.14
Citrulline	-15.4	-15.5	-5.3	6.9	6.72	0.07	0.17	0.29	0.20
Aspartate	-0.5	0.2	0.9	-1.4	0.87	0.29	0.65	0.60	0.07
Arginine metabolism AA[3]									
Ornithine	3.5	6.1	8.2	2.4	2.73	0.45	0.51	0.59	0.14
Citrulline	-15.4	-15.5	-5.3	6.9	6.72	0.07	0.17	0.29	0.20
Proline	6.0	11.4	8.1	-2.0	4.58	0.21	0.96	0.61	0.12
Glutamate	8.7	15.0	22.9	9.9	4.51	0.12	0.17	0.22	0.05
Glutamine	-172.6	-168.7	-152.5	-177.0	13.89	0.62	0.68	0.41	0.22
Polyamine AA[3]									
Ornithine	3.5	6.1	8.2	2.4	2.73	0.45	0.51	0.59	0.14
Methionine	0.8	0.0	0.7	-1.8	1.14	0.35	0.36	0.65	0.13
Cationic transporter AA[3]									
Lysine	4.7	8.5	8.4	-12.2	7.53	0.18	0.72	0.99	0.06
Ornithine	3.5	6.1	8.2	2.4	2.73	0.45	0.51	0.59	0.14
Histidine	1.3	1.6	3.5	-6.6	2.85	0.07	0.58	0.64	0.02

[1] Treatments were administered to ewes daily for 14 d mixed in a 50 g fine ground corn carrier. Treatments were 0, 90, 180, and 360 mg of RP-ARG/kg BW daily and are coded 0 (0 mg/RP-ARG/kg BW), 90 (90 mg/RP-ARG/kg BW), 180 (180 mg/RP-ARG/kg BW), and 360 (360 mg/RP-ARG/kg BW). Control ewes (0) were fed the 50 g of fine ground corn carrier without additions of RP-ARG.

[2] There were four treatments (n = 15/treatment), these data were calculated based on final (d 15) amino acid concentrations – baseline (d 0) amino acid concentrations. Contrasts were used to address specific questions: 0 (0 mg/RP-ARG/kg BW) vs. RP-ARG (CON vs. 90 + 180 + 360) to test for an overall effect of RP-ARG, 180 vs. 90, and 180 vs. 360 to test for the optimal rumen-protected arginine supplementation dose. For the standard error of the mean (SEM), n = 60.

[3] All amino acids absent already-stated arginine.

Doppler ultrasound analysis

All ewes receiving arginine supplement had lower PI, which indicates greater distal tissue perfusion, than CON ewes (*P* = 0.02; Table 2.6). This data was similar to findings by Meyer et al. (2011b), in which steers receiving 180 mg/kg BW rumen-protected arginine had lower PI than steers receiving no supplement. In the case of this experiment, the tissue of interest for increased vascular perfusion is the placenta.

There was a quadratic effect of supplementation on RI, indicating that ewes receiving 90 or 180 mg/kg BW of arginine supplement had the least resistance of blood flow in the carotid artery (*P* = 0.01). This was also similar to previous data demonstrating that steers receiving 180 mg/kg BW rumen-protected arginine had decreased carotid RI as compared to steers receiving 360 mg/kg BW (Meyer et al., 2011b).

The mean velocity of blood flow in the carotid artery increased linearly with supplementation (*P* = 0.02). It is interesting that coupled with this, we note a linear decrease in CSA due to arginine supplementation (*P* = 0.001). It is expected that vasodilation effects would be the reverse of what was demonstrated. In previous research, both of these parameters were unaffected by arginine supplementation (Meyer et al., 2011b).

In addition, we observed a linear increase in heart rate due to arginine supplementation (*P* = 0.001), coupled with a linear decrease in stroke volume (*P* = 0.05). These opposing effects may explain why we did not observe changes in total cardiac output, as cardiac output is determined based on both heart rate and stroke volume. Previous research has noted no change in heart rate, stroke volume, or cardiac output due to rumen-protected arginine supplementation in steers (Meyer et al., 2011b).

This blood flow data also agrees with the amino acid data in that 180 mg/kg BW is the most appropriate dose tested for rumen-protected arginine supplementation. This dose demonstrated a low RI to permit blood flow with less resistance, and improved distal tissue perfusion (lower PI) due to arginine supplementation. Both of these parameters agree with previous research in rumen-protected arginine supplementation in steers (Meyer et al., 2011b).

Table 2.6. Carotid artery hemodynamics in nulliparous ewes supplemented with varying doses of rumen-protected arginine (RP-ARG)[1]

Item	Treatments[2]				SEM	Contrast *P* - values[3]		
	0	90	180	360		0 vs. 90, 180, 360	Linear	Quadratic
	% change from baseline							
Pulsatililty index	0.00	-6.99	-8.30	-5.10	2.53	0.02	0.27	0.03
Resistance index	0.00	-3.28	-3.60	-0.92	0.93	0.02	0.85	0.01
Peak systolic velocity	0.00	5.49	2.88	10.04	2.99	0.08	0.03	0.89
End diastolic velocity	0.00	15.84	13.99	15.71	4.80	0.01	0.06	0.11
Mean velocity	0.00	11.74	10.20	14.53	3.72	0.01	0.02	0.23
Cross-sectional area	0.00	-7.03	-9.99	-11.51	1.82	0.001	0.001	0.03
Flow volume	0.00	2.75	-4.12	0.74	4.06	0.97	0.91	0.61
Heart rate	0.00	0.80	3.49	8.92	1.70	0.02	0.001	0.52
Stroke volume	0.00	3.25	-7.12	-8.88	4.22	0.38	0.05	0.99
Cardiac output	0.00	1.81	-4.24	-0.12	4.09	0.86	0.83	0.61

[1] Doppler ultrasonography was performed on nulliparous ewe lambs fed complete pelleted diets and increasing level of rumen-protected arginine (RP-ARG).

[2] Treatments were administered to ewes daily mixed in a 50 g fine ground corn carrier. Treatments were 0, 90, 180, and 360 mg of RP-ARG/kg BW daily and are coded CON (0 mg/RP-ARG/kg BW), RP-ARG90 (90 mg/RP-ARG/kg BW), RP-ARG180 (180 mg/RP-ARG/kg BW), and RP-ARG360 (360 mg/RP-ARG/kg BW). Control ewes (CON) were fed the 50 g of fine ground corn carrier without additions of RP-ARG.

[3] There were four treatments (n = 15/treatment) and four sampling periods (after baseline hemodynamic measurements). Data were calculated as percent change from baseline and then normalized to the control treatment by setting it to zero. No treatment × sampling period interactions were present, so main effect least square means are reported (n = 60). Contrasts were used to address specific questions and were CON vs. RP-ARG (CON vs. RP-ARG90 + RP-ARG180 + RP-ARG360) to test for an overall effect of RP-ARG, Linear to test for a linear effect of increasing level of RP-ARG, and quadratic to test for a quadratic effect of increasing level of arginine. For linear and quadratic contrasts unequal spacing coefficients were generated and used for contrast statements. For the standard error of the mean (SEM) n = 60.

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CHAPTER 3. EFFECTS OF MATERNAL NUTRITION AND RUMEN-PROTECTED ARGININE SUPPLEMENTATION ON EWE AND POSTNATAL LAMB PERFORMANCE

Abstract

Our hypothesis was that arginine supplementation would overcome the negative effects of restricted maternal intake during the last two-thirds of gestation on ewe and lamb performance. To test this hypothesis, multiparous, Rambouillet ewes (n = 32) were allocated to 3 treatments in a completely random design at 54 ± 3.9 d of gestation. Dietary treatments were 100% of requirements (control, **CON**), 60% of control (restricted, **RES**), or RES plus a rumen-protected arginine supplement dosed at 180 mg/kg BW once daily (**RES-ARG**). Ewes were penned individually in a temperature-controlled facility. At parturition, lambs were immediately removed from their dam and reared independently. At d 54 ± 3 of age, lambs were stunned using captive bolt and exsanguinated, and organs were collected and weighed. Ewe BW from d 75 of gestation through parturition was greater ($P \leq 0.05$) in CON compared with RES or RES-ARG. Similarly, ewe BCS from d 82 of gestation through parturition was greater ($P \leq 0.02$) in CON than either RES or RES-ARG. Total ewe colostrum mass (g) at 3 h after parturition was greater ($P \leq 0.001$) in CON than RES or RES-ARG. Lamb birth weight was greater ($P = 0.04$) in CON than RES ewes, and tended ($P = 0.10$) to be greater in CON vs. RES-ARG. Lambs born to CON ewes had greater ($P \leq 0.03$) BW than lambs from RES ewes at 7, 14, and 33 d postpartum. On d 19, lambs from CON and RES-ARG ewes both had greater ($P \leq 0.04$) BW than lambs from RES ewes (12.0 and 11.5 vs. 10.3 ± 0.41 kg, respectively). Lambs born to CON and RES-ARG ewes had greater ($P \leq 0.04$) average daily gain (**ADG**) than lambs from RES ewes on d 19 (355.0 and

354.0 vs. 306.4 ± 15.77 g, respectively). Lambs from CON and RES-ARG ewes also had greater ($P \leq 0.02$) girth circumference than lambs from RES ewes on d 19 (55.4 and 54.6 vs. 51.3 ± 0.97 cm, respectively). On d 54, lambs from RES-ARG ewes had greater ($P = 0.003$) curved crown rump length than lambs from RES ewes (99.8 vs. 93.9 ± 1.28 cm, respectively). Adrenal glands in lambs from CON dams had greater ($P = 0.04$) mass than adrenal glands in lambs from restricted dams (RES + RES-ARG). Livers in lambs from RES-ARG ewes weighed more ($P = 0.05$) than lambs from RES ewes. These results confirm our hypothesis that arginine supplementation during the last two-thirds of gestation can mitigate some negative consequences associated with restricted maternal nutrition in the offspring, but not in the underfed dams themselves.

Introduction

Fetal growth restriction (**FGR**) has been implicated as the cause of many deleterious postnatal offspring performance defects or traits, including lower birth weights and poor neonatal growth and body composition (Wu et al., 2006; Caton and Hess, 2010; Reynolds and Caton, 2012). One of the major causes of FGR is compromised maternal nutrition, which may occur in extensive grazing systems. In the Western U.S., grazing ewes often receive less than 50% of NRC recommendations, resulting in loss of body weight during pregnancy and reduced lactation performance (Wu et al., 2006; Meyer et al., 2011; Long et al., 2009).

A potential supplement to offset FGR is arginine, a semi-essential amino acid. Arginine contributes to NO and polyamine production, both of which play key roles in placental growth and function (Martin et al., 2001; Kwon et al., 2003; Wu et al., 2009). Nitric oxide and polyamines play unique roles in regulating fetal development throughout gestation, and

contribute to nutrient delivery to, and use by, the developing fetus despite maternal undernutrition. Improved fetal growth has been demonstrated in ovine models of FGR in response to intravenous arginine administration (Wu et al., 2009). Arginine may also enhance offspring growth via insulin stimulation or other avenues of glucose metabolism (Floyd et al., 1966; Schmidt et al., 1992; Gannon et al., 2002). Lassala et. al. (2010) found that arginine administration to underfed ewes enhanced offspring birth weights by 21% as compared to saline-infused underfed ewes; birth weights of offspring from arginine-infused underfed ewes were equal to those from control-fed ewes.

Use of current rumen protection technologies allows for oral administration of specific amino acids with subsequent delivery to the small intestine, which is a practical approach for strategic supplement delivery to ruminants. In this study, we tested the hypothesis that arginine supplementation would mitigate the negative effects of compromised maternal nutrition during the last two thirds of gestation on both ewe and lamb performance. We expected lambs from nutrient restricted, arginine supplemented dams to present as normal and therefore be similar to lambs from control-fed ewes.

Materials and methods

Animals

Protocols described herein were approved by the North Dakota State University Institutional Animal Care and Use Committee. Multiparous Rambouillet-cross ewes (n = 32; 67.7 ± 6.2 kg initial BW) were confirmed pregnant via ultrasound on 41 ± 6.0 d after mating. Ewes were housed individually in a climate controlled facility with free access to water. Ewes were fed a pelleted diet daily at 0800. Weekly ewe BW measurements allowed monitoring of

ewe BW change to determine if dietary adjustments were needed. Body condition scores were assessed every two weeks by two or three independent observers.

Experimental design and treatments

This experiment was a completely randomized design. Ewes were randomly assigned to one of three treatments at 54 ± 3.9 d of gestation: 100% of dietary requirements (control, **CON**; based on NRC, 1985, 2007), 60% of control (restricted, **RES**), or RES with the addition of a rumen-protected arginine (**RP-ARG**, Kemin Industries, Des Moines, IA) supplement (**RES-ARG**). Supplement provided to the RES-ARG ewes contained 180 mg arginine/kg BW (based on initial BW). Arginine was mixed with 50 g of fine ground corn and fed once daily at 0800 before offering the pelleted diet. Both CON and RES ewes were also provided 50 g of fine ground corn daily, without the added rumen protected arginine. Pelleted diets (Table 2.1 from Chapter 2, same diet as the previous study) were fed once daily to ewes on an individual basis, with amounts specific to ewe BW and targeted nutrient supply. Pelleted diets were consumed within 2 h of feeding. Treatments continued until parturition. Two CON and one RES ewe died (2 unknown causes and 1 pneumonia) before parturition. Their data were included in the analyses up to removal from the study.

Parturition and lamb management

A closely monitored, 24 h lambing protocol was implemented during the expected dates of parturition. At parturition, lambs were not permitted to suckle from ewes, and were removed from dams immediately and reared independently. There were four sets of twins (2 CON, 1 RES, and 1 RES-ARG). At 3 h post parturition, ewes were administered a 1 mL (20 USP units)

intramuscular injection of Oxytocin (Vet Tek, Blue Springs, MO) and milked out to determine colostrum weight.

Following removal from the ewe, lambs were towel dried and weighed. Lambs received an intramuscular injection of vitamin A, D, and E (0.5 mL/lamb; 100,000 IU of A, 10,000 IU of D3, 300 IU of E/mL; Stuart Products, Bedford, TX), and 1 mL of *Clostridium perfringens* types C and D and tetanus vaccine (Essential 3+T, Colorado Serum, Denver, CO) subcutaneously. Finally, the umbilical cord was clipped and dipped in 7% iodine tincture.

Lambs received artificial colostrum (Lifeline Rescue Colostrum, APC, Ankeny, IA), administered at 19.1 mL/kg of lamb birth weight at 0 and 2 h post birth, and 25.5 mL/kg of lamb birth weight at 4, 8, 12, 16, and 20 h post birth to achieve 10.64 g IgG/kg lamb birth weight, as previously described (Meyer et al., 2010; Neville et al., 2010).

Lambs were group housed in a climate controlled facility with free access to water. At 24 h post birth, lambs received milk replacer (Super Lamb Milk Replacer, Merrick's Inc., Middleton, WI; DM basis: 24% CP, 30% fat, 0.10% crude fiber, 0.5 to 1.0% Ca, 0.65% P, 0.3 ppm Se, 66,000 IU/kg vitamin A, 22,000 IU/kg vitamin D, and 330 IU/kg vitamin E) for ad libitum intake via bottle until a strong suckling response was observed. Lambs then transitioned to a teat bucket system (Meyer et al., 2010; Neville et al., 2010). In addition to milk replacer, a mixture of long stem mid-bloom alfalfa hay and creep feed (DM basis: 20% CP, 6% fat, 8% crude fiber, 1.4 to 1.9% Ca, 0.4% P, 0.5% to 1.5% NaCl, 0.3 ppm Se, 11,000 IU/kg vitamin A, 6,000 IU/kg vitamin D, and 100 IU/kg vitamin E) were available ad libitum. At 7 d, all tails were docked and male lambs were castrated by banding. At 40 ± 3 d, lambs received an additional 2-mL injection of vitamin A, D, and E as previously described. Lamb BW was measured at birth, 24 h, and 3, 7, and 14, 19, 33, 40, 47, and 54 ± 3 d. Curved crown rump length, measured as the

distance from the crown of the head to the rump along the backbone, and girth, measured as the circumference around the rib cage just behind the forelegs, were determined at birth, and at 19 and 54 d. Two lambs died during the experiment of unrelated causes, one at 7 d (RES-ARG), and another at 45 d (RES). Their data were included in the analyses up to removal from the study.

Necropsy

At d 54 ± 3 of age, lambs were stunned using captive bolt and exsanguinated. Viscera were removed and dissected, and the liver and pancreas were separated from the viscera to obtain individual organ weights. The digestive tract was stripped of digesta and fat, and the ileum, duodenum, and jejunum were separated from the large intestine as described by Soto-Navarro et al. (2004), and each component weighed. The heart was also removed and measured for ventricle dimensions and thickness. In addition, the brain, adrenal glands, kidneys, gonads, spleen, stomach complex, and thyroid were removed and each component weighed.

Jejunal histological analyses

Jejunal tissue was further dissected to a 15 cm segment for mucosal analysis; the segment was cut open flat along the mesenteric side and the luminal surface washed with PBS. After rinsing, the mucosa was scraped from the section using a glass microscope slide. Differential weights were taken on the segment before and after mucosal removal to determine percent mucosa in the jejunum.

Further dissection of jejunal tissue yielded sections (< 1 cm wide) for immersion-fixation in neutral buffered formalin (**NBF**), and tissues were ultimately stored in 70% ethanol solution. Following fixation, these tissue sections were embedded in paraffin (Reynolds and Redmer,

1992) and cut to 5-µm cross-sections. These 5-µm cross-sections were mounted on glass slides, deparaffinized in Histoclear (Electron Microscopy Services, Hatfield, PA), and rehydrated through a series of ethanol/water solutions. Antigen retrieval was performed for 15 minutes with a 10 mM sodium citrate 0.05% tween (pH = 6) buffer in a 2100 retriever (Electron Microscopy Sciences, Hatfield, PA); following antigen retrieval slides were cooled to room temperature for 20 minutes. Tissues were rinsed twice with tris buffered saline (**TBS**) containing 0.1% Triton X-100 (**TBST**) prior to treatment with a blocking buffer consisting of TBS and 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 20 minutes. Primary antibody against Ki67 (1:100; Clone MM1; Vector Laboratories, CA) was used to treat slides overnight at 4°C to mark those cells that are proliferating (Freetly et al., 2014). The following day, slides were washed in TBST prior to treatment in total darkness using a goat anti-mouse CF633 secondary antibody (1:200; 89138-632; VWR, Radnor, PA) for 30 minutes. Finally, slides were washed in distilled water prior to coverslip application using Vectashield Hardset mounting medium (Vector Laboratories, Inc., Burlingame, CA) containing 4',6-diamidino-2-phenylindole (**DAPI**).

Slides were visualized via photomicrographs taken on Zeiss Imager.M2 epifluorescence microscope using 10x objective and AxioCam HRm camera with a Zeiss piezo automated stage. Photomicrographs were taken at six random locations per animal visualizing the crypt region of the intestine, as this is the primary location of intestinal cell proliferation. Images were analyzed using Image-ProPlus 5.0 software (MediaCybernetics Inc., Silver Spring, MD) for percentage of proliferating Ki-67–positive cells of the total number of cells within the analyzed crypt region. Of the six photomicrographs, the three proliferation percentage calculations within the smallest range of each other were used to calculate an average percent proliferation for each lamb.

Statistical analysis

Data were analyzed as a completely random design using the GLM procedure of SAS (SAS Inst. Inc., Cary, NY) with ewe or lamb serving as the experimental unit. Fetal number was included in the model statement and retained if significant ($P \leq 0.10$). After protection with an overall F-test for treatment ($P \leq 0.10$) means were separated using the PDIFF procedure of SAS and P - values ≤ 0.05 were considered different.

Results and discussion

Ewe performance

Restricted (RES and RES-ARG) ewes weighed less ($P \leq 0.05$) than CON ewes from d 75 of pregnancy until parturition (Table 3.1). Similarly, RES and RES-ARG ewes had lower ($P \leq 0.02$) BCS than CON ewes from d 82 throughout parturition, and by d 68 CON ewes had greater ($P \leq 0.03$) BCS than RES-ARG ewes (Table 3.2). These results are similar to those reported by Meyer et al. (2010). Changes in ewe BW and BCS in CON and RES ewes indicate our experimental model responded as predicted and was appropriate for testing our hypothesis regarding supplementation of rumen-protected arginine. In this study, the arginine treatment had no rescue effect ($P \geq 0.82$) on ewe BW or BCS. Similar maternal performance based on arginine supplementation was observed by Satterfield et al. (2013).

Colostrum produced at 3 h postpartum by CON ewes was greater ($P \leq 0.001$) compared with RES and RES-ARG ewes (753.7 vs. 298.6 and 105.4 ± 88.31 g, respectively). Differences observed between CON and RES were expected and reported previously (Wu et al., 2006; Swanson et al., 2008; Meyer et al., 2011). Results also indicated that rumen-protected arginine

supplementation did not rescue colostrum yield in restricted ewes. Effects of the arginine supplement used in this study on longer-term lactation responses were not determined.

Table 3.1. Influence of nutrient restriction and arginine supplementation on ewe BW (kg) throughout gestation

d	Treatment[1]			SEM	*P* - value
	CON	RES	RES-ARG		
54	63.8	63.7	64.2	1.95	0.98
61	64.1	60.3	60.9	1.95	0.32
68	62.9	57.8	57.5	1.94	0.09
75	62.3^{b}	56.8^{a}	56.7^{a}	2.00	0.07
82	64.1^{b}	57.9^{a}	57.3^{a}	1.99	0.03
89	65.3^{b}	58.1^{a}	57.6^{a}	2.09	0.02
96	65.5^{b}	57.9^{a}	57.9^{a}	2.20	0.02
103	65.7^{b}	57.7^{a}	57.4^{a}	2.13	0.01
110	66.4^{b}	57.7^{a}	57.7^{a}	2.06	0.005
117	67.0^{b}	56.9^{a}	56.5^{a}	2.16	0.002
124	67.6^{b}	56.3^{a}	56.7^{a}	2.17	0.001
131	67.9^{b}	56.0^{a}	56.3^{a}	2.13	<0.001
138	69.6^{b}	56.6^{a}	56.9^{a}	2.12	<0.001
145	69.6^{b}	56.3^{a}	56.1^{a}	2.23	<0.001
152	67.0^{b}	56.5^{a}	56.6^{a}	3.30	0.06

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 10).
[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Table 3.2. Influence of nutrient restriction and arginine supplementation on ewe BCS throughout gestation[1]

d	Treatment[2]			SEM	*P* - value
	CON	RES	RES-ARG		
54	2.90	2.91	2.88	0.075	0.94
68	2.94^{b}	2.78^{ab}	2.71^{a}	0.073	0.08
82	2.99^{b}	2.65^{a}	2.66^{a}	0.094	0.02
96	2.90^{b}	2.47^{a}	2.42^{a}	0.093	0.001
110	2.93^{b}	2.40^{a}	2.34^{a}	0.137	0.006
124	2.98^{b}	2.26^{a}	2.26^{a}	0.108	<0.001
138	2.90^{b}	2.01^{a}	2.05^{a}	0.133	<0.001
152	2.75^{b}	1.65^{a}	1.79^{a}	0.199	0.001

[1]BCS structured by scale of 1 = thin to 5 = over conditioned
[2]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 10).
[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Lamb birth weight and performance

Lambs from CON ewes had greater ($P = 0.04$) BW at birth than lambs from RES ewes, with lambs from RES-ARG fed ewes being intermediate and similar to both CON and RES (Table 3.3). This same response was observed for lamb BW on d 7, 14, and 33. On d 3, lambs from CON ewes weighed more ($P \leq 0.02$) than lambs from RES and RES-ARG ewes. On d 19, lambs from CON and RES-ARG ewes weighed more ($P \leq 0.04$) than lambs from RES ewes. Keeping with our hypothesis, these data indicate that arginine may play a role in recovering postnatal BW in lambs from nutritionally compromised dams. This hypothesis is further supported in data by Lassala et al. (2010), where lambs from ewes on a restricted nutrient plane receiving IV L-arginine were similar in birth weights to lambs from adequately fed ewes, both with heavier birth weights than lambs from ewes on nutrient restriction (50% NRC requirements).

Table 3.3. Influence of maternal nutrient restriction and arginine supplementation on offspring BW (g) over time

	Maternal Treatment[1]				
d	CON	RES	RES-ARG	SEM	*P* - value
0	5,228^{b}	4,449^{a}	4,603^{ab}	257.1	0.09
3	6,045^{b}	4,692^{a}	4,990^{a}	298.2	0.008
7	7,112^{b}	6,100^{a}	6,321^{ab}	312.7	0.07
14	9,882^{b}	8,811^{a}	9,459^{ab}	308.8	0.05
19	11,973^{b}	10,272^{a}	11,500^{b}	405.3	0.01
33	17,360^{b}	15,278^{a}	16,202^{ab}	574.5	0.04
40	19,971	18,072	19,488	705.7	0.14
47	21,765	20,606	22,028	796.9	0.41
54	23,830	21,870	23,656	889.9	0.24

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 11).

[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Lamb ADG followed a similar pattern as BW, with lambs from CON and RES-ARG ewes having greater ($P \leq 0.04$) ADG than lambs from RES ewes on d 19 (Table 3.4). Girth measurements at birth and d 54 were greater ($P = 0.03$) in lambs from CON compared with RES ewes, with RES-ARG being intermediate and similar to both CON and RES (Table 3.5). However, on d 19 lambs from CON and RES-ARG ewes had greater ($P \leq 0.02$) girth measurements than lambs from RES ewes. Lambs from RES-ARG ewes had greater ($P = 0.003$) curved crown rump measurements than lambs from RES ewes on d 54, with lambs from CON ewes being intermediate and similar to both RES and RES-ARG. These data support the potential role arginine may play in enhancing offspring growth from underfed dams.

Table 3.4. Influence of maternal nutrient restriction and arginine supplementation on offspring ADG (g) over time

	Maternal Treatment[1]				
d[2]	CON	RES	RES-ARG	SEM	*P* - value
3	272.4	81.0	129.1	72.96	0.17
7	269.1	235.9	245.4	19.49	0.47
14	332.4	311.5	334.7	13.14	0.37
19	355.0[b]	306.4[a]	354.0[b]	15.77	0.05
33	367.6	328.1	346.3	13.29	0.11
40	368.6	340.6	367.9	14.45	0.28
47	351.9	342.3	367.1	15.02	0.51
54	344.5	321.4	349.7	14.98	0.37

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 11).
[2]Interval of d for ADG measured between two subsequent dates (i.e. d 3 through 7 = d 7 ADG)
[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Table 3.5. Influence of maternal nutrient restriction and arginine supplementation on offspring girth (cm) and curved crown rump (cm) length over time

	Maternal Treatment[1]				
Item	CON	RES	RES-ARG	SEM	P - value
Girth					
0d	42.2[b]	38.6[a]	39.4[ab]	1.14	0.08
19d	55.4[b]	51.3[a]	54.6[b]	0.97	0.01
54d	70.8[b]	67.0[a]	69.7[ab]	1.23	0.10
CCR					
0d	54.9	52.6	55.1	1.49	0.43
19d	73.7	69.4	72.9	1.86	0.21
54d	96.3[ab]	93.9[a]	99.8[b]	1.28	0.01

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 11).

[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Lamb organ mass

After organ collection and weighing, no differences ($P \geq 0.44$) were observed based on nutritional plane or arginine supplementation in total GI tract weight (Table 3.6). These results contradict Meyer et al. (2013), where lambs from adequately-fed ewes had greater empty GI tract weights than lambs from nutrient-restricted ewes. These measurements were taken in lambs 19 to 22 d of age, which are significantly younger than in the present study. It is possible that these differences may have been present during fetal development and at birth, but corrected during growth. To support this, Reed et al. (2007) observed several differences in organ mass of offspring from restricted ewes compared to offspring from control-fed ewes when offspring were harvested just before birth at d 135 ± 5 d of gestation. These differences may have disappeared if offspring were followed to a later age.

Adrenal glands in lambs from CON dams had greater ($P = 0.01$) mass than adrenal glands in lambs from RES dams, and tended to have greater ($P = 0.07$) mass than lambs from RES-ARG dams. Also, pancreases in lambs from CON ewes were greater ($P = 0.04$) in mass than lambs from RES ewes. These organs are major users of glucose: the pancreas uses glucose for

insulin and the adrenal glands as synthesis of glucocorticoids. This suggests a possible difference in our offspring in circulating glucose, which has been documented to be lower in bovine FGR offspring in previous literature (Long et al., 2009). In offspring of smaller size like our RES offspring, there will be less maintenance costs for glucose to feed, so smaller adrenal glands and pancreas are adequate for their health and maintenance. In addition, the pancreas also houses proteolytic enzymes which RES offspring may use less of due to slower or lesser metabolism of protein.

Livers in lambs from RES-ARG ewes weighed more ($P = 0.05$) than lambs from RES ewes. This is intriguing, as the liver is the primary organ for ureagenesis, a process that arginine is essential for. Ureagenesis is especially important in ruminants, as urea synthesized in the liver can be released for urea recycling to the digestive tract to meet protein needs of the animal. This could be an essential process in a nutrient-restricted gestational environment, when the animal is most likely not receiving enough dietary protein to meet requirements. The crucial role of maternal protein in fetal development has been well documented (Wu et al, 2006; Larson et al., 2009). A smaller liver for RES offspring may be sufficient for the amount of arginine they have to contribute to the urea cycle, whereas a RES-ARG lamb may have more arginine to contribute, and therefore would perform the urea cycle more readily. This in turn could allow them to recycle more urea to meet protein requirements more efficiently.

Jejunal characteristics

Further analysis of the jejunum showed that on a g/kg BW basis, offspring from CON ewes tended ($P = 0.06$) to have greater jejunal mass than offspring from RES ewes. This may

contribute to the difference in gain between maternal nutritional treatments, as the jejunum is the major location of the small intestine for nutrient absorption.

There were no differences in total jejunum weight, percent mucosa, mucosal tissue weight, or percent cell proliferation in the jejunum ($P \geq 0.28$). This is inconsistent with Yunusova et al. (2013), in which offspring from nutrient restricted and overfed ewes were compared to those from control-fed ewes in terms of small intestinal biology. Offspring from both nutrient restricted and overfed ewes had decreased ($P = 0.09$) crypt cell proliferation compared to offspring from control-fed ewes. However, tissues were harvested from these offspring at 180 d. It is possible that if we had followed our offspring to a later age we may have seen this effect develop. This may also contribute to offspring with fewer differences in organ weights among treatments as age progresses. The intestine of offspring from restricted ewes may adapt to expend less energy on crypt cell regeneration in order to allow organ development to progress.

Table 3.6. Influence of maternal nutrient restriction and arginine supplementation on offspring organ mass at d 54 of age

	Maternal Treatments				
Organ	CON	RES	RES-ARG	SEM	*P* - value
Brain, g	93.6	93.4	92.7	2.69	0.97
g/kg BW	4.05	4.31	3.96	0.224	0.52
Adrenals, g	1.72[a]	1.46[b]	1.54[ab]	0.072	0.03
g/kg BW	0.074	0.067	0.065	0.0035	0.18
Thyroid, g	1.80	1.81	1.93	0.139	0.77
g/kg BW	0.076	0.083	0.082	0.0056	0.61
Heart, g	147.0	132.3	138.4	5.38	0.16
g/kg BW	6.19	6.08	5.87	0.169	0.40
Liver, g	481.2	429.5	490.0	21.08	0.11
g/kg BW	20.34	19.63	20.70	0.616	0.47
Pancreas, g	28.7	24.3	27.2	1.53	0.12
g/kg BW	1.20	1.10	1.16	0.058	0.47
Kidneys, g	106.1	102.6	108.6	4.67	0.66
g/kg BW	4.46	4.68	4.58	0.084	0.17
Total visceral adiposity, g	1,021.7	851.2	888.1	102.40	0.45
g/kg BW	41.5	38.9	37.5	3.83	0.75
Omental fat, g	511.6	424.4	487.8	53.87	0.49
g/kg BW	20.69	19.38	20.55	2.013	0.88
Perirenal fat, g	510.0	426.8	400.3	55.98	0.34
g/kg BW	20.79	19.52	16.97	2.182	0.45
Full GI tract, g	3,732.2	3,330.8	3,471.6	186.73	0.30
g/kg BW	156.4	152.5	147.2	5.82	0.52
Empty GI tract, g	955.9	891.2	920.2	35.35	0.44
g/kg BW	40.59	40.91	39.00	1.172	0.48
Stomach, g	304.5	267.8	259.0	15.67	0.10
g/kg BW	12.78	12.31	11.10	0.630	0.16
Small intestine, g	454.7	442.8	470.5	20.55	0.64
g/kg BW	19.37	20.31	19.85	0.707	0.65
Large intestine, g	190.4	180.6	190.7	9.74	0.71
g/kg BW	8.09	8.28	8.05	0.394	0.91

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 11).

[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Table 3.7. Influence of maternal nutrient restriction and arginine supplementation on offspring small intestine characteristics at d 54 of age

Item	Maternal treatments			SEM	*P* - value
	CON	RES	RES-ARG		
Small intestine, g	454.7	442.8	470.5	20.55	0.64
g/kg BW	19.37	20.31	19.85	0.707	0.65
Duodenum, g	43.9	41.2	43.5	7.88	0.97
g/kg BW	1.84	1.88	1.85	0.316	1.00
Ileum, g	263.5	207.6	256.9	21.98	0.16
g/kg BW	11.07	9.53	10.70	0.782	0.35
Jejunum, g	63.9	95.8	78.0	14.09	0.29
g/kg BW	2.72	4.46	3.39	0.629	0.16
Jejunal Characteristics					
Jejunum, g	63.9	95.8	78.0	14.09	0.29
g/kg BW	2.72	4.46	3.39	0.629	0.16
% mucosa	81.5%	82.0%	84.3%	1.43	0.34
Mucosal weight	52.0	79.6	65.9	11.95	0.28
Percent cell proliferation	29.9	36.8	30.2	4.100	0.41

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 11).

[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Conclusions

Ewe performance outcomes were inconsistent with our hypothesis and were not responsive to supplemental rumen-protected arginine during gestation. However, in keeping with our hypothesis, lamb BW, ADG, and body size measurements were responsive while organ masses were not responsive to maternal rumen-protected arginine supplementation. Additional research is needed to further define the effects of supplementation of rumen-protected arginine during gestation on offspring heath and performance outcomes.

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CHAPTER 4. EFFECTS OF MATERNAL NUTRITION AND RUMEN-PROTECTED ARGININE SUPPLEMENTATION ON MATERNAL CAROTID ARTERIAL HEMODYNAMICS AND CIRCULATING AMINO ACIDS IN DAMS AND OFFSPRING

Abstract

Our hypothesis was that rumen-protected arginine supplementation during gestation would induce improved gestational hemodynamics in ewes by increasing distal tissue perfusion while decreasing vascular resistance. We further hypothesized that maternal supplementation of this amino acid would affect circulating concentrations of related amino acids: citrulline, ornithine, lysine, and histidine, in both dams and their offspring. To test our hypothesis, we randomly assigned (n = 32) multiparous Rambouillet ewes to one of three treatments: control (**CON**) received 100% nutrient requirements during gestation, restricted (**RES**) received 60% nutrient requirements during gestation, and a third group received the RES diet with 180 mg/kg daily rumen-protected arginine supplement (**RES-ARG**). Ewes were penned individually in a temperature-controlled facility. Hemodynamics were measured via Doppler ultrasound with baseline measurements at d 50, followed by scans on d 90 and 130 of gestation. We observed no change in hemodynamics during gestation based on arginine supplementation; however, CON ewes did have increased ($P = 0.05$) distal tissue perfusion compared to both groups of restricted ewes (RES and RES-ARG). Blood samples were taken from ewes on d 54, 82, 110, and 138 of gestation, along with immediately post parturition. We observed that throughout gestation RES-ARG ewes had significantly more circulating citrulline than RES ewes ($P = 0.002$). At parturition, CON and RES-ARG ewes had similar ($P = 0.29$), while RES ewes had lower circulating citrulline concentrations ($P \leq 0.03$). We did not see overall gestation differences ($P =$

0.17) in circulating arginine concentrations among treatments in ewes. At parturition, lambs were immediately removed from their dam and reared independently. Blood samples were taken from lambs on d 1, 3, 7, 33, and 54 of age. At 24 h (d 1) of age, lambs from CON dams had greater ($P = 0.008$) circulating arginine concentrations than lambs from RES dams, with lambs from RES-ARG dams intermediate to both ($P \geq 0.09$). By d 54 of age, lambs from RES-ARG dams had greater ($P = 0.02$) circulating arginine concentrations than CON, while RES was intermediate to both ($P \geq 0.19$). These results do not follow our hypothesis that arginine supplementation will improve hemodynamics during gestation, but do confirm that maternal rumen-protected arginine supplementation will alter maternal arginine and related amino acid concentrations during gestation and in their offspring.

Introduction

Numerous arginine supplementation strategies have been studied for their effectiveness in delivering arginine to the bloodstream; however, in ruminants, this presents a more unique challenge. Dietary supplementation of amino acids to ruminants is, as a standard, not effective due to the ability of the rumen to break down the amino acids before they can reach the small intestine for absorption. Rumen protection technologies have made amino acid supplementation to ruminants possible (Oke et al., 1986). With proper protection technology, a dietary amino acid supplement should be able to pass through the rumen intact and be absorbed into the bloodstream via the small intestine. Several rumen-protected amino acid supplements are available and in use, especially methionine and lysine (Oke et al., 1986; Sun et al., 2007; Schwab and Ordway); however, rumen-protected arginine is not currently commercially available.

Arginine has been an amino acid of interest for supplementation primarily due to its effects on blood flow. Arginine provides the N to synthesize NO from NOS enzymes (Morris et al., 2007; Wu et al., 2009; Wu et al., 2013). Nitric oxide is a vasodilator that will increase blood flow by affecting vascular tone (Martin et al., 2001). Meyer et al. (2011b) found that rumen-protected arginine supplementation to steers can influence carotid arterial hemodynamics by increasing distal tissue perfusion and decreasing resistance in the vessel. These blood flow effects are especially of interest during gestation, as increased blood flow to the placenta may increase nutrient transport to the developing fetus. It has been demonstrated that at-risk pregnancies are often associated with impaired blood flow during gestation; however, previous studies have demonstrated that therapeutics targeting the NO system may be effective in increasing blood flow to help to rescue the pregnancy (Reynolds et al., 2006).

A common cause of at risk pregnancies is FGR in utero, which can be induced by poor maternal nutrition during gestation (Wu et al., 2006). Lassala et al. (2010) administered arginine infusions via jugular catheter to gestating ewes receiving 50% of their daily nutrient requirements, and saline infusions to half of the underfed ewes along with ewes receiving 100% of their daily nutrient requirements from d 60 of gestation through parturition. This arginine infusion to underfed ewes enhanced birth weights by 21% as compared to underfed ewes receiving saline. Lambs from arginine-infused underfed dams were not different than lambs from control-fed saline-injected ewes. Their study demonstrates that arginine administration during gestation to at-risk pregnant dams may rescue the fetus from deleterious life effects.

It is prudent to note that administration of any amino acid will effect metabolism of other amino acids – some positively and some negatively. Arginine interacts with some key amino acids in specific ways. Arginine can both be synthesized from and play a role in synthesis of

citrulline; during times of low protein citrulline can be recycled to provide more arginine to the animal (Cynober, 2002). Citrulline supplementation has been shown to increase plasma arginine, and is relatively bioavailable (Cynober, 2007). Arginine can also synthesize ornithine in the gut via arginases, and ornithine can in turn synthesize polyamines downstream (Cynober, 2002; Flynn et al., 2002). Polyamines also play important roles in pregnancy due to their regulation of angiogenesis and embryonic development (Kwon et al., 2003). Lysine and histidine can have an antagonist relationship with arginine due to competition for the same y^+ intestinal transporter (Flynn et al., 2002; Wu et al., 2009). Most of this lysine research has been performed in chicks, as chicks cannot synthesize arginine to correct this imbalance. Chicks supplemented with lysine have shown decreased growth rate, symptoms of arginine deficiency, and decreased plasma arginine concentrations due to an increase in arginine requirements (Jones et al., 1967). These results have been mixed in other studies, as most mammals can synthesize arginine from citrulline to counteract this imbalance.

The objectives of this study are to evaluate the ability of rumen-protected arginine supplementation to positively impact carotid arterial hemodynamics during gestation. To aid in this, we also evaluated circulating amino acids in both pregnant dams and their offspring. We hypothesize that arginine supplementation will cause increased circulating arginine in supplemented ewes, along with increased ornithine and citrulline and decreased lysine. We further hypothesize that arginine supplementation will cause increased distal tissue blood perfusion and decreased resistance to vascular flow.

Materials and methods

Animals

Protocols described herein were approved by the North Dakota State University Institutional Animal Care and Use Committee. Multiparous Rambouillet-cross ewe lambs (n = 32; 67.7 ± 6.2 kg initial BW) from NDSU Hettinger Research Extension Center were housed individually in a temperature-controlled facility (12 to 21°C; Animal Nutrition and Physiology Center; Fargo, ND) with free access to water. Facility lighting was timed to mimic normal daylight patterns.

Experimental design and treatments

These procedures are described in more detail in Chapter 3. Ewes were fed a complete pelleted diet daily at 0800 (Table 2.1; Chapter 2, same diet as previous study). Ewe BW was monitored weekly and BCS every two weeks (by two or three independent observers) to determine if dietary adjustments were needed. Dietary intake for each individual ewe was calculated based on BW and ME requirements, and adjusted appropriately based on nutritional treatment. Diets were analyzed for DM, ash, and CP following AOAC (1990), and NDF and ADF using an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY).

Ewes were randomly assigned to one of three dietary treatments. Dietary treatments included 100% of dietary requirements (control, **CON**; based on NRC, 1985, 2007), 60% of control (restricted, **RES**), or RES with the addition of a rumen protected arginine (**RP-ARG**, Kemin Industries, Des Moines, IA) supplement (**RES-ARG**). The rumen-protected arginine dose was administered at 180 mg/kg BW based on previous research done by our lab (Meyer et al., 2011b; also experiment described in Chapter 2), and delivered in a 50 g fine ground corn carrier

fed before the pelleted feed. This supplement was only received by the RES-ARG ewes, however the 50 g of fine ground corn was received by all ewes regardless of treatment.

Parturition and lamb management

At parturition, lambing was closely monitored 24 h per day as lambs were separated from dams immediately post birth. We sought to eliminate any bias in development due to maternal lactation. Lambs continued to be reared independent of dams for the remainder of the study. These procedures were described in more detail in Chapter 3 of this thesis.

There were four sets of twins (2 CON, 1 RES, and 1 RES-ARG). Two lambs died during the experiment of unrelated causes, one at 7 d (RES-ARG), and another at 45 d (RES). Their data were included in the analyses up to removal from the study.

Doppler ultrasonography

The procedure for Doppler ultrasound used in this experiment was followed using the same procedure described for the previous dose titration experiment in Chapter 2. Carotid arterial hemodynamics were evaluated using duplex B-mode and D-mode programs of the color Doppler ultrasound instrument (model SSD-3500; Aloka America, Wallingford, CT) with an attached 5.0-MHz finger transducer probe (Aloka UST-672). Ultrasound assessments were obtained without use of anesthesia, beginning with a baseline measurement on d 50, followed by measurements on d 90 and 130 of gestation. All scans were performed 10 cm below the mandible on the left carotid artery after shearing of wool and cleaning of the affected area. Aquasonic transmission gel (Parker Laboratories, Fairfield, NJ) was utilized as needed.

A cross-sectional view of the carotid artery was visualized first using B mode, and arterial pulsatility was confirmed using a duplex view of B-mode and D-mode. By using B-mode for visualization while simultaneously using D-mode for recording pulsatile waves, duplex mode was also used to obtain final hemodynamic measurements.

As described in Lemley et al., 2012, three separate ultrasonography assessments yielding three analogous cardiac cycle waveforms were used to average hemodynamics for each ewe within a gestational day (9 measurements per day). Hemodynamic measurements were calculated using peak systolic velocity (**PSV**), end-diastolic velocity (**EDV**), mean velocity (**MnV**), and cross-sectional area of the vessel (**CSA**). These parameters were used to calculate final hemodynamic measurements including pulsatility index (**PI**; PI = [PSV (cm/s) – EDV (cm/s)]/MnV (cm/s)), an indicator of tissue blood perfusion, and resistance index (**RI**; RI = [PSV (cm/s) – EDV (cm/s)]/PSV (cm/s)), an indicator of vascular resistance.

Blood sample collection and analysis

Blood samples from dams were attained via right jugular venipuncture (to avoid puncturing the left carotid artery used for Doppler ultrasonography) at 0700 on d 54, 82, 110, 138, of gestation, and immediately following parturition. Blood was collected from lambs immediately post birth, and at d 3, 7, 33, and 54 of age.

All samples were collected in 10 mL Corvac serum separator vacuum tubes with thrombin (Tyco Healthcare, Mansfield, MA), which were placed on ice immediately post sample collection and held a minimum of 45 minutes to permit clotting. Whole blood samples were centrifuged for 30 minutes at 1,500 × g at 4°C. Following centrifugation supernatant was pipetted into 2-mL screw-cap vials and stored at 20°C.

Amino acid concentrations in serum were determined using ultra performance liquid chromatography (UPLC) methods similar to Meyer et al., 2011a, and the analysis performed in the previous experiment in Chapter 2 of this thesis.

Statistical analysis

Amino acid concentrations in both ewes and lambs along with Doppler measurements were analyzed as repeated measures over time using the MIXED procedure if SAS (SAS Inst. Inc., Cary, NC). The factors measured included maternal dietary treatment, d of gestation, and interactions between treatment and d of gestation. Ante-dependence was used to appropriately describe the covariate structure. After protection with an overall F-test for treatment ($P \leq 0.10$) means were separated using the method of least significant difference and statistical procedures of SAS and *P* - values ≤ 0.05 were considered different.

Results and discussion

Ewe Doppler measurements

Following our hypothesis that arginine will improve hemodynamics during gestation, we expected RES-ARG ewes to respond similarly to CON ewes, with RES ewes having a disadvantage in hemodynamic measurements. Contrary to our hypothesis, we did not see an effect ($P \geq 0.24$; Table 4.1) on maternal carotid hemodynamics due to arginine supplementation. However, we did observe differences due to level of nutrition in PI measurements. Ewes receiving adequate nutrients (CON) had lower ($P = 0.05$) PI than both RES and RES-ARG ewes. This means that CON ewes had greater distal tissue perfusion than RES ewes, which follows our original hypothesis. Previous studies have noted that in at risk pregnancies similar to those of

RES ewes, essential blood flow alterations during gestation may not occur and offspring may suffer as a result (Reynolds et al., 2006). It was our thinking that arginine supplementation may induce these blood flow increases regardless of nutritional plane, but our data did not support this theory.

It may seem most intuitive that blood flow to organs should increase as pregnancy progresses, but this is not necessarily the case for all individual tissues. Our data shows that as pregnancy progresses, PI, as measured in the carotid artery, increases ($P < 0.001$); this increase in PI was an effect of an increase in PSV ($P < 0.001$), EDV ($P < 0.001$), and MnV ($P < 0.001$). An increase in PI signifies a decrease in distal tissue perfusion; in the case of the carotid artery, the tissue of perfusion is the brain. This increase in PI despite arginine supplementation is contrary to previous data from our lab; in a previous study, rumen-protected arginine supplementation at 180 mg/kg BW to steers caused both decreased carotid PI and RI compared to those steers receiving no arginine supplement (CON), and therefore increased distal tissue perfusion with lowered vascular resistance (Meyer et al., 2011b). We hypothesize that the major difference between the previous study and our current study is the status of pregnancy; the arginine supplement could not overcome the effect of alterations in blood flow during pregnancy. Rosenfeld (1977) found that, in pregnant ewes, blood flow to the brain as a percentage of cardiac output decreases as pregnancy progresses, along with blood flow to the liver and kidney In contrast, blood flow to the uterus and mammary gland increases markedly as pregnancy progresses. Further, Rosenfeld found that since cardiac output as a whole increases throughout pregnancy, actual blood flow to the brain remains the same. In either case, blood flow to the brain did not increase in either our ewes or the ewes in Rosenfeld’s study.

In addition to PI and its components, we observed an increase across all treatments in flow volume ($P < 0.001$) and cardiac output ($P < 0.001$) throughout pregnancy, which is similar to what has been observed in pregnant ewes in previous studies (Rosenfeld et al., 1977). Similar to our current study, the previous steer study from our lab cited no change in flow volume and cardiac output due to arginine supplementation (Meyer et al., 2011b).

Table 4.1. Influence of nutrient restriction and arginine supplementation on ewe carotid arterial hemodynamics throughout gestation

	Treatment[1]					Day of Pregnancy					Treatment* Day of Pregnancy
Item	CON	RES	RES-ARG	SEM	*P* - value	50	90	130	SEM	*P* – value	*P* – value
Pulsatility index	1.781^{a}	2.034^{b}	2.032^{b}	0.0807	0.05	1.462^{a}	2.136^{b}	2.249^{b}	0.0788	<0.001	0.17
Resistance index	0.671	0.755	0.674	0.0511	0.41	0.700	0.708	0.691	0.0429	0.38	0.53
Peak systolic velocity	131.6	138.1	122.6	12.45	0.67	123.0^{a}	118.3^{a}	151.1^{b}	12.43	<0.001	0.70
End diastolic velocity	42.9	44.7	39.7	4.00	0.66	36.6^{a}	38.7^{a}	52.0^{b}	4.29	<0.001	0.66
Mean velocity	53.6	49.0	43.8	4.11	0.24	61.1^{a}	40.4^{b}	44.8^{b}	3.90	<0.001	0.85
Cross-sectional area	0.27	0.28	0.27	0.020	0.77	0.27	0.27	0.28	0.016	0.78	0.87
Flow volume	824	744	703	68.5	0.44	960^{a}	623^{b}	688^{b}	61.5	<0.001	0.55
Heart rate	94	94	84	7.0	0.50	94	89	90	6.9	0.80	0.66
Stroke volume	8.8	8.7	8.4	0.80	0.93	10.3^{a}	7.4^{b}	8.2^{b}	0.63	<0.001	0.70
Cardiac output	0.82	0.73	0.70	0.067	0.41	0.95^{a}	0.61^{b}	0.68^{b}	0.060	<0.001	0.56

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 10).

[a, b]Means within a row with different superscripts differ ($P \leq 0.05$) within main effect means.

Ewe circulating amino acids

As we hypothesized, throughout gestation RES-ARG ewes had significantly more (P = 0.002; Figure 4.1) circulating citrulline than RES ewes. However, CON ewes had significantly more (P = 0.05; Figure 4.1) circulating citrulline throughout gestation than RES-ARG ewes. At parturition particularly, CON and RES-ARG ewes had similar (P = 0.29), while RES ewes had lower ($P \leq 0.03$) circulating citrulline concentrations. Because of the much intertwined relationship between arginine and citrulline (Cynober, 2002), these results are not surprising. Since RES-ARG ewes are intermediate (CON > RES-ARG > RES; $P = < 0.001$) in circulating citrulline concentrations throughout gestation, this may signify more efficient arginine mobilization than CON ewes, which may be converting excesses of arginine to citrulline. Ewes receiving restricted nutrients (particularly protein) may be mobilizing citrulline to synthesize arginine where necessary.

Circulating ornithine concentrations were also different among treatments at specific points of gestation (P = 0.04; Figure 4.1). Despite CON ewes having greater ($P \leq 0.02$) circulating ornithine than both RES and RES-ARG ewes at d 110 and d 138 of gestation, at parturition RES-ARG ewes had greater (P = 0.03) circulating ornithine than RES ewes, with CON ewes being intermediate to both ($P \geq 0.08$).

These differences in citrulline and ornithine may help to explain why we don't see overall gestation differences (P = 0.17; Table 4.2) in circulating arginine concentrations among treatments. Because arginine can be metabolized to both ornithine and citrulline, we may be measuring effects of arginine which aren't contributing the measurements of arginine itself, but measurements of ornithine or citrulline instead.

We did not see any effects of antagonism in circulating lysine or histidine concentrations throughout gestation among treatments in these ewes ($P \geq 0.13$; Figure 4.2). This shows that the dose of arginine that we are providing (180 mg/kg) is not too potent to inhibit the animal's ability to overcome the potential amino acid imbalance induced by supplementation. Also, we know that sheep have different amino acid metabolism capabilities than poultry, which is the primary species showing evidence of arginine/lysine antagonism.

Table 4.2. Serum arginine concentrations in ewes supplemented with rumen-protected arginine from d 54 to term of gestation[1]

	Treatment				*P* - values		
d of gestation	CON	RES	RES-ARG	SEM	Trt	d of gestat-ion	d of gestat-ion *Trt
54	183.47	192.61	208.44	18.722	0.17	<0.001	0.01
82	139.34	127.67	126.76	7.400			
110	138.24	119.42	139.29	9.737			
138	183.96	144.86	148.12	8.162			
Parturition	124.17	106.50	125.65	9.655			

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 10).

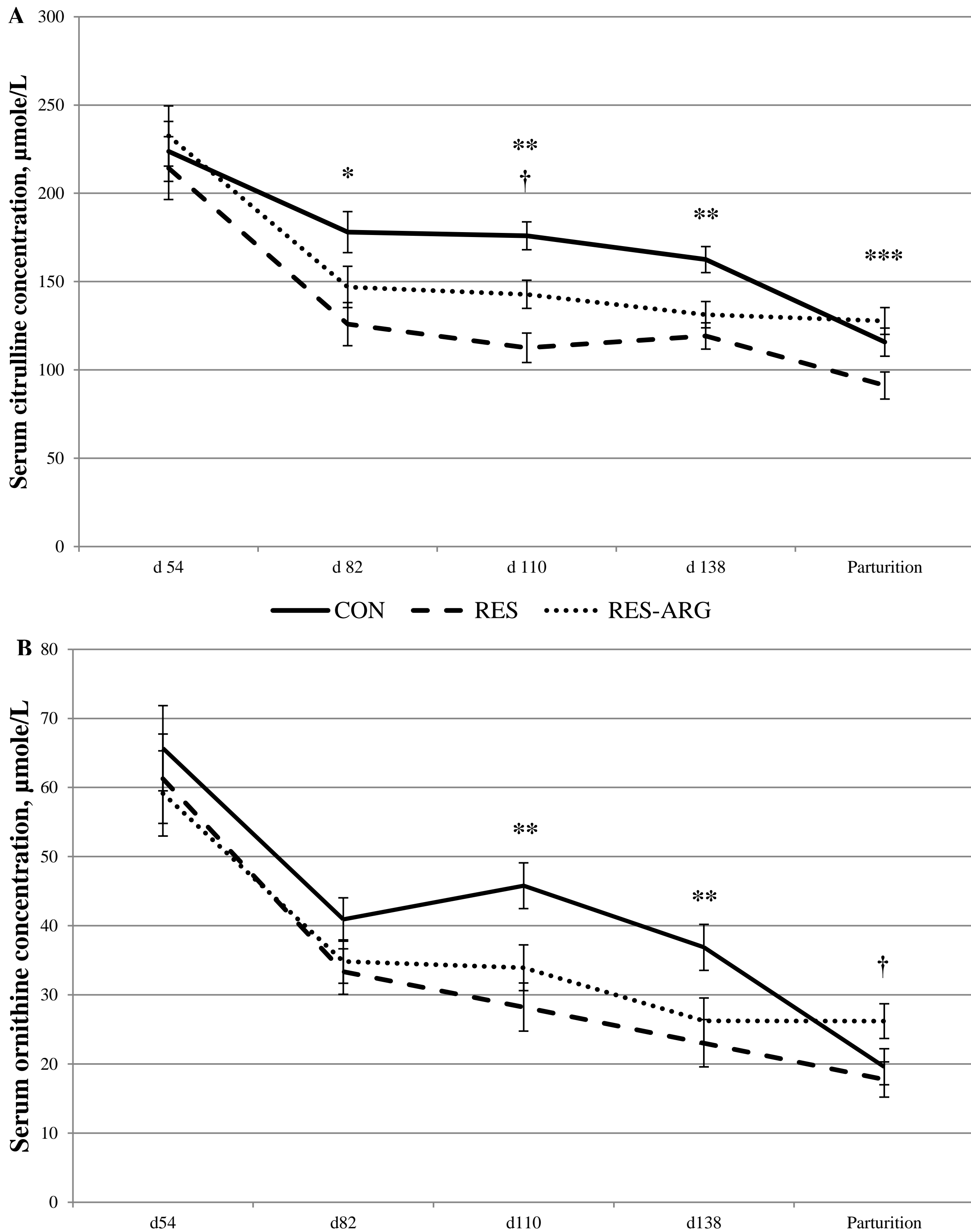

Figure 4.1. Influence of nutrient restriction and arginine supplementation on serum concentrations of (A) citrulline and (B) ornithine, amino acids related to arginine metabolism. * = CON > RES, ** = CON > RES and RES-ARG, *** = CON and RES-ARG > RES, † = RES-ARG > RES (Differences at $P \leq 0.05$)

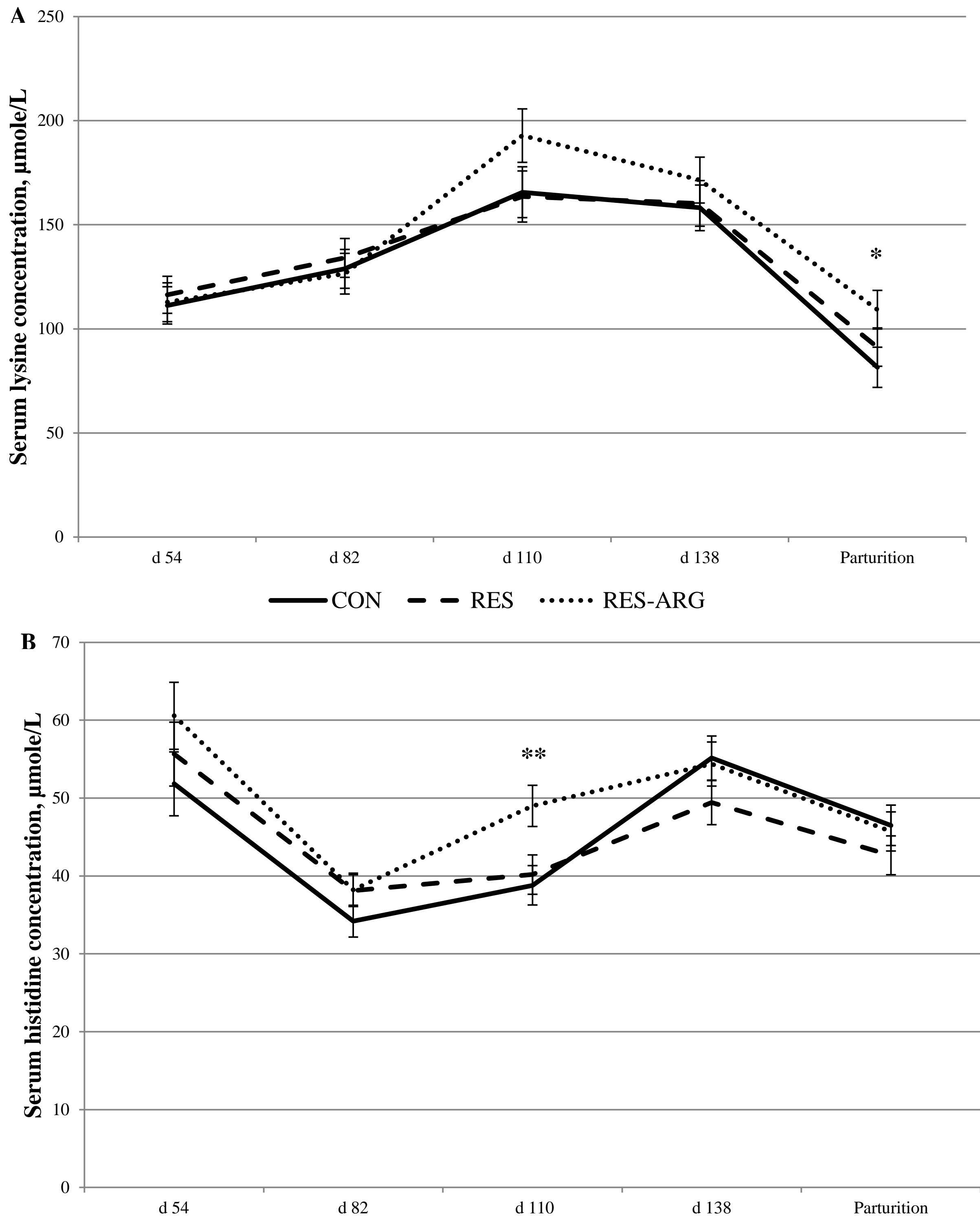

Figure 4.2. Influence of nutrient restriction and arginine supplementation on serum concentrations of (A) lysine and (B) histidine, potential amino acid transporter antagonists. * = RES-ARG > CON, ** = RES-ARG > CON and RES (Differences at $P \leq 0.05$)

Lamb circulating amino acids

Differences in circulating arginine concentrations were observed among offspring from dams on different nutritional treatments at different ages (*P* = 0.04; Figure 4.3). At 24 h of age, lambs from CON dams had greater (*P* = 0.008) circulating arginine concentrations than lambs from RES dams, with lambs from RES-ARG dams intermediate to both (*P* ≥ 0.09). This agrees with previous data from Kwon et al. (2004), in which fetal plasma from ewes fed 50% of requirements from d 28 to d 135 of gestation had reduced amino acid concentrations (including arginine, citrulline, and ornithine) as compared to offspring from ewes fed 100% of requirements throughout gestation. This previous data suggests that our maternal arginine supplementation improved circulating amino acids in offspring from nutrient restricted dams.

Strangely, it seems that offspring from RES ewes maintained arginine concentrations over time while offspring from CON ewes dropped in arginine concentration over time. At d 33 of age, lambs from RES dams had greater (*P* = 0.03) circulating arginine concentrations than CON, while RES-ARG was intermediate to both (*P* ≥ 0.16; Figure 4.3). Also at d 33 of age, lambs from CON dams had greater (*P* ≤ 0.03; Table 4.3) circulating citrulline concentrations than lambs from both RES and RES-ARG dams. It is possible that the arginine in CON offspring was used to synthesize citrulline because it may have reached a point of excess.

By d 54 of age, lambs from RES-ARG dams had greater (*P* = 0.02) circulating arginine concentrations than CON, while RES was intermediate to both (*P* ≥ 0.19; Figure 4.3). These trends over time seem to describe a more steady state metabolism of arginine in offspring from RES-ARG dams compared to the more volatile serum arginine concentration changes observed in offspring from RES or CON dams. This steady state metabolism could provide more advantageous use of arginine for vascular modulation and polyamine metabolism, therefore

potentially offering the offspring a more healthy life than offspring from at-risk nutritional pregnancies may normally have (Reynolds et al., 2006). In Chapter 3 of this thesis, I have described previous data from our lab that shows maternal arginine supplementation during gestation improves BW and ADG in offspring from nutrient restricted dams. A contributor to this effect could be more efficient amino acid metabolism in offspring induced by maternal supplementation.

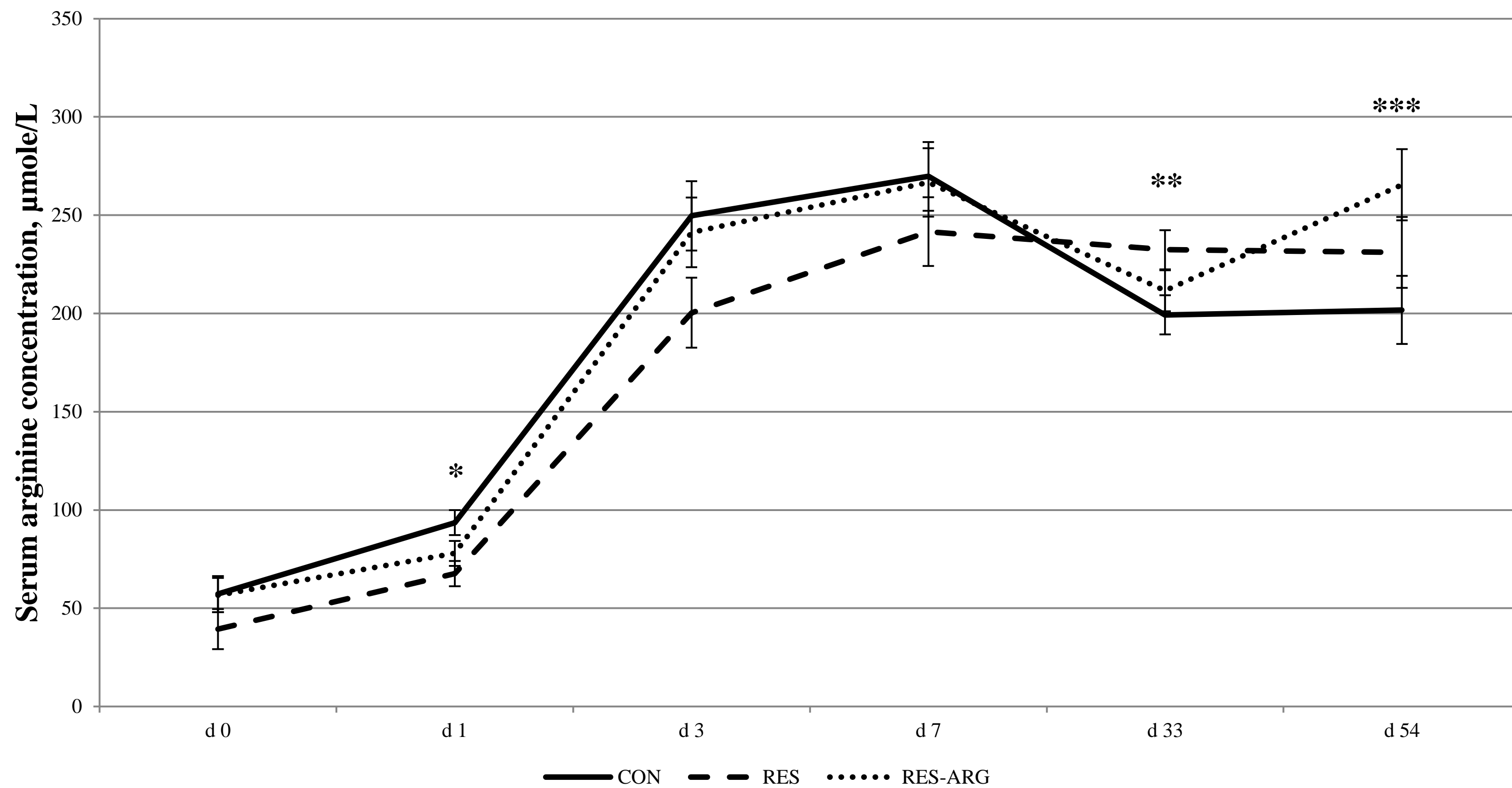

Figure 4.3. Influence of maternal nutrient restriction and arginine supplementation on offspring serum arginine concentration over time. CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 11). * = CON > RES, RES-ARG = CON and RES; ** = RES > CON, RES-ARG = CON and RES; *** = RES-ARG > CON, RES = CON and RES-ARG. Differences determined at $P \leq 0.05$.

Table 4.3. Serum amino acid concentrations in lambs from ewes supplemented with rumen-protected arginine from d 54 to term of gestation

	Maternal Treatment				*P* - values		
Age	CON	RES	RES-ARG	SEM	Trt	Age	Age*Trt
	Ornithine serum concentration (µmole/L)						
d 0	75.84	64.67	74.98	6.435	0.17	<0.001	0.82
d 1	30.54	28.52	24.12	4.655			
d 3	56.29	53.09	46.94	5.278			
d 7	54.63	45.10	47.43	4.858			
d 33	93.77	87.27	72.92	10.083			
d 54	91.41	76.76	76.05	8.963			
	Citrulline serum concentration (µmole/L)						
d 0	138.01	128.42	157.21	13.374	0.02	<0.001	0.05
d 1	96.84	74.16	67.71	14.727			
d 3	418.58[a]	301.46[b]	366.43[ab]	29.979			
d 7	331.24	325.29	310.22	20.765			
d 33	350.71[a]	271.81[b]	281.89[b]	22.260			
d 54	355.79	282.18	322.94	32.109			
	Lysine serum concentration (µmole/L)						
d 0	34.30[ab]	33.04[a]	44.99[b]	3.958	0.84	<0.001	0.71
d 1	43.84	37.77	34.57	4.644			
d 3	90.77	87.62	83.43	7.570			
d 7	99.27	96.35	91.61	7.949			
d 33	146.99	144.56	138.79	12.869			
d 54	168.46	163.19	175.37	14.722			
	Histidine serum concentration (µmole/L)						
d 0	53.26	51.82	55.08	6.120	0.80	<0.001	0.29
d 1	77.30[a]	62.34[b]	61.21[b]	4.329			
d 3	80.27	84.19	79.64	6.038			
d 7	54.27	48.75	56.11	4.532			
d 33	50.10	55.24	54.81	3.666			
d 54	64.65	64.61	69.01	5.447			

[1]CON = control, 100% NRC requirements (n = 11); RES = restricted, 60% CON nutrients (n = 11); RES-ARG = restricted + arginine, 60% CON nutrients with rumen-protected arginine supplement (n = 11).
[a, b]Means within a row with different superscripts differ ($P \leq 0.05$).

Summary

Data from this study demonstrates that maternal arginine supplementation during the last two-thirds of gestation has potential to improve offspring amino acid metabolism for arginine and related amino acids. We noted a more steady state metabolism effect on fetal arginine over time in RES-ARG lambs as opposed to offspring from CON and RES ewes. However, we reject our hypothesis that arginine supplementation during gestation improves maternal carotid arterial hemodynamics in at-risk pregnancies.

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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Arginine has been an amino acid at the center of biomedical research for justified reasons; its potential effects on blood flow and polyamine synthesis along with essential biological (especially reproductive and fetal development) processes have been well documented (Wu et al., 2009). Our goal was to establish a dietary supplementation protocol for gestating ruminants that would enhance life of offspring postnatally.

Our dose titration study (Chapter 2) left us confident that in ewes, 180 mg/kg BW is the most efficient dose tested for the ewe to metabolize and utilize the supplement to induce an effect on blood flow. We know from previous data in our lab (Meyer et al., 2011) that this same dose has been shown to be most appropriate for steers. Taken together these data indicated that 180 mg/kg BW daily is the most effective level of rumen-protected arginine tested.

When we applied this dose to a pregnant ewe nutrient restriction model, we found novel data suggesting that dietary arginine supplementation to restricted nutrient dams can result in enhanced postnatal fetal growth, despite not improving maternal BW or BCS (chapter 3). This data had been previously supported via arginine injection to ewes (Lassala et al., 2010); however, not via dietary consumption, which is more practical in production settings.

Taking our analyses a step further, we learned that the effects we saw based on supplementation regarding blood flow of non-pregnant ewes (Chapter 2) were not replicated in pregnant ewes (Chapter 4). The remarkable shift in blood flow during pregnancy (Rosenfeld et al., 1974; Rosenfeld, 1977; Reynolds et al., 2006) likely overshadowed any response from the arginine supplement. However, we did see that circulating serum arginine concentrations, along with related amino acids (citrulline, ornithine, lysine, and histidine), appeared to reflect more

efficient, steady-state metabolism in both ewes and their offspring. This could contribute to an enhancement in postnatal development of offspring.

Because this supplement is novel and not yet commercially available, more research should be conducted to determine how this effect of enhanced fetal growth despite maternal undernutrition is occurring. Specifically, nutrient transporters in dams and offspring should be examined to see if this data supports the serum amino acid concentration data: do those arginine supplemented dams and offspring up regulate arginine transporters to enhance metabolism?

In addition, the effect of this supplement in other ruminant species, especially cattle, should be studied. Does this supplement have the same effect across species, or only in sheep? Taking this a step forward, would arginine supplementation to pregnant mothers receiving inadequate nutrition help to encourage normal fetal development and postnatal life of human babies? In areas of the world where protein sources are scarce, specifically, should this amino acid be considered for supplementation to pregnant women?

All of these are questions for researchers moving forward in arginine research to address. It is my hope that the data we collected may provide information for these potential studies in the future.

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