The Impact of Dietary Arginine and Citrulline in the Neonatal Gut

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Abstract

Citrulline is a non-protein amino acid that is converted to arginine in the urea cycle through the activities of argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL); as such, citrulline supplementation may increase arginine availability. Arginine deficiency in preterm infants is associated with the serious gastrointestinal infection, necrotizing enterocolitis (NEC). Low arginine may contribute to NEC via inadequate nitric oxide (NO) synthesis, which would impair blood flow to the gut. Arginine is converted to NO by nitric oxide synthase (NOS), but citrulline may be a more effective precursor of NO, as ASS and ASL are co-localized with NOS. Preterm infants are more susceptible to NEC during the transition from parenteral nutrition (PN) to enteral feeding. In piglets with PN-induced intestinal atrophy, we hypothesized that supplemental citrulline would lead to greater NO production and higher blood flow to the intestine. Piglets (7-10 days, n = 20) received venous and gastric catheters and a probe to measure superior mesenteric artery (SMA) blood flow. Intestinal atrophy was induced with PN feeding. On day 4, piglets were randomized to enteral refeeding of an elemental formula with arginine concentration equivalent to 1) sow milk (Low Arg), 2) 2.5 times sow milk (High Arg) or 3) Low Arg plus citrulline (Cit) with arginine plus citrulline equimolar to High Arg. At the initiation of enteral feeding, SMA blood flow was measured for 10 hours. On day 5, whole body protein and NO synthesis were measured with tracers. Whole body protein synthesis was highest in High Arg group compared to Low Arg and Cit groups, suggesting that the concentration of dietary arginine supplied by milk does not meet the whole body needs of a neonate with compromised intestinal function; furthermore, citrulline was not able replace arginine for protein synthesis. NO synthesis and SMA blood flow were highest in the Cit group, suggesting that citrulline was a better precursor for NO synthesis than arginine, and supported better blood flow

to the gut. Citrulline supplementation with arginine at concentrations greater than that in milk may be a means to improve arginine status and enhance intestinal recovery in neonates who are at high risk for NEC during the transition from parenteral to enteral feeding.

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Abbreviations

AA - Amino Acid

AFBM - Analytical Facility for Bioactive Molecules

Arg - Arginine

ASL - Argininosuccinate Lyase

ASS - Argininosuccinate Synthase

BCAA - Branched-Chain Amino Acids

cGMP - Cyclic Guanosine Monophosphate

Cit - Citrulline

CSCCD - Center for the Study of Complex Childhood Diseases

EN - Enteral

eNOS - Endothelial Nitric Oxide Synthase

FSR - Fractional Synthetic Rate

FT- Full-term

GC-MS - Gas Chromatography-Mass Spectrometry

GI - Gastrointestinal

GTP - Guanosine Triphosphate

HILIC - Hydrophilic Interaction Chromatography

HPLC - High Performance Liquid Chromatography

ID - Internal Diameter

IL - Interleukin

INF- γ - Interferon Gamma

iNOS - Inducible Nitric Oxide Synthase

IP - Intraportal

IV - Intravenous

l-NMMA - NG-monomethyl-1-arginine

LCMS-MS - Liquid Chromatography-Mass Spectrometry-Mass Spectrometry

MeOH - Methanol

NEC - Necrotizing Enterocolitis

nNOS - Neuronal Nitric Oxide Synthase

NO - Nitric Oxide

NOS - Nitric Oxide Synthase

NRC - National Research Council

NSAID - Non-steroidal Anti-inflammatory Drug

NT - Near-term

OD - Outer Diameter

Ox - Oxidation

P5C - 1-Pyrroline-5-Carboxylic-acid

PB - Protein Breakdown

PCA - Perchloric Acid

PN - Parenteral Nutrition

PS - Protein Synthesis

PT - Preterm

ROS - Reactive Oxygen Species

SMA - Superior Mesenteric Artery

TLR4 - Toll-like Receptor 4

TNF-α - Tumor Necrosis Factor-α

TPN - Total Parenteral Nutrition

1. Introduction

1.1 Importance of Arginine in Health and Metabolism

Arginine is a conditionally essential amino acid in adults, as it can be synthesized from a number of precursor amino acids including glutamine, glutamate and proline, with the final step involving the conversion of citrulline to arginine in the kidney (Featherston *et al.,* 1973). However, in the fetus and neonate, arginine is an essential amino acid because *de novo* synthesis cannot maintain whole body demands (Wu *et al.,* 2000). Arginine contains four nitrogen atoms per molecule making it the most abundant carrier of nitrogen in humans and animals (Wu *et al.,* 1999). Wu *et al.* (2000) reviewed the role of arginine in development, health and disease stating that arginine administration is beneficial in improving reproductive, cardiovascular, pulmonary, renal, gastrointestinal, liver and immune functions as well as facilitating wound healing (Wu *et al.,* 2000). Arginine is involved in many synthetic processes, including the synthesis of proteins, ornithine, proline, creatine, glutamate, agmatine, urea, nitric oxide and polyamines. Ornithine, a metabolite of arginine, is an immediate precursor for polyamine synthesis. Polyamines stimulate the assembly of 30S ribosomal units therefore playing a key role in translation as well as tissue growth and cell differentiation. In addition, polyamines protect cells from oxidative damage by acting as antioxidants (Igarashi & Kashiwagi, 2000).

1.2 Arginine Metabolism: Neonate vs Adult

Arginine is synthesized from citrulline in the liver through the urea cycle (Morris, 2004a). However, arginase activity in hepatocytes is high, causing rapid hydrolysis of arginine to ornithine and urea resulting in no net arginine synthesis (Flynn *et al.,* 2002). *De novo* synthesis

of arginine in adults involves the intestinal-renal axis. Dietary arginine (Marini, 2012), glutamine and proline (Tomlinson *et al.,* 2011) are the major precursors for citrulline synthesis that takes place in intestinal enterocytes. As such, citrulline is the major precursor for arginine synthesis that occurs in the kidney (Borsook *et al.,* 1941; Wu & Morris, 1998). Citrulline that is synthesized in the intestine is released into the portal venous system where it bypasses the liver to be taken up by the kidney (Windmueller & Spaeth, 1981). Citrulline "bypasses" the liver because hepatocytes are not readily permeable for plasma citrulline (Meijer *et al*., 1990). Once in the kidney, argininosuccinate synthase and argininosuccinate lyase located in the proximal renal tubules convert citrulline to arginine (Morris, 2004b). The low activity of renal arginase in the kidney allows arginine to enter into the circulation where it is utilized by other tissues (Bertolo & Burrin, 2008).

In contrast to adults, *de novo* arginine synthesis occurs predominantly in intestinal enterocytes for both human neonates (Tomlinson *et al.,* 2011) and neonatal piglets (Flynn & Wu, 1996) due to the low activity of arginase (Bertolo & Burrin, 2008). The metabolic requirements for arginine in neonatal piglets is estimated to be 1.1 $g \cdot kg^{-1} \cdot d^{-1}$ (Wu *et al.,* 2004a). However, for one-week old piglets, sow's milk provides approximately 0.40 $g \cdot kg^{-1} \cdot d^{-1}$ (Wu *et al.,* 2004a). Therefore, suckling pigs rely heavily on *de novo* arginine synthesis to meet their metabolic requirements. Proline acts as the major precursor for *de novo* arginine synthesis in the neonatal piglet (Brunton *et al.,* 1999) as well as human neonates (Tomlinson *et al.,* 2011). In the neonate, dietary and newly synthesized arginine is released into the portal blood as arginine (Bertolo & Burrin, 2008). However, as animals age, the small intestine releases more citrulline compared to arginine. A study conducted by Blachier *et al.* (1993) using pig intestine from one day old pigs demonstrated efficient conversion of citrulline to arginine in enterocytes. However,

argininosuccinate synthase and argininosuccinate lyase activities decrease in enterocytes as piglets age (Wu & Knabe, 1995). Wu *et al.* (1994) demonstrated that 29-58 day old piglets release more citrulline from the small intestine that was synthesized from dietary arginine, as opposed to releasing it as arginine as in newborns. Thus, it is evident that *de novo* arginine synthesis changes with age.

Despite the evidence that suggests the intestinal-renal axis is not predominant in neonates, a recent study conducted by Marini *et al.* (2017) demonstrated it does exist in neonates. Marini *et al*. (2017) quantified *in vivo* citrulline production in premature (10 days preterm), neonatal (7 days old), and young pigs (35 days old) using citrulline tracers. The researchers demonstrated that the neonatal pigs had higher citrulline fluxes and plasma concentrations than premature and young pigs. In addition, they found that citrulline was released from the gut and utilized by the kidney in both neonatal and young pigs. The experimental approach and frailty of the premature pigs prevented the quantification of citrulline release from the gut and utilization by the kidney in this group. Furthermore, there was a lack of colocalization of intestinal ASS and ASL with the enzymes that synthesize citrulline, which allowed for the release of citrulline and its utilization by the kidney for arginine synthesis. Therefore, the researchers concluded that the intestinal-renal axis for arginine synthesis is present in the neonatal pig. Another study conducted by Marini and colleagues (2012), using amino acid tracers, demonstrated that arginine was not directly utilized by the gut in neonatal piglets however, was the main precursor for plasma ornithine and subsequently, citrulline synthesis. The researchers noted that the contribution of arginine to the rate of appearance of citrulline is through plasma ornithine with little to no direct use of arginine by the gut in both fed and fasted states. Instead, ornithine is generated by arginine in other organs, enters the circulation and is taken up by enterocytes for citrulline production.

Even with abundant precursors in mammary milk and normal gut function, it appears that piglets fed sow milk fail to acquire the whole body arginine requirement for maximal growth and metabolism (Wu *et al.,* 2004a). A study conducted by Boyd *et al.* (1995) demonstrated that artificially fed piglets can potentially reach growth rates of ≥400 g/d. Compared to sow fed piglets, growth was 74% greater. Another study reported an improvement in piglet weight gain of 28% when fed a diet supplemented with 0.2% arginine, and of 66% with a 0.4% arginine diet, compared to the control piglets fed unsupplemented sow-milk replacer. The diets with additional 0.2% and 0.4% arginine led to final body weights that were 15% and 32% higher, respectively (Kim & Wu, 2004). In weaning piglets, expression of vascular endothelial growth factor and intestinal development were enhanced when supplemented with dietary arginine as reported by Yao *et al.* (2011). In that study using 21 day old piglets, supplementation with 1% L-arginine for 7 days led to improvements in relative weight of the small intestine (33%), body weight (38%), and feed efficiency (28%). There was also greater villus height throughout the entire length of the intestine (Yao *et al.,* 2011). Cellular and humoral immunity in piglets was enhanced when a milk-based formula was supplemented with 0.4-0.8% arginine and fed for two weeks (Tan *et al.,* 2009). These types of results could translate into important improvements in feeding strategies for human infants with compromised intestinal function, considering that mammary milk from sows and humans is low in arginine concentration relative to whole body needs and the neonate relies on *de novo* arginine synthesis in the intestinal epithelium (Tomlinson *et al.,* 2011, Flynn & Wu, 1996). In situations where intestinal metabolism is compromised, dietary arginine supplementation may be necessary for maximal growth and to optimize metabolic processes.

1.3 The Role of Dietary Nutrients in Intestinal Growth and Development

The most potent stimulus for intestinal mucosal growth is the presence of nutrients within the lumen. These nutrients are involved in the growth and oxidative metabolism of the mucosal epithelial cells and can trigger the release of local growth factors, gut hormones as well as activate different neural pathways (Johnson, 1981, Steiner *et al*., 1968). The neonatal gut has a high metabolic requirement, making it highly susceptible to nutrient deficiencies. A decline in protein synthesis, gut mass and villus height is evident during prolonged fasting. Not surprisingly, these effects are more pronounced in neonates, especially low birth weight and premature infants, due to greater growth rates compared to any other time in the life cycle. This stresses the importance of nutritional support for neonates to meet high metabolic demands (Jacobi & Odle, 2012).

1.4 Nutritional Support of Neonates

Newborns have low nutrient stores combined with immature gastrointestinal tracts, degradative pathways and kidney function for nitrogen excretion (Heird *et al.,* 1972). The lack of nutrient stores and immaturity of the gut is a greater concern when infants are born as low birth weight or preterm, with very limited protein and energy reserves; the duration of survival without a source of nutrition is approximately 7 days (Heird *et al.,* 1972). Therefore, early nutritional support is essential and may take the form of enteral (via the gut) or parenteral (intravenous) administration, depending on confounding medical conditions and the level of prematurity. However, these methods of feeding differ substantially in quality and quantity of nutrients being delivered to the extra-splanchnic tissues. When *in utero*, the majority of nutrients are supplied through the umbilical circulation, with only a small contribution from the

swallowing of the amniotic fluid in the last weeks before birth (Buddington & Sangild, 2011). This creates a challenge for adapting to enteral nutrition; however, the enteral delivery of nutrients is well tolerated in the majority of term-born infants. GI homeostasis is achieved well with mother's milk because normal digestion, nutrient absorption, gut barrier function and immune tolerance are maintained (Klein *et al.,* 2000). In contrast, many preterm infants have an intolerance to enteral feeding which poses a risk to their survival (Burrin *et al.,* 2000, Bjornvad *et al.,* 2008), and will delay nutrient-induced intestinal growth. Direct nutrient supply to the gut stimulates growth of the small intestine. Studies in animals and humans have shown that luminal nutrients led to improved intestinal growth, mucosal integrity and function (Levine *et al*., 1974, Morgan *et al*., 1987, Shulman, 1988, Zaloga *et al*., 1992, Sax *et al*., 1996). Reductions (<60% total caloric intake) or complete exclusion of enteral nutrition have been demonstrated to induce gut mucosal atrophy in neonatal and adult pigs (Burrin *et al.*, 2000, Levine *et al*., 1974). This induced atrophy has also been documented to occur in human neonates (Rossi *et al*., 1993). Breakdown of the gut microenvironment reduces the ability of the gut to metabolize essential nutrients. In addition, there is an increase in inflammation and intestinal stress resulting in increased incidences of intestinal dysfunction, necrotizing enterocolitis, intestinal permeability, bacterial translocation, sepsis, stunted growth and even death (Jacobi & Odle, 2012). As such, the successful initiation of enteral feeding is an important clinical goal in the nutritional management of newborn and premature infants.

1.5 Total Parenteral Nutrition

When enteral feeding is not tolerated or contraindicated because of medical instability, then total parenteral nutrition (TPN) may be necessary. TPN is the intravenous provision of

nutrients, and is used as a lifesaving technique, particularly for preterm or low birth weight infants. TPN may be administered solely or with minimal enteral nutrition (EN) in an attempt to maintain intestinal integrity (Jacobi & Odle, 2012). Prolonged TPN feeding can lead to intestinal atrophy due to the lack of luminal nutrients stimulating trophic factors. TPN-induced mucosal atrophy is characterized by morphological changes such as reduced villus height and mucosal surface area, decreased crypt cell proliferation, decreased protein synthesis and increased apoptosis (Goldstein *et al*. 1985, Shulman *et al*. 1988, Burrin *et al*. 2000, Kansagra *et al*. 2003). TPN-induced gut atrophy has been well established in animal models. Kansagra *et al*. (2003) demonstrated that TPN feeding resulted in impaired gut barrier function and higher intestinal permeability in piglets. In addition, Morgan and colleagues (1987) demonstrated 70% reduction in proximal small bowel weight in piglets on long-term TPN compared to those fed enterally. Bertolo *et al.* (1999) conducted a piglet study that describes the metabolic and physiologic effects of first-pass metabolism by the small intestine. The researchers fed identical diets to 3 day old piglets for 8 days via gastric (IG), portal (IP) or central venous (IV) catheters. Villous atrophy was observed in the duodenum and jejunum of IV and IP pigs and crypt depth were lower in IV pigs compared to IG. Neonatal intestinal mucosal breakdown that occurs with IV feeding is linked to immaturity of the intestine and the lack of enteral nutrients being supplied (Sangild, 2006). When medical management deems it necessary to halt enteral feeding, the transition from EN to parenteral nutrition is accompanied by a rapid decrease in intestinal blood flow. Niinikoski *et al*. (2004) demonstrated that blood flow rapidly decreased in less than eight hours after the transition from EN to PN. They reported very rapid tissue responses to the decline in blood flow, with suppression of protein synthesis and villous atrophy, as well as reduced cell proliferation and survival measured at 24 and 48 hours post TPN initiation, respectively.

1.6 Route of Feeding and Gut Health

The route of feeding, whether delivered into the gut or intravenously, will have an impact on intestinal metabolism as well as whole body nitrogen utilization (Duffy & Pencharz, 1986, Jeevanandam *et al*., 1987, Bertolo *et al*., 1999). To demonstrate this, the study by Bertolo *et al.* (1999) provided piglets with identical diets that were delivered intravenously either through a central line (to bypass the gut and liver) or intraportally (to bypass the gut only) or was delivered into an intragastric catheter. Piglets receiving the IV delivery had significantly lower whole body protein deposition, body protein content, total small intestinal and mucosa weights. In addition, free urea cycle amino acids were altered in mucosa and plasma suggesting that gut atrophy, as induced by IV feeding, limited arginine synthesis, translating into decreased protein deposition (Bertolo *et al*., 1999). Thus, it is clear that TPN negatively impacts gut metabolism through by-passing splanchnic organ metabolism. Moreover, gut atrophy as a result of prolonged IV feeding further complicates the establishment of enteral feeding. This becomes an even greater concern with premature or very low birth weights infants because intestinal immaturity, injury and/or infection almost always complicate the adequate provision of enteral nutrition (Wilson, 1997). Unfortunately, these circumstances cause the premature infant to be even more reliant on parenteral nutrition leading to a cycle of enteral feeding intolerance and greater dependence on PN support.

Stoll *et al*. (2012) demonstrated that the composition of EN has a significant influence on neonatal piglet metabolic dysfunction and trophic polypeptide production. Therefore, this would mean further investigation is required to determine the optimal macronutrient composition and chemical nutrients to promote direct and/or indirect stimulation of trophic and cytoprotective pathways for the developing intestine. A better understanding of the individual nutrients, mode

of nutrient supplementation, type of nutrient supplementation, and the cellular and immunological adaptations is needed to optimize infant nutrition for growth and development as well as long-term quality of life.

To summarize, neonates require an adequate supply of arginine through *de novo* synthesis and the diet to meet whole body requirements. TPN bypasses intestinal metabolism resulting in gut atrophy and diminished *de novo* synthesis of arginine. Thus, a greater amount of dietary arginine is required when infants are supported by TPN. TPN-associated gut atrophy causes the gut to be more susceptible to damage, further perpetuating the impact on intestinal arginine synthesis and dietary arginine demand. Further damage can occur in situations such as re-introduction of enteral feeding following TPN feeding. Unfortunately, some preterm infants may proceed to develop an even more severe complication, necrotizing enterocolitis.

1.7 Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is the most prevalent and lethal gastrointestinal complication in premature infants. NEC affects 1–3 live births per 1,000 in North America, and mortality reaches ~50% in the most severe cases (Papillon *et al*., 2013, Stoll *et al*., 2015). As many as 15 million babies are born preterm every year, which accounts for 11% of live births worldwide (Blencowe *et al*., 2012).

Prematurity leads to microbial dysbiosis and colonization often following administration of artificial infant formulas. The premature intestine is thought to have an increased baseline reactivity with microbial ligands, increasing the susceptibility for the development of NEC (Niño *et al*., 2016). Moreover, enteral feeding is a risk factor for NEC, leading to intestinal edema, hemorrhage, leukocyte infiltration, transmural necrosis, and pneumatosis intestinalis (build-up of

gas in the intestinal wall) (Neu & Walker, 2011, Niño *et al*., 2016, Tanner *et al*., 2015). Therefore, NEC pathophysiology is likely multifactorial involving innate immunity, especially the expression and activity of Toll-like receptor signaling, more specifically Toll-like receptor 4 (TLR4) (Claud & Walker, 2001, Nanthakumar *et al*., 2000, Niño *et al*., 2016). TLR4 is an innate immune receptor that recognizes lipopolysaccharides found in Gram-negative bacteria. Evidence suggests that increased susceptibility to NEC is related to an upregulation of downstream signalling regulators of TLR4. The key affected regulators in question are nuclear factor κB1, single Ig IL-1- related receptor, the co-receptor molecule lymphocyte antigen and the small glycolipid transport protein ganglioside GM2 activator. Furthermore, a single nucleotide polymorphism and genetic variants encoding proteins have been described in the literature to be linked to the regulation of the immune phenotype shift from type 1 to type 2 T helper cells. The genetic variant causes an enhanced IL-4 effect which regulates the phenotypic shift and this shift may help defend against the development of NEC (Sampath *et al*., 2011, 2015, Zhou *et al*., 2015, Héninger *et al*., 2002, Treszl *et al*., 2003).

Development of NEC is also inversely related to gestational age, for example, infants born at approximately 27 weeks are more susceptible and tend to develop NEC around 4-5 weeks after birth, whereas infants closer to 37-weeks are less susceptible and develop it around 2 weeks after birth (Beeby & Jeffery, 1992). Delayed microbial colonization of the gut and establishment of virulent microbial agents may explain the reason for the late onset of NEC in 27-week infants compared to 37-week infants. This delayed colonization of gut microbiota results in longer hospital stays and the use of a wide array of antibiotics (Claud & Walker, 2008).

Modeling the elements of prematurity in animals can be challenging, but the preterm piglet replicates these key elements of human NEC, including spontaneous development with

infant formula feeding (Sangild *et al*., 2006, Sodhi *et al*., 2008). Touloukian *et al*. (1971, 1972) developed animal models to mimic neonatal human conditions and demonstrated that severe gut ischemia in piglets and dogs caused intestinal injury similar to NEC (Touloukien *et al*., 1971, 1972). It was hypothesized that significant perturbations in intestinal blood flow resulted in NEC. Early observations showed that hypoxia in piglets reproduced NEC-like lesions (Touloukien *et al*., 1972). Using a neonatal piglet asphyxiation model, the researchers discovered that blood flow was redirected away from the intestine allowing sustained blood flow toward the brain and heart protecting theses organs from hypoxia. This situation is termed the "dive reflex" and ultimately resulted in intestinal injury. Additional studies have demonstrated that intestinal hypoxia precedes NEC-derived inflammation (Caplan & Fanaroff, 2017, Gamsu & Kempley, 1997, Nowicki, 2005, Nowicki *et al*., 2005). In creating rodent models of NEC, it is necessary that the animals undergo hypoxia for the development of NEC (Sodhi *et al*., 2008). Gay *et al*. (2011) & Zamora *et al*. (2015) demonstrated using non-invasive, continuous abdominal near-infrared spectroscopy that low intestinal oxygenation precedes the onset of NEC in preterm piglets. Given its role in endothelial relaxation, inhibition of platelet aggregation and regulation of inflammation, it has been speculated that nitric oxide may be the link between hypoxia and NEC pathogenesis (Berchner-Pfannschmidt *et al*., 2007, Chokshi *et al*., 2008, Ignarro *et al*., 1987, Liu *et al*., 2015). Nitric oxide is produced from arginine by nitric oxide synthase (NOS) and is associated with inflammation and NEC in human infants (Ford *et al*., 1997, Yazji *et al*., 2013). Importantly, arginine supplementation has been shown to successfully reduce NEC incidence in clinical trials (Amin *et al*., 2002, El-Shimi *et al*., 2015, Polycarpou *et al*., 2013, Shah & Shah, 2007) and animal studies (Di Lorenzo *et al*., 1995).

Not surprisingly, NEC is associated with low plasma arginine and vascular dysfunction. Arginine status is associated with NEC risk and it is known that infants who develop NEC have low plasma arginine concentrations as identified by a number of studies (Becker *et al*., 2000, Wilmore, 2004, Zhou *et al*., 2015). The presence of IV nutrition lowers *de novo* arginine synthesis as a result of gut atrophy. Thus, it is important to establish (or re-establish) enteral feeding as soon as possible since it is crucial for the restoration of both gut function and structure. Low plasma concentrations of arginine in preterm infants with NEC suggest that supplemental arginine may be important to prevent the onset of the disease. Indeed, a clinical study conducted by Amin *et al*. (2002) demonstrated that supplementing arginine through parenteral and enteral routes lowered the incidence of NEC in premature infants. The study consisted of 152 premature infants who were placed onto either an arginine supplemented or a low arginine diet. Arginine was initially given with the parenteral diet and later, by enteral route (40% of the infants′ requirement). The researchers reported that five infants in the arginine treated group compared to 21 in the control group developed NEC, indicating that poor arginine status is assuredly a factor in the development of NEC in premature infants.

Arginine is required for the detoxification of ammonia, which is an extremely toxic substance for the central nervous system. Therefore, poor arginine status also contributes to elevated ammonia levels. It has been demonstrated that elevated ammonia levels can decrease mucosal cell viability and contribute to mucosal damage (Tsujii *et al*., 1992). It was reported in a study conducted by Batshaw *et al*. (1984) that ammonia levels were elevated in ~50% of premature infants compared to normal weight infants. When investigating plasma arginine and ornithine levels, the researchers discovered that arginine levels were much lower in the hyperammonemic group. Arginine supplementation resulted in a 25% decrease in ammonia

levels in the preterm infants compared to those not treated. Therefore, it is evident that arginine supplementation can positively impact ammonia levels and may alleviate ammonia induced mucosal damage.

In comparison to arginine status in preterm infants, little research is available to describe the role of intestinal citrulline production and gut health in neonates. As the precursor for arginine synthesis, low citrulline availability for arginine synthesis could also predispose an infant to NEC. Circulating citrulline concentration is commonly used as a biomarker of intestinal functionality in both adults and neonates (Crenn *et al*., 2000, Papadia *et al*., 2007, Fragkos *et al*., 2017). Low plasma concentrations of citrulline may indicate reduced enterocyte function (Celik *et al*., 2013). Moreover, studies have indicated that plasma levels of citrulline are low in preterm infants suffering from NEC compared to healthy preterm infant controls (Wu *et al*., 2004b, Ioannou *et al*., 2012, Celik *et al*., 2013). Interestingly, the importance of citrulline for NO production is garnering increasingly more attention since its discovery as a precursor for NO synthesis in *in vitro* studies (Hecker *et al*., 1990, Xie *et al*., 2000, Flam *et al*., 2001, Solomonson *et al*., 2003, Shen *et al*., 2005, Flam *et al*., 2007). Numerous *in vivo* studies have demonstrated that citrulline supplementation will increase/enhance NO synthesis (Luiking *et al*., 2008, Schwedhelm *et al*., 2008, Wijnands *et al*., 2012, Wijnands *et al*., 2015). However, *in vivo* studies that describe the impact of citrulline on nitric oxide synthesis specifically in models related to preterm infants have only recently begun appearing in the literature (Robinson *et al*., 2018); more research is necessary to characterize the importance of citrulline. Arginine availability may be more limited than citrulline for NO production because unlike citrulline, arginine has numerous metabolic fates. The limited availability of arginine for processes such as NO production is further exacerbated in premature infants, due to reduced *de novo* arginine synthesis

as a result of intestinal injuries. Thus, citrulline supplementation may be an ideal therapeutic target to increase NO synthesis and reduce the incidence of necrotizing enterocolitis.

1.8 Importance of Nitric Oxide in Health and Metabolism

Nitric oxide is synthesized by the oxidation of arginine to equimolar amounts of citrulline and NO through one of the three different isoforms of the nitric oxide synthase enzymes. Inducible-NOS (iNOS), endothelial-NOS (eNOS) and neuronal-NOS (nNOS) are the three different isoforms that are expressed at different levels depending on the location within the body (Moncada & Higgs, 1993, Cho, 2001). iNOS is expressed predominantly in macrophages and neutrophils but can also be found in epithelial cells and neurons (Wallace & Miller, 2000). eNOS and nNOS are constitutively expressed and at the gastrointestinal level, eNOS is expressed basally at the vascular endothelium and nNOS is expressed at the enteric nervous system of the gastrointestinal tract (Cho, 2001). During an inflammatory response, iNOS is up-regulated and results in a sustained increase in NO production leading to formation of reactive oxygen species (ROS) and oxidative stress (Nathan, 1997). The interaction between NO and ROS can result in either beneficial or negative outcomes depending on the conditions in which the process takes place (Wallace & Miller, 2000). NO can bind to a heme group of soluble guanylyl cyclase, subsequently resulting in the conversion of GTP to cGMP and increasing the intracellular concentrations of cGMP. Next, cGMP acts to bind and modify its targets (proteins, kinases, ion channels or phosphodiesterases) to elicit a response. NO can also engage in cGMP independent processes where its redox derivatives can post-translationally modify or oxidize proteins or lipids to mediate various cellular activities (Moncada & Higgs, 1993).

NO derived from nNOS and eNOS acts as a neurotransmitter and a vasodilator, respectively (Moncada & Higgs, 1993). In the brain, NO acts to regulate physiological processes related to behavior, cognitive function and synaptic plasticity. Moreover, NO controls blood flow in the brain, promotes angiogenesis, and maintains cellular redox state, cell immunity, and neuronal survival. However, NO regulation is crucial as overproduction of NO may lead to neurodegeneration (Moncada & Higgs, 2006). Elevated circulating cytokines concentrations (TNF-α and IL-1, IL-6, and IL-8) and/or microbial products like lipopolysaccharide leads to iNOS activation during an inflammatory response and results in increased NO synthesis (Groeneveld *et al*., 1997, Knowles & Moncada, 1994, Moncada & Higgs, 1993, Nakae *et al*., 2000). The NO produced in this circumstance has important immune regulatory functions; it can act as a free radical scavenger, control or kill pathogens, regulate cytokine production, and promote T-helper cell development (Titheradge, 1999). Interestingly, localized NO responses are concentration and exposure time dependent (Thomas *et al*., 2008) but in general, lower NO levels promote cell survival and proliferation with higher concentrations promoting cell cycle arrest, apoptosis, and senescence. In conclusion, arginine can indirectly influence NO-mediated functions, such as immune modulation (Daly *et al*., 1990, Reynolds *et al*., 1988) and enhancement of the immune response during an immunological challenge (Li *et al*., 2007).

1.9 Nitric Oxide and Intestinal Health

NO plays an important role in maintaining and protecting the gastrointestinal tract. NO has been demonstrated to maintain gastric mucosal integrity, stimulate mucus secretion, inhibit leukocyte adherence, maintain vascular tone and mediate mucosal blood flow. NO can mediate blood flow since it acts as a powerful vasodilator. In a study conducted by Pique *et al*., (1989) it

was demonstrated that the NOS inhibitor NG-monomethyl-l-arginine (l-NMMA) induced a dose-dependent increase in rat systemic blood pressure and a decrease in resting gastric mucosal blood flow. In addition, exogenous forms of NO, such as a nitroglycerin patch, can protect rat gastric mucosa from indomethacin-induced gastric damage (Calatayud *et al*., 1999). This is likely due to maintained mucosal blood flow from the patch and inhibition of leukocyte-endothelial interactions (Calatayud *et al*., 1999).

It is well known that mucus secretion helps to contribute to gastrointestinal defenses by acting as a physical barrier to damage and protection from pepsin and acidic damage (Wallace & Miller, 2000, Allen *et al*., 1993). Interestingly, incubation of rat gastric mucosal cells with NO stimulated mucus secretion. This mechanism was demonstrated to be dependent on both dose and cGMP (Brown *et al*., 1993). Brown and colleagues (1992) investigated this mechanism *in vivo* using the NO-donor isosorbide dinitrate. Again, in rat gastric lumen they found a dose-dependent increase in mucus gel thickness, thus mediating mucus secretion and helping to contribute protection to gastric epithelium.

Although NO is required for normal gastrointestinal function, a large excess of NO may have deleterious effects on the gastrointestinal tract. The enzyme iNOS is induced in the presence of gastrointestinal mucosal inflammation and it has been demonstrated that NO derived from iNOS is involved in gastric ulcer repair (Schmassmann *et al*., 2006). Despite this, NO also contributes to the perpetuation of gastrointestinal inflammation (Fiocchi, 1998, Lauritsen *et al*., 1986). Likewise, different gastrointestinal pathologies are associated with the induction of NOS. Activation of iNOS occurs in *Helicobacter pylori*-induced gastritis, inflammatory bowel diseases and nonsteroidal anti-inflammatory drug-induced ulcerogenesis (Wallace & Miller, 2000, Martin *et al*., 2001, Mannick *et al*., 1996). Therefore, the role NO plays in these conditions and the

potential effects of excess NO are still unclear. Using rats, Calatayud *et al*., (1999) demonstrated that the addition of an exogenous source of NO can protect against or reduce the severity of indomethacin-induced gastric damage. Interestingly, when indomethacin was administered to iNOS knockout mice there was a reduction in the amount of gastric damage compared with that in wild-type animals (Souza *et al*., 2004), suggesting that NO generated from iNOS contributed to indomethacin-induced gastric lesions. These studies contradict each other and illustrate how difficult it can be to predict the role NO plays in different scenarios. Moreover, studies investigating NSAID-induced ulcerogenic damage have demonstrated that high levels of NO result in damage (Gurbuz *et al*., 1999, Lamarque & Whittle, 1995) whereas a gastroprotective effect is seen with lower levels (Calatayud *et al*., 1999, Elliot *et al*., 1995). Similarly, experimental models of colitis have reported the same contradictory effect (Wallace & Miller, 2000).

Homeostatic functions in the cardiovascular system include maintenance of blood flow and pressure, regulating macromolecule exchange between blood and tissues, and preventing the improper activation of leukocytes (Pober & Sessa, 2015). This vascular endothelium system synthesizes and secretes NO to modulate the function and health of arteries and surrounding tissues (Seals *et al*., 2014). In healthy arteries, vasodilation, coagulant and inflammatory processes are favourably modulated (Seals *et al*., 2014). In their basal state, endothelial cells prevent interactions with leukocytes and the basal production of NO by the endothelium exerts an anti-inflammatory and anti-adhesive effect (Hickey & Kubes, 1997). Failure of endothelial cells to adequately perform any of these basal functions constitutes ‘endothelial cell dysfunction’. Inadequate production of NO is a major cause of endothelial cell dysfunction and results in vessel constriction (Pober & Sessa, 2007). Intestinal endothelial dysfunction limits or

even eliminates NO production resulting in intestinal ischemia; this may predispose preterm infants to NEC development (Ito *et al*., 2007).

1.10 Importance of Citrulline in Health and Metabolism

Citrulline is a non-protein amino acid synthesized *de novo* from ornithine and carbamoyl phosphate in the urea cycle by the enzyme ornithine transcarbamylase. As a urea cycle intermediate, citrulline plays an important role in the excretion of toxic ammonia through urea production. Glutamine acts as a precursor to citrulline synthesis because it can be converted to ornithine by the subsequent actions of 1-Pyrroline-5-Carboxylic-acid (P5C) synthase (generating P5C from glutamine) followed by ornithine aminotransferase (P5C to ornithine). This newly synthesized ornithine is then converted to citrulline. Net synthesis of citrulline in the adult occurs in the small intestinal epithelium due to the low enzymatic activity of ASS and ASL releasing it into portal circulation (Bertolo *et al*., 2008). As mentioned previously, the intestinal-renal axis may be present in the neonate thus, citrulline can also be released by the neonatal intestine. Due to the minimal uptake of citrulline by the liver it remains in systemic circulation where it can enter the kidney for conversion to arginine (Bertolo *et al*., 2008). This has important implications because it has been demonstrated that citrulline supplementation can increase arginine availability. An example of citrulline's potential as a supplement was demonstrated in a study conducted by Luiking *et al.* (2009), in which arginine metabolism was restored in sepsis patients. Compared to arginine, bioavailability of citrulline is greater due to its ability to be handled by a larger number of AA transporters (Bahri *et al*., 2008, Moinard *et al*., 2008). Citrulline has been utilized as a supplement, since it can be used as a substrate from which arginine can be synthesized or as a NO precursor. The "arginine paradox" refers to the idea that the availability

of extracellular arginine is rate-limiting for NO production by NOS (Flam *et al*., 2001, Shen *et al*., 2005), despite normal intracellular concentrations that are in excess of the Km (3 μM) for arginine (Pollock *et al*., 1991, Hecker *et al*., 1990, Hardy & May, 2002, Kurz & Harrison, 1997). Intracellular concentrations of arginine in endothelial cells have been reported to range from 100 to 800 μM (Shen *et al*., 2005, Hecker *et al*., 1990), supporting the arginine paradox theory. As such, low extracellular arginine will limit NO synthesis, and, higher extracellular arginine availability is required to elicit maximal NO release from cells. The mechanisms behind the arginine paradox are still unknown and may involve citrulline. Therefore, supplementing with citrulline may be a means to increase arginine availability for NOS enzymes and thus improve NO production. Interestingly, in endothelial cells, citrulline can be used or recycled by NOS as a result of its co-localization with the enzymes ASS and ASL, in order to tightly couple arginine synthesis to NO production (Flam *et al*., 2007, Flam *et al*., 2001). This is in contrast with ASS and ASL in the liver, where the enzymes are co-localized with mitochondria to complete the urea cycle (Cohen & Kuda, 1996). Therefore, citrulline recycling may provide an important substrate for NOS when arginine is limiting and in demand for other processes such as protein synthesis. Citrulline has fewer "metabolic fates" compared to arginine, and therefore may be a better means to supply NO.

Citrulline metabolism is "split" between two organs and is related to the effective capture of arginine by the liver. Without a metabolic adaptation, nearly all exogenous arginine would be extracted from the portal circulation by the liver, minimalizing the availability of arginine for other organs. Additionally, arginine is a positive regulator of ureagenesis and other amino acids may be over-metabolized (Meijer *et al*., 1990). However, citrulline can be considered as a "masked" form of arginine, allowing it to bypass the liver because the liver is unable to extract

citrulline from the portal circulation (Windmueller and Spaeth, 1981). Beyond the liver, citrulline is converted to arginine by the kidney, and is released and made available for the entire body.

1.11 Citrulline and NO

Citrulline can serve as a precursor for NO synthesis because NOS is co-localized with ASS and ASL in some cell types. The citrulline-NO cycle (Figure 1.1) was first described by Hecker *et al*., (1990), who incubated arginine-depleted endothelial cells with Krebs' solution for 60 min and found a 9.7 fold increase in arginine levels. This coincided with a 90% decrease in intracellular glutamine, while all other amino acids had negligible changes. Endothelial cells labelled with [^{14}C]-arginine however, demonstrated significant generation of unlabelled arginine, as evidenced by an 8.8 fold decrease in specific activity of the intracellular arginine pool. Interestingly, there was no formation of urea and no conversion of ornithine to arginine in the arginine-depleted cells but there was conversion of citrulline to arginine, likely through formation of argininosuccinic acid. Lastly, when non-depleted cells were stimulated there was only a transient accumulation of citrulline. These data indicate that citrulline is recycled to arginine during the biosynthesis of NO. Flam *et al*., (2007) provided evidence to support the recycling of citrulline to arginine for NO production and hypothesized that recycling of citrulline would result in greater NO production compared to *de novo* citrulline formation due to the efficiency of citrulline recycling through the citrulline-NO cycle. Bovine aortic endothelial cells were incubated with [^{14}C]-arginine and stimulated using bradykinin and sodium orthovanadate to produce NO. NO (as nitrite) and citrulline (as [^{14}C]-arginine to [^{14}C]-citrulline conversion) production were measured. The magnitude of the apparent ratio of endothelial NO to citrulline produced was used as an indicator of the degree of coupling of the citrulline–NO cycle to

endothelial NO production. The researchers observed a significantly higher (8 to 1) production of NO compared to [^{14}C]-citrulline under both basal and stimulated conditions. Furthermore, ASS is an important rate limiting step of the citrulline-NO cycle, so to further support their findings, they used an ASS inhibitor (α-methyl-DL-aspartate). The addition of inhibitor reduced NO production in a dose dependent manner. In addition, the ratio of NO (as nitrite) to citrulline produced was still high (7:1) at inhibition levels of 80%. This indicates a direct correlation between NO production and ASS inhibition thus, further supporting the tight coupling of the citrulline–NO cycle to NO production.

In a recently published paper, Robinson *et al*., (2018) demonstrated that prematurity reduces nitric oxide production and precedes the onset of NEC in neonatal piglets. Using piglets delivered by caesarean section at 103 days [preterm (PT)], 110 days [near-term (NT)], or 114 days [full-term (FT)] they administered TPN for two days followed by oral administration of an infant formula for 42 h, to induce NEC. Gestational age was linked to the incidence and severity of NEC, with the highest incidence and mortality rates present in premature pigs. The researchers reported that the PT pigs had lower citrulline production and arginine fluxes ($P < 0.05$) throughout the study compared to the pigs at a more advanced gestational age (NT and FT). At birth, PT piglets demonstrated a lower expression of genes associated with arginine and citrulline synthesis compared to the NT and FT groups. In addition, the PT piglets had shorter jejunum and ileum villi compared to the NT and FT piglets. Thus, a reduced citrulline production due to intestinal damage precedes the development of NEC and may impact the citrulline-NO cycle for NO synthesis. Overall, they reported that lower citrulline, arginine availability and NO production in preterm infants results in poor intestinal perfusion and hypoxia and are central toward the development of NEC. Therefore, citrulline and arginine supplementation may be

crucial for preterm infants because it has the potential to overcome these metabolic deficiencies and ultimately reduce the incidence of NEC.

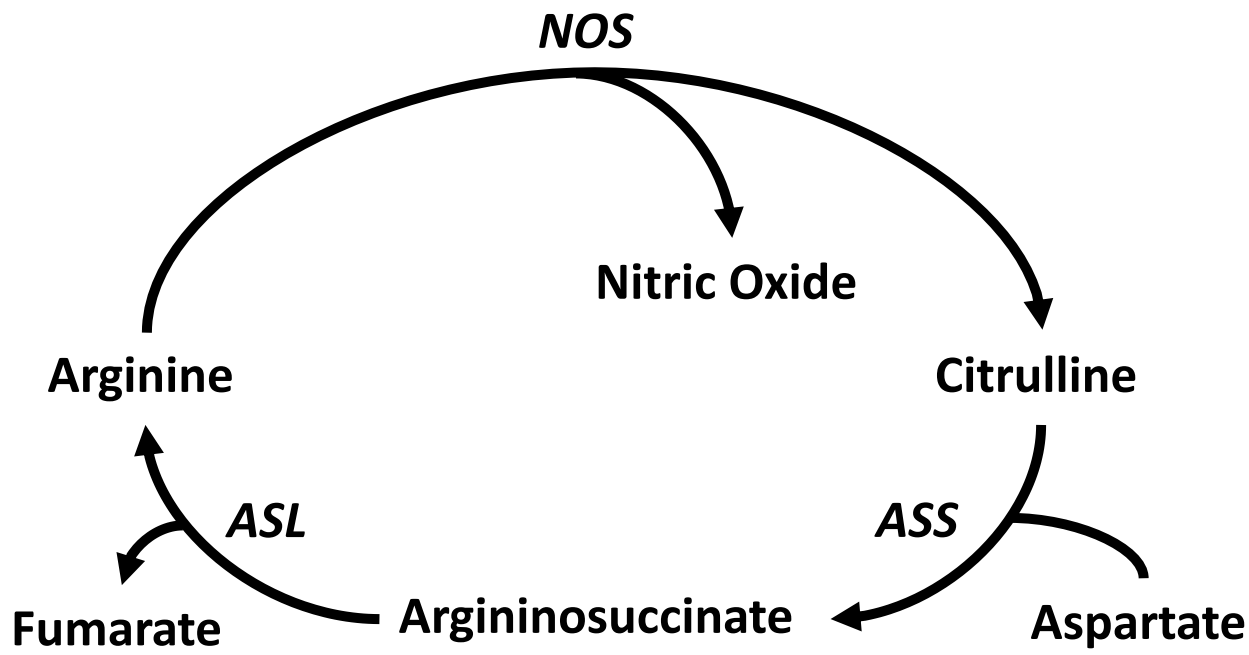

Figure 1.1 Citrulline-NO cycle. The cycle is composed of NO synthase (NOS), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL).

1.12 Rationale

Arginine is an essential amino acid in the neonate because *de novo* synthesis cannot maintain whole body demands. In situations leading to intestinal damage such as prolonged TPN feeding, *de novo* arginine synthesis is decreased. Therefore, dietary arginine supplementation may be necessary to increase availability of arginine for maximal growth and to optimize metabolic processes. Citrulline is converted to arginine through the activities of ASS and ASL. Thus, citrulline supplementation may increase arginine availability. Arginine deficiency in preterm infants is associated with NEC and low arginine may contribute to NEC via inadequate NO synthesis and impaired blood flow to the gut. Arginine is converted to NO by NOS, but citrulline may be a more effective precursor of NO because ASS and ASL are co-localized with NOS. Preterm infants are more susceptible to NEC during the transition from PN to enteral feeding. Thus, in piglets with PN-induced intestinal atrophy, we believe that supplemental citrulline will result in greater NO production and higher blood flow to the intestine. In turn, this would lead to greater structural and functional improvements in the recovering intestine.

Hypothesis

We hypothesized that providing dietary arginine at a concentration greater than in sow milk or providing a dietary source of citrulline would lead to greater NO production, higher blood flow to the intestine and structural and functional improvements in the intestinal mucosa in neonatal piglets with TPN-induced gut atrophy.

Objectives

In neonatal piglets, the objectives were:

- to determine if high versus low dietary arginine, or the addition of citrulline to a low arginine diet would lead to greater NO synthesis;
- to determine if dietary citrulline would lead to a higher rate of blood flow to the intestine compared to high arginine;
- to determine if high versus low dietary arginine would support greater whole body protein synthesis and whether the addition of citrulline to low dietary arginine would support whole body protein synthesis similar to high arginine;
- to determine whether high arginine or low arginine with citrulline would improve gut morphology in the recovering intestine.

2. Material & Methods

2.1 Surgical Procedure

Sow-fed Yucatan miniature piglets (7-10 d of age) were obtained from Animal Care Services, Memorial University of Newfoundland. All animal care and handling procedures were approved by the Institutional Animal Care Committee of Memorial University and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Upon arrival of the piglet to the laboratory, the body weight was measured and an intramuscular injection of ketamine hydrochloride (0.22 mL/kg; Rogarsetic Rogar STB, Montreal Canada) and acepromazine (0.2 mL/kg; Atravet:Ayerst Laboratories, Montreal Canada) was given to induce anesthesia. Atropine (0.1 mL/kg; Rafter Dex Canada, Calgary, Canada) was also administered through a subcutaneous injection to reduce airway secretions. Once the piglet was anesthetized, an endotracheal tube was inserted to facilitate general anesthesia that was maintained throughout the entire procedure using 0.8%-1.5% isoflurane (Abbot Laboratories Ltd.) delivered with 1.5 L/min medical grade oxygen. Under general anesthesia, the animal was cleaned with Prepodyne scrub solution (West Penetone Inc., Montreal Canada) and 70% isopropanol prior to being moved to the surgery table. The piglet was monitored for body temperature, oxygen saturation, heart rate and respiratory rate during the procedure.

A small incision was made on the back, slightly off centre but parallel to the spine, in order to insert and anchor two catheters which were tunneled under the skin toward the neck or the leg, to incision sites close to the entry points for the jugular and femoral veins. The gastric catheter was also tunnelled under the skin from a second small incision on the back to near the abdominal cavity. The jugular and femoral veins were isolated by blunt dissection, and a silicone

catheter was inserted and secured locally to the surrounding muscle using a grommet and suture. One catheter (ID 0.76 mm, OD 1.65 mm) was introduced into the left external jugular vein and advanced to the cranial vena cava immediately cranial to the heart. The second catheter (ID 0.63 mm, OD 1.19 mm) was introduced into the left femoral vein and advanced to the caudal vena cava immediately caudal to the heart. Both jugular and femoral incisions were closed at the skin with interrupted sutures. Subsequently, an incision was made along the edge of the rib cage and blunt dissection of the muscle layers was performed to reach the peritoneum. Further blunt dissection was done to separate the peritoneum from the renal fascia to locate the superior mesenteric artery (SMA). The SMA was isolated, and 4 mm back or side perivascular ultrasonic blood flow probe (Transonic Systems Inc., Ithaca, USA) was secured around the vessel. The space between the probe and vessel was filled with ultrasound gel to facilitate ultrasonic transmission. The wire from the probe was exteriorized from the body by tunnelling under the skin to an incision on the back. To ensure the probe was secure, a loop of the lead wire was sutured into a "pocket" that was created between the skin and underlying muscle, prior to closing the incision. Next, a mid-ventral incision was made to open the abdominal cavity followed by isolation of the stomach. A purse-string suture was created on the stomach and pierced in the middle with an 18-gauge needle. The gastric catheter (ID 1.60 mm, OD 3.20 mm) was then inserted through the purse-string suture into the stomach where it was secured with the suture. Finally, another purse-string suture was created and secured again. The peritoneum was closed with interrupted sutures, muscle layers were closed with a blanket suture, and the skin was closed with interrupted sutures. All of the catheters (jugular, femoral and gastric) and flow probe wire were exteriorized from the body close together. The piglet had antibacterial cream (Hibitane) applied to incision sites and was given a dose of Borgal antibiotic (0.1 mL/kg

(trimethoprim 40 mg/mL and sulfadoxine 200 mg/mL) Intervet Canada Ltd, Canada) diluted with 10 mL of saline through the femoral vein. The piglet was placed in a jacket and the catheters and probes were threaded through a port in the jacket, and temporarily stored in a pocket attached to the jacket.

2.2 Diet and Daily Animal Care During the Adaptation Period

Immediately following surgery, piglets were transferred to the animal housing room, with a 12-h light and 12-h dark cycle. Temperature in the room was maintained at 28 °C with supplemental heat provided by heat lamps attached to each cage. Piglets were housed individually in metabolic cages which allowed visual and aural contact with other piglets. The intravenous (IV) and intra-gastric (IG) lines were connected to a dual-port swivel and tether system (Lomir Biomedical, Montreal, Quebec, Canada). This system facilitates continuous infusions while allowing the piglets to move freely in the cages. Complete elemental diet (adaptation diet) was continuously infused IV at 50% of the maximal rate (13.5 mL/kg/h) by dual channel infusion pumps (Baxter Healthcare Corporation, Deerfield, USA). On the morning of the following day, the rate was increased to 75% for 6 h then increased to 100% of the target rate in the afternoon of Day 1. Piglets were kept on a TPN diet for 4 days with diet supplied through the jugular catheter to induce intestinal atrophy. Piglets received an IV injection of 0.1 mL/kg of Borgal diluted with saline every morning for the duration of the study. Antibacterial veterinary ointment was applied on the incision sites until they healed. Blood samples were taken from the femoral catheter and SMA blood flow was measured every morning using a Transonic T403 flow meter (Transonic Systems).

2.3 Experimental Protocol

On the morning of day 4, piglets were randomized to one of three experimental enteral diets (described below). The enteral diets were initiated at 50% of intake (with 50% of nutrients provided by TPN), and advanced to 75:25 after 3 h, and then to 100% enteral feeding at 5 h. SMA blood flow was measured prior to the initiation of enteral feeding, and then hourly for 10 h following the initiation of the enteral diets. On the morning of day 5 piglets underwent a 6 h primed constant IG infusion of stable isotopes (Table I). The constant infusion was delivered as half of the hourly dose and was infused intragastrically every 30 min (Urschel *et al*., 2007, Van Eijk *et al*., 2007). Baseline blood samples were taken from the femoral catheter and subsequent blood sampling occurred every 30 min. Blood samples were immediately collected into a heparinized vacutainer and centrifuged at 1300 *g* (VWR Clinical 200, Germany) for 5 min to separate plasma. The plasma samples were stored at -80°C until needed for analyses.

Table I: Composition of the Prime and Constant Dose for Stable Isotopes

Stable Isotope	Prime (mg/kg)	Constant (mg/kg/h)	Purpose
L-Arginine:HCl (15N4, 99%;13C6, 99%)*	2.00	3.40	Arg (M+10) to Cit (M+9) for NO determination
L-Citrulline (Ureido-13C, 99%; 3,3,4-D3, 98%)#	1.61	1.61	To determine Cit flux
L-Phenylalanine (Ring-D5, 98 %)*	1.09	3.40	Phe (M+5) to Tyr (M+4) for whole body protein synthesis
L-Tyrosine (Ring-D4, 98%)*	0.51	N/A	To prime the Tyr (M+4) product pool
L-Tyrosine (Ring-3,5-D2, 98%)*	0.50	1.65	To determine Tyr flux

*****Sourced from Cambridge Isotopes Laboratories, Inc., **#** Sourced from Advanced Research in Chemistry, A.R.C. Laboratories

On the morning of day 6, a flooding dose of unlabelled (1.5 mmol per kg of body weight; Sigma-Aldrich) and labelled phenylalanine (D8, 98%) (0.15 mmol per kg of body weight; Cambridge Isotopes Laboratories, Inc.) was given as an intravenous bolus and infused over 5 min. Phenylalanine (labeled and unlabeled) was dissolved in pyrogen-free water (11 mL per kg of body weight) and warmed before administering. Thirty min after the initiation of the IV flooding dose, piglets were anaesthetized with 5% isoflurane mixed with oxygen (1.5 L/min) by mask. The abdomen was opened and organ and tissues samples (liver, kidney, jejunal mucosa, gastrocnemius and longissimus dorsi muscles) were collected. These samples were immediately frozen using a freeze-clamp and were placed in liquid nitrogen. Small intestinal morphology was assessed by weighing a 50 cm length of proximal jejunum, and slitting the section open to measure the circumference of the gut. The opened section was flushed with ice-cold saline, and the mucosa was scraped from the muscularis externa using a glass microscope slide and even pressure. Another small sample of jejunum was placed in 10% buffered formalin for histology. The freezing time for each tissue was recorded and samples were stored at -80°C until needed for analysis.

2.4 Parenteral and Enteral Diet Preparation and Delivery

All of the parenteral and enteral diets were complete elemental diets that were prepared in our laboratory using crystalline L-amino acids (Evonik Industries AG, Hanau-Wolfgang, Germany or Sigma, St. Louis, MO, USA). The amino acid profile was based on a commercially available parenteral nutrition solution for infants (Vaminolact; Fresenius Kabi, Germany) that was modified for piglets (Wykes *et al*., 1993). To prepare the diets, dry amino acids (Table II) were weighed out and thoroughly mixed together in a 4 L beaker. Pyrogen-free water was

heated to 55-65 °C, and the dry amino acid mixture was added slowly under a cover of nitrogen gas (to avoid oxidation) until completely dissolved. Next, D-glucose and major minerals (Appendix I) were added and the solution was brought up to the final volume with pyrogen-free water. Once the diets were cooled, they were cold sterilized by filtering through a 0.22 μm filter (ACROPAK, Pall Corporation, Switzerland) into sterile IV bags (Baxter Corporation, Mississauga, ON, Canada) while working in a laminar flow hood. Each bag was filled to 750 mL and kept refrigerated and protected from light until required. Just prior to using the diet bag, the diets were made complete with the addition of 3 mL of a vitamin mixture. For the adaptation TPN diet, Multi-12/K1 (Baxter, Canada) was used to supply the vitamins. A laboratory-made multivitamin mixture patterned after Multi-12/K1 was used with the experimental enteral diets (Appendix II), 1 mL of iron dextran (Vetoquinol Canada Inc., Canada), providing 2 mg of iron per kg of body weight, 3 mL of trace element mix (Appendix III) and 145 mL of 20% SMOF lipid (Fresenius Kabi, Uppsala, Sweden) (Appendix IV). The complete diet provided 15 g amino acids/kg/day and 1.1 MJ of metabolizable energy/kg/day with lipids and carbohydrate supplying 50% of the non-protein energy.

Table II: Amino Acid Profiles of Adaptation Diet and Experimental Diets

Amino Acid	Adaptation Diet		Low Arg		High Arg		Cit	
	g/kg/day	g/L	g/kg/day	g/L	g/kg/day	g/L	g/kg/day	g/L
Alanine	1.41	5.89	1.41	5.89	1.01	4.21	1.27	5.29
Arginine	0.87	3.65	0.37	1.54	0.87	3.65	0.37	1.54
Citrulline	0.00	0.00	0.00	0.00	0.00	0.00	0.51	2.12
Aspartic Acid	0.80	3.32	0.80	3.32	0.80	3.32	0.80	3.32
Cysteine	0.18	0.76	0.18	0.76	0.18	0.76	0.18	0.76
Glutamic Acid	1.37	5.72	1.37	5.72	1.37	5.72	1.37	5.72
Glycine	0.35	1.47	0.35	1.47	0.35	1.47	0.35	1.47
Histidine	0.40	1.69	0.40	1.69	0.40	1.69	0.40	1.69
Isoleucine	0.60	2.51	0.60	2.51	0.60	2.51	0.60	2.51
Leucine	1.36	5.67	1.36	5.67	1.36	5.67	1.36	5.67
Lysine-HCl	1.34	5.58	1.34	5.58	1.34	5.58	1.34	5.58
Methionine	0.25	1.04	0.25	1.04	0.25	1.04	0.25	1.04
Phenylalanine	0.72	3.00	0.72	3.00	0.72	3.00	0.72	3.00
Proline	1.08	4.52	1.08	4.52	1.08	4.52	1.08	4.52
Serine	0.74	3.11	0.74	3.11	0.00	0.00	0.00	0.00
Taurine	0.06	0.27	0.06	0.27	0.06	0.27	0.06	0.27
Tryptophan	0.27	1.14	0.27	1.14	0.27	1.14	0.27	1.14
Tyrosine	0.11	0.44	0.11	0.44	0.11	0.44	0.11	0.44
Valine	0.69	2.89	0.69	2.89	0.69	2.89	0.69	2.89
Threonine	0.53	2.23	0.53	2.23	0.53	2.23	0.53	2.23

To test our hypotheses, on day 4 piglets were randomized to one of three experimental diets (described below) which were delivered intragastrically, starting slowly at 50% of requirement (with 50% of total nutrition delivered by TPN), and increased to 75:25 after 3 h, then advanced to full EN after 5 h. The diets were delivered via the gastric catheter at a rate of 13.5 mL/kg/h and continued for 2 days. During this period, the body weight of each animal was measured every day in the morning and the diet infusion rate was adjusted to the body weight.

2.4.1 Enteral Diet Treatments

Low arginine (Low Arg): The purpose of this diet was to deliver arginine to piglets at a concentration similar to that of sow milk levels (Davis *et al*., 1994). The concentration of arginine that was used in this diet provided 0.37 g arginine/kg/d (Table II).

High arginine (High Arg): This diet was formulated to be identical to the Low Arg diet, but with arginine added to deliver approximately 2.5 times more arginine (Table II); to maintain isonitogenous diets, serine was removed from the diet, and the alanine concentration was also reduced to accommodate the high nitrogen content of arginine. The concentration of arginine in the high diet was limited to providing 0.87 g arginine/kg/d because any additional arginine would require the removal of greater amounts of non-essential alanine, which is involved in nitrogen trafficking (Felig, 1973) and could impact other study outcomes. The arginine supplied in this diet matched the amount in our TPN adaptation, which may still be limiting in arginine when intestinal metabolism is by-passed (Brunton *et al*., 2003), but is more than double the National Research Council (NRC) recommendation for arginine intake. The major goal of this diet was to assess the effect of more dietary arginine on its availability as a substrate for NOS and NO production.

Citrulline (Cit): Because citrulline can be used by NOS or can increase arginine availability for NOS we included a third diet treatment containing citrulline. This diet contained arginine at the same concentration as the Low Arg group, with added citrulline. The citrulline combined with the arginine in this treatment was equimolar to the arginine in the High Arg treatment. The Cit diet investigated whether citrulline supplementation to an arginine deficient diet was more

effective than arginine at increasing NO synthesis and overall gut recovery from TPN-induced gut atrophy.

2.5 Analytical Procedures

2.5.1 Preparation of Jejunal Samples for Histological Assessment

The day following each necropsy, the jejunal intestine samples that were previously placed in formalin were cut into 2-3 mm thick sections using a scalpel blade and then loaded into a tissue processing cassette. The cassettes were immediately lowered into a formalin bath in the tissue processor (Citadel, Thermo Scientific, United Kingdom). The tissue processing was initiated and followed the program detailed in Appendix V, ending in a hot paraffin wax bath. To facilitate embedding tissue samples into paraffin blocks, metal base moulds were heated on a hot plate up to, but not in excess of, 85 °C. Wax was poured into the metal mould from a wax dispenser (Thermo Scientific, England) and an intestinal sample was placed in the center of the mould. The cassette was then placed into the mould followed by the addition of more wax. The mould was placed on ice to cool and solidify the wax. The hardened wax cassette containing the embedded sample was removed from the mould. To create histology slides, the cassette with the embedded tissue sample was fixed in the microtome (Shandon Finesse 325, Thermo Electron Corporation, United Kingdom), and 5 μm sections of jejunal sample were cut. The cut sections were submerged in 70% ethanol for ~ 1 min then placed in a warm water bath to remove any wrinkles in the wax that were formed during the cutting procedure. Next, the wax slices were placed on gelatin coated slides (Appendix VI) and allowed to dry overnight. The next day, the mounted samples were placed in a rack and put through a series of hematoxylin and eosin staining steps, as described in Appendix VII. The stained slides were left to dry for

approximately 2 h. Lastly, the cover slips were mounted and secured with Permount (Fisher Scientific, NJ, USA) and left to dry overnight before observation under a microscope (Axiostar Plus, Carl Zeiss microimaging) at 10X magnification. The microscope was fitted with an OMAX camera which allowed villus height and crypt depth to be measured in µm using OMAX ToupView software. Ten measurements over three intestinal sections were recorded and averaged per animal in each treatment. Measurements were conducted by a single investigator (MFH) who was masked to the treatments.

2.5.2 Preparation of Plasma Samples for Whole Body Protein and NO Synthesis Quantification

To prepare plasma samples for mass spectrometer analysis, 200 µL of 100% MeOH was added to an Eppendorf tube with 25 µL of plasma and vortexed for 10 sec. Tubes were then centrifuged for 10 min at 13000 *g*. Supernatant was collected into borosilicate glass tubes (13 x 100 mm, VWR disposable culture tubes), covered with parafilm and frozen at -80°C for 20 min. Samples were then freeze dried for ~ 1 hr. Samples were then derivatized using 100 µL of 3N HCl in n-butanol, capped and put in an oven at 65°C for 20 min and were dried down for ~ 1 hr and stored at -20°C until ready for shipment. The n-butanol derivatization procedure was used to increase the sensitivity for detecting the citrulline isotope, however it impacted the sensitivity of the phenylalanine isotope measurement. Therefore, underivatized samples were also analysed to accurately determine phenylalanine and tyrosine enrichments for the calculation of whole body protein synthesis.

Analyses were performed at the Analytical Facility for Bioactive Molecules (AFBM) of the Centre for the Study of Complex Childhood Diseases (CSCCD) at the Hospital for Sick Children, Toronto, Ontario.

2.5.3 LC-MS-MS Analysis

Personnel at the AFBM extracted and dried the plasma samples in test tubes and reconstituted with them with 1 mL of 10/90 Water/Acetonitrile, 5 mM ammonium formate, pH 3.2. Samples were analysed on a Sciex QTrap5500 mass spectrometer (Framingham, Massachusetts, USA) and Agilent 1290 HPLC system (Agilent Technologies: Santa Clara, California, USA). The sample injection volume was 5 µL, and chromatography was run using a flow rate of 500 µL/min through a Kinetex HILIC column (4.6 x 50mm, 2.6 µm, Phenomenex, Torrance, California, USA) with a gradient starting at 5% "A" (90/10 water/acetonitrile 5 mM ammonium formate pH 3.2) and 95% "B" (10/90 water/acetonitrile 5 mM ammonium formate pH 3.2) and then increased to 100% A at 5 min and returning to initial conditions at 6 min for re-equilibration and a total run time of 10 min. Acquisition was performed in positive mode electrospray ionization with a source temperature of 600 °C and an ion spray voltage setting of 5300. Multiple reaction monitoring mass transitions (m/z) were as follows for underivatized samples: phenylalanine 166.1-120.0, phenylalanine (M+5) 171.1-125.0, tyrosine 182.1-136.0, tyrosine (M+2) 184.1-138.0, tyrosine (M+4) 186.1-140.0. For derivatized samples the mass transitions were: arginine 231.0-70.0, arginine (M+10) 241.1-75.1, citrulline 232.15-70.0, citrulline (M+4) 236.15-73.1, citrulline (M+9) 241.11-75.1. Peak integration and data analysis were performed using Analyst 1.6.3 software spectrometer (Sciex, Framingham, Massachusetts,

USA). Area ratios were calculated by plotting peak areas of the labelled mass transition to peak areas of the unlabelled mass transition for each analyte.

2.5.3.1 Calculations

Whole body rate of appearances (flux) for each isotope was calculated as follows:

$$\mathbf{Q_{Phe\ or\ Tyr} = I_{Phe\ or\ Tyr} \bullet [(E_i / E_p) - 1]}$$

where $Q_{Phe\ or\ Tyr}$ is the rate of appearance for phenylalanine or tyrosine in μmol/(kg · h); $I_{Phe\ or\ Tyr}$ is the tracer infusion rates in μmol/(kg · h); E_i is the enrichment of either phenylalanine (ring-D5) or tyrosine (ring-3,5-D2) in the infusates expressed as the tracer:tracee ratio; and E_p is the plasma enrichment of individual tracer at plateau expressed as the tracer:tracee ratio (van Eijk *et al*., 2007) .

The steady state equation of total whole body flux of phenylalanine is:

$$\mathbf{Q_{Phe} = I_{diet} + PB = Ox + PS}$$

where Q_{Phe} is the entry rate of unlabeled phenylalanine into the central plasma pool equating to phenylalanine absorbed from the diet and phenylalanine appearance from protein breakdown (PB). The disposal rate of phenylalanine from the plasma pool is represented by its

disappearance from the plasma for protein synthesis (PS) and its irreversible loss through oxidation to tyrosine (Ox).

Phenylalanine to tyrosine conversion (Ox) was determined as follows:

$$\mathbf{Ox = Q_{Tyr(M+2)} \bullet [E_{Tyr(M+4)} / E_{Phe(M+5)}]}$$

where $Q_{Tyr(M+2)}$ is the plasma tyrosine flux, estimated from the primed constant infusion of labeled tyrosine (ring-3,5-D2). $E_{Tyr(M+4)}$ and $E_{Phe(M+5)}$ are the respective tracer:tracee ratios of the tyrosine (ring-D4) product and phenylalanine (ring-D5). Protein synthesis in μmol/(kg · h) was derived from the steady-state flux equation as follows:

$$\mathbf{PS = Q_{Phe} - Ox}$$

Lastly, protein synthesis was expressed as g of protein/kg of BW/day assuming the phenylalanine content of protein to be 280 umol/g (Thompson, 1989).

Similarly, the plasma arginine-to-citrulline flux (NO production) can be calculated as described previously for the phenylalanine to tyrosine conversion:

$$\mathbf{Q_{Arg \rightarrow Cit} = Q_{Cit} \bullet [E_{Cit(M+9)} / E_{Arg(M+10)}]}$$

where Q_{Cit} is the plasma citrulline flux, estimated from the primed constant infusions of labeled citrulline (Ureido-^{13}C, 3,3,4-D3). $E_{Cit(M+9)}$ and $E_{Arg(M+10)}$ are the respective tracer:tracee ratios of the citrulline (^{15}N3, ^{13}C6) product and arginine (^{15}N4, ^{13}C6).

Lastly, the plasma citrulline-to-arginine flux can be calculated as follows:

$$\mathbf{Q_{Cit \rightarrow Arg} = Q_{Arg} \bullet [E_{Arg(M+4)} / E_{Cit(M+4)}]}$$

where Q_{Arg} is the plasma arginine flux, estimated from the primed constant infusions of labeled arginine (^{15}N4, ^{13}C6). $E_{Arg(M+4)}$ and $E_{Cit(M+4)}$ are the respective tracer:tracee ratios of the arginine (Ureido-^{13}C, 3,3,4-D3) product and citrulline (Ureido-^{13}C, 3,3,4-D3).

2.5.4 Liver, Mucosa and Muscle Preparation for GC-MS Analysis of Amino Acids to Measure Tissue Specific Rates of Protein Synthesis

Tissue phenylalanine was extracted as per the method of Lamarre *et al*. (2015). Tissue was homogenized (200 mg liver, muscle, 100 mg mucosa) with 4 mL (2 mL for 100 mg of tissue) of deionized water at 50% speed for approximately one min using a mechanical homogenizer. Next, 0.5 mL of 2 M perchloric acid (PCA) was added and the sample was vortexed followed by centrifugation (3000 x *g*) for 20 min at 4 °C. After centrifugation the supernatant was collected and represents the free amino acid portion of the tissue (tissue free). The protein-bound (protein derived amino acids) portion is contained with the remaining pellet. To this pellet 5 mL of 0.2 M PCA was added and the sample was vortexed and centrifuged (3000

x *g*) again for 20 min. Supernatant was discarded and the centrifugation step that was just described was repeated two more times. The pellet was transferred to a glass tube and 4 mL of 6 M HCl was added, the tube was capped and incubated at 110 °C for 18 hr. The remaining solution was filtered using a 0.45 μm filter and ready for conditioning. Both the tissue-free and protein-bound fractions were applied to a hydrophobic solid-phase extraction cartridge (Bond Elute C18, 100 mg, 1 mL; Agilent Technologies, Santa Clara, CA) to isolate the phenylalanine. To this end, the column was primed using 1 mL of 100% MeOH followed by 1 mL of 0.1 M PCA, and any eluent was discarded. Next, the tissue free (0.5 mL) or protein-bound (1 mL) sample was loaded onto the column. Aromatic amino acids such as phenylalanine will bind to the column and any other amino acids will be found in the eluent therefore, the eluent was discarded. Finally, the column was rinsed with 1 mL 0.1 M PCA followed by 1 mL 100% MeOH, which elutes the bound phenylalanine for collection. This process was repeated again for protein-bound samples. The collected sample was dried in a vacuum oven (protein-bound) or freeze dried (tissue free) for approximately 10 h. The dry samples were resuspended in 500 μL of HPLC water with 50 μL used for derivatization. Derivatization was accomplished by adding 21.5 μL of 0.5 M phosphate buffer (0.4303 M HNa_2PO_4, 0.0697 M H_2NaPO_4, pH 8) followed by 50 μL of resuspended sample and lastly, 133.3 μL of 100 mM 2,3,4,5,6-Pentafluorobenzylbromide (dissolved in acetone) to a glass tube. After capping and mixing the sample was incubated at 60° C for 1 h using a heating block. Immediately after incubation the sample was left to cool at room temperature for 5 min and then 333.33 uL of hexane was added. The vial was capped, mixed and allowed to settle for 5 min until two separate layers formed. The organic phase (top layer) was extracted and transferred (~200 μL) into gas chromatography-mass spectrometry (GC-MS) vials fitted with an insert. Finally, to determine the isotopic enrichment of L-[D8, 98%] phenylalanine

in tissue-free and protein-bound fractions, 1 uL of sample was injected into a GC-MS and pentafluorobenzyl bromide derivatives were quantified (Sigma-Aldrich). Quantification was made using a model 6890 GC linked to a 5976N quadrupole MS (Agilent Technologies) operating in the electron ionization mode. A mixed sample of L-[D8, 98%] phenylalanine and unlabeled phenylalanine was processed in scan mode and 91 and 99 ions or 300 and 308 were identified as potential ions. A standard curve was run before analyzing the samples to identify the linear ranges. The ions 300 and 308 were selected for the following calculations. However, it was later identified that two deuterium on the D8 phenylalanine are unstable, thus the ions of 306 and 307 were quantified and summed with the 308 ion to determine an accurate D8 phenylalanine total. Percent molar enrichment (mol%) was determined, and the fractional synthesis rate (%/d) of protein was calculated as follows:

$$FSR = IE_{bound} / IE_{free} \bullet 1{,}440 / t \bullet 100$$

where IE_{bound} and IE_{free} are the isotopic enrichments (mol%) of L-[D8, 98%] phenylalanine of the perchloric acid-insoluble (protein-bound) and perchloric acid-soluble (tissue free) phenylalanine pool; *t* is the time of labeling in min; and 1,440 is the number of minutes per day.

2.6 Statistical Analysis

Data were analyzed by one-way ANOVA with Bonferroni post-tests or one-way repeated measures ANOVA with Bonferroni post-tests with time and treatment as the dependent variables. Grubb's test was used to identify significant outliers. When group variances were significantly different, the data were log transformed for statistical analyses, but are presented in the figures as untransformed values. Data are presented as means and standard deviation (SD). P

< 0.05 was considered to be significant. Statistical analyses were performed using GraphPad Prism 5 or 9 (Graph Pad Software, San Diego, CA, USA).

3. Results

Data from a total of 20 piglets are included in this study. Based on a sample size calculation using blood flow data from previous studies, we determined that a sample size of n=6 was sufficient for the current study. However, two animals from the Low Arg group failed to provide blood flow data after recovery from surgery. As such, we studied two additional piglets on this treatment. This resulted in a sample size of n=8 for the Low Arg treatment group for most outcomes, but only n=6 for the blood flow data. Only male piglets were included in this study because there was limited availability of female pigs due to concurrent studies requiring female animals. All piglets were healthy, playful and explorative of their surroundings throughout the study period. However, the enteral diet induced minor vomiting in seven piglets during the enteral phase.

3.1 Body Weight

Piglet body weights were not significantly different between treatments at entry into the study or on the final day (Figure 3.1). Similarly, the daily mean body weight was not different among treatment groups (Figure 3.2). However, there was a significant change over time for all treatments (Figure 3.2) ($P<0.0001$).

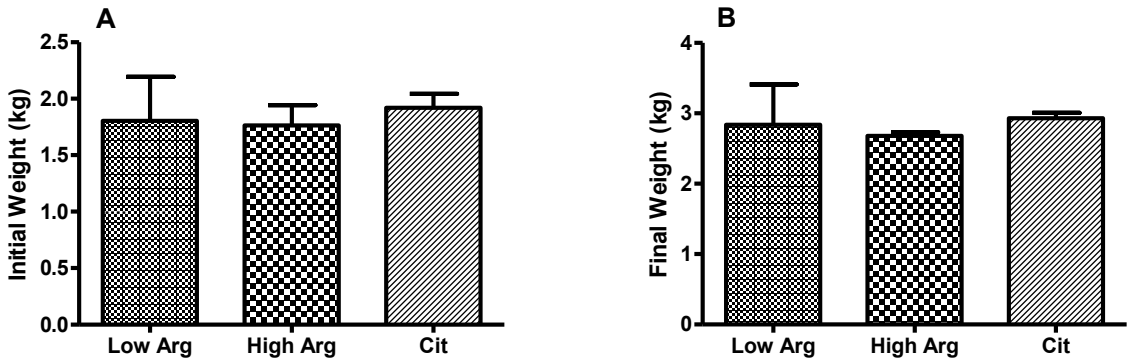

Figure 3.1 A: piglet weight at entry into the study. B: piglet weight at necropsy. Data are means and SD and were analyzed by one-way non-repeated measures ANOVA. Low Arg (n=8), High Arg and Cit (n=6).

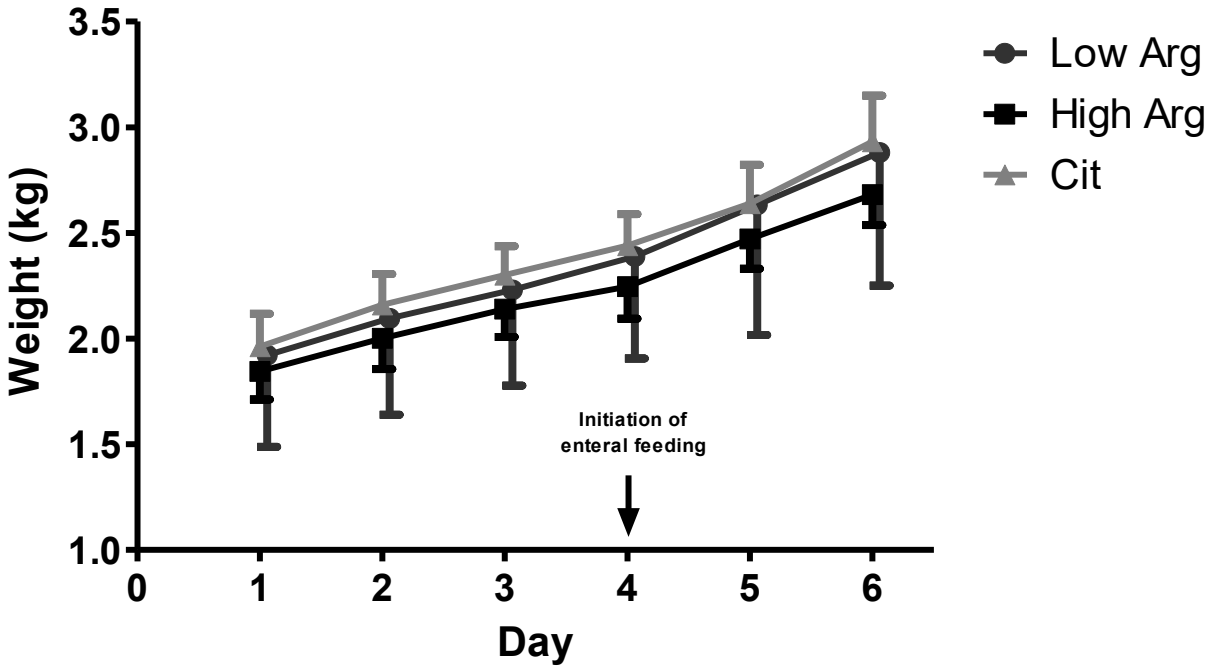

Figure 3.2 Daily piglet weight. Data are means and SD and were analyzed by one-way repeated measures ANOVA. Low Arg (n=8), High Arg and Cit (n=6).

3.2 Organ Morphology

3.2.1 Small Intestinal Morphology

The jejunal mucosa weight (per cm gut length) was not significantly different between treatment groups (ANOVA, P=0.20) (Figure 3.3, A). Interestingly, there was a diet effect on jejunum circumference (ANOVA, P=0.002), which was significantly higher in both the High Arg and Cit group compared to the Low Arg group, suggesting better growth in these groups (Figure 3.3, B). The mucosal weight was corrected by the circumference to obtain mucosal weight per surface area (Figure 3.3, C). Correcting for the circumference resulted in no difference between the High Arg and Low Arg groups; the Cit and High Arg group were also not different. Interestingly, the Low Arg group was higher than the Cit group (P=0.003). The mucosa was dried to correct for potential differences in tissue water content. There was no difference among groups for percent dry mucosal weight (Figure 3.4).

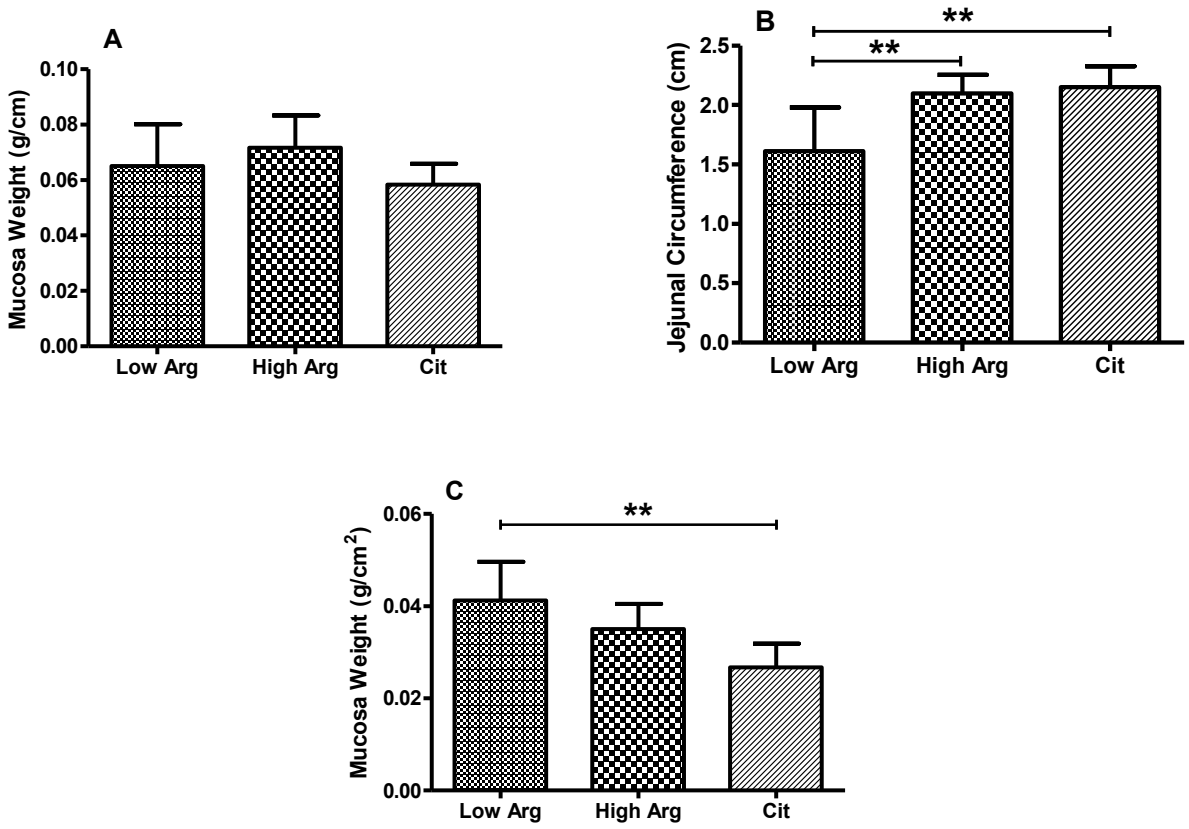

Figure 3.3 Small intestinal morphology. A: jejunal mucosa weight (g/cm). B: jejunum circumference (cm). C: jejunal mucosa weight per surface area (g/cm^2). Data were analyzed by one-way non-repeated measures ANOVA, followed by a Bonferroni multiple comparisons test, **P<0.01; Low Arg (n=8), High Arg and Cit (n=6).

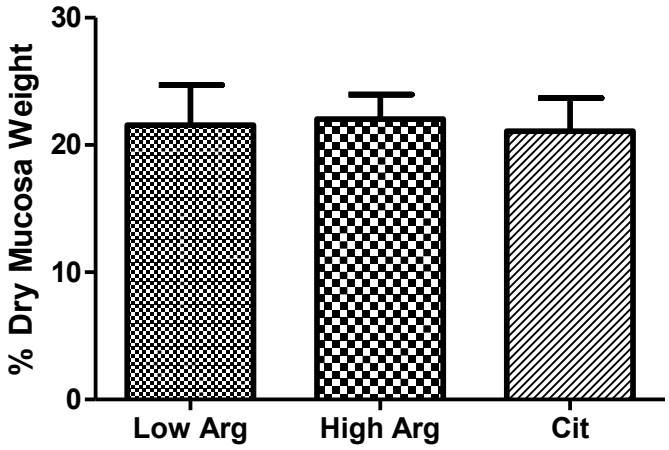

Figure 3.4 Percent dry mucosa weight. Data were analyzed by one-way non-repeated measures ANOVA, Low Arg (n=8), High Arg and Cit (n=6).

3.2.2 Liver

The entire liver was removed from the pig, blotted dry and weighed, to provide a gross liver weight. The effect of diet on absolute liver weight neared significance (ANOVA, P=0.055) (Figure 3.5). When corrected for body weight there was an overall effect of diet treatment (ANOVA, P=0.003) and the livers from piglets that received the Cit diet were significantly heavier compared to the other treatments (Figure 3.5 B).

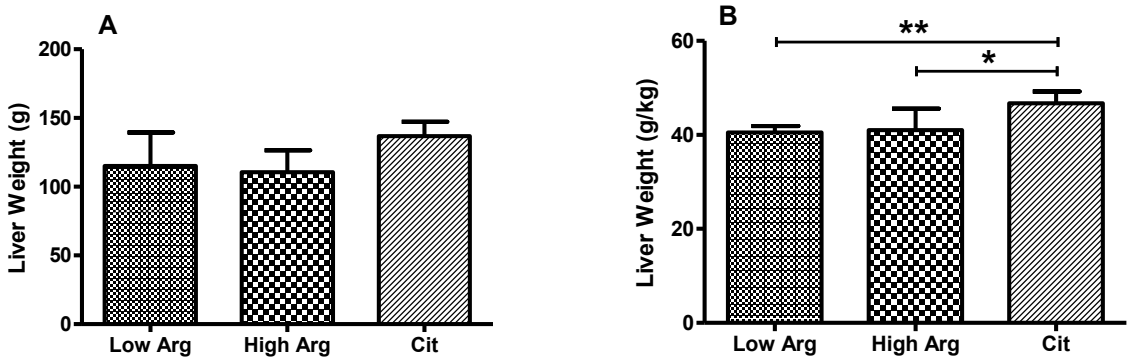

Figure 3.5 Mean liver absolute weight (A) and corrected for body weight (B). Data were analyzed by one-way non-repeated measures ANOVA, followed by a Bonferroni multiple comparisons test, *P<0.05, **P<0.01: Low Arg (n=8), High Arg and Cit (n=6).

3.2.3 Jejunal Histology

Mucosal villus length and crypt depth were significantly affected by treatment (P for overall ANOVA, <0.0001 and 0.0016, respectively). Piglets provided with high arginine presented with greater villus height than the other two treatment groups (Figure 3.6); similarly, the High Arg group had a greater crypt depth compared to the other two treatments (Figure 3.6).

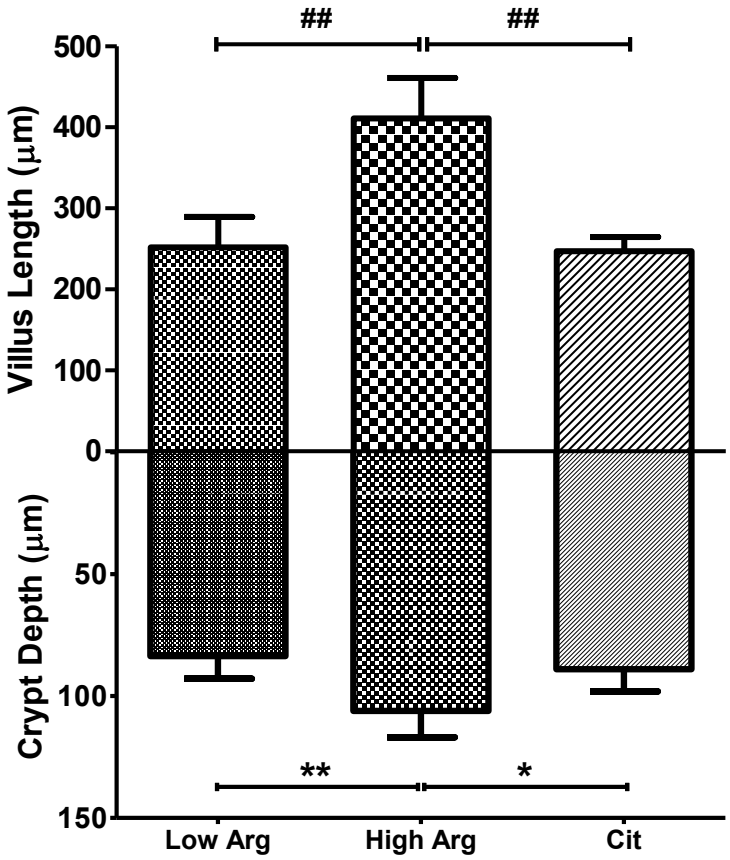

Figure 3.6 Jejunal villus length and crypt depth. Data were analyzed by one-way non-repeated measures ANOVA, followed by a Bonferroni multiple comparisons test, *P<0.05, **P<0.01, ##P<0.0001; Low Arg (n=8), High Arg and Cit (n=6).

3.3 Plasma and Tissue Free Amino Acids

A significant treatment effect was present for the jejunal mucosa free arginine concentration (Table 3.1), with differences between the High Arg and Low Arg treatments, and between the High Arg and Cit groups. Citrulline concentrations were also different between Cit and Low Arg and the Cit and High Arg treatments. Lastly, glutamine was significantly different between the Low Arg and High Arg and the Low Arg and Cit groups. The other arginine related amino acids glutamate, proline and ornithine were not different between treatments (Table 3.1). We also observed differences in some amino acids that are not involved in arginine biosynthesis, including valine, isoleucine, hydroxyproline and serine (Table 3.1).

Surprisingly, there were no differences in plasma arginine or citrulline concentrations among treatment groups (Appendix VIII); however, when amino acids were grouped by those related to arginine metabolism, the High Arg treatment had significantly greater total amino acids related to arginine metabolism compared to the Cit group (Table 3.2). Individual amino acid concentrations for study days 4 to 6 are presented in Appendix VIII.

Table 3.1 Tissue free amino acids (nmol/g) in jejunal mucosa samples.				
	Low Arg[+]	**High Arg**	**Cit**	**P-value[++]**
Arginine-related				
Arginine	129 ± 72[b]	336 ± 122[a]	131 ± 34[b]	**P = 0.0004**
Citrulline	640 ± 188[b]	434 ± 228[b]	981 ± 256[a]	**P = 0.002**
Glutamic Acid	2209 ± 368	1845 ± 747	1810 ± 225	P > 0.05
Glutamine	609 ± 100[a]	342 ± 20[b]	441 ± 88[b]	**P < 0.0001**
Proline	2550 ± 698	3076 ± 890	2363 ± 449	P > 0.05
Ornithine	133 ± 24	135 ± 23	163 ± 31	P > 0.05
Essential Amino Acids				
Histidine	207 ± 99	215 ± 94	133 ± 43	P > 0.05
Threonine	823 ± 203	870 ± 260	711 ± 141	P > 0.05
Valine	1027 ± 231[ab]	1313 ± 211[a]	827 ± 188[b]	**P = 0.01**
Methionine	77 ± 27	52 ± 20	78 ± 9	P > 0.05
Isoleucine	1498 ± 409[a]	922 ± 235[b]	689 ± 131[b]	**P = 0.0007**
Leucine	1571 ± 459	1859 ± 535	1355 ± 352	P > 0.05
Phenylalanine	250 ± 23	250 ± 24	259 ± 32	P > 0.05
Tryptophan	317 ± 223	428 ± 154	368 ± 62	P > 0.05
Lysine	1014 ± 292	1052 ± 287	935 ± 200	P > 0.05
Other Non-essential Amino Acids				
Aspartic Acid	1332 ± 268	1075 ± 378	1090 ± 226	P > 0.05
Hydroxyproline	1496 ± 407[ab]	1074 ± 455[a]	1688 ± 307[b]	**P = 0.04**
Serine	1548 ± 641[a]	369 ± 75[b]	475 ± 59[b]	**P = 0.0002**
Glycine	5443 ± 1248	4326 ± 537	4204 ± 647	P > 0.05
Taurine	3364 ± 843	2888 ± 581	3348 ± 696	P > 0.05
Alanine	3640 ± 488	3443 ± 989	2785 ± 269	P > 0.05
Tyrosine	261 ± 90	184 ± 31	205 ± 56	P > 0.05

[+]Treatments were Low Arg, n=8, High Arg and Cit, n=6.

[++] P-value represents overall treatment effect by one-way ANOVA.

Values with different letters are significantly different ($P < 0.05$).

Table 3.2 Total plasma amino acids (μmol/L).				
	Low Arg	**High Arg**	**Cit**	**P-value**
Day 4#				
Total Arginine-related Amino Acids	2700 ± 681^{ab}	3380 ± 534^{a}	2347 ± 316^{b}	**P = 0.015**
Total Essential Amino Acids	2565 ± 566^{a}	2716 ± 325^{a}	1763 ± 480^{b}	**P = 0.006**
Total Other Non-Essential Amino Acids	3292 ± 1038^{ab}	4510 ± 1137^{a}	2740 ± 413^{b}	**P = 0.013**
Total BCAA*	618 ± 298^{ab}	780 ± 127^{a}	460 ± 180^{b}	**P = 0.05**
Day 5#				
Total Arginine-related Amino Acids	3588 ± 430^{a}	3664 ± 462^{a}	1705 ± 320^{b}	**P < 0.000001**
Total Essential Amino Acids	3613 ± 270^{a}	3400 ± 765^{a}	1467 ± 565^{b}	**P = 0.0007**
Total Other Non-Essential Amino Acids	4571 ± 544^{a}	4550 ± 411^{a}	1783 ± 432^{b}	**P < 0.000001**
Total BCAA	687 ± 115^{a}	873 ± 181^{a}	390 ± 98^{b}	**P = 0.00003**
Day 6#				
Total Arginine-related Amino Acids	2740 ± 832^{ab}	3510 ± 279^{a}	2360 ± 772^{b}	**P = 0.031**
Total Essential Amino Acids	2792 ± 549^{a}	3216 ± 926^{a}	1434 ± 478^{b}	**P = 0.0006**
Total Other Non-Essential Amino Acids	4221 ± 1139^{ab}	4431 ± 686^{a}	2733 ± 1270^{b}	**P = 0.025**
Total BCAA	585 ± 180^{ab}	844 ± 134^{a}	446 ± 244^{b}	**P = 0.007**

#Individual plasma amino acid concentrations for each day can be found in Appendix VIII.

*BCAA - Branched-chain amino acids

3.4 Superior Mesenteric Artery Blood Flow

The mean SMA blood flow was not significantly different among treatment groups when measured daily from day 1 to day 6 (Figure 3.7). Piglets receive the same TPN diet up until day 4 to induce atrophy. A blood flow signal was not obtainable immediately following surgery for most pigs, likely because natural settling had to occur within the cavity; however, blood flow was measurable in all animals the following morning of day 1. Immediately following implantation, intra-operative blood flow readings were obtained in most animals and were approximately ~25% than readings measured on day 1 (data not shown). The introduction of enteral feeding induced a rapid rise in blood flow in all treatment groups.

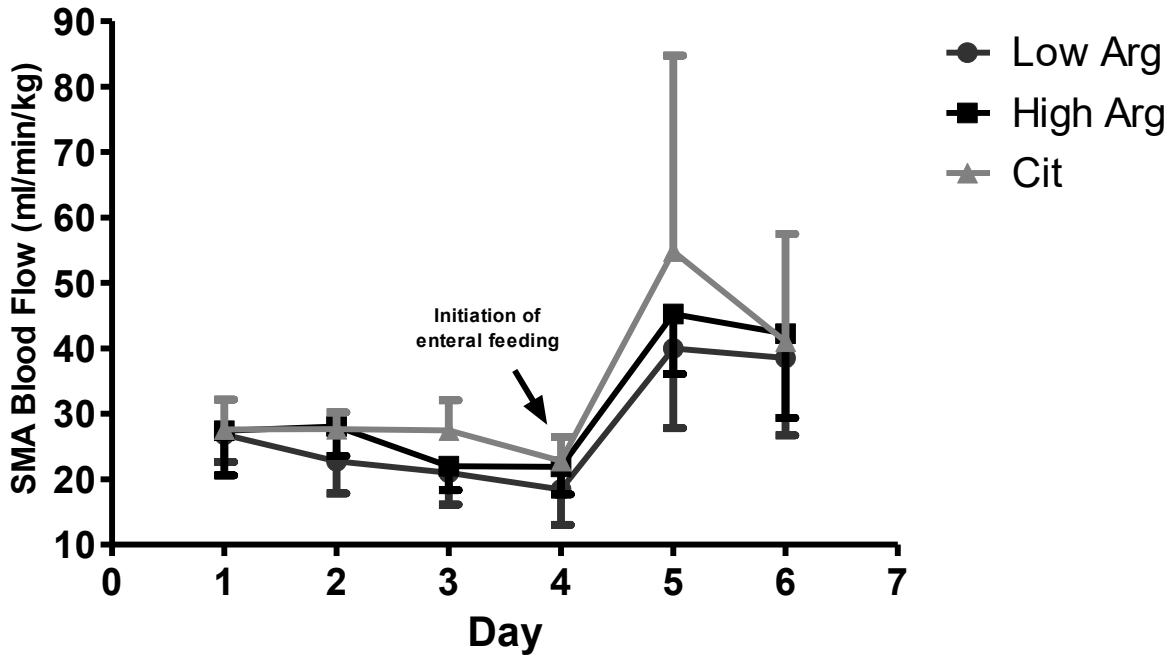

Figure 3.7 Daily recorded SMA blood flow (mL/min/kg). Data are means and SD and were analyzed by one-way repeated measures ANOVA, Low Arg, High Arg and Cit (n=6).

3.5 SMA Blood Flow Following the Initiation of Enteral Feeding

On day 4 of the study, the SMA blood flow was measured hourly starting before and following the initiation of enteral diet delivery. The enteral diet was well tolerated by all piglets during the first day of enteral feeding. There was some vomiting that occurred in a small number of animals throughout the remainder (days 5 to 6) of the enteral feeding period. At the initiation of the enteral feed, all piglets experienced a rapid response, demonstrating a steady rise in blood flow (Figure 3.7). Interestingly, SMA flow in the Cit group was greater than the Low Arg group at hours 7, 8, 9 and 10 after the initiation of EN feeding; the Cit group was also greater than the High Arg group by hour 10 (Figure 3.8). The addition of citrulline to the enteral diet enhanced blood flow by approximately 36% over the short term. The slope of the rate of blood flow determined over the 10 hour period was significantly higher in the Cit group compared to the Low and High Arg groups (Figure 3.9). Because it visually appeared that the Cit treatment had a late effect on blood flow, the slope from 5 to 10 hours was also compared (Figure 3.10). The Cit group had a significantly greater slope compared to both the High Arg and Low Arg groups.

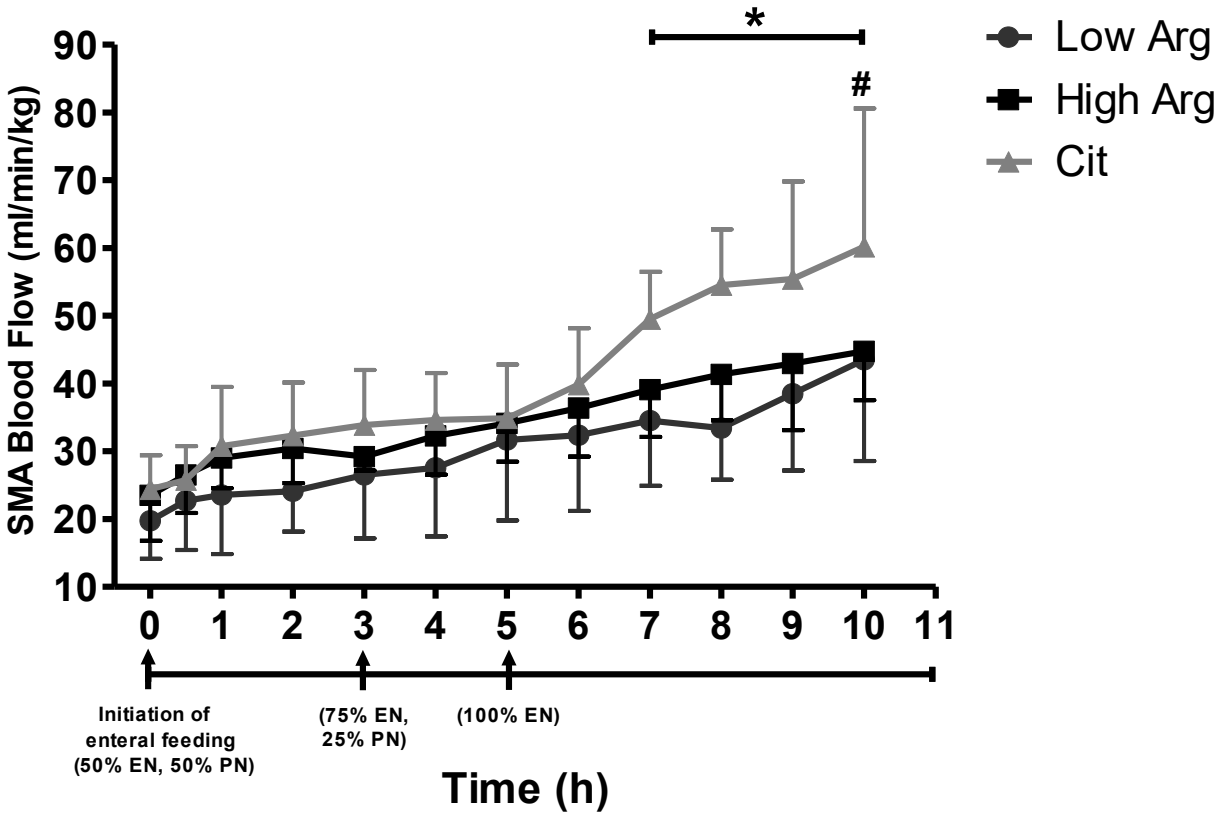

Figure 3.8 Mean SMA blood flow (ml/min/kg) during the 10 hour period immediately after the initiation of enteral feeding (EN). Data are means and SD and were analyzed by one-way repeated measures ANOVA (P=0.023). Low Arg, High Arg and Cit (n=6). *Cit vs Low Arg, P<0.05; #Cit vs High Arg, P<0.05.

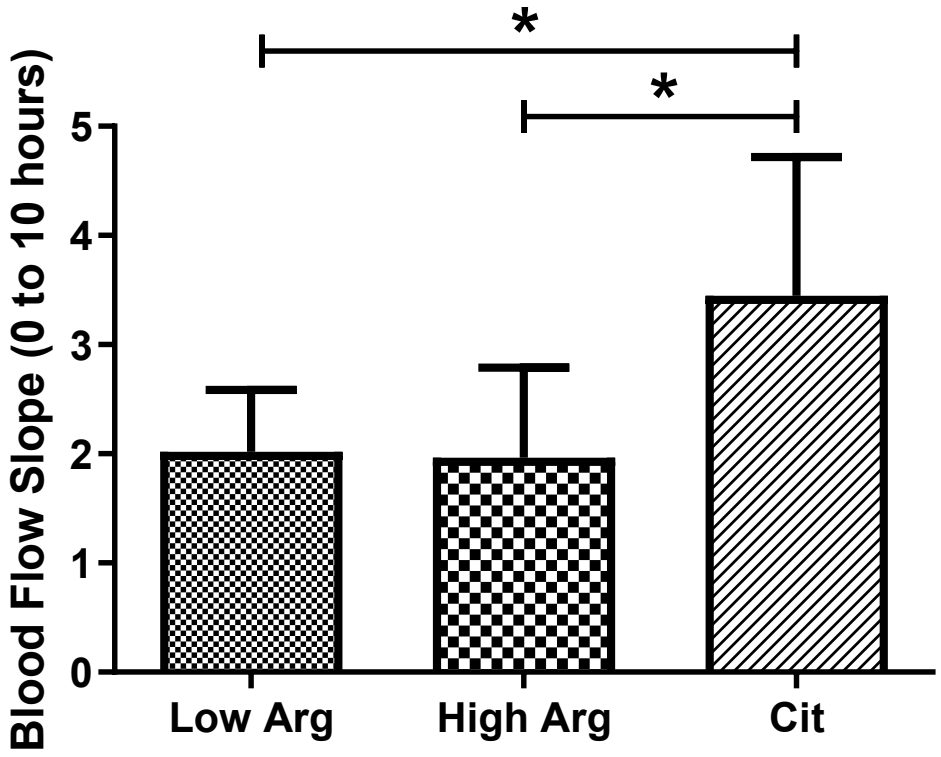

Figure 3.9 Mean SMA blood flow slopes calculated from the measurements during the 10 hour period immediately after the initiation of enteral feeding (EN). Data are means and SD and were analyzed by one-way non-repeated measures ANOVA, followed by a Bonferroni multiple comparisons test. *P<0.05, Low Arg, High Arg and Cit (n=6).

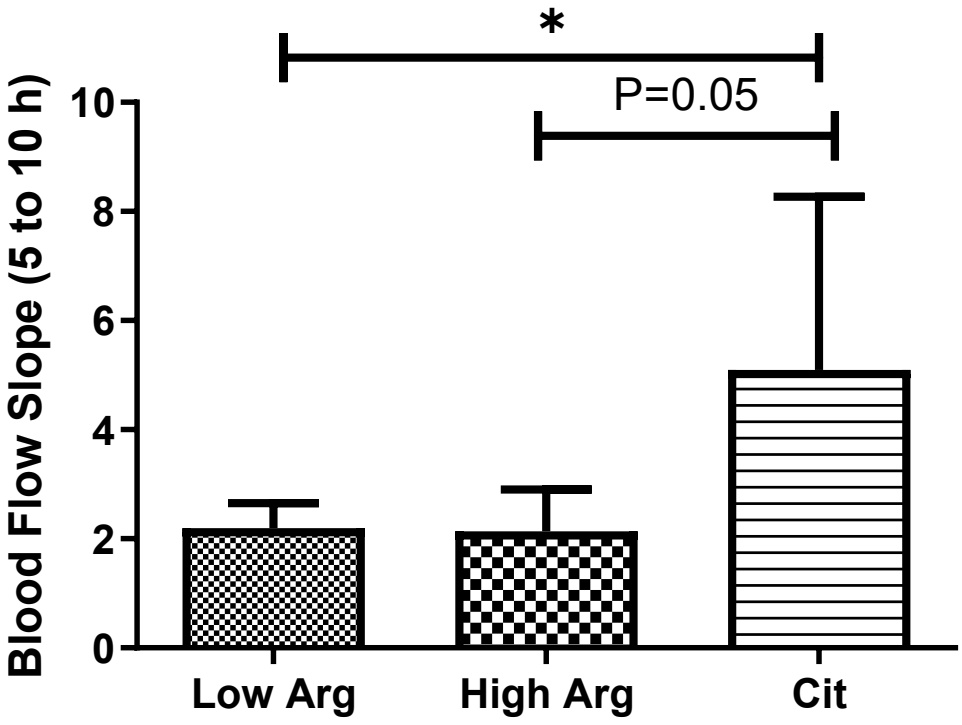

Figure 3.10 SMA blood flow slopes calculated from the measurements during the 5 to 10 hour period immediately after the initiation of enteral feeding (EN). Data are means and SD and were analyzed by one-way non-repeated measures ANOVA (P=0.019), followed by a Bonferroni multiple comparisons test. *P<0.05; Low Arg, High Arg and Cit (n=6).

Table 3.3 Isotopic Enrichments (%) and fluxes (μmol/kg/hr) on day two of enteral feeding.				
	Low Arg	**High Arg**	**Cit**	**P-value**
Enrichment (%)				
Arginine (M+10)	21.36 ± 8.93	16.81 ± 5.06	18.16 ± 4.52	P > 0.05
Citrulline (M+9)	0.45 ± 0.60	0.19 ± 0.07	0.16 ± 0.07	P > 0.05
Citrulline (M+4)	25.91 ± 7.02[b]	22.60 ± 6.81[b]	9.77 ± 1.53[a]	**P < 0.05**
Phenylalanine (M+5)	5.78 ± 0.62	4.94 ± 0.98	5.65 ± 0.31	P > 0.05
Tyrosine (M+4)	1.58 ± 0.50	1.15 ± 0.43	1.75 ± 0.50	P > 0.05
Tyrosine (M+2)	6.14 ± 2.57	7.51 ± 4.81	6.34 ± 0.43	P > 0.05
Flux (μmol/kg/hr)				
Arginine (M+10)	65.35 ± 26.44	82.32 ± 27.17	73.63 ± 23.63	P > 0.05
Citrulline (M+4)	28.42 ± 11.64[b]	34.02 ± 13.24[b]	85.52 ± 18.45[a]	**P < 0.0001**
Phenylalanine (M+5)	328.92 ± 35.40	399.43 ± 90.83	334.89 ± 20.24	P > 0.05
Tyrosine (M+2)	158.03 ± 63.44	155.62 ± 90.05	132.20 ± 8.64	P > 0.05

3.6 NO Synthesis

The composition of the enteral diet impacted the rate of NO synthesis (Figure 3.11). Higher NO synthesis was measured in the citrulline supplemented animals compared to both the Low and High Arg groups (Figure 3.11). Because the citrulline treatment resulted in greater mean blood flow rates and NO synthesis, we correlated NO synthesis rate with the blood flow rate measured at the tenth hour after the initiation of enteral feeding, but no significant correlation was evident (data not shown).

The addition of citrulline to the diet resulted in a greater citrulline flux (Table 3.3); however, the same effect did not occur with arginine supplementation, as arginine flux was not different between groups (Table 3.3). The citrulline supplemented piglets had a higher conversion rate compared to the other two groups (Figure 3.12). The proportion of citrulline that was converted to arginine was quantified by dividing the citrulline to arginine conversion by the flux of citrulline then converting to a percentage. We found that there was an overall effect of diet on the percentage of citrulline converted to arginine; there was a trend towards the Low Arg group being higher than the other two groups (Figure 3.13).

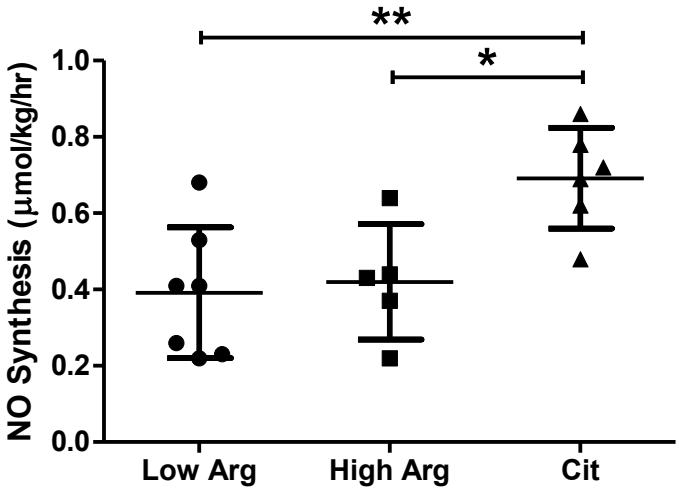

Figure 3.11 Nitric oxide synthesis (µmol/kg/hr) on day two of enteral feeding. Data are means ± SD and were analyzed by one-way non-repeated measures ANOVA (P=0.0068), followed by a Bonferroni multiple comparison test, *P<0.05, **P<0.01; Low Arg (n=7), High Arg (n=5) and Cit (n=6).

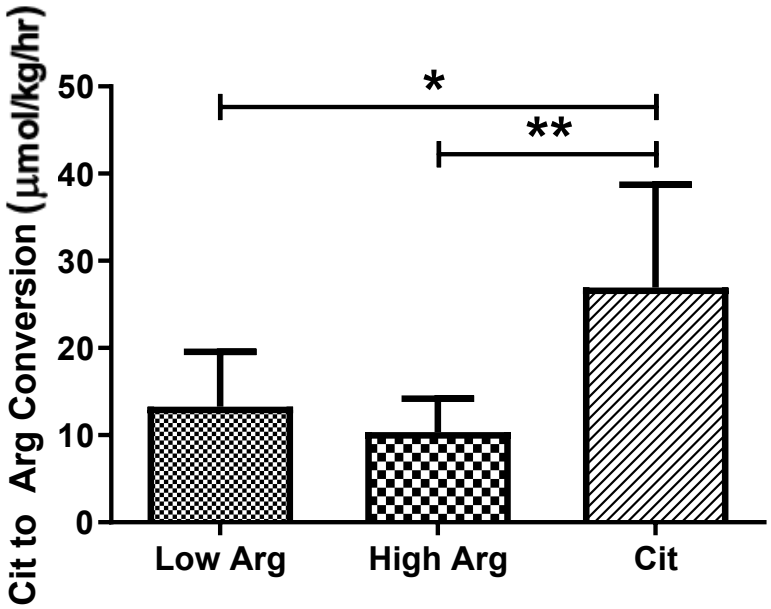

Figure 3.12 Citrulline to arginine conversion (µmol/kg/hr) on day two of enteral feeding. Data are means and SD and were analyzed by one-way non-repeated measures ANOVA (P=0.0038), followed by a Bonferroni multiple comparisons test, *P<0.05, **P<0.01; Low Arg (n=8), High Arg and Cit (n=6).

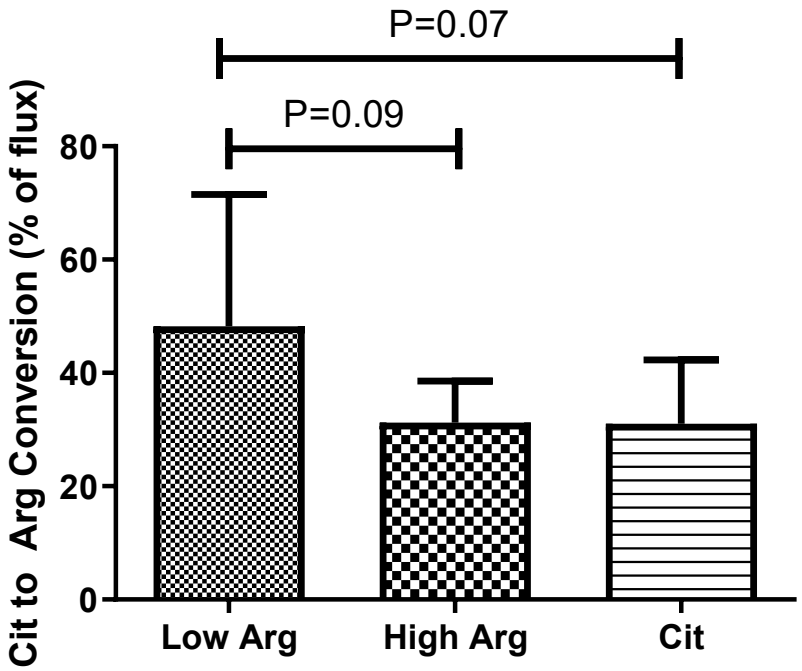

Figure 3.13 Proportion of citrulline converted to arginine (%) on day two of enteral feeding. Data are means and SD and were analyzed by one-way non-repeated measures ANOVA (P=0.033), followed by a Bonferroni multiple comparisons test. Low Arg (n=8), High Arg and Cit (n=6).

3.7 Whole Body Protein Synthesis

There was a greater rate whole body protein synthesis in the High Arg group compared to the Low Arg and Cit groups (Figure 3.14). Phenylalanine fluxes were not different between treatment groups (ANOVA, P=0.07) (Table 3.3).

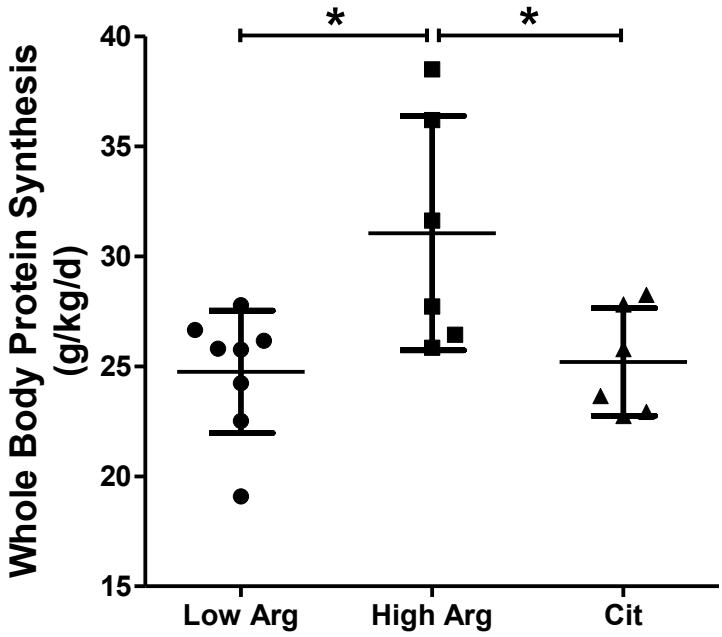

Figure 3.14 Mean whole body rate of protein synthesis on day two of enteral feeding (24 hr after introducing the enteral diet). Data were analyzed by one-way non-repeated measures ANOVA (P=0.011), followed by a Bonferroni multiple comparisons test, *P<0.05; Low Arg (n=8), High Arg and Cit (n=6).

3.8 Tissue Specific Protein Synthesis

No effect of diet was found in the rate of protein synthesis in jejunal mucosa, liver or longissimus dorsi muscle (Figure 3.15A-C).

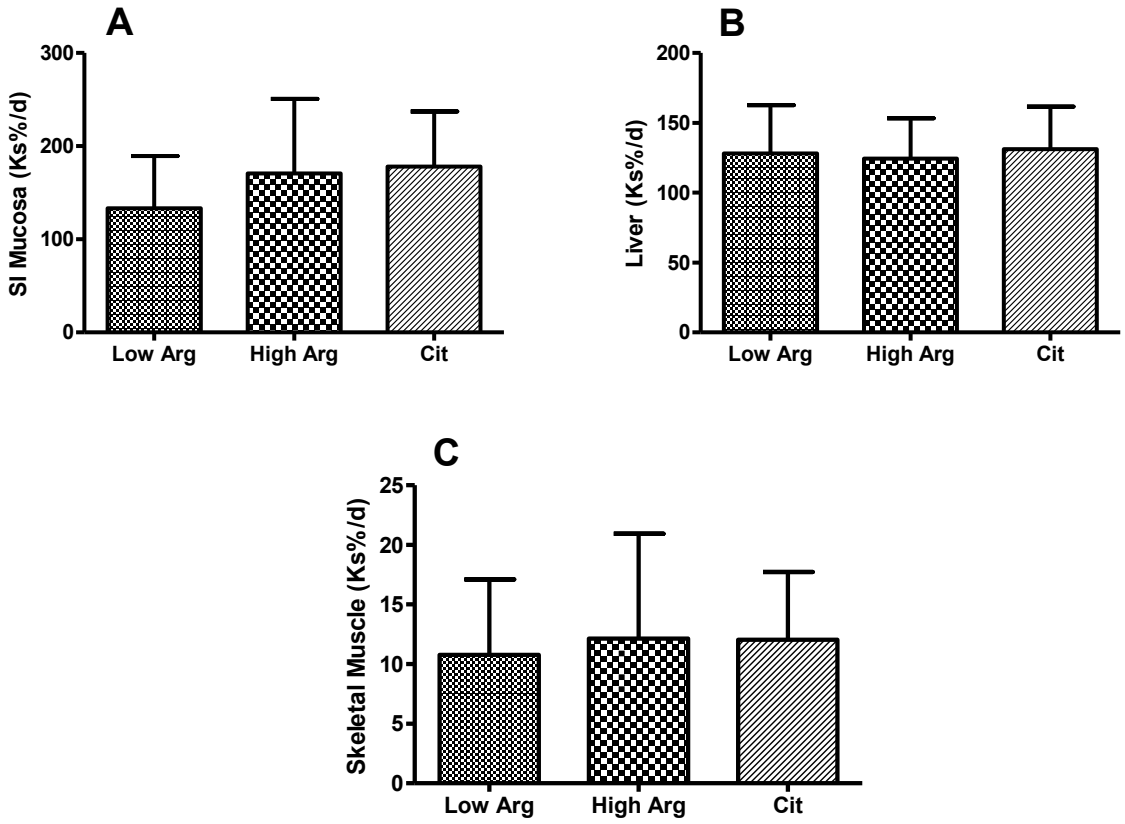

Figure 3.15 Tissue specific rate of protein synthesis presented as percent per day in A) proximal jejunal mucosa, B) liver and C) longissimus dorsi muscle at necropsy, after two days of enteral feeding. Data are means and SD and were analyzed by one-way non-repeated measures ANOVA, Low Arg (n=8), High Arg and Cit (n=6).

4. Discussion

The purpose of this study was to determine if arginine or citrulline supplementation in piglets with TPN-induced gut atrophy would result in an increased blood flow to the gut and enhanced whole body NO synthesis. Furthermore, we wanted to determine whether these enhancements would improve gut recovery. More specifically, we addressed the question of whether the concentration of arginine that occurs naturally in sow milk was adequate to optimize NO synthesis and blood flow to the recovering gut. We compared this to a diet that supplied arginine at a concentration two and a half times higher than sow milk levels and closer to predicted whole body requirements (Wu *et al*., 2004a). Our third treatment provided supplemental citrulline instead of additional arginine. We included this group to assess whether the citrulline-NO cycling pathway (Hecker *et al*., 1990) translates into citrulline being a more effective precursor for NO synthesis than arginine and thus, supports greater blood flow to the gut. Our most important novel finding was that citrulline supplementation did induce a higher rate of nitric oxide synthesis and supported a greater rate of blood flow to the intestine; however, this did not clearly translate into morphological benefits at the level of the mucosa in our time period.

SMA Blood Flow: The pattern of blood flow that we measured throughout the atrophy phase of the feeding trial was typical of parenteral feeding (Niinikoski *et al*., 2004). Acute in-surgery blood flow readings were recorded for 16 of the 18 piglets during the intra-operative period (data not shown). For some piglets, the in-surgery blood flow readings were missed due to difficulty attaining a stable reading. Based on the values we did record, it appeared that the piglets experienced a 25% decline in blood flow within the first 12 hours, prior to the first standardized

measure on the morning of day 1. After this initial decline, blood flow remained relatively constant or marginally declined throughout the atrophy period of the study. At the end of the atrophy phase (Day 4), the mean blood flow in each group was lower (~ 25 - 30%) than the post-surgery (Day 1) blood flow but was not significantly different among treatments. Reduction in blood flow is a normal response to TPN feeding as evidenced in previous studies. Burrin *et al*., (2003) demonstrated that in piglets which were administered chronic TPN (6 day period) had lower portal blood flow rates than enterally fed piglets. In addition, Niinikoski *et al*., (2004) found that SMA and portal blood flow decreases by about 30% after the initiation of TPN (8 hours) compared to enterally fed pigs. Lastly, in a study conducted by Dinesh *et al*., (2014) it was reported that all piglets experienced a steady decline in SMA blood flow during a TPN adaptation period (4 days). In our study, the transition to enteral feeding on day 4 induced a rapid response in rate of blood flow for all treatments with increases of approximately 200%, 160% and 140% in the Cit, High Arg and Low Arg groups, respectively.

Hourly blood flow measurements taken over the 10 hours following the initiation of enteral feeding identified different patterns of response among treatment groups. The enhancement in blood flow with citrulline supplementation appeared to occur after 5 hours of EN diet delivery, which was the point at which full enteral diet delivery was achieved. Visual inspection of the blood flow data versus time (Figure 3.8) suggested that the slope of the line for the Cit group deviated from the other two groups after 5 hours, so we analyzed whether the mean slopes were different during this period, and indeed the Cit group slope was significantly higher. This may be indicative of a dose or threshold response. Blood flow differences between the citrulline and low arginine treatment groups were evident from 7 hours onwards; this is an important finding, when considering that the low arginine group was receiving arginine at the

concentration in sow milk. Even with more than double the arginine intake supplied by the high arginine diet, piglets still demonstrated a blood flow rate that was lower than the citrulline supplemented group at 10 hours.

Our initial hypothesis was that both supplemental arginine and citrulline would lead to greater NO synthesis and thus higher blood flow. It is worth noting that the Low Arg treatment group became significantly lower than the Cit treatment group three hours before a difference was evident between the High Arg and Cit groups. It wasn't until late in the experiment that the difference in absolute blood flow rates between citrulline and arginine supplemented pigs became significant; this suggests that NO synthesis was better in the arginine supplemented animals compared to the Low Arg animals. However, there was no differences in blood flow between the High and Low Arg groups at any point. In addition, the Low Arg group appears to be recovering in the final hours of our day 4 blood flow readings. Thus, it is difficult to conclude with our findings that arginine supplementation can improve blood flow. The blood flow data on day 4 was only measured up to ten hours following the initiation of enteral feeding but it is likely that blood flow may continue to increase after ten hours in all groups. Moreover, if we had measured beyond ten hours we may have seen a greater separation in blood flow rate between the Cit and High Arg groups. Unfortunately, the first measurement point that was significantly different between the supplemental citrulline and high arginine group was also our last measurement point of the experiment day. The mean blood flow on the morning of day 5 was similar to the mean flow measured at the 10 h point on day four. This could mean that blood flow had neared a plateau; however, we do not know if blood flow continued to increase and then decline or if it had indeed reached a plateau by 10 hours. There may be a short window in which blood flow is enhanced by citrulline and this could be anywhere between 7 hours and the blood

flow reading measured on day 5. Without continued measurements beyond ten hours, it is unknown whether citrulline supplementation continued to support greater blood flow compared to the other groups for any length of time.

The enhanced blood flow with citrulline may be facilitated by limited intestinal metabolism of citrulline, allowing it to enter the portal circulation to be utilized for NO synthesis. Multiple *in vitro* and *in vivo* studies have suggested that the intestinal-renal axis does not exist in the neonatal gut and citrulline is preferentially converted to arginine and then released as arginine (Bertolo *et al*., 2003, Urschel *et al*., 2005, Wu *et al*., 1994). However, a recently published paper has provided evidence that the intestinal-renal axis does exist and that there is a significant flux of citrulline out of the neonatal piglet intestine (Marini *et al*., 2017). In our study, we did not quantify citrulline appearance into the portal circulation but based on our NO synthesis and blood flow data from our citrulline group, we speculate that some citrulline must be released into circulation to be used by NOS enzymes. Moreover, the Low and High Arg groups had no differences in blood flow. The Cit group elicited systemic effects as demonstrated by higher NO synthesis compared to the High Arg treatment. Arginine is the predominant substrate for NOS, but recent literature has also clearly shown that NOS can also utilize citrulline as a precursor (Hecker *et al*., 1990, Flam *et al*., 2007). The study conducted by Marini *et al*., 2017 in which they found a significant flux of citrulline out of the intestine supports our speculation of citrulline release into circulation for NOS utilization.

There is likely an interplay of mechanisms leading to greater blood flow that includes luminal nutrient stimulation and metabolic responses (NO induced vasodilation). Mostly likely, the large increase in blood flow on day 4 after initiating enteral feeding occurred predominantly in response to gut stimulation. However, any additional stimuli for blood flow such as NO

production may become apparent as the nutrient-induced blood flow effect reaches a threshold. At this point greater blood flow elicited from NO induced activity may be possible. We cannot know whether this NO induced blood flow was a short-lived response, or whether a new threshold was reached and matched over time by the piglets not provided with citrulline. Rapid increase in blood flow towards the gut is a normal physiological response when luminal contents are present (Niinikoski *et al*., 2004). Luminal contents act to stimulate increases in blood flow to the gut as was seen during the first 10 hours of enteral feeding (Van Goudoever *et al*., 2001). There were no differences found between groups on days 5 and 6 therefore, suggesting that the Cit group reached a peak in blood flow rate more rapidly than the High or Low Arg groups. This could be very important, because introduction of enteral feeding represents a high-risk period for neonates and there may be an important benefit associated with early increases of blood flow, as was identified in the piglets supplemented with citrulline. Eventually, the other treatment groups "caught-up" with the citrulline treated pigs. It is important that we have identified this short window of enhanced blood flow because this may be enough of an advantage to elicit positive or protective outcomes for preterm infants that are susceptible to NEC.

Inflammation is a main component involved in the pathogenesis of NEC. NO can have anti-inflammatory effects when present at an appropriate concentration. In contrast, induction of too much NO could inflict greater damage in a compromised gut. To this end, it would give us valuable information about the inflammatory status of the tissue if we were to measure inflammatory markers in the gut tissue. It would be important to know whether greater NO synthesis with citrulline supplementation results in improved inflammatory status; this could be highly beneficial in preventing the pathogenesis of NEC. In addition, it is known that vasoconstriction (mediated through endothelin-1) promotes intestinal injury, whereas

vasodilation (where NO has a role) has protective effects against NEC development (Watkins & Besner, 2013).

Whole Body Protein Synthesis: Interestingly, whole body protein synthesis was highest in piglets fed the greatest amount of arginine; this was likely because the dietary arginine was closer to predicted whole body requirements (Wu *et al*., 2004a). The Low Arg and Cit diets provided arginine at the concentration found in sow-milk (Davis *et al*., 1994), and it is important to note that the diets also contained abundant precursor amino acids, in particular proline and glutamate, that could be used for arginine synthesis (Wu *et al*., 2004a, Brunton *et al*., 1999, Tomlinson *et al*., 2011, Bertolo & Burrin, 2008). Neonates rely heavily on *de novo* synthesis of arginine to meet whole body requirements. The rest of the amino acids were provided at concentrations that may have been similar or greater than sow milk levels, but we ensured they were present in adequate amounts to meet whole body requirements, including to serve as precursors for arginine synthesis. This is an important point to emphasize because despite adequate arginine precursors in the Low Arg and Cit groups there was still a significantly lower whole body protein synthesis, suggesting that arginine was the limiting amino acid. It seems clear that the presence of gut atrophy impeded *de novo* synthesis of arginine in these animals. This study clearly demonstrated that the arginine concentration supplied in milk does not meet the needs of a neonate with compromised intestinal function.

When citrulline was added to the diet, a greater rate of conversion of citrulline to arginine was apparent. We could speculate that the greater conversion rate should have led to more arginine available to maintain whole body protein synthesis, but this is not what we found. In absolute terms, the rate of arginine appearing in the plasma pool from citrulline was more than

double in the Cit group compared to the Low Arg animals, but this was still not enough to support a rate of protein synthesis similar to that of the group provided with high dietary arginine. Not surprisingly, the citrulline flux was highest with citrulline supplementation, so we used these data to quantify the proportion of citrulline that was partitioned to arginine synthesis. We found that almost half of citrulline flux was directed towards arginine in the Low Arg group (P=0.07) compared to only 31% in the other two groups, so some compensation for the low arginine was occurring. It would follow that when arginine availability is limited, more citrulline would be captured as arginine in protein; however even with citrulline supplementation, the additional contribution from citrulline could not make up for the arginine deficit. The isotope kinetics technique that we used identified that 30 to 50% of the citrulline flux was appearing in the plasma as arginine, so the question that remains is what is happening to the remaining 50 to 70% of the citrulline moving through the pool.

What was not known previously, but is clear from our data, is that citrulline cannot replace arginine for protein synthesis. Another route of citrulline metabolism in the neonate may be through its conversion to arginine and to guanidinoacetic acid (GAA) to supply creatine. *De novo* creatine synthesis involves the conversion of arginine to GAA via the enzyme l-arginine:glycine amidinotransferase and the subsequent conversion of GAA to creatine through S-adenosylmethionine and guanidinoacetate N-methyltransferase (Dinesh *et al*., 2018). Growing infants must rapidly accrue creatine as tissue pools expand, to serve as a short-term energy reservoir in tissues. *De novo* creatine synthesis supplies ~75% of the daily requirement (Brosnan *et al*., 2009); however, parenteral nutrition products are devoid of creatine which means that the metabolic burden related to creatine synthesis is even greater with these feeding strategies (Dinesh *et al*., 2020). A recent study by Dinesh *et al*. (2020) from our lab group demonstrated

that a significant proportion (~12.5%) of citrulline that enters the kidney is released as GAA rather than as arginine. The citrulline to GAA pathway must first synthesize arginine and then be converted into guanidinoacetic acid (GAA) in the kidney. This newly synthesized GAA is released from the kidney and converted to creatine in the liver. Our research group has shown that even with very low intakes of arginine that limited protein synthesis, there was still an obligatory conversion of arginine to creatine (Dinesh *et al*., 2021). As such, it is likely that in this study, some citrulline was utilized for GAA and creatine synthesis. During an acute *in situ* study in piglets, we measured mass balance of GAA and amino acids across the kidney in response to citrulline and arginine infusions. Interestingly, we found that the GAA released from the kidney during the citrulline treatment was ∼1.5 fold greater than the amount released during an equimolar arginine treatment. So citrulline was a better precursor for GAA (and subsequently creatine) synthesis than the more direct precursor, arginine. Related to this study, it would be interesting to determine how much of the citrulline isotope could be found in GAA, in order to give us a more complete picture. It may be that to optimize NO and protein synthesis, a combination supplemental arginine and citrulline group is ideal. The additional arginine may spare citrulline for NO synthesis, and citrulline may spare arginine for protein synthesis. Our Low Arg and High Arg diets were elemental diets that were designed to mimic the pattern of amino acids in sow milk, except that both diets were devoid of citrulline. The citrulline concentrations reported in human and sow milk are very low relative to other arginine-related amino acids (Wu & Knabe *et al*., 1994, Sánchez *et al*., 2013, Davis *et al*., 1994). The citrulline intake from milk by piglets and infants are ~0.002 and 0.0078 g/kg/d, respectively (Wu & Knabe *et al*., 1994); in this study we supplemented citrulline at 0.5 g/kg/d, far in excess of natural

dietary sources. TPN and EN formulations are virtually devoid of citrulline therefore, its inclusion may be useful for preterm infants that are susceptible to intestinal complications.

Tissue Specific Protein Synthesis: We investigated tissue specific protein synthesis in mucosa, liver and muscle under the hypothesis that supplemental arginine and/or citrulline would result in greater whole body protein synthesis and tissue protein synthesis, particularly mucosal protein synthesis. The provision of additional arginine did support an overall higher whole body protein synthesis, but we did not find greater protein synthesis in the mucosa of the arginine supplemented animals. This is surprising, given that the intestinal tissue should extract its arginine requirement on first pass. In addition, we thought there may be improved mucosal protein synthesis in the piglets supplemented with citrulline because of the improved blood flow that should have resulted in a more efficient nutrient supply. The timing of the various measurements in this study may be the reason no differences in mucosal protein synthesis rates were detected. Mucosal protein synthesis was measured on day 6, two days after the initiation of enteral feeding. We may have missed a window of higher protein synthesis that occurred during the early period of enteral feeding. Support for this is evident in the greater jejunal circumference and gut size and villus height crypt depth in the High Arg group. The High Arg group had better intestinal growth outcomes, so it may be that an accelerated response occurred early on, prior to the study end. An enhanced rate of protein synthesis with high arginine intake, measured as percent per day, would translate into greater absolute protein synthesis in this group over a short time. This would explain why we observed better structural outcomes in the intestine of these animals despite no differences in the rate of mucosal protein synthesis two days after enteral initiation. Reducing the duration of enteral feeding with an earlier collection of tissue may allow

us to detect a difference between groups. Alternatively, we could have also waited longer before performing the necropsy to determine if more differences in gut morphology became apparent. However, the villus height and crypt depth data support the concept of an early response, because we did measure better outcomes in the high arginine treated piglets compared to the other groups, suggesting that there was likely better protein synthesis at some point.

The lack of apparent differences between the Cit and Low Arg group is likely explained in the same way it was for the whole body data. Presumably arginine was the amino acid that was limiting tissue specific protein synthesis in the Cit and Low Arg groups. We speculated that if any differences were to be found among treatments, the mucosa was most likely to be different, followed by the liver and finally the muscle. The gut mucosa can extract nutrients from the diet prior to entering the portal circulation, taking priority over other tissues (Goodman, 2010).

Tissue and Plasma Amino Acid Profiles: The profile of the tissue free amino acids identified a number of differences related to the diet treatments. As expected, the high arginine diet resulted in the highest concentration of arginine in the mucosa; similarly, the addition of citrulline to the diet resulted in a greater concentration of citrulline in the mucosa compared to the other treatment groups. Interestingly, we discovered that the Low Arg group had a higher mucosal glutamine concentration compared to the other groups. Glutamine is not a component of our enteral diets, because it is unstable in aqueous solutions in its free form. Our enteral diets contained substantial amino acid precursors for glutamine, including glutamate, but the gut can also readily extract glutamine from the arterial blood supply (Reeds & Burrin, 2001). It is a metabolically important amino acid for epithelial growth and proliferation, but studies in piglets

have suggested that the gut can readily use glutamate as well (Janeczko *et al*., 2007). It may be that the high tissue glutamine in the Low Arg mucosa was extracted from the systemic circulation to support both mucosal and immune cell functions. Intestinal intraepithelial lymphocytes utilize glutamine as an important energy source and require glutamine for proliferation (Wu *et al*., 1996). Glutamine is utilized for the expression of key lymphocyte cell surface markers, such as CD25, CD45RO, and CD71. In addition, glutamine acts to modulate the production of cytokines, such as interferon-gamma (IFN-γ), TNF-α, and IL-6 effectively reducing pro-inflammatory responses. Therefore, glutamine is an energy substrate for leukocytes, playing a role in cell proliferation and tissue repair process activity (Cruzat *et al*., 2018).

There were also differences in several non-urea cycle amino acids among treatment groups. The citrulline supplemented animals had higher amounts of hydroxyproline in the gut mucosa compared to the high arginine group. Hydroxyproline is a product of the post-translational modification of collagen (Barbul, 2008). Collagen consists of approximately 14% hydroxyproline and hydroxyproline is derived from small peptides that were cleaved from procollagen during its synthesis (Xia *et al*., 2006, Cundy *et al*., 2014, Barbul, 2008). NO has been demonstrated to enhance collagen synthesis and wound healing and iNOS may be partly involved in this improvement in wound healing through arginine delivery (Xia *et al*., 2006). NO synthesis was highest in the Cit group, suggesting that it may be enhancing collagen synthesis and turnover in the recovering intestinal mucosa.

Mucosal serine concentration was higher in the Low Arg group compared to the High Arg and Cit groups. The difference can be explained by diet composition, as serine concentration in the High Arg and Cit groups was reduced to make the diets isonitrogenous. Serine was chosen

as the amino acid to balance the nitrogen content among the diets because it is dispensable, and previous studies using low serine or serine-free diets have reported no deleterious effects. Reeds (2000) mentions that only glutamic acid and serine are truly nonessential due to their ease of synthesis from nitrogenous sources and being primary precursors to all other non-essential amino acids. In studies of amino acid requirements in neonatal piglets, alanine was commonly manipulated in the diets to maintain isonitrogenous intakes (House *et al*., 1997, Kim & Wu, 2004). However, alanine has a large plasma pool, and it is involved in nitrogen trafficking and recycling. Because arginine contributes significant nitrogen in the diet (four nitrogen atoms per molecule of arginine), we were concerned that removal of alanine would alter nitrogen metabolism or affect its disposal in a study that was manipulating a urea cycle metabolite. Valine concentration was also higher in the mucosa of the High Arg group compared to the Cit group. This is not what we would have expected since low dietary arginine would limit protein synthesis in the intestinal cell; this in turn, could result in an accumulation of indispensable amino acids to occur because of a lower rate of incorporation into protein. The isoleucine concentrations support this concept, as the low arginine group had higher mucosal free isoleucine concentration compared to the high arginine and citrulline groups.

Gross Morphology: Our hypothesis predicted that the piglets supplemented with either arginine or citrulline would have a higher mucosal weight and overall better gut growth; however, this was not the case. While mucosal weight was not different, the piglets supplemented with citrulline or arginine had a greater jejunum circumference, that does suggest enhanced overall intestinal growth in these animals. We also corrected the mucosal weight by the jejunal circumference to give us the mucosal weight per surface area of the gut; using this parameter the

Low Arg group had significantly higher weight per surface area compared to the piglets fed citrulline, with no difference between the arginine groups. Our initial thoughts were that there may be more inflammation in the Low Arg group, leading to tissue edema and artificially high mucosal weight. A well characterized response to TPN feeding and intestinal atrophy is increased inflammation in the gut (Ganessunker *et al*., 1999). To assess whether tissue hydration potentially confounded the mucosal tissue weight data, we measured the dry weight of the tissue; however, we found no differences between treatments.

The absolute liver weights were not different between groups; however, when liver weight was corrected for the piglet body weight, the piglets treated with citrulline had higher liver weights. This is surprising, as the diets were fed for only two days, which seems like a remarkably short duration to induce a measurable change in the liver mass.

5. Future Directions

Inflammation in the intestinal mucosa is a well-known complication of parenteral feeding, but to date we have not measured any markers of inflammation. NO production is known to have both pro and anti-inflammatory effects, particularly in the gut. Our citrulline supplemented animals did not demonstrate positive intestinal growth outcomes when compared to the arginine supplemented group. Measuring inflammatory markers will provide some insight into whether dietary citrulline supplementation supports an anti- or pro-inflammatory state; this could help explain lack of intestinal recovery. Inflammatory markers that could provide useful information would be myeloperoxidase, TNF-α, INF-γ and tissue antioxidant capacity.

We provided citrulline as substrate for NO synthesis and assessed blood flow to the gut. Some of the citrulline supplied in the diets may be partitioned towards GAA (and creatine) synthesis. In turn, this would result in less arginine release from the kidney for peripheral use.

We infused a stable isotope of citrulline to measure NO synthesis but we could also use this isotope to estimate how much GAA was being produced from citrulline. By combining these new data with the NO and arginine synthesis data we can generate a more complete picture of the metabolism of citrulline in the neonate.

In order to help us understand why the liver weight in the citrulline supplemented animals was heavier than the other groups, we could quantify lipid content. The literature suggests that citrulline does play a role in regulating non-alcoholic fatty liver disease (NAFLD) progression. In a study conducted by Rajcic *et al*., (2021) using mice, their data suggests that the protective effects of citrulline on the development and progression of NAFLD are related to alterations of intestinal arginase activity and intestinal permeability. Citrulline has been shown to improve hepatic lipid metabolism, insulin sensitivity and steatosis in models of fructose-induced NAFLD (Jegatheesan *et al*., 2015a, Jegatheesan *et al*., 2015b). However, this would be contrary to our findings, suggesting that citrulline can attenuate the development of NAFLD. Thus, any potential lipid accumulation in the livers of our citrulline supplemented piglets would be unlikely. Despite this, the involvement of citrulline in lipid status of the liver warrants investigation.

Future studies should include a treatment group that supplements citrulline with dietary high arginine. We determined that the citrulline and arginine supplemented piglets both had beneficial outcomes that differed between treatments. The arginine group had greater whole body protein synthesis and villus and crypt growth. Whereas the citrulline treated piglets had higher NO synthesis and SMA blood flow. Therefore, supplementing the piglets with citrulline and arginine may allow us to capitalize on all these benefits. Interestingly, in a study using rabbits (Hayashi *et al*., 2005), the researchers found that a combination of citrulline and arginine produced a synergistic response in elevating plasma NO and improved rabbit ear artery blood

flow. Moreover, a key observation of the study was an up-regulation of eNOS with chronic administration of citrulline. Thus, a prolonged citrulline supplemented diet may have resulted in more measurable improvements in gut growth and function.

6. Summary and Conclusions

In the current study we have clearly shown that dietary arginine at the concentration found in sow milk is not sufficient to support recovery of a compromised gut. We have also discovered that citrulline is an effective precursor for NO synthesis and can improve blood flow to the gut. However, arginine supplementation did not produce the same result. Thus, citrulline is a more effective precursor for NO synthesis and can improve blood flow to the gut more effectively than equimolar amounts of arginine. Interestingly, these improvements in both NO synthesis and blood flow did not translate into measurable structural and functional improvements in the gut over the short term, in piglets with TPN-induced atrophy. Despite this, citrulline may have an early positive impact during the transition to enteral feeding; it is possible that protection was afforded by the high NO production that we could not measure in this study. Benefits also might have become more apparent if the gut was challenged with a bacterial load, as can happen in the clinical scenario with formula or human milk feeding. Timing may also be crucial for infants that are susceptible to NEC early or later in the re-feeding period. Prolonged citrulline supplementation may result in a gut that is less susceptible to NEC. Arginine supplementation supported higher whole body protein synthesis and improved intestinal outcomes despite no significant enhancements in NO production and blood flow. Although we did not measure greater mucosal protein synthesis in this group, we suspect that we collected the mucosa too late, as better growth in intestinal villi and crypts suggest accelerated protein synthesis. In conclusion, citrulline supplementation and arginine provided at concentrations well

above sow milk levels both resulted in unique beneficial outcomes, so combining the treatments into one should be investigated. This strategy will spare citrulline to be available as a precursor for NO (and GAA/creatine) synthesis and will provide enough arginine for important growth and recovery processes such as protein and polyamine synthesis.

7. References

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8. Appendices

Appendix I: D-Glucose & Major Minerals in Complete Adaptation and Treatment Diets

	g/L
D-Glucose	90.30
K_2HPO_4 Trihydrate	1.57
KH_2PO_4 Monobasic	1.09
Potassium Acetate	1.47
NaCl	2.17
$MgSO_4$	0.78
$ZnSO_4$ Heptahydrate	0.09
Calcium Gluconate	6.41

Appendix II: Commercial Pediatric IV-Multi-Vitamin Mixture

Solution 1 Vitamin

	Amount per 4 mL	Dose (per kg BW/day)
Ascorbic Acid (Vitamin C)	80 mg	17.41
Palmitate (Vitamin A)	2300 IU	500.48
Cholecalciferol (Vitamin D_3)	400 IU	87.04
Thiamine (Vitamin B_1/hydrochloride form)	1.2 mg	0.26
Riboflavin (Vitamin B_2/riboflavin-5-phosphate sodium)	1.4 mg	0.30
Pyridoxine HCl (Vitamin B_6)	1 mg	0.22
Niacinamide	17 mg	3.70
Dexapanthenol (d-panthenyl alcohol)	5 mg	1.10
dl-α-tocopherol acetate (Vitamin E)	7 IU	1.52
Vitamin K_1	0.2 mg	0.04

Inactive Ingredients: 50 mg polysorbate 80, sodium hydroxide and/or hydrochloric acid to adjust pH.

Multi-12/K1 Pediatric multivitamins, Baxter, Canada

Solution 2 Vitamin

	Amount per 2 mL	Dose (per kg BW/day)
Biotin	140 ug	4.35
Folic Acid	20 ug	30.46
Cyanocobalamin (Vitamin B_{12})	1 ug	0.22

Inactive Ingredients: 75 mg mannitol, citric acid and/or sodium citrate to adjust pH.

Procedure: Vial 1 and 2 were mixed, and 3 mL was added to each 750 mL diet bag.

The vitamin mixture was designed based on Multi-12/K1 (Baxter, Canada) a commercial pediatric multivitamin solution for parenteral nutrition. The amount added to the parenteral or enteral diets provided 120% of NRC requirements for piglets (Wykes *et al*., 1993). All water-soluble vitamins and the water-soluble form of vitamin K were dissolved in pyrogen free water. The fat-soluble vitamins A, D, E and K were dissolved in polysorbate-80 and gradually added to the water-soluble vitamins. The pH was adjusted to 7.4 using acetic acid/NaOH. The final solutions were filtered through a 0.2 μm syringe filter into a sterile IV bag. The bag was kept in a refrigerator at 4°C and protected from light until required.

Appendix III: Trace Mineral Concentrations in Complete Adaptation and Treatment Diets

Elemental Name	Supplied Mineral Formula	Dose (mg/kg BW/day)	Amount Supplied (g/L diet)
Zinc	$ZnSO_4 \cdot 7H_2O$	10.09	40.69
Copper	$CuSO_4 \cdot 5H_2O$	0.86	3.12
Manganese	$MnSO_4 \cdot H_2O$	0.66	1.86
Chromium	$CrCl_3 \cdot 6H_2O$	0.01	0.05
Selenium	SeO_2	0.05	0.06
Iodine	NaI	0.02	0.02

The trace element mixture was prepared in the lab and designed to supply at least 120% of NRC requirements for piglets (National Research Council, 1998). Minerals were dissolved using pyrogen free water and filtered through a 0.2 μm syringe filter (baslx, Fisher Scientific, Pittsburgh) into a sterile IV bag. It was kept refrigerated at 4°C and away from light until needed.

Appendix IV: Commercial IV Fat Emulsion (Fresenius Kabi SMOF lipid)

	Percent (%)
Total Lipid	
Soybean Oil	30
Olive Oil	25
Fish Oil	15
Medium-chain Triglycerides	3
Key Fatty Acids	
Palmitic Acid	9
Oleic Acid	27
Linoleic Acid	18
α-Linolenic Acid	2
Arachidonic Acid	0.5
Eicosapentaenoic Acid	3
Docosahexaenoic Acid	2

Appendix V: Tissue Processor Program

Position	Reagent	Time (hr:min)
1	Formalin	0:01
2	Formalin	0:01
3	70% Ethanol	0:45
4	80% Ethanol	0:45
5	100% Ethanol	0:45
6	100% Ethanol	0:45
7	100% Ethanol	1:00
8	100% Ethanol	1:00
9	Xylene	1:00
10	Xylene	1:00
11	Hot Wax	1:30
12	Hot Wax	1:30

Appendix VI: Gelatin Coating for Microscope Slides

Two solutions:

1) 0.3 g gelatin dissolved in 290 mL deionized water
2) 0.03 g potassium chromate dissolved in 10 mL deionized water

Both solutions were mixed and slides coated for 1 min and allowed to dry for a couple hours.

Appendix VII: Hematoxylin and Eosin Staining Procedure

Time	Solution	Incubation time
0	xylene	5min
5	xylene	5min
10	100% ethanol	2min
12	95% ethanol	2min
14	80% ethanol	2min
16	70% ethanol	2min
18	running water	2min
20	hematoxylin	15min
35	running water	9min
44	eosin	4min
48	95% ethanol	2min
50	95% ethanol	2min
52	100% ethanol	2min
56	xylene	2min

Appendix VIII: Amino Acid Analysis of Plasma Samples on Days 4-6

Absolute amounts of amino acids in plasma were determined using high performance liquid chromatography (HPLC) (Waters, Mississauga, ON).

Sample Preparation for HPLC Analyses

HPLC analysis was performed in order to determine the absolute amounts of free amino acids in femoral plasma samples. 100 µl of plasma sample was mixed with 20 µl of 2.5 mM norleucine standard (Sigma-Aldrich Canada Ltd, Oakville, ON). 1 ml of 0.5% trifluoroacetic acid (TFA) (Sigma-Aldrich Canada Ltd, Oakville, ON) in methanol (MeOH) (Fisher Scientific, Whitby, ON) was added to the samples to precipitate any protein that was present. Supernatant was separated after the samples were centrifuged at 5000 rpm for 5 min. The supernatant was frozen in liquid nitrogen and vacuum dried on the Thermo Scientific® Digital Series SpeedVac Systems (Thermo Fisher Scientific, Nepean, ON). Subsequently 50 µl of a mixture of triethylamine (TEA) (Sigma Aldrich Canada Ltd, Oakville, ON), MeOH (Fisher Scientific, Whitby, ON) and water (2:2:6 ratio respectively) was added to each sample. Again, samples were vacuum dried using the SpeedVac system. When dry, the samples were labeled with a mixture of TEA, PITC (Thermo Scientific, USA), MeOH and water in a 1:1:7:1 ratio. 20 µl of the PITC solution was added to each sample and incubated for a period of 35 minutes at room temperature; freezing in liquid nitrogen halted the process. This derivatization step allows PITC to bind with free amino acids to form phenylthiocarbamyl amino acids. The samples were then dried down again. Prior to HPLC analyses, the samples were re-suspended in 200 µl of sample diluent which was 5 mM Na_2HPO_4 (Sigma Aldrich Canada Ltd, Oakville, ON) titrated to pH 7.4 with 10% H_3PO_4 acid and 10% acetonitrile (Fisher Scientific, Whitby, ON). After addition of the diluent, samples were

vortexed and then centrifuged at 5000 rpm for 5 min and the supernatant was transferred to HPLC vials.

HPLC Analysis of Plasma Samples

HPLC samples were placed in Waters 717 Plus Auto Sampler system (Waters, Mississauga, ON). Aliquots of sample (20 µl plasma) were injected into the reverse phase C18 column which was maintained at 46 ºC to facilitate the separation procedure. The phenylthiocarbamyl amino acids were separated during a 90 min run time, at 1 ml/min and quantified by a UV absorbance of 254 nm. The peaks in the chromatogram for each amino acid were integrated using Breeze software (Waters, Version 3.3, 2002, Waters Corporation, Woburn, MA), and the peaks associated with each amino acid was compared to the peak area of the internal norleucine standard to determine the amino acid concentration.

Plasma amino acids (μmol/L) on day 4.				
	Low Arg	**High Arg**	**Cit**	**P-value**
Arginine-related				
Arginine	132 ± 89	146 ± 57	82 ± 28	P > 0.05
Citrulline	196 ± 97	468 ± 129	414 ± 94	P > 0.05
Glutamic Acid	322 ± 102	390 ± 121	162 ± 95	P > 0.05
Glutamine	299 ± 120	149 ± 52	206 ± 68	P > 0.05
Proline	1536 ± 431[b]	2058 ± 324[a]	1416 ± 338[b]	**P < 0.001**
Ornithine	215 ± 158	169 ± 113	67 ± 34	P > 0.05
Essential Amino Acids				
Histidine	204 ± 75	328 ± 79	212 ± 53	P > 0.05
Threonine	155 ± 73	226 ± 62	98 ± 24	P > 0.05
Valine	66 ± 25	132 ± 107	52 ± 31	P > 0.05
Methionine	269 ± 101	397 ± 161	251 ± 107	P > 0.05
Isoleucine	164 ± 77	187 ± 54	138 ± 86	P > 0.05
Leucine	396 ± 228	461 ± 124	270 ± 75	P > 0.05
Phenylalanine	735 ± 217[a]	425 ± 179[ab]	327 ± 91[b]	**P < 0.01**
Tryptophan	215 ± 158	169 ± 112	67 ± 34	P > 0.05
Lysine	553 ± 210	391 ± 225	364 ± 148	P > 0.05
Other Non-essential Amino Acids				
Aspartic Acid	123 ± 36	191 ± 112	119 ± 63	P > 0.05
Hydroxy-Proline	175 ± 58	347 ± 89	218 ± 61	P > 0.05
Serine	698 ± 258[b]	1023 ± 172[a]	502 ± 175[b]	**P < 0.001**
Asparagine	163 ± 80	293 ± 59	175 ± 55	P > 0.05
Glycine	1258 ± 435	1452 ± 834	1208 ± 143	P > 0.05
Taurine	178 ± 49	245 ± 49	193 ± 44	P > 0.05
Alanine	494 ± 142	547 ± 176	336 ± 48	P > 0.05
Tyrosine	391 ± 146	413 ± 210	191 ± 49	P > 0.05

Plasma amino acids (μmol/L) on day 5.				
	Low Arg	**High Arg**	**Cit**	**P-value**
Arginine-related				
Arginine	48 ± 16[b]	184 ± 29[a]	49 ± 16[b]	**P < 0.00001**
Citrulline	290 ± 89	414 ± 74	338 ± 118	P > 0.05
Glutamic Acid	448 ± 269[a]	425 ± 173[a]	154 ± 56[b]	**P < 0.01**
Glutamine	633 ± 96[a]	356 ± 88[b]	40 ± 11[c]	**P < 0.001**
Proline	2100 ± 212[a]	2222 ± 467[a]	1033 ± 229[b]	**P < 0.001**
Ornithine	68 ± 30	93 ± 36	113 ± 68	P > 0.05
Essential Amino Acids				
Histidine	270 ± 37	384 ± 99	163 ± 85	P > 0.05
Threonine	204 ± 40	267 ± 41	88 ± 70	P > 0.05
Valine	72 ± 35	103 ± 49	36 ± 27	P > 0.05
Methionine	311 ± 68	376 ± 51	193 ± 54	P > 0.05
Isoleucine	196 ± 42	229 ± 60	109 ± 30	P > 0.05
Leucine	419 ± 89[a]	541 ± 124[a]	244 ± 52[b]	**P < 0.01**
Phenylalanine	1587 ± 171[a]	1079 ± 636[b]	185 ± 132[c]	**P < 0.001**
Tryptophan	68 ± 30	93 ± 37	113 ± 68	P > 0.05
Lysine	485 ± 86	540 ± 253	334 ± 202	P > 0.05
Other Non-essential Amino Acids				
Aspartic Acid	73 ± 48	107 ± 87	45 ± 18	P > 0.05
Hydroxy-Proline	183 ± 38[a]	424 ± 70[b]	182 ± 96[ab]	**P < 0.05**
Serine	968 ± 94[a]	544 ± 69[b]	264 ± 147[c]	**P < 0.001**
Asparagine	231 ± 52	230 ± 43	112 ± 63	P > 0.05
Glycine	1880 ± 416[a]	1866 ± 159[a]	706 ± 140[b]	**P < 0.001**
Taurine	160 ± 21	222 ± 41	116 ± 46	P > 0.05
Alanine	649 ± 104[a]	656 ± 194[a]	300 ± 157[b]	**P < 0.001**
Tyrosine	426 ± 89[a]	502 ± 111[a]	176 ± 106[b]	**P < 0.01**

Plasma amino acids (μmol/L) on day 6.				
	Low Arg	**High Arg**	**Cit**	**P-value**
Arginine-related				
Arginine	37 ± 20^{b}	167 ± 37^{a}	64 ± 21^{b}	**P < 0.00001**
Citrulline	232 ± 97	315 ± 109	455 ± 145	P > 0.05
Glutamic Acid	315 ± 228	417 ± 90	206 ± 163	P > 0.05
Glutamine	419 ± 112	374 ± 192	122 ± 53	P > 0.05
Proline	1639 ± 443^{b}	2087 ± 288^{a}	1520 ± 536^{b}	**P < 0.001**
Ornithine	98 ± 57	150 ± 111	41 ± 21	P > 0.05
Essential Amino Acids				
Histidine	250 ± 109	342 ± 158	306 ± 137	P > 0.05
Threonine	188 ± 70	296 ± 68	132 ± 22	P > 0.05
Valine	54 ± 33	89 ± 25	67 ± 45	P > 0.05
Methionine	290 ± 65	301 ± 82	222 ± 33	P > 0.05
Isoleucine	156 ± 74	224 ± 47	106 ± 68	P > 0.05
Leucine	375 ± 100	531 ± 74	272 ± 144	P > 0.05
Phenylalanine	1247 ± 212^{a}	818 ± 671^{b}	161 ± 128^{c}	**P < 0.001**
Tryptophan	98 ± 57	150 ± 111	44 ± 21	P > 0.05
Lysine	446 ± 157	464 ± 264	202 ± 125	P > 0.05
Other Non-essential Amino Acids				
Aspartic Acid	76 ± 38	121 ± 61	48 ± 12	P > 0.05
Hydroxy-Proline	109 ± 40	316 ± 52	157 ± 79	P > 0.05
Serine	969 ± 323^{a}	643 ± 177^{a}	407 ± 237^{b}	**P < 0.001**
Asparagine	215 ± 89	221 ± 50	143 ± 88	P > 0.05
Glycine	1678 ± 582^{a}	1651 ± 346^{a}	1113 ± 590^{b}	**P < 0.001**
Taurine	136 ± 37	221 ± 47	147 ± 65	P > 0.05
Alanine	665 ± 158	786 ± 105	578 ± 288	P > 0.05
Tyrosine	375 ± 97	472 ± 69	178 ± 57	P > 0.05