Effect of immune system stimulation on protein metabolism and amino acid utilization in growing pigs

by

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A Dissertation

In

Animal Science

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

DOCTOR OF PHILOSOPHY

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ACKNOWLEDGMENTS

First and foremost, I owe all that I am and all that I will be to my Heavenly Father; for without him I wouldn't be where I am today. He has blessed me immensely with opportunities in life and gaining my doctorates from Texas Tech University doesn't fall short of that list.

Next, I would like to express my deepest gratitude to my advisor and mentor Dr. Anoosh Rakhshandeh for taking a chance on me and giving me the opportunity to work in his lab as one of his very first graduate students. His continuous support, encouragement, and guidance has helped shaped me into the person I am today. More than anything I am forever grateful for your patience and motivation, and for always believing in me even when I didn't believe in myself. It goes without question to say that the completion of this research and dissertation would not have been possible without you. Furthermore, a sincere thank you to my committee members Dr. Michael Ballou, Dr. Jhones Sarturi, Dr. David Klein, Dr. Teresa Davis, and Dr. Shu Wang. You have all provided me with invaluable guidance and advice along this journey and have influence me to become a better scientist. Furthermore, thank you to Dr. Bradley Johnson and his lab group; Kimberly Wellmann, Zach Smith, Jessica Baggerman, and Jongkyoo Kim, for for helping me in the muscle biology portion of this dissertation. I truly enjoyed working with all of you.

To the following graduate students and undergraduate students that helped make this research a success, thank you. Deltora Hewitt, we started this grad school journey together and I will forever be thankful that God crossed our paths during our time at TTU. Thank you for always lending a helping during my research but mostly thank you

for always believing in me and encouraging me to continue. I am proud to call you a life-long friend. Hailey Wooten, Amy Petry, and Kimberly Wellmann graduate school would not have been nearly as fun without you! Thank you for all your help in making this research successful but most importantly thank you for the friendships and memories. I look forward to seeing what the future holds all of you. Andrea Krieg, thank you for all your patience in the lab and for always lending a helping hand. Thank you to the following undergraduate students: Treyson Antonick, Marie Traylor, and Tate Leatherwood for always helping with feeding, sample collections, and lab analysis.

Lastly, some very special thanks goes out to my family. To my parents, Mike and June, you have always encouraged me to chase after my dreams and never once held me back from them, even when I had the crazy idea of moving half way across the country. Thank you for always being there and supporting me no matter what. Finally, to my husband Max McGilvray. Thank you for your patience and unconditional love during this time.

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ABSTRACT

Immune system stimulation (ISS) results in metabolic alterations characterized by distinct changes in protein and amino acid (AA) metabolism that impact the pigs' productivity and efficiency of nutrient utilization for growth. This occurs, in part, by repartitioning AA away from body protein deposition (PD) towards process involved in an immune response, thus impacting AA requirements both quantitatively and qualitatively. Furthermore, the metabolism of threonine (Thr) during ISS has gained attention due to its role in the synthesis of Thr-rich immune system metabolites, such as immunoglobulins, acute phase proteins and, in particular, intestinal mucins. The research herein was conducted to evaluate the effect of ISS on the aspects of protein metabolism and AA utilization in the pig.

In experiment 1 (n=10; BW 9.4 ± 1.1 kg), a stable isotopic tracer technique was used to evaluate the impact of *Escherichia coli* Lipopolysaccharide (LPS) induced-ISS on plasma free AA (Lys, Met, Thr, Trp, Ile, Leu, Val, Phe, Gln) flux as an indicator of AA utilization in growing pigs during the fed state using. In this study, Blood chemistry, hematology, and BT results indicated that LPS induced effective ISS in pigs ($P<0.05$). Immune system stimulation tended to reduce N retention ($P=0.09$) and the N retention-to-N intake ratio ($P=0.08$). Apparent ileal digestibility of N and apparent total tract digestibility of dietary energy were reduced by ISS ($P<0.05$). Plasma flux (µmol/kg BW/h) for Ile and Phe was reduced by ISS ($P<0.05$). A strong tendency of decreased Lys flux was observed in ISS pigs ($P=0.08$). Immune system stimulation increased the pool size for Leu ($P<0.05$) but reduced the pool size for Ile ($P<0.05$). In experiment 2 (n=20; BW 9.4 ± 0.9 kg), using the same stable isotopic tracer technique, the effects of porcine

reproductive and respiratory syndrome virus (PRRSv) infection on PD and AA metabolism was evaluated. Blood chemistry, hematology, BT, and serum viral load results indicated that PRRSv inoculation induced effective ISS in pigs ($P<0.05$). Immune system stimulation significantly reduced the ADFI by 21.7 ± 4.58 g/kg BW/d (P=0.01). Immune system stimulation had no effect on N retention ($P=0.99$) and the N retention-to-N intake ratio ($P=0.85$) after controlling for ADFI. Apparent ileal digestibility of N was reduced by ISS ($P<0.05$), but had no effect on apparent total tract digestibility of dietary energy (P=0.12). Plasma flux (μmol/kg BW/h) for Met and Thr was increased by ISS (P<0.05). A strong tendency of increased Val flux was observed in ISS pigs ($P=0.08$). Immune system stimulation increased the pool size for Ile, Leu, Lys, Phe, and Val (P<0.05). Collectively, these results suggest that ISS alters the utilization of dietary N and AA flux, as well as pool size in growing pigs. The increase in Thr and Met flux in PRRSv-infected pigs may be associated with enhanced utilization of Met and Thr for the synthesis of immune system metabolites and increased catabolism of these AA. Thus, dietary Met and Thr requirements may increase in health challenged pigs, relative to the requirements for other AA.

The observed 1.5 times increase in plasma Thr flux in PRRSv challenged pigs, suggested an increased metabolic demand for Thr during ISS. Therefore, in experiment 3 (n=39; initial BW 32 ± 2.1 kg) the impact of ISS on dietary Thr requirements in growing pigs was evaluated using the LPS model of ISS. Based on the relationship between standardized ileal digestible Thr intake and whole-body PD, the marginal efficiency of SID Thr utilization for PD was not affected by ISS. However, ISS substantially increased

the extrapolated maintenance SID Thr requirements, represented by the intercept at zero PD (ISS- vs. ISS+, −11.2 vs. −56.3 SE 14.8; $P < 0.05$).

In experiment 4 (n=12; initial BW 31 ± 4.8 kg), the impact of immune system stimulation (ISS) on whole-body nitrogen metabolism and muscle fiber characteristics was evaluated in growing pigs. Immune system stimulation significantly reduced whole-body protein synthesis (10.77 vs. 7.12 g N/kg $BW^{0.60}$/d; SE 0.44), degradation (9.41 vs. 6.64 g N/kg $BW^{0.60}$/d; SE 0.04), and retention (1.38 vs. 0.58 g N/kg $BW^{0.60}$/d; SE0.08) and increased the protein synthesis to retention ratio (7.61 vs. 12.26; SE 1.4) in ISS+ pigs relative to ISS- pigs ($P<0.01$). Furthermore, ISS showed a general decrease in skeletal muscle nuclei and fiber cross-sectional area and a shift from myosin heavy chain (MHC)-IIX towards MHC-I fibers ($P<0.05$).

Keywords: growing pigs, immune system stimulation, muscle fibers, plasma amino acid flux, protein metabolism, threonine requirement

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LIST OF ABBREVIATIONS

AA	Amino Acids
ACTH	Adrenocorticotropic hormone
ADFI	Average daily feed intake
AID	Apparent ileal digestibility
AnionGAP	Anion Gap
ANS	Autonomic nervous system
APP	Acute phase proteins
APR	Acute phase response
ATTD	Apparent total tract digestibility
BT	Body temperature
BUN	Blood urea nitrogen
BW	Body weight
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Corticotropin releasing hormone
dpi	Days post inoculation
DM	Dry matter
EAAL	Endogenous amino acid loss
eIF	eukaryotic initiation factors
GCMS	Gas chromatography mass spectrometry
GE	Gross energy
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GIT	Gastrointestinal tract
Gln	Glutamine
Hb	Hemoglobin

HCT	Hematocrit
HPAA	hypothalamic-pituitary-adrenal axis
IGF-1	Insulin like growth factor 1
IL	Interleukin
Ile	Isoleucine
IM	Intramuscular
IR	Infrared
ISS	Immune System Stimulation
Leu	Leucine
LPS	Lipopolysaccharide
Lys	Lysine
N-balance	Nitrogen balance
mRNA	Messenger RNA
MAPK	Mitogen activated protein kinase
Met	Methionine
mTORC	Mammalian target of rapamycin
N	Nitrogen
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
PAMP	Pathogen-associated molecular receptors
PD	Protein deposition
PEDv	Porcine epidemic diarrhea virus
Phe	Phenylalanine
PRRs	Pathogen recognition receptors
PRRSv	Porcine reproductive and respiratory syndrome virus
ROS	Reactive oxygen species
SE	Standard error
SID	Standardized ileal digestibility
SIM	Selected ion monotoring

S6K1	Ribosomal S6 kinase 1
Thr	Threonine
TLR	Toll-like receptors
TNF	Tumor necrosis factor
Trp	Tryptophan
TTR	Tracer-to-tracee ratio
Val	Valine

CHAPTER I

INTRODUCTION

In spite of extensive biosecurity measures implemented in modern pork production units, pigs are routinely exposed to infectious agents that highly impact the profitability and sustainability of pork production. Immune system stimulation (ISS) is accompanied by a common pattern of response in cellular and metabolic events that alter the animals nutrient utilization and efficiency for optimal growth (Williams et al., 1997; Colditz, 2002; Colditz, 2004). Mediated largely by pro-inflammatory cytokines, major metabolic changes are characterized by induced sickness behavior, and alterations in hormone secretion, protein turnover and lipid metabolism (Johnson, 1997; Buchanan and Johnson, 2007; Rakhshandeh and de Lange, 2011). Consequently, a redirection of nutrients away from growth and reproduction towards the activation of pathways involved in the hyper-activation of the immune system occur, compromising the animal's productivity (Reeds and Jahoor, 2001; Obled, 2003).

Proteins and amino acids (AA) are usually the second most expensive ingredient in swine diets. Immune system stimulation modifies the metabolism of protein and AA (Le Floc'h et al., 2004). It is assumed that ISS increases whole-body protein turnover (i.e. synthesis + degradation) through the increased rates of protein synthesis and protein degradation in various tissues (Klasing and Leshchinsky, 2000). Hallmark to disease is the reduction in lean gain as a result of reduced dietary AA influx coupled with reduced protein synthesis and increased protein degradation in skeletal muscle (Orellana et al., 2004). Increased protein degradation of skeletal muscle serves to maximize the release of AA for the support of increased protein synthesis in visceral organs due to increased

synthesis of immune system proteins and metabolites important in host defense (Reeds and Jahoor, 2001; Rakhshandeh and de Lange, 2011). However, the profile of AA released from skeletal muscle differs from profile of AA needed by the immune system, thus, impacting AA requirements both quantitatively and qualitatively (Reeds and Jahoor, 2001; Obled, 2003). Current understanding on the effect of ISS on protein and AA metabolism and utilization is limited and often ignores the relationship among other AA (NRC Swine, 2012). Development of effective nutritional strategies could help mitigate the negative impact of disease on productivity of pigs. Therefore, formulation of optimal diets that closely match the AA requirements during disease requires further quantitative and qualitative knowledge.

Previously, measures of plasma free AA concentrations have been used to evaluate the effects of ISS on AA metabolism. However, concentrations of plasma free AA can be maintained by AA influx from dietary intake and protein degradation, and AA efflux toward protein synthesis and catabolism, suggesting results based on measures of concentration could be misleading (Waterlow, 2006). Alternatively, changes in plasma free AA flux (i.e. kinetics) can occur without changes in plasma AA concentrations, reflecting modifications of AA metabolism in pigs during different metabolic states such as disease. Measures of plasma free AA flux could provide a better alternative for evaluating the impact of ISS on AA metabolism (Holtrop et al., 2004; Rakhshandeh et al., 2010; Rakhshandeh and de Lange, 2010).

This dissertation presents studies in which the impact of ISS on AA metabolism in growing pigs was evaluated. First, a stable isotopic dilution technique was used to evaluate the impact of ISS on the kinetics of multiple AA simultaneously in growing pigs

challenged with either *E. coli* lipopolysaccharide or porcine reproductive and respiratory syndrome virus (PRRSv). Second, the impact of ISS on dietary threonine requirements was determined in a nitrogen balance study and using a linear regression comparison of dietary SID threonine intake for protein deposition. Lastly, the effect of SID threonine intake on whole body protein turnover was assessed with a single oral administration of ^{15}N -glycine. In addition, the effect of ISS skeletal muscle fiber type composition was evaluated.

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CHAPTER II

REVIEW OF LITERATURE

INTRODUCTION

Despite the extensive biosecurity measures used in modern swine production facilities, pigs are routinely exposed to disease causing agents that negatively impact the profitability and sustainability of pork production. It is well known that pigs reared in commercial settings are up to 30% slower growing and less efficient than those housed in clean environments (Williams et al., 1997; Le Floc'h et al., 2009; Renaudeau, 2009; de Ridder et al., 2012). This performance deficit imposes a significant economic loss for the United States swine industry. For example, infection with porcine reproductive and respiratory syndrome virus (PRRSv) represents nearly $664 million annually (Holtkamp et al., 2013). Immune system stimulation (ISS) and subsequent release of pro-inflammatory cytokines results in cellular and metabolic events that are characterized by distinct changes in protein and amino acid (AA) utilization in both the absorptive and post-absorptive states (Johnson, 1997; Spurlock, 1997; Colditz, 2002; Faure et al., 2006; Rémond et al., 2009). Consequently, AA are redirected away from growth and reproduction towards processes involved in immunological defense (Reeds and Jahoor, 2001; Obled, 2003; Le Floc'h et al., 2004)

Immune system stimulation and simultaneous decreases in feed intake drive the mobilization of skeletal muscle protein to supply AA needed for utilization by immune system activated metabolic pathways. However, the profile of AA required by the immune system differs markedly from that released by skeletal muscle protein, creating an internal AA imbalance (Reeds et al., 1994; Reeds and Jahoor, 2001). Therefore, AA

that are typically first limiting for growth in a healthy state may become in excess, and those AA typically in excess may become first limiting for immune system activation suggesting ISS-induced quantitative (i.e. dietary AA requirements) and qualitative (i.e. metabolic demand or AA use) consequences on AA requirements (Le Floc'h et al., 2004). Current knowledge about the effects of disease on protein and AA metabolism, utilization and requirements in swine is limited, and often ignores the relationship among other AA (NRC, 2012). Development of effective nutritional strategies, such as those that more closely match the AA requirements of pigs during disease, could help mitigate the negative impact of disease on the animal's productivity. In this review, immune system regulation of AA utilization during the absorptive and post absorptive states is discussed and the implications of its effects for dietary AA requirements are considered.

THE IMMUNE SYSTEM AND IMMUNE RESPONSE

All vertebrates are equipped with a highly adaptable immune system that has evolved a range of complex and highly efficient recognition and destruction mechanisms capable of maintaining host homeostasis during an immune challenge (Elgert, 2009). The immune response is regulated through two highly interconnected systems, the innate and adaptive immune systems, which are classified based upon their speed and specificity towards an insult (Parkin and Cohen, 2001). This section will briefly discuss the key features of the immune system and its response as it pertains to modifications of nutrient utilization, especially that of AA, in the pig. For a more detailed description of the immune system and immune response the reader is referred to several excellent reviews (Elgert, 2009; Alberts et al., 2015; Iwasaki and Medzhitov, 2015).

Immune response

Innate immunity plays a critical role in the early detection and subsequent triggering of an inflammatory response towards invading pathogens. Innate immune cells, primarily those of the mononuclear phagocytic lineage, rely on germline encoded receptors (e.g. toll-like receptors; TLRs), that recognize a wide range of pathogen-associated molecular patterns (PAMPs) foreign to the mammalian organism (Iwasaki and Medzhitov, 2015). Ligation of TLRs induces downstream signaling that activates nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) and mitogen-activated protein kinase (MAPK) pathways, inducing the expression of a wide variety of genes involved in host defense, and eliciting the release of key pro-inflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, that augment the inflammatory response (Mogensen, 2009; Iwasaki and Medzhitov, 2015). The initial stages of inflammation localize the infection by directing immune system components to the sight of infection to eliminate the invader and initiate the healing process (Elgert, 2009). If the immune response is sufficiently large, the concentration of pro-inflammatory cytokines in the blood increase and act systemically to trigger the acute phase response (APR; Klasing and Leshchinsky, 2000). The systemic release of pro-inflammatory cytokines is of particular interest in pig production due to their ability to act on disparate organ systems, antagonizing the growth and feed efficiency of the animal. Pro-inflammatory cytokines can act directly, *via* their receptors expressed in virtually all tissues, and indirectly, by stimulating hormonal and neurological responses, to alter the metabolism, physiology, and behavior of the animal. This leads to an integrated host response that directs nutrients, in particular AA, towards host defense (Johnson, 1997; Colditz, 2004).

During an immune response of sufficient severity, cells of the innate immune system activate the adaptive immune response, and work in concert with each other to effectively eliminate the pathogen. Adaptive immunity is a sophisticated and highly specific defense system that is dependent on B and T lymphocytes containing antigen receptors generated by somatic recombination, thus, allowing them to recognize almost any conceivable invading pathogen (Elgert, 2009; Iwasaki and Medzhitov, 2015).

Nutritional cost of an immune response

Activation of the immune system is both highly complex and metabolically active, which can impose substantial nutritional and metabolic costs on the animal. Klasing (2004, 2007) has attempted to quantify the nutritional costs of the immune system in poultry by subdividing it into three components: 1) costs associated with the development of the immune system 2) costs associated with maintaining the immune system and 3) costs associated with the use of the immune system to clear an invading pathogen. The costs associated with the immune system can differ significantly between species and breeds depending on which branch of the immune system (innate or adaptive) they are more likely to use (Lee and Klasing, 2004). In general, the metabolic costs associated with the development of the innate immune system are low due to the lack of a diversification process in innate cell development, low rates of cell turnover during quiescence, and the small tissue mass needed by constitutive innate cells and proteins. However, the metabolic costs of using the innate immune system are high due to the physiological, metabolic and behavioral changes associated with a systemic inflammatory response and potential autoimmune damage. Alternatively, the metabolic costs associated with the development of the adaptive immune system are high due to the inefficient

process of lymphocyte diversification and antigen receptors, but the costs of using it is low, becoming highly efficient upon subsequent exposure to the same pathogen (Lee and Klasing, 2004; Lee, 2006; Klasing, 2007)

REGULATION OF PROTEIN AND AMINO ACID METABOLISM DURING IMMUNE SYSTEM STIMULATION

It is well established that immune challenged animals eat less, grow slower, and have reduced feed efficiency compared to healthy pigs, even when no obvious clinical signs of disease exist (Williams et al., 1997; Le Floc'h et al., 2004, 2009). Activation of the immune system, and subsequent release of key pro-inflammatory cytokines, results in physiological and metabolic alterations that compromise the pig's efficiency of nutrient utilization for optimal growth. These alterations are systematically characterized by distinct changes in protein metabolism and AA utilization (Obled, 2003; Le Floc'h et al., 2004). This section will discuss how immune system stimulation regulates and alters protein metabolism in the animal.

Impact of ISS on dry matter and protein intake

Immune system stimulation is accompanied by reduced voluntary feed intake (i.e. anorexia) and, thus, reduced protein intake. Feed intake is critical for the normal growth of animals; thus, depressed feed intake is largely responsible for reduced growth rates in immunologically stressed animals. An early study by Klasing et al. (1987) determined that approximately 70% of reduced growth rates (i.e. lean gain) observed in ISS animals can be attributed to reduced feed intake. While costly to the producer, evidence suggests that loss of appetite during disease is an adaptive response that is beneficial to the survival of the animal, provided it does not persist to long (Murray and Murray, 1979).

Disease induced reductions in feed intake is complex and involves the close interplay between myriad factors, circulating levels of hormones and metabolites, and physical responses of the gastrointestinal system, all of which is integrated by the central nervous system (CNS) to induce or suppress the physical act of eating (Sartin et al., 2011).

During ISS, pro-inflammatory cytokines, particularly IL-1, disrupt normal feeding behavior and suppress feed intake *via* mechanisms that are predominantly CNS-specific (Buchanan and Johnson, 2007). In the brain, inflammatory cytokines exert their anorexigenic effects by directly stimulating hypothalamic neurons and increasing the release of neuropeptides (e.g. pro-opiomelanocortin) and neurotransmitters (e.g. norepinephrine) involved in appetite suppression (Plata-Salamán, 2000; Johnson and Escobar, 2005). Moreover, pro-inflammatory cytokines reduce appetite through regulation of gastrointestinal activity (e.g. reduce gastric motility and emptying), as well as inducing the secretion of satiety signaling hormones, such as cholecystokinin (CCK), glucagon, insulin and leptin (Plata-Salamán, 2000). Periods of suppressed feed intake can vary from small reductions to complete cessation of eating depending on the severity of the disease and robustness of the host (Kyriazakis and Doeschl-wilson, 2009). Limited nutrient intake requires the host to rely more on endogenous stores and reallocate resources to supply the needed nutrients, specifically AA, for immune system activated pathways.

Impact of ISS on digestive capacity

The gastrointestinal tract (GIT) is a highly adaptive organ that can quickly change its function during various physiological states. During systemic inflammation, the GIT prioritizes host defense over digestion and absorption, and undergoes major physiological

changes that impact intestinal motility, barrier function and permeability, microflora, expression of digestive enzymes, and nutrient transport mechanisms (Burkey et al., 2009; Turner, 2009). Digestion and absorption represent critical factors in assessing the efficiency of nutrient utilization. Therefore, when developing management and nutritional strategies to optimize swine health and performance, it is imperative to evaluate the influence of ISS on gastrointestinal function (Willing et al., 2013; Liu, 2015).

Increased intestinal dysfunction during systemic inflammation is heavily regulated by pro-inflammatory cytokines, especially TNF-α and interferon (INF)-γ. These cytokines act synergistically to disrupt intestinal permeability through the redistribution and altered expression of tight junction proteins, along with cytoskeletal rearrangements mediated primarily by myosin light chain kinase phosphorylation of myosin light chain (Groschwitz and Hogan, 2009; Suzuki, 2013). Furthermore, pro-inflammatory cytokines induce extensive apoptosis and inflammation in intestinal epithelial cells, further increasing intestinal permeability (Lee, 2015).

The production and secretion of intestinal mucins, the main structural component of mucus, plays a critical role in maintaining mucosal homeostasis. During systemic inflammation, mucin synthesis and secretion is enhanced in an attempt to maintain and effective mucosal barrier (Faure et al., 2007). This increase is primarily mediated by pro-inflammatory cytokines and occurs through receptor mediated activation of the stress-activated protein kinase/ c-Jun NH2-terminal kinase or janus kinase/ signal transducers and activators of transcription pathways (Dharmani et al., 2009; Cornick et al., 2015). Because intestinal mucins are particularly rich in threonine, serine, proline, and cysteine,

their enhanced synthesis and secretion during ISS could potentially limit the availability of these AA, for other body functions (Faure et al., 2007).

Pro-inflammatory cytokines have also been shown to reduce gut motility during ISS *via* suppression of intestinal contractile activity (Akiho, 2011). These pro-inflammatory cytokines augment hypomotility directly by suppressing the contractility of circular muscle *via* downregulating key enzymes involved in smooth muscle contraction, and indirectly by stimulating the production and release of nitric oxide and upregulation of cyclooxygenase-2 and prostaglandin E_2 from endothelial cells (Gonzalo et al., 2011; Ruan et al., 2011). Pro-inflammatory cytokines further regulate intestinal motility during ISS by inducing the secretion of gut derived satiety peptide CCK (Palazzo et al., 2007).

In a healthy state, symbiosis of intestinal microflora is controlled in part by intestinal motility through the clearing of excess bacteria from the gut lumen (Barbara et al., 2005; Ringel and Maharshak, 2013). Therefore, cytokine-induced reductions in gut motility may, in part, be responsible for altered intestinal microflora, and thus, intestinal bacterial overgrowth, during ISS. Moreover, cytokine-induced suppression of antimicrobial peptides (e.g. β-defensins) secreted by intestinal epithelial cells further increases the risk of intestinal colonization by potentially harmful bacterial (Kolls et al., 2008). Increases in the level of pathogenic microbes in the intestine can further enhance GIT inflammation, dysregulation of gut barrier integrity and function, decreases in gut motility, and decreased absorptive capacity of dietary nutrients (Kolls et al., 2008).

Findings on the changes in the production of digestive enzymes during systemic inflammation are somewhat inconsistent. However, they seem to be dependent upon the severity of the disease and occur primarily through regulation of exocrine pancreatic

function (Tribl et al., 2003; Sendler et al., 2012; Chaari et al., 2017). Several studies suggest that inflammatory mediators act upon the pancreas to induce a state of pancreatitis, which causes impaired exocrine function and reduced enzyme secretion. Other studies suggest a common increase in pancreatic enzyme production during sepsis (Sendler et al., 2012; Chaari et al., 2017). Finally, some evidence indicates that inflammatory mediators increase the secretion of pancreatic secretory trypsin inhibitors during the APR. However, it is not well understood whether or not this increase in trypsin inhibitors impacts the digestibility of nutrients in the GIT (Wang and Xu, 2010).

It is well known that systemic inflammation causes changes in intestinal morphology of the pig. Pro-inflammatory mediators induce these changes by acting directly on the tissue through their receptors, or indirectly through ISS mediated reduced feed intake (Wijtten et al., 2011; Liu, 2015). In growing pigs, infection with porcine epidemic diarrhea virus (PEDv) or coinfection with porcine reproductive and respiratory syndrome virus (PRRSv) and PEDv induced villus atrophy and a reduced villus to crypt ratio across all segments of the small intestine. Interestingly, in the same study, pigs inoculated with PRRSv alone showed no significant change the morphology of the intestine (Schweer et al., 2016a). Differences in intestinal morphology seen in PEDv and PRRSv challenged pigs may be explained by differences in the pathogenesis of an enteric (PEDv) vs a systemic/respiratory (PRRSv) viral infection. Furthermore, pigs challenged with intraperitoneal *E. coli* lipopolysaccharide (LPS) resulted in reduced villus heights and morphology disruption in the small intestine (Zhu et al., 2013; Pi et al., 2014). Alterations in intestinal morphology during ISS can the efficiency of GIT function such

as nutrient digestion and absorption. However, this may be dependent on the type and severity of the disease.

Inflammation induced changes in intestinal morphology can have consequences on the expression and activity of intestinal nutrient transporters as reported in various animal models (Tappenden, 2008). However, reports on ISS-induced changes in absorptive capacity are inconsistent. For example, Hang et al. (2007) reported an upregulation in the peptide transporter, PepT-1, in rats with systemic inflammation, suggesting that the gut can maintain absorptive function of di- and tripeptides, independent of changes in intestinal mucosa surface area. Similarly, rats challenged with *Staphylococcus aureus* had no effect on the transport rates of L-methionine, L-leucine, and L-lysine, but it impaired jejunal SLGT-1 glucose transport activity (Moretó and Pérez-Bosque, 2009). Alternatively, LPS induced systemic inflammation has been shown to impair absorptive functions of AA by inhibiting the transport capacity (Vmax) and Na^+/K^+-ATPase activity of Na^+-dependent transporters in the jejunum of rabbits (Abad et al., 2001). Pigs infected with PRRSv saw increased jejunal glucose transport activity, while PEDv infection caused decreased jejunal glucose transport activity, without altering the expression of sodium-glucose linked transporter-1 (SGLT-1) (Schweer et al., 2016a). Furthermore, PRRSv and PEDv infection in pigs tended to increase jejunum mRNA abundance of AA transporter SLC6a14 ($ATB0^+$, a $Na+/Cl^-$ dependent AA transporter; Schweer et al., 2016a). Similar to the PRRSv pigs, growing pigs challenged with LPS had increased ileal glucose transport, as well as increased phosphorous transport (Rakhshandeh et al., 2012). On the other hand, while pigs exposed to chronic social stress did show an increase in the expression of SGLT-1, they had impaired

glucose transport activity relative to control pigs, suggesting compromised SGLT-1 transporter function (Li et al., 2017). Interestingly, Albin et al. (2007) showed that the nutrient transporter activity across the small intestine in two pig breeds responded differently to an LPS challenge, with responses being dependent upon the nutrient evaluated (i.e. glucose and AA) as well as the segment of the intestine examined (Albin et al., 2007). Effects of disease on intestinal nutrient transport systems are inconsistent and may be dependent upon the type and severity of disease, as well as the genetics of the animal. Nonetheless, these findings indicate that systemic inflammation alters the expression and activity of nutrient transporters which could impact the overall absorption of various nutrients needed for other processes such as growth. Further studies are needed to evaluate the impact of systemic ISS on intestinal nutrient transport systems.

Taken together, ISS induced morphological and functional changes in the GIT likely perturb the digestion and absorption of dietary nutrients and thus, compromise the pig's efficiency of nutrient utilization for growth. Several recent studies have begun to elucidate the impact of ISS on nutrient digestibility. Studies in growing pigs challenged with LPS has shown to decrease the apparent ileal digestibility (AID) of dietary protein (Rakhshandeh et al., 2012). However, in separate studies, LPS had no effect on the AID of dietary protein or select AA (Rakhshandeh et al., 2010, 2014). Similarly, in PRRSv challenged pigs, one study reported a decreased in apparent total tract digestibility (ATTD) of dietary protein (Schweer et al., 2017), while another study conducted in the same lab reported no effect on ATTD of dietary nitrogen as well as no effect of AID of specific AA in PRRSv infected pigs (Schweer et al., 2016). Although inconsistent, these studies suggest that nutrient digestibility may be impacted during ISS, and thus, becomes

a limiting factor for the utilization of dietary protein and AA for various body functions, meriting further investigations.

Impact of ISS on protein metabolism: neuroendocrine regulation

Many aspects of altered protein and AA metabolism during ISS are mediated by pro-inflammatory cytokine regulation of the neuroendocrine system. Pro-inflammatory cytokines have been shown to regulate hormonal and neuronal signaling pathways *via* activation of the hypothalamic-pituitary adrenal axis (HPAA) and the autonomic nervous system (ANS; Karrow, 2006). The CNS contains cytokine producing cells, as well as cytokine receptors, which allow for direct and indirect stimulation of the HPAA and induction of sickness behaviors, including lethargy, reduced appetite, and fever (Johnson and Escobar, 2005; Karrow, 2006; Buchanan and Johnson, 2007; Miguel et al., 2010). The activation of the HPAA is caused primarily by IL-1 stimulation of hypothalamic neurons, although, its effects are enhanced by the synergistic effects of TNF-α and IL-6 (Broussard et al., 2001; Karrow, 2006).

Pro- inflammatory cytokines exert their effects on the hypothalamus, in part, by inducing the secretion of corticotropin releasing hormone (CRH), which causes a subsequent increase in pituitary-derived adrenocorticotropic hormone (ACTH). Release of ACTH into the circulation results in the stimulation and release of adrenal glucocorticoids, such as cortisol (Broussard et al., 2001). Increased cortisol levels can be beneficial in regulating the immune response, providing a negative feedback loop to inhibit inflammatory cytokine production. However, cortisol's negative impact on nutrient metabolism, especially protein and AA metabolism, is often a more important consideration (Carroll, 2008). Cortisol has been shown to induce a catabolic response in

skeletal muscle by decreasing protein synthesis and increasing protein degradation. This decreased muscle protein synthesis is the result of reduced AA uptake into the muscle, and suppression of the stimulatory actions of insulin, insulin like growth factor 1 (IGF-1), and AA (in particular leucine), on the phosphorylation of key eukaryotic initiation factors (eIFs) involved in protein synthesis. At the same time, cortisol also induces muscle proteolysis by directly activating major cellular proteolytic systems, including ubiquitin proteasome, lysosomal, and calcium dependent (calpains) systems (Schakman et al., 2013). Amino acids released from skeletal muscle are then reallocated to the liver, where the pleiotropic effect of cortisol increases AA uptake. Furthermore, glucocorticoids alter the local production of growth factors by inhibiting IGF-1 synthesis and stimulating the production of myostatin, a negative regulator of muscle mass through the inhibition of myogenesis. The negative effects of myostatin on muscle mass is seen in both mice (McPherron et al., 1997; Szabo et al., 1998) and cattle (Grobet et al., 1997; Kambadur et al., 1997), as well as pigs. Specifically, growing pigs infected with PRRSv showed an increase in myostatin mRNA, which was accompanied by a decrease in weight gain and protein accretion (Escobar et al., 2004).

Systemic inflammation alters the production and activity of anabolic and catabolic hormones. Pro-inflammatory cytokines regulate hormonal signaling pathways, including insulin, growth hormone (GH), IGF-1, glucagon, leptin, and cortisol (discussed previously), inducing a state of hormone resistance to regulate the utilization of the animals' limited resources (Kelley et al., 2007; Schakman et al., 2013). For example, TNF-α acts directly on skeletal muscle to induce a state of insulin resistance by inhibiting the post-receptor activity of insulin signaling, eliciting a reduction in skeletal muscle

protein synthesis through suppression of mammalian target of rapamycin (mTOR)-dependent translation initiation (Wei et al., 2008). However, it has been shown that the effects of systemic inflammation on insulin resistance may vary depending on the age and nutritional status of the animal. For example, during endotoxin challenge, young growing pigs in the fed state maintain a high sensitivity and responsiveness for insulin stimulated muscle protein synthesis, relative to mature septic animals, despite suppression of mTOR-dependent translation initiation suggesting a mTOR-independent regulation of muscle protein synthesis (Orellana et al., 2006a; b, 2008).

Pro-inflammatory cytokines adversely affect the somatotropic axis, a major regulator of animal growth, which consists of GH, IGF-1, and their associated carrier proteins and receptors (Remaville et al., 2002). In immunologically challenged animals, there becomes and uncoupling of the GH-IGF-1 axis through reduced circulating IGF-1 levels and reduced sensitivity of receptors for growth hormone releasing hormone (GHRH), GH, and IGF-1 (Johnson and Escobar, 2005). It has been suggested that inflammatory cytokines, especially IL-1β, impede the release of GH from the anterior pituitary through the suppression of GHRH (Spurlock, 1997). However, the effect of systemic inflammation on circulating GH levels seems species-specific and dependent on disease type. For example, when administered LPS, humans and sheep show an increase in circulating GH while cattle, chickens, and rats show decreased plasma GH levels (Broussard et al., 2001; Carroll, 2008; Borghetti et al., 2009). In pigs, when injected intraperitoneally with LPS or orally with *Salmonella typhimurium* there was either moderate increases or no changes in circulating GH levels, respectively (Hevener et al., 1997; Balaji et al., 2000; Wright et al., 2000; Borghetti et al., 2009). Despite this

variability among species, circulating IGF-1 levels appear to be consistently decreased among immunologically challenged animals. This uncoupling of the GH-IGF-1 axis can be explained by TNF-α and IL-1 induced decreases in the expression of hepatic GH receptors and inhibited post-receptor signaling cascade necessary for the synthesis and release of IGF-1 (Johnson and Escobar, 2005; Carroll, 2008). In addition to reducing the synthesis of IGF-1, inflammatory cytokines induce IGF-1 resistance in the skeletal muscle inhibiting its anabolic effects on muscle protein synthesis (Johnson and Escobar, 2005).

Evidence of elevated plasma glucagon levels immediately after acute inflammatory challenges have been observed in various animal models (Jones et al., 2012). During ISS, glucagon secretion is stimulated *via* direct islet sympathetic innervation or stimulation of pancreatic α-cells by systemic catecholamine ligation to β-adrenergic receptors expressed on the pancreas. Increased glucagon levels serve to increase hepatic glucose output by stimulating both glycogenolysis and gluconeogenesis pathways, which supports enhanced glucose utilization during ISS associated with immune system activation and inflammatory processes (Jones et al., 2012).

Regulation of the neuroendocrine system serves a critical role aimed to control and regulate systemic inflammation and subsequent immune response. Alterations in anabolic and catabolic hormones serve to maximize the mobilization of AA from peripheral tissues towards visceral organs for increased gluconeogenesis and synthesis of APP and other immune system metabolites. Loss of anabolic stimuli coupled with the catabolic effects of proinflammatory cytokines implies major consequences on protein and AA metabolism and impact the productivity of the animal.

Modifications of protein synthesis and degradation during ISS

Disease induced modifications of skeletal muscle protein metabolism is multifaceted and presumably the most extensively studied among body tissues. Skeletal muscle serves as the largest reservoir of protein and AA in the body, thus, changes in muscle protein metabolism can result in significant changes to whole body protein metabolism. Derangements in skeletal muscle protein metabolism generally result from both decreased protein synthesis and increased protein degradation, in attempt to establish homeostasis by maximizing the flow of AA to visceral tissues, especially the liver, for increased gluconeogenesis and hepatic APP synthesis.

Systemic ISS reduces muscle protein synthesis by decreasing the synthesis of both myofibrillar and sarcoplasmic proteins, preferentially in muscle primarily composed of fast-twitch glycolytic fibers. However, insults of sufficient severity results in a more generalized decrease in muscle protein synthesis across all fiber types is observed (Vary and Kimball, 1992; Lang et al., 2007; Lang and Frost, 2007). Sepsis-induced inhibition of muscle protein synthesis primarily results from a decrease in translational efficiency during the initiation phase of translation (Lang et al., 2007; Lang and Frost, 2007). Translation initiation is regulated by various protein eukaryotic initiation factors (eIF's), most of which are sensitive to various anabolic and catabolic hormones, such as insulin, IGF-1, and glucocorticoids, as well as nutrients, including AA (Lang et al., 2007). It has been suggested TNF-a and glucocorticoids (previously discussed) work in a cooperative manner to suppress the activity of protein kinase mTOR and the subsequent phosphorylation of key eIFs and binding proteins that regulate translation initiation. This inhibition suppresses the formation of the 43S pre-initiation complex, as well as the

assembly of the initiation factor complex (eIF4F) necessary for the loading of the 43S pre-initiation complex onto capped mRNA (Lang et al., 2007; Lang and Frost, 2007).

Accelerated skeletal muscle proteolysis serves to maximize the release of AA towards visceral organs in support of increased synthesis of metabolites involved in host defense. Intracellular protein breakdown also contributes to reduced PD seen during ISS. This breakdown is regulated by three proteolytic pathways: ubiquitin proteasome-dependent, the calcium-dependent (calpain), and lysosomal systems. Among these, the calpain and ubiquitin proteasome-dependent pathways play a significant role in skeletal muscle protein breakdown. Glucocorticoids are likely the primary mediators of muscle proteolysis. However, pro-inflammatory cytokines, especially TNF- α, act synergistically to further enhance muscle protein degradation (Hasselgren, 2000).

Protein synthesis substantially increases in the liver during ISS due to the synthesis of both secretory and structural proteins involved in host defense (Obled, 2003; Le Floc'h et al., 2004). Increased hepatic APP synthesis is stimulated primarily by the direct pleiotropic effects of IL-6 and indirect effects of IL-1β and TNF-α, through their stimulated release of glucocorticoids (Jacobi et al., 2006; Van Hall et al., 2008). Stimulation of hepatocytes by pro-inflammatory cytokines, glucocorticoids, and growth factors, such as AA, results in increased mTOR activity (Kimball et al., 2003). Hepatic synthesis of APP increases several-fold during ISS which may incur a metabolic cost to the animals due to the mismatch in AA composition between skeletal muscle and various APP (Reeds et al., 1994). For example, approximately 2g of skeletal muscle must be catabolized to support the synthesis of 1g of the typical APP mixture. Thus, difference in

AA profiles of APP relative to skeletal muscle, may result in altered AA requirements of specific AA, according to their composition (Reeds et al., 1994).

Protein turnover is described as the continual synthesis and breakdown of body proteins in which the difference between protein synthesis and breakdown represents net protein gain, reflecting the partition of AA between anabolic and catabolic processes in farm animals. It is a fundamental and highly efficient biological process that proves advantageous for homeothermy, plasticity (i.e. interorgan exchange of AA) and metabolic function in mammals (Lobley, 2003). Over the last 50 years, researchers have attempted to quantify rates of turnover at the tissue and whole-body level. Rates of protein synthesis and degradation during ISS have been measured in various tissues (Breuillé et al., 1994; Bruins et al., 2000). However, little to no information is available on the impact of infectious disease on whole-body protein turnover in pigs. Gaining this knowledge will help to determine the metabolic costs of hyper-activation of the immune system at the whole-animal level.

IMPACT OF IMMUNE SYSTEM STIMULATION ON AMINO ACID UTILIZATION

Threonine

The metabolism of threonine (Thr) during ISS has gained attention due to its role in the synthesis of Thr-rich immune system metabolites, such as immunoglobulins, acute phase proteins and, in particular, intestinal mucins (Faure et al., 2007; Rémond et al., 2009; Rakhshandeh and de Lange, 2011). Threonine plays a critical role in the maintenance of intestinal health, which is reflected by its high rate of intestinal uptake relative to other essential AA (Le Floc'h and Sève, 2005; Mao et al., 2011). Furthermore,

intestinal catabolism of dietary threonine through the threonine dehydrogenase pathways does not appear to account for its relatively high first pass extraction rate in pigs (Le Floc'h and Sève, 2005). Thus, high rates of intestinal threonine extraction may be associated with its utilization for mucosal protein synthesis, in particular the synthesis of mucins, which are particularly rich in threonine (Faure et al., 2006; Faure et al., 2007; Law et al., 2007). It has previously been demonstrated that intestinal inflammation increases gastrointestinal threonine uptake and mucin synthesis (Rémond et al., 2009).. This increase can largely be associated with an increase in the synthesis of mucin 2, the main secreted intestinal mucin, which is particularly rich in threonine (>30%) relative to other mucins (13-20%), during inflammation (Faure et al., 2003; Faure et al., 2006; Rémond et al., 2009). In pigs, Rakhshandeh *et al*. observed a 1.6-foldincrease in the expression of mucin 2, a major proteinous component of mucus, at the transcriptional level in the small intestine of ISS pigs (Rakhshandeh et al., 2013). Because mucins are major components of the basal and specific endogenous AA losses (EAAL) in pigs, accounting for up to 60% of total intestinal nitrogen losses (Moughan, 1999; NRC, 2012) and considering that Thr accounts for 16-20% of crude mucin, increased synthesis and secretion of mucins may impact Thr requirements in growing pigs during ISS (Lien et al., 1997).

Sulfur amino acids

The increased metabolic need for cysteine seems to be of particular importance for increased hepatic glutathione (GSH) synthesis, and to a lesser extent the enhanced APP production (Rakhshandeh and de Lange, 2011). For a detailed review on the metabolism of SAA during ISS the reader is referred to Rakhshandeh and de Lange,

2011. In general, reports of ISS-induced increased utilization of methionine has been attributed to the increased need of cysteine for the synthesis of other essential metabolites such as GSH and taurine. Increased GSH turnover and plasma cysteine flux, and reduced plasma cysteine and total SAA levels have been reported in LPS challenged growing pigs (A. Rakhshandeh et al., 2010; Rakhshandeh and de Lange, 2010). Furthermore, LPS-induced endotoxemia in has been shown to increase the overall irreversible loss of cysteine to taurine (Rakhshandeh and de Lange, 2011). Collectively, these studies demonstrate the metabolic need for cysteine substantially increases during inflammatory states. Beneficial effects of supplemental L-cysteine on immune function has been reported in pigs. Supplementation with L-cysteine during experimental induced colitis in pigs resulted in improved gut health and suppression of inflammatory responses (Kim et al., 2009). Similarly, LPS challenged piglets fed a L-cysteine supplemented diet resulted in a higher average daily gain, improved GIT barrier function, and suppressed LPS-induced intestinal inflammation and oxidative stress (Song et al., 2016). Alternatively, Litvak et al., (2013) suggests that there is a preferential use of dietary methionine during ISS as a result of an increase in the optimal dietary methionine to total SAA ratio seen in growing pigs (Litvak et al., 2013). This finding is further supported by Rakhshandeh et al., (2014) who reported an approximately 23% increase in SAA maintenance requirements per unit of PD in LPS challenged pigs.

Tryptophan

Tryptophan is an essential AA that not only serves as a building block for whole body protein synthesis, but it also plays important biological roles including serving as a precursor for the synthesis of kynurenine, serotonin, and melatonin, appetite regulation,

anti-oxidative defense and suppression of inflammatory response (Le Floc'h and Seve, 2007). Reports of decreased plasma tryptophan concentrations have been observed in pigs and other species during systemic inflammation, and has been associated with the increase in metabolic demand for tryptophan (Melchior et al., 2004). During ISS, increased utilization of tryptophan is likely associated with the catabolism of tryptophan to its metabolite kynurenine through the activation of the tryptophan catabolism enzyme indoleamine 2,3-dioxygenase (IDO; Melchior et al., 2004; Capozzalo et al., 2015). de Ridder et al. (2012) observed a decrease in the partial efficiency of tryptophan utilization for PD during ISS, which suggested that dietary tryptophan requirements may increase during ISS. The reduced in efficiency in tryptophan utilization in LPS-induced pigs may be explained by the increased catabolism of tryptophan to kynurenine. However, incorporation of tryptophan into APP may also have an effect (Reeds et al., 1994; de Ridder et al., 2012). Moreover, several studies have shown a beneficial effect of tryptophan supplementation in immune challenged pigs (Le Floc'h et al., 2012). Pigs infected orally with enterotoxigenic *E. coli* had improved growth performance, feed efficiency, and immune function when the ratios of SID tryptophan was increased above the levels recommended by the National Research Council 2012 (Capozzalo et al., 2015; Capozzalo et al., 2017).

Lysine

Lysine is typically the first limiting AA in swine diets and the first limiting for protein deposition. Thus, the requirements of AA are usually expressed relative to the requirements for lysine, and is recognized as the "ideal protein" (van Milgen and Dourmad, 2015). The concept of the ideal protein is to provide the animal with the

optimum balance of all AA required for maintenance and production for clearly defined physiological states (NRC, 2012). The health status of the pig does not alter the efficiency of lysine utilization for protein deposition, but instead reduces daily dietary lysine requirements in ISS pigs (Williams et al., 1997). These changes in lysine requirements reflects the changes in the animal's ability to accrete skeletal muscle protein during an immune challenge. These finding could suggest a lower protein requirement for the animal during ISS due to its compromised ability for growth.

Leucine

Leucine is a potent anabolic stimulator of skeletal muscle, impacting both protein synthesis and protein degradation processes. Leucine increases protein synthesis in skeletal muscle *via* direct simulation of the mTOR signaling pathway (Kimball and Jefferson, 2006). Therefore, it is reasonable to assume that supplementation of leucine in the diet of challenged pigs could attenuate the adverse effects of ISS on skeletal muscle. However, leucine supplementation in LPS challenged pigs did not affect nitrogen retention or whole body protein turnover during ISS (Rudar et al., 2016; Rudar et al., 2017). This result is likely due to an ISS-induced resistance to leucine's anabolic effects through disruption of mTOR signaling (Laufenberg et al., 2014). Conversely, young growing pigs exposed to sustained endotoxemia largely maintain muscle protein synthesis in the presence of exogenous substrate supply. This result is likely due to their high anabolic capacity and sensitivity to AA, especially leucine, and insulin. However, as the animal matures it loses this capacity and becomes more susceptible to profound changes in muscle protein synthesis during sepsis (Orellana et al., 2004; R. A. Orellana et al., 2006; Orellana et al., 2007). Thus, the extent to which ISS alters skeletal muscle

protein metabolism is dependent on the severity of the insult, as well as the age and nutritional state of the animal (Lang et al., 2007).

Systemic inflammation clearly alters the protein metabolism and AA utilization in disparate organs during pre- and post-absorptive metabolism and is highly regulated by both pro-inflammatory cytokines, as well as anabolic and catabolic hormones. These changes in AA utilization during ISS impact the dietary requirements of specific AA, and supplementation of these AA may mitigate the negative impact of disease on PD.

CONCLUSIONS AND IMPLICATIONS

Immune system stimulation negatively impacts the behavior and productivity of growing pigs and thus the animals' well-being and nutrient utilization, along with the profitability of commercial pork production. Hyper activation of the immune system, and subsequent release of pro-inflammatory cytokines, results in physiological and metabolic shifts that that alter amino acid utilization and, thus, increasing the dietary requirements of SAA, threonine, and tryptophan, but decreases the requirements of lysine. Therefore, the ideal profile of AA to optimize performance efficiency in healthy animals does not meet the needs of an immunologically challenged animal. better understanding on how ISS impacts the AA requirements of pigs will allow feed manufactures and commercial pig producers to optimize diet formulations that will more closely match the AA requirements of animals during disease, thus improving animal welfare and production efficiency.

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CHAPTER III

IMMUNE SYSTEM STIMULATION INDUCED BY ESCHERICHIA COLI LIPOPOLYSACCHARIDE ALTERS AMINO ACID KINETICS AND PROTEIN DEPOSITION IN GROWING PIGS

ABSTRACT

Changes in plasma free amino acid (AA) kinetics reflect the modification of AA metabolism in different metabolic states. Immune system stimulation (ISS) in growing pigs redistributes AA from protein retention towards processes involved in the immune response, thus impacting AA utilization. The aim of the current study was to evaluate the effect of ISS on whole-body nitrogen (N) utilization and the kinetics of plasma free AA. Ten gilts (BW 9.4 ± 1.1 kg) were surgically fitted with jugular vein catheters, individually housed in metabolism crates, and feed-restricted (550 g/d). Repeated intramuscular injections of increasing amounts of *Escherichia coli* lipopolysaccharide (LPS) was used to induce ISS (30 and 36 µg/kg BW, given 48 h apart). Whole-body N-balance was conducted for 3 d before ISS (ISS-) and 3 d during ISS (ISS+). At the end of each N-balance period a bolus dose of labeled [U-^{13}C, U-^{15}N] AA mixture was infused intravenously, followed by serial blood collection for determination of isotopic enrichment. A double exponential model was fitted with plasma enrichment data for each pig and each AA, and equation parameters were used to estimate plasma free AA flux and pool size. Apparent ileal digestibility of N was determined using the slaughter technique and an indigestible marker. Blood samples were collected before and 72 h after the initiation of ISS and assayed for hematology and blood chemistry. Body temperature (BT) was monitored during the course of study. Blood chemistry, hematology, and BT results indicated that LPS induced effective ISS in pigs ($P<0.05$). Immune system

stimulation tended to reduce N retention (*P=0.09*) and the N retention-to-N intake ratio (*P=0.08*). Apparent ileal digestibility of N and apparent total tract digestibility of dietary energy were reduced by ISS (*P<0.05*). Plasma flux (μmol/kg BW/h) for Ile and Phe was reduced by ISS (*P*<0.05). A strong tendency of decreased Lys flux was observed in ISS pigs (*P=0.08*). Immune system stimulation increased the pool size for Leu (*P*<0.05) but reduced the pool size for Ile (*P<0.05*). Collectively, these results suggest that ISS alters the utilization of dietary N and AA flux, as well as pool size in growing pigs. The decrease in Lys, Phe, and Ile flux during ISS may be attributed to a reduction in whole body protein synthesis or decreased catabolism of these AA.

Keywords: growing pigs, immune system stimulation, kinetics, LPS, plasma amino acid flux

INTRODUCTION

Exposure to chronic sub-clinical or mild-clinical immune system stimulation (ISS) results in altered protein and amino acid (AA) utilization in growing pigs (Williams et al., 1997; Le Floc'h et al., 2009). The latter results in the redirection of AA away from growth and reproduction towards mounting an immune response (Obled, 2003). Increases in the synthesis of newly synthesized immune system proteins and metabolites, such as acute phase proteins (APP), glutathione, and immunoglobulins, may require additional needs for specific AA (Reeds and Jahoor, 2001). The latter can impact AA requirements qualitatively and quantitatively.

Previous studies have suggested that requirements for sulfur containing AA (SAA), branched chain AA (BCAA), aromatic AA, threonine (Thr), and glutamine (Gln) increase during ISS (Reeds and Jahoor, 2001; Obled, 2003; Rakhshandeh and de Lange,

2011). However, it is important to note that these studies were conducted using different models of ISS with some eliciting a more severe immune response than the others, various nutritional approaches, and various methods of measurements, with some studies using plasma AA concentration to interpret the results. However, changes in AA metabolism can occur without simultaneous alterations in the concentrations of free AA in the extracellular compartments, including plasma (Waterlow, 2006). Furthermore, the majority of the studies that evaluated the effect of ISS on AA utilization focused only on one AA at a time, which potentially ignores the possible interactions between AA and their effect on AA needs during ISS. Moreover, some of these studies based their conclusions on observed increased in metabolic demand for an AA without considering that an increase in the metabolic demand does not always affect dietary requirements for AA. In some case, enhanced metabolic demands for AA can be met from endogenous sources of AA, such as skeletal muscle (Rakhshandeh and de Lange, 2011). Quantitative information about the effect of ISS on the utilization of multiple AA simultaneously is largely lacking in the literature.

The development of nutritional strategies that can reduce the negative impacts of ISS on the pig's productivity requires an understanding of the quantitative effects of ISS on AA needs. Plasma free AA flux reflects the amount of free AA that disappears per unit of time from the plasma pool for protein synthesis and catabolism. Changes in plasma free AA flux, thus, can better reflect the modification of AA metabolism in different metabolic states (Waterlow, 2006; Kampman-van De Hoek et al., 2015). Therefore, measuring plasma free AA flux can provide a more accurate insight into the metabolic changes that are brought about by ISS. The objective of the current study was

to evaluate the effects of ISS induced by *E. coli* lipopolysaccharide (LPS) on flux and pool size of multiple AA simultaneously in the plasma, as well as on whole-body nitrogen (N) utilization in growing pigs.

METHODS AND MATERIALS

The experimental protocol was approved by the Texas Tech University (TTU) Animal Care and Use Committee (IACUC approval number 13049-07).

Animals, housing, diet and feeding, and general experimental design

Ten PIC gilts free of major swine pathogens (Pig Improvement Company North America, TN, USA; initial body weight 9.4 ± 1.1 kg) were obtained from the TTU swine herd, surgically fitted with jugular catheters, individually housed in metabolism crates, and feed-restricted (550 g/d) a corn-soy bean meal based diet (ME 14.0 MJ/kg, standardized digestible Lys 10.4 g/kg; Table 3.1). After recovery from surgery, pigs were subjected to a 3 d pre-ISS (ISS-) and a 3 d post-ISS (ISS+) N-balance study. At the completion of each N-balance period an isotope tracer study was conducted to determine plasma free AA flux. Daily feed allowance was allocated into two equal feedings per day at 08:00 am and 05:00 pm. Pigs had free access to water throughout the study. Twenty-four hours before isotopic infusion, the feeding intervals were changed to every 4 h to minimize diurnal patterns in AA metabolism. At the end of the study, pigs were euthanized by intravenous injection of a lethal dose of sodium pentobarbital (FATAL PLUS, Vortech Pharmaceutical, Ltd., Dearborn, MI, USA).

Surgical catheterization and immune system stimulation

Silicon catheters (Micro-Renathane, 0.095 O.D. × 0.066 I.D., Braintree Scientific Inc., Braintree, MA, USA) were surgically inserted in the left and right external jugular veins according to the procedures originally described by Weirich et al., (1970) and modified by de Lange et al., (1989). Gilts were allowed to recover for at least seven days. During recovery, each pig was treated with one dose of penicillin (25,000 units intramuscular, IM), one dose of anti-inflammatory banamine (2.2 mg/kg body weight IM) and one dose of the painkiller buprenorphine (0.01 mg/kg subcutaneously). General health, feed intake and eye temperature of pigs were monitored frequently during the recovery period. Following recovery and 3 d pre-ISS N-balance, pigs were subjected to a 3 d pre- ISS (ISS-) N-balance study. Immune system stimulation was induced by repeated IM injections of increasing amounts of *Escherichia coli* lipopolysaccharide (30 and 36 µg/kg body weight; LPS strain 055:B5; Sigma Aldrich) given 48 h apart. The second dose was increased to overcome potential tolerance to LPS (Rakhshandeh and de Lange, 2012).

Isotopic infusion

At the completion of each N-balance period, a single bolus sterile dose (0.9 ml/kg body weight) of universally labeled [U-^{13}C, U-^{15}N] AA mixture (97-99 atom percent, Cambridge Isotope Laboratories, Tewksbury, MA, USA) suspended in saline was infused *via* one of the indwelled jugular catheters over a 30 second period. The AA mixture (mg/ml sterile saline) contained isoleucine (Ile), 2.3, leucine (Leu), 6.0, lysine (Lys), 10, methionine (Met), 0.8, phenylalanine (Phe), 4.3, threonine (Thr), 1.2, tryptophan (Trp), 3.6 and glutamine (Gln), 2.1.

Observations and sampling

The initial body weight (BW) of each pig was measured prior to entering metabolism crates and final BW was determined at the conclusion of the study. After recovery from surgery, pigs were feed restricted and subjected to a 3 d pre-ISS and 3 d post-ISS N-balance as described by Rakhshandeh et al., (2014). Briefly, urine was collected *via* collection trays underneath each crate that funneled urine into tared, lidded buckets containing sufficient amounts of 3 N HCl to maintain urine pH below 3. From each 24 h urine collection, 10% of the urine volume was pooled for each pig at each N-balance period and stored at 4°C until analyzed for total N contents. Feed waste and vomit for each pig was collected, oven dried, cooled in a desiccator and weighed to accurately determine daily feed intake. Fecal samples were collected daily and stored in sealed plastic bags at -20°C until processed further. At the conclusion of the study, fecal samples were thawed, pooled and mixed together per pig and per N-balance period and stored at -20°C.

After each isotopic infusion serial blood samples were then taken before isotopic infusion started and then at time 2.5, 5, 7.5, 10, 15, 20, 30, and 45 minutes after infusion to measure the change in plasma isotopic enrichment of each AA. A 3ml blood sample was drawn at each time point into a heparinized tube (BD Vacutainer, BD Franklin Lakes, NJ, USA) *via* one of the indwelled catheters. Following the completion of each isotope tracer study, blood samples were centrifuged at 1500 × g for 15 minutes at 4°C. The plasma fraction was then aliquoted and stored at -80°C until further analysis.

Eye temperature was monitored daily during the pre-ISS period and at times 2, 6, 12, 24, 48, 72, and 76 hours post-ISS. Thermography of the eye was performed using a FLIR E40 (FLIR® Systems, Inc.,Wilsonville, Oregon) digital camera, as previously

described by Petry et al., (2017). The resolution for each IR image was set at 160 x 120 pixels and the emissivity value was set to the recommended value of 0.98 for biological tissues. Multiple IR pictures were taken approximately 50 cm away from the eye and an average of the best three pictures, in terms of focus and precision, were selected for determination of BT. Infrared pictures were interpreted using FLIR Tools software (FLIR® Systems, Inc., Wilsonville, Oregon). To evaluate the effect of ISS on measures of blood chemistry and hematology, fresh whole blood samples were collected at times 0 and 76 h post ISS. Samples were collected from jugular catheters and immediately analyzed using i-STAT Handheld Analyzer (Abaxis Inc., CA, USA) with i-STAT EG7+ test cartridges.

Apparent ileal digestibility of dietary N was determined using the slaughter technique and titanium dioxide (TiO_2) as an indigestible marker (Low, 1977). Immediately following the conclusion of the post-ISS isotope tracer study, pigs were euthanized, a ventral abdominal incision was made, the ileocecal junction was located, and the last 150 cm of the small intestine was isolated and clamped to prevent digesta movement. The ileum was then excised and the ileal digesta was gently expelled, collected and stored at -20 °C until further processing. A separate group of gilts (BW11.5 ±0.45 kg; n=9) were feed restricted, treated with sterile saline and used to determine AID of dietary N using the slaughter technique described above.

Analytical Procedures

Fecal and ileal digesta samples were lyophilized and pulverized before analysis for nutrient contents. Nitrogen content of feces, digesta and urine was quantified in duplicate and diet samples were quantified in triplicate using a LECO-Trumac N (Leco

Co., Henderson, NV, USA). Dry matter (DM) content of feces, digesta and diet samples were determined by oven drying for 24 hours at 120°C according to the Association of Official Analytical Chemists procedures (AOAC, 1997). Titanium dioxide levels of fecal and diet samples were determined in duplicate and triplicate, respectively, and according to standard Association of Official Analytical Chemists procedures (AOAC, 1997). Samples of the diet and lyophilized feces were analyzed in duplicate for the determination of gross energy (GE) using a 6300 model calorimeter bomb (Parr Instruments, Moline, IL) according to Association of Analytical Chemists procedures (AOAC, 1990).

Plasma free labeled and unlabeled AA isolation and quantification were achieved using a Phenomenex™ EZ:Faast Amino Acid Analysis Kit (Torrance, CA, USA) and Gas Chromatography Mass Spectrometry (GC-MS). Before derivatization with a propyl chloroformate derivatizing agent, plasma samples were deproteinized using a 3-KDa centrifugal filter (VWR international, Randor, PA, USA). Samples were then freeze dried, reconstituted in Milli-Q water and gently vortexed until sample residue was completely dissolved. Derivatization of samples was completed according to the Phenomenex™ EZ:Faast Amino Acid Analysis Kit and according to the manufacturer's instructions. Samples were kept at -20°C until further analysis by GC-MS. Quantification of derivatized unlabeled and labeled AA in standard and plasma samples was achieved by GC-MS (Agilent 6890 GC coupled with an Agilent 5973 mass selective detector). This method employed selective ion monitoring (SIM) to identify and quantify multiple unlabeled and labeled AA simultaneously. The method utilizes differences in mass between labeled and unlabeled AA (McGilvray et al., 2017).

Calculations and statistical analysis

The AID of crude protein (CP; N × 6.25) and GE were calculated using the indicator method and TiO_2 as an indigestible marker. The enrichment of labeled AA in the plasma was expressed as the tracer-to-tracee ratio (TTR). Plasma samples were collected from pigs before each isotopic infusion to determine the possible background enrichment for each AA. The plasma free AA flux was calculated from the change in the enrichment of each isotopically labeled AA in the plasma after the infusion of bolus dose of universally labeled [U-^{13}C, U-^{15}N]-AA, as described by Holtrop et al., (2004). The following standard double-exponential model was fitted to the TTR for each AA *via* nonlinear least-squares, using the following equation:

$$y = \alpha_1 \, exp(\alpha_2 t) + \alpha_3 \, exp(\alpha_4 t)$$

where *y* is the predicted TTR for each AA in the plasma at time t (min) and α_1, α_2, α_3, and α_4 are parameter estimates. These parameter estimates were used to calculate the flux (Q) of each AA (μmol/kg BW/h) in an individual pig, using the following equation:

$$Q = \frac{D}{\alpha_1/\alpha_2 + \alpha_3/\alpha_4} \times 60$$

where D is the dose of the infused [U-^{13}C, U-^{15}N] AA (mmol/kg BW). In this calculation Q represents the sum of outflow (i.e. efflux) of free AA from the plasma pool toward incorporation of the AA into protein and other peptides (i.e. protein synthesis) and the loss of the AA through catabolism (Holtrop et al., 2004; Waterlow, 2006).

The pool size of for each AA and pig was calculated using the following equation:

$$Pool\ size = \frac{D}{\alpha_1 + \alpha_3}$$

where D is the dose of the infused [U-^{13}C, U-^{15}N] AA (mmol/kg BW), and α_1 and α_3 are parameter estimates that were acquired from fitting a double exponential model fitted to the TTR for each AA and pig (Holtrop et al., 2004).

The rate of inflow (i.e. influx) of AA into the plasma pool from proteolysis was calculated using the steady-state model of Waterlow (2006) in the following equation:

$$Q = I + B = S + U$$

where I and B are the rate of AA inflow into the plasma pool from the diet (standardized ileal digestible AA) and proteolysis, respectively. S and U represent the rate of AA outflow toward whole-body protein synthesis and catabolism, respectively. All values are expressed as μmol/kg BW/h.

Statistical analysis was carried out using SAS software version 9.4 (SAS Institute, NC, USA). Normality and homogeneity of variances was confirmed using the Univariate procedure (PROC UNIVARIATE). Outliers were determined as any value that differed from the treatment mean by ± 2 standard deviation. Data were analyzed in a complete randomized design with health status as fixed effects and pig within crate as a random effect using Mixed procedure (PROC MIXED). Average daily feed intake (ADFI) was used as a co-variate for determining the effect of ISS on measured parameters and when appropriate ($P>0.10$), a reduced model was used. For parameters such as BT that were measured over time, repeated measurements analysis of variance was used. An appropriate covariance structure was selected for analyses by fitting the model with the structure, which provided the 'best' fit, based on Akaike information criterion (AIC) and Schwarz Bayesian criterion (BIC). Tukey-Kramer was used for multiple comparisons test. Values are reported as least square means with their standard errors (SE). Treatment

effects were considered significant at P≤0.05. A tendency towards a significant difference between treatment means was considered at P≤0.10.

RESULTS

General observations

Prior to the study, all pigs showed signs of good health and consumed all of their daily feed allowance. During the to 7 d recovery period, and before ISS, all pigs readily consumed their daily feed allowance and showed signs of good health with no fluctuation in BT. Shortly after the first LPS injection, pigs started to show sickness behaviors, such as lethargy, fever and vomiting. Dry matter from vomit was less than 1.0 % of feed allowance. Immune system stimulation tended to decrease ADFI by 5.4 ± 2.58 g/kg BW/d (*P=0.07*). Data from one pig was excluded from the post-ISS period because of its severe reaction to the LPS challenge. Due to dysfunctional catheters, the isotope tracer study was conducted on 7 ISS- and 6 ISS+ pigs. Analyzed diet nutrient contents were generally in agreement with the calculated values that were derived from feed ingredient composition and the nutrient levels in feed ingredients, according to Swine NRC (2012). In the interpretation of results and for calculations of AA intake for individual pigs, calculated diet nutrient contents were used.

Measures of immune function

The main effects of ISS on measures of immune function, hematology and blood chemistry are presented in Table 3.2. Hematocrit (HCT) and hemoglobin (Hb) levels were not affected by ISS. Repeated injection with increasing amounts of LPS increased eye temperature by 0.8°C (*P*<0.05) relative to ISS- pigs and remained elevated

throughout the duration of the study (Figure 3.1). No treatment effect on the blood concentration levels of hemoglobin and hematocrit were observed. Glucose levels were lower and BUN levels were higher in the blood of ISS+ pigs (*P<0.03*). Levels of Na^+, K^+, Cl^-, and HCO_3^- were not affected by the health status of pigs, but anion gap (AnionGAP), a measure of acid/base-balance, was higher in ISS + pigs (*P<0.02*).

Nitrogen balance and nutrient digestibility

Data on N utilization and AID during ISS are shown in Table 3.3. Statistical differences between ISS- and ISS+ pigs were detected after controlling for ADFI. Immune system stimulation did not affect N intake and total N excretion (*P>0.05*). However, ISS tended to decrease N retention (*P=0.10*) and the N retention-to-N intake ratio (*P=0.06*). Apparent ileal digestibility (AID) of dietary N was reduced by 17.3 ±4.20 % in ISS+ pigs compared to saline treated control pigs (*P<0.01*). Relative to ISS- pigs, the ATTD of GE decreased by 11.4 ± 5.59 % in ISS+ pigs (*P=0.05*).

Plasma free amino acid kinetics

Data on plasma free AA kinetics are presented in Table 3.4. The double exponential model precisely defined the decrease in the TTR of individual plasma AA over time after infusion of the bolus dose of [U-^{13}C, U-^{15}N]-labeled AA. Immune system stimulation decreased the plasma flux of Ile (*P=0.01*) and Phe (*P=0.01*) and tended to decrease plasma Lys flux (*P=0.08*), after controlling for ADFI. Flux of other AA was not affected by ISS. After controlling for ADFI, a decrease in the release of Phe (*P=0.01*) and Val (*P=0.04*) from proteolysis was observed. In addition, a tendency toward the reduced release of Lys from proteolysis in ISS+ was observed after accounting for difference for ADFI between ISS groups (*P=0.09*). No significant effect of ISS on the

release of other AA from proteolysis was observed, after controlling for ADFI. Immune system stimulation, after controlling for ADFI, decreased free Ile (*P=0.05*), but increased free Leu pool size (*P =0.05*). The pool size of other free AA was not affected by ISS.

DISCUSSION

The main objective of the study was to evaluate the effects of ISS induced by LPS on the plasma flux of AA, whose metabolism putatively become more important during ISS. In the current study, a pre-established model of repeated IM injections with increasing amounts of LPS was used to induce ISS. This model induces a sustained and relatively mild ISS, allowing for the study of nutrient utilization during ISS (Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Litvak et al., 2013). In the present study, ISS resulted in elevated eye temperature and changes in blood chemistry. Eye temperature serves as an indicator of core body temperature, which is orchestrated by the release of pro-inflammatory cytokines, in particular IL-1β, from immune cells during systemic ISS (Hughes et al., 1985; Johnson, 1998; Karrow, 2006; Petry et al., 2017). Furthermore, pro-inflammatory cytokines serve as chief stimulators of APP synthesis during ISS Rakhshandeh et al. (2012, 2014) has demonstrated a positive correlation between eye temperature and plasma Il-1β, haptoglobin, and fibrinogen using the same model of ISS (Rakhshandeh and de Lange, 2012; Rakhshandeh et al., 2014). A 25% increase in BUN levels in the current study can most likely be associated with increases in preferential AA catabolism and catabolism of AA in excess, a characteristic often seen during systemic ISS. Systemic ISS is often accompanied by AA imbalances due to differences between the AA composition of immune system proteins and that of skeletal muscle. This imbalance leads to increased catabolism of AA that are in excess (Johnson,

1997; Webel et al., 1997; Rakhshandeh and de lange, 2011; NRC Swine, 2012). In addition, in the present study, a tendency for a reduction in the N retention: N intake ratio was observed in ISS+ pigs, probably due to an increase in the preferential catabolism of AA and catabolism of excess AA. This result agrees with the increased BUN levels observed in the same group of pigs (Rakhshandeh and de Lange, 2011). The decrease in blood glucose levels during ISS can likely be attributed to increased uptake of glucose by immune cells, since glucose is the preferred source of energy for mounting an immune response during ISS (Spurlock, 1997; Rigobelo and Ávila, 2011). Immune system stimulation also resulted in an increase in AnionGAP in ISS+ pigs relative to ISS- pigs. The higher AnionGAP in ISS+ pigs was most likely caused by an increased level of lactic acid in the blood, since the levels of Na^+, K^+, Cl^- and HCO_3^- were not affected by ISS. Thus, this result suggests a shift from aerobic to anaerobic glycolytic metabolism, which often occurs during ISS (De Backer, 2003). Long term and severe inflammatory responses in various species are characterized by reduced Hb levels and HCT. In the current study, however, ISS did not affect Hb levels and HCT, likely because only a mild and short term immune response was stimulated by our model of ISS. Collectively, these results indicated that repeated injection with increasing amount of LPS resulted in an effective ISS in pigs in the current study.

Several studies have suggested that physiological changes occur in the gastrointestinal tract, which disrupt digestion and absorption during ISS (Liu, 2015). In this study, systemic ISS induced by LPS decreased AID of dietary N by 17% relative to control feed restricted pigs (n=10). This result is in agreement with our previous finding using the same model of ISS (Rakhshandeh et al., 2012) and can likely and in part be

associated with increased intestinal endogenous AA losses (EAAL). Several studies have shown increased synthesis and secretion of intestinal mucins in immune challenged pigs (Rakhshandeh et al., 2013; Wilberts et al., 2014; Schweer et al., 2016). Mucins are the main component of the intestinal EAAL, which can influence the estimation of AID for dietary N and AA (Nyachoti et al., 1997). Thus, these findings warrant further studies to evaluate the impact of ISS on intestinal EAAL in growing pigs.

Whole-body protein (N×6.25) retention is the balance between protein synthesis and proteolysis. Reduced protein gain during ISS is brought about by reduced protein synthesis and increased proteolysis in skeletal muscles (Orellana et al., 2004). Previously our team and others have shown that repeated IM injections of increasing amounts of LPS moderately reduced the N retention in growing pigs (Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Litvak et al., 2013; Rakhshandeh et al., 2014). In the current study, however, only a tendency for a decrease in N retention was observed in ISS+ pigs compared to ISS- pigs. This more moderate reduction in N retention can likely be associated with resistance to the LPS-induced decline in skeletal muscle protein synthesis that is often observed in very young animals. Specifically, Orellana et al. (2004) reported that in the absorptive state LPS-induced ISS did not affect the high rate of protein synthesis in the skeletal muscle of nursery pigs, which was in contrast to what was observed in older pigs (Orellana et al., 2004). Additionally, ISS is often associated with increased N excretion per unit of N intake, which leads to a reduced N retention: N intake ratio (Rakhshandeh and de Lange, 2011). In the current study, a strong tendency was observed for a reduction in the N retention: N intake ratio in ISS+ pigs compared to ISS- pigs. This result can likely be attributed to increased AA catabolism and reduced

efficiency of AA utilization for N retention in ISS+ pigs (Rakhshandeh and de Lange, 2011). Taken together, these results suggest that ISS altered dietary N utilization in nursery pigs.

In the current study, an isotope tracer infusion technique was used to quantify the rate of disappearance of free AA, i.e. flux, from the plasma as the general free AA pool in ISS- and ISS+ pigs. We made the following assumptions using the current isotope tracer technique: 1) the transfer of labeled AA (tracer) and unlabeled AA (tracee) between body compartments (i.e. plasma and tissues) occurs indiscriminately, 2) no recycling of the tracer into the plasma pool occurs once the tracer has been incorporated into the body protein or peptide pool, 3) the flux for an AA occurs as an outflow from the plasma pool toward the incorporation of the AA into synthesized protein and other peptides, or by the loss of the AA through catabolism, 4) pigs were in a physiological steady state during the course of the study, i.e. the inflow of free AA into the plasma pool equaled the outflow from the plasma pool. It is of importance to note that the measured flux in this study reflects the rate of AA efflux toward both protein synthesis and catabolism and does not differentiate between these two fluxes (Holtrop et al., 2004; Waterlow, 2006). We hypothesized that changes in plasma free AA kinetics can reflect modification of AA metabolism during ISS.

It is well established that the daily requirements for Lys are determined by protein retention (Möhn et al., 2000; NRC, 2012). It has been shown that ISS does not impact Lys utilization efficiency and changes in Lys requirements reflect changes in body protein gain (Klasing and Barnes, 1988; Williams et al., 1997). In the present study, ISS tended to decrease Lys flux and numerically increased the Lys pool size, suggesting a

reduction in the metabolic need for Lys during ISS. The latter can predominantly be associated with the moderate decrease in N retention, and thus whole-body protein synthesis (Kampman-van De Hoek et al., 2015). Immune system stimulation also tended to decrease Lys release from proteolysis which can be attributed to reduced protein degradation in skeletal muscle (Orellana et al., 2004; Norton and Layman, 2006). These results are in contrast to those of Kampman-van De Hoek et al. (2015), who reported no change in Lys flux in ISS pigs. This contrast is likely due to the lack of effect of the use of complete Freud's adjuvant (CFA)-induced ISS in that study.

Some studies have suggested that BCAA (Leu, Ile, and Val) are essential for proliferation, growth and the normal function of cells of the immune system, primarily lymphocytes (Calder, 2006; Monirujjaman and Ferdouse, 2014). A number of reports have shown beneficial results on immune function and health when supplementing BCAA above daily requirements, while others failed to show a relationship between measures of immune function and BCAA supplementation (Cerra et al., 1983; Hale et al., 2004; Thornton et al., 2006). Nevertheless, in the current study, a concomitant decrease in Ile flux and pool size in ISS+ pigs suggested a reduction in Ile utilization for protein synthesis and/or catabolism. In other words, our findings suggested that the metabolic demand for Ile was decreased during ISS. Decrease in Ile flux due to a reduction in Ile catabolism is a more probable scenario because N retention, and thus protein synthesis, was only slightly affected by ISS in this study. The reduced Ile pool size can likely be associated with the decrease in Ile release from proteolysis. Although not statistically different, the Ile release from proteolysis was decreased by 36% in the present study. Alternatively, the reduction in Ile pool size might be attributed to the reduced

bioavailability of dietary Ile due to reduced ileal digestibility of dietary N. Again, these results are not in agreement with the findings of Kampman-van De Hoek et al. (2015), who reported no change in the flux or pool size of Ile in feed-restricted pigs challenged with CFA (Kampman-van De Hoek et al., 2015). Leucine flux and release from proteolysis was not affected by ISS, suggesting no change in the metabolic need for Leu during ISS. Therefore, increases in the Leu pool size can be associated with Leu intake in the current study and that Leu may have been in excess of its requirement. These findings are in general agreement with findings of Kampman-van De Hoek et al. (2015). Reduced Val release from proteolysis was concomitant with no change in Val flux and pool size in the current study. This result suggested that the pool size of Val was maintained *via* dietary Val intake during ISS and that the dietary supply of Val was sufficient to support protein retention during ISS. Taken together, these results provide no evidence for an increased metabolic demand or requirement for BCAA during ISS. These results are in general agreement with the findings of Rudar et al. (2017), who recently reported no change in N flux, protein synthesis and protein degradation in ISS pigs fed supplemental levels of Leu (Rudar et al., 2017).

Reeds et al. (1994) suggested an increase in the utilization of aromatic AA (Phe, Tyr and Trp) during ISS due to increased turnover of aromatic AA-rich APP during the acute phase response of ISS (Reeds et al., 1994). Other investigators also have suggested an increase in both the metabolic need and dietary requirements for Trp due to enhanced catabolism of Trp during ISS (Melchior et al., 2004; de Ridder et al., 2012). However, in this study flux of two essential aromatic AA, Phe and Trp, toward protein synthesis and catabolism did not increase during ISS. Indeed, ISS reduced the flux and release of Phe

from proteolysis by 37 and 68%, respectively, and had no effect on Phe pool size. These results suggested a significant reduction in metabolic demand for Phe during ISS. The reduction in Phe flux may have occurred as the result of reduced utilization of Phe for protein synthesis, decreased Phe catabolism and /or decline in the synthesis of tyrosine (Tyr) from Phe. The latter is of importance because it has been shown that during ISS pro-inflammatory cytokines downregulate the activity of Phenylalanine-hydroxylase, a key enzyme that catalyzes the synthesis of Tyr from Phe (Capuron et al., 2011). In the present study the lack of change in the pool size of Phe can be attributed to Phe intake and that the intake was sufficient to meet the requirement for Phe during ISS. Taken together, these results suggested that ISS reduced the metabolic need for Phe and had no effect of on needs for Trp.

It has been suggested that requirements for sulfur containing amino acids, i.e. Met + cysteine (Cys), increase during ISS due to the enhanced utilization of Cys for synthesis of APP, glutathione, taurine and other immune system metabolites (Malmezat et al., 2000; Rakhshandeh and de Lange, 2010; Litvak et al., 2013). Rakhshandeh et al. (2010) reported a 26% increase in plasma Cys flux in pigs challenged with LPS (Rakhshandeh et al., 2010). However, in the current study Met flux and pool size was not affected by ISS, suggesting no quantitative change in the metabolic demand for Met during ISS. This result is in agreement with the findings of Kampman-van De Hoek et al. (2015), who reported no changes in Met flux in pigs challenged with CFA. Additionally, in the current study we did not observe a change in Thr flux, pool size and release from proteolysis suggesting no change in the Thr requirement during ISS in nursery pigs. Overall, these results suggest that increased metabolic demand for Met and Thr to support the synthesis

of immune system metabolites was not quantitatively large enough to influence Met and Thr utilization, as determined by measure of flux in the current study.

CONCLUSIONS AND IMPLICATIONS

Collectively, these results suggest that repeated injections of increasing amounts of LPS induced a relatively mild ISS in nursery pigs, altering immune function. Measures of plasma AA kinetics provide a better insight into AA metabolism during ISS, when compared to other measures such as plasma AA concentration or pool size as no association was found between measures of AA flux and that of pool size in the current study. Immune system stimulation, at the level that was induced in this study, reduced the AID of dietary N and tended to reduce N retention, as well as the efficiency of dietary N utilization. The decrease in Phe and Ile flux, as well as a tendency for reduced Lys flux, in ISS pigs may be attributed to a reduction in whole body protein synthesis or decreased catabolism of these AA.

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TABLES AND FIGURES

Table 3.1 Diet composition (as-fed basis) and calculated nutrient contents in diet

Item	Amount
Ingredients, g/kg	
Corn	613
Soy-bean meal	343
Vitamins and minerals pre-mix[1]	20
Limestone	7.0
Dicalcium phosphate	14
Salt	3.0
TiO_2 (marker)	5.0
Calculated nutrient contents, g/kg	
Dry matter, %	90.30
ME, kcal/kg	3323
Crude Protein[2]	181
Lysine	10.4
Methionine	3.0
Methionine + Cysteine	6.0
Threonine	6.7
Tryptophan	2.3
Leucine	16.4
Isoleucine	8.2
Phenylalanine	9.5
Valine	9.1
Calcium	7.3
Phosphorous	4.5

[1]Providing the following amounts of vitamins and trace minerals (per kg of diet): vitamin A, 10075 IU; vitamin D3,1100 IU; vitamin E, 83 IU; vitamin K (as menadione), 3.7 mg; D-pantothenic acid, 58.5 mg; riboflavin, 18.3 mg; choline, 2209.4 mg; folic acid, 2.2 mg; naicin, 73.1 mg; thiamin, 7.3 mg; pyridoxine, 7.3 mg; vitamin B12, 0.1 mg; D-biotin, 0.4; Cu, 12.6 mg; Fe, 100 mg; Mn, 66.8 mg; Zn, 138.4 mg; Se, 0.3 mg; I, 1.0 mg; S, 0.8 mg; Mg, 0.0622%; Na, 0.0004%; Cl, 0.0336%; Ca, 0.0634%, P, 0.003%; K, 0.0036%
[2] Protein and amino acids are Standard Ileal Digestibility (SID) basis.

Table 3.2 Effect of immune system stimulation (ISS) induced by *E. coli* lipopolysaccharide (LPS) on blood parameters in growing pigs[1]

Blood Parameter	ISS-	ISS+	SE	*P* <
n	10	9		
Hematology				
Hemoglobin, g/dl	12.1	11.2	1.33	*0.62*
Hematocrit, %PCV[2]	27.3	30.1	5.49	*0.64*
Blood Chemistry				
Blood Urea Nitrogen, mg/dl	8.7	11.6	1.08	*0.03*
Glucose, mg/dl	89.4	61.7	4.44	*0.01*
Acid/Base				
Na, mmol/L	142.5	140.8	0.93	*0.21*
K, mmol/L	4.2	4.6	0.32	*0.21*
Cl, mmol/L0	103.1	104.2	1.35	*0.51*
HCO_3, mmol/L	26.2	25.3	2.56	*0.79*
Anion gap, mEg/L	12.5	16.1	1.01	*0.02*

[1]The data presented are least square means after controlling for average daily feed intake as a co-variable ± the largest SE standard error (SE) of mean and represents the best estimate of mean that obtained immediately before ISS (ISS-) and 72 h after start of ISS (ISS+). Immune system stimulation was induced by injection of E. coli lipopolysaccharide (30 and 36 µg/kg BW), given 48-h apart.
[2] PCV: packed cell volume

Table 3.3 Effect of immune system stimulation (ISS) on dietary nutrient utilization in growing pigs[1]

Parameter	ISS-	ISS+	SE	*P*-value
n	10	9		
Final body weight, kg	12.3	13.1	0.57	0.26
Nitrogen (N) utilization, mmol/kg BW/d				
N intake	101.9	101.9	1.14	*0.83*
N Excretion	42.6	44.7	3.12	*0.61*
N Retention	61.0	54.4	2.92	*0.10*
N intake: N retention	0.58	0.52	0.03	*0.06*
Nutrient digestibility[2], %				
AID of N	76	54	4.70	*0.01*
ATTD of energy	79	68	5.35	*0.05*

[1]The data presented are least square means after controlling for average daily feed intake as a co-variable ± the largest SE standard error (SE) of mean. Whole-body N balance was conducted for 3 d before ISS (ISS-) and 3 d during ISS (ISS+). Immune system stimulation was induced by injection of *E. coli* lipopolysaccharide (30 and 36 µg/kg BW), given 48-h apart.

[2]AID: Apparent ileal digestibility determined using slaughter technique and titanium dioxide (TiO_2) as an indigestible marker. To determine the AID of N in non-immune challenged pigs (ISS-) a separate group of pigs (n=10) were feed restricted and injected with sterile saline. ATTD: Apparent total tract digestibility.

Table 3.4 Effect of immune system stimulation (ISS) on flux (μmol/kg BW/h) released from protein degradation (μmol/kg BW/h), and pool size (μmol/kg BW) of selected plasma free amino acids (AA)[1 and 2]

		ISS-	ISS+	SE	*P* ≤
n		7	6	-	-
Ile					
	Flux	112	75	11.7	*0.01*
	Proteolysis	47	30	15.3	*0.33*
	Pool size	12	9	1.1	*0.05*
Leu					
	Flux	561	532	64.9	*0.70*
	Proteolysis	452	420	92.5	*0.77*
	Pool size	25	38	5.1	*0.05*
Lys					
	Flux	394	325	31.9	*0.08*
	Proteolysis	285	216	32.1	*0.09*
	Pool size	30	36	7.1	*0.48*
Met					
	Flux	108	111	29.7	*0.92*
	Proteolysis	81	77	32.0	*0.90*
	Pool size	6	6	1.7	*0.83*
Phe					
	Flux	126	79	12.2	*0.01*
	Proteolysis	50	16	10.0	*0.01*
	Pool size	26	23	4.4	*0.66*
Thr					
	Flux	83	76	4.1	*0.19*
	Proteolysis	23	17	4.4	*0.25*
	Pool size	19	15	4.0	*0.46*
Trp					
	Flux	150	138	53.3	*0.86*
	Proteolysis	131	118	34.9	*0.80*
	Pool size	10	22	6.5	*0.14*
Val					
	Flux	185	164	18.2	*0.36*
	Proteolysis	96	54	13.4	*0.04*
	Pool size	16	18	2.5	*0.59*
Gln					
	Flux	275	247	39.6	*0.56*
	Proteolysis	-	-	-	-
	Pool size	19	26	4.4	*0.18*

[1]The data presented are least square means after controlling for average daily feed intake as a co-variable ± the largest SE standard error (SE) of mean. Ten gilts were surgically fitted with venous catheters and feed restricted (550 g/d) on a corn-SBM based diet. Immune system stimulation was induced by injection of E. coli lipopolysaccharide (30 and 36 µg/kg BW), given 48-h apart. N-balances were determined during a 3-d pre-ISS and a 3-day ISS period. At the end of each N-balance period a single dose of [U-13C, U-15N] AA mixture was infused intravenously, and serial blood samples were taken to determine isotopic enrichment. A double exponential model was fitted to the plasma enrichment for each pig and AA, and equation parameters were used to estimate plasma AA flux and pool size.
[2]Proteolysis: Amino acid released from protein proteolysis was calculated as the difference between the AA flux and intake, using the steady-state model of Waterlow (Waterlow, 2006).

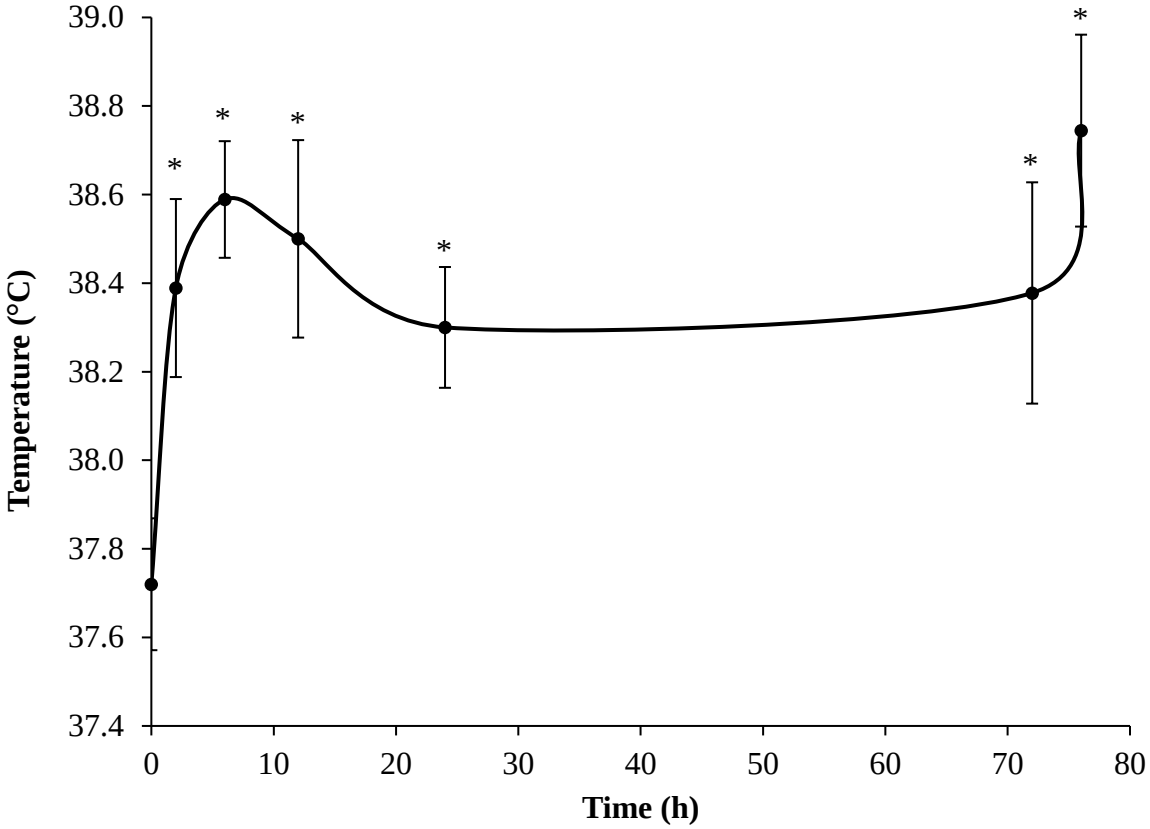

Figure 3.1 Changes in eye temperature (°C ± standard error of mean) of immune system stimulated (ISS) pigs over time. The data presented are least square means ± the largest standard error (SE) of mean and represents the best estimate of mean that obtained immediately before ISS and during 72 h post-ISS. Immune system stimulation was induced by injection of *E. coli* lipopolysaccharide (30 and 36 µg/kg BW), at time 0 and 48 post-ISS.
* indicates significant differences ($P < 0.05$) in eye temperature from time 0.

CHAPTER IV

IMMUNE SYSTEM STIMULATION INDUCED BY PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ALTERS AMINO ACID KINETICS AND DIETARY NITROGEN UTILIZATION IN GROWING PIGS

ABSTRACT

Changes in plasma free amino acid (AA) kinetics reflect the modification of AA metabolism in different metabolic states. Immune system stimulation (ISS) in growing pigs redistributes AA from protein retention towards processes involved in the immune response, thus impacting AA utilization. The aim of the current study was to evaluate the effect of ISS induced by a live pathogen on whole-body nitrogen (N) utilization and the kinetics of plasma free AA. Twenty gilts (Body weight; BW; 9.4 ± 0.9 kg) were surgically fitted with jugular vein catheters, individually housed in metabolism crates, and feed-restricted (550 g/d). Immune system stimulation was induced by intramuscular inoculation of a live field porcine reproductive and respiratory syndrome virus (PRRSv). Whole-body N balance was conducted for 3 d pre-inoculation (ISS-) and 3 d post-inoculation (ISS+). At the end of each N balance period a bolus dose of labeled [U-^{13}C, U-^{15}N]-AA mixture was infused intravenously, followed by serial blood collection for determination of isotopic enrichment. A double exponential model was fitted with plasma enrichment data for each pig and each AA, and equation parameter estimates were used to estimate plasma free AA flux and pool size. Apparent ileal digestibility (AID) of N was determined using the slaughter technique and an indigestible marker. Blood samples were collected before and at 2, 4, 6, 8, and 10 days post inoculation, and assayed for hematology and blood chemistry. Body temperature (BT) was monitored throughout the

study. Blood chemistry, hematology, BT, and serum viral load results indicated that PRRSv inoculation induced effective ISS in pigs (*P<0.05*). Immune system stimulation significantly reduced the ADFI by 21.7 ± 4.58 g/kg BW/d (*P*=0.01). Immune system stimulation had no effect on N retention (*P=0.99*) and the N retention-to-N intake ratio (*P=0.85*) after controlling for ADFI. Apparent ileal digestibility of N was reduced by ISS (*P<0.05*), but had no effect on apparent total tract digestibility of dietary energy (*P*=0.12). Plasma flux (μmol/kg BW/h) for Met and Thr was increased by ISS (*P*<0.05). A strong tendency of increased Val flux was observed in ISS pigs (*P=0.08*). Immune system stimulation increased the pool size for Ile, Leu, Lys, Phe, and Val (*P*<0.05). Collectively, these results suggest that ISS alters the utilization of dietary N and AA flux, as well as pool size in growing pigs. The increase in Thr and Met flux in PRRSv-infected pigs may be associated with enhanced utilization of Met and Thr for the synthesis of immune system metabolites and increased catabolism of these AA. Thus, dietary Met and Thr requirements may increase in health challenged pigs, relative to the requirements for other AA.

Keywords: growing pigs, immune system stimulation, kinetics, PRRSv, plasma amino acid flux

INTRODUCTION

Exposure to chronic sub-clinical or mild-clinical immune system stimulation (ISS) results in altered protein and amino acid (AA) utilization in growing pigs (Williams et al., 1997a; Le Floc'h et al., 2009). The latter results in the redirection of AA away from growth and reproduction towards mounting an immune response (Obled, 2003). Increases in the synthesis of immune system proteins and metabolites, such as acute phase proteins,

glutathione, and immunoglobulins, may require additional needs for specific AA (Reeds and Jahoor, 2001). The latter can impact AA requirements qualitatively (metabolic demand; i.e. AA use) and quantitatively (dietary AA requirements).

Previous studies have suggested that requirements for sulfur containing AA (SAA; Methionine and cysteine), branched chain AA, aromatic AA, threonine (Thr), and glutamine (Gln) increase during ISS (Reeds and Jahoor, 2001; Obled, 2003; Rakhshandeh and de Lange, 2011). Note that these studies were conducted using different models of ISS with some eliciting a more severe immune response than the others, various nutritional approaches, and various methods of measurements, with some studies using plasma AA concentration to interpret the results. However, change in AA metabolism can occur without simultaneous alterations in the concentrations of free AA in the extracellular compartments, including the plasma (Waterlow, 2006). Furthermore, the majority of the studies that evaluated the effect of ISS on AA utilization focused only on one AA at a time, which potentially ignores the possible interactions between AA and their effects on AA needs during ISS. Thus, minimal information is available on the quantitative effect of AA on the utilization of multiple AA simultaneously.

The development of nutritional strategies that can reduce the negative impacts of ISS on pig productivity requires an understanding of the quantitative effects of ISS on AA needs. Plasma free AA flux reflects the amount of free AA that disappears per unit of time from the plasma pool for protein synthesis and catabolism. Changes in plasma free AA flux, thus, can better reflect the modification of AA metabolism in different metabolic states (Waterlow, 2006; Kampman-van De Hoek et al., 2015). Therefore, measuring plasma free AA flux can provide a more accurate insight into the metabolic

changes that are brought about by ISS. The objective of the current study was to evaluate the effects of ISS induced by porcine reproductive and respiratory syndrome virus (PRRSv) on flux and pool size of multiple AA simultaneously in the plasma, as well as on whole-body nitrogen (N)-utilization in growing pigs.

METHODS AND MATERIALS

The experimental protocol was reviewed and approved by the Texas Tech University Animal Care and Use Committee (IACUC approval number 13049-07).

Animals, housing, diet and feeding, and general experimental design

Twenty PIC PRRSv-naïve gilts (Pig Improvement Company North America, TN, USA; initial body weight 9.4 ± 0.9 kg) were obtained from the TTU swine herd, surgically fitted with jugular catheters, individually housed in metabolism crates, and feed-restricted (550g/d) a corn-soy bean meal based diet (ME 14.0 MJ/kg, standardized digestible Lys 10.4 g/kg; Table 4.1). After recovery from surgery, pigs were subjected to a 3 d pre-inoculation (ISS-) and a 3 d post-inoculation (ISS+) N-balance study. At the completion of each N-balance period an isotope tracer study was conducted to determine plasma free AA flux. Daily feed allowance was allocated into two equal feedings per day at 08:00 am and 05:00 pm. Pigs had free access to water throughout the study. Twenty-four hours before isotopic infusion, the feeding intervals were changed to every 4 h to minimize diurnal patterns in AA metabolism. At the end of the study, pigs were euthanized by an intravenous injection of a lethal dose of sodium pentobarbital (FATAL PLUS, Vortech Pharmaceutical, Ltd., Dearborn, MI, USA).

Surgical catheterization and immune system stimulation

Silicon catheters (Micro-Renathane, 0.095 O.D. × 0.066 I.D., Braintree Scientific Inc., Braintree, MA, USA) were surgically inserted into the left and right external jugular veins according to the procedures originally described by Weirich et al., (1970) and modified by de Lange et al., (1989). Gilts were allowed to recover for at least seven days. During recovery, each pig was treated with one dose of penicillin (25,000 units intramuscular, IM), one dose of anti-inflammatory banamine (2.2 mg/kg body weight IM) and one dose of the painkiller buprenorphine (0.01 mg/kg subcutaneously). General health, feed intake and eye temperature of pigs were monitored frequently during the recovery period. Pigs were inoculated IM with a live field PRRSv isolated from pigs in northern Iowa to induce ISS.

Isotopic infusion

At the completion of each N-balance period, a single bolus sterile dose (0.9 ml/kg body weight) of universally labeled [U-^{13}C, U-^{15}N] AA mixture (97-99 atom percent, Cambridge Isotope Laboratories, Tewksbury, MA, USA) suspended in saline was infused *via* one of the indwelled jugular catheters over a 30 second period. The AA mixture (mg/ml sterile saline) contained isoleucine (Ile), 2.3, leucine (Leu), 6.0, lysine (Lys), 10, methionine (Met), 0.8, phenylalanine (Phe), 4.3, threonine (Thr), 1.2, tryptophan (Trp), 3.6 and glutamine (Gln), 2.1.

Observations and sampling

The initial body weight (BW) of each pig was measured prior to entering metabolism crates and final BW was determined at the conclusion of the study. After recovery from surgery, pigs were feed restricted and N-balance was measured for 72

hours before (ISS-) and after (ISS+) inoculation. The post-ISS N-balance measurement was started at day 7 post-inoculation (dpi) to allow for sufficient viremia to develop, as observed and described by Rakhshandeh et al. (2013) using the the same strain of PRRSv in previous studies. Briefly, urine was collected *via* collection trays underneath each crate that funneled urine into tared, lidded buckets containing sufficient amounts of 3 N HCl to maintain urine pH below 3. From each 24-h urine collection, 10% of the urine volume was pooled for each pig at each N-balance period and stored at 4°C until analyzed for total N contents. Feed waste and vomit for each pig was collected, oven dried, cooled in a desiccator and weighed to accurately determine daily feed intake. Fecal samples were collected daily and stored in sealed plastic bags at -20°C until processed further. At the conclusion of the study, fecal samples were thawed, pooled and mixed together per pig and per N-balance period and stored at -20°C.

At the end of each N-balance an isotopic infusion study was conducted. Serial blood samples were then taken before isotopic infusion started and then at time 2.5, 5, 7.5, 10, 15, 20, 30, and 45 minutes after infusion to measure the change in plasma isotopic enrichment of each AA. A 3 ml blood sample was drawn at each time point into a heparinized tube (BD Vacutainer, BD Franklin Lakes, NJ, USA) *via* one of the indwelled catheters. Following the completion of each isotope tracer study, blood samples were centrifuged at 1500 × g for 15 minutes at 4°C. The plasma fraction was then aliquoted and stored at -80°C until further analysis.

Eye temperature was monitored daily during the pre-ISS and post-ISS periods. Thermography of the eye was performed using a FLIR E40 (FLIR® Systems, Inc.,Wilsonville, Oregon) digital camera, as previously described by Petry et al., (2017).

The resolution for each IR image was set at 160 x 120 pixels and the emissivity value was set to the recommended value of 0.98 for biological tissues. Multiple IR pictures were taken approximately 50 cm away from the eye and an average of the best three pictures, in terms of focus and precision, were selected for determination of BT. Infrared pictures were interpreted using FLIR Tools software (FLIR® Systems, Inc., Wilsonville, Oregon). To evaluate the effects of ISS on measures of blood chemistry, hematology, and serum viral load, fresh whole blood samples were collected at 0, 2, 4, 6, 8, and 10 dpi. Samples were collected from jugular catheters and immediately analyzed using an i-STAT Handheld Analyzer (Abaxis Inc., CA, USA) with i-STAT CHEM8+ test cartridges.

Apparent ileal digestibility of dietary N was determined using the slaughter technique and titanium dioxide (TiO_2) as an indigestible marker (Low, 1977). Immediately following the conclusion of the post-ISS isotope tracer study, pigs were euthanized, a ventral abdominal incision was made, the ileocecal junction was located, and the last 150 cm of the small intestine was isolated and clamped to prevent digesta movement. The ileum was then excised and the ileal digesta was gently expelled, collected and stored at -20 °C until further processing. A separate group of gilts (BW11.5 ±0.45 kg; n=9) was feed restricted (550 g/d), treated with sterile saline and used to determine AID of dietary N using the slaughter technique described above.

Analytical Procedures

Fecal and ileal digesta samples were lyophilized and pulverized before analysis for nutrient contents. Nitrogen content of feces, digesta and urine was quantified in duplicate and diet samples were quantified in triplicate using a LECO-Trumac N (Leco

Co., Henderson, NV, USA) analyzer. Dry matter (DM) content of feces, digesta and diet samples were determined by oven drying for 24 hours at 120°C according to the Association of Official Analytical Chemists procedures (AOAC, 1997). Titanium dioxide levels of fecal and diet samples were determined in duplicate and triplicate, respectively, and according to standard Association of Official Analytical Chemists procedures (AOAC, 1997). Samples of the diet and lyophilized feces were analyzed in duplicate for the determination of gross energy (GE) using a 6300 model calorimeter bomb (Parr Instruments, Moline, IL) according to procedures described Association of Analytical Chemists (AOAC, 1990).

Plasma free labeled and unlabeled AA isolation and quantification were achieved using a Phenomenex^{TM} EZ:Faast Amino Acid analysis kit (Torrance, CA, USA) and Gas Chromatography Mass Spectrometry (GC-MS). Before derivatization with a propyl chloroformate derivatizing agent, plasma samples were deproteinized using a 3-KDa centrifugal filter (VWR international, Randor, PA, USA). Samples were then freeze dried, reconstituted in Milli-Q water and gently vortexed until sample residue was completely dissolved. Derivatization of samples was completed according to the Phenomenex^{TM} EZ:Faast Amino Acid analysis kit and according to the manufacturer's instructions. Samples were kept at -20°C until further analysis by GC-MS. Quantification of derivatized unlabeled and labeled AA in standard and plasma samples was achieved by GC-MS (Agilent 6890 GC coupled with an Agilent 5973 mass selective detector). This method employed selective ion monitoring (SIM) to identify and quantify multiple unlabeled and labeled AA simultaneously. The method utilizes differences in mass between labeled and unlabeled AA (McGilvray et al., 2017).

Calculations and statistical analysis

The AID of crude protein (CP; N × 6.25) and GE were calculated using the indicator method and TiO_2 as an indigestible marker. The enrichment of labeled AA in the plasma was expressed as the tracer-to-tracee ratio (TTR). Plasma samples were collected from pigs before each isotopic infusion to determine the possible background enrichment for each AA. The plasma free AA flux was calculated from the change in the enrichment of each isotopically labeled AA in the plasma after the infusion of a bolus dose of universally labeled [U-^{13}C, U-^{15}N]-AA, as described by Holtrop et al., (2004). The following standard double-exponential model was fitted to the TTR for each AA *via* nonlinear least-squares, using the following equation:

$$y = \alpha_1 \, exp(\alpha_2 t) + \alpha_3 \, exp(\alpha_4 t)$$

where *y* is the predicted TTR for each AA in the plasma at time t (min) and α_1, α_2, α_3, and α_4 are parameter estimates. These parameter estimates were used to calculate the flux (Q) of each AA (μmol/kg BW/h) in an individual pig, using the following equation:

$$Q = \frac{D}{\alpha_1/\alpha_2 + \alpha_3/\alpha_4} \times 60$$

where D is the dose of the infused [U-^{13}C, U-^{15}N] AA (mmol/kg BW). In this calculation Q represents the sum of outflow (i.e. efflux) of free AA from the plasma pool toward incorporation of the AA into protein and other peptides (i.e. protein synthesis) and the loss of the AA through catabolism (Holtrop et al., 2004; Waterlow, 2006).

The pool size of for each AA and pig was calculated using the following equation:

$$Pool\ size = \frac{D}{\alpha_1 + \alpha_3}$$

where D is the dose of the infused [U-^{13}C, U-^{15}N] AA (mmol/kg BW), and α_1 and α_3 are parameter estimates that were acquired from fitting a double exponential model fitted to the TTR for each AA and pig (Holtrop et al., 2004).

The rate of inflow (i.e. influx) of AA into the plasma pool from proteolysis was calculated using the steady-state model of Waterlow (2006) in the following equation:

$$Q = I + B = S + U$$

where I and B are the rate of AA inflow into the plasma pool from the diet (standardized ileal digestible AA) and proteolysis, respectively. S and U represent the rate of AA outflow toward whole-body protein synthesis and catabolism, respectively. All values are expressed as μmol/kg BW/h.

Statistical analysis was carried out using SAS software version 9.4 (SAS Institute, NC, USA). Normality and homogeneity of variances was confirmed using the Univariate procedure (PROC UNIVARIATE). Outliers were determined as any value that differed from the treatment mean by ± 2 standard deviation. Data were analyzed in a complete randomized design with health status as fixed effects and pig within crate as a random effect using Mixed procedure (PROC MIXED). Average daily feed intake (ADFI) was used as a co-variate for determining the effect of ISS on measured parameters and when appropriate (P>0.10), a reduced model was used. For parameters such as BT that were measured over time, repeated measurements analysis of variance was used. An appropriate covariance structure was selected for analyses by fitting the model with the structure, which provided the 'best' fit, based on Akaike information criterion (AIC) and Schwarz Bayesian criterion (BIC). Tukey-Kramer was used for multiple comparisons test. Values are reported as least square means with their standard errors (SE). Treatment

effects were considered significant at P≤0.05. A tendency towards a significant difference between treatment means was considered at P≤0.10.

RESULTS

General Observations

All pigs were naïve to PRRSv before inoculation (Figure 4.2), showed signs of good health and readily consumed experimental diets after recovery from surgery and before initiation of post-ISS period. The ISS+ pigs displayed clinical signs of disease such as lethargy and fever after inoculation. It is important to note that infection with PRRSv elicited a more severe response in some pigs relative to others. Immune system stimulation significantly reduced the ADFI by 21.7 ± 4.58 g/kg BW/d (P=0.01). Data from 4 pigs were excluded from the study due to a severe immune response to PRRSv. Due to dysfunctional catheters the isotope tracer study was conducted on 15 ISS- and 6 ISS+ pigs. Analyzed diet nutrient contents were generally in agreement with anticipated calculated values, which were derived from feed ingredient composition and nutrient levels in feed ingredients according to Swine NRC (2012). In the interpretation of results and for calculation of AA intake for individual pigs, calculated diet nutrient contents were used.

Measures of Immune function, hematology, blood chemistry, and viral load

The effect of ISS on blood parameters are presented in Table 4.2. Immune system stimulation increased the levels of creatinine and anion gap (AnionGap; P<0.01) and reduced levels of hemoglobin (Hb; P<0.01). Hematocrit, blood urea nitrogen (BUN), glucose, and other measures of electrolyte balance were not affected by ISS (P>0.05)

after controlling for ADFI. The viral load (genomic copy/uL) of ISS+ pigs is shown in Figure 4.1. The viral load began to increase at 2 dpi, reached a maximum peak at 4 dpi, and remained elevated for the rest of the study ($P<0.05$). Inoculation with PRRSv increased eye temperature by 0.9°C ($P<0.01$) relative to ISS- pigs (Figure 4.2). Eye temperature began to rise 24 h post inoculation, reached its maximum peak 2 dpi and remained elevated for the duration of the study. [ ]

Body weight, N utilization and nutrient digestibility

The effects of ISS on growth performance and N utilization are presented in Table 4.3. The statistical difference between ISS- and ISS+ pigs was detected after controlling for ADFI. No significant differences between ISS groups existed in the final BW of the pigs ($P>0.05$). Immune system stimulation had no effect on total N excretion, retention, or the N intake: N retention ratio ($P>0.05$). PRRSv infection reduced the AID of dietary N ($P<0.01$), but had no effect on the ATTD of energy.

Plasma free Amino acid kinetics

The effects of ISS on plasma free AA flux, proteolysis, and pool size after controlling for ADFI are presented in Table 4.4. Immune system stimulation increased the plasma flux of Met and Thr ($P<0.01$) and tended to increase Val ($P=0.08$), but it did not affect plasma flux of other AA ($P>0.05$). During the ISS+ period, pigs had an increased release of Met and Thr ($P<0.01$) and tended to increase the release of Phe ($P=0.09$) from protein breakdown relative to the ISS- period. The Ile, Leu, Lys, Trp, and Val release from protein breakdown was not affected by ISS ($P>0.05$). The pool size for Ile, Lys, Leu, Phe, and Val were increased during the ISS+ period ($P<0.05$)

DISCUSSION

The main objective of the present study was to evaluate the effects of ISS induced by porcine reproductive and respiratory syndrome virus (PRRSv) on flux and pool size of multiple AA simultaneously in the plasma, as well as on whole-body nitrogen (N)-utilization in growing pigs. In the current study, pigs were inoculated IM with a live, field PRRSv to induce ISS. Porcine reproductive and respiratory syndrome virus-induced ISS elicited a febrile response, as determined by eye temperature. This technique was used as a less invasive measure of the febrile response, which indicates the effectiveness of ISS, and correlates to core BT (Petry et al., 2017). Eye temperature remained elevated for the entire course of the ISS+ period, suggesting persistent, elevated core BT, an indicator of a systemic immune response (Petry et al., 2017). The pro-inflammatory cytokine IL-1β serves as the main endogenous pyrogen in initiating the febrile response and works synergistically with IL-6 and TNF-α (Johnson, 1997). Therefore, an increase in eye temperature suggests that ISS in the current study may have been mediated by pro-inflammatory cytokines. We also observed a significant increase in the levels of creatinine in the blood during PRRSv infection. Measures of creatinine, an indicator for increased skeletal muscle proteolysis, can be associated with a reduced efficiency of AA utilization and protein accretion during ISS (Hosten, 1990). In the current study, we observed a decrease in the level of Hb during ISS. Long term and severe inflammatory responses in various species are characterized by reduced Hb levels. The latter occurs as a result of pro-inflammatory cytokine mediated interference with reticuloendothelial iron transport, decreased sensitivity of erythron to erythropoietin, and reduced erythrocyte survival during severe inflammation (Goyette et al., 2004). We also observed an increase in AnionGap during the ISS+ period. Importantly, ISS did not impact the plasma

electrolyte balance as indicated by the levels of Na^+, K^+, and Cl^-. Therefore, higher AnionGAP in ISS+ pigs most likely indicates an increased level of lactic acid in the blood, which would reflect a shift from aerobic to anaerobic glycolytic metabolism. This shift usually occurs during the acute phase response of systemic ISS (De Backer, 2003), further indicating the presence of ISS. These metabolic changes during ISS are often characterized by reduced blood glucose levels during the post-absorptive state, due to enhanced glucose uptake by immune cells as their preferred source of energy (Kominsky et al., 2010; Delmastro-Greenwood and Piganelli, 2013; Pearce and Pearce, 2013). However, in the current study, ISS had no effect on blood glucose levels, since our pigs were in the absorptive state when blood samples were collected. Collectively, these results indicated that inoculation with a live, field PRRSv induced an effective ISS in our study and is in agreement with other studies employing the same model of disease (Mastromano et al., 2013; Rakhshandeh et al., 2013a).

Immune system stimulation is commonly associated with reduced voluntary feed intake (Williams et al., 1997b). Based on the experimental design in this study pigs were feed restricted in an attempt to separate the effect of N feed intake from that of the immune system on N and AA utilization. However, PRRSv infection resulted in a relatively severe response that further reduced the ADFI during the post-ISS period. Therefore, in this study feed intake was used as a co-variate when analyzing the effect of PRRSv on N and AA utilization. In analysis of co-variance, we assumed that a linear relationship between the co-variate (ADFI) and the responses (measures of N and AA utilization) existed.

In the current study the effect of ISS on N intake, retention and urinary excretion can solely be explained by reduced feed intake since after controlling for ADFI the effect of ISS on these parameters became insignificant. Apparent ileal digestibility of N, however, was reduced by ISS. An increase in the synthesis and secretion of intestinal mucins has been reported in pigs under immunological challenge (Rakhshandeh et al., 2013b; Wilberts et al., 2014; Schweer et al., 2016). Mucins represent a major component of basal and specific endogenous AA loss (EAAL) in pigs, accounting for up to 60% of total intestinal N losses in pigs (Moughan, 1999; NRC, 2012). Therefore, the reduced AID in ISS pigs can likely be associated with increased EAAL in these pigs. However, further studies are warranted to investigate the impact of ISS on intestinal EAAL in growing pigs. Infection with PRRSv also had no impact on the N retention: N intake ratio, a measure of the efficiency of dietary N utilization for whole body N retention (Rakhshandeh et al., 2014).

Plasma free AA flux reflects the amount of free AA that disappears per unit of time from the plasma pool toward protein synthesis and catabolism, and does not distinguish between the two fluxes. Therefore, changes in plasma free AA flux, in combination with the pool size, can better reflect the modification of AA metabolism in different metabolic states, than merely plasma AA concentrations alone (Waterlow, 2006; Kampman-van De Hoek et al., 2015). It is now well known that changes in AA metabolism can occur without simultaneous alterations in the concentrations of plasma free AA, since AA concentrations can be maintained from AA influx (i.e. AA intake and protein breakdown) and AA efflux (i.e. protein synthesis and AA oxidation). Even so, measures of plasma AA concentrations have previously been used to evaluate the effects

of ISS of AA metabolism (Melchior et al., 2004; Melchior et al., 2005; Le Floc'h et al., 2006). In the current study PRRSv increase the plasma flux of Met, suggesting an increase in the utilization of Met during ISS. This finding is consistent with several studies that have reported an enhanced utilization of SAA for the synthesis of important immune system metabolites such as the intracellular antioxidant, glutathione, as well as taurine, during ISS (Rakhshandeh et al., 2010; Rakhshandeh and de Lange, 2010). Rakhshandeh et al. (2014) also showed a substantial increase in SAA maintenance requirements, suggesting an increase in dietary SAA requirements per unit of PD (Rakhshandeh et al., 2014). Furthermore, Litvak et al. (2013) reported a substantial increase in the optimal dietary Met to SAA ratio in growing pigs for whole body PD, suggesting a preferential use of Met from a dietary SAA source during ISS (Litvak et al., 2013).

Similarly, PRRSv infection increased the plasma free Thr flux in ISS+ relative to ISS- pigs. This result is likely due to the role of Thr in the synthesis of Thr-rich immune system metabolites, such as immunoglobulins, acute phase proteins and intestinal mucins (Faure et al., 2007; Rémond et al., 2009; Rakhshandeh and de Lange, 2011). Considering that Thr accounts for 16-20% of crude mucin, increased Thr utilization for mucin synthesis is probably the pathway that is the greatest quantitative contributor to increased Thr utilization during ISS (Lien et al., 1997; Faure et al., 2006; Rémond et al., 2009). This idea is supported by a study conducted by Rakhshandeh *et al*. (2013) where they observed a 1.6-fold increase in the transcription of mucin 2, a major proteinous component of mucus, in the small intestine of ISS pigs (Rakhshandeh et al., 2013b). Additionally, mucins are major components of the basal and specific EAAL in pigs, accounting for up to 60% of total intestinal N losses (Moughan, 1999; NRC, 2012) which further supports the reduced AID of

dietary nitrogen seen in the PRRSv pigs. Increased synthesis and secretion of mucins may impact dietary Thr requirements in growing pigs during ISS and warrant further study.

In the current study, we also observed a greater release of Met and Thr from protein breakdown in ISS+ relative to ISS- pigs, even without a concomitant change in N retention. Measures of N retention using traditional N balance methods reflect the total whole-body N retention and does not distinguish between N retained for lean accretion versus synthesis of other body proteins and non-protein nitrogenous compounds. Therefore, increases in the release of Met and Thr from protein breakdown could be associated with prioritization of these AA for the synthesis of immune system metabolites during ISS (Kampman-van De Hoek et al., 2015). An increase in pool size for Ile, Leu, and Lys was observed in PRRSv challenged pigs with no associated changes in the flux or release of these AA from protein breakdown. This finding suggests that the oxidation of these AA may be reduced during ISS. If so, reduced oxidation may be a compensatory mechanism that spares these AA from catabolism when AA for protein synthesis is limiting (Kampman-van De Hoek et al., 2015). We also observed an increase in the pool size of Phe, which is likely explained by the increased release of Phe from protein breakdown and reduced utilization of this AA during ISS.

CONCLUSIONS AND IMPLICATIONS

Collectively, results of this study suggested that the intramuscular inoculation with PRRSv elicited an effective but relatively severe immune system stimulation. Immune system stimulation reduced apparent ileal digestibility of dietary N but had no effect on other aspects of N utilization. Immune system stimulation increased the plasma

Met and Thr flux and increased the release of these AA from protein breakdown. Increased flux of these AA may be largely attributed to an increase in their utilization for the synthesis of immune system metabolites and may impact Met and Thr dietary requirements. These findings warrant further studies that directly evaluate the impact of ISS on the dietary requirements of Met and Thr in growing pigs.

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TABLES AND FIGURES

Table 4.1 Diet composition (as-fed basis) and calculated nutrient contents in diet

Item	Amount
Ingredients, g/kg	
Corn	613
Soy-bean meal	343
Vitamins and minerals pre-mix[1]	20
Limestone	7.0
Dicalcium phosphate	14
Salt	3.0
TiO_2 (marker)	5.0
Calculated nutrient contents, g/kg	
Dry matter, %	90.30
ME, kcal/kg	3323
Crude Protein[2]	181
Lysine	10.4
Methionine	3.0
Methionine + Cysteine	6.0
Threonine	6.7
Tryptophan	2.3
Leucine	16.4
Isoleucine	8.2
Phenylalanine	9.5
Valine	9.1
Calcium	7.3
Phosphorous	4.5

[1]Providing the following amounts of vitamins and trace minerals (per kg of diet): vitamin A, 10075 IU; vitamin D3,1100 IU; vitamin E, 83 IU; vitamin K (as menadione), 3.7 mg; D-pantothenic acid, 58.5 mg; riboflavin, 18.3 mg; choline, 2209.4 mg; folic acid, 2.2 mg; naicin, 73.1 mg; thiamin, 7.3 mg; pyridoxine, 7.3 mg; vitamin B12, 0.1 mg; D-biotin, 0.4; Cu, 12.6 mg; Fe, 100 mg; Mn, 66.8 mg; Zn, 138.4 mg; Se, 0.3 mg; I, 1.0 mg; S, 0.8 mg; Mg, 0.0622%; Na, 0.0004%; Cl, 0.0336%; Ca, 0.0634%, P, 0.003%; K, 0.0036%
[2] Protein and amino acids are Standard Ileal Digestibility (SID) basis.

Table 4.2 Effect of immune system stimulation (ISS) induced by porcine reproductive and respiratory syndrome virus (PRRSv) on blood parameters in growing pigs[1]

Blood Parameter	ISS-	ISS+	SE	*P* <
n	20	16		
Hematology				
Hemoglobin, g/dl	12.1	8.1	0.95	*0.01*
Hematocrit, % PCV[2]	24.8	22.9	2.65	*0.60*
Blood Chemistry				
Blood Urea Nitrogen, mg/dl	11.8	11.0	0.74	*0.49*
Glucose, mg/dl	78.2	84.8	5.68	*0.38*
Creatinine, mg/dl	0.68	0.86	0.05	*0.01*
Acid/Base				
Na, mmol/L	142.2	142.2	1.04	*0.96*
K, mmol/L	4.0	4.2	0.14	*0.32*
Cl, mmol/L0	102.9	102.0	1.44	*0.56*
Anion gap, mEg/L	13.0	16.6	1.11	*0.01*

[1]The data presented are least square means after controlling for average daily feed intake as a co-variable ± the largest standard error (SE) of mean and represents the best estimate of mean that were obtained immediately before ISS (ISS-) and 2, 4, 6, 8, and 10 days post inoculation with an intramuscular injection of a live field porcine reproductive and respiratory syndrome virus (ISS+).
[2] PCV: packed cell volume

Table 4.3 Effect of immune system stimulation (ISS) on dietary nutrient utilization in growing pigs[1]

Parameter	ISS-	ISS+	SE	*P*-value
n	20	16		
Final body weight, kg	13.5	14.1	1.00	0.67
Nitrogen (N) utilization, mmmol/kg BW/d				
Intake	75.4	75.4	0.09	*0.67*
Excretion	40.5	36.6	2.49	*0.27*
Retention	38.2	38.1	3.88	*0.99*
N intake: N retention	0.52	0.50	0.12	*0.85*
Nutrient digestibility[2], %				
AID of N	76	54	4.70	*0.01*
ATTD of energy	79	81	0.74	*0.12*

[1]The data presented are least square means after controlling for average daily feed intake as a co-variable ± the largest standard error (SE) of mean. Whole-body N-balance was conducted for 3 d before ISS (ISS-) and 3 d during ISS (ISS+). Immune system stimulation was induced by injection of a live porcine reproductive and respiratory syndrome virus.

[2]AID: Apparent ileal digestibility determined using slaughter technique and titanium dioxide (TiO_2) as an indigestible marker. To determine the AID of N in non-immune challenged pigs (ISS-) a separate group of pigs (n=10) were feed restricted and injected with sterile saline. ATTD: Apparent total tract digestibility.

Table 4.4 Effect of immune system stimulation (ISS) on flux (μmol/kg BW/h), release from protein degradation (μmol/kg BW/h), and pool size (μmol/kg BW) of selected plasma free amino acids (AA)[1 and 2]

		ISS-	ISS+	SE	*P* ≤
n		15	6	-	-
Ile					
	Flux	112	126	27.7	*0.67*
	Proteolysis	47	50	22.1	*0.90*
	Pool size	12	18	2.2	*0.02*
Leu					
	Flux	561	673	68.2	*0.21*
	Proteolysis	452	510	58.8	*0.45*
	Pool size	25	40	5.6	*0.03*
Lys					
	Flux	394	453	34.7	*0.21*
	Proteolysis	285	335	22.5	*0.12*
	Pool size	30	60	3.5	*0.01*
Met					
	Flux	108	228	23.7	*0.01*
	Proteolysis	81	216	25.4	*0.01*
	Pool size	6	14	6.3	*0.19*
Phe					
	Flux	126	150	21.4	*0.36*
	Proteolysis	50	82	15.2	*0.09*
	Pool size	26	58	8.5	*0.01*
Thr					
	Flux	83	130	16.0	*0.01*
	Proteolysis	23	128	28.6	*0.01*
	Pool size	19	33	9.9	*0.19*
Trp					
	Flux	150	117	44.8	*0.60*
	Proteolysis	131	110	34.9	*0.67*
	Pool size	10	16	5.0	*0.39*
Val					
	Flux	185	216	13.0	*0.08*
	Proteolysis	96	116	17.3	*0.43*
	Pool size	16	31	4.1	*0.01*
Gln					
	Flux	275	272	70.1	*0.96*
	Proteolysis	-	-	-	-
	Pool size	19	24	5.8	*0.40*

[1]The data presented are least square means after controlling for average daily feed intake as a co-variable ± the largest SE standard error (SE) of mean. Twenty gilts were surgically fitted with venous catheters and feed restricted (550 g/d) on a corn-SBM based diet. Immune system stimulation was induced by injection of porcine reproductive and respiratory syndrome virus. N-balances were determined during a 3-d pre-ISS and a 3-day ISS period. At the end of each N-balance period a single dose of [U-13C, U-15N] AA mixture was infused intravenously, and serial blood samples were taken to determine isotopic enrichment. A double exponential model was fitted to the plasma enrichment for each pig and AA, and equation parameters were used to estimate plasma AA flux and pool size.
[2]Proteolysis: Amino acid released from protein proteolysis was calculated as the difference between the AA flux and intake, using the steady-state model of Waterlow (Waterlow, 2006).

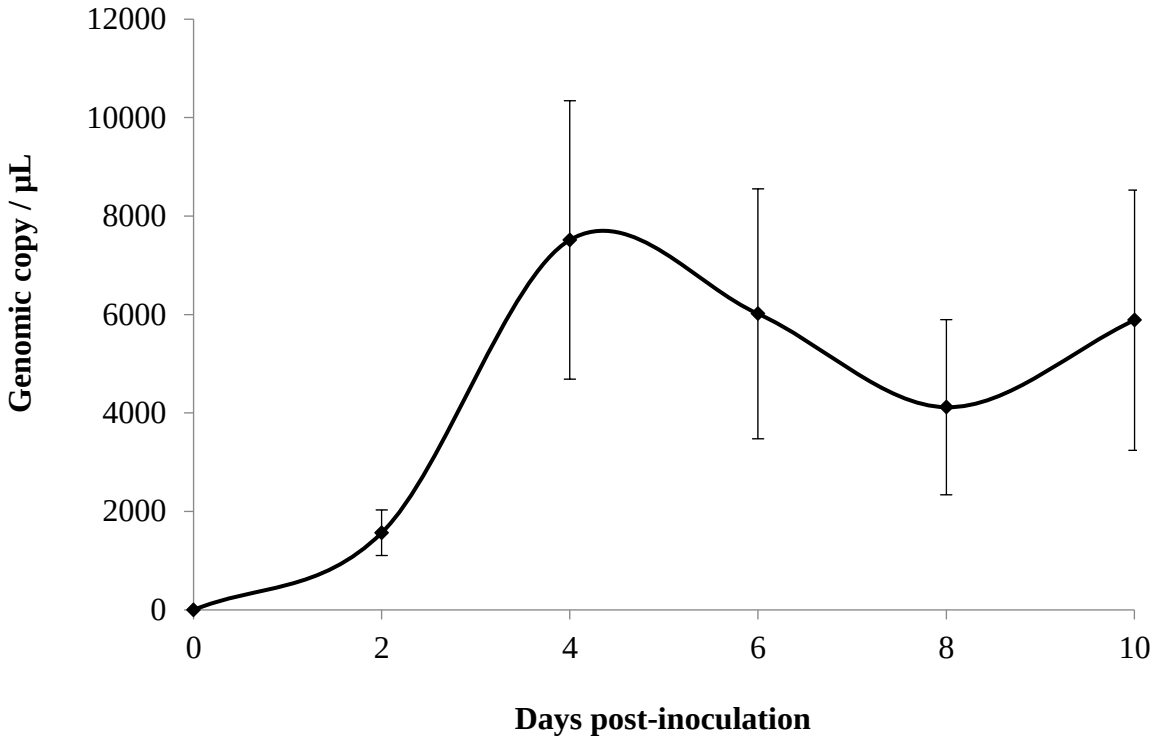

Figure 4.1 Changes in serum viral load (genomic copy/µL) in pigs challenged with porcine reproductive and respiratory syndrome virus (PRRSv) during the 10 days post inoculation (dpi).

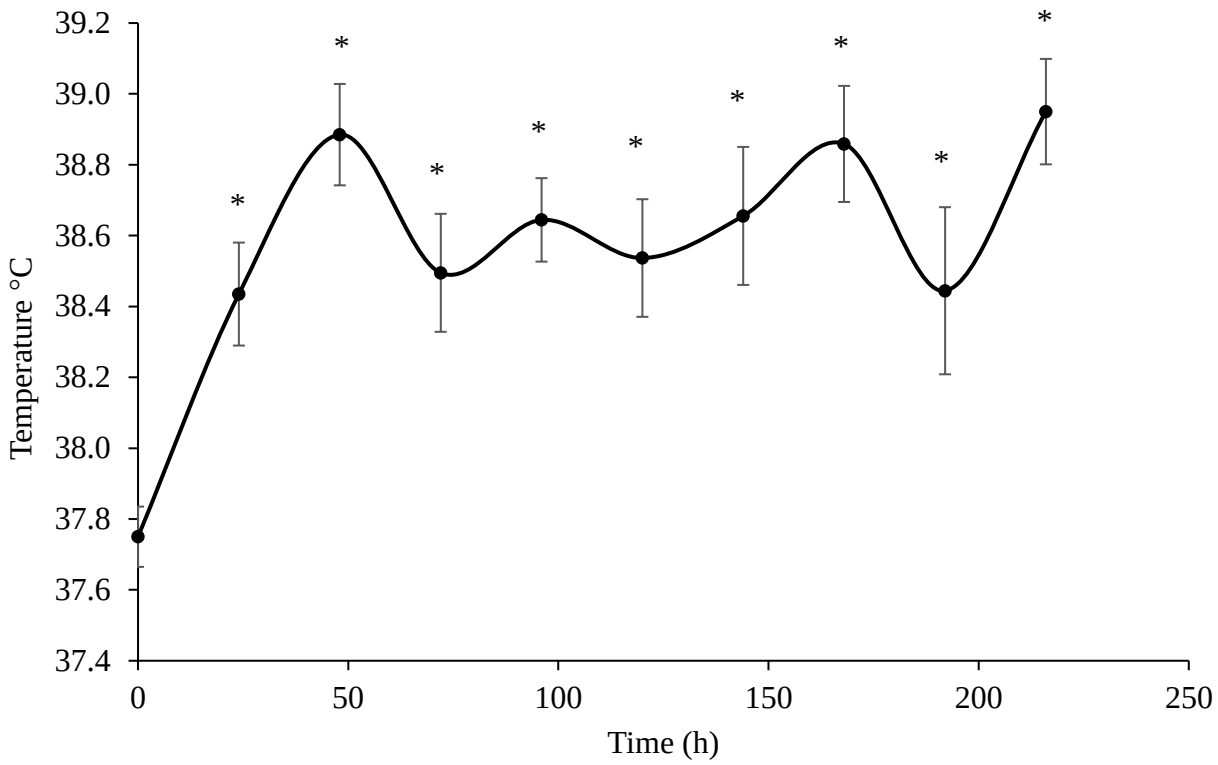

Figure 4.2 Changes in eye temperature (°C ± standard error of mean) of immune system stimulated (ISS) pigs challenged with porcine reproductive and respiratory syndrome virus over time. The data presented are least square means ± the largest standard error (SE) of mean and represents the best estimate of mean that obtained immediately before ISS and during 72 h post-ISS.
* indicates significant differences ($P < 0.05$) in eye temperature from time 0.

CHAPTER V

IMMUNE SYSTEM STIMULATION INCREASES DIETARY THREONINE REQUIREMENTS FOR PROTEIN DEPOSITION IN GROWING PIGS

ABSTRACT

The impact of immune system stimulation (ISS) on the utilization of dietary threonine (Thr) for whole-body protein deposition (PD) was evaluated in growing pigs. Thirty-nine gilts (initial BW 32 ± 2.1 kg) of commercially relevant genetics were individually housed in metabolism crates and fed one of six experimental diets in which Thr was the first limiting among other amino acids (AA). Diets were formulated to contain 70, 90, and 110 % of daily Thr requirements, which were estimated based on the potential of each ISS group for PD. Following adaptation to the experimental diets, pigs from each dietary treatment group were injected with either increasing amounts of *Escherichia coli* lipopolysaccharide (ISS+; 25 and 35 µg/kg BW) or saline (ISS-). Injections were given 48-h apart and whole-body nitrogen balance was measured for 72-h following the first injection. Body temperature (BT) was monitored and blood samples were collected 24-h post-ISS and evaluated for measures of blood chemistry. Blood chemistry and BT results indicated an effective ISS in pigs ($P<0.03$). Threonine intake increased PD in a linear fashion in both ISS groups ($P<0.01$). The marginal efficiency of SID Thr utilization for PD, represented by the slope, was not affected by ISS. However, ISS substantially increased the extrapolated maintenance SID Thr requirements, represented by the intercept at zero PD (ISS- vs. ISS+, -11.2 vs. -56.3 SE 13.2; $P<0.05$). Collectively, our results indicated that the physiological changes associated with ISS

increased the dietary SID Thr requirements for PD due to an increase in maintenance requirements.

Keywords: Immune system stimulation, threonine requirement, growing pigs

INTRODUCTION

Immune system stimulation (ISS) alters amino acid (AA) utilization in part by repartitioning AA towards process involved in an immune response, thus impacting AA requirements both quantitatively and qualitatively (i.e. AA ratio to lysine; Lys; Reeds and Jahoor, 2001; Obled, 2003; Rakhshandeh and de Lange, 2011). The metabolism of threonine (Thr) during ISS has gained attention due to its role in the synthesis of Thr-rich immune system metabolites, such as immunoglobulins, acute phase proteins (APP) and, in particular, intestinal mucins (Faure et al., 2007; Rémond et al., 2009; Rakhshandeh and de Lange, 2011). Rakhshandeh *et al*. observed a 1.6-foldincrease in the expression of mucin 2, a major proteinous component of mucus, at the transcriptional level in the small intestine of ISS pigs (Rakhshandeh et al., 2013). This result was significant because mucins are major components of the basal and specific endogenous AA losses (EAAL) in pigs, accounting for up to 60% of total intestinal nitrogen (N) losses (Moughan, 1999; NRC, 2012). Considering that Thr accounts for 16-20% of crude mucin, increased synthesis and secretion of mucins may impact Thr requirements in growing pigs during ISS (Lien et al., 1997). This idea was supported by a study from Stuart *et al.* that reported a 1.5-foldincrease in plasma Thr flux, a measure of Thr utilization, in pigs challenged with porcine reproductive and respiratory syndrome virus (Stuart et al., 2015). This study also reported a significant reduction in apparent ileal digestibility of dietary N, which can be associated with an increase in EAAL in these pigs. Thus, increases in the utilization of Thr for synthesis of immune

system metabolites contribute, in part, to reduced lean tissue accretion during ISS and may impact sustainable pork production. Despite these findings, no study, to our knowledge, has directly evaluated the impact of systemic ISS on the specific measures of Thr requirements in growing pigs, such as the requirements for maintenance and protein deposition (PD). Therefore, the current study evaluated the effects of ISS on dietary Thr requirements for PD in growing pigs. We hypothesized that ISS increases Thr utilization for biological processes involved in an immune response, therefore impacting dietary Thr requirements for PD.

METHODS AND MATERIALS

All methods and procedures for this experiment were approved by the Texas Tech University (TTU) Animal Use and Care Committee. All animal trials were conducted at the TTU Swine Research Center (New Deal, TX, USA).

General experimental design, housing, treatments and diets

A total of thirty-nine PIC pigs (Pig Improvement Company North America, TN, USA; body weight 22 ±2.9 kg) were obtained from the TTU breeding herd, housed in floor pens in an environmentally controlled facility and fed a commercial grower corn-SBM based diet *ad libitum* prior to being transferred to the metabolism crates. Pigs were transferred to stainless steel adjustable metabolism crates (initial body weight, BW; 32 ± 2.1 kg) 4-d before starting the N-balance study and assigned to one of six experimental diets in which Thr was the first limiting among other AA. Following adaptation to the experimental diets for 4-d, pigs from three dietary treatment groups were injected intramuscularly (IM) with increasing amounts of *Escherichia coli* lipopolysaccharide

(ISS+; 25 and 35 µg/kg BW; LPS strain 055:B5; Sigma Aldrich; n=24), while pigs from the other three dietary treatment groups were received sterile saline (ISS-; n=15). Injections were given 48-h apart and whole-body nitrogen balance (N-balance) was measured for 72-h following the first injection. The initial dose of LPS was increased by 29% for the subsequent injection to overcome tolerance to LPS (Rakhshandeh and de Lange, 2012). Pigs in the ISS- group received IM injections of sterile saline to account for the stress induced by injection. Blood samples were collected 24-h post-ISS by jugular venipuncture and assayed for measures of blood chemistry. Infrared (IR) thermography was performed to monitor body temperature (BT) during the course of the study. For each ISS group, diets were formulated based on the nutrient requirements that were predicted using the NRC Swine model and based on performance variables determined in previous studies (NRC, 2012; Rakhshandeh et al., 2012; Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014; Stuart et al., 2016). The performance variables included a mean initial BW of 25 kg, an average daily feed intake (ADFI) of 1.3 kg/d for ISS- and 0.9 kg/d for ISS+, and PD of 100 and 60 g/d for ISS- and ISS+, respectively (NRC, 2012). Within each ISS group three levels of dietary standardized ileal digestible (SID) Thr were evaluated 70% (L1), 90% (L2), and 110% (L3) of established Thr requirements for maximum PD. In all of the experimental diets, ratios of essential SID AA to Thr (AA:Thr) exceeded the recommendations of NRC Swine by 20% (Table 5.1 and 5.2; NRC, 2012). Diets L1 and L3 for each ISS group were prepared in single batches and L2 diets were achieved by blending equal parts of L1 and L3. A constant ratio between dietary N and Lys, as well as dietary N and Thr was maintained across all experimental diets. All experimental diets were isoenergetic and

contained 14 MJ/kg of metabolizable energy. The experimental diets were fortified with vitamins and minerals to surpass the requirements recommended by NRC Swine (Table 1; NRC, 2012). Titanium dioxide (0.25% TiO_2; Bobette Boyer Hall Technologies, St. Louis USA.) was included in all diets as an indigestible marker to determine nutrient digestibility. Pigs were fed either 1250 or 860 g/d divided into two equal meals, which was fed twice per day, and allowed free access to water. The feed allowance was slightly feed restricted (5%) to avoid feed refusal and minimize feed waste.

Observations, sampling and chemical analysis

Eye temperature was measured at times 0, 2, 4, 6, 8, 10, 24, 48, 50 and 72 hours post LPS injection using a FLIR E40 digital camera (FLIR Systems, Inc., Wilsonville, OR) as described by Petry et al. (2017). The IR resolution for each picture was set at the maximum resolution of 160 ×120. The emissivity value used was 0.98, which is the recommendation for biological tissues. Multiple pictures were taken and an average of three pictures with the best quality, in terms of focus and precision, were analyzed. Nitrogen balance was conducted as described by Rakhshandeh et al. (2014). In brief, urine was collected via collection trays underneath each crate that funneled urine into tared lidded buckets containing sufficient amounts of 3 N HCl to maintain urine pH below 3. From each 24-h urine collection, 10% of the urine volume was pooled for each pig and stored at 4°C until analyzed for total N contents. Feed waste and vomit for each pig was collected in trays beneath each feeder, oven dried, cooled in a desiccator and weighed to accurately determine daily feed intake. Fecal samples were collected twice daily and stored in sealed bags at -20°C until further processing. At the end of the N-balance period, feces were thawed, pooled for each pig, thoroughly homogenized using a

mixer and kept at -20°C until subsampled and lyophilized. Blood samples were collected by external jugular venipuncture 24-h post initial LPS or saline injection. To evaluate blood chemistry parameters, fresh whole blood samples were immediately analyzed using an i-STAT Handheld Analyzer (Abaxis Inc., CA, USA) with an i-STAT CHEM8+ cartridge.

Fecal samples were lyophilized, pulverized and thoroughly mixed before further analysis. Titanium dioxide and DM of fecal and feed samples were determined in triplicate and according to standard Association of Official Analytical Chemists procedures (AOAC, 1997). The nitrogen content of feces, urine and feed samples were quantified in triplicate using a LECO-Trumac N analyzer (Leco Co., NV, USA) according to standard Association of Official Analytical Chemists procedures (AOAC, 1997). Amino acid analysis of the diets was performed at the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia, MO, USA. Dietary AA were determined by cation-exchange chromatography (cIEC-HPLC) coupled with post-column ninhydrin derivatization and quantitation (AOAC, 1997).

Calculations and statistical analysis

Apparent total tract digestibility of dietary N, which was determined using the indicator technique and TiO_2 as an indigestible marker, was used to compute fecal N excretion. Nitrogen retention (g/d) was calculated as balance between the net N intake (feed N minus feed waste N) and N excretion (fecal plus urinary N). Protein deposition was calculated as retained N × 6.25 (Rakhshandeh et al., 2014). The following linear regression model was used to estimate maintenance SID Thr requirements and the marginal efficiency of SID Thr utilization for PD for each ISS group:

$$\text{PD (g/d)} = a + b \times \textit{(SID Thr intake)}$$

The regression coefficient *a* was used to estimate extrapolated maintenance SID Thr requirements (g/d), i.e. SID Thr intake when PD equals zero, calculated as $-a/b$. The regression coefficient *b* (g PD/g SID Thr intake) represents the marginal efficiency of utilization of SID Thr intake for PD (Fuller et al., 1989; Rakhshandeh et al., 2014).

Statistical analysis was carried out using SAS software version 9.4 (SAS Institute, NC, USA). Normality and homogeneity of variances was confirmed using the Univariate procedure (PROC UNIVARIATE). Outliers were determined as any value that differed from the treatment mean by ± 2 standard deviation. Data were analyzed in a complete randomized block design with diet and block as fixed effects and pig within crate as a random effect using Mixed procedure (PROC MIXED). Dietary Thr intake was used as a co-variate for determining the effect of ISS on measures of blood chemistry and BT and when appropriate ($P>0.10$), a reduced model was used. For parameters such as BT that were measured over time, repeated measurements analysis of variance was used. An appropriate covariance structure was selected for analyses by fitting the model with the structure, which provided the 'best' fit, based on Akaike information criterion (AIC) and Schwarz Bayesian criterion (BIC). Tukey-Kramer was used for multiple comparisons test. To evaluate the effect of SID Thr intake on measures of dietary N utilization, a polynomial orthogonal contrast was tested. Regression procedure (PROC REG) was used to test the differences in regression parameters (a and b) between ISS- and ISS+ groups when PD was regressed on SID Thr intake. Values are reported as least square means with their standard errors (SE). Treatment effects were considered significant at $P\leq0.05$.

A tendency towards a significant difference between treatment means was considered at $P \leq 0.10$.

RESULTS

General observations

All pigs readily consumed experimental diets and showed signs of good health prior to the study. The ISS+ pigs displayed clinical signs of disease such as lethargy, fever and vomiting. The vomitus and feed waste collected amounted to less than 2% of the feed allowance. Data from one pig was excluded from the study due to a severe immune response to LPS injection. Analyzed dietary nutrient contents fell within anticipated values ($\pm$15 %) that were derived from NRC Swine, 2012 (NRC, 2012). Calculated values of dietary contents were used for the interpretation of results.

Measures of immune function, hematology and blood chemistry

The main effects of ISS on measures of immune function, hematology and blood chemistry are presented in Table 5.3. Immune system stimulation increased eye temperature by 0.62 ±0.117 °C ($P<0.01$). Relative to ISS- pigs, blood urea nitrogen (BUN) and creatinine levels, as well as anion gap (AnionGAP), were higher in ISS+ pigs ($P<0.03$). Hemoglobin (Hb), hematocrit (HCT) and blood glucose levels were not affected by ISS ($P>0.10$).

Growth performance and N utilization

The effects of ISS on growth performance and N utilization are presented in Table 5-4. No significant differences between ISS groups existed in initial BW (32 ±2.2 kg; $P>0.20$). Final BW was lower in the ISS+ group compared to the ISS- group, as

determined by a multiple range comparison test (average of 33 vs. 36 ±1.2 kg; $P<0.04$). Daily dry matter intake (DMI) was affected by daily dietary allowance ($P<0.01$), but not by ISS ($P>0.15$). As anticipated, increasing the daily Thr allowance increased the SID Thr intake in a linear fashion in both ISS groups ($P<0.01$). An increase in SID Thr intake improved the ATTD of N in ISS+ pigs linearly ($P=0.02$), but it did not affect the ATTD of N in ISS- pigs ($P=0.70$). In both ISS groups, dietary N intake increased linearly with SID Thr intake ($P<0.01$). Higher SID Thr intake increased the urinary N excretion in ISS- pigs in a linear fashion ($P<0.04$), while it had no effect on urinary N excretion in ISS+ pigs ($P>0.69$). No significant difference was observed in urinary N excretion between ISS groups ($P>0.20$). Total N excretion (i.e. urine and feces) linearly increased with SID Thr intake in ISS- pigs ($P<0.01$). Dietary SID Thr intake had no effect on total N excretion in ISS+ pigs ($P>0.30$). In both ISS groups, SID Thr intake increased the daily PD in a linear fashion ($P<0.01$). Nitrogen retention-to-N intake (N retention: N intake) was significantly lower (38%) in ISS+ pigs as determined by a multiple range comparison (P=0.03). Nitrogen retention: N intake was not different among levels of Thr intake in the ISS- group ($P=0.42$); however, the ratio was significantly lower in pigs with the lowest level of Thr intake in the ISS+ group ($P<0.01$). The comparison of linear regression parameters (Table5) seen when relating PD (g/d) to SID Thr intake (g/d) indicated that the marginal efficiency of SID Thr utilization for PD, represented by the slope, was not affected by ISS ($P=0.10$). However, ISS substantially increased the extrapolated maintenance SID Thr requirements, represented by the intercept at zero PD in Fig. 5-1 ($P<0.05$; Table 5-5).

DISCUSSION

The main objective of the present study was to evaluate the impact of mild clinical ISS on dietary SID Thr requirements for PD in growing pigs. Since the efficiency of the first limiting AA for PD can be affected by the dietary AA balance, we used serial dilutions of dietary protein to generate dietary treatments that avoided confounding Thr intake levels with changes in dietary AA balance (Wang and Fuller, 1989). In this study Thr was first limiting AA in all diets, which was achieved by ensuring that the ratio of all other essential SID AA to Thr exceeded the recommendations of NRC Swine (NRC, 2012). Previous studies have confirmed that when the ratio of essential AA to a target essential AA exceed the optimum ratio for maximum PD by 20%, the target AA become first limiting among all other essential AA (Wang and Fuller, 1989; de Ridder et al., 2012; Rakhshandeh et al., 2014). Therefore, in the present study, the observed change in PD was a function of the change in available Thr and not the overall supply of essential AA. In this study, we did not pair-feed the control (ISS-) animals since reduced feed intake can influence the maintenance requirements and efficiency of the utilization of the first limiting AA for PD in non-immune challenged pigs (Fuller et al., 1989; NRC, 2012). Therefore, pigs in both the ISS- and ISS+ groups were fed 1250 and 860 g/d, respectively. This level of daily feed allowance for the ISS+ pigs was determined using previous studies that observed a constant quantitative decrease in feed intake with the same model of ISS (Rakhshandeh et al., 2012; Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014; Stuart et al., 2016).

In the current study, ISS was induced by repeated IM injections of increasing amounts of LPS. We previously have shown that this model of ISS induces a relatively mild immune response that mimics the impact of mild clinical chronic disease on protein

and AA utilization (Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014). In the present study, LPS-induced ISS elicited a febrile response, as determined by eye temperature. This technique was used as a less invasive measure of the febrile response, which indicates the effectiveness of ISS, and correlates to core BT (Petry et al., 2017). Eye temperature remained elevated for the entire course of the study in ISS+ pigs, suggesting persistent, elevated core BT, an indicator of a systemic immune response (Petry et al., 2017). The pro-inflammatory cytokine IL-1β serves as the main endogenous pyrogen in initiating the febrile response and works synergistically with IL-6 and TNF-α (Johnson, 1997). Therefore, an increase in eye temperature suggests that ISS in the current study may have been mediated by pro-inflammatory cytokines. We also observed a significant increase in the levels of urea nitrogen and creatinine in the blood of ISS+ pigs. Measures of BUN, the primary metabolite derivative of AA catabolism, and blood creatinine, an indicator for increased skeletal muscle degradation, have been associated with a reduced efficiency of AA utilization and protein accretion during ISS (Hosten, 1990). The latter is consistent with our findings, in which we observed a significant reduction in N retention: N intake ratio, and suggest a decrease in the efficiency of dietary N utilization for whole-body PD in ISS+ pigs (Rakhshandeh et al., 2014). We also observed an increase in AnionGAP in ISS+ pigs. Importantly, ISS did not impact the plasma electrolyte balance that was calculated from levels of Na^+, K^+, and Cl^- (data not shown). Therefore, higher AnionGAP in ISS+ pigs most likely indicates an increased level of lactic acid in the blood, which would reflect a shift from aerobic to anaerobic glycolytic metabolism. This shift usually occurs during the acute phase response of systemic inflammation (De Backer, 2003), further indicating the presence of

ISS. These metabolic changes during ISS are often characterized by reduced blood glucose levels during the post-absorptive state, due to enhanced glucose uptake by immune cells as their preferred source of energy (Kominsky et al., 2010; Delmastro-Greenwood and Piganelli, 2013; Pearce and Pearce, 2013). However, in the current study, ISS had no effect on blood glucose levels, since our pigs were in the absorptive state when blood samples were collected. Long term and severe inflammatory responses in various species are characterized by reduced Hb levels and HCT. The latter occurs as a result of pro-inflammatory cytokine mediated interference with reticuloendothelial iron transport, decreased sensitivity of erythron to erythropoietin, and reduced erythrocyte survival during severe inflammation (Goyette et al., 2004). The lack of effect of ISS on Hb levels and HCT in our study is likely because only a mild inflammatory response was stimulated by our model of ISS. Collectively, these results indicated that repeated injection of increasing amounts of LPS induced an effective ISS in our study.

Reduced protein gain and increased N loss are characteristics of an immune response (Reeds and Jahoor, 2001; Obled, 2003). Based on the experimental design in the current study, pigs in each ISS group were fed to their estimated requirements, thus mitigating the potentially confounding effect lower SID Thr intake on PD. However, reduced PD in the ISS+ pigs can be attributed, predominantly, to hyper-activation of the immune system, since N retention per unit of N intake was significantly lower in these animals compared to ISS- pigs. In previous studies using the same model of ISS, we have shown that while PD was reduced in ISS+ pigs, it remained unaffected over time in pair-fed ISS- pigs (de Ridder et al., 2012; Rakhshandeh et al., 2014). The efficiency of utilization of the first limiting AA becomes reduced at the levels required for maximum

PD (Williams et al., 1997; Möhn et al., 2000). Therefore, in the present study, the marginal efficiency of dietary SID Thr utilization for PD was determined at levels of Thr intake that were below those needed to maximize PD in each ISS group. The marginal efficiency of utilization of Thr for PD, represented by the slope, was numerically higher in ISS+ pigs than ISS- pigs, but it did not reach the levels of statistical significance (1 g of additional SID Thr intake supported 19.5 ±2.6 and 28.0 ±3.9 g/d PD in the ISS- and ISS+ pigs, respectively; P=0.10). The efficiency of SID Thr utilization in ISS- pigs was probably affected by the energy intake being slightly limiting in this group, especially at L3[(28)]. These results are in general agreements with the findings of Fuller et al. who used N-balance to estimate the marginal efficiency of Thr intake for PD (Fuller et al., 1989). According to NRC Swine (NRC, 2012), whole-body protein contains approximately 3.8% Thr in non-immune challenged pigs. Assuming that this percentage remained unchanged in ISS+ pigs, the mean efficiency of utilization of SID Thr intake for Thr retention in PD can be estimated at 0.90 for both ISS groups. This value is much higher than estimates suggested by NRC Swine and de Lange *et al.* (de Lange et al., 2001; NRC, 2012) and can be attributed predominantly to the systemic overestimation of PD values when using conventional N-balance methods (Fuller et al., 1989; de Lange et al., 2001). However, the above assumption may require further verification as we and others have shown that ISS can alter the AA profile of whole-body PD (Breuillé et al., 2006; Rakhshandeh et al., 2008).

The daily requirement for Thr represents the sum of the requirements for maintenance functions and for PD (Fuller et al., 1989; NRC, 2012). In the current study, the relationship between PD and SID Thr intake generated a higher estimate of

maintenance SID Thr requirements in the ISS+ pigs than in the ISS- pigs (1.9 ±0.40 g/d; using a common partial efficiency of Thr utilization of 23.8 for the ISS- and ISS+; average b coefficients in Table 4). This higher estimate directly contributes to the observed impact of ISS on PD at all levels of dietary Thr intake. Maintenance requirements for absorbed Thr serve to replace gut endogenous and integument Thr losses, replace losses due to the minimum plus inevitable catabolism of Thr, and the use of Thr for synthesis of immune system metabolites (Fuller et al., 1989; Hahn and Baker, 1995; Rakhshandeh and de Lange, 2011). Among these, increased Thr utilization for mucin synthesis, a major component of gut EAAL, is likely the pathway that is the greatest quantitative contributor to increased Thr requirements during ISS (Faure et al., 2006; Rémond et al., 2009). Threonine-rich mucins are the major component of mucus produced by Brunner's glands and goblet cells, which are found in the epithelial lining of the intestine and the respiratory tract. Greater numbers of goblet cells are found in the large intestine than in the small intestine (Kim and Ho, 2010). Therefore, when evaluating the effects of ISS on gut endogenous Thr losses, and thus Thr requirements, the contribution of the large intestine to total intestinal Thr loses must be considered. Overall, the observed increase in the Thr requirement for maintenance in the current study agrees with reports from other studies that showed enhanced Thr utilization at intestinal tissue and whole-body levels during ISS (Faure et al., 2003; Faure et al., 2006; Dharmani et al., 2009; Rémond et al., 2009). Collectively, our results suggest that an increase in the dietary SID Thr requirements for PD during ISS is due to increased extrapolated Thr maintenance requirements, which is probably due to enhanced utilization of Thr for the synthesis of mucins and other immune system metabolites.

CONCLUSIONS AND IMPLICATIONS

Repeated injection with increasing amounts of LPS elicited effective immune system stimulation, allowing evaluation of dietary threonine utilization in growing pigs. Immune system stimulation increased dietary standardized ileal digestible threonine requirements for protein deposition by increasing the maintenance requirements for threonine in growing pigs at a fixed level of energy intake. Thus, immune challenged pigs require higher levels of threonine in their diets to maintain PD levels similar to those during non-immune challenged states. Increased maintenance requirements during ISS may be largely attributed to the enhanced utilization of absorbed threonine for synthesis of immune system metabolites, particularly mucins. These findings warrant further studies to directly evaluate the impact of ISS on endogenous amino acid losses from both the small and large intestines in growing pigs.

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TABLES AND FIGURES

Table 5.1 Ingredient composition and nutrient contents of experimental diets

	Treatment			
	ISS-		ISS+	
	L1[1]	L3[2]	L1[1]	L3[2]
Ingredient composition, (g/kg as-fed basis)				
Corn	823	656	860	716
Soybean meal	121	290	86.5	232
Hydrogenated vegetable fat	10	10	10	10
Lysine HCl	7.9	12.4	6.8	10.7
DL-Methionine	2.3	3.6	1.96	3.08
L-Threonine	4.0	6.3	3.53	5.55
L-Tryptophan	1.4	2.1	1.19	1.86
Limestone	9.6	8	9	7.5
Dicalcium phosphate	8.6	7.1	8	6.8
Salt	5	5	5	5
Vitamin and mineral premix[2]	15	15	15	15
Titanium dioxide	2.5	2.5	2.5	2.5
Calculated nutrient content (g/kg as-fed basis)				
Metabolizable energy MJ/kg	13.76	13.77	13.79	13.79
Crude protein (N × 6.25)	106.3	166.9	93.1	146.4
Calcium	6.1	6.1	5.6	5.6
STTD P[3]	2.8	2.9	2.6	2.7

[1]Thirty-nine gilts (initial BW 32 ±2.1 kg) were fed one of six experimental diets in which Thr was the first limiting among other AA. Gilts were injected with either *E. coli* lipopolysaccharide (immune system stimulated, ISS+) or saline (ISS-). Diets were formulated to contain 70% (level 1, L1), 90% (L2), and 110% (L3) of daily standardized ileal digestible (SID) Thr requirements, which were estimated based on the potential of each ISS group for PD according to the NRC Swine model(NRC, 2012). Diets L1 and L3 for each ISS group were prepared in single batches and L2 diets were achieved by blending equal parts of L1 and L3.

[2]Provided the following amounts of vitamins and trace minerals (per kg of diet): vitamin A, 10075 IU; vitamin D3,1100 IU; vitamin E, 83 IU; vitamin K (as menadione), 3.7 mg; D- pantothenic acid, 58.5 mg; riboflavin, 18.3 mg; choline, 2209.4 mg; folic acid, 2.2 mg; naicin, 73.1 mg; thiamin, 7.3 mg; pyridoxine, 7.3 mg; vitamin B12, 0.1 mg; D-biotin, 0.4; Cu, 12.6 mg; Fe, 100 mg; Mn, 66.8 mg; Zn, 138.4 mg; Se, 0.3 mg; I, 1.0 mg; S, 0.8 mg; Mg, 0.0622%; Na, 0.0004%; Cl, 0.0336%; Ca, 0.0634%, P, 0.003%; K, 0.0036%.

[3]Standardized total tract digestible phosphorous.

Table 5.2 Formulated and analyzed total amino acid (AA) content of experimental diets (%; as-fed basis)[1 and 2]

	ISS-				ISS+			
	L1 Formulated	L1 Analyzed	L3 Formulated	L3 Analyzed	L1 Formulated	L1 Analyzed	L3 Formulated	L3 Analyzed
Isoleucine	0.52	0.54	0.87	0.85	0.44	0.45	0.75	0.75
Leucine	1.26	1.22	1.75	1.68	1.16	1.11	1.58	1.52
Lysine	0.89	0.94	1.40	1.38	0.77	0.79	1.21	1.26
Methionine	0.28	0.27	0.46	0.39	0.24	0.21	0.40	0.36
Cysteine	0.25	0.25	0.35	0.32	0.23	0.21	0.32	0.29
Phenylalanine	0.63	0.63	1.01	0.95	0.56	0.53	0.88	0.84
Threonine	0.50	0.51	0.78	0.78	0.44	0.43	0.69	0.68
Tryptophan	0.16	0.16	0.25	0.24	0.14	0.12	0.22	0.21
Valine	0.61	0.59	0.97	0.90	0.54	0.50	0.85	0.81

[1]Thirty-nine gilts (initial BW 32 ±2.1 kg) were fed one of six experimental diets in which Thr was the first limiting among other AA and injected with either *E.coli* lipopolysaccharide (immune system stimulated, ISS+) or saline (ISS-). Diets were formulated to contain 70% (level 1, L1), 90% (L2), and 110% (L3) of daily standardized ileal digestible (SID) Thr requirements, which were estimated based on the potential of each ISS group for PD according to the NRC Swine model (NRC, 2012). Diets L1 and L3 for each ISS group were prepared in single batches and L2 diets were achieved by blending equal parts of L1 and L3.
[2]Analysis of the amino acid contents for the diets was performed at the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia, MO, USA.

Table 5.3 Main effects of immune system stimulation (ISS) on eye temperature and blood parameters in growing pigs[1, 2]

Parameter	ISS-	ISS+	SE	*P*-value
Number of pigs	15	23		
Eye temperature, °C	38.4	39.0	0.08	*0.01*
Hematology				
Hemoglobin, g/dL	10.8	9.5	1.19	*0.33*
Hematocrit, % PCV[3]	31.8	27.9	3.51	*0.34*
Blood chemistry				
Blood Urea Nitrogen, mg/dL	4.7	24.2	7.61	*0.03*
Glucose, mg/dL	97.4	100.3	4.79	*0.74*
Creatinine	1.1	2.6	0.67	*0.05*
Acid/Base				
AnionGAP, mEg/L	20.4	22.6	0.59	*0.03*

[1]Data are the least square means ± the largest standard error of mean (SE). Pigs were injected with either increasing amounts of *E. coli* lipopolysaccharide (ISS+; 25 and 35 µg/kg BW) or saline (ISS-), given 48-h apart.
[2]Results for eye temperature represent the best estimate of mean obtained during ISS based on the repeated-measures ANOVA. Infrared thermography was used to monitor eye temperature at 0, 2, 4, 6, 8, 10, 24, 48, 50, and 72 hour post-ISS. Hematology, blood chemistry and acid/base data was obtained 48 h post-ISS.
[3]PCV: packed cell volume

Table 5.4 Effects of immune system stimulation (ISS) and standardized ileal digestible (SID) threonine (Thr) intake on final body weight (BW) and dietary nitrogen utilization in growing pigs[1]

	Treatment							
Health status	ISS-			ISS+			SE	*P* <
Dietary Thr level	L1	L2	L3	L1	L2	L3		
n	5	5	5	8	7	8	-	-
Final BW, kg	35	37	37	33	34	33	1.18	*0.01*
Dry matter intake, kg/d	1.09	1.10	1.10	0.68	0.75	0.70	0.047	*0.01*
SID Thr intake, g/d	4.4	5.6	6.9	2.4	3.4	3.9	0.24	*0.01*
ATTD[2] of N, %	70.2	76.5	73.8	56.7	69.4	68.9	3.96	*0.01*
N utilization, g/d								
N intake	23.0	29.7	36.4	13.0	18.1	20.4	1.28	*0.01*
Urinary N excretion	4.5	6.5	7.4	5.7	6.4	5.8	1.02	*0.28*
Total N excretion	11.3	13.5	16.9	11.1	11.9	12.1	0.95	*0.01*
PD[3], g/d	72.9	101.3	121.7	11.8	38.7	52.1	5.77	*0.01*
N retention: N intake	0.51	0.54	0.54	0.15	0.34	0.41	0.044	*0.01*

[1]Data are the least square means ± the largest standard error of mean (SE). Thirty-nine gilts (initial BW 32 ±2.1 kg) were fed one of six experimental diets in which Thr was the first limiting among other AA. Diets were formulated to contain 70% (level 1, L1), 90% (L2), and 110% (L3) of daily SID Thr requirements, which were estimated based on the potential of each ISS group for PD according to the NRC Swine model(NRC, 2012). Pigs were injected with either increasing amounts of *E. coli* lipopolysaccharide (ISS+; 25 and 35 µg/kg BW) or saline (ISS-), given 48-h apart. Whole-body N-balance was measured for 72-h.
[2]ATTD: apparent total tract digestibility of dietary N. The indicator technique was used to determine dietary N digestibility using titanium dioxide as an indigestible marker.
[3]PD: Protein (N × 6.25) deposition.

Table 5.5 Impact of immune system stimulation (ISS) on parameters representing the linear relationship between standardized ileal digestible (SID) Threonine (Thr) intake and protein deposition (PD) in growing pigs [1 and 2]

	ISS-	ISS+	SE	*P*
Intercept (PD at 0 SID Thr intake)	-11.2	-56.3	13.20	*0.05*
Slope (g PD/g SID Thr intake)	19.547	28.0	3.94	*0.10*
R^2	0.81	0.71		

[1]Data of regression analysis using one-slope model: $y = a + b(x)$, where y is the PD (g/d), a (intercept) can be used for mathematical estimation of extrapolated maintenance requirement ($-a/b$), b (slope) represents the marginal efficiency of Thr utilization for PD and x is the daily Thr intake (g/d) when SAA intake is below the requirements for maximum PD.

[2]Thirty-nine gilts (initial BW 32 ±2.1 kg) were fed one of six experimental diets in which Thr was the first limiting among other AA. Diets were formulated to contain 70 (level 1, L1), 90 (L2), and 110 (L3) % of daily SID Thr requirements that were estimated based on the potential of each ISS group for PD according to NRC Swine model(Ref). Pigs were injected with either increasing amounts of *E. coli* lipopolysaccharide (ISS+; 25 and 35 µg/kg BW) or saline (ISS-), given 48-h apart, while measuring whole-body N-balance for 72-h.

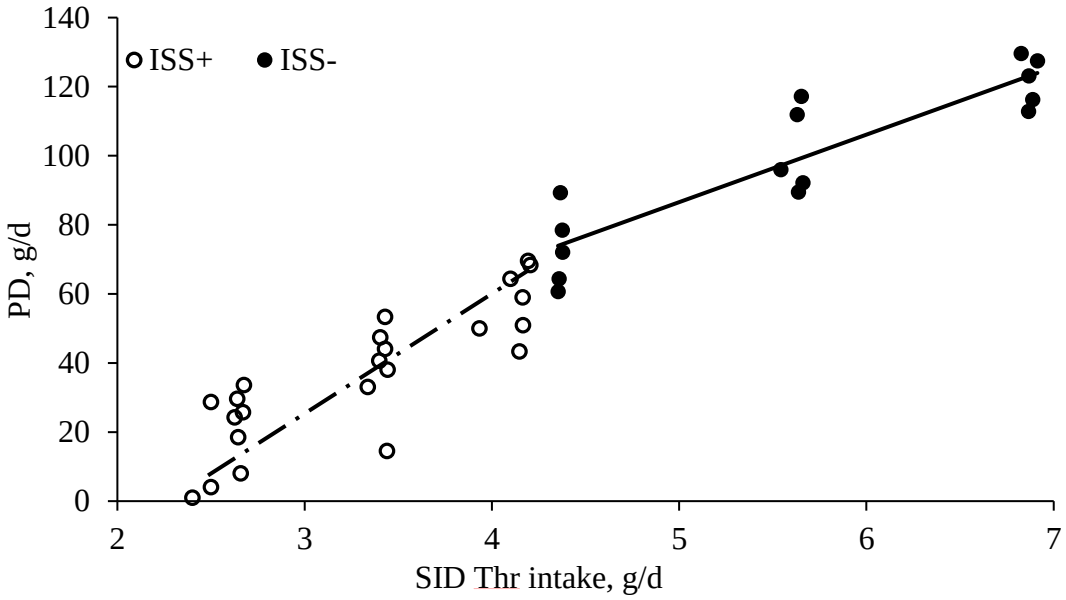

Figure 5.1 Effects of immune system stimulation (ISS) and standardized ileal digestible threonine (SID Thr) intake on whole-body protein deposition (PD) in growing pigs. Thirty-nine gilts (initial BW 32 ±2.1 kg) were fed one of six experimental diets in which Thr was the first limiting among other AA. Diets were formulated to contain 70 (level 1, L1), 90 (L2), and 110 (L3) % of daily SID Thr requirements that were estimated based on the potential of each ISS group for PD according to NRC Swine model(Ref). Pigs were injected with either increasing amounts of *E. coli* lipopolysaccharide (ISS+, ○; 25 and 35 µg/kg BW) or saline (ISS-,●) , given 48-h apart, while measuring whole-body N-balance for 72-h.

CHAPTER VI

IMPACT OF IMMUNE SYSTEM STIMULATION ON WHOLE-BODY PROTEIN METABOLISM AND MUSCLE FIBER CHARACTERISTICS

ABSTRACT

The impact of immune system stimulatiofn (ISS) on whole-body nitrogen (N) metabolism and muscle fiber characteristics was evaluated in growing pigs. Twelve gilts (initial BW 31 ± 4.8 kg) of commercially relevant genetics were individually housed in metabolism crates and fed one of two experimental diets in which a constant ratio between dietary N to standardized ileal digestible (SID) lysine was maintained across both diets (2.7g/d). Diets were formulated based on the potential of each ISS group for protein deposition. Following adaption to the experimental diets, pigs from each dietary treatment group were administered a single oral dose of ^{15}N-glycine and injected with either increasing amounts of *Escherichia coli* lipopolysaccharide (ISS+; 25 and 35 µg/kg BW) or saline (ISS-). Injections were given 48-h apart and whole-body nitrogen flux was measured for 72-h following the first injection. Body temperature (BT) was monitored and blood samples were collected 24-h post-ISS and evaluated for measures of blood chemistry. Following the 72-h collection period, pigs were euthanized with a lethal dose of sodium pentobarbital and muscle samples were collected from *longissimus dorsi* (LD), *psoas major* (PM), *semitendinosus (*ST), and *serratus ventralis* (SV) for measures of fiber type characteristics. Blood chemistry and BT results indicated an effective ISS in pigs (P<0.03). Immune system stimulation significantly reduced whole-body protein synthesis (10.77 vs. 7.12 g N/kg BW$^{0.60}$/d; SE 0.44), degradation (9.41 vs. 6.64 g N/kg BW$^{0.60}$/d; SE 0.04), and retention (1.38 vs. 0.58 g N/kg BW$^{0.60}$/d; SE0.08) and increased the protein

synthesis to retention ratio (7.61 vs. 12.26; SE 1.4) in ISS+ pigs relative to ISS- pigs (P<0.01). Furthermore, ISS showed a general decrease in skeletal muscle nuclei and fiber cross-sectional area and a shift from myosin heavy chain (MHC)-IIX towards MHC-I fibers (P<0.05). Collectively, these results suggest that ISS suppresses whole-body protein synthesis and degradation and impacts the efficiency of whole-body protein turnover. Furthermore, ISS induces atrophy in the skeletal muscle and favors a slow-twitch oxidative fiber type composition.

Keywords immune system stimulation, muscle fibers, pigs, protein metabolism

INTRODUCTION

Pigs reared in commercial settings are continuously exposed to disease causing pathogens that impact the profitability and sustainability of pig production. Immune system stimulation (ISS) and subsequent release of pro-inflammatory cytokines reduce the overall rate of whole-body protein deposition (PD), negatively impacting the pigs productivity and efficiency of nutrient utilization for growth (Johnson, 1997; Obled, 2003; de Ridder et al., 2012; Rakhshandeh et al., 2014). The latter results from alterations in whole-body protein and amino acid (AA) metabolism, in which AA are partitioned away from the peripheral tissues towards visceral organs (Le Floc'h et al., 2004). This shift prioritizes AA, for utilization in gluconeogenesis and the enhanced synthesis of acute phase proteins and other immune system metabolites (Reeds and Jahoor, 2001). Thus, ISS causes reduced protein synthesis and increased protein degradation in skeletal muscle and increased visceral protein synthesis (Breuillé et al., 1998; Lang et al., 2007). Immune system stimulation induced decrease in skeletal muscle protein synthesis likely occurs by decreasing the synthesis of both myofibrillar and sarcoplasmic proteins,

preferentially in skeletal muscle primarily composed of fast-twitch glycolytic fibers (Vary and Kimball, 1992; Orellana et al., 2004; Lang et al., 2007; Lang and Frost, 2007; Orellana et al., 2007).

Although the rates of protein synthesis in various individual tissues have been reported (Breuillé et al., 1994; Breuillé et al., 1998; Bruins et al., 2000), little information is available on rates of body protein turnover at the whole animal level, especially under immunological stress. Furthermore, limited information is available on the effects of ISS on muscle fiber characteristics. Insight on the impact of ISS on protein and AA metabolism at the whole animal level could help in determining the metabolic costs of hyperactivation of the immune system. Therefore, the specific goal of this study was to determine the impact of ISS and protein intake on the rate of whole-body protein turnover (i.e. synthesis and degradation) and impact on muscle fiber characteristics of various muscle types in growing pigs.

METHODS AND MATERIALS

All methods and procedures for this experiment were approved by the Texas Tech University (TTU) Animal Use and Care Committee. All animal trials were conducted at the TTU Swine Research Center (New Deal, TX, USA).

General experimental design, housing, treatments and diets

A total of twelve PIC pigs (Pig Improvement Company North America, TN, USA) were used as previously described in Chapter V. Briefly, pigs were transferred to stainless steel adjustable metabolism crates (initial body weight 31 ± 4.8 kg) 4-d prior to the experimental period and assigned to one of two dietary treatments. Following the 4-d

adaption period, and 2 h prior to ISS, pigs were administered a single oral dose of ^{15}N-glycine (10 mg/kg BW; Glycine ^{15}N, 98%, Cambridge Isotope Laboratories, Inc.). Pigs were injected intramuscularly (IM) with either increasing amounts of *Escherichia coli* lipopolysaccharide (ISS+; 25 and 35 µg/kg BW; LPS strain 055:B5; Sigma Aldrich; n=7), or sterile saline (ISS-; n=5). Injections were given 48-h apart and whole-body nitrogen (N) flux was measured for 72-h using the end-product method, following the first injection. The initial dose of LPS was increased by 29% for the subsequent injection to overcome tolerance to LPS (Rakhshandeh and de Lange, 2012). Pigs in the ISS- group received IM injections of sterile saline to account for the stress induced by injection. Blood samples were collected 24 h post ISS by jugular venipuncture and assayed for measures of blood chemistry. Infrared (IR) thermography was performed to monitor body temperature (BT) during the course of the study

For each ISS group, diets were formulated based on the nutrient requirements that were predicted using the NRC Swine model and based on performance variables determined in previous studies (NRC, 2012; Rakhshandeh et al., 2012; Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014). The performance variables included a mean initial BW of 25 kg, an average daily feed intake (ADFI) of 1.3 kg/d for ISS- and 0.9 kg/d for ISS+, and PD of 100 and 60 g/d for ISS- and ISS+, respectively (NRC, 2012). Within each ISS group, levels of dietary standardized ileal digestible (SID) essential AA were adjusted to meet the potential of each group for maximum PD. A constant ratio of dietary N to SID Lysine (Lys) was maintained across both experimental diets (Table 6.1 ; NRC, 2012). All experimental diets were isoenergetic and contained 14 MJ/kg of metabolizable energy. The experimental diets

were fortified with vitamins and minerals to surpass the requirements recommended by NRC Swine (NRC, 2012). Titanium dioxide (0.25% TiO_2; Bobette Boyer Hall Technologies, St. Louis USA.) was included in all diets as an indigestible marker to determine nutrient digestibility. Pigs were fed either 1250 or 850 g/d divided into two equal meals, which was fed twice per day, and allowed free access to water. The feed allowance was slightly feed restricted (5%) to avoid feed refusal and minimize feed waste. At the end of the study, pigs were euthanized by intravenous injection of a lethal dose of sodium pentobarbital (FATAL PLUS, Vortech Pharmaceutical, Ltd., Dearborn, MI, USA), and muscle samples were collected.

Observations and sampling

Body temperature (BT), blood chemistry, feed waste and vomit were measured as previously described (see Chapter V, methods and materials, *observations, sampling, and chemicals).* Total urinary output was collected in 6 consecutive 12-h collections for a total of 72-h for the determination of urinary ammonia and urea nitrogen and ^{15}N excretion. Twenty-four hours prior to the 72-h collection period, urine was collected for 2 consecutive 12-h collections, to determine natural abundance of ^{15}N in urinary ammonia and urea. Urine was collected as previously described in Chapter V. Briefly, for each 12-h collection, urine was weighed, and a 150mL aliquot was sampled, placed in a sealed collection cup and stored at 4°C until further analysis. At the conclusion of the study, urine samples were pooled by pig and then by ISS group to create three samples: baseline, 12-60 h and then 72 h, for each ISS- group.

The impacts of ISS on muscle fiber characteristics were determined by collecting muscle samples from the *longissimus dorsi* (LD), *serratus ventralis* (SV), *semitendinosus*

(ST), and *psoas major* (PM) immediately following euthanasia. Following sample collection, muscle fiber orientation was identified and sectioned into a 2cm x 2cm x 3cm portion, placed into a mold, and embedded in clear frozen section compound (VWR International, Randor, PA). The sample was frozen using dry ice-chilled 2-methyl-butane and placed in a cooler of dry ice for transport. Samples were then stored at -80°C until further analysis.

Analytical procedures

Urinary ammonia and urea N concentrations were determined for each pooled urine sample (BioAssay Systems: QuantiChrom™ Urea Assay Kit DIUR-100 and EnzyChrom ™ ammonia/ammonium Assay kit ENH3-100). Methods for ^{15}N analysis, as described by (Rivera-Ferre et al., 2006), were followed with slight modifications. Briefly, isolation of urinary ammonia and urea was achieved using a cation exchange resin (Dowex® 50W X8 H^+ form, 200-400 mesh, Sigma-Aldrich) converted to the Na/K form. Extraction of urinary ammonia N was achieved by passing an aliquot of urine containing approximately 3,000 µg ammonia N through a chromatography column (Poly-Prep 731-1550, BioRad, Madrid, Spain) containing 0.5 mL of the Na/K resin. The eluate contained the urea fraction. Ammonia bound to the column was then eluted by adding 1 mL of 1M KOH to the column and collected into a solution containing 35 µL of 12 M sulfuric acid and 20 µL bromphenol blue (1% in Milli-Q water). Samples were then stored at −20°C until further analysis.

The elute containing the urea fraction was then subjected to enzymatic hydrolysis using a urease solution containing 400 units/mL suspended in a sodium phosphate buffer, pH 7 (Urease Type C-3 Jack Bean, ≥ 600,000 units/g solid, Sigma Aldrich). A portion of

the elute, containing approximately 3,000 µg of urea N, was added to a 10 mL Vacutainer (BD Vacutainer, BD Franklin Lakes, NJ, USA). Vacutainers were recapped, and 100 µL if urease solution was injected through the rubber stopper. Samples were then incubated at 30°C for 1 h in an inverted position to ensure no ammonia loss. Following incubation, 0.5 mL of 1 M HCL was injected through the stopper to stop the reaction. The solution was then passed through the chromatography columns as previously described for ammonia N extraction to obtain the urea N fraction (Rivera-Ferre et al., 2006). Lastly, both ammonia N and urea N fractions were prepared for combustion in an elemental analyzer. Briefly, 35 µL of each sample was absorbed in 2mg Chromosorb W (30-60 mesh Acid Washed 10gm; Elemental Microanalysis, Okehampton, UK) in tin capsules (Elemental Microanalysis, Okehampton, UK). Isotopic enrichment of urinary ammonia and urea N was analyzed by the University of California Davis Stable Isotope Facility using an Elementar Vario Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The ^{15}N enrichment in each fraction was obtained after correcting for the natural abundance of ^{15}N in urine.

Preparation of embedded muscle samples for immunohistochemical sectioning and staining were carried out as described by Hergenreder et al. (2016). Briefly, embedded muscle samples were transferred from −80 °C to −20 °C for 24-h prior to sectioning. Embedded samples were then cut into 10-µm cross sections at −20°C using a Leica CM1950 cryostat (Lieca Biosystems, Buffalo Grove, IL). Two cryosections were mounted on positively charged glass slides (Superfrost Plus; VWR International) for the determination of muscle fiber distribution, area, and nuclei density.

Immunohistochemical staining of cross sections was done as described by Hergenreder et al. (2016) with the exception of the antibodies used. Cryosections were incubated in the following primary antibodies (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA): a 1:100 (vol:vol) ratio of anti-myosin heavy chain (MHC)-IIB IgM (10F5 Supernatant), a 1:75 (vol:vol) ratio of anti-MHC-IIA and anti-MHC-I IgG1 (BF-35 Supernatant), and a 1:100 (vol:vol) ratio of anti-MHC-I IgG2b (BA-D5 Supernatant). After slides were rinsed three times for 5 min each in a phosphate buffered solution (PBS), cryosections were incubated for 30 min at room temperature in opaque boxes in the following secondary antibodies: a 1:1000 (vol:vol) ratio of goat α-mouse IgM, Alexa-Fluor 488 (Invitrogen); a 1:1000 (vol:vol) ratio of goat α-mouse IgG1, Alexa-Fluor 546 (Invitrogen); and a 1:1000 (vol:vol) ratio of goat α-mouse IgG2b, Alexa-Fluor 633 (Invitrogen). Slides were then rinsed three times for 5 min each in PBS. Finally, cover-slips were added with ProLong® Gold mounting media (ThermoFisher) containing 4’6-diamidino-2-phenylindole (DAPI) and allowed to cure horizontally in opaque boxes for 36-h at room temperature. Images were taken within 48-h of curing, as described by Hergenreder et al. (2016). All slides were imaged at 200X working difference magnification using an inverted fluorescence microscope (Nikon Eclipse, Ti-E; Nikon Instruments, Inc., Mellville, NY) with a UV light source (Intensilight C-HGFIE; Nikon Instruments, Inc.) and a CoolSnap ES^2 monochrome camera (Photometrics, Tucson, AZ). Images were artificially colored and analyzed using NIS Elements Imaging software (Nikon Instruments, Inc.). Five random images were taken of each slide fixed with two cryosections. For each image, all MHC-I, MHC-IIA, and MHC-IIX fiber types were identified and expressed as a percentage of the total fiber number.

The cross-sectional area of each fiber type in each image was measured using the NIS Elements software (Nikon Instruments Inc.). Nuclei density was determined by enumerating the total number of DAPI-stained cells in each image.

Calculations and statistical analysis

Whole-body protein turnover was determined using the end-product method (Waterlow et al., 1978) and a single oral dose of ^{15}N-glycine. This method assumes that the ^{15}N supplied by ^{15}N-glycine is partitioned between protein synthesis and AA oxidation in the same proportion as unlabeled AA and that the ^{15}N released from protein degradation is not reincorporated into whole-body protein during the collection period. Based on these assumptions, the proportion of ^{15}N excreted in the end product (urinary ammonia or urea) to the total dose given reflects the contribution of unlabeled N excreted as that end-product to the total flux (Waterlow et al., 1978; Rivera-Ferre et al., 2006):

$$d/ex = Q/Ex$$

where d is the dose of ^{15}N given orally (g); ex is the total amount of ^{15}N excreted as urinary ammonia or urea (g) during the collection period; Q is the total whole-body N flux (g/d), and Ex is the amount of N excreted in urinary ammonia or urea (g/d).

Rates of whole-body N flux was calculated separately for urinary ammonia and urea as end products as they often have different ^{15}N enrichment, and then estimated by the arithmetic mean (Fern et al., 1981), using the standard equation of Waterlow et al. (1978):

$$Q = Ex\ (d/ex)$$

Absolute rates of whole-body protein synthesis and degradation were estimated from the following equation:

$$Q = I + B = S + U$$

where Q represents the total whole-body nitrogen flux (g/kg $BW^{0.60}$/d), I is the rate of dietary nitrogen entering the pool (g/kg $BW^{0.60}$/d), B is the rate of whole-body protein breakdown (g N/kg $BW^{0.60}$/d), S is the rate of whole-body protein synthesis (g N/kg $BW^{0.60}$/d), and U is the total urinary nitrogen excretion (g/kg $BW^{0.60}$/d).

Statistical analysis was carried out using SAS software version 9.4 (SAS Institute, NC, USA). Normality and homogeneity of variances was confirmed using the Univariate procedure (PROC UNIVARIATE). Outliers were determined as any value that differed from the treatment mean by ± 2 standard deviation. Data on whole-body N metabolism were analyzed in a complete randomized block design with ISS and block as fixed effects and crate as a random effect using Mixed procedure (PROC MIXED). Nuclei density and muscle fiber type (FT) data were analyzed in a complete randomized design (CRD) with ISS and muscle type (MT) and the interaction between ISS and MT (ISS × MT) as fixed effects and crate as a random effect using Mixed procedure (PROC MIXED). Fiber area data were analyzed in CRD with ISS, MT and FT and the interaction among the main effects as fixed and crate as random effect using Mixed procedure (PROC MIXED). Dietary N intake was used as a co-variate for determining the effect of ISS on measures of whole body N flux and muscle fiber characteristics and when appropriate (P>0.10), a reduced model was used. For parameters such as BT that were measured over time, repeated measurements analysis of variance was used. An appropriate covariance structure was selected for analyses by fitting the model with the structure, which provided

the 'best' fit, based on Akaike information criterion (AIC) and Schwarz Bayesian criterion (BIC). Treatment effects were considered significant at $P \leq 0.05$. A tendency towards a significant difference between treatment means was considered at $P \leq 0.10$.

RESULTS

General observations

All pigs readily consumed experimental diets and showed signs of good health prior to the study. Clinical signs of disease were observed in ISS+ as described in Chapter V, Results, *Measures of immune function, hematology and blood chemistry*). indicators of ISS were previously presented (see Table 5.3) and described in Chapter V and confirmed effectiveness of ISS. Data from one pig was excluded from the study due to a severe immune response to LPS injection. Analyzed dietary nutrient contents fell within anticipated values ($\pm$15 %) that were derived from NRC Swine, 2012 (NRC, 2012). Calculated values of dietary contents were used for the interpretation of results.

Whole-body N metabolism

The effects of ISS on whole body N metabolism are presented in Table 6.2. Final BW was lower in ISS+ group compared to the ISS- pigs ($P<0.02$). As anticipated, ISS+ pigs had a lower dietary N intake relative to ISS- pigs ($P<0.01$). However, ISS had no impact on urinary N excretion ($P=0.75$). Nitrogen flux, whole-body protein synthesis and protein degradation were all reduced in ISS+ relative to ISS- pigs ($P<0.01$). As a result, ISS reduced whole-body N retention ($P<0.01$). Nitrogen retention-to-N intake (N-Retention: N-Intake) was significantly lower (51%) in ISS+ compared to ISS- pigs

(P<0.01). Furthermore, ISS reduced the protein synthesis: degradation ratio (P<0.01) and increased the protein synthesis: retention ratio (P<0.05) in ISS+ relative to ISS- pigs.

Immunohistochemistry

Data on the effect of ISS on nuclei density and muscle fiber type distribution in the LD, PM, ST, and SV are presented in Table 6.3. An ISS × MT interaction was observed for nuclei (P<0.01). In the PM, ISS significantly reduced the density of nuclei (P<0.01) but had no effect on the other muscle types. An interaction between ISS × MT was seen in the percentage of MHC-I and MHC-IIX fiber types (P<0.05), but not for MHC-IIA fibers (P=0.16). Immune system stimulated pigs showed a greater percentage of MHC-I fibers (P<0.01) and a decreased percentage of MHC-IIX fibers (P<0.04) in LD relative to ISS- pigs. Similarly, in the SV, ISS resulted in a greater percentage of MHC-I fibers (P<0.01) and a decreased percentage of MHC-IIX fibers (P<0.01). The percentage of MHC-IIA fibers were reduced in the ST (P<0.05) and tended to reduce in the PM (P=0.09) of ISS+ pigs relative to ISS- pigs.

Data on the effect of ISS on fiber cross sectional area are presented in Figure 6.1. There was a significant interaction between ISS, MT, and FT for fiber cross-sectional area (P<0.01). Immune system stimulation significantly reduced the cross-sectional area of MHC-I fibers in the LD and ST (P<0.05) but increased the MCH-I cross sectional area in the SV (P<0.05). Immune system stimulation reduced MHC-IIX cross-sectional area in the LD, ST, and SV (P<0.05) but increased it in the PM (P<0.01). Lastly, ISS significantly reduced the MHC-IIA cross-sectional area in the ST (P<0.01) but had no effect in the LD, PM and SV.

DISCUSSION

The main objective of this study was to evaluate the effect of protein intake on whole-body protein turnover and skeletal muscle fiber characteristics during ISS in growing pigs. In this study, we did not pair-feed the control (ISS-) animals since reduced feed intake can influence whole-body protein turnover and efficiency of dietary AA utilization for various body functions in non-immune challenged pigs (Fuller et al., 1989; NRC, 2012). Therefore, pigs in both the ISS- and ISS+ groups were fed 1250 and 860 g/d, respectively. This level of daily feed allowance for the ISS+ pigs was determined using previous studies that observed a constant quantitative decrease in feed intake with the same model of ISS (Rakhshandeh et al., 2012; Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014; Stuart et al., 2016). In the current study, ISS was induced by repeated injections of increasing amounts of LPS. We previously have shown that this model of ISS induces a relatively mild immune response that mimics the impact of mild clinical chronic disease on protein and AA utilization (Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014). The effectiveness of this model at inducing ISS in these pigs was reported and discussed previously (see Chapter V, Discussion and Table 5.3).

Traditional N-balance methods, measured as the difference between N-intake and N-output (i.e. fecal-N and urinary-N), do not allow for individual estimates on the contribution of protein synthesis and protein degradation to whole-body PD. Therefore, in this study a single dose of ^{15}N-glycine was used to measure these aspects of whole-body protein turnover. Advantages to this approach include it being a simple, non-invasive, and monetarily feasible method that provides precise estimates in the determination of whole-body N flux. However, because the urea pool turns over more

slowly than the ammonia pool, it essential to have a collection period that allows for complete clearance of the tracer into protein synthesis and excretion. If the time is to short the whole-body N flux will be underestimated. Alternatively, if the collection period is too long, tracer from the breakdown of labeled protein may re-enter the amino-N pool (Waterlow, 2006). In this study, the period of total urine collection was determined based upon a study previously conducted in our lab using this same technique in which we determine the rate of ^{15}N excretion in urinary urea and ammonia separately (Hewitt et al., 2016). These results indicated that complete ^{15}N excretion in urinary urea and ammonia occurs between 60 and 72-h after administration of ^{15}N-glycine (Hewitt et al., 2016). This finding was in agreement with Rivera-Ferre at al. (2006) who suggested that more than 95% of ^{15}N is excreted by 60 h after isotope administration (Rivera-Ferre et al., 2006). In the current study, the total excretion of ^{15}N at the end of 72 h accounted for >95% of the oral dose given (data not shown), indicating that our collection period allowed for complete ^{15}N excretion. Because the ^{15}N enrichment of urinary ammonia and urea vary inversely, the best estimate of whole-body N flux was calculated using the arithmetic mean, which is the average of the two fluxes (i.e. urinary ammonia and urea), and assumes that the dose of the tracer is equally partitioned between the two pathways (Fern et al., 1981). In this study, N-retention, calculated as the balance between protein synthesis and protein degradation, was in close agreement with the N-balance observations conducted simultaneously in these pigs (Table 6.2). The Higher N retention estimates associated with the N-balance method can be attributed to the systematic overestimation of N retention values due to an unavoidable N losses through feed waste,

urinary and or fecal excretion, and sample processing (Möhn et al., 2000; de Lange et al., 2001).

Reduced protein gain and increased N loss are characteristics of an immune response (Reeds and Jahoor, 2001; Obled, 2003). Distinct changes in whole-body protein and AA metabolism during ISS are multifaceted and include increases and decreases in both protein synthesis and degradation in disparate organ systems (Breuillé et al., 1994; Mercier et al., 2002; Orellana et al., 2004). In this study, ISS reduced whole-body N flux, which corresponded to reductions in both whole-body protein synthesis and degradation. A similar response was seen in *Escherichia coli* lipopolysaccharide (LPS) challenged growing pigs relative to non-pair-fed control pigs, when administered a single oral dose of ^{15}N. Interestingly, in this same study ISS only increased whole-body protein degradation, but had no effect on whole-body N flux or protein synthesis relative to the pair-fed group (Daiwen et al., 2008). These results seen in the pair-fed group may be explained by the influence of reduced feed intake on whole-body protein turnover and efficiency of dietary AA utilization for various body functions in healthy animals (Fuller et al., 1989; NRC, 2012; Hewitt et al., 2016). In another study, measures of whole-body turnover in LPS challenged pigs using a continuous infusion of ^{15}N-glycine resulted in a reduction in whole-body protein synthesis, but had no effect on whole-body N flux or protein degradation (Rudar et al., 2017). Based on the experimental design in the current study, pigs in each ISS group were fed to their estimated requirements base on their potential for PD, thus mitigating the potentially confounding effect lower SID AA and energy intake on the efficiency of N utilization and estimates of whole-body protein turnover in healthy pigs (Reeds et al., 1980; Daiwen et al., 2008; Hewitt et al., 2016).

However, reduced N retention in the ISS+ pigs can be attributed, predominantly, to hyperactivation of the immune system, since N retention per unit of N intake was significantly lower in these animals compared to ISS- pigs. The observed reduction in N retention is in agreement with previous studies conducted in pigs using the same model of ISS (Rakhshandeh et al., 2012; Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014). Contrary to previous studies using a pair-fed control group, these observations suggest that when protein intake is adjusted to meet the potential of the animal for growth, ISS reduces whole-body protein synthesis and degradation. Because skeletal muscle represents the largest reservoir of protein and AA in the body, alterations in skeletal muscle protein turnover likely give rise to these changes in whole body-body protein turnover (Cooney et al., 1997; Wray et al., 2002; Van Hall et al., 2008).

In the present study, ISS reduced the ratio of synthesis to degradation and the increased the protein synthesis per unit of retention, suggesting a reduced efficiency of protein metabolism and N utilization in ISS animals. The increase in protein degradation per unit of protein synthesis may be explained by the need to increase the mobilization of AA away from growth processes towards an immune response (Obled, 2003; Le Floc'h et al., 2004). Furthermore, the ISS-induced increase in protein synthesis per unit of retention could be explained by an increase in the synthesis of both proteinaceous and non-proteinaceous metabolites involved in the immune response. For example, components of the basal and specific gut endogenous N losses in pigs, account for up to 60% of total intestinal N losses (Moughan, 1999; NRC, 2012). Endogenous N losses primarily consist of mucins, digestive enzymes, sloughed cells, and immunoglobulins and antimicrobial

peptides (Nyachoti et al., 1997). Among these, ISS has been shown to significantly increase the synthesis of intestinal mucins aimed at maintaining an effective intestinal barrier function during disease (Faure et al., 2003; Faure et al., 2006; Dharmani et al., 2009; Rémond et al., 2009). Mucin proteins are highly resistant to digestion and thus represent a net loss of N from the body. Therefore, mucin synthesis may be contributing to the observed inefficiency of protein metabolism in ISS pigs (Law et al., 2007). Taken together, our results suggest that ISS reduces the efficiency of whole-body protein turnover, which is probably attributed to the increase in the synthesis of immune system metabolites.

Systemic ISS reduces muscle protein synthesis by decreasing the synthesis of both myofibrillar and sarcoplasmic proteins, particularly in skeletal muscle primarily composed of fast-twitch glycolytic fibers. However, severe systemic insults result in a more generalized decrease in muscle protein synthesis across all fiber types (Vary and Kimball, 1992; Lang et al., 2007; Lang and Frost, 2007). Sepsis-induced inhibition of muscle protein synthesis, mediated by the direct and indirect actions of pro-inflammatory cytokines, primarily results from a decrease in translational efficiency during the initiation phase of translation via the suppression of protein kinase mammalian target of rapamycin (mTOR) activity, and the subsequent phosphorylation of key eukaryotic initiation factors and binding proteins that regulate translation initiation. (Lang et al., 2007; Lang and Frost, 2007). In the current study, ISS reduced the percentage of MHC-IIX fibers and increased the percentage MHC-I fibers, suggesting a transition away from glycolytic (MHC-IIX) to more oxidative fiber types (MHC-I) in the various muscles of ISS+ pigs. In previous studies conducted in neonatal pigs, LPS-induced ISS reduced

skeletal muscle protein synthesis, preferentially in fast-twitch glycolytic muscles relative to oxidative muscle types (Orellana et al., 2002; Orellana et al., 2004; Orellana et al., 2007). These responses may be explained by ISS-induced inhibition of the stimulatory effects of anabolic hormones on skeletal muscle and the availability of substrates for protein synthesis and energy metabolism. It has been shown that fast-twitch glycolytic fiber types are particularly sensitive to insulin stimulated protein synthesis (Davis et al., 2001). During ISS, pro-inflammatory cytokines, in particular tumor necrosis factor (TNF-α), has been shown to induce a transient state of insulin resistance by inhibiting the post-receptor activity of insulin signaling, eliciting a reduction in skeletal muscle protein synthesis through suppression of mTOR-dependent translation initiation (Lang et al., 2007; Wei et al., 2008). Moreover, ISS-induced insulin resistance in the skeletal muscle also inhibits glucose uptake, likely to conserve glucose availability for enhanced glucose utilization by immune cells as their preferred source of energy (Kominsky et al., 2010; Delmastro-Greenwood and Piganelli, 2013; Pearce and Pearce, 2013). Pro-inflammatory cytokines act on adipocytes to induce a state of lipolysis, thus increasing plasma levels of free fatty acids (Jacobi et al., 2006), which would support a predominant slow-twitch oxidative fiber type composition. Collectively, these results suggest an ISS-induced shift in fiber type composition in favor of slow-twitch oxidative fiber type (MHC-I) likely due to the inhibited protein synthesis in glycolytic fibers and availability of energy substrates during ISS. Thus, the animals are likely more sustainable in an oxidative state within the skeletal muscle. In this study, ISS also resulted in a general reduction of nuclei density and fiber cross-sectional area in both MHC-I and MHC-II fiber types. These results can be attributed to ISS-induced atrophy in the skeletal muscle, which is in agreement with

our findings of ISS-induced decreases in whole-body protein synthesis and degradation and reduced N retention.

CONCLUSIONS AND IMPLICATIONS

Repeated injection with increasing amounts of LPS elicited an effective immune response that allowing for the evaluation of impact of ISS on whole-body protein turnover and skeletal muscle fiber type characteristics. When protein intake was adjusted to the potential of the animal for PD, ISS reduced whole-body protein synthesis and degradation, reduced the efficiency of dietary N utilization for protein synthesis and deposition, increased the protein synthesis-to-retention ratio and decreased the protein synthesis-to-degradation ratio. Because skeletal muscle represents the largest reservoir of protein and AA in the body, alterations in skeletal muscle protein turnover likely give rise to these changes in whole body-body protein turnover and reduced efficiencies of N utilization. Furthermore, ISS induced skeletal muscle atrophy and caused a shift in fiber type composition toward an oxidative fiber type composition. These results warrant further studies to evaluate the efficacy of nutritional intervention to mitigate the negative impact on whole-body protein turnover and pork quality.

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TABLES AND FIGURES

Table 6.1 Ingredient composition and nutrient contents of experimental diets[1]

	Treatment	
	ISS-	ISS+
Ingredient composition, (g/kg as-fed basis)		
Corn	740	788
Soybean meal	205.5	159
Hydrogenated vegetable fat	10	10
Lysine HCl	10.2	8.8
DL-Methionine	3.0	2.5
L-Threonine	5.2	4.5
L-Tryptophan	1.8	1.5
Limestone	8.8	8.3
Dicalcium phosphate	7.9	7.4
Salt	5	5
Vitamin and mineral premix[2]	15	15
Titanium dioxide	2.5	2.5
Calculated nutrient content (g/kg as-fed basis)		
Metabolizable energy MJ/kg	13.8	13.8
Crude Protein (N × 6.25)	136.6	119.8
Calcium	6.1	5.6
STTD P[3]	2.9	2.7

[1] Diets were formulated based upon the potential of each ISS group for protein deposition according to the NRC Swine model

[2] Provided the following amounts of vitamins and trace minerals (per kg of diet): vitamin A, 10075 IU; vitamin D3,1100 IU; vitamin E, 83 IU; vitamin K (as menadione), 3.7 mg; D- pantothenic acid, 58.5 mg; riboflavin, 18.3 mg; choline, 2209.4 mg; folic acid, 2.2 mg; naicin, 73.1 mg; thiamin, 7.3 mg; pyridoxine, 7.3 mg; vitamin B12, 0.1 mg; D-biotin, 0.4; Cu, 12.6 mg; Fe, 100 mg; Mn, 66.8 mg; Zn, 138.4 mg; Se, 0.3 mg; I, 1.0 mg; S, 0.8 mg; Mg, 0.0622%; Na, 0.0004%; Cl, 0.0336%; Ca, 0.0634%, P, 0.003%; K, 0.0036%.

[3] Standardized total tract digestible phosphorous.

Table 6.2 The impact of immune system stimulation (ISS) on whole-body nitrogen (N) metabolism in growing pigs[1]

	Health Status			
	ISS-	ISS+	SE	*P*<
Animals, *n*	5	7		
Final BW, kg $BW^{0.60}$	8.64	8.14	0.083	*0.02*
N Intake. g/kg $BW^{0.60}$/d	2.83	2.02	0.068	*0.01*
N Excretion,	1.48	1.52	0.117	*0.75*
Flux[2], g/kg $BW^{0.60}$/d	12.25	8.66	0.337	*0.01*
Synthesis, g N/kg $BW^{0.60}$/d	10.77	7.12	0.437	*0.01*
Degradation, g N/kg $BW^{0.60}$/d	9.41	6.64	0.402	*0.01*
Retention[3], g N/kg $BW^{0.60}$/d	1.38	0.58	0.083	*0.01*
N-balance[4], g/kg $BW^{0.60}$/d	1.64	0.72	0.115	*0.01*
N-Retention:N-intake	0.49	0.24	0.051	*0.01*
Synthesis:Degradation	1.15	1.08	0.018	*0.01*
Synthesis:Retention	7.61	12.26	1.407	*0.05*

[1]Data are the least square means ± the largest standard error of mean (SE). Pigs were injected with either increasing amounts of Escherichia coli lipopolysaccharide (ISS+; 25 and 35 µg/kg BW) or saline (ISS-), given 48-h apart. Nitrogen intake was tested as co-variate for determining the effect of ISS on whole-body N flux and when appropriate (P>0.01), a reduced model was used.
[2]Calculated as the arithmetic mean of urinary urea and ammonia N flux
[3]Calculated as the balance between protein synthesis and protein degradation
[4]Calculated from nitrogen balance (N-balance) study (see Chapter V)

Table 6.3 Effect of immune system stimulation (ISS) on nuclei density and muscle fiber composition in different muscle types of growing pigs[†]

	Health Status			*P* <		
	ISS-	ISS+	SE	ISS	MT	ISS x MT
Nuclei density, mm^2						
LD	1158	1054	77.6	0.09	0.01	*0.01*
PM	1390	1136	55.6			
ST	1143	1168	88.3			
SV	1089	1119	41.7			
MHC-I, %						
LD	9	13	1.1	0.01	0.01	*0.03*
PM	21	25	2.4			
ST	21	26	4.9			
SV	17	29	2.3			
MHC-IIA, %						
LD	17	16	2.0	0.05	0.01	*0.16*
PM	28	23	2.0			
ST	26	19	2.0			
SV	12	13	2.0			
MHC-IIX, %						
LD	73	68	1.5	0.04	0.01	*0.05*
PM	50	47	4.6			
ST	52	52	6.7			
SV	71	55	3.3			

† Data are the least square means ± the largest standard error of mean (SE). Pigs were injected with either increasing amounts of Escherichia coli lipopolysaccharide (ISS+; 25 and 35 µg/kg BW) or saline (ISS-), given 48-h apart. Pigs were euthanized 72-h post-ISS with muscle samples being collected immediately following from longissimus dorsi (LD), psoas major (PM), semitendinosus (ST), and serratus ventralis (SV)
Nitrogen intake was tested as co-variate for determining the effect of ISS on nuclei density and muscle fiber composition and when appropriate (P>0.01), a reduced model was used.

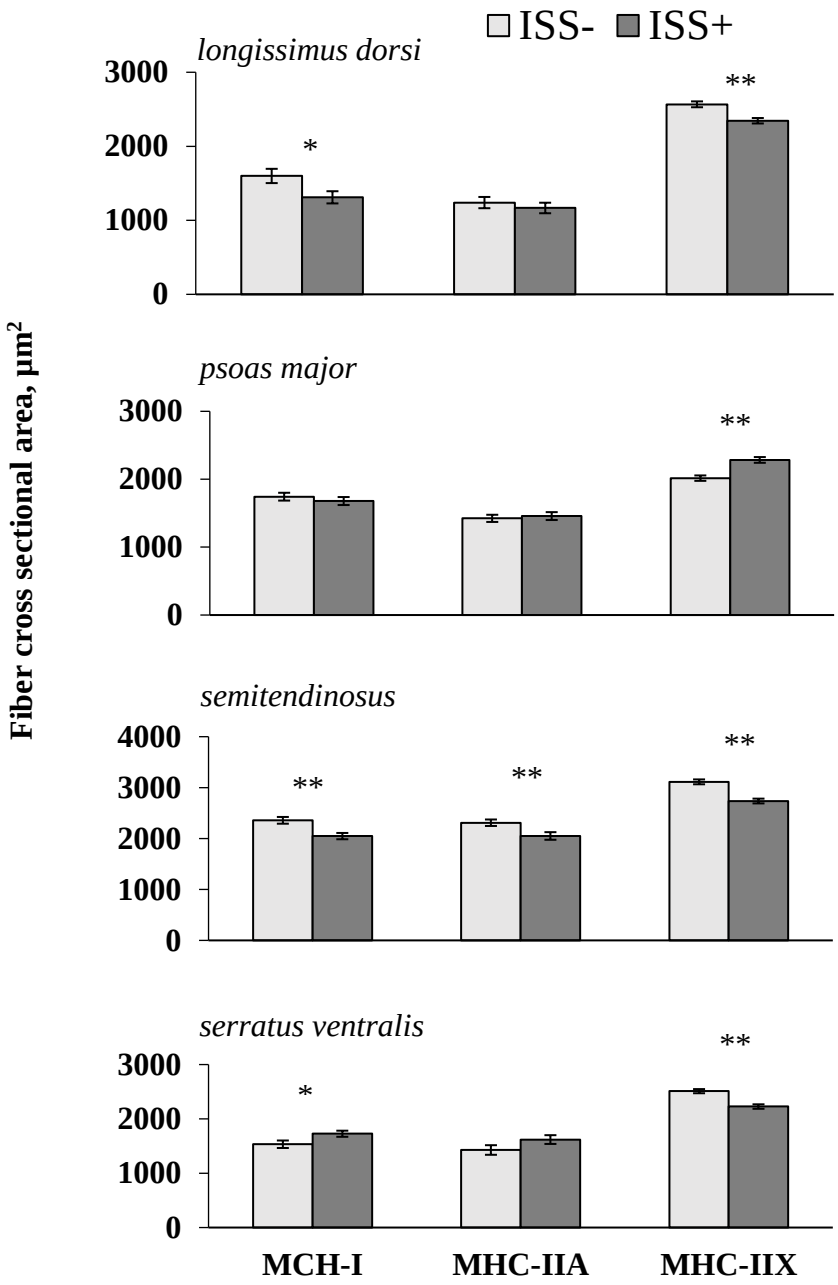

Figure 6.1 Effect of immune system stimulation (ISS) on fiber cross-sectional area, (μm^2) on *longissimus dorsi*, *psoas major*, *semitendinosus*, and *serratus ventralis*. *, $P<0.05$; **, $P<0.01$. Nitrogen intake was tested as co-variate for determining the effect of ISS on fiber cross sectional area and when appropriate ($P>0.01$), a reduced model was used

CHAPTER VII

GENERAL CONCLUSIONS AND DISCUSSION

Exposure to chronic sub-clinical or mild-clinical immune system stimulation (ISS) results in altered protein and amino acid (AA) utilization in growing pigs (Williams et al., 1997; Le Floc'h et al., 2009). The latter results in the redirection of AA away from growth and reproduction towards mounting an immune response, thus impacting AA requirements both quantitatively and qualitatively, i.e. AA ratio to lysine; Lys (Reeds and Jahoor, 2001; Obled, 2003; Rakhshandeh and de Lange, 2011). Previous studies have suggested that requirements for sulfur containing AA, branched chain AA, aromatic AA, threonine (Thr), and glutamine (Gln) increase during ISS (Reeds and Jahoor, 2001; Obled, 2003; Rakhshandeh and de Lange, 2011).

The first study presented here evaluated the effects of ISS induced by *E. coli* lipopolysaccharide (LPS) on flux and pool size of multiple AA simultaneously in the plasma, as well as on whole-body nitrogen (N) utilization in growing pigs (Chapter III). Because measures of AA concentration and traditional N balance studies alone do not provide insight into mechanisms that control AA metabolism and thus, AA requirements, we measured the changes in plasma free AA flux, in combination with the pool size, to better assess changes in AA metabolism in different metabolic states (Waterlow, 2006; Kampman-van De Hoek et al., 2015). ISS induced by repeated intramuscular (IM) injections of increasing amounts of LPS induced a relatively mild immune response that mimicked the impact of mild clinical chronic disease on protein and AA utilization (Rakhshandeh and de Lange, 2012; de Ridder et al., 2012; Rakhshandeh et al., 2014). Immune system stimulation reduce the apparent ileal digestibility (AID) of dietary

nitrogen (N) and efficiency of N utilization. Furthermore, LPS decrease the plasma flux of Phe and Ile and tended to reduce Lys. This reduction could indicate that the uptake of these AA into body protein synthesis and/or the catabolism of the AA is reduced during ISS.

In the second study, N utilization and plasma free AA kinetics were evaluated in growing pigs inoculated IM with a live field strain of PRRSv (Chapter IV). In this study, infection with PRRSv had no effect on aspects of N utilization however, these results were likely confounded by the significant reduction in average daily feed intake of PRRSv challenged pigs. Regardless, PRRSv increased the plasma Met and Thr flux, as well as the release of these AA from protein breakdown into the plasma free AA pool, suggesting an increase in the utilization of these AA during ISS. Collectively, in the first two studies, LPS injections and PRRSv exposure effectively induced an immune response and altered the AA kinetics of pigs during the fed state. However, their effects on the utilization of specific AA were varied. Given that PRRSv induced a more severe immune response than LPS injections, the differences in AA flux between these two studies may be a function of disease severity, or perhaps the specific disease itself.

The metabolism of threonine (Thr) is important during ISS due to its role in the synthesis of Thr-rich immune system metabolites, such as immunoglobulins, acute phase proteins (APP) and, in particular, intestinal mucins (Faure et al., 2007; Rémond et al., 2009; Rakhshandeh and de Lange, 2011). Considering that Thr accounts for 16-20% of crude mucin, increased synthesis and secretion of mucins may impact Thr requirements in growing pigs during ISS (Lien et al., 1997). This idea was supported by the study presented here, where a 1.5-fold increase in plasma Thr flux was observed in the PRRSv challenged pigs. However, no study had directly evaluated the impact of systemic ISS on the specific

measures of Thr requirements in growing pigs, such as the requirements for maintenance and protein deposition (PD). Therefore, the effects of ISS on dietary Thr requirements for PD in growing pigs was evaluated (Chapter V). In this study at Thr intakes below those needed to maximize PD, the marginal efficiency of Thr utilization was similar between ISS+ and ISS- pigs. However, the linear relationship between PD and standardized ileal digestible (SID) Thr intake generated a higher estimate of maintenance SID Thr requirements in ISS+ pigs. The observed increase in maintenance requirements during ISS may be largely attributed to the enhanced utilization of absorbed Thr for synthesis of immune system metabolites, particularly mucins. These findings warrant further studies to directly evaluate the impact of ISS on endogenous amino acid losses from both the small and large intestines in growing pigs.

Clearly, ISS alters whole-body protein and amino acid (AA) metabolism. It is well known that ISS increases protein synthesis in the liver, and decreases protein synthesis and increases protein degradation in the skeletal muscle (Breuillé et al., 1998; Lang et al., 2007). However, little to no information was available on the impact of infectious disease on whole-body protein turnover in pigs or on the specific impact of ISS on skeletal muscle fiber type characteristics. Hence, in another study presented here, the impact of ISS and protein intake on the rate of whole-body protein turnover and on skeletal muscle fiber characteristics in various muscles was evaluated in growing pigs (Chapter VI). In this study, control pigs were not pair-feed since reduced feed intake can influence whole-body protein turnover and the efficiency of dietary AA utilization in non-immune challenged pigs (Fuller et al., 1989; NRC, 2012). Rather, pigs in each ISS group were fed to their estimated requirements base on their potential for PD. In this

context, ISS reduced whole-body protein synthesis and degradation, which corresponded with lower N retention in ISS+ relative to ISS- pigs. More importantly, ISS significantly lowered the N retention per unit of N intake and the synthesis to degradation ratio. It also significantly increased the protein synthesis to retention ratio. Collectively, these findings suggest that when intake requirements are adjusted to the potential of the animal for growth, ISS reduced the efficiency of N utilization and whole-body protein turnover. In the skeletal muscle, ISS induced a transition away from glycolytic fiber types towards a more oxidative fiber type composition. This shift may be associated with hormone resistance and the lack of available substrates for protein synthesis and energy metabolism in the fibers. Furthermore, ISS reduce the nuclei density and cross-sectional fiber area across all fiber types, indicating ISS-induced skeletal muscle atrophy and a reduced capability for protein synthesis. Collectively, these findings suggest an ISS-induced decline in pork quality and should be investigated further.

Taken together, the results of the experiments in this dissertation indicate that ISS alters the metabolic demand for various AA and reduces the pig's potential for PD relative to healthy pigs. A reduced capability for PD in ISS+ pigs lowers the daily dietary SID Thr requirements for maximum growth performance; however, ISS increases the extrapolated Thr requirement for body maintenance functions. Furthermore, ISS reduces the efficiency of N utilization and whole-body protein turnover and highly impacts the metabolism of skeletal muscle protein. These findings support the idea that ISS likely alters the optimal dietary ratio among AA, especially Thr. Furthermore, these results indicate that PD is likely still the main determinant of AA requirements in immune

challenged pigs. Thus, formulation of diets that better meet the requirements of an animal during ISS could optimize PD potential and whole-body protein turnover.

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