Immune system stimulation induced by porcine reproductive and respiratory syndrome virus alters plasma free amino acid flux and dietary nitrogen utilization in starter pigs¹

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ABSTRACT: Changes in plasma free amino acid (AA) flux reflect the modification of AA metabolism in different metabolic states. Infectious diseases repartition AA away from protein retention toward processes involved in immune defense, thus impacting AA utilization in pigs. The current study sought to evaluate the effects of disease induced by a live pathogen on plasma free AA flux and whole-body nitrogen (N) utilization. Twenty gilts (BW 9.4 ± 0.9 kg) were surgically catheterized into the jugular vein, individually housed in metabolism crates, and feed-restricted (550 g/d). Intramuscular inoculation of a live field strain of porcine reproductive and respiratory syndrome virus (PRRSV) was used to induce disease. Whole-body N-balance was conducted across 3 d both before PRRSV inoculation (PRRSV⁻) and also after PRRSV inoculation (PRRSV⁺). At the end of each N-balance period, a bolus dose of a labeled [U-¹³C, U-¹⁵N]-AA mixture (Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, and Gln) was infused intravenously, followed by serial blood collection for measurement 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 (AID) of dietary N was determined using the slaughter technique and an indigestible marker. Blood chemistry, hematology, body temperature, and serum viremia indicated that PRRSV induced effective immune response in pigs ($P < 0.05$). Challenge with PRRSV reduced the AID of N ($P < 0.05$), but had no effect on apparent total tract digestibility of dietary energy ($P = 0.12$). Plasma flux ($\mu\text{mol/kg BW/h}$) for Met and Thr was increased by PRRSV infection ($P < 0.05$). A strong tendency of increased Val flux was observed in PRRSV⁺ pigs ($P = 0.06$). Infection with PRRSV increased the pool size for Lys, Met, Thr, Trp, Leu, Val, and Gln ($P < 0.05$). Collectively, these results suggest that PRRSV 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⁺ pigs may be associated with enhanced utilization of these AA for the synthesis of immune system metabolites and increased catabolism of these AA. Thus, dietary Met, Thr, and Val requirements may increase in pigs infected with PRRSV, relative to the requirements for other AA.

Key words: amino acid kinetic, growing pigs, plasma amino acid flux, porcine reproductive and respiratory syndrome virus

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INTRODUCTION

Exposure to infectious disease results in altered protein and amino acid (AA) utilization in growing pigs (Williams et al., 1997; Le Floc'h et al., 2009), which leads to reduced overall productivity. This reduced productivity occurs when AAs are directed away from lean tissue accretion toward mounting an immune response (Obled, 2003). Increases in the synthesis of immune system proteins and metabolites, such as acute phase protein (APP), glutathione, mucins, and immunoglobulins, may increase the need for specific AA, impacting AA requirements qualitatively (i.e., the AA profile) and quantitatively (Reeds and Jahoor, 2001). Some previous studies have suggested that the requirements for Met, Cys, branched-chain AA (BCAA), aromatic AA, Thr, and Gln increase during immune system stimulation (ISS; i.e., disease), while some suggest otherwise (Reeds et al., 1994; Melchior et al., 2004; Calder, 2006; Rakhshandeh and de Lange, 2011; Rakhshandeh et al., 2014). These inconsistencies may result from the use of different models of ISS with various levels of severity, various nutritional approaches such as various balances of dietary AA, and various methods of measurement. Additionally, many of these studies used plasma AA concentration to formulate their conclusions, which can be misleading since changes in AA metabolism can occur without simultaneous alterations in the concentrations of free AA in the extracellular compartments, including the plasma (Waterlow, 2006). Plasma free AA flux, on the other hand, reflects the amount of free AA that disappears per unit of time from the plasma pool for protein synthesis and catabolism. Thus, changes in plasma free AA flux can better reflect the alteration in whole-body AA metabolism during infectious diseases (Waterlow, 2006; Kampman-van De Hoek et al., 2015). Furthermore, most of these previous studies focused on the effect of ISS on the utilization of only one AA at a time, which ignores the possible interactions among AA and how these interactions may affect AA needs during ISS. The goal of the present study was to evaluate the effects of infection with porcine reproductive and respiratory syndrome virus (PRRSV), a major swine pathogen, on the plasma kinetics of multiple AA whose metabolism putatively becomes more important during ISS. We

hypothesized that PRRSV impacts AA utilization, and hence, free AA flux in the plasma.

MATERIALS AND METHODS

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

General Experimental Design, Animals, Housing, Diet, and Feeding

Twenty PIC PRRSV-naive gilts (Pig Improvement Company North America, Hendersonville, TN; initial BW 9.4 ± 0.9 kg) were obtained from the TTU swine herd, surgically fitted with jugular catheters, individually housed in metabolism crates, and fed a corn–soybean meal (SBM)-based diet (ME 14.0 MJ/kg, standardized ileal digestible Lys 10.4 g/kg; Table 1). Experimental diets were fed to the animals during recovery from surgery (for 6 ± 1 d). At the end of the recovery period, pigs were acclimatized to a daily feed allowance of 550 g/d for 3 d. Pigs were then subjected to 2 nitrogen (N)-balance studies, each lasting for 3 d, one of which occurred pre-inoculation (PRRSV–) and one of which occurred post-inoculation (PRRSV+; de Lange et al., 1989). The post-PRRSV N-balance study was started at 7 d post-inoculation (dpi) to allow sufficient viremia to develop, as observed by Rakhshandeh et al. (2013a) with the same strain of PRRSV. At the completion of each 3-d N-balance period, an isotope tracer study was conducted to determine plasma free AA flux. The daily feed allowance was allocated into 2 equal feedings per day at 08:00 a.m. and 05:00 p.m., except for the 24 h prior to isotope tracer infusion. Twenty-four hours before isotopic infusion, the feeding intervals were changed to every 4 h to minimize diurnal patterns in AA metabolism (Möhn et al., 2003; Rakhshandeh, 2011; Kampman-van De Hoek et al., 2015). Experimental diets contained 0.5% titanium dioxide (TiO_2) as an indigestible marker and pigs had free access to water throughout the study. 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).

Table 1. Diet composition (as-fed basis), calculated and analyzed nutrient contents

Item	Amount
Ingredients, g/kg	
Corn	613
Soybean 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	18.1
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
Analyzed nutrient contents, g/kg	
Dry matter, %	90.7
Crude protein ³	20.6
Lysine	11.9
Methionine	3.0
Methionine + cysteine	6.3
Threonine	7.9
Tryptophan	2.5
Leucine	17.6
Isoleucine	9.3
Phenylalanine	10.3
Valine	9.9

¹Amounts of vitamins and trace minerals (per kilogram of diet): vitamin A, 10,075 IU; vitamin D3, 1,100 IU; vitamin E, 83 IU; vitamin K (as menadione), 3.7 mg; D-pantothenic acid, 58.5 mg; riboflavin, 18.3 mg; choline, 2,209.4 mg; folic acid, 2.2 mg; niacin, 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%.

²Protein and amino acids are standard ileal digestibility basis.

³Protein and amino acids are total basis.

each pig was treated with 1 dose of penicillin [25,000 units intramuscularly (**i.m.**)], anti-inflammatory banamine (2.2 mg/kg BW **i.m.**), and the analgesic buprenorphine [0.01 mg/kg subcutaneously (**s.c.**)]. General health, feed intake (**FI**), and eye temperature were monitored frequently during the recovery period. To induce ISS, pigs were inoculated **i.m.** with ~600 genomic units of a live field strain of PRRSV (isolated from pigs in northern Iowa), which was suspended in phosphate buffer (Rakhshandeh et al., 2013a).

Isotope Infusion

At the completion of each N-balance period, a single bolus sterile dose (0.9 mL/kg BW) of a universally labeled [U-¹³C, U-¹⁵N] AA mixture (97 to 99 atom percent, Cambridge Isotope Laboratories, Tewksbury, MA) suspended in saline was infused via one of the indwelled jugular catheters over a 30-s period. The AA mixture (μmol/mL sterile saline) contained isoleucine (**Ile**), 5.5; leucine (**Leu**), 15; valine (**Val**), 14.4; lysine (**Lys**), 32; methionine (**Met**), 4.3; phenylalanine (**Phe**), 55; threonine (**Thr**), 6.3; tryptophan (**Trp**), 5.5; and glutamine (**Gln**), 9.6. The following assumptions were made 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; and 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 important 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 2 fluxes (Holtrop et al., 2004; Waterlow, 2006).

Observations and Sampling

The initial BW of each pig was measured prior to entering metabolism crates and final BW was determined at the conclusion of the study. The N-balance studies were conducted as described by Rakhshandeh et al. (2014). Briefly, urine was quantitatively collected for 24 h via collection trays underneath each crate that funneled urine into tared,

Surgical Catheterization and ISS

Silicon catheters (Micro-Renathane, 0.095 OD × 0.066 ID, Braintree Scientific Inc., Braintree, MA) 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). During recovery,

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 were collected, oven dried, cooled in a desiccator, and weighed to accurately determine daily DM intake. Fecal samples were collected daily and stored 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 until further analyzed for N, an indigestible marker, and GE contents.

At the end of each 3-d N-balance period, an isotopic infusion study was conducted. Serial blood samples were then taken (~1.5 mL) immediately before isotopic infusion started and then at times 2.5, 5, 7.5, 10, 15, 20, 30, and 45 min after infusion to measure the change in plasma isotopic enrichment of each AA. Blood samples were drawn at each time point into a heparinized tube (BD Vacutainer, Franklin Lakes, NJ). To avoid contamination with infusate, blood samples were taken via the indwelled catheter that was not used for infusion. Following the completion of each isotope tracer study, blood samples were centrifuged at $1,500 \times g$ for 15 min 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, OR) digital camera, as previously described by [Petry et al. \(2017\)](#). The resolution for each infrared (IR) image was set at 160×120 pixels and the emissivity value was set to the recommended value of 0.98 for biological tissues. Multiple IR pictures were taken ~50 cm away from the eye and an average of the best 3 pictures, in terms of focus and precision, were selected for determination of body temperature (BT). Infrared pictures were interpreted using FLIR tools software. To confirm that an effective ISS occurred, measures of blood chemistry, hematology, serum viral load, and serology, fresh whole blood samples were collected (~3 mL) at 0, 2, 4, 6, 8, and 10 dpi from jugular catheters. Blood chemistry and hematology parameters were immediately analyzed using an i-STAT Handheld Analyzer (Abaxis Inc., Union City, CA) with i-STAT CHEM8+ test cartridges. Serum viremia was determined at Iowa State Diagnostic Laboratories (Ames, IA) using qPCR-PRRSV Tetra-Core Quantitation (USA type; Tetracore, Inc., Rockville, MD) and a PRRS X3

virus ELISA commercial kit (IDEXX Laboratories, Inc., Westbrook, ME).

Apparent ileal digestibility (AID) of dietary N was determined using the slaughter technique and TiO_2 ([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 (BW 11.5 ± 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) analyzer. Dry matter (DM) content of feces, digesta, and diet samples were determined by oven drying for 24 h at 120 °C according to the Association of Official Analytical Chemists procedures (method no. 930.15; [AOAC, 1997](#)). Titanium dioxide levels of fecal and diet samples were determined in duplicate and triplicate, respectively, and according to standard AOAC procedures (method no. 973.36; [AOAC, 1997](#)). Samples of the diet and lyophilized feces were analyzed in duplicate for the determination of GE using a 6300 model calorimeter bomb (Parr Instruments, Moline, IL) according to procedures of the [AOAC \(1997\)](#).

Plasma free labeled and unlabeled AA isolation and quantification were achieved using a Phenomenex EZ:Faast Amino Acid Analysis Kit (Torrance, CA) 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, Radnor, PA). 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 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). Amino acid analysis of the diets was performed at the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia, MO. Dietary AAs were determined by cation-exchange chromatography coupled with post-column ninhydrin derivatization and quantitation (AOAC, 1997).

Calculations and Statistical Analysis

A power test was used to determine the number of animals per treatment group. The coefficient of variance (CV %) was 5; the percent difference from the control was decided at 10; and $P \leq 0.05$ with a power of 90%. The AID of crude protein (CP; $N \times 6.25$), AA, and GE were calculated using the indicator method and TiO_2 as an indigestible marker. Standardized ileal digestibility (SID) of AA was estimated as described by Stein et al. (2007). 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 (nls), 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. For model identifiability, $\alpha_1 > \alpha_3$ was enforced. The robustness of the estimates obtained from nls and the accuracy of the resulting model fits are strongly influenced by the initial parameter values provided to the nls machinery. Therefore, for each AA outcome, the initial values for α_1 , α_2 , α_3 , and α_4 were set to be the best result obtained from a maximum-likelihood-based grid search from 5,000 constrained but randomly chosen initial values for α_1 , α_2 , α_3 , and α_4 , using the

optimum function in R and the aforementioned standard double exponential model. These parameter estimates were then used to calculate the flux (Q) of each AA ($\mu\text{mol/kg BW/h}$) 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 each AA and pig was calculated using the following equation:

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

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 released from proteolysis, respectively. S and U represent the rate of AA outflow toward whole-body protein synthesis and catabolism, respectively. This equation assumes that at steady state, the rate of AA inflow (influx) into the plasma pool is equal to the rate outflow (efflux) of AA from the plasma pool. All values are expressed as micromole per kilogram BW per hour.

Statistical analysis was carried out using SAS software version 9.4 (SAS Institute, Cary, NC). Normality and homogeneity of variances were confirmed using the univariate procedure (PROC UNIVARIATE). Outliers were determined as any value that differed from the treatment mean by ± 2 SD. Data were analyzed in a complete randomized design with health status as fixed effects and pig within treatment as a random effect using mixed procedure (PROC MIXED). Average daily feed intake (ADFI) was used as a covariate for determining the effect of ISS on AID of dietary N and apparent

total tract digestibility (ATTD) of dietary energy. For parameters, such as BT, measures of blood chemistry, hematocrit, and viremia, 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 and Schwarz Bayesian criterion. Tukey–Kramer was used for a comparison test. Values are reported as least square means with the largest SE. Treatment effects were considered significant at $P \leq 0.05$. A tendency toward a significant difference between treatments was considered at $P \leq 0.10$.

RESULTS

General Observations

All pigs were naive to PRRSV before inoculation (Fig. 1), showed signs of good health, and readily consumed experimental diets during recovery from surgery and before inoculation with PRRSV. No fluctuation in BT was observed prior

Figure 1. Effects of porcine reproductive and respiratory syndrome virus (PRRSV) on viremia (a, ●) and serum antibody titers (b, ▲) during the 10-d post-inoculation period. The data presented are least square means $\pm$ the largest SEM and represents the best estimate of mean that was obtained immediately before PRRSV inoculation ($n = 15$ pigs) and during the 10-d period after virus infection ($n = 6$ pigs). PRRSV viremia (genomic copies equivalent) was determined using quantitative real-time qPCR. *Significant differences ($P < 0.05$) from day 0.

to PRRSV challenge. After inoculation with PRRSV, pigs displayed clinical signs of disease such as lethargy, with some being more severe than the others. Challenge with PRRSV reduced the ADFI by 19.8 ± 4.17 g/kg BW/d ($P = 0.01$). Data from 4 pigs were excluded from the study due to a severe immune response to PRRSV challenge. Therefore, BW, ADFI, and N-balance data were determined using 16 PRRSV+ pigs. The final BW, ADFI, N-balance, measures of blood chemistry and hematocrit were not different between the six pigs that were given the post-ISS isotopic infusion and the rest of the PRRSV+ pigs ($P > 0.47$). Thus, data on all 16 PRRSV+ pigs were reported. Due to dysfunctions in 1 or both catheters, measures of blood chemistry and hematocrit were determined in 15 PRRSV– and 11 PRRSV+ pigs. Also, due to dysfunctional catheter, the isotope tracer study was conducted on 15 PRRSV– and 6 PRRSV+ pigs. Analyzed diet nutrient contents were generally in agreement with the calculated values (Table 1), which were derived from feed ingredient composition and nutrient levels in feed ingredients according to Swine National Research Council (NRC, 2012). Thus, when interpreting results and when calculating AA intake for individual pigs, calculated diet nutrient contents were used.

Measures of Immune Function, Hematology, Blood Chemistry, and Viremia

The interaction effects between time, viral load (genomic copy per milliliter serum), and sample-to-positive ratio (S:P ratio) are shown in Fig. 1. The viral load increased at 2 dpi and remained elevated for the rest of the study ($P < 0.05$). The S:P ratio began to increase at 6 dpi and reached a maximum at 10 dpi ($P < 0.05$). The interaction between eye temperature and time (i.e., day) was significant (Fig. 2; $P < 0.01$). Inoculation with PRRSV increased eye temperature by 0.9 °C ($P < 0.01$) relative to PRRSV– pigs. Eye temperature started to rise at 2 dpi, maximized at 4 dpi, and remained elevated throughout the course of the study ($P < 0.05$). The main effects of ISS on hematology and blood chemistry are presented in Table 2. Challenge with PRRSV increased the levels of creatinine and anion gap (AnionGap; $P < 0.05$) and reduced the levels of hemoglobin (Hb; $P < 0.01$). Hematocrit, blood urea nitrogen (BUN), and other measures of electrolyte balance were not affected by PRRSV ($P > 0.05$). Blood glucose levels were lower in PRRSV+ pigs than in PRRSV– pigs ($P < 0.05$).

Figure 2. Changes in the eye temperature ($^{\circ}\text{C} \pm \text{SEM}$) of pigs challenged with porcine reproductive and respiratory syndrome virus (PRRSV) over time. The data presented are least square means $\pm$ the largest SEM and represents the best estimate of mean that was obtained immediately before PRRSV inoculation ($n = 15$ pigs) and during the 10-d period after virus infection ($n = 6$ pigs). *Significant differences ($P < 0.05$) in eye temperature from time 0.

Table 2. Effects of immune system stimulation induced by porcine reproductive and respiratory syndrome virus (PRRSV) on blood parameters in growing pigs¹

Blood parameter	PRRSV–	PRRSV+	SE	<i>P</i>
<i>N</i>	15	11		
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	90.6	82.1	2.99	≤ 0.05
Creatinine, mg/dL	0.68	0.77	0.04	≤ 0.05
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/L	102.9	102.0	1.44	≤ 0.56
Anion gap, mEq/L	13.0	16.6	1.11	≤ 0.01

¹The data presented are least square means after controlling for average daily feed intake $\pm$ the largest SEM and represent the best estimates of mean that were obtained immediately before PRRSV inoculation (PRRSV–) and across 10 d after PRRSV inoculation (PRRSV+). Immune system stimulation was induced by intramuscular injection of a live PRRSV.

²PCV = packed cell volume.

Body Weight, N Utilization, and Nutrient Digestibility

The effects of ISS on BW, N utilization, and nutrient digestibility are presented in Table 3. Pigs challenged with PRRSV did not gain weight, as BW showed no significant difference between the pre-challenge and post-challenge periods ($P = 0.11$). Infection with PRRSV reduced N intake ($P < 0.01$), N retention ($P < 0.01$), and fecal and total N excretion ($P < 0.01$) but it had no effect on urinary N excretion ($P = 0.47$). The N intake-to-N retention

Table 3. Effects of immune system stimulation induced by porcine reproductive and respiratory syndrome virus (PRRSV) on dietary nutrient utilization in growing pigs¹

Parameter	PRRSV–	PRRSV+	SE	<i>P</i>
<i>N</i>	20	16		
Final BW, kg	13.4	14.3	0.39	≤ 0.11
Nitrogen (N) utilization, mmol/kg BW/d				
Intake	93.3	47.6	8.49	≤ 0.01
Urinary excretion	19.6	21.2	1.86	≤ 0.47
Fecal excretion	23.5	11.2	0.99	≤ 0.01
Total excretion	43.2	32.3	2.30	≤ 0.01
Retention	50.5	17.7	7.71	≤ 0.01
N intake: N retention	0.53	0.37	0.05	≤ 0.01
Nutrient digestibility², %				
AID of N	76	54	4.70	≤ 0.01
ATTD of energy	79	81	0.74	≤ 0.12

¹The data presented are least square means $\pm$ the largest SEM. Two whole-body N-balance studies were conducted for 3 d, both before PRRSV infection (PRRSV–) and after PRRSV infection (PRRSV+). Immune system stimulation was induced by intramuscular injection of a live PRRSV.

²AID and ATTD: Apparent ileal digestibility of N and apparent total tract digestibility of energy are presented as least square means after correcting for average daily feed intake. AID determined using slaughter technique and titanium dioxide (TiO_2) as an indigestible marker. To determine the AID of N in non-immune challenged pigs (PRRSV–), a separate group of pigs ($n = 10$) were feed-restricted and injected with sterile saline.

ratio (N intake:N retention) was substantially lower in PRRSV+ pigs compared to their unchallenged counterparts ($P < 0.01$). After controlling for ADFI, infection with PRRSV reduced the AID of dietary N ($P < 0.01$), but had no effect on the ATTD of energy ($P = 0.12$).

Amino Acid Kinetics

The effects of PRRSV on plasma free AA flux, proteolysis, and pool size are presented in Table 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 [^{13}C , ^{15}N]-labeled AA. Challenge with PRRSV increased the plasma flux of Met and Thr ($P < 0.01$) and tended to increase Val ($P = 0.06$), but it did not affect plasma flux of other AA ($P > 0.05$). During the PRRSV challenge period, pigs had an increased release of Met, Thr, Lys, and Val ($P < 0.04$) from protein breakdown relative to the pre-challenge period. The Ile, Leu, Trp, and Val release from protein breakdown was not affected by PRRSV infection ($P > 0.05$). The pool size for Leu, Lys, Met, Thr, Trp, Val, and Gln was increased during infection with PRRSV ($P < 0.05$). Infection with

Table 4. Effects of immune system stimulation induced by porcine reproductive and respiratory syndrome virus (PRRSV) on flux ($\mu\text{mol/kg BW/h}$), release from protein degradation ($\mu\text{mol/kg BW/h}$), and pool size ($\mu\text{mol/kg BW}$) of selected plasma free amino acids (AAs)^{1,2}

	PRRSV–	PRRSV+	SE	P
N	15	6	–	–
Lys				
Flux	410	380	29.1	≤ 0.41
Proteolysis	272	335	22.5	≤ 0.04
Pool size	25	40	5.6	≤ 0.04
Met				
Flux	108	228	23.7	≤ 0.01
Proteolysis	81	216	25.4	≤ 0.01
Pool size	30	60	3.5	≤ 0.01
Thr				
Flux	83	130	16.0	≤ 0.01
Proteolysis	19	95	12.3	≤ 0.01
Pool size	26	58	8.5	≤ 0.01
Trp				
Flux	150	117	28.0	≤ 0.15
Proteolysis	98	83	21.4	≤ 0.54
Pool size	13	19	2.0	≤ 0.05
Leu				
Flux	586	558	41.6	≤ 0.21
Proteolysis	472	510	37.8	≤ 0.31
Pool size	12	18	2.2	≤ 0.02
Ile				
Flux	120	90	14.6	≤ 0.12
Proteolysis	47	50	22.1	≤ 0.90
Pool size	18	24	5.8	≤ 0.40
Val				
Flux	185	216	13.0	≤ 0.06
Proteolysis	93	167	21	≤ 0.01
Pool size	7	18	3.0	≤ 0.04
Phe				
Flux	133	118	16.5	≤ 0.36
Proteolysis	55	82	15.2	≤ 0.15
Pool size	5	8	1.5	≤ 0.08
Gln				
Flux	275	272	70.1	≤ 0.96
Proteolysis	–	–	–	–
Pool size	16	31	4.1	≤ 0.01

¹The data presented are least square means $\pm$ the largest SEM. Twenty gilts were surgically fitted with venous catheters and feed-restricted (550 g/d) on a corn–soybean meal-based diet. Immune system stimulation was induced by injection of porcine reproductive and respiratory syndrome virus. N-balances were determined across 3 d, both before (PRRSV–) and after (PRRSV+) infection with PRRSV. At the end of each N-balance period, a single dose of an [^{13}C , ^{15}N]-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 data for each pig and AA, and equation parameters were used to estimate plasma AA flux and pool size.

²Proteolysis: AA released from protein proteolysis was calculated as the difference between the AA flux and intake, using the steady-state model of Waterlow (Waterlow, 2006).

PRRSV tended to increase the pool size of Phe ($P = 0.08$).

DISCUSSION

PRRSV continues to be the most costly swine disease worldwide by causing decreased weight gain in growing pigs, predominantly due to muscle wasting (Benfield et al., 1992). The development of nutritional strategies that can reduce the negative impacts of infectious disease on pig productivity requires a quantitative understanding of the effects of ISS on AA needs. Thus, the main objective of the present study was to discover the effects of PRRSV-induced ISS on the plasma kinetics of the AA whose metabolism putatively becomes more important during ISS. We also explored the effect of PRRSV on whole-body N utilization in starter pigs. We previously have shown that the PRRSV model of ISS induces a sustained and relatively moderate immune response, allowing for the study of nutrient utilization (Mastromano et al., 2013; Rakhshandeh et al., 2013a).

In the current study, challenge with PRRSV resulted in changes in measures of blood chemistry and elevated eye temperature that was concomitant with increased serum viremia and the development of anti-PRRSV serum antibodies. Using the same model of ISS, we have previously shown that an increase in BT correlates with elevated levels of IL-1 β , TNF- α , haptoglobin, and C-reactive protein, and reduced white blood cell counts and neutrophil-to-lymphocyte ratios (Mastromano et al., 2013; Rakhshandeh et al., 2013a). Thus, elevated eye temperature in the current study likely reflects the same changes in these measures of immune function. In support of this idea, we observed a decrease in the level of Hb during ISS in the present study, which is often associated with a pro-inflammatory cytokine-mediated reduction in the survival rate of erythrocytes (Goyette et al., 2004). Furthermore, PRRSV infection increased the AnionGap, but had no effect on the plasma electrolyte balance, as indicated by the levels of Na⁺, K⁺, and Cl[–]. Therefore, higher AnionGap in PRRSV+ pigs most likely indicates an increased level of lactic acid in the blood, which would reflect a shift from aerobic to anaerobic glycolytic metabolism (De Backer, 2003). Moreover, the observed lack of difference in the BUN level between the ISS groups can be associated with the higher rate of AA catabolism, and thus, reduced efficiency of N utilization in PRRSV+ pigs, since the N intake was significantly lower in this group

of pigs compared to the unchallenged counterpart (McGilvray et al., 2019). Finally, 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). Thus, reduced blood glucose level in the current study, can, in part, be associated with enhanced glucose uptake by immune cells and, to a larger extent, to reduced ADFI. Collectively, these results indicated that inoculation with a live, field strain of PRRSV induced an effective ISS in our study.

It has been suggested that physiological changes occur in the gastrointestinal tract during ISS, which may interfere with normal digestion and absorption processes (Liu, 2015). In the current study, we evaluated the impact of PRRSV-induced ISS on the ileal digestibility of dietary N using a slaughter technique. We preferred this technique over cannulation to avoid the additional stress caused by surgical procedures, possible secondary infections, and any interference with the digestive function (e.g., intestinal motility) of the animals. Characteristic technical problems associated with the slaughter technique were minimized by controlling the sampling time relative to the feeding schedule, clearly identifying the intestinal segment from which the samples were obtained (i.e., the distal ileum), and minimizing mucosal cell shedding by using sodium pentobarbital for euthanizing the pigs (Donkoh et al., 1994; Rakhshandeh et al., 2014). In addition, ADFI was used as a covariate when analyzing the effect of PRRSV on measures of N and energy digestibility, because it is well established that the measure of apparent nutrient digestibility, especially AID of N, is a function of FI in swine (NRC, 2012). In the present study, systemic ISS decreased the AID of dietary N by 29% relative to control, feed-restricted pigs. This reduction can likely and in part be associated with increased intestinal Thr-rich endogenous AA losses (EAAL). Several studies have shown increased synthesis and secretion of intestinal mucins in immune challenged pigs (Rémond et al., 2009; Rakhshandeh et al., 2013c). Mucins are the main component of the intestinal EAAL, which can affect the estimation of AID for dietary N and AA (Faure et al., 2007). The findings of the current study are in agreement with the findings of other workers who used the slaughter technique to determine AID of dietary N and AA (Lee, 2012; Rakhshandeh et al., 2013b; McGilvray et al., 2019). Thus, the need for further studies to directly

evaluate the impact of ISS on intestinal EAAL, intestinal integrity, and ileal digestibility of AA in growing pigs is warranted.

Whole-body protein ($N \times 6.25$) retention is the balance between protein synthesis and proteolysis. Reduced protein retention during ISS is brought about by reduced protein synthesis and increased proteolysis, predominantly in the skeletal muscles (Orellana et al., 2004). It has been suggested that in a moderate ISS, approximately 70% of reduced protein retention (i.e., lean gain) can be attributed to the reduced FI that often accompanies ISS, and the rest to the cost of ISS (Klasing et al., 1987). However, it is not possible to separate the effects of FI from that of ISS on N utilization since the infection reduced the FI beyond the level that was expected in the present study. The lack of difference between the 2 ISS groups in urinary N excretion indicated a higher rate of AA catabolism in infected pigs, as the N intake was significantly lower than the uninfected pigs in the current study. It is well established that N resulting from the deamination of AA is quantitatively excreted via urine (Rakhshandeh et al., 2014). In addition, the significant reduction in N retention: N intake in PRRSV+ pigs suggested a lower efficiency of dietary N utilization in PRRSV-infected pigs, which is often evident in immune challenged pigs (Rakhshandeh and de Lange, 2011). These results are consistent with elevated blood creatinine and BUN in PRRSV-infected pigs, suggesting a reduction in the efficiency of N utilization in these animals (Hosten, 1990). Taken together, these results suggest that PRRSV challenge altered dietary N utilization by reducing the efficiency of N utilization for N retention.

In the present 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 PRRSV- and PRRSV+ pigs. We hypothesized that changes in plasma free AA kinetics can reflect modifications of AA metabolism during ISS caused by PRRSV infection. It is well known that the daily requirements for Lys are determined by protein retention (NRC, 2012). It has been reported that ISS does not impact the efficiency of Lys utilization for N retention, and changes in Lys requirements reflect changes in body protein gain (Williams et al., 1997). In the present study, PRRSV had no effect on Lys flux, but increased the Lys pool size and Lys release from proteolysis by 60% and 23%, respectively. Thus, these results suggest that a reduction in the metabolic need for Lys occurred during ISS caused by PRRSV infection and can predominantly

be associated with decreased Lys utilization for whole-body protein synthesis (Waterlow, 2006). An increase in the Lys pool size can mainly be attributed to increased Lys release from proteolysis, most likely in skeletal muscle, since the SID Lys intake was lower PRRSV+ pigs than in PRRSV- pigs (Orellana et al., 2004). These results are in general agreement with the findings of McGilvray et al. (2019), but in contrast to those of Kampman-van De Hoek et al. (2015), who reported no change in Lys kinetics in ISS pigs. This contrast is likely due to the use of complete Freud's adjuvant (CFA) in the latter study, which did not elicit a sufficiently strong immune response to shift body metabolism. Taken together, the current findings suggest a reduction in metabolic demand for Lys in PRRSV+ pigs.

In the current study, challenge with PRRSV resulted in a concomitant and significant increase in Met flux (111%), Met release from proteolysis (167%), and the pool size (100%), suggesting an enhanced metabolic demand for Met in ISS pigs. Increased Met pool size reflects the higher rate of met influx into the plasma pool than the efflux of Met from the same pool (Waterlow, 2006). Increased demand for Met during ISS can mainly be associated with the enhanced utilization of Met for Cys synthesis via transsulfuration, since N retention, and thus protein synthesis, was reduced in this study (Malmezat et al., 2000; Rakhshandeh et al., 2010b). Cysteine becomes conditionally essential during ISS, because Cys utilization for synthesis of immune system metabolites such as glutathione, taurine, APP, and mucins increases significantly in pigs and other species (Faure et al., 2006; Rakhshandeh and de Lange, 2010, 2011; Rakhshandeh et al., 2010a,b). In addition, Rakhshandeh et al. (2014) reported an increase in the dietary sulfur-containing amino acids (SAA) requirement per unit of protein deposition (PD) in pigs challenged with escherichia coli lipopolysaccharide (LPS) (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 dietary Met during ISS (Litvak et al., 2013). An increase in the release of Met from proteolysis provides further evidence for prioritization of this AA for the synthesis of immune system metabolites during ISS (Kampman-van De Hoek et al., 2015). Taken together, the results of the current study suggest an enhanced Met requirement in PRRSV-infected pigs, most likely due to irreversible loss of Met for synthesis of Cys. The latter may impact the daily dietary requirements of Met and total SAA.

In the present study, challenge with PRRSV resulted in a 1.6-, 5.0-, and 2.2-fold increase in Thr flux, release from proteolysis, and pool size, respectively, suggesting a substantial increase in the metabolic demand for Thr. This result can most likely be attributed to the enhanced utilization of Thr for synthesis of Thr-rich immune system metabolites, such as immunoglobulins, APP, and especially intestinal mucins, since N retention, and thus protein synthesis, was decreased in PRRSV-infected pigs (Faure et al., 2007; Rémond et al., 2009; Rakhshandeh and de Lange, 2011). 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). Considering that Thr accounts for 16% to 20% of crude mucin, increased synthesis and secretion of mucins can substantially impact Thr requirements in growing pigs 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. (2013c) where they observed a 1.6-fold increase in the expression of mucin 2, a major proteinous component of mucus, in the small intestine of pigs challenged with LPS (Rakhshandeh et al., 2013b). Thus, an increase in the release of Thr from proteolysis could be associated with prioritization of this AA for the synthesis of immune system metabolites during PRRSV infection (Kampman-van De Hoek et al., 2015). Moreover, PRRSV infection is specifically associated with an increase in the synthesis and secretion of mucus in respiratory ducts, bronchioles, and the oropharynx (Plagemann, 2003), providing yet a further explanation for enhanced Thr flux in PRRSV-infected pigs. Collectively, the findings of the current study suggest a substantial enhancement in the metabolic demand for Thr during PRRSV challenge, which may increase the dietary requirement for Thr. These results warrant further studies to directly evaluate the effect of ISS on dietary SID Thr requirements.

Previous studies have suggested that BCAAs are essential for proliferation, growth, and the normal function of cells of the immune system, primarily lymphocytes (Calder, 2006; Monirujjaman and Ferdouse, 2014). Some 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., 1984; Hale et al., 2004; Thornton et al., 2006). In the current study, challenge with PRRSV affected neither the flux nor the release from proteolysis, but increased the Ile pool

size, suggesting a decrease in Ile utilization. In other words, our findings suggested that the metabolic demand for Ile may decrease during ISS. Leucine flux and release from proteolysis were not affected by PRRSV challenge, suggesting no change in the metabolic need for Leu under these conditions. Therefore, increases in the Leu pool size can be associated with SID Leu intake in the current study and that Leu may have been in excess of its requirement in PRRSV+ pigs. These findings are consistent with the findings of [Kampman-van De Hoek et al. \(2015\)](#) and [Rudar et al. \(2017\)](#), who reported no change in Leu flux in ISS pigs ([Kampman-van De Hoek et al., 2015](#); [Rudar et al., 2017](#)). Infection with PRRSV had no effect on Ile flux, release from proteolysis, and pool size, suggesting that the utilization of this AA was not affected by ISS and that the supply of Ile in the diet was sufficient to meet requirements. Our results are in general agreement with the findings of [Kampman-van De Hoek et al. \(2015\)](#), who reported no change in the flux of Ile in feed-restricted pigs challenged with CFA ([Kampman-van De Hoek et al., 2015](#)). Challenge with PRRSV resulted in a concomitant increase in Val release from proteolysis and the Val pool size, and a strong tendency for increased Val flux. Increased Val flux in PRRSV+ pigs can mainly reflect the enhanced Val efflux toward irreversible loss of Val as protein retention, and thus, protein synthesis was significantly lower in these pigs. The enhanced irreversible loss of Val in PRRSV+ pigs can likely be associated with 1) increased utilization of Val for the synthesis of mucins since Val is the second most abundant essential AA, after Thr, in the structure of these proteins, and/or 2) increased catabolism of Val. It is now well known that an imbalance between BCAA, caused by even a moderate excess of Leu, can increase the Val catabolism, similar to that we observed in PRRSV+ pigs in the current study ([Langer and Fuller, 2000](#); [Faure et al., 2006](#)). The increase in the Val pool size likely occurred because the rate of Val efflux from the pool was lower than the influx of Val from proteolysis and intake in PRRSV+ pigs ([Waterlow, 2006](#)). Taken together, these results provide no evidence for an increased metabolic demand or requirement for BCAA, with the exception of Val, during ISS. Increased metabolic demand for Val during ISS may increase dietary Val requirement. These results warrant further studies to establish an optimum balance between BCAA for ISS pigs.

Based on the calculation of [Reeds et al. \(1994\)](#), an increase in the utilization of aromatic AA (Phe, Tyr, and Trp) during ISS can be expected, due to

increased turnover of aromatic AA-rich APP during the acute phase response of ISS ([Reeds et al., 1994](#)). Other investigators, using plasma concentration or N-balance as the sole measurements, suggested an increase in both the metabolic need and dietary requirements for Trp, probably due to enhanced catabolism of Trp during ISS ([Melchior et al., 2004](#); [de Ridder et al., 2012](#)). In the current study, we observed a tendency for increased the Phe pool size with no changes in flux or release from proteolysis in PRRSV-infected pigs. These results suggest a decrease in the utilization of Phe in PRRSV-challenged pigs. The increased Phe pool size may have occurred as the result of decline in the synthesis of tyrosine (Tyr) from Phe. The latter is important because pro-inflammatory cytokines downregulate the activity of phenylalanine-hydroxylase, a key enzyme that catalyzes the synthesis of Tyr from Phe, during ISS ([Capuron et al., 2011](#)). Alternatively, the increased Phe pool size could be explained by an increased release of Phe from protein breakdown during ISS. Although not statistically different, the Phe release from proteolysis was 49% higher in PRRSV+ pigs than PRRSV- pigs in the current study. These results also suggest that the utilization of Phe for the synthesis of APP during ISS might not be great enough to impact Phe flux. Additionally, infection with PRRSV did not affect Trp flux or release from proteolysis, but increased the Trp pool size, suggesting that any change in the metabolic demand for Trp during PRRSV infection is not quantitatively large enough to affect Trp kinetics. Furthermore, PRRSV infection did not affect the flux of Gln, but increased the Gln pool size, suggesting that any putative increase in the Gln requirement during disease is not large enough to affect Gln utilization. Increased Gln pool size can mainly be associated with increased AA catabolism in the peripheral tissues (i.e., skeletal muscle) of PRRSV+ pigs, since Gln is synthesized predominantly in the muscle and act as a carrier for transport of excess N ([Pitts and Pilkington, 1966](#)). Taken together, these results suggested that ISS has no effect on metabolic need for Phe, Trp, and Gln when pigs fed a corn-SBM-based diet formulated to optimize growth.

SUMMARY AND CONCLUSIONS

Collectively, the results of this study suggested that i.m. inoculation with PRRSV elicited an effective but relatively severe ISS, which led to a significant reduction in FI. Despite this reduction, 3 important results were found. First, challenge with PRRSV reduced AID of dietary N but had

no effect on ATTD of dietary energy. The reduced AID of N in PRRSV-challenged pigs is likely, and in part, associated with increased intestinal EAAL, since enhanced production of protective mucins/mucus is a part of the innate immune response. This idea can be further supported by the enhanced flux of Thr and Val, the 2 most abundant essential AAs in the structure of mucins that was observed. Second, in nursery pigs fed a conventional corn-SBM diet that was formulated for optimum growth, infection with PRRSV increased the plasma Met, Thr, and Val flux and increased the release of these AA from proteolysis. Increased Val flux to certain extent can be associated with enhanced catabolism of Val caused by an ISS-induced imbalance between BCAA. Increased flux of these AA may be largely attributed to an increase in their utilization for the synthesis of immune system proteins and metabolites and may affect their daily dietary requirements. Finally, in starter pigs that fed a commercial style corn-SBM-based diet, PRRSV infection does not increase the metabolic demand and probably the dietary requirements for Lys, Leu, Ile, Phe, Trp, and Gln. Indeed, the findings of the current study provide evidence for a reduction in the demand for these AA. Therefore, an optimum ratio between the AA studied here needs to be established for PRRSV-challenged pigs. In addition, further studies are needed to evaluate the effects of PRRSV infection on basal and specific intestinal endogenous loss of AAs.

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