Excess dietary leucine in diets for growing pigs reduces growth performance, biological value of protein, protein retention, and serotonin synthesis[1]

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ABSTRACT: An experiment was conducted to test the hypothesis that excess dietary Leu affects metabolism of branched-chain AA (**BCAA**) in growing pigs. Forty barrows (initial BW: 30.0 ± 2.7 kg) were housed individually in metabolism crates and allotted to 5 dietary treatments (8 replicates per treatment) in a randomized complete block design. The 5 diets were based on identical quantities of corn, soybean meal, wheat, and barley and designed to contain 100, 150, 200, 250, or 300% of the requirement for standardized ileal digestible Leu. Initial and final (d 15) BW of pigs were recorded. Daily feed consumption was also recorded. Urine and fecal samples were collected for 5 d following 7 d of adaptation to the diets. At the end of the experiment, blood and tissue samples were collected to analyze plasma urea N, plasma and hypothalamic serotonin, tissue BCAA, serum and tissue branched-chain α-keto acids, and mRNA abundance of genes involved in BCAA metabolism. Results indicated that ADG, ADFI, and G:F decreased (linear, $P < 0.05$) as dietary Leu increased. A trend (linear, $P = 0.082$) for decreased N retention and decreased (linear, $P < 0.05$) biological value of dietary protein was also observed, and plasma urea N increased (linear, $P < 0.05$) as dietary Leu increased. A quadratic reduction ($P < 0.05$) in plasma serotonin and a linear reduction ($P < 0.05$) in hypothalamic serotonin were observed with increasing dietary Leu. Concentrations of BCAA in liver increased (linear, $P < 0.001$) whereas concentrations of BCAA in skeletal muscle decreased (linear, $P < 0.05$) as dietary Leu increased. Concentration of α-ketoisovalerate was reduced (linear and quadratic, $P < 0.001$) in liver, skeletal muscle, and serum, and α-keto-β-methylvalerate was reduced (linear, $P < 0.001$; quadratic, $P < 0.001$) in skeletal muscle and serum. In contrast, α-keto isocaproate increased (linear, $P < 0.05$) in liver and skeletal muscle, and also in serum (linear and quadratic, $P < 0.001$) with increasing dietary Leu. Expression of mitochondrial BCAA transaminase and of the E1α subunit of branched-chain α-keto acid dehydrogenase increased (linear, $P < 0.05$) in

skeletal muscle as dietary Leu increased. In conclusion, excess dietary Leu impaired growth performance and nitrogen retention, which is likely a result of increased catabolism of Ile and Val, which in turn reduces availability of these AA resulting in reduced protein retention and excess dietary Leu also reduced hypothalamic serotonin synthesis.

Key words: branched-chain amino acids, leucine, pigs, serotonin, tryptophan

INTRODUCTION

Leucine, Val, and Ile are categorized as the branched-chain AA (**BCAA**) because of the structural similarity of their side chains (Harper et al., 1984). All 3 BCAA share the enzymes that are involved in the first 2 steps of their catabolic pathway (Harris et al., 2005). The first step of catabolism of BCAA is a transamination step catalyzed by BCAA transaminase (**BCAT**). This step produces branched-chain α-keto acids (**BCKA**) from BCAA in a reversible reaction. The branched-chain α-keto acid dehydrogenase (**BCKDH**) complex is the second common enzyme complex that is needed for the irreversible degradation of BCKA to produce the acyl-CoA derivatives from the BCKA. The α-keto acids that are the results of metabolism of Leu, Val, and Ile are α-keto isocaproate (**KIC**), α-ketoisovalerate (**KIV**), and α-keto-β-methylvalerate (**KMV**), respectively. Leucine is considered a key regulator of the metabolism of BCKA, because KIC stimulates activation of the BCKDH complex in the liver (Harper et al., 1984). As corn and corn co-products have relatively high Leu concentrations compared with other protein sources, it is more likely that diets have excess Leu if large amounts of these ingredients are used. For example, if a corn-based diet with 30% corn distillers dried grain with solubles is fed to growing pigs, dietary Leu will be 150 to 200% of the requirement. If excess Leu is included in diets for pigs, the metabolism of all 3 BCAA may increase because of increased activities of BCAT. Higher activity of BCAT may then produce more KIC, which activates BCKDH with increased metabolism of Ile and Val as a result. Excess Leu, therefore, may decrease the quantities of Val and Ile available for protein synthesis and cause reduction in protein retention (Wiltafsky et al., 2010). However, limited data are available on effects of dietary Leu on expression of BCAT and BCKDH and on requirements for Val and Ile in growing pigs.

Leucine may also have an inhibitory effect on feed intake by stimulating the mechanistic target of rapamycin in the brain (Cota et al., 2006). Tryptophan is involved in feed intake regulation partly by enhancing serotonin signaling in the brain (Henry et al., 1992). Leucine and Trp are both categorized as large neutral AA (**LNAA**), and they share a common uptake pathway across the blood-brain barrier (Barea et al., 2009). As a consequence, it is possible that excessive Leu may result in reduced Trp uptake into the brain due to competition for transporters, resulting in reduced serotonin synthesis (Wessels et al., 2016a,b). Therefore, the objective of this experiment was to test the hypothesis that excess dietary Leu may affect N balance, growth performance, plasma urea N (**PUN**), plasma and hypothalamic serotonin, tissue BCAA, serum and tissue BCKA, and abundance of genes related to BCAA metabolism in growing pigs.

MATERIALS AND METHODS

Animal care procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois.

Animals, Diets, and Experimental Design

Forty growing barrows with an initial BW of 30.0 ± 2.7 kg were allotted to 5 dietary treatments with 8 replicate pigs per treatment in a randomized complete block design. There were 4 blocks of 10 pigs with 2 pigs per diet in each block and diets were fed for 15 d. The 5 experimental diets were formulated to contain identical quantities of corn, soybean meal, wheat, and barley (Table 1), but L-Leu was included in the 5 diets at 0, 0.5, 1.0, 1.5, or 2.0% (Tables 2 and 3). The requirement for standardized ileal digestible (**SID**) Leu for 25 to 50 kg pigs is estimated to be 0.99% (NRC, 2012), which corresponds to a SID Leu:Lys ratio of 1.01:1.0. The

basal diet contained 0.98% SID Leu and 1.0% SID Lys, and thus was believed to provide Leu at the requirement. By adding crystalline Leu to this diet, experimental diets containing 150, 200, 250, or 300% of the requirement for SID Leu were formulated. Glycine inclusion in the diets was reduced as Leu inclusion increased to maintain a constant concentration of dietary CP at 15%.

Housing and Feeding

Pigs were individually housed in metabolism crates that were equipped with a slatted floor, a feeder, and a nipple drinker. A screen for fecal collections was placed below the slatted floor and a pan for urine collection was placed under the screen. Pigs were fed at 3 times the energy requirement for maintenance (i.e., 197 kcal/kg × $BW^{0.60}$; NRC, 2012), which was provided each day in 2 equal meals at 0800 and 1600 h. Water was provided on an *ad libitum* basis.

Sample Collection and Data Recording

The initial 7 d of the experiment were considered the adaptation period to the experimental diets and conditions. Urine and fecal samples were collected during the following 5 d according to standard procedures for the marker to marker method (Adeola, 2001). Fecal collection was ended with the appearance of the second marker on d 14. Urine was collected in buckets containing 50 mL of 3*N* HCl as a preservative. Fecal samples and 20% of the collected urine were stored at -20°C immediately after collection. At the conclusion of the experiment, urine samples were thawed and mixed within animal and diet. The BW of pigs was recorded at the beginning and at the conclusion of the experiment. The amount of feed supplied and feed refusals were recorded daily.

Blood and Tissue Collection and Analysis

On d 15 in the morning, pigs were fed 400 g of their experimental diet 2.5 h prior to blood sampling. Three blood samples were collected from the jugular vein of all pigs using heparinized vacutainers, vacutainers containing EDTA, and serum-separating vacutainers (BD, Franklin Lakes, NJ). Plasma and serum were obtained by centrifugation at 1,500 × *g* at 4°C for 15 min. Plasma from blood in heparinized tubes was used to analyze for PUN using a Beckman Coulter Clinical Chemistry AU analyzer (Beckman Coulter Inc., Brea, CA). Platelet-free plasma was prepared from anticoagulated blood containing EDTA by double centrifugation according to the protocol described by Shen et al. (2012). The supernatant was filtered with a 0.45 µm syringe filter to remove remaining platelets from the plasma and was stored at -80°C until analysis. Serum samples were used to determine serum BCKA.

After blood sampling, all pigs were euthanized by electrocution and then exsanguinated. Samples of liver and skeletal muscle (longissimus dorsi) tissue were collected into 2mL cryogenic tubes and snap-frozen in liquid N. Brain tissue was also removed, and the hypothalamus was isolated and frozen in liquid N. All tissue samples were stored at -80°C until analysis. Concentration of serotonin in the platelet-free plasma and hypothalamus were analyzed using ELISA kits developed for porcine tissues according to the manufacturer's protocol (GenWay Biotech, Inc., San Diego, CA). For plasma analysis, 50 µL of platelet-free plasma was used. To obtain homogenates from the hypothalamus, frozen samples were weighed (0.5 g) and homogenized with buffer solution on ice using a hand-held Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). The homogenate was centrifuged at 15,000 × g at 4°C for 30 min and the supernatant was used to determine the concentration of tissue-free serotonin in the hypothalamus.

Liver, skeletal muscle, and plasma samples were lyophilized in a vacuum-freeze dryer (Lyo Screen Control Plus; IMA Life, Tonawanda, NY) and homogenized. Amino acid analysis (method 999.13; AOAC Int., 2007) was conducted using HPLC (L-8900 AA Analyzer; Hitachi, Japan) by Ajinomoto Animal Nutrition North America Laboratory (Eddyville, IA) to measure BCAA composition in liver and skeletal muscle tissues and Trp concentration in plasma, respectively.

Branched-Chain α-Keto Acid Analysis

Quantification of BCKA in serum and tissues was carried out by liquid chromatography-mass spectrometry (LC/MS) analysis using a Sciex 5500 QTrap with Agilent 1200 LC (AB Sciex, Framingham, MA) according to the protocol described by Olson et al. (2013). Frozen liver and skeletal muscle tissues were maintained in liquid N, and then powdered one at a time using a stainless steel mortar and pestle. High-performance liquid chromatography grade methanol was used as an extraction solvent to remove interfering proteins from serum and tissue homogenates (Zhang et al., 2018). To increase recoveries of the 3 BCKA, a 5-min ultrasonic treatment was conducted using an ultrasonic generator (Qsonica Q700; Qsonica, Newtown, CT) at maximum amplitude (100%).

Gene Expression

Total RNA was extracted from liver tissue using the RNeasy Mini Kit and from skeletal muscle tissue using the RNeasy Fibrous Tissue Kit (Qiagen, Valencia, CA) according to protocols from the manufacturer. Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA quality was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and all RNA samples used for reverse transcription had an RNA integrity number greater than 8.

Total RNA (100 ng/μL) was reverse transcribed by means of a SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA) to synthesize the double-stranded complementary DNA (**cDNA**). Double-stranded cDNA was diluted and used for quantitative reverse transcription polymerase chain reaction (**qRT-PCR**). Each 10 μL reaction consisted of 5 μL SYBR® Green (Applied Biosystems, Foster City, CA), 4 μL diluted cDNA sample, 0.4 μL of 10 μM forward and reverse primers (Table 4), and 0.2 μL DNase/RNase free water. The qRT-PCR were performed in a QuantStudio™ 7 Flex (Applied Biosystems, Foster City, CA) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. An additional dissociation stage was added to verify the presence of a single PCR product. All reactions were run in triplicate. Data were analyzed using the QuantStudio™ 6 and 7 Flex Software (Applied Biosystems, Foster City, CA).

Two internal control genes, glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) and hydroxymethylbilane synthase (**HMBS**), were used to normalize the expression of tested genes (Vigors et al., 2014). The GAPDH gene was used because it is constitutively expressed at high levels in most tissues and it was expected that glycolysis would not be different among pigs fed experimental diets. The HMBS gene was used because it was expected that heme synthesis would not be different among pigs fed experimental diets.

The tested genes included mitochondrial BCAT (**BCATm**), BCKDH E1βα, BCKDH E1β, BCKDH E2, and BCKDH kinase (**BCKDK**). The BCKDH complex consists of 3 subunits (E1, E2, and E3). However, the E3 unit was not included in the analysis of gene expression because it is not BCKDH-specific, whereas the BCKDK was included because it causes inactivation of the BCKDH complex. To obtain the value of relative gene expression, the

average of triplicate samples was used and divided by the geometric mean values from the 2 internal control genes.

Chemical Analyses

Prior to analysis, frozen fecal samples were dried in a forced-air drying oven at 55°C until constant weight and ground for analysis. Ingredients, diets, fecal samples, and urine samples were analyzed for CP (method 984.13; AOAC Int., 2007) using a Kjeltec 8400 apparatus (FOSS Inc., Eden Prairie, MN). Samples of corn, SBM, wheat, and barley, which were the main ingredients in the diets, and all experimental diets were analyzed for AA [method 982.30 E (a, b, c); AOAC Int., 2007] using an Amino Acid Analyzer (model L 8800; Hitachi High Technologies America Inc., Pleasanton, CA). All diets were analyzed for DM (method 930.15; AOAC Int., 2007), ash (method 942.05; AOAC Int., 2007), and concentration of acid hydrolyzed ether extract (method AM 5-04; AOAC Int., 2007) was measured by ANKOM HCl hydrolysis system and an ANKOM XT15 fat extractor (ANKOM Technologies, Macedon, NY). All diets were also analyzed for GE using a bomb calorimeter (model 6400; Parr Instruments, Moline, IL).

Calculations and Statistical Analyses

The apparent total tract digestibility of N in each experimental diet and retention of N for each pig were calculated based on the method described by Pedersen et al. (2007). The apparent total tract digestibility of N was calculated using Eq. [1]:

$$\text{ATTD of N} = [(\text{Ni} - \text{Nf})/\text{Ni}] \times 100\%, \quad [1]$$

where ATTD of N is the apparent total tract digestibility of N (%); Ni is the N intake (g) from d 7 to 12; and Nf is the N output (g) in feces originating from the feed that was fed from d 7 to 12.

The retention of N (Nr) for each pig was calculated using Eq. [2]:

$$Nr = \{[Ni - (Nf + Nu)]/Ni\} \times 100\%, \quad [2]$$

where Nr is the retention of N (%), Ni is the N intake (g) from d 7 to 12, Nf and Nu are N output (g) in feces and urine originating from the feed that was fed from d 7 to 12, respectively.

The biological value of the protein in the diets was also calculated by expressing the retention of N as a percentage of the difference between N intake and N output in feces. (Rojas and Stein, 2013). Normality of data was verified and outliers were identified using the UNIVARIATE procedure (SAS Inst. Inc., Cary, NC). Data were analyzed using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC). The experimental unit was the pig and the model included dietary treatment as a fixed variable and block and replicate within block as random variables. Treatment means were separated by using the LSMEANS statement. Orthogonal polynomial contrasts were used to determine linear and quadratic effects of increasing levels of SID Leu in experimental diets. Statistical significance and tendency were considered as $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

RESULTS

All animals were healthy throughout the experiment. Analyzed values for Leu and Lys were in agreement with calculated values in experimental diets. However, calculated CP values

appeared to be overestimated in experimental diets. Final BW, ADG, ADFI, and G:F decreased (linear, $P < 0.05$) as dietary SID Leu increased (Table 5). Although all pigs were fed similar amounts of feed throughout the experimental period, feed refusals increased linearly ($P < 0.05$) as dietary SID Leu increased (data not shown).

During the 5-d collection period, there was a tendency (linear, $P = 0.056$) for decreasing feed intake as dietary SID Leu increased (Table 6), but there were no linear or quadratic effects of dietary Leu on total N intake, fecal and urinary N excretion, apparent total tract digestibility of N, or retention of N (% of intake). A trend (linear, $P = 0.082$) for decreased N retention (g/5 d) was observed with increasing SID Leu in experimental diets. However, if daily feed intake was used as a co-variate in the analysis, (adjusted mean = 6,678 g/5 d), no linear or quadratic effects of excess Leu on N retention (g/5 d) was observed. The biological value of protein was reduced (linear, $P < 0.05$) as dietary Leu increased and this reduction was observed in the original analysis as well as if feed intake was used as a co-variate in the analysis.

A linear increase ($P < 0.05$) in PUN was observed with increasing SID Leu in the diets (Fig. 1) and increasing dietary Leu resulted in a quadratic reduction ($P < 0.05$) in plasma serotonin (Fig. 2). Likewise, hypothalamic serotonin linearly decreased ($P < 0.05$) with increasing SID Leu in the diets (Fig. 3).

Concentrations of BCAA in the liver increased linearly ($P < 0.001$) with increasing SID Leu in the diets (Table 7). In contrast, concentrations of BCAA in skeletal muscle decreased linearly ($P < 0.05$) with increasing SID Leu in the diets.

Concentrations of plasma free Ile, Trp, Val, Ala, and Cys decreased (linear, $P < 0.05$; quadratic, $P < 0.05$) as dietary SID Leu increased (Table 8). Likewise, concentrations of Gly, Pro, and Ser in plasma linearly decreased ($P < 0.05$) as dietary SID Leu increased. In contrast,

Plasma free Leu concentration increased (linear and quadratic, $P < 0.001$) as dietary SID Leu increased. There were linear increases ($P < 0.05$) in concentrations of His and Phe in plasma with increasing dietary SID Leu, but the calculated Trp to LNAA ratio in plasma linearly decreased ($P < 0.05$) as dietary SID Leu increased.

Linear and quadratic reductions (linear, $P < 0.001$; quadratic, $P < 0.001$) in KIV in the liver, skeletal muscle, and serum was observed as dietary SID Leu increased (Table 9). Likewise, there were linear and quadratic decreases (linear, $P < 0.001$; quadratic, $P < 0.001$) in the concentration of KMV in skeletal muscle and serum with increasing dietary SID Leu. In contrast, concentrations of KIC in the liver and skeletal muscle increased linearly ($P < 0.05$) with increasing SID Leu in the diets. In the serum, increases (linear, $P < 0.001$; quadratic, $P < 0.001$) in KIC concentration were observed as dietary SID Leu increased. Calculated Trp to NLAA ratio linearly decreased as dietary

Expression of BCATm, BCKDH E1α, BCKDH E1β, BCKDH E2, and BCKDK in the liver was not affected by increasing SID Leu in the diets (Table 10). In skeletal muscle, linear increases ($P < 0.05$) in the expression of BCATm and the E1α subunit of BCKDH were observed as dietary SID Leu increased. However, expression of BCKDH E1β, BCKDH E2, and BCKDK in skeletal muscle was not affected by increasing SID Leu in the diets.

DISCUSSION

The objective of the current study was to test the hypothesis that excess dietary Leu may affect N balance, growth performance, PUN, plasma and hypothalamic serotonin, tissue BCAA, serum and tissue BCKA, and abundance of genes related to BCAA metabolism. The SID Val:Lys ratio needed to maximize growth performance of pigs is around 0.70:1 (Gloaguen et al.,

2011; Waguespack et al., 2012; Soumeh et al., 2015) although the ratio suggested by NRC (2012) is 0.65:1. Dose-response experiments with Ile have been conducted in growing pigs to determine the optimal SID ratio of Ile to Lys and a ratio of approximately 0.53:1 in diets without spray-dried blood cells appears to maximize pig growth performance (Wiltafsky et al. 2009; Waguespark et al., 2012). This ratio is close to the estimated requirement of NRC (2012). Therefore, a SID Val:Lys ratio of 0.70:1 and a SID Ile:Lys ratio of 0.53:1 were used in the formulation of experimental diets.

Because no differences in N intake and in the apparent total tract digestibility of N were observed, the reduced retention of N and the reduced biological value of N that was observed as the SID Leu:SID Lys increased is indicative of the reduced utilization of dietary N for protein deposition as dietary Leu increased. The negative correlation between dietary Leu and N utilization may be due to an increased degradation of all 3 BCAA, which then resulted in a deficiency of Val and Ile (Wiltafsky et al., 2010).

The concentration of PUN is often used as a response criterion in AA requirement studies, because PUN is considered a rapid parameter of both changes in dietary AA concentration and efficiency of AA utilization in pigs (Coma et al., 1995). The increased PUN that was observed as pigs were fed increasing dietary Leu is most likely a result of increased catabolism of Ile and Val, which in turn reduces availability of these AA and causes an imbalance among other indispensable AA (Gatnau et al., 1995). If these or other AA reduced protein retention as indicated by the reduced N retention, an imbalance among indispensable AA may have been generated, which likely resulted in increased deamination of AA and a subsequent increase in PUN. Increased intake of Leu likely also contributed to the increase in PUN.

The linear reductions in ADFI, ADG, and G:F that were observed in the present study as dietary Leu increased is in agreement with reported responses to excess dietary Leu (Harper et al., 1984; Gatnau et al., 1995; Wiltafsky et al., 2010; Wessels et al., 2016c). This may be a result of the imbalanced supply of BCAA that resulted from the reduced availability of Val and Ile in diets with excess Leu. Gloaguen et al. (2012) indicated that pigs can detect BCAA imbalances in diets within 1 h after a meal is provided and that they will avoid eating that diet, which indicates that there is an innate mechanism against imbalanced supply of indispensable AA in the diet. Sensing AA deficiency by the anterior piriform cortex along with reduced feed intake of AA-deficient diets is considered a protective mechanism to prevent degradation of protein in the brain (Hao et al., 2005).

Tryptophan is a precursor for serotonin, which is a cerebral neurotransmitter that plays an important role in appetite regulation (Zhang et al., 2007). High Trp intake increases pigs feed intake by pigs (Henry et al 1992; Ettle and Roth, 2004), and this may be partly attributed to increased serotonin synthesis (Shen et al., 2012). There is a positive correlation between hypothalamic Trp and hypothalamic serotonin, whereas hypothalamic Trp and plasma Leu are negatively correlated (Wessels et al., 2016a). Thus, the decreased serotonin concentration in both plasma and hypothalamus that was observed in the present study as dietary Leu increased, indicates that excess dietary Leu may reduce Trp uptake into the brain, resulting in decreased serotonin synthesis in the hypothalamus. It is possible that reduced Trp uptake in the brain increases Trp in plasma, but the current data indicated that Trp in plasma was linearly decreased as dietary Leu increased. This inconsistency is likely a result of the reduced Trp intake that was observed as dietary Leu increased. Henry et al. (1992) indicated that low Trp to LNAA ratio in plasma decreased serotonin synthesis in the hypothalamus, resulting in reduced voluntary feed

intake in pigs. Therefore, reduced Trp to LNAA ratio in plasma, which is mainly a result of increased dietary Leu, may also have contributed to the reduced feed intake that was observed in this experiment as dietary Leu increased.

Branched-chain α-keto acids are derived from the first step in metabolism of BCAA via BCAT, which is a reversible transaminase that is mainly present in skeletal muscle. The enzymatic transfer of the amino group of BCAA to pyruvate or Glu to synthesize Ala or Gln results in the carbon skeleton from the 3 BCAA being turned into 3 α-keto acids that are specific to each of the 3 BCAA. The BCAT consists of mitochondrial BCAT (**BCATm**) and cytosolic BCAT isoenzymes, but only BCATm was analyzed in this experiment because this enzyme has high activity in skeletal muscle (Wiltafsky et al., 2010). The increased mRNA abundance of BCATm only in skeletal muscle that was observed indicates that the transamination of BCAA was increased in skeletal muscle, but not in the liver, as dietary Leu increased. Wiltafsky et al. (2010) reported that expression of BCATm in skeletal muscle is greater than in the liver, but we were not able to confirm that observation.

Because BCAT is primarily located in skeletal muscle, BCAAs represent about 50 % of skeletal-muscle AA uptake, and most of the other plasma AA do not undergo catabolism in muscle (Hutson et al., 2005). In the present experiment, sampling of tissues occurred in the early postprandial phase (2.5 h after eating), and the observed concentrations of AA may, therefore, reflect an intermediate state of BCAA metabolism, which may be the reason greater concentration of AA in the muscle than in the liver was observed. However, additional research is required to determine if concentrations change over time after a meal.

The second step in the BCAA catabolic pathway is irreversible and involves the BCKDH complex (Harper et al.,1984), which catalyzes the decarboxylation of the α-keto acids. The

complex is mainly located in the liver and consists of 3 catalytic subunits (E1, E2, and E3 subunits). This enzyme complex catabolizes all 3 BCKA to form the corresponding branched-chain acyl-CoA, and this step is considered the most important step in BCAA catabolism (Wiltafsky et al., 2009; Wessels et al., 2016b). Crowell et al. (1990) indicated that dietary supplementation of KIC to a low CP diet fed to rats resulted in increased KIC concentration in plasma, whereas KIV and KMV concentrations were reduced. This indicates that KIC is the key regulator of the BCAA catabolic process (Langer et al., 2000; Wiltafsky et al., 2010). In the present study, the increased KIC in liver, muscle, and serum of pigs fed increasing dietary Leu is most likely a result of the increased expression of BCATm. The reduced concentrations of KIV and KMV that were observed in serum and tissues as dietary Leu increased are in accordance with data obtained in pigs (Langer et al., 2000; Wiltafsky et al., 2010), broiler chickens (Calvert et al., 1982), and rats (Block and Harper, 1984; Harper and Benjamin, 1984). It is likely that the increased stimulation of BCKDH that was a result of increased KIC by excess dietary Leu increased decarboxylation of KIV and KMV, which resulted in the reduced KIV and KMV concentrations that were observed. However, there were no clear changes in the abundance of genes related to the BCKDH complex in the skeletal muscle or in the liver as dietary Leu increased. This observation is in agreement with Wiltafsky et al. (2010) who concluded that the abundance of genes related to the BCAA catabolic pathway may not be drastically changed by alterations in dietary Leu or KIC, because mechanisms that adapt to high concentrations of Leu and KIC are believed to be regulated post-transcriptionally. Activity of BCKDH is also regulated by phosphorylation of BCKDK (Zhou et al., 2012). However, there were also no clear changes in the abundance of BCKDK genes in skeletal muscle or liver. It is possible that endocrine regulations are involved in expression of BCKDK (Shimomura et al., 2001; Harris et al., 2001).

Recently, Wessels et al. (2016b) reported that excess dietary Leu increased BCKDH activity in several tissues including pancreas, kidney, liver, cardiac muscle, and brain, and indicated that the most significant increase of BCKDH activity was detected in the brain. This indicates that the cellular post-transcriptional and post-translational regulations play important roles in the BCAA catabolic pathway in response to excess Leu.

The current study confirms that excess dietary Leu has negative impact on growth performance, N utilization, protein retention, and serotonin synthesis in growing pigs, although the effect of excess Leu on metabolism of BCAA remains unclear. To clarify the mode of action of excess dietary Leu in pigs, it is necessary to determine the activities of BCAT, BCKDH, and BCKDK. Additional research, therefore, is needed to determine the antagonism of Leu on BCAA metabolism and investigate the interaction between Leu and Trp on appetite regulation in pigs. The observation that increasing dietary Leu from 100 to 150% of the requirement resulted in a reduction in liver, muscle, and serum α-keto-β-methylvalerate and α-keto isovalerate, without changing N-retention may be a result of downstream Leu metabolites stimulating protein synthesis. Human volunteers consuming the Leu metabolite β-hydroxy-β-methylbutyrate (**HMB**) had reduced urinary nitrogen excretion, but no changes in plasma urea compared with a placebo-supplemented control group (Nissen and Abumrad, 1997). Likewise, human subjects consuming HMB had reduced muscle breakdown and increased lean body mass gain compared with controls consuming a placebo (Nissen and Abumrad, 1997). It may, therefore, be speculated that protein degradation, protein synthesis, and protein retention may be influenced by Leu metabolites, but research to address if this is taking place in pigs is needed.

In conclusion, N retention and biological value of N were decreased as dietary Leu exceeded the requirement, which indicates that excess dietary Leu may increase catabolism of

Val and Ile, and thereby create an AA imbalance. Growth performance of pigs was reduced because of reduced ADFI, lack of free Val and Ile as substrates for protein synthesis, and consequently reduced protein retention as dietary SID Leu increased. The PUN concentration in pigs fed increasing levels of dietary Leu increased as a consequence of the increased dietary Leu as well as catabolism of Ile and Val, which may have reduced the availability of these AA for protein synthesis and caused an imbalance among other indispensable AA. Changes in BCAA and BCKA concentrations were observed, whereas changes in abundance of genes related to the BCAA catabolic pathway were not observed as dietary Leu increased. Plasma and hypothalamic serotonin decreased because of excess dietary Leu, which likely resulted in reduced Trp uptake into the brain, which subsequently may have impaired appetite regulation. Overall, it appears that excess dietary Leu may have negative impacts on protein retention and feed intake and the likely reason for this is antagonism between Leu and Val, Ile, and Trp.

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Table 1. Chemical composition of ingredients used in experimental diets (as-fed basis)[1]

Item	Corn	Soybean meal	Wheat	Barley
CP, %	7.09	44.42	11.74	10.98
Indispensable AA, %				
Arg	0.33	3.03	0.50	0.48
His	0.22	1.16	0.27	0.24
Ile	0.27	2.01	0.38	0.36
Leu	0.85	3.29	0.71	0.67
Lys	0.27	2.70	0.36	0.43
Met	0.14	0.58	0.15	0.16
Phe	0.37	2.16	0.49	0.49
Thr	0.26	1.65	0.31	0.34
Trp	0.06	0.60	0.14	0.10
Val	0.35	2.11	0.48	0.51
Dispensable AA, %				
Ala	0.52	1.85	0.41	0.43
Asx[2]	0.49	4.74	0.58	0.65
Cys	0.17	0.62	0.25	0.22
Glx[3]	1.31	7.86	2.94	2.18
Gly	0.30	1.80	0.49	0.44
Pro	0.66	2.38	1.05	1.03
Ser	0.32	2.02	0.43	0.37
Tyr	0.19	1.56	0.23	0.22

[1]Ingredients were analyzed in duplicate.

[2]Asx = Asp and Asn.

[3]Glx = Glu and Gln.

Table 2. Ingredient composition of experimental diets (as-fed basis)

	SID[1] Leu relative to requirement[2], %				
Ingredient, %	100	150	200	250	300
Ground corn	20.74	20.74	20.74	20.74	20.74
Soybean meal, 44% CP	12.50	12.50	12.50	12.50	12.50
Wheat	33.00	33.00	33.00	33.00	33.00
Barley	25.00	25.00	25.00	25.00	25.00
Cornstarch	0.80	0.60	0.40	0.20	-
Soybean oil	3.00	3.00	3.00	3.00	3.00
L-lysine·HCl	0.55	0.55	0.55	0.55	0.55
DL-methionine	0.10	0.10	0.10	0.10	0.10
L-threonine	0.24	0.24	0.24	0.24	0.24
L-tryptophan	0.04	0.04	0.04	0.04	0.04
L-leucine	-	0.50	1.00	1.50	2.00
L-isoleucine	0.02	0.02	0.02	0.02	0.02
L-valine	0.11	0.11	0.11	0.11	0.11
Glycine	1.20	0.90	0.60	0.30	-
Limestone	1.20	1.20	1.20	1.20	1.20
Monocalcium phosphate	0.80	0.80	0.80	0.80	0.80
Salt	0.40	0.40	0.40	0.40	0.40
Vitamin-mineral premix[3]	0.30	0.30	0.30	0.30	0.30

[1]SID = standardized ileal digestible.

[2]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[3]The vitamin-micromineral premix provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B12, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.

Table 3. Analyzed and calculated nutrient composition of experimental diets (as-fed basis)[1]

Item	SID[2] Leu relative to requirement[3], %				
	100	150	200	250	300
Analyzed composition					
DM, %	88.54	88.46	88.71	88.46	88.34
GE, kcal/kg	4,003	4,008	4,014	4,020	4,028
CP, %	15.15	15.24	15.23	15.23	15.48
Acid hydrolyzed ether extract, %	6.02	5.98	6.06	5.85	5.93
Ash, %	4.47	4.51	4.59	4.45	4.23
Indispensable AA, %					
Arg	0.70	0.71	0.73	0.71	0.74
His	0.34	0.34	0.33	0.34	0.34
Ile	0.51	0.54	0.54	0.53	0.53
Leu	0.97	1.53	1.89	2.49	3.00
Lys	0.98	0.97	1.05	1.05	1.01
Met	0.27	0.24	0.28	0.27	0.26
Phe	0.59	0.62	0.63	0.63	0.63
Thr	0.69	0.65	0.65	0.71	0.65
Trp	0.23	0.20	0.20	0.19	0.21
Val	0.69	0.72	0.73	0.72	0.72
Dispensable AA, %					
Ala	0.58	0.57	0.58	0.55	0.58
Asx[4]	0.99	0.98	1.02	1.01	1.02

Cys	0.24	0.24	0.25	0.23	0.24
Glx[5]	2.63	2.64	2.73	2.56	2.67
Gly	1.81	1.35	1.12	0.85	0.56
Pro	0.99	1.01	1.05	0.99	1.03
Ser	0.52	0.50	0.50	0.50	0.51
Tyr	0.36	0.40	0.38	0.39	0.40
Calculated composition					
CP, %	17.56	17.54	17.53	17.51	17.49
SID indispensable AA, %					
Arg	0.76	0.76	0.76	0.76	0.76
His	0.34	0.34	0.34	0.34	0.34
Ile	0.52	0.52	0.52	0.52	0.52
Leu	0.98	1.48	1.97	2.47	2.96
Lys	0.98	0.98	0.98	0.98	0.98
Met	0.31	0.31	0.31	0.31	0.31
Phe	0.63	0.63	0.63	0.63	0.63
Thr	0.66	0.66	0.66	0.66	0.66
Trp	0.20	0.20	0.20	0.20	0.20
Val	0.69	0.69	0.69	0.69	0.69

[1]Diets were analyzed in duplicate.

[2]SID = standardized ileal digestible.

[3]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[4]Asx = Asp and Asn.

[5]Glx = Glu and Gln.

Table 4. Primer sequences utilized for quantitative reverse transcription-PCR

Gene[1]	Direction[2]	Primer sequence	Reference
Internal control gene			
GAPDH	F	5′-CAG CAA TGC CTC CTG TAC CA-3′	Vigors et al. (2014)
	R	5′-ACG ATG CCG AAG TTG TCA TG-3′	
HMBS	F	5′-CTG AAC AAA GGT GCC AAG AAC A-3′	Vigors et al. (2014)
	R	5′-GCC CCG CAG ACC AGT TAG T-3′	
Target gene			
BCATm	F	5′-GCC TGA AGG CGT ACA AAG G-3′	Wiltafsky et al. (2010)
	R	5′-GAT GCA CTC CAG CAA CTC G-3′	
BCKDH E1α	F	5′-CCA GAT GCC CGT CCA CTA C-3′	Wiltafsky et al. (2010)
	R	5′-CCC CCT CTC CGA AGT AAC AG-3′	
BCKDH E1β	F	5′-GCC GAA GTC ATC CAA GAA GG-3′	Wiltafsky et al. (2010)
	R	5′-TGA CCT CAC AGG ACA CTC CAA G-3′	
BCKDH E2	F	5′-ACG ATA CTG CTT ATG TGG GAA AG-3′	Wiltafsky et al. (2010)
	R	5′-TGT GGC CCT TTA TCT CTT GG-3′	

BCKDK	F	5′-TCC GAC CAT GAT GCT CTA TTC-3′	Wiltafsky et al. (2010)
	R	5′-GAA GTC CTT GAT GCG GTG AG-3′	

[1]*GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *HMBS* = hydroxymethylbilane synthase; *BCATm* = mitochondrial branched-chain amino transferase; *BCKDH E1α* = branched-chain α-keto acid dehydrogenase E1α subunit; *BCKDH E1β* = branched-chain α-keto acid dehydrogenase E1β subunit; *BCKDH E2* = branched-chain α-keto acid dehydrogenase E2 subunit; *BCKDK* = branched-chain α-keto acid dehydrogenase kinase.

[2]Direction of primer (F = forward; R = reverse).

Table 5. Growth performance of pigs fed diets with graded levels of standardized ileal digestible (SID) Leu relative to requirement[1] (as-fed basis)[2]

Item	SID Leu relative to requirement, %					SEM	*P*-values		
	100	150	200	250	300		ANOVA	Linear	Quadratic
BW, kg									
Initial BW (Day 0)	30.2	30.0	30.4	29.9	29.8	1.0	0.840	0.516	0.680
Final BW (Day 15)	40.6	39.6	40.4	38.8	38.2	1.2	0.051	0.009	0.504
ADG, g/d	698	645	673	593	559	47	0.009	< 0.001	0.522
ADFI, g/d	1,416	1,409	1,411	1,360	1,278	31	0.003	< 0.001	0.050
G:F	0.50	0.46	0.48	0.44	0.44	0.03	0.128	0.023	0.835

[1]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[2]Each least squares mean represents 8 observations.

Table 6. Nitrogen balance of growing pigs fed diets with graded levels of standardized ileal digestible (SID) Leu relative to requirement[1] during a 5-d collection period (as-fed basis)[2]

Item	SID Leu relative to requirement, %					SEM	*P*-values		
	100	150	200	250	300		ANOVA	Linear	Quadratic
Feed intake, g/5 d	6,827	6,766	6,693	6,675	6,428	243	0.349	0.056	0.553
N intake, g/5 d	165	165	163	163	159	5.9	0.730	0.187	0.729
N output in feces, g/5 d	29	29	27	29	26	1.7	0.338	0.151	0.732
N output in urine, g/5 d	28	30	30	30	31	2.5	0.648	0.235	0.528
ATTD[3] of N, %	82.4	82.7	83.3	82.1	83.7	0.7	0.381	0.315	0.776
N retention, g/5 d	108	106	106	103	102	3	0.504	0.082	0.994
N retention, %	65.4	64.3	64.9	63.6	64.3	1.3	0.286	0.136	0.447
Biological value[4], %	79.4	77.7	77.8	77.5	76.8	1.4	0.149	0.021	0.579

[1]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[2]Each least squares mean represents 8 observations.

[3]ATTD = apparent total tract digestibility.

[4]Biological value was calculated as [N retained/(N intake – N output in feces)] × 100 (Rojas and Stein, 2013).

Table 7. Effects of dietary Leu concentration on tissue branched-chain AA and plasma Trp of growing pigs fed diets with graded levels of standardized ileal digestible (SID) Leu relative to requirement[1] (as-fed basis)[2]

Item	SID Leu relative to requirement, %					SEM	*P*-values		
	100	150	200	250	300		ANOVA	Linear	Quadratic
Calculated AA intake, g/d									
Lys	13.4	13.1	14.1	14.0	13.0	0.5	0.035	0.915	0.026
Ile	6.96	7.31	7.23	7.08	6.81	0.26	0.167	0.273	0.030
Leu	13.2	20.7	25.3	33.2	38.6	1.0	< 0.001	< 0.001	0.710
Val	9.42	9.74	9.77	9.61	9.26	0.35	0.347	0.479	0.052
Trp	3.14	2.71	2.68	2.54	2.70	0.10	< 0.001	< 0.001	< 0.001
Liver, %									
Ile	2.36	2.45	2.50	2.63	2.65	0.12	0.011	< 0.001	0.775
Leu	4.99	5.27	5.33	5.52	5.54	0.24	0.021	0.001	0.391
Val	3.15	3.30	3.32	3.43	3.47	0.15	0.039	0.003	0.662
Muscle, %									
Ile	3.57	3.41	3.43	3.41	3.37	0.05	0.065	0.014	0.344

Leu	6.15	5.90	5.99	5.92	5.87	0.09	0.127	0.041	0.394
Val	3.70	3.54	3.56	3.53	3.50	0.05	0.049	0.011	0.218

[1]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[2]Each least squares mean represents 8 observations.

Table 8. Effects of dietary Leu concentration on plasma free AA profile of growing pigs fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement[1] (as-fed basis)[1]

	SID Leu relative to the requirement, %						*P*-values		
Item	100	150	200	250	300	SEM	ANOVA	Linear	Quadratic
Indispensable AA, %									
Arg	21.9	20.7	22.8	20.6	26.3	2.5	0.382	0.221	0.258
His	5.2	4.8	5.4	5.3	6.1	0.4	0.186	0.046	0.187
Ile	16.3	6.0	5.5	4.5	4.6	1.0	< 0.001	< 0.001	< 0.001
Leu	15.5	37.5	53.8	58.5	81.0	3.2	< 0.001	< 0.001	0.380
Lys	36.8	33.5	39.3	34.6	47.0	4.5	0.194	0.110	0.179
Met	8.7	8.2	8.3	6.9	8.7	0.8	0.315	0.580	0.222
Phe	10.8	10.7	11.3	12.9	12.2	0.6	0.010	0.002	0.999
Thr	53.6	44.0	46.0	63.9	58.3	6.9	0.080	0.102	0.254
Trp	10.4	7.7	7.7	7.3	8.7	0.7	0.001	0.028	0.001
Val	75.4	19.7	17.5	14.4	15.6	3.7	< 0.001	< 0.001	< 0.001
Dispensable AA, %									

Ala	92.8	66.0	60.1	70.4	68.5	6.5	0.001	0.010	0.001
Asx[2]	4.7	4.3	4.0	3.6	3.9	0.4	0.299	0.072	0.330
Cys	3.3	2.5	1.6	1.3	1.3	0.3	< 0.001	< 0.001	0.029
Glx[3]	53.5	47.3	52.8	43.0	48.6	5.7	0.675	0.446	0.702
Gly	219	181	162	122	107	11	< 0.001	< 0.001	0.462
Pro	59.1	48.5	47.0	50.0	45.0	2.6	0.003	0.002	0.091
Ser	24.5	20.6	19.7	22.7	18.3	1.8	0.021	0.022	0.553
Tyr	12.5	11.4	11.6	11.9	13.3	0.9	0.388	0.401	0.067
Trp:LNAA[4] ratio[6], %	8.0	9.1	7.9	7.3	7.0	0.7	0.065	0.023	0.282
Trp:BCAA[6] ratio, %	9.8	12.3	10.3	9.7	8.9	1.0	0.039	0.060	0.068

[1]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[2]Asx = Asp and Asn.

[3]Glx = Glu and Gln.

[4]LNAA = large neutral amino acids; Ile, Leu, Phe, Trp, Val, and Tyr (Henry et al., 1992).

[5]The ratio of Trp to the sum of other LNAA.

[6]BCAA = branched-chain amino acids.

Table 9. Effects of dietary Leu concentration on tissue and serum branched-chain α-keto acids of growing pigs fed diets with graded levels of standardized ileal digestible (SID) Leu relative to requirement[1] (as-fed basis)[2]

	SID Leu relative to requirement, %						*P*-values		
Item[3]	100	150	200	250	300	SEM	ANOVA	Linear	Quadratic
Liver, ng/mg									
α-keto-β-methylvalerate	0.16	ND[4]	ND	ND	ND	-	-	-	-
α-keto isocaproate	0.31	0.50	0.63	0.65	0.85	0.11	0.011	< 0.001	0.850
α-keto isovalerate	0.26	0.11	0.10	0.10	0.08	0.02	< 0.001	< 0.001	< 0.001
Muscle, ng/mg									
α-keto-β-methylvalerate	1.04	0.24	0.24	0.19	0.19	0.08	< 0.001	< 0.001	< 0.001
α-keto isocaproate	0.51	1.94	3.67	4.26	6.14	0.62	< 0.001	< 0.001	0.916
α-keto isovalerate	0.72	0.14	0.20	0.18	0.17	0.06	< 0.001	< 0.001	< 0.001
Serum, μg/mL									
α-keto-β-methylvalerate	9.56	3.40	2.80	2.62	2.25	0.55	< 0.001	< 0.001	< 0.001
α-keto isocaproate	5.12	13.45	18.70	20.48	24.92	1.22	< 0.001	< 0.001	0.014
α-keto isovalerate	3.28	0.79	0.66	0.56	0.53	0.19	< 0.001	< 0.001	< 0.001

[1]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[2]Each least squares mean represents 8 observations.

[3]α-keto-β-methylvalerate = α-keto acid of Ile; α-keto isocaproate = α-keto acid of Leu; α-keto isovalerate = α-keto acid of Val.[4]ND = not detected.

Table 10. Effects of dietary Leu concentration on relative mRNA abundance of genes related to branched-chain AA metabolism of growing pigs fed diets with graded levels of standardized ileal digestible (SID) Leu relative to requirement[1] (as-fed basis)[2]

Item[3]	SID Leu relative to requirement, %					SEM	*P*-values		
	100	150	200	250	300		ANOVA	Linear	Quadratic
Liver									
BCATm	1.35	1.58	1.66	1.64	1.63	0.19	0.793	0.325	0.433
BCKDH E1α	0.64	0.66	0.88	0.81	0.75	0.13	0.659	0.351	0.352
BCKDH E1β	0.81	0.86	1.29	1.04	0.99	0.15	0.133	0.232	0.104
BCKDH E2	0.91	1.30	1.19	1.28	1.21	0.21	0.526	0.307	0.271
BCKDK	1.10	1.09	1.09	0.90	0.94	0.18	0.869	0.366	0.885
Muscle									
BCATm	0.81	1.20	1.20	1.20	1.23	0.19	0.080	0.033	0.102
BCKDH E1α	0.58	1.03	0.97	0.98	1.02	0.14	0.043	0.029	0.085
BCKDH E1β	0.90	0.97	1.11	0.92	1.04	0.08	0.372	0.366	0.471
BCKDH E2	0.82	1.04	1.03	1.20	1.14	0.19	0.686	0.191	0.590
BCKDK	0.73	0.78	0.90	0.68	0.78	0.10	0.095	0.994	0.244

[1]The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012).

[2]Each least squares mean represents 6, 7, or 8 observations.

[3]*BCATm* = mitochondrial branched-chain amino transferase; *BCKDH E1α* = branched-chain α-keto acid dehydrogenase E1α subunit; *BCKDH E1β* = branched-chain α-keto acid dehydrogenase E1β subunit; *BCKDH E2* = branched-chain α-keto acid dehydrogenase E2 subunit; *BCKDK* = branched-chain α-keto acid dehydrogenase kinase.

Figure Legends

Figure 1. Plasma urea nitrogen (PUN) of growing pigs (N = 40; n = 8) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

Figure 2. Plasma serotonin of growing pigs (N = 30; n = 6) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

Figure 3. Hypothalamic serotonin of growing pigs (N = 30; n = 6) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

Figure 1. Plasma urea nitrogen (PUN) of growing pigs (N = 40; n = 8) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

Figure 1

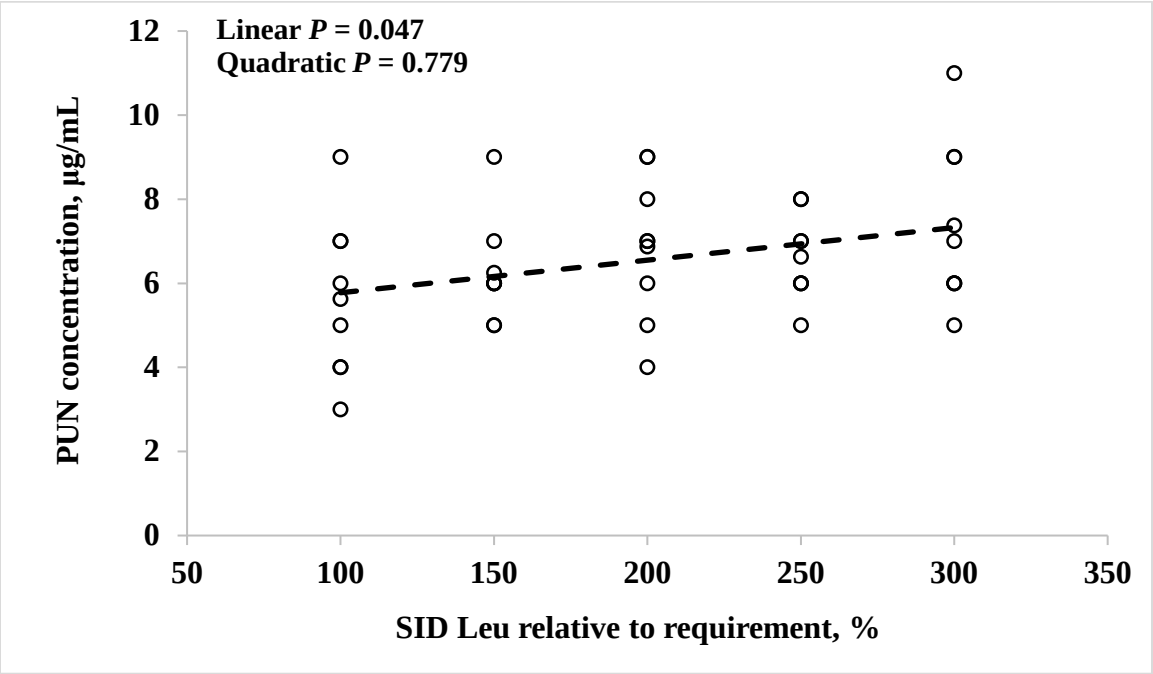

Figure 2. Plasma serotonin of growing pigs (N = 30; n = 6) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

Figure 2

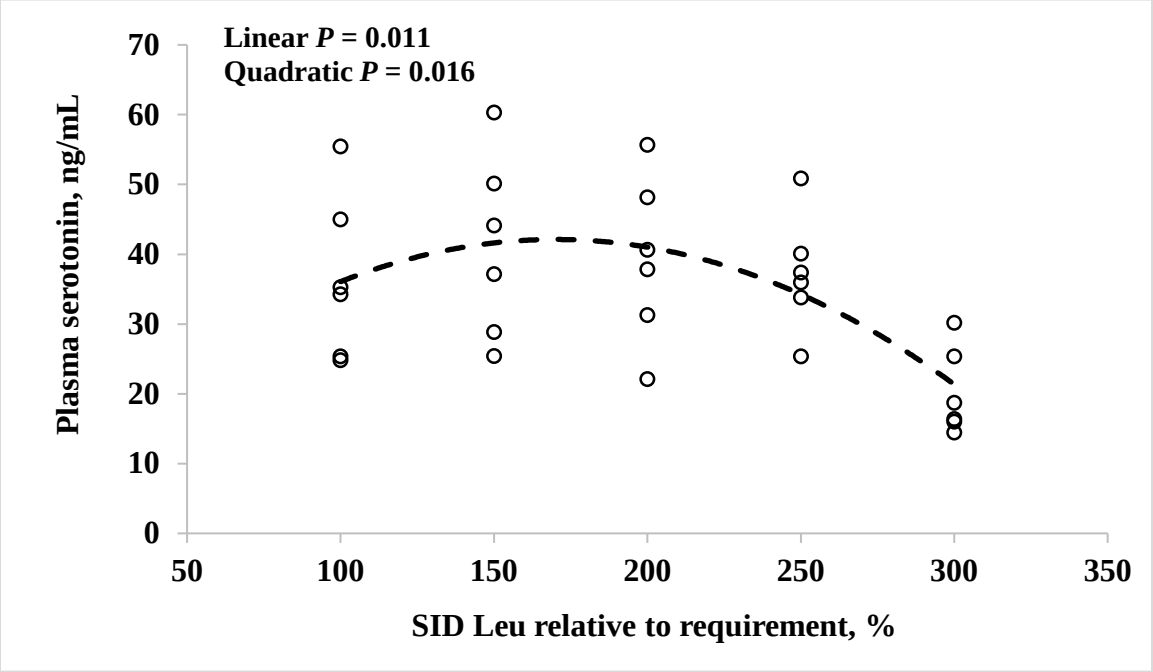

Figure 3. Hypothalamic serotonin of growing pigs (N = 30; n = 6) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

Figure 3