EXCESS LEUCINE IN DIETS FOR GROWING PIGS NEGATIVELY AFFECTS GROWTH PERFORMANCE, NITROGEN BALANCE, AND METABOLISM OF BRANCHED-CHAIN AMINO ACIDS AND TRYPTOPHAN

BY

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DISSERTATION

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ABSTRACT

Five experiments were conducted to determine the effects of excess dietary Leu on metabolism of branched-chain amino acids (**BCAA**) and to demonstrate the interactions among BCAA and Trp in diets fed to growing pigs. In experiment 1, the objective was to determine the effects of excess dietary Leu on growth performance, N balance, protein retention, and serotonin synthesis of growing pigs. Results indicated that average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio decreased (linear, $P < 0.05$) as dietary Leu increased. Decreased (linear, $P < 0.05$) biological value of dietary protein was also observed, and plasma urea N (**PUN**) increased (linear, $P < 0.05$) as dietary Leu increased. A linear reduction ($P < 0.05$) in hypothalamic serotonin was observed with increasing dietary Leu. In experiment 2, the objective was to determine effects of dietary Ile and Val supplementation to diets with adequate or excess Leu on N balance and BCAA metabolism of growing pigs. Results indicated that excess Leu in diets reduced ($P < 0.05$) N retention and biological value of diets and increased ($P < 0.05$) PUN, but PUN was reduced ($P < 0.05$) as dietary Val increased. Concentrations of BCAA in liver were greater ($P < 0.05$) in pigs fed excess-Leu diets than in pigs fed adequate-Leu diets, but concentrations of BCAA in muscle were greater ($P < 0.05$) in pigs fed adequate-Leu diets. Increasing dietary Ile increased ($P < 0.05$) plasma free Ile and plasma concentration of the Ile metabolite, α-keto-β-methylvalerate, but the increase was greater in diets with adequate Leu than in diets with excess Leu (interaction, $P < 0.001$). Likewise, plasma concentrations of Val and the Val metabolite, α-keto isovalerate, increased more with increasing dietary Val in diets with Leu at the requirement than in diets with excess Leu (interaction, $P < 0.001$). Increasing dietary Leu increased ($P < 0.05$) plasma free Leu and plasma concentration of the Leu metabolite, α-keto isocaproate. In contrast, increased dietary Val reduced ($P < 0.05$) plasma concentration of α-keto

isocaproate. Experiment 3 was conducted to determine interactions between dietary Trp and dietary Leu on plasma serotonin and hypothalamic serotonin concentrations and growth performance of growing pigs. Results indicated that increasing dietary Trp increased ($P < 0.05$) ADG, ADFI, and hypothalamic serotonin whereas increasing dietary Leu reduced ($P < 0.05$) ADG, ADFI, and hypothalamic serotonin, but the increase caused by dietary Trp was greater if Leu was provided at 300% of the requirement than if it was provided at the requirement (interaction, $P < 0.05$). Experiments 4 and 5 were conducted to test the hypothesis that increasing concentrations of dietary Val, Ile, or Trp in diets containing excess Leu from corn protein mitigate negative effects of excess dietary Leu on N balance, PUN, and growth performance of growing pigs. Results from experiment 4 indicated that fecal N output increased if Ile was added to diets without added Val, but that was not the case if Val was added (interaction, $P < 0.05$). Addition of Ile to diets reduced N retention, and N retention increased with Trp addition to diets without Val addition, but not in diets with added Val (interaction, $P < 0.05$). The biological value of protein increased if Trp was added to diets without addition of Ile, but if Ile was added, Trp addition did not increase the biological value of protein (interaction, $P < 0.05$). Results from experiment 5 indicated that final body weight and ADG of pigs fed a diet with exces Leu were reduced compared with pigs fed a corn-soybean meal control diet. However, if Val and Trp were added to the diet with excess Leu ADG and final body weight were not different from that obtained for pigs fed the corn-soybean meal control diet. In conclusion, results of these experiments indicate that excess dietary Leu may have negative impacts on N balance, metabolism of BCAA, and growth performance of pigs. Excess dietary Leu also may reduce serotonin synthesis in the hypothalamus, which may have contributed to the reduced ADFI observed for pigs fed diets with excess Leu. However, increasing concentrations of dietary Trp

and Val alone or in combination may have the potential to alleviate the negative effects of excess dietary Leu.

Key words: branched-chain amino acids, growth performance, nitrogen balance, serotonin, tryptophan

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CHAPTER 1: 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 used 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. This step produces branched-chain α-keto acids (**BCKA**) from BCAA in a reversible reaction. The branched-chain α-keto acid dehydrogenase 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. Among BCAA, Leu is considered a key regulator of the metabolism of BCKA, because its metabolite stimulates activation of the branched-chain α-keto acid dehydrogenase complex in the liver (Harper et al., 1984). When excess Leu is included in diets for pigs, degradation of all 3 BCAA may increase by stimulating effects of Leu or its metabolite on BCAA degrading enzymes (Wiltafsky et al., 2010). Duan et al. (2016) confirmed that serum Ile and Val concentrations were reduced by excess dietary Leu in growing pigs and high dietary Leu reduces feed intake and growth performance in pigs (Gatnau et al., 1995; Wiltafsky et al., 2010), which may be a result of the imbalanced supply of BCAA that result from increased metabolism of Val and Ile.

Morales et al. (2016) demonstrated that supplementations of Ile and Val above their requirements in high Leu diets appears to correct the negative effect of excess Leu on absorption and degradation of BCAA without growth reduction. According to a recent meta-analysis by Cemin et al. (2019), increasing concentrations of dietary Val and Ile alone or in combination has the potential to alleviate the negative effects of excess dietary Leu on growth performance of pigs.

Leucine may have an inhibitory effect on feed intake by stimulating the mechanistic target of rapamycin in the brain (Cota et al., 2006). Excess dietary Leu may also reduce synthesis of serotonin in the brain (Wessels et al., 2016a,b), because excess Leu may prevent Trp, which is the precursor for serotonin, from being transported from blood to the brain, and therefore reduce the availability of Trp for serotonin synthesis in the brain. High Trp intake increases feed intake (Henry et al., 1992), and this is partly attributed to increased serotonin synthesis, because serotonin plays an important role in appetite regulation (Zhang et al., 2007).

Therefore, the objectives of this dissertation are to determine the effects of excess dietary Leu on growth performance, N balance, protein retention, and serotonin synthesis by growing pigs, to evaluate the effects of dietary Ile and Val supplementations to high or low Leu diets on N balance and BCAA metabolism of growing pigs, to determine the effects of dietary Leu and Trp supplementations on serotonin metabolism and growth performance of growing pigs, and to determine the effects of dietary Ile, Val, and Trp supplementations to high Leu diets on N balance and growth performance of growing pigs.

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CHAPTER 2: BRANCHED-CHAIN AMINO ACID NUTRITION OF PIGS: LITERATURE REVIEW

INTRODUCTION

Amino acids (**AA**) are essential nutrients and are precursors for body protein synthesis as well as basic components of hormones and enzymes in animals (Lewis, 2001; NRC, 2012). Each AA is composed of both amino- and carboxyl groups with a functional R group, which is often referred to as the side chain. This side chain represents the characteristic of each AA. There are 20 primary AA that are necessary for protein synthesis in animal. For swine, 9 of them are not synthesized or not adequately synthesized by the animal; therefore, they are classified as nutritionally indispensable AA which must be supplied through diets (Wu, 2009). The remaining 11 AA are considered dispensable or semi-dispensable as they can be synthesized by the animal; so they are not required in the diets.

The branched-chain amino acids (**BCAA**) are indispensable AA for swine. Leucine together with Val and Ile are categorized as BCAA because of the structural similarity of their side chains (Harper et al., 1984). Due to the structural similarity, all 3 BCAA share enzymes that are involved in the first 2 steps of their catabolic pathway (Harris et al., 2005). In addition, the BCAA are unique among AA, because deamination of BCAA primarily occurs in skeletal muscle (Cole, 2015).

Excessive excretion of N from swine manure is a major concern in the animal industry (Kornegay and Harper, 1997). There have been many efforts to reduce excretion of N from pigs (Kerr and Easter, 1995; Han et al., 2001). The most effective way to address this concern is the use of reduced crude protein (**CP**) diets that are fortified with crystalline indispensable AA. The use of crystalline AA also provides the opportunity to reduce total feed cost. In the past, there

were 4 feed-grade indispensable AA (L-Lys HCl, DL-Met, L-Thr and L-Trp) available for poultry and livestock diets, and these AA have been used to reduce CP in diet for swine. Recently, feed grade L-Val and L-Ile have become commercially available as well. Therefore, the use of crystalline Val and Ile in pig diets has increased because Val and Ile may be the next limiting AA for pigs after Lys, Thr, Met, and Trp in reduced CP diets (Liu et al., 2000; Lordelo et al., 2008; Htoo et al; 2014). In contrast, Leu is usually in excess in corn-soybean meal-based practical diets due to its high concentration in both corn and soybean meal. However, corn protein has greater Leu to CP ratio than soybean meal. Thus, even if the intact protein source such as soybean meal is replaced by feed-grade crystalline AA, reduced CP diets will contain excess Leu because more corn is needed to compensate for the reduced inclusion of soybean meal. However, Leu also may become a limiting AA if low-protein diets are formulated based on wheat and barley, which have lower Leu to CP ratios than corn protein (Gloagen et al., 2013). It is, therefore, necessary to know the exact requirement of BCAA and its metabolism and to determine the interactive effects among the 3 BCAA.

GENERAL ASPECTS OF BRANCHED-CHAIN AMINO ACIDS

Valine

Valine is an indispensable AA for growth and body protein synthesis in swine. Valine is generally considered to be the fifth limiting AA after Lys, Thr, Met, and Trp in corn-soybean meal (**SBM**)-based practical diets for pigs (Mavromichalis et al., 1998; Figueroa et al., 2003). For lactating sow, Val has been suggested as the second limiting AA after Lys in corn-SBM-based diet, because optimum Val requirement is considered greater for milk synthesis (Touchette et al., 1998).

Valine is closely related to feed intake in pigs (Gloaguen et al., 2011, 2012). If Val is deficient in low CP diets, the feed intake will decrease resulting in the negative impact on growth, and Val may be related to appetite regulation as a signal of dietary deficiency of indispensable AA (Gloaguen et al., 2011). It has also been suggested that pigs have an ability to detect Val deficiency in the diets after 1h of ingestion (Gloaguen et al., 2012). However, reduced feed intake caused by Val deficiency was mitigated when the other 2 BCAA were also deficient in the diets (Gaines et al., 2011). Therefore, the BCAA imbalance in the diets might be a main reason for reduction of feed intake regardless of Val deficiency status in pigs.

Isoleucine

Isoleucine along with His is often considered to be the sixth limiting AA in corn-SBM-based diets for pigs (Figueroa et al., 2003). Together with Trp, Val and His, Ile may equally be the fourth limiting AA after Lys, Met, and Thr in corn-SBM-based practical diets (Brudevold and Southern, 1994; Mavromichalis et al., 1998).Therefore, it is necessary to consider Ile as a potential limiting AA in reduced CP diets.

Isoleucine is classified as BCAA and also belongs to the group of large neutral AA (**LNAA**) which contain Trp, His, Phe, Tyr and BCAA (Fernstrom, 2013; Cole, 2015). All of these LNAA are transported by the same transporter through the blood brain barrier into the brain (Smith, 2000; Fernstrom, 2013). Because of different affinity of the transporter to each LNAA, the rate of uptake for these AA into the brain may be in a competitive way. Due to the relatively low affinity of the transporter to Ile, Ile uptake is negatively affected when the other LNAA, which has high affinity for the transporter, are high in the diets (Smith, 2000; van Milgen et al., 2012). Therefore, imbalanced supplementations of BCAA may affect Ile uptake and its requirement in pigs.

Historically, blood-containing ingredients such as spray dried animal blood or spray dried blood meal were used in Ile dose-response studies including Ile requirement for pigs because of its unique AA profile (Bergström et al., 1996; Kerr et al., 2004; Wiltafsky et al., 2009; Table 2.1). Generally, blood products have high CP concentration and are rich in most indispensable AA, but with a low Ile content. The use of blood product as a protein source makes it possible to formulate Ile-deficient diets in requirement studies (van Milgen et al., 2012). However, large variability in requirement estimates for Ile has been repeated which possibly is explained by the severe imbalance among the BCAA in blood products (Wiltafsky et al., 2009). Therefore, it is necessary to determine the optimum Ile requirement based on the balanced BCAA containing diets without the impact of blood products.

Leucine

Although Leu is a nutritionally indispensable AA for swine, additional supplementation of L-Leu is usually not required in pig diets, because most feed ingredients that are commonly used in pig diets are high in Leu (Stein et al., 2016), so practical diets for pigs are rarely deficient in Leu. In particular, diets based on corn and corn co-products and sorghum and sorghum co-products are rich in Leu (Sotak et al., 2015). This is likely the main reason why only a few previous studies were conducted to determine the Leu requirements for pigs (Gatnau et al., 1995; Augspurger and Baker, 2004). However, regardless of nutritional needs, Leu has received great attention during the last 30 years because of its biological significance. Many *in vivo* studies were conducted to identify the physiological role of Leu in protein synthesis and its regulatory effect on BCAA metabolism.

Leucine serves not only as an inhibitor of protein degradation in skeletal muscle (Nakashima et al., 2005), but also as a stimulator of protein synthesis in cell, via activation of the

mammalian target of the rapamycin signaling pathway in animals (Sans et al., 2006). Among BCAA, only Leu has a stimulatory effect on the mammalian target of the rapamycin phosphorylation in terms of muscle protein synthesis (Escobar et al., 2006). However, there is was no effect of Leu supplementation on protein accretion or average daily gain in weanling pigs when graded levels of dietary Leu was provided (Edmonds and Baker, 1987). It is possible that the stimulatory effects of Leu on protein synthesis is not always activated, and this might be partly influenced by BCAA imbalance in the diets (Wu, 2009).

The BCAA are unique because they share 2 common metabolic steps (Cole, 2015). Among the BCAA, Leu can regulate the activity of the branched-chain α-keto acid dehydrogenase (**BCKDH**) which is used in the second step of the BCAA catabolic pathway (Murakami et al., 2005; Brosnan and Brosnan, 2006). Excess dietary Leu may result in increased metabolism of Val and Ile, which may cause a reduction in available Val and Ile because of the activated BCKDH that is a result of the excess Leu in the diet (Wessels et al., 2016b). However, excess Val and Ile seem to have less stimulation effect on increasing the metabolism of BCAA (D'Mello and Lewis, 1970). Therefore, it is necessary to identify the exact effects of excess dietary Leu and to determine the optimum ratio of BCAA in diets for pigs.

METABOLISM OF BRANCHED-CHAIN AMINO ACIDS

Metabolism of BCAA have been studied extensively because of the uniqueness and the biological significance. Generally, AA catabolism can be classified into 2 groups based on the degradation pathway of its carbon skeleton (D'Mello, 2003). Some AA that can be converted into glucose via gluconeogenesis in the liver are referred to as glucogenic AA. Contrary to glucogenic AA, some AA that can be converted into ketone bodies are referred to as ketogenic

AA. Leucine is a strictly ketogenic AA whereas Val is a strictly glucogenic AA. Isoleucine can be both glucogenic and ketogenic AA. 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).

Branched-chain α-keto acids are derived from the first step of catabolic pathway via BCAT, which is reversible transaminase that mainly occurs in skeletal muscle. This enzyme transfers the amino group ($–NH_3^{+}$) of BCAA to α-ketoglutarate in order to form three different α-keto acids such as α-keto isocaproate (**KIC**; the corresponding α-keto acid of Leu), α-keto isovalerate (**KIV**; the corresponding α-keto acid of Val), and α-keto-β-methylvalerate (**KMV**; the corresponding α-keto acid of Ile). The BCAT is mainly located in skeletal muscle in pigs. The activity of BCAT is regulated by the concentration of substrate, because it is not a rate-limiting enzyme, thus increased BCAA intake results in increased corresponding α-keto acids in plasma (Harper et al., 1984; Wiltafsky et al., 2010).

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). Among BCAA, 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. Increased stimulation of BCKDH that was a result of increased KIC concentration in plasma increased decarboxylation of KIV and KMV, which resulted in the reduced KIV and KMV concentrations.

This indicates that KIC is the key regulator of the BCAA catabolic process (Langer et al., 2000; Wiltafsky et al., 2010). Therefore, increased concentration of Leu will increase the catabolism of all BCAA by stimulation of enzymatic activity of BCKDH. When excess Leu in diets is offered to pigs, degradation of all 3 BCAA may increase by Leu or its metabolite (Wiltafsky et al., 2010). Results of previous studies indicate that excess dietary Leu can reduce pig feed intake and growth performance (Gatnau et al., 1995; Wiltafsky et al., 2010), which may be a consequence of the imbalanced supply of BCAA that resulted from the increased degradation of Val and Ile.

Unlike BCAT, the BCKDH is a rate-limiting enzyme, thus the activity of BCKDH is highly regulated. Basically, activity of BCKDH is regulated by phosphorylation of BCKDH kinase (Zhou et al., 2012). Phosphorylated BCKDH is inactive from whereas dephosphorylated enzyme is active form. The BCKDH might also be regulated by concentrations of its end products (Harper et al., 1984). Wiltafsky et al. (2010) reported that the abundance of BCKDH genes was not drastically changed by alterations in dietary Leu or KIC. It is possible the mechanisms that adapt to high concentrations of Leu and KIC are believed to be regulated post-transcriptionally. 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.

IDEAL PROTEIN

Historically, the concept of using an ideal protein was first introduced by Mitchell (1964). The ultimate goal of using this concept was to create an ideal profile of AA required for

maximizing growth performance of pigs and provide the correct amount of all AA to pigs without excess or deficiency of AA (Easter, 1994). Basically, the ideal protein profile for pigs was designed as a ratio of all indispensable AA relative to Lys. Lysine was selected because it is the first limiting AA in most swine diets. The advantage of using the expression relative to Lys is that the requirement for all indispensable AA can be simply calculated based on the considerable amount of information about the Lys requirement. However, it is necessary to consider the growth stages and calculate a ratio for the varying stages of growth in pigs (NRC, 2012; van Milgen and Dourmad, 2015). The practical application of ideal protein concept in diet formulation for pigs was initiated by the Agricultural Research Council (ARC, 1981). Currently, the ideal protein ratios are available from different institutes (Table 2.2).

THE OPTIMAL BRANCHED-CHAIN AMINO ACIDS TO LYSINE RATIO

Most BCAA requirement studies has been used growth performance data such as average daily gain, average daily feed intake, and gain to feed ratio and plasma urea N as the response criteria to determine their requirement estimates. The levels that maximize gain to feed ratio and average daily gain and minimize plasma urea N are proposed as optimum requirement of each AA. Historically, many of requirement studies has been conducted to determine accurate requirement for BCAA over 60 years ago. To estimate the BCAA requirements, standardized ileal digestible (**SID**) Lys should be the second-limiting AA after tested BCAA. If it is not the case and other AA are second limiting, the requirement estimates for BCAA will be underestimated. According to the summarized data by NRC (2012), the optimum ratio of Val, Ile, Leu to Lys ratio was 63, 51, and 100%, respectively.

Valine to lysine ratio

Most of previous research on the optimum Val:Lys ratio focused on weanling pigs (Table 2.3). Despite many experiments were conducted to determine Val requirement in weanling pigs, the results has shown a wide range (65 to 75%) of Val:Lys ratio among the different studies. Based on the meta-analysis study by van Milgen et al. (2013), the recommended Val:Lys ratio to maximized growth performance of growing pigs was 0.69.

Isoleucine to lysine ratio

One of the earliest requirement estimates for Ile in pigs was proposed by Brinegar et al. (1950). Among BCAA, relatively large number of requirement studies was conducted to determine the Ile requirement for pigs (Table 2.4). Based on the meta-analysis study by van Milgen et al. (2012), the minimum level of Val:Lys ratio to maximized growth performance of growing pigs was 0.50. However, using blood products such as blood meal or blood cells in diets for growing pigs tends to increase the requirement for Ile.

Leucine to lysine ratio

Based on the recommendation of NRC (2012), SID Leu:Lys ratio of 100% is suggested for optimal growth of weanling pigs. Gloaguen et al. (2013) demonstrated that the requirement estimate for SID Leu:Lys ratio of weanling pigs was 102% based on the curvilinear-plateau model. However, Soumeh et al. (2015) estimated and suggested that SID Leu:Lys ratio to maximize growth weanling pigs was 93%. Recently, a study was conducted by Wessels et al. (2016c) to determine optimum ratio of SID Leu:Lys in the low-CP diets (15%) fed to 10 to 30kg pigs. The authors concluded that SID Leu:Lys ratio needed to maximize growth was 108% when using the curvilinear-plateau model.

TRYPTOPHAN AND SEROTONIN IN BRAIN

Tryptophan is one of indispensable amino acids (AA) that is often limiting for growth in pigs fed corn-soybean meal-based diets (Lewis, 2001; Petersen, 2011). Tryptophan is involved in feed intake regulation partly by enhancing serotonin signaling in the brain (Henry et al., 1992). Tryptophan has to compete with all other LNAA such as Val, Leu, Ile, Tyr, and Phe for a common uptake transporter (L-type AA transporter 1) to cross the blood-brain barrier (Le Floc'h and Sève, 2007). Fernstrom (2013) indicated that increasing levels of one of the LNAA elevates its brain uptake and decreases the uptake of the other LNAA. Since Trp and BCAA are both categorized as LNAA, it is possible that there is competition for the transporters and excess Leu may result in reduced Trp uptake into the brain.

Trptophan is the precursor of serotonin, and biosynthesis of serotonin is regulated by tryptophan 5-hydroxylase though a hydroxylazion step of Trp in the methoxyindole pathway (Fernstrom and Wurtman, 1971).Tryptophan hydroxylase, which is the rate-limiting enzyme of serotonin synthesis has 2 different isoforms (Walther and Bader, 2003). Tryptophan hydroxylase 1 is responsible for the circulating serotonin in blood whereas brain serotonin is mainly regulated by tryptophan hydroxylase 2. Brain serotonin is a neurotransmitter that plays an important role in feed intake 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 in the brain (Shen et al., 2012a). Availability of dietary Trp in the brain is considered a rate-limiting factor for hypothalamic serotonin synthesis (Meunier-Salaün et al., 1991; Shen et al., 2012b). Excess dietary Leu may also reduce synthesis of serotonin in the brain (Wessels et al., 2016a). Because excess Leu may prevent Trp being transported from blood to brain, which may result in reduced availability of Trp for serotonin synthesis in the brain. There is a positive

correlation between hypothalamic Trp and hypothalamic serotonin, whereas hypothalamic Trp and plasma Leu are negatively correlated (Wessels et al., 2016a). 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. However, Wessels et al (2016b) indicated that pigs fed diets containing excess Leu had lower feed intake than pigs fed diets containing SID Leu at the requirement (NRC, 2012), but pigs had similar brain Trp concentration regardless of dietary Leu concentrations in diets. Thus, it is possible that Trp concentration in the brain might not be the only driver of reduced feed intake.

CONCLUSIONS

In recent years, use of co-products from the grain processing industries has become more common in swine diets as alternative sources of energy and protein because of the reduced cost of these ingredients. Co-products from corn and sorghum usually have high Leu concentrations and if large amounts of corn or sorghum co-products are used in swine diets, pigs will have excess dietary Leu, which may result in reduced feed intake and growth performance. Therefore, it is important to determine how excess dietary Leu affects feed intake and growth performance and it is necessary to identify solutions to overcome the negative effects of excess Leu in pigs. The current dissertation will, therefore, focus on conducting research to determine interactions among Leu, Ile, Val, and Trp and responses to graded inclusion levels of these AA in diets containing excess Leu will be determined.

TABLES

Table 2.1. Crude protein, Lys, and branched-chain amino acids concentrations (%) and Leu to crude protein ratio (%) of selected feed ingredients[1]

Ingredient	CP[2]	Lys	Branched-chain amino acids			Leu:CP[2]
			Val	Ile	Leu	
Avian blood meal[2]	88.40	7.47	5.80	3.65	9.60	10.86
Barley	11.33	0.40	0.52	0.37	0.72	6.35
Blood meal	88.65	8.60	7.96	0.97	11.45	12.92
Blood plasma	77.84	6.90	5.12	2.69	7.39	9.49
Canola meal, expelled	35.19	1.58	1.63	1.67	1.95	5.54
Corn, yellow dent	8.24	0.25	0.38	0.28	0.96	11.65
Corn gluten meal	58.25	0.93	2.42	2.23	9.82	16.86
Corn DDGS, 6-9% oil	27.36	0.90	1.39	1.06	3.25	11.88
Porcine blood meal[2]	94.60	8.24	8.46	0.55	12.79	13.52
Sorghum	9.36	0.20	0.46	0.36	1.21	12.93
Soybean meal, dehulled, solvent extracted	47.73	2.96	2.23	2.14	3.62	7.58
Soy protein concentrate	65.20	4.09	3.14	2.99	5.16	7.91
Spray dried animal blood[2]	89.40	8.26	7.54	0.69	11.37	12.72
Spray dried blood cells[2]	92.60	8.88	8.35	0.28	12.62	13.63
Spray dried plasma protein[2]	77.00	6.95	5.16	2.44	7.64	9.92

[1]Values from NRC (2012).

[2]CP = crude protein.

[2]Values adapted from Almeida et al. (2013).

Table 2.2. The ideal protein ratios for weanling pigs from different institutes

SID[1] values, %	BSAS[2], 2003	NRC, 2012	INRA[3], 2013
Lys	100	100	100
Thr:Lys	65	59	61
Met:Lys	30	29	32
(Met+Cys):Lys	59	55	54
Trp:Lys	19	16	20-22
Val:Lys	70	63	67
Ile:Lys	58	51	53
Leu:Lys	100	100	102
His:Lys	34	34	32
Phe:Lys	57	58	57
(Phe+Tyr):Lys	100	93	111
Tyr:Lys	-	-	-

[1]SID = standardized ileal digestible.

[2]The British Society of Animal Science, UK.

[3]France.

Table 2.3. Proposed standardized Ileal digestible (SID) Val:Lys (%) ratios for pigs

Reference	BW[1] range, kg	Diet composition[2]	Response parameters	Statistical model	SID Val:Lys
Wiltafsky et al., 2009	8 to 25	Corn, SBM, Wheat, Barley	Growth performance	Linear-plateau	66
Wiltafsky et al., 2009	8 to 25	Corn, SBM, Wheat, Barley	N-balance	Linear-plateau	65
Barea et al., 2009	12 to 25	Corn, SBM, Wheat, Barley	Growth performance	Linear-plateau	68 to 74
Barea et al., 2009	12 to 25	Corn, SBM, Wheat, Barley	Growth performance	Curvilinear-plateau	72 to 81
Gaines et al., 2011	13 to 32	Corn, SBM	Growth performance	Linear-plateau	64 to 65
Gaines et al., 2011	13 to 32	Corn, SBM	Growth performance	Quadratic function	71 to 72
Gloaguen et al., 2011	12 to 22	Corn, SBM, CGM	Growth performance	Curvilinear-plateau	71 to 73
Waguespack et al., 2012	20 to 45	Corn, SBM	Growth performance	Linear-plateau	67 to 70
Soumeh et al., 2015	8 to 15	Wheat, Barley, SPC	Growth performance	Linear-plateau	67
Soumeh et al., 2015	8 to 15	Wheat, Barley, SPC	Growth performance	Curvilinear-plateau	71

[1]BW = body weight.

[2]SBM = soybean meal; CGM = corn gluten meal; SPC = soy protein concentrate.

Table 2.4. Proposed standardized Ileal digestible (SID) Ile:Lys (%) ratios for pigs

Reference	BW[1] range, kg	Diet composition[2]	Response parameters	Statistical model	SID Ile:Lys
Wiltafsky et al., 2009	8 to 25	Wheat, Barley, Corn, SDBC	Growth performance	Linear-plateau	59
Wiltafsky et al., 2009	8 to 25	Wheat, Barley, Corn, SDBC	Growth performance	Linear-plateau	54
Waguespark et al., 2012	22 to 44	Corn, SBM	Growth performance	Linear-plateau	68 to 74
Norgaard et al., 2013	8 to 18	Wheat, Barley, Corn, SPC	Growth performance	Curvilinear-plateau	72 to 81
Soumeh et al., 2014	8 to 15	Wheat, Barley, SPC	Growth performance	Linear-plateau	64 to 65
Htoo et al., 2014	10 to 22	Wheat, Barley, SBM, SDBC	Growth performance	Curvilinear-plateau	46 to 47
Htoo et al., 2014	24 to 39	Wheat, Barley, SBM, SDBC	Growth performance	Curvilinear-plateau	50 to 54
Htoo et al., 2014	24 to 39	Wheat, Barley, SBM, SDBC	Plasma urea N	Curvilinear-plateau	53

[1]BW = body weight.

[2]SDBC = spray-dried blood cells; SBM = soybean meal; SPC = soy protein concentrate.

Table 2.5. Proposed standardized Ileal digestible (SID) Leu:Lys (%) ratios for pigs

Reference	BW[1] range, kg	Diet composition[2]	Response parameters	Statistical model	SID Leu:Lys
Gloaguen et al., 2013	11 to 22	Wheat, Barley, SBM	Growth performance	Curvilinear-plateau	102
Soumeh et al., 2015	8 to 12	Wheat, Barley, SPC	Growth performance	Curvilinear-plateau	93
Wessels et al., 2016	10 to 30	Corn, SBM, Wheat, Barley	Growth performance	Curvilinear-plateau	108

[1]BW = body weight.

[2]SBM = soybean meal; SPC = soy protein concentrate.

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CHAPTER 3: EXCESS DIETARY LEUCINE IN DIETS FOR GROWING PIGS REDUCES GROWTH PERFORMANCE, BIOLOGICAL VALUE OF PROTEIN, PROTEIN RETENTION, AND SEROTONIN SYNTHESIS

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 body weight: 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) body weights 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 average daily gain, average daily feed intake, and gain to feed ratio 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 N retention, which is likely a result of increased catabolism of Ile and Val. This results 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 amino acids (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 body weight (**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 3.1), but L-Leu was included in the 5 diets at 0, 0.5, 1.0, 1.5, or 2.0% (Tables 3.2 and 3.3). 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 crude protein (**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.

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 single-stranded complementary DNA (**cDNA**). Single-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 3.4), 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]:

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

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. (Mitchell, 1924). 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 3.5). 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 3.6), 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 (Figure 3.1) and increasing dietary Leu resulted in a quadratic reduction ($P < 0.05$) in plasma serotonin (Figure 3.2). Likewise, hypothalamic serotonin linearly decreased ($P < 0.05$) with increasing SID Leu in the diets (Figure 3.3).

Concentrations of BCAA in the liver increased linearly ($P < 0.001$) with increasing SID Leu in the diets (Table 3.7). 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 3.8). 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 3.9). 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.

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 3.10). 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 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 N 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 occurs in pigs is needed.

CONCLUSION

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 increased PUN concentration in pigs is 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 and a subsequent reduction in Trp uptake into the brain, which 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.

TABLES

Table 3.1. Chemical composition of ingredients used in experimental diets, as-fed basis[1]

Item	Corn	Soybean meal	Wheat	Barley
CP[2], %	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[3]	0.49	4.74	0.58	0.65
Cys	0.17	0.62	0.25	0.22
Glx[4]	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

Table 3.1. (Cont.)

Tyr	0.19	1.56	0.23	0.22
BCAA[5]:CP ratio, %				
Ile:CP	3.81	4.52	3.24	3.28
Leu:CP	11.99	7.41	6.05	6.10
Val:CP	4.94	4.75	4.09	4.64

[1]Ingredients were analyzed in duplicate.

[2]CP = crude protein.

[3]Asx = Asp and Asn.

[4]Glx = Glu and Gln.

[5]BCAA = branched-chain amino acids.

Table 3.2. Ingredient composition of experimental diets, as-fed basis

	SID[2] Leu relative to requirement[3], %				
Item	100	150	200	250	300
Ground corn	20.74	20.74	20.74	20.74	20.74
Soybean meal, 44% crude protein	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).

Table 3.2. (Cont.)

[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 D_3 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 B_{12}, 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 3.3. Analyzed nutrient composition of experimental diets, as-fed basis[1]

	SID[2] Leu relative to requirement[3], %				
Item	100	150	200	250	300
Analyzed composition					
Gross energy, kcal/kg	4,003	4,008	4,014	4,020	4,028
Crude protein, %	15.15	15.24	15.23	15.23	15.48
Dry matter, %	88.54	88.46	88.71	88.46	88.34
Ash, %	4.47	4.51	4.59	4.45	4.23
Acid hydrolyzed ether extract, %	6.02	5.98	6.06	5.85	5.93
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

Table 3.3 (Cont.)

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

[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 3.4. 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′	

Table 3.4. (Cont.)

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 3.5. Growth performance of 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	100	150	200	250	300	SEM	ANOVA	Linear	Quadratic
Body weight, kg									
Day 1	30.2	30.0	30.4	29.9	29.8	1.0	0.840	0.516	0.680
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 3.6. 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 (Mitchell, 1924).

Table 3.7. 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

Table 3.7. (Cont.)

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 3.8. Effects of dietary Leu concentration on plasma free AA profile 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	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

Table 3.8. (Cont.)

Asx[3]	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[4]	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[5] ratio[6], %	8.0	9.1	7.9	7.3	7.0	0.7	0.065	0.023	0.282
Trp:BCAA[7] 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]Each least squares mean represents 8 observations.

[3]Asx = Asp and Asn.

[4]Glx = Glu and Gln.

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

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

[7]BCAA = branched-chain amino acids.

Table 3.9. 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).

Table 3.9. (Cont.)

[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 3.10. 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]

	SID Leu relative to requirement, %						*P*-values		
Item[3]	100	150	200	250	300	SEM	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).

Table 3.10. (Cont.)

[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.

FIGURES

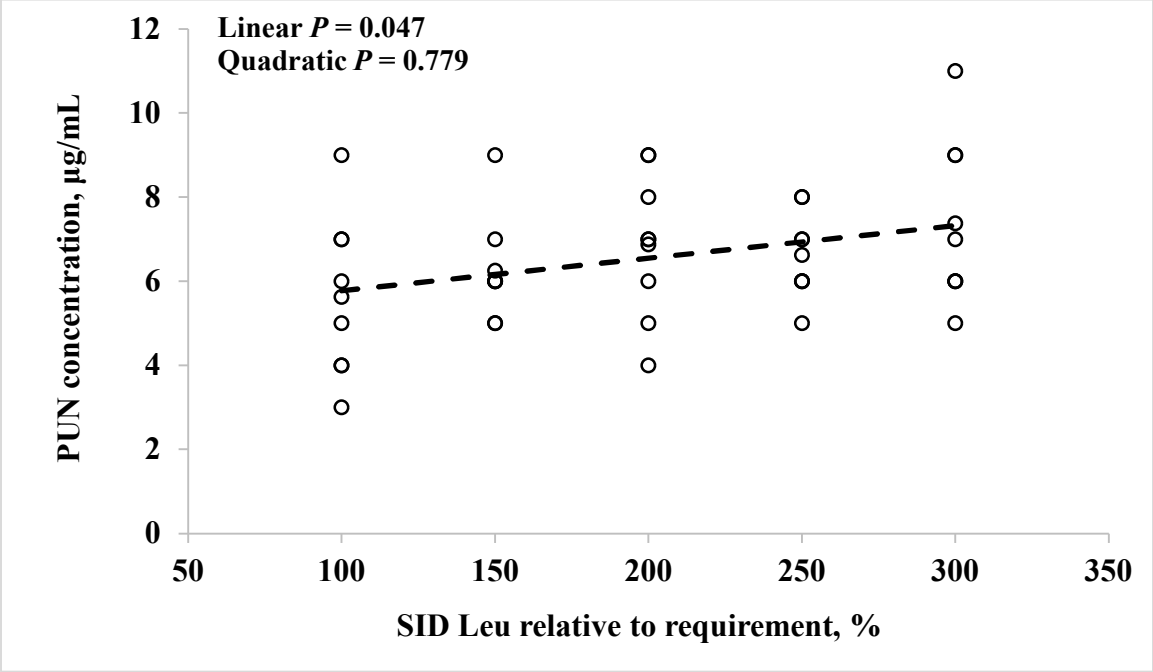

Figure 3.1. Plasma urea nitrogen (PUN) of growing pigs (N = 40; n = 8) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

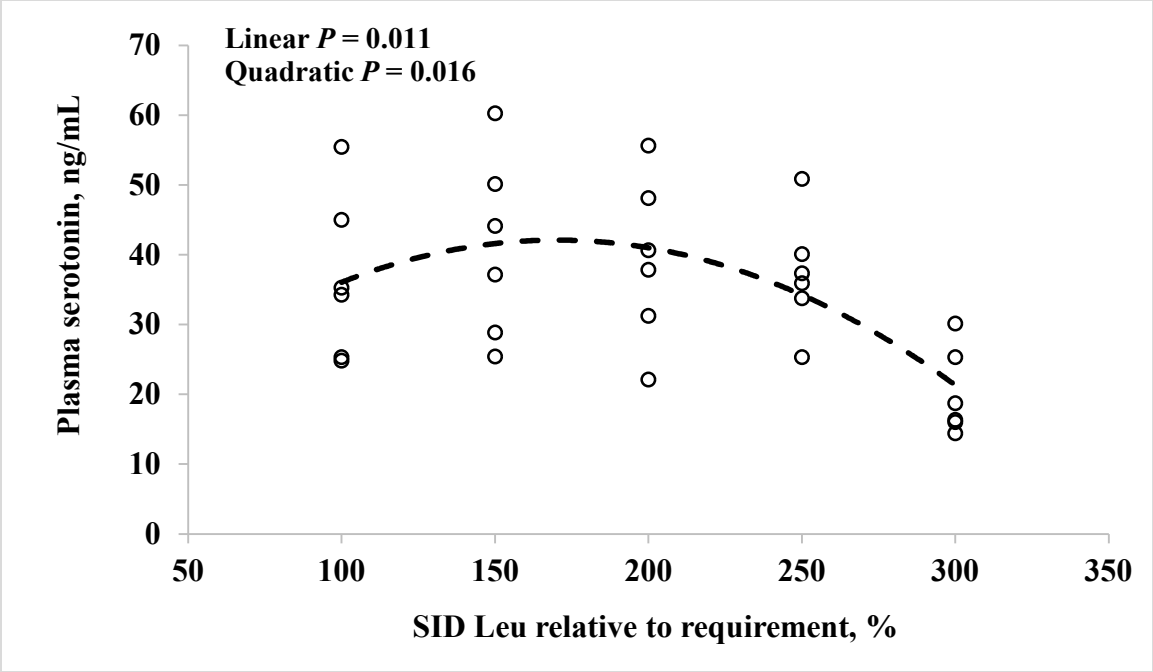

Figure 3.2. Plasma serotonin of growing pigs (N = 30; n = 6) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

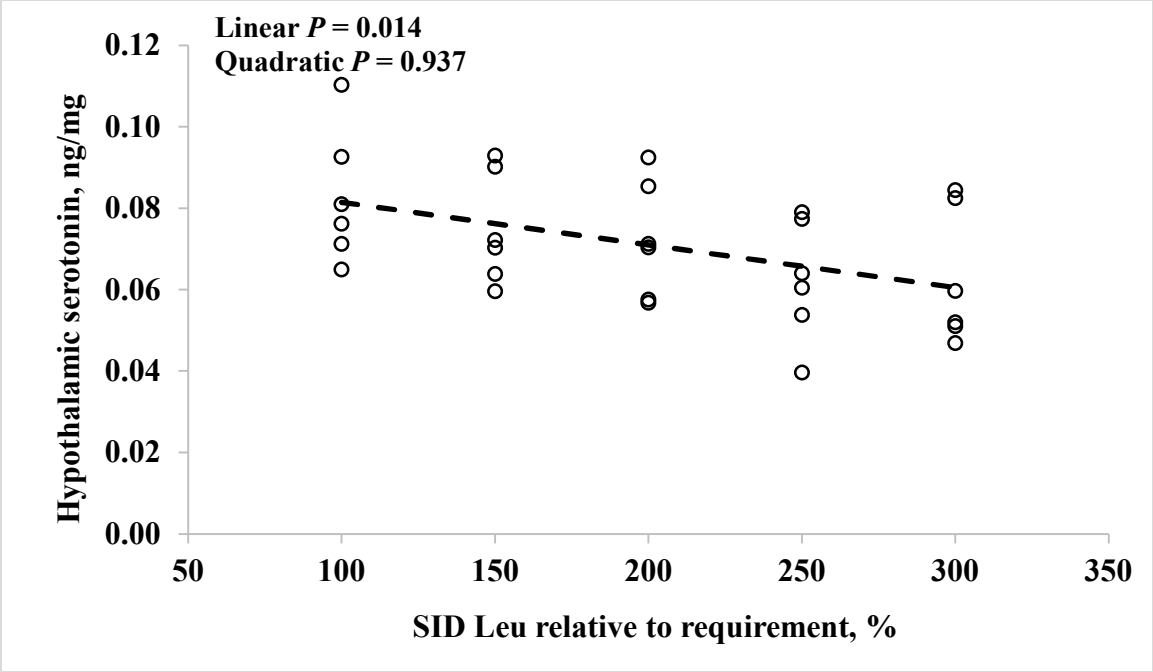

Figure 3.3. Hypothalamic serotonin of growing pigs (N = 30; n = 6) fed diets with increasing concentrations of standardized ileal digestible (SID) Leu relative to the requirement (NRC, 2012).

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CHAPTER 4: EFFECTS OF DIETARY ISOLEUCINE AND VALINE SUPPLEMENTATIONS TO EXCESS OR LOW LEUCINE DIETS ON NITROGEN BALANCE AND METABOLISM OF BRANCHED-CHAIN AMINO ACIDS IN GROWING PIGS

ABSTRACT

An experiment was conducted to test the hypothesis that Ile and Val supplementations may overcome detrimental effects of excess dietary Leu on N balance and metabolism of branched-chain amino acids (**BCAA**) in growing pigs. A total of 144 barrows (initial body weight: 28.5 ± 2.5 kg) were housed in metabolism crates and randomly assigned to 18 diets. The basal diet contained 0.98% standardized ileal digestible (**SID**) Lys and had SID Leu, Val, and Ile ratios to SID Lys of 100, 60, and 43%, respectively. Two levels of synthetic L-Leu (0 or 2.0%), 3 levels of synthetic L-Ile (0, 0.1, or 0.2%), and 3 levels of synthetic L-Val (0, 0.1, or 0.2%) were added to the basal diet for a total of 18 diets that were arranged in a 2 × 3 × 3 factorial. Urine and fecal samples were collected for 5 d after 7 d of adaptation. Blood, skeletal muscle, and liver samples were collected at the conclusion of the experiment. Results indicated that there were no 3-way interactions among main effects. Excess Leu in diets reduced ($P < 0.05$) N retention and biological value of diets and increased ($P < 0.001$) plasma urea N (**PUN**). However, PUN was reduced ($P < 0.05$) as dietary Val increased. Concentrations of BCAA in liver were greater ($P < 0.001$) in pigs fed excess-Leu diets than in pigs fed low-Leu diets, but concentrations of BCAA in muscle were greater ($P < 0.05$) in pigs fed low-Leu diets. Increasing dietary Ile increased ($P < 0.001$) plasma free Ile and plasma concentration of the Ile metabolite, α-keto-β-methylvalerate, but the increase was greater in diets without excess Leu than in diets with excess Leu

(interaction, $P < 0.001$). Likewise, plasma concentration of Val and the Val metabolite, α-keto isovalerate, increased ($P < 0.001$) more with increasing dietary Val in diets with Leu at the requirement than in diets with excess Leu (interaction, $P < 0.001$). Increasing dietary Leu increased ($P < 0.001$) plasma free Leu and plasma concentration of the Leu metabolite, α-keto isocaproate. In contrast, increased dietary Val reduced ($P < 0.05$) plasma concentration of α-keto isocaproate. In conclusion, excess dietary Leu reduced N retention and biological value of diets and increased PUN in growing pigs, but Val supplementation to high Leu diets may increase the efficiency of amino acid utilization for protein synthesis as indicated by reduced PUN. Excess dietary Leu also changed BCAA profiles in skeletal muscle, liver, and plasma, but Val supplementation decreased the Leu metabolite, α-keto isocaproate, in plasma.

Key words: branched-chain amino acids, isoleucine, leucine, nitrogen balance, pigs, valine

INTRODUCTION

Leucine is a key regulator that stimulates catabolism of branched-chain amino acids (**BCAA**; i.e., Leu, Ile, and Val) in skeletal muscle and liver (Harper et al., 1984; Cemin et al., 2019a). If diets fed to pigs contain excess Leu, catabolism of all 3 BCAA may increase because of the stimulating effect of the Leu metabolite, α-keto isocaproate (**KIC**) on the branched-chain α-keto acid dehydrogenase (**BCKDH**) enzyme complex, which is responsible for degradation of the 3 branched-chain α-keto acids (**BCKA**) that originate from metabolism of the 3 BCAA (Wiltafsky et al., 2010). Serum Ile and Val concentrations were reduced by excess dietary Leu in growing pigs (Duan et al., 2016; Wessels et al., 2016), and high dietary Leu reduces feed intake and growth performance in pigs (Gatnau et al., 1995; Wiltafsky et al., 2010), which may be a result of the imbalanced supply of BCAA that result from increased metabolism of Val and Ile.

Recent data confirmed that excess dietary Leu reduced growth performance and biological value of protein and tended to reduce N balance of growing pigs, which is likely a result of reduced availability of Val and Ile (Kwon et al., 2019).

Recently, Morales at al. (2016) demonstrated that supplementations of Ile and Val above their requirements in high Leu diets corrected the negative effect of excess Leu on absorption and degradation of BCAA without growth reduction. This indicates that excess dietary Leu reduces the availability of Ile and Val, and as a consequence, addition of extra Ile and Val may help prevent negative effects of excess Leu. Therefore, the objective of this experiment was to test the hypothesis that inclusion of dietary Ile and Val above the requirement may overcome detrimental effects of excess Leu on N balance and metabolism of BCAA in growing pigs.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at the University of Illinois reviewed and approved the protocol for the experiment before the animal work was initiated.

Animals, Diets, and Experimental Design

A total of 144 growing barrows (initial body weight = 28.5 ± 2.5 kg) that were the offspring of Line 359 boars and Camborough sows (Pig Improvement Company, Henderson, TN) were assigned to 18 dietary treatments with 8 replicates pigs per treatment in a randomized complete block design. There were 8 blocks with 1 pig per diet in each block. A basal diet with a standardized ileal digestible (**SID**) Leu:Lys ratio of 100%, a SID Ile:Lys ratio of 43%, and a SID Val:Lys ratio of 60% was formulated based on corn, wheat, barley, soy protein concentrate, and spray-dried blood cells (Tables 4.1 and 4.2). Two levels of synthetic L-Leu (0 or 2.0%), 3 levels of synthetic L-Ile (0, 0.1, or 0.2%), and 3 levels of synthetic L-Val (0, 0.1, or 0.2%) were added

to the basal diet – for a total of 18 diets that were used in a 2 × 3 × 3 factorial arrangement of treatments.

All diets were formulated to be isoenergetic (3,350 kcal ME/kg) and to contain 1.00% SID Lys, which was assumed to be slightly above the SID Lys requirement for 25 to 50 kg pigs (NRC, 2012). Other indispensable amino acids (**AA**) except the 3 BCAA were included in all diets in excess of the requirements (NRC, 2012). Glycine was included to maintain a constant concentration of dietary crude protein at 15.50%.

Housing and Feeding

Pigs were individually housed in metabolism crates that were equipped with a feeder and a nipple drinker. Pigs were fed experimental diets for 13 days and the BW of pigs was recorded at the start and at the conclusion of the feeding period. Pigs were fed 2.3 times the maintenance requirement for metabolizable energy (i.e., 197 kcal/kg × $BW^{0.60}$; NRC, 2012), which was provided in 2 daily meals at 0800 and 1600 h. Water was provided on an *ad libitum* basis.

Sample Collection

After 5 d of adaptation to the experimental diets, urine and fecal samples were collected during the following 5 d according to the marker to marker method (Adeola, 2001). Urine was collected in buckets with 50 mL of 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.

On d 13, blood samples were collected from the jugular vein of all pigs using vacutainers containing heparin (BD, Franklin Lakes, NJ). All samples were centrifuged at 1,500 × g at 4°C for 15 min to collect plasma, which were frozen at -80ºC until analyzed. After blood sampling,

all pigs were euthanized. Liver and skeletal muscle (longissimus dorsi) were collected and immediately frozen in liquid N.

Sample Analyses

Samples of corn, wheat, barley, soy protein concentrate, and blood cells, 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). Ingredient samples were also analyzed for dry matter (Method 930.15; AOAC Int, 2007). The 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 thawed urine samples were analyzed for crude protein (method 984.13; AOAC Int., 2007) using a Kjeltec 8400 apparatus (FOSS Inc., Eden Prairie, MN). Plasma from blood in heparinized tubes was analyzed for plasma urea N (**PUN**) using a Beckman Coulter Clinical Chemistry AU analyzer (Beckman Coulter Inc., Brea, CA). Lyophilized and homogenized liver and skeletal muscle samples were analyzed by Ajinomoto Animal Nutrition North America Laboratory (Eddyville, IA) for BCAA concentration (method 999.13; AOAC Int., 2007) using an Amino Acid Analyzer (model L-8900; Amino Acid Analyzer; Hitachi High Technologies America Inc., Pleasanton, CA). Concentrations of BCAA and BCKA in plasma from blood in EDTA tubes were measured 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 Beals et al. (2016).

Calculations and Statistical Analyses

The apparent total tract digestibility (**ATTD**) 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

biological value of protein in the diets was calculated by expressing the retention of N as a percentage of the difference between N intake and N output in feces (Mitchell, 1924).

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) as a 2 × 3 × 3 factorial arrangement of treatments. The experimental unit was the pig and the model included SID Leu:Lys ratio, SID Ile:Lys ratio, SID Val:Lys ratio, and the interactions among SID Leu:Lys, SID Ile:Lys, and SID Val:Lys as fixed variables and block (replicate) as a random variable. Treatment means were separated by using the LSMEANS statement. Statistical significance and tendency were considered as $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

RESULTS

All animals remained healthy throughout the experiment. Crude protein and AA concentrations in corn, wheat, barley, soy protein concentrate, and spray-dried blood cells were in agreement with expected values (NRC, 2012). Analyzed values for Lys and BCAA were in agreement with formulated values in all experimental diets (Tables 4.3 and 4.4).

There were no 3-way interactions among main effects throughout the study. No effects of adding dietary Ile, Leu, or Val were observed for feed intake and N intake of pigs, but excess dietary Leu in diets increased ($P < 0.05$) urinary N excretion (Table 4.5). Fecal N excretion and ATTD of N were not affected by dietary Ile, Leu, or Val supplementations, but N retention (g/ 5d and % of intake) and the biological value of diets were reduced ($P < 0.05$) as dietary Leu increased.

Adding Leu to diets increased ($P < 0.05$) PUN, but PUN was reduced ($P < 0.05$) as

dietary Val increased (Table 4.6). Concentrations of Ile, Leu, and Val in liver were greater ($P < 0.05$) in pigs fed excess-Leu diets than in pigs fed diets where Leu was at the requirement, but concentrations of Ile, Leu, and Val in skeletal muscle were greater ($P < 0.05$) in pigs fed diets with Leu at the requirement than in pigs fed excess-Leu diets.

Concentrations in plasma of free Ile and α-keto β-methylvalerate (**KMV**) increased ($P < 0.001$) as dietary Ile increased in the diet, but the increase was greater if dietary Leu was at the requirement than if Leu was at 300% of the requirement (interaction, $P < 0.001$; Figure 4.1). Likewise, plasma free Val and α-keto isovalerate (**KIV**) increased ($P < 0.001$) as dietary Val increased, but the increase was greater if dietary Leu was in excess of the requirement than at the requirement (interaction, $P < 0.001$; Figure 4.2). Increasing dietary Leu increased ($P < 0.001$) plasma free Leu and plasma KIC and increasing dietary Val increased ($P < 0.001$) plasma KIC but not plasma free Leu (Figure 4.3).

DISCUSSION

The objective of the current study was to test the hypothesis that increasing dietary Ile and Val may overcome detrimental effects of excess Leu on N balance and metabolism of BCAA in growing pigs. Therefore, it was necessary to formulate basal diets that were co-limiting in Ile and Val to generate clear responses to increasing dietary Ile and Val supplementations. Spray-dried blood cells were used to formulate diets containing SID Ile:Lys ratio of 0.43:1. Blood by-products such as spray-dried blood cells are sometimes used in diets for nursery pigs because of their high protein quality (van Dijk et al., 2001; DeRouchey et al., 2002). However, most blood by-products have imbalanced AA patterns with low Ile concentration (van Milgen et al., 2012), and have therefore, been extensively used in dose-response experiments for Ile (Parr

et al., 2004; Wiltafsky et al., 2009; Htoo et al., 2014). In addition, soy protein concentrate was used because of its low Val concentration (NRC, 2012) and diets with a SID Val:Lys ratio of 0.60:1 could therefore be formulated.

Results confirmed that increased dietary Leu reduced retention of N and the biological value of diets, which is indicative of reduced N utilization for protein deposition. The negative effect of increased dietary Leu on protein deposition may be due to increased degradation of all 3 BCAA because of increased expression of the 2 shared enzymes, BCAA aminotransferase and BCKDH (Kwon et al., 2019). This may then result in a deficiency of Ile and Val for protein synthesis (Wiltafsky et al., 2010; Kwon et al., 2019). Current results also confirmed that increased dietary Leu reduced plasma free Ile and Val concentrations whereas increased plasma free Leu concentration was observed. Thus, excess dietary Leu can create an imbalanced supply of Ile and Val for protein synthesis especially when dietary Ile and Val are close to the requirements. A SID Leu:Lys ratio of 1.00:1, a SID Val:Lys ratio of 0.70:1, and a SID Ile:Lys ratio of 0.53:1 are believed to provide all 3 BCAA at the requirement levels for 25 to 50 kg growing pigs (NRC, 2012; van Milgen et al., 2012; 2013). Indeed, pigs fed the diet containing all 3 BCAA at the requirement levels had the greatest retention of N and the greatest biological value of protein (data not shown) among the 18 treatments, indicating that this diet provided AA that were closer to the requirement than other diets.

The increased PUN that was observed as dietary Leu increased is most likely a result of increased catabolism of Ile and Val, which in turn reduces availability of these AA for protein synthesis and causes an imbalance among other indispensable AA (Gatnau et al., 1995). A deficiency in Ile and Val also may have reduced protein synthesis as indicated by the reduced N retention, which may have resulted in increased deamination of other AA and a subsequent

increase in PUN. This observation is in agreement with previous data (Kwon et al., 2019). Whereas no effect of Ile supplementation on PUN was observed, the reduced PUN that was observed as dietary Val increased indicates that Val addition is more beneficial in preventing negative effects of excess Leu on efficiency of AA utilization than increased Ile addition. The reason for the lack of a beneficial effect of Ile supplementation may be that Ile is not degraded as much as Val if Leu is in excess or that the requirement for SID Ile is less than 53% relative to SID Lys that was used in formulation of the control diet. It is also possible that Ile supplementation above the requirement impairs growth performance of pigs (Soumeh et al., 2014), indicating that excess dietary Ile can also create BCAA antagonism. Results of a recent meta-analysis, however, indicates that increasing concentrations of dietary Val and Ile alone or in combination have the potential to alleviate the negative effects of excess dietary Leu on growth performance of pigs (Cemin et al., 2019b).

The first step in the catabolism of BCAA, which is catalyzed by BCAA aminotransferase, results in synthesis of the 3 corresponding BCKA, KIV, KIC, and KMV, from Ile, Leu, and Val, respectively (Harris et al., 2005). If excess Leu is included in diets for pigs, the catabolism of all 3 BCAA may increase because of increased activities of BCAA aminotransferase. But higher activity of the transamination enzyme may produce more KIC, which activates BCKDH and changes concentrations of BCKA in blood. The changes in BCAA and BCKA that were observed as dietary Leu increased are in agreement with Langer et al. (2010) who reported that excess dietary Leu reduced plasma concentrations of Ile and Val, as well as of KIV, KIC, and KMV. In addition, whole-body Val oxidation was increased due to increasing BCKDH activity. In rats, dietary supplementation of KIC to a low crude protein diet resulted in increased KIC in plasma, whereas concentrations of KIV and KMV were reduced (Crowell et al., 1990). However,

the current data indicated that adding more Val to the diets reduced KIC in plasma regardless of dietary Leu concentration, which may reduce the activation of BCKDH, and therefore, prevent metabolism of KIV and KMV. Because KIV and KMV can be reaminated to form Ile and Val, respectively, a reduced metabolism of KIV and KMV may negate some of the negative effects of excess Leu. Results of previous studies indicated that excess dietary Leu greatly decreased overall growth performance of pigs, but increasing Val alleviated the reductions of average daily gain and gain to feed ratio (Gloaguen et al., 2011; Millet et al., 2015). Thus, the current data indicating that dietary Val can partly ameliorate effects of excess Leu are in agreement with data from previous research. It is, therefore, possible that excess dietary Leu changes the Val requirement and adding more Val is needed to ensure optimal growth performance of pigs.

CONCLUSION

Decreased N retention and biological value of diets and the increased PUN that were observed as dietary Leu increased indicate that excess dietary Leu increased catabolism of Val and Ile and created an AA imbalance with reduced protein synthesis as consequence. However, Val supplementation may increase the efficiency of AA utilization for protein synthesis, which was indicated by reduced PUN. Concentrations of BCAA in skeletal muscle and liver, and profiles of BCAA and their metabolites in plasma were changed because of excess dietary Leu, but Val supplementation decreased concentrations of KIC, which is considered the key regulator of the BCAA catabolic process. Overall, it appears that excess dietary Leu may have negative impacts on N balance and metabolism of BCAA, but Val supplementation may partially overcome the negative impacts of excess dietary Leu.

TABLES

Table 4.1. Chemical composition of ingredients used in experimental diets, as-fed basis[1]

Item[2]	Corn	Wheat	Barley	SPC	SDBC
CP[3], %	7.09	11.74	10.98	64.48	93.81
Dry matter, %	87.14	88.64	89.24	94.82	91.78
Indispensable amino acids, %					
Arg	0.32	0.52	0.55	4.63	3.65
His	0.21	0.24	0.24	1.68	6.79
Ile	0.26	0.39	0.38	3.01	0.35
Leu	0.78	0.69	0.72	4.97	12.81
Lys	0.26	0.36	0.48	4.10	8.62
Met	0.14	0.19	0.19	0.92	0.98
Phe	0.30	0.45	0.43	1.85	6.92
Thr	0.32	0.45	0.49	3.21	3.54
Trp	0.06	0.13	0.12	0.87	1.60
Val	0.26	0.32	0.38	2.51	8.52
Dispensable amino acids, %					
Ala	0.50	0.41	0.47	2.75	7.93
Asx[4]	0.46	0.58	0.72	7.08	10.21
Cys	0.16	0.26	0.24	0.93	0.54
Glx[5]	1.21	2.70	2.19	11.37	7.52
Gly	0.29	0.45	0.45	2.66	4.12
Pro	0.62	0.90	0.93	3.20	3.27

Table 4.1. (Cont.)

Ser	0.33	0.44	0.42	2.72	4.12
Tyr	0.19	0.27	0.27	2.23	2.21
BCAA[6]:CP ratio, %					
Ile:CP	3.67	3.32	3.46	4.67	0.37
Leu:CP	11.00	5.88	6.56	7.71	13.66
Val:CP	3.67	2.73	3.46	3.89	9.08

[1]Ingredients were analyzed in duplicate.

[2]SPC = soy protein concentrate; SDBC = spray-dried blood cells.

[3]CP = crude protein.

[4]Asx = Asp and Asn.

[5]Glx = Glu and Gln.

[6]BCAA = branched-chain amino acids.

Table 4.2. Ingredient composition of experimental diets containing 100% standardized ileal digestible (SID) Leu:Lys ratio, as-fed basis[1]

	SID Ile:Lys , %:	43			53			63		
Item, %	SID Val:Lys , %:	60	70	80	60	70	80	60	70	80
Ground corn		32.35	32.35	32.35	32.35	32.35	32.35	32.35	32.35	32.35
Ground wheat		22.00	22.00	22.00	22.00	22.00	22.00	22.00	22.00	22.00
Ground barley		28.00	28.00	28.00	28.00	28.00	28.00	28.00	28.00	28.00
Soy protein concentrate		7.60	7.60	7.60	7.60	7.60	7.60	7.60	7.60	7.60
Spray-dried blood cells		1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Soybean oil		3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
L-Lys·HCl		0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Met		0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-Thr		0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
L-Trp		0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
L-Leu		-	-	-	-	-	-	-	-	-
L-Ile		-	-	-	0.10	0.10	0.10	0.20	0.20	0.20
L-Val		-	0.10	0.20	-	0.10	0.20	-	0.10	0.20

Table 4.2. (Cont.)

Gly	1.35	1.30	1.25	1.30	1.25	1.20	1.25	1.20	1.15
Cornstarch	1.05	1.00	0.95	1.00	0.95	0.90	0.95	0.90	0.85
Limestone	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Monocalcium phosphate	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin-mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[1]Nine additional diets that were similar to the above diets with the exception that the SID Leu:Lys ratio was 300% instead of 100% were also formulated. This was accomplished by including 2.0% L-Leu in all diets and reducing both Gly and cornstarch inclusion to 0.20%.

[2]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 D_3 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 B_{12}, 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 4.3. Analyzed nutrient composition of experimental diets containing 100% standardized ileal digestible (SID) Leu:Lys ratio, as-fed basis[1]

	SID Ile:Lys , %:	43			53			63	
Item	SID Val:Lys , %:	60	70	80	60	70	80	60	70	80
Crude protein, %		15.48	15.55	15.41	15.46	15.43	15.56	15.51	15.39	15.52
Indispensable amino acids, %										
Arg		0.77	0.76	0.70	0.77	0.80	0.76	0.76	0.76	0.75
His		0.38	0.38	0.36	0.38	0.39	0.37	0.37	0.37	0.37
Ile		0.49	0.51	0.50	0.63	0.64	0.62	0.70	0.70	0.69
Leu		1.16	1.13	1.09	1.15	1.19	1.15	1.14	1.13	1.13
Lys		1.12	1.05	1.04	1.06	1.11	1.03	1.06	1.06	1.09
Met		0.27	0.28	0.27	0.28	0.29	0.27	0.26	0.27	0.26
Phe		0.71	0.70	0.67	0.71	0.72	0.70	0.69	0.69	0.69
Thr		0.67	0.65	0.66	0.68	0.67	0.65	0.66	0.68	0.69
Trp		0.15	0.15	0.16	0.16	0.16	0.15	0.16	0.16	0.15
Val		0.74	0.82	0.89	0.73	0.84	0.90	0.71	0.81	0.89

Table 4.3. (Cont.)

Dispensable amino acids, %									
Ala	0.71	0.69	0.66	0.69	0.72	0.69	0.69	0.70	0.68
Asx[1]	1.16	1.13	1.05	1.14	1.18	1.13	1.12	1.12	1.10
Cys	0.23	0.24	0.23	0.26	0.25	0.24	0.24	0.24	0.24
Glx[2]	2.59	2.55	2.39	2.54	2.62	2.56	2.53	2.53	2.49
Gly	1.90	1.86	2.04	1.89	1.77	1.79	1.85	1.76	1.69
Pro	1.00	0.97	0.96	0.94	1.01	0.93	1.00	0.98	0.98
Ser	0.55	0.54	0.51	0.54	0.57	0.55	0.55	0.56	0.55
Tyr	0.40	0.40	0.38	0.41	0.42	0.41	0.41	0.41	0.40

[1]Asx = Asp and Asn.

[2]Glx = Glu and Gln.

Table 4.4. Analyzed nutrient composition of experimental diets containing 300% standardized ileal digestible (SID) Leu:Lys ratio, as-fed basis[1]

	SID Ile:Lys , %:	43			53			63	
Item	SID Val:Lys , %:	60	70	80	60	70	80	60	70	80
Crude protein, %		15.45	15.53	15.49	15.52	15.49	15.45	15.52	15.43	15.56
Indispensable amino acids, %										
Arg		0.71	0.69	0.69	0.79	0.76	0.75	0.76	0.76	0.76
His		0.36	0.35	0.35	0.38	0.38	0.37	0.38	0.37	0.37
Ile		0.49	0.49	0.49	0.62	0.62	0.58	0.69	0.68	0.67
Leu		3.10	3.10	3.16	3.11	3.14	3.06	3.09	3.05	3.18
Lys		1.17	1.11	1.10	1.13	1.06	0.97	1.03	1.00	1.12
Met		0.26	0.29	0.30	0.28	0.30	0.28	0.32	0.31	0.25
Phe		0.67	0.65	0.65	0.71	0.70	0.69	0.69	0.69	0.69
Thr		0.69	0.63	0.63	0.67	0.66	0.64	0.66	0.61	0.72
Trp		0.15	0.16	0.16	0.15	0.16	0.16	0.16	0.16	0.15
Val		0.67	0.76	0.85	0.74	0.81	0.89	0.71	0.78	0.94

Table 4.4. (Cont.)

Dispensable amino acids, %									
Ala	0.67	0.65	0.66	0.71	0.69	0.69	0.69	0.69	0.70
Asx[1]	1.07	1.02	1.03	1.17	1.14	1.10	1.13	1.12	1.12
Cys	0.22	0.23	0.23	0.27	0.26	0.25	0.26	0.24	0.24
Glx[2]	2.44	2.36	2.39	2.61	2.52	2.54	2.53	2.53	2.51
Gly	0.74	0.68	0.63	0.70	0.67	0.62	0.66	0.62	0.58
Pro	0.93	0.99	0.96	1.02	0.98	1.02	1.02	0.97	1.01
Ser	0.56	0.49	0.51	0.56	0.55	0.55	0.56	0.55	0.57
Tyr	0.39	0.38	0.38	0.42	0.41	0.40	0.41	0.41	0.41

[1]Asx = Asp and Asn.

[2]Glx = Glu and Gln.

Table 4.5. Main effects of dietary Leu, Ile, and Val concentrations on N balance of growing pigs, as-fed basis

Main effect:	SID[1] Leu:Lys, %		SID Ile:Lys, %			SID Val:Lys, %			Pooled	*P*-values		
Item	100	300	43	53	63	60	70	80	SEM	Leu	Ile	Val
Feed intake, g/5 d	4,839	4,810	4,808	4,832	4,833	4,823	4,817	4,833	62	0.140	0.516	0.807
N intake, g/5 d	120	119	119	120	120	120	119	120	1.5	0.212	0.498	0.593
N output in feces, g/5 d	20.1	19.7	20.1	19.6	19.9	20.3	19.5	19.8	1.3	0.425	0.684	0.361
N output in urine, g/5 d	19.4	21.6	20.9	20.4	20.2	21.3	20.3	19.9	2.2	0.009	0.750	0.403
ATTD[2] of N, %	83.2	83.5	83.1	83.6	83.4	83.0	83.6	83.5	1.1	0.535	0.565	0.374
N retention, g/5 d	80.4	78.0	78.1	79.8	79.8	78.0	79.5	80.2	2.8	0.024	0.356	0.236
N retention, %	67.1	65.3	65.5	66.6	66.6	65.2	66.6	66.9	2.1	0.041	0.488	0.212
Biological value[3], %	80.6	78.2	78.8	79.7	79.8	78.6	79.6	80.1	2.2	0.008	0.620	0.346

[1]SID = standardized ileal digestible.

[2]ATTD = apparent total tract digestibility.

[3]Biological value was calculated as [N retained/(N intake – N output in feces)] × 100 (Mitchell, 1924).

Table 4.6. Main effects of dietary Ile, Leu, and Val concentrations on plasma urea N and tissue branched-chain AA (BCAA) concentrations of growing pigs, as-fed basis

Main effect:	SID[1] Leu:Lys, %		SID Ile:Lys, %			SID Val:Lys, %			Pooled	*P*-values		
Item	100	300	43	53	63	60	70	80	SEM	Leu	Ile	Val
Plasma urea N, μg/mL	5.26	6.51	6.06	6.02	5.58	6.40	5.73	5.54	0.57	< 0.001	0.277	0.027
Liver BCAA												
Ile, %	2.72	2.92	2.86	2.81	2.80	2.81	2.82	2.83	0.08	< 0.001	0.195	0.905
Leu, %	5.32	5.60	5.51	5.45	5.42	5.77	5.47	5.47	0.13	< 0.001	0.208	0.853
Val, %	3.19	3.36	3.31	3.27	3.26	3.26	3.29	3.28	0.08	< 0.001	0.287	0.705
Muscle BCAA												
Ile, %	3.57	3.47	3.50	3.52	3.55	3.54	3.50	3.52	0.08	< 0.001	0.255	0.321
Leu, %	5.99	5.90	5.92	5.94	5.98	5.97	5.91	5.96	0.09	0.003	0.223	0.199
Val, %	3.54	3.29	3.35	3.50	3.39	3.46	3.34	3.44	0.28	0.006	0.405	0.540

[1]SID = standardized ileal digestible.

FIGURES

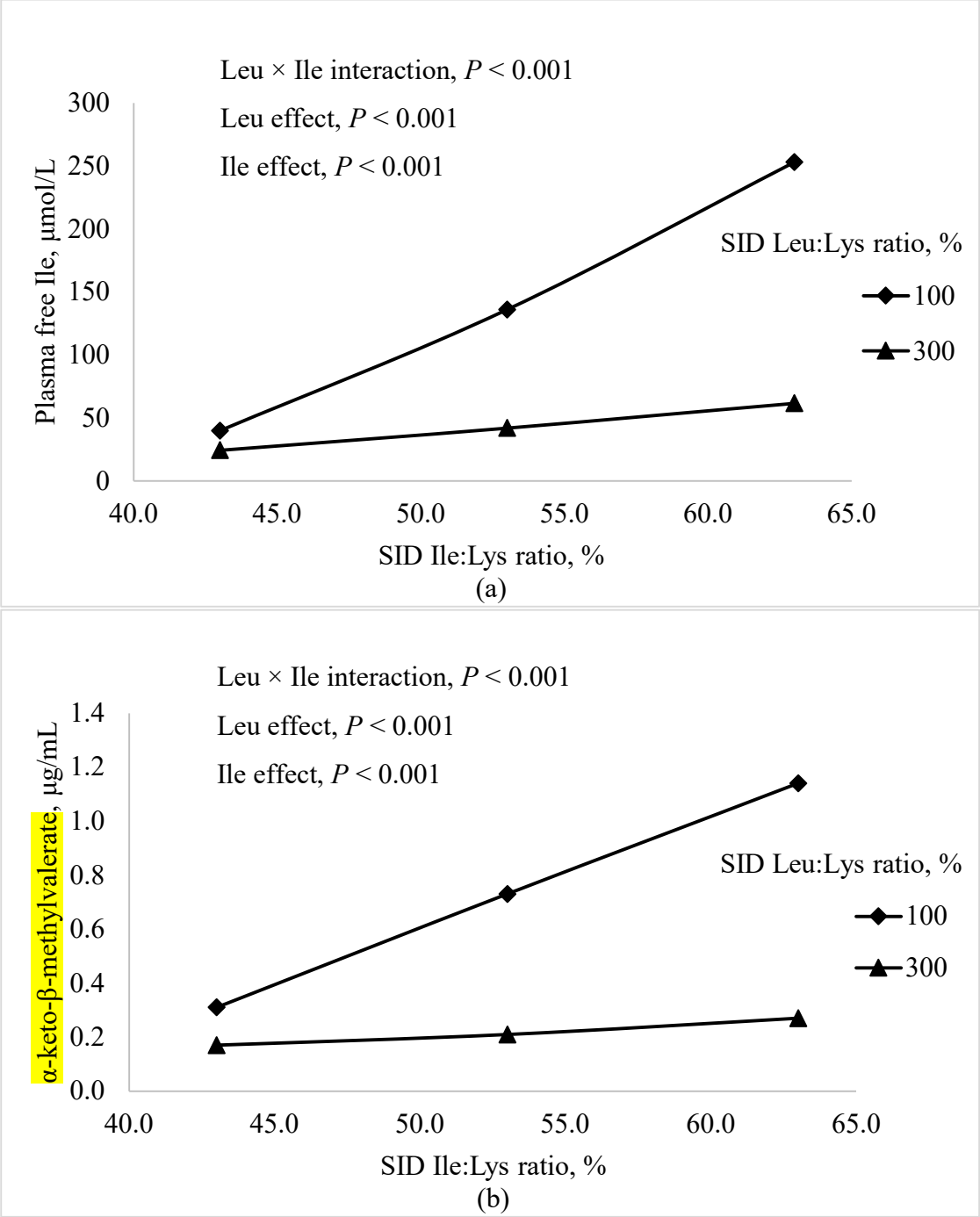

Figure 4.1. Effects of dietary Leu and Ile concentrations on (a) plasma free Ile and (b) α-keto-β-methylvalerate (Ile metabolite) in plasma of growing pigs fed diets containing from 43 to 63% standardized ileal digestible (SID) Ile:Lys and from 100 to 300% SID Leu:Lys.

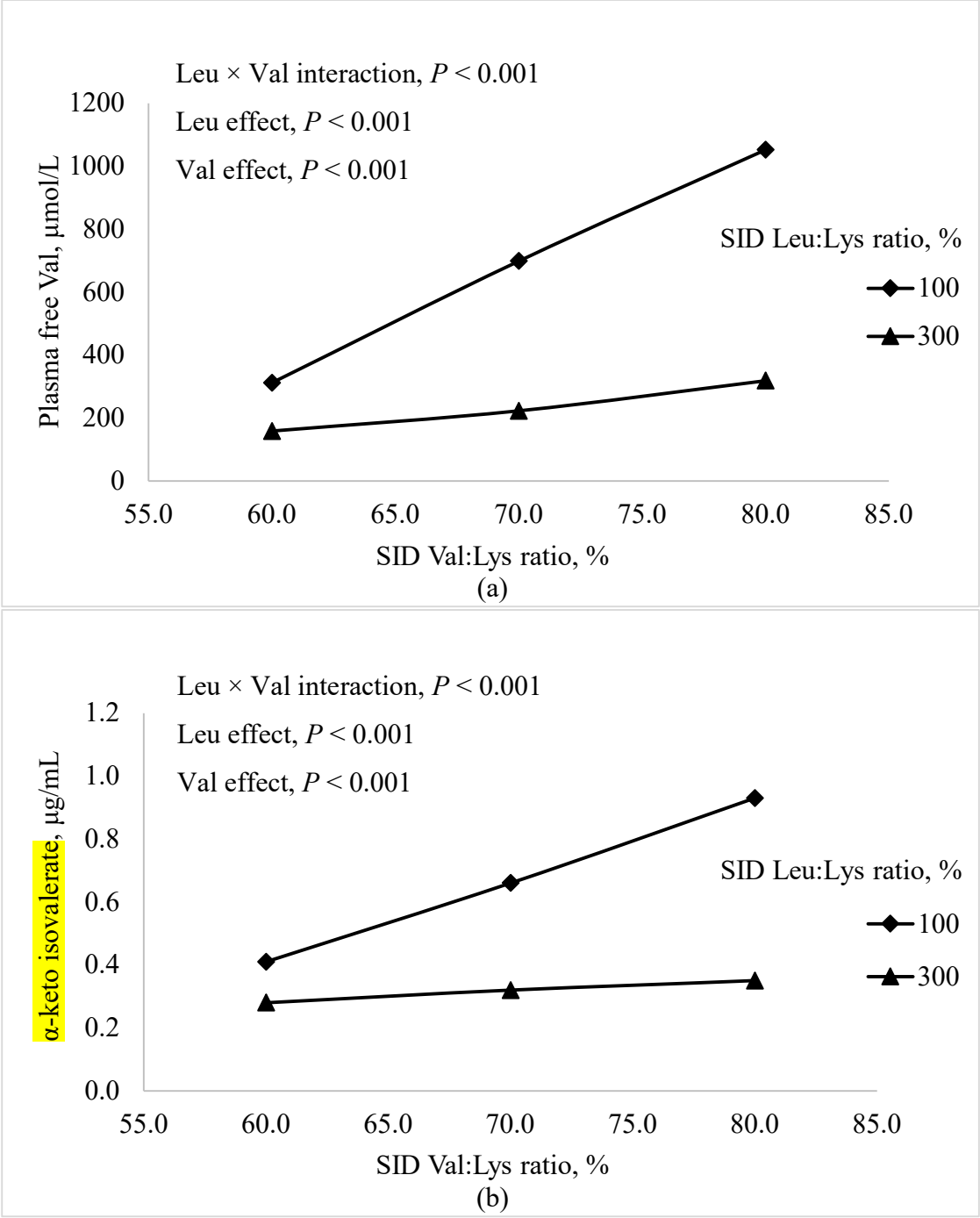

Figure 4.2. Effects of dietary Leu and Val concentrations on (a) plasma free Val and (b) α-keto isovalerate (Val metabolite) in plasma of growing pigs fed diets containing from 60 to 80% standardized ileal digestible (SID) Ile:Lys and from 100 to 300% SID Leu:Lys.

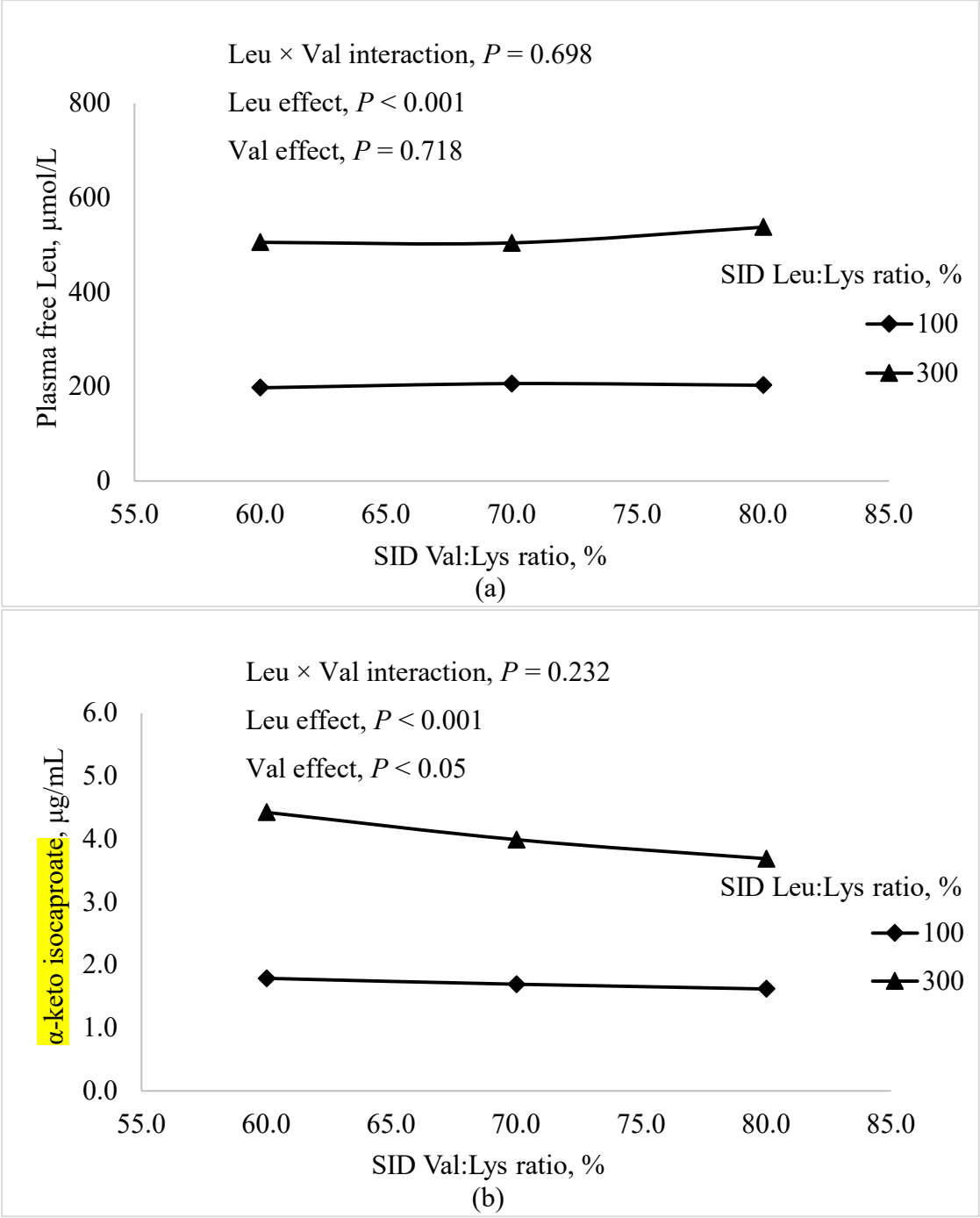

Figure 4.3. Effects of dietary Leu and Val concentrations on (a) plasma free Leu and (b) α-keto isocaproate (Leu metabolite) in plasma of growing pigs fed diets containing from 60 to 80% standardized ileal digestible (SID) Ile:Lys and from 100 to 300% SID Leu:Lys.

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CHAPTER 5: EFFECTS OF DIETARY LEUCINE AND TRYPTOPHAN SUPPLEMENTATIONS ON SEROTONIN METABOLISM AND GROWTH PERFORMANCE OF GROWING PIGS

ABSTRACT

An experiment was conducted to test the hypothesis that increased dietary Trp is needed in high-Leu diets for growing pigs to prevent a drop in plasma serotonin and hypothalamic serotonin concentrations and to maintain growth performance of animals. A total of 144 growing pigs (initial body weight: 28.2 ± 1.9 kg) were divided into 2 blocks of 72 pigs and randomly assigned to 9 dietary treatments in a randomized complete block design. There were 2 pigs per pen and 4 replicate pens per block for a total of 8 replicate pens per treatment. The 9 diets were formulated in a 3 × 3 factorial with 3 levels of dietary Leu (100, 200, or 300% of the requirement for standardized ileal digestible [**SID**] Leu), and 3 levels of dietary Trp (18, 23, or 28% SID Trp:Lys). A basal diet that met requirements for SID Leu and SID Trp was formulated and the other 8 diets were formulated by adding crystalline L-Leu and (or) L-Trp to the basal diet. Individual pig weights were recorded at the beginning of the experiment and at the conclusion of the 21-d experiment. On the last day of the experiment, one pigs per pen was sacrificed and blood and hypothalamus samples were collected to measure plasma urea N, plasma serotonin, and hypothalamic serotonin concentrations. Results indicated that increasing dietary Trp increased ($P < 0.05$) average daily gain (**ADG**), average daily feed intake (**ADFI**), and hypothalamic serotonin, whereas increasing dietary Leu reduced ($P < 0.05$) ADG, ADFI, and hypothalamic serotonin, but the increase caused by dietary Trp was greater if Leu was provided at 300% of the requirement than if it was provided at the requirement (interaction, $P < 0.05$).

Plasma Leu concentration was negatively affected by both dietary Leu and dietary Trp, but the negative effect of Trp was greater if Leu was at 300% of requirement than that at 100% of requirement (interaction, $P < 0.05$). Plasma concentration of Trp was positively affected by increased dietary Trp and increased dietary Leu, but the increase in plasma concentration of Trp was greater if Leu was at the requirement than at 300% of the requirement (interaction, $P < 0.05$). In conclusion, increased dietary Leu reduced ADG, ADFI, and hypothalamic serotonin concentration, and influenced the metabolism of several indispensable amino acids, but Trp supplementation partly overcame the negative effect of excess dietary Leu, demonstrating the importance of Trp in the regulation of hypothalamic serotonin, and therefore, feed intake of growing pigs.

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

INTRODUCTION

Tryptophan is one of indispensable amino acids (**AA**) that is often limiting for growth in pigs fed corn-soybean meal-based diets (Lewis, 2001; Petersen, 2011). Tryptophan may act as a regulator of feed intake by enhancing serotonin signaling in the brain (Henry et al., 1992), because Trp 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 feed intake (Henry et al 1992; Ettle and Roth, 2004), and this is partly attributed to increased serotonin synthesis (Shen et al., 2012a). Availability of dietary Trp in the brain is considered the rate-limiting step in hypothalamic serotonin synthesis (Meunier-Salaün et al., 1991; Shen et al., 2012b). However, to be transported into the brain, Trp competes with other large neutral AA

such as Val, Leu, Ile, Tyr, and Phe for a common transporter (L-type AA transporter 1) to cross the blood-brain barrier (Le Floc'h and Sève, 2007).

Diets based on corn and corn co-products and sorghum and sorghum co-products are rich in Leu and excess dietary Leu reduces pig feed intake and growth performance (Gatnau et al., 1995; Wiltafsky et al., 2010). Excess dietary Leu may also reduce synthesis of serotonin in the brain (Wessels et al., 2016b), because excess Leu may prevent Trp from being transported from the blood to the brain, which may result in reduced availability of Trp for serotonin synthesis in the brain. Indeed, excess dietary Leu reduces hypothalamic serotonin concentration (Kwon et al., 2019). As a consequence, it is possible that if dietary Leu is in excess of the requirement, extra dietary Trp may be needed to overcome the reduction in serotonin concentrations, but to our knowledge, no information about effects of extra Trp on growth performance and serotonin synthesis have been presented for pigs fed diets containing excess Leu. Therefore, the objective of this experiment was to test the hypothesis that increased dietary Trp is needed in high-Leu diets for growing pigs to prevent drops in both plasma serotonin and hypothalamic serotonin concentrations and to maintain growth performance of animals.

MATERIALS AND METHODS

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

Animals, Diets, and Experimental Design

A total of 144 growing pigs with an initial body weight of 28.2 ± 1.9 kg were divided into 2 blocks of 72 pigs and randomly assigned to 9 dietary treatments in a randomized complete block design. There were 2 pigs (1 barrow and 1 gilt) per pen and 4 replicate pens per block for a

total of 8 replicate pens per treatment. Pigs were the offspring of Line 359 boars and Camborough sows (Pig Improvement Company, Henderson, TN). A basal diet based on corn, soybean meal, wheat, and barley was formulated to contain 100% of the requirement for standardized ileal digestible (**SID**) Leu (NRC, 2012; Table 5.1). Two additional diets were formulated by adding crystalline L-Leu to the basal diet to increase the concentration of SID Leu to 200 or 300% of the requirement. These 3 diets were formulated to have a SID Trp:Lys ratio of 18%. Six additional diets were formulated by adding either 0.05% or 0.10% crystalline L-Trp to each of the 3 original diets. Thus, there was a total of 9 diets that were arranged in a 3 × 3 factorial design with 3 levels of Leu (100, 200, or 300% of the requirement) and 3 levels of SID Trp (18, 23, or 28% SID Trp:Lys).

All diets were formulated to be isoenergetic (3,300 kcal ME/kg) and to contain 1.00% SID Lys, which was assumed to be slightly above the SID Lys requirement for 25 to 50 kg pigs (NRC, 2012). Requirements for SID Leu and Trp for 25 to 50 kg pigs are estimated to be 0.99% and 0.17%, respectively (NRC, 2012). These values correspond to 1.01 SID Leu:Lys ratio and 0.17 SID Trp:Lys ratio. Therefore, diets containing 100% of the requirement for SID Leu and 18% SID Trp:Lys were believed to provide dietary Trp slightly above the requirement. Other indispensable AA were included in all diets in excess of the requirement (NRC, 2012). Glycine was included in all diets to maintain a constant concentration of dietary crude protein at 18% among all diets.

Pigs were housed in pens with concrete slats. Each pen (0.9 × 1.8 m) was equipped with a feeder and a nipple drinker, and pigs had free access to feed and water throughout the experiment. The individual body weight of pigs was recorded at the beginning and at the end of the 21-d experiment. Daily feed allotments were recorded, and the weight of feed left in the

feeders was recorded on the last day of the experiment to calculate feed consumption. The average daily gain (**ADG**), average daily feed intake (**ADFI**), and average gain to feed ratio were calculated for each pen of pigs and for each treatment group at the conclusion of the experiment.

Sample Collection

At the beginning and on d 11 of the experiment, 1 blood sample was collected from the jugular vein of 1 barrow in each pen using a heparinized vacutainer (BD, Franklin Lakes, NJ). On the last d of the experiment, 2 blood samples were collected in heparinized vacutainers and vacutainers containing EDTA (BD, Franklin Lakes, NJ) from the same pig in each pen that was used for bleeding at the beginning and on d 11 of the experiment. All samples were centrifuged at 1,500 × g at 4°C for 15 min to collect plasma and samples were then frozen at -80ºC until analyzed. After bleeding, one pig per pen was euthanized by electrocution and then exsanguinated. Brain tissue was removed, and the hypothalamus was isolated and frozen in liquid N and then stored at -80 ºC until analysis.

Sample Analyses

Samples of corn, soybean meal, 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). These samples were also analyzed for crude protein (method 984.13; AOAC Int., 2007) using a Kjeltec 8400 apparatus (FOSS Inc., Eden Prairie, MN). All diets and ingredients were also analyzed for dry matter (method 930.15; AOAC Int., 2007) and all diets were analyzed for ash (method 942.05; AOAC Int., 2007). The concentration of acid hydrolyzed ether extract (method AM 5-04; AOAC Int., 2007) in all diets was measured using the ANKOM HCl hydrolysis system and an ANKOM XT15 fat extractor (ANKOM Technologies, Macedon,

NY). All diets were also analyzed for gross energy using a bomb calorimeter (model 6400; Parr Instruments, Moline, IL). Plasma from blood in the heparinized tubes was analyzed for plasma urea N using a Beckman Coulter Clinical Chemistry AU analyzer (Beckman Coulter Inc., Brea, CA). The heparinized plasma samples were also analyzed for free AA using an Amino Acid Analyzer (model L 8900; Hitachi High Technologies America Inc., Pleasanton, CA) equipped with a high-performance cation exchange column (Agricultural Experiment Station Chemical Laboratories in University of MO).

Platelet-free plasma was prepared from anticoagulated blood in the tubes containing EDTA by double centrifugation according to the protocol described by Shen et al. (2012a). The supernatant was filtered with a 0.45um syringe filter to remove remaining platelets from the plasma. Concentrations of serotonin in the platelet-free plasma and in the hypothalamus were analyzed using ELISA kits developed for porcine according to the manufacturer's protocol (GenWay Biotech, Inc., San Diego, CA). 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.

Statistical Analyses

Normality of data was verified, and outliers were identified using the UNIVARIATE procedure of SAS (SAS Institute. Inc., Cary, NC). Data were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute. Inc., Cary, NC). The experimental unit was the pen and the model included dietary concentration of SID Leu, dietary concentration of SID Trp, and the interaction between SID Leu and SID Trp as fixed effects and block and replicate within

block as random effect. Assumptions of the model were tested using PROC GPLOT and influence options of SAS. Effects of dietary concentration of SID Leu, dietary concentration of SID Trp, and the interaction between SID Leu and SID Trp were considered significant at $P \leq 0.05$. If the interaction or one of the main effects was significant, the software NLREG version 6.5 (Sherrod, 2008) was used to determine parameter estimates for the second-order response surface model to increasing dietary concentrations of SID Leu and SID Trp as described by Khuri and Cornell (1996). Parameter estimates of the model that were not significant ($P > 0.10$), and were not included in a significant interaction, were removed from the model and the estimates were recalculated. The surface response full model was:

$$Y = a + b \times \text{SID Trp} + c \times \text{SID Trp}^2 + d \times \text{SID Leu} + e \times \text{SID Leu}^2 + f \times \text{SID Trp} \times \text{SID Leu} + g \times \text{SID Trp}^2 \times \text{SID Leu} + h \times \text{SID Trp} \times \text{SID Leu}^2 + i \times \text{SID Trp}^2 \times \text{SID Leu}^2,$$

where Y is the dependent variable, a is the intercept, b, c, d, e, f, g, h, and i are the coefficients, and SID Trp and SID Leu are the percentage concentrations of dietary SID Trp and SID Leu.

RESULTS

Crude protein and AA concentrations in corn, soybean meal, wheat, and barley were in agreement with reported values (NRC, 2012; Table 5.2) and analyzed values for crude protein, Leu, Trp, Lys, and other AA in experimental diets were also in agreement with calculated values (Table 5.3). All animals were healthy and readily consumed their assigned diets throughout the experimental period. The reduced models ($P < 0.05$) were used to predict ADG and ADFI (Table 5.4). However, the average gain to feed ratio could not be predicted from dietary SID Trp or SID Leu. For ADG and ADFI, the negative linear SID Trp and SID Leu terms, and the interaction

between SID Trp and SID Leu were included in the final model (Figures 5.1 and 5.2). An interaction ($P < 0.05$) between SID Leu and SID Trp was observed for ADG and ADFI, because increasing SID Trp increased ADG and ADFI more if SID Leu was at 300% of the requirement than at the requirement.

For prediction of serotonin in the hypothalamus, the reduced model ($P < 0.05$) was used (Table 5.5). An interaction ($P < 0.05$) between SID Trp and SID Leu was also observed because increasing SID Trp at high SID Leu concentrations increased hypothalamic serotonin less if Leu was at the requirement than at 300% of the requirement (Figure 5.3). However, plasma urea N and plasma serotonin could not be predicted from dietary SID Trp or SID Leu using the chosen model.

For prediction of free AA concentrations in plasma, reduced models ($P < 0.05$) were used (Table 5.6). For Ile, both negative linear SID Trp and SID Leu terms and the interaction between SID Trp and SID Leu were included in the model (Figure 5.4). Increasing SID Trp at the highest SID Leu level increased Ile concentration in plasma but increasing SID Trp at the lowest SID Leu levels decreased Ile concentration in plasma (interaction, $P < 0.05$). For Leu and Trp, positive linear SID Trp and SID Leu terms and the interaction between SID Trp and SID Leu were included in the final model (Figures 5.5 and 5.6). Plasma concentration of Leu was reduced by increasing dietary Trp, but the reductionwas greater if Leu was at 300% of the requirement than at the requirement (interaction, $P < 0.05$; Figure 5.5). Plasma concentration of Trp increased with increasing dietary Trp but the increase was greater if SID Leu was at the requirement than at 300% of the requirement (interaction, $P < 0.05$; Figure 5.6).

Increasing SID Trp at the highest SID Leu level increased Val concentration in plasma but increasing SID Trp at lower SID Leu levels decreased Val concentration in plasma

(interaction, $P < 0.05$; Figure 5.7). Increasing SID Trp if SID Leu was at the requirement increased Thr concentration in plasma, but increasing SID Trp if SID Leu was above the requirement decreased Thr concentration in plasma (interaction, $P < 0.05$; Figure 5.8).

DISCUSSION

Corn and corn co-products and sorghum and sorghum co-products have high Leu:crude protein ratio compared with other cereal grains and the Leu:Lys ratio in corn and sorghum protein is also greater than in most oilseed meals (NRC, 2012; Sotak et al., 2015). If corn- or sorghum-coproducts supply a large amount of protein in diets, concentrations of dietary Leu will, therefore, be elevated, which may result in an imbalanced supply of branched-chain AA (**BCAA**) to pigs (Rojo-Gomez, 2011; Yang et al., 2019). For example, if a corn-based diet with 30% conventional corn distillers dried grains with solubles (**DDGS**) is fed to growing pigs, dietary SID Leu will exceed the requirement by 50 to 100%. If greater inclusion of DDGS or if high-protein DDGS is used, the excess of dietary Leu may be even greater (Espinosa and Stein, 2018). It is, therefore, possible that practical diets under certain circumstances contain 200% Leu or more compared with the requirement.

Results of this experiment confirm that increased dietary Leu reduced ADG and ADFI, but the interactions between SID Trp and SID Leu in the model demonstrated that the negative effect of excess Leu may partially be ameliorated by increasing dietary Trp. This observation is in agreement with reported responses to excess dietary Leu (Gatnau et al., 1995; Wiltafsky et al., 2010; Wessels et al., 2016a). However, the observation that both ADG and ADFI were maximized at the lowest Leu concentration indicates that excess Trp cannot completely overcome the negative effects of excess Leu. Pigs fed diets containing excess Leu had lower

ADFI than pigs fed diets containing SID Leu at the requirement (NRC, 2012), but pig brain Trp concentrations were not influenced by dietary Leu (Wessels et al., 2016a). Thus, it is possible that Trp concentration in the brain is not the only reason for the reduced ADFI. Imbalanced supply of AA may induce metabolic losses of specific indispensable AA, resulting in AA deficiency (Jansman et al., 2019). Sensing AA deficiency by the anterior piriform cortex in the brain may be another reason for reduced feed intake if AA supply is inadequate, and this may be a protective mechanism to prevent protein breakdown in the brain (Hao et al., 2005).

Plasma urea N is considered a measure for changes in dietary AA balance and efficiency of AA utilization in pigs (Coma et al., 1995). Excess dietary Leu increased plasma urea N, which likely was a result of increased catabolism of Ile and Val, and therefore imbalances among other indispensable AA (Kwon et al., 2019). Supply of AA in excess of the requirement may result in increased urea synthesis, because the excess AA are catabolized (Eggum, 1970). However, the lack of responses for plasma urea N in this experiment indicates that adding additional dietary Leu or dietary Trp to the diet was not effective in improving the efficiency of protein synthesis.

The increased concentration of serotonin in the hypothalamus that was observed as dietary Trp increased confirms the importance of Trp as a precursor for serotonin. Availability of Trp in the brain is the rate-limiting factor for serotonin biosynthesis (Meunier-Salaün et al., 1991; Shen et al., 2012b). Results also demonstrated the negative effect of Leu on serotonin synthesis, which is likely because excess Leu reduces Trp uptake in the brain due to competition for the shared L-type AA transporter from blood to brain (Henry et al., 1992; Wessels et al., 2016a). A positive correlation between hypothalamic Trp and hypothalamic serotonin and a negative correlation between hypothalamic Trp and plasma Leu were reported (Wessels et al., 2016b). Reduced availability of Trp in plasma also decreased serotonin synthesis in the

hypothalamus, resulting in reduced voluntary feed intake in pigs (Henry et al., 1992). Therefore, the reduced feed intake caused by excess dietary Leu that was observed in the present study may be a result of decreased serotonin concentration in the brain, which was also demonstrated.

Platelets in blood and in the gastrointestinal tracts are the greatest pools of body serotonin in animals (Le Floc'h et al., 2017). Serotonin in blood is mostly synthesized by the enterochromaffin cells of the gastrointestinal tract (Watanabe et al., 2010) and approximately 95% of blood serotonin is stored in the platelets. Blood serotonin concentration did not affect intake of food in humans (Anderson et al., 1985), and dietary Trp did not affect plasma serotonin in pigs although serotonin in the hypothalamus was increased (Shen et al., 2012a;b). Thus, the observation that serotonin concentration in the brain was not related to serotonin concentration in blood, and that plasma serotonin was not affected by dietary Leu or Trp is in agreement with previous data.

The current results confirm that increased dietary Leu reduced availability of free Val and Ile in plasma, which is in agreement with previous data (Duan et al., 2016; Wessels et al., 2016a; Kwon et al., 2019). Leucine stimulates catabolism of BCAA in skeletal muscle and liver (Harper et al., 1984). If diets fed to pigs contain excess Leu, catabolism of all 3 BCAA may increase because of the stimulating effect of the Leu metabolite, α-keto isocaproate on BCAA catabolizing enzymes (Wiltafsky et al., 2010). Therefore, excess dietary Leu decreases the quantities of Val and Ile that are available for protein synthesis.

Because crystalline L-Leu or L-Trp were added to the basal diet that was believed to provide dietary Leu or Trp at the requirement, increased concentrations of free Leu or Trp in plasma was expected as additional Leu or Trp was added to diets. However, the interaction between SID Trp and SID Leu for both AA in plasma may be a result of improved ADG and

ADFI in pigs fed diets with additional Trp, indicating that inclusion of additional Trp is needed in diets with excess dietary Leu.

The reason the concentration of Thr in plasma was positively affected by addition of Leu to the diet may be that the Thr dehydratase pathway, which may be used in Thr metabolism, may be affected by BCAA catabolizing enzymes in rats (House et al., 2001) and it is possible that is also the case in pigs. The observation that addition of Trp to the diet containing 300% Leu increased concentrations of plasma Thr may be a result of the greater feed intake in pigs fed diets with greater Trp concentration. However, if pigs were fed diets containing Leu at the requirement or at 200% of the requirement, addition of Trp had a negative effect of plasma Thr. It is possible that the reason for this observation is that additional dietary Trp results in increased microbial synthesis of indoles in the intestinal tract, which in turn may initiate increased synthesis of mucin, which is rich in Thr. Inclusion of fiber in diets to pigs results in increased requirement for dietary Thr because of increased synthesis of mucin (Mathai et al., 2016), and it is, therefore, possible that Thr requirement is also increased if the concentration of indoles in the intestinal tract is increased. However, additional research is needed to test this hypothesis.

CONCLUSION

Increased dietary Leu reduced ADG and ADFI, but Trp supplementation partially overcame the negative effect of excess dietary Leu. Hypothalamic serotonin concentration was decreased as dietary Leu increased as a consequence of reduced uptake of Trp, but hypothalamic serotonin concentration was increased as dietary Trp increased. Changes in BCAA, Trp, and Thr concentrations in plasma were observed by addition of Leu or Trp to the diet, indicating that excess dietary Leu influenced the metabolism of several indispensable AA.

TABLES

Table 5.1. Ingredient composition of experimental diets, as-fed basis

SID[1] Leu relative to requirement[2], %:	100			200			300		
Item SID Trp:Lys , %:	18	23	28	18	23	28	18	23	28
Ground corn	27.91	27.91	27.91	27.91	27.91	27.91	27.91	27.91	27.91
Soybean meal, 46% crude protein	17.60	17.60	17.60	17.60	17.60	17.60	17.60	17.60	17.60
Ground wheat	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Ground barley	34.00	34.00	34.00	34.00	34.00	34.00	34.00	34.00	34.00
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Cornstarch	0.90	0.90	0.90	0.45	0.45	0.45	-	-	-
L-Lys·HCl	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
DL-Met	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-Thr	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
L-Trp	0.02	0.07	0.12	0.02	0.07	0.12	0.02	0.07	0.12
L-His	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
L-Leu	–	–	–	1.00	1.00	1.00	2.00	2.00	2.00

Table 5.1. (Cont.)

L-Val	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-Gly	1.20	1.15	1.10	0.65	0.60	0.55	0.10	0.05	0.00
Limestone	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Monocalcium phosphate	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin-mineral premix[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[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 D_3 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 B_{12}, 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 5.2. Analyzed nutrient composition of ingredients, as-fed basis

Item	Corn	SBM[1]	Wheat	Barley
CP[2], %	7.02	46.24	11.01	11.25
Dry matter, %	85.56	88.80	86.58	87.64
Indispensable AA, %				
Arg	0.32	3.28	0.52	0.55
His	0.21	1.19	0.24	0.24
Ile	0.26	2.15	0.39	0.38
Leu	0.78	3.51	0.69	0.72
Lys	0.26	2.91	0.36	0.48
Met	0.14	0.61	0.19	0.19
Phe	0.32	2.36	0.45	0.49
Thr	0.26	1.79	0.32	0.38
Trp	0.06	0.63	0.13	0.12
Val	0.33	2.20	0.46	0.53
Dispensable AA, %				
Ala	0.50	1.97	0.41	0.47
Asp	0.46	5.04	0.58	0.72
Cys	0.16	0.62	0.26	0.24
Glu	1.21	8.08	2.70	2.19
Gly	0.29	1.92	0.45	0.45
Pro	0.62	2.28	0.90	0.93
Ser				

Table 5.2. (Cont.)

Tyr	0.19	1.71	0.27	0.27
BCAA[3]:CP ratio, %				
Ile:CP	3.70	4.65	3.54	3.38
Leu:CP	11.11	7.59	6.27	6.40
Val:CP	4.70	4.76	4.18	4.71

[1]SBM = soybean meal.

[2]CP = crude protein.

[3]BCAA = branched-chain amino acids.

Table 5.3. Analyzed nutrient composition of experimental diets, as-fed basis

SID[1] Leu relative to requirement[2], %:	100			200			300		
Item SID Trp:Lys , %:	18	23	28	18	23	28	18	23	28
Gross energy, kcal/kg	4,000	4,011	4,006	4,055	4,051	4,052	4,082	4,074	4,073
Crude protein, %	16.46	16.63	16.40	16.63	16.61	16.70	16.62	16.58	16.58
Dry matter, %	88.68	88.73	88.55	88.59	88.78	88.70	88.56	88.56	88.51
Ash, %	4.65	4.56	4.49	4.37	4.46	4.32	4.37	4.65	4.43
Acid hydrolyzed ether extract, %	4.43	4.46	4.56	4.64	4.55	4.61	4.49	4.58	4.43
Indispensable AA, %									
Arg	0.95	0.91	0.89	0.95	0.92	0.93	0.84	0.88	0.87
His	0.42	0.41	0.40	0.42	0.41	0.42	0.39	0.40	0.39
Ile	0.68	0.66	0.63	0.67	0.65	0.66	0.60	0.63	0.61
Leu	1.21	1.19	1.13	2.16	2.14	2.21	3.27	3.05	3.20
Lys	1.12	1.09	1.12	1.12	1.09	1.12	1.07	1.08	1.08
Met	0.31	0.29	0.29	0.31	0.28	0.29	0.26	0.31	0.27
Phe	0.77	0.75	0.72	0.77	0.74	0.76	0.69	0.73	0.72

Table 5.3. (Cont.)

Thr	0.65	0.69	0.69	0.70	0.69	0.69	0.65	0.69	0.66
Trp	0.18	0.24	0.28	0.19	0.23	0.28	0.20	0.23	0.27
Val	0.84	0.84	0.82	0.86	0.83	0.84	0.79	0.81	0.80
Dispensable AA, %									
Ala	0.73	0.71	0.68	0.72	0.69	0.71	0.66	0.69	0.68
Asp	1.37	1.32	1.31	1.37	1.32	1.35	1.22	1.28	1.25
Cys	0.29	0.27	0.26	0.28	0.27	0.27	0.25	0.26	0.24
Glu	2.99	2.85	2.75	2.94	2.82	2.90	2.67	2.78	2.73
Gly	1.66	1.73	1.89	1.29	1.22	1.24	0.71	0.70	0.61
Pro	1.12	1.05	0.99	1.10	1.02	1.00	1.00	1.02	1.04
Tyr	0.49	0.46	0.46	0.49	0.48	0.48	0.44	0.46	0.47

[1]SID = standardized ileal digestible.

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

Table 5.4. Least squares means for growth performance of growing pigs fed diets with varying ratios between dietary standardized ieal digestible (SID) Leu and SID Trp, as-fed basis

SID Leu relative to requirement[1], %:	100			200			300			SEM
SID Trp:Lys , %:	18	23	28	18	23	28	18	23	28	
No. of pens	8	8	8	8	8	8	8	8	8	
Body weight, kg										
Day 1	28.8	28.9	29.1	28.9	28.8	29.0	28.8	28.9	29.0	0.9
Day 21	47.0	47.8	47.0	46.6	47.0	48.0	44.5	46.0	45.3	1.9
ADG, g/d[2]	867	898	852	845	869	905	750	815	777	61
ADFI, g/d[3]	1,675	1,724	1,630	1,657	1,656	1,720	1,519	1,584	1,506	95
Gain to feed ratio[4]	0.52	0.52	0.52	0.51	0.52	0.53	0.49	0.51	0.51	0.02

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

[2]Results indicated that ADG from d 0 to d 21 at different combinations of SID Trp and SID Leu could be described by the following model: 975.196 – 1.792 × SID Trp – 0.944 × SID Leu + 0.021 × SID Trp × SID Leu ($P < 0.05$).

[3]Results indicated that ADFI from d 0 to d 21 at different combinations of SID Trp and SID Leu could be described by the following model: 1839.196 – 0.299 × SID Trp – 1.062 × SID Leu + 0.016 × SID Trp × SID Leu ($P < 0.05$).

[4]Results indicated that average gain to feed ratio could not be predicted from dietary SID Trp or SID Leu.

Table 5.5. Least squares means for concentrations of plasma urea N (PUN), plasma serotonin, and hypothalamic serotonin of growing pigs fed diets with varying ratios between dietary standardized ileal digestible (SID) Leu and SID Trp, as-fed basis

SID Leu relative to requirement[1], %:	100			200			300			SEM
SID Trp:Lys , %:	18	23	28	18	23	28	18	23	28	
No. of pens	8	8	8	8	8	8	8	8	8	
PUN, μg/mL[2]										
Day 1	7.5	7.3	7.8	7.6	7.4	7.6	6.9	7.3	7.3	0.6
Day 11	7.5	7.5	7.4	8.4	7.5	8.0	7.8	8.8	7.6	0.7
Day 21	8.8	7.4	7.9	8.6	7.9	8.3	7.6	8.9	8.1	1.0
Serotonin										
Hypothalamus, ng/mg[3]	0.139	0.138	0.142	0.128	0.132	0.136	0.122	0.126	0.125	0.006
Plasma, ng/mL[4]	62.8	40.1	68.8	57.2	53.6	41.1	67.8	59.7	39.7	19.1

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

[2]Results indicated that PUN could not be predicted from dietary SID Trp or SID Leu.

[3]Results indicated that serotonin concentration in hypothalamus at different combinations of SID Trp and SID Leu could be described by the following model: 0.135 + 0.001 × SID Trp – 0.00007 × SID Leu – 0.0000002 × SID Trp × SID Leu ($P < 0.05$).

[4]Results indicated that serotonin concentration in plasma could not be predicted from dietary SID Trp or SID Leu.

Table 5.6. Least squares means for concentrations of amino acids (AA) in plasma of growing pigs fed diets with varying ratios between dietary standardized ileal digestible (SID) Leu and SID Trp, as-fed basis

SID Leu relative to requirement[1], %:	100			200			300			SEM
SID Trp:Lys , %:	18	23	28	18	23	28	18	23	28	
Indispensable AA, μg/mL[2]										
Arg	24.8	21.4	23.9	22.9	20.5	23.0	23.7	23.6	19.4	2.5
His	8.4	6.8	7.1	7.9	7.7	9.6	8.0	8.3	7.1	0.8
Ile[3]	16.1	14.3	14.8	6.4	7.4	6.4	5.7	5.4	5.9	0.8
Leu[4]	22.2	19.1	19.1	34.8	32.9	32.9	44.9	46.5	37.2	4.2
Lys	22.1	22.1	23.2	25.1	27.5	26.9	27.1	26.5	23.1	3.4
Met	7.0	6.0	6.6	6.4	5.9	6.6	7.0	6.6	6.1	0.8
Phe	12.3	10.2	10.9	10.8	11.5	11.7	12.3	11.9	12.3	0.7
Thr[5]	22.3	19.2	18.2	20.2	17.6	19.6	24.6	26.7	25.0	2.5
Trp[6]	7.3	8.6	10.6	7.9	9.3	10.8	8.3	9.9	9.4	0.9
Val[7]	43.0	41.6	42.5	16.1	17.3	16.3	13.4	13.6	13.6	1.6
Dispensable AA, μg/mL[8]										

Table 5.6. (Cont.)

Ala	41.3	43.1	40.9	38.5	45.9	45.5	45.4	46.9	54.5	4.3
Asp	3.5	3.6	3.3	3.5	3.8	3.7	3.6	4.0	3.8	0.3
Cys	0.3	ND	0.3	ND	0.6	0.1	0.5	0.4	0.2	0.2
Glu	38.1	40.1	37.4	37.6	41.5	43.9	41.6	39.3	44.7	3.0
Gly	131.7	164.4	137.8	124.2	137.1	115.0	139.4	140.7	145.7	16.5
Pro	29.1	31.3	31.6	29.0	30.4	30.8	31.5	32.1	38.3	2.4
Ser	16.6	20.2	17.6	16.1	19.3	16.0	18.4	17.9	20.8	1.8
Tyr	11.9	13.0	12.4	11.9	13.4	12.9	13.8	12.0	14.6	1.0

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

[2]Results indicated that concentrations of indispensable AA except Ile, Leu, Thr, Trp, and Val in plasma could not be predicted from dietary SID Trp or SID Leu.

[3]Results indicated that Ile concentration in plasma at different combinations of SID Trp and SID Leu could be described by the following model: 22.702 – 0.181 × SID Trp – 0.064 × SID Leu + 0.001 × SID Trp × SID Leu ($P < 0.05$).

[4]Results indicated that Leu concentration in plasma at different combinations of SID Trp and SID Leu could be described by the following model: 8.628 + 0.034 × SID Trp + 0.166 × SID Leu – 0.002 × SID Trp × SID Leu ($P < 0.05$).

[5]Results indicated that Thr concentration in plasma at different combinations of SID Trp and SID Leu could be described by

Table 5.6. (Cont.)

the following model: 29.408 – 0.586 × SID Trp – 0.024 × SID Leu + 0.002 × SID Trp × SID Leu ($P < 0.05$).

[6]Results indicated that Trp concentration in plasma at different combinations of SID Trp and SID Leu could be described by the following model: – 2.014 + 0.467 × SID Trp + 0.028 × SID Leu – 0.001 × SID Trp × SID Leu ($P < 0.05$).

[7]Results indicated that Val concentration in plasma at different combinations of SID Trp and SID Leu could be described by the following model: 54.997 – 0.087 × SID Trp – 0.153 × SID Leu + 0.001 × SID Trp × SID Leu ($P < 0.05$).

[8]Results indicated that concentrations of dispensable AA in plasma could not be predicted from dietary SID Trp or SID Leu.

FIGURES

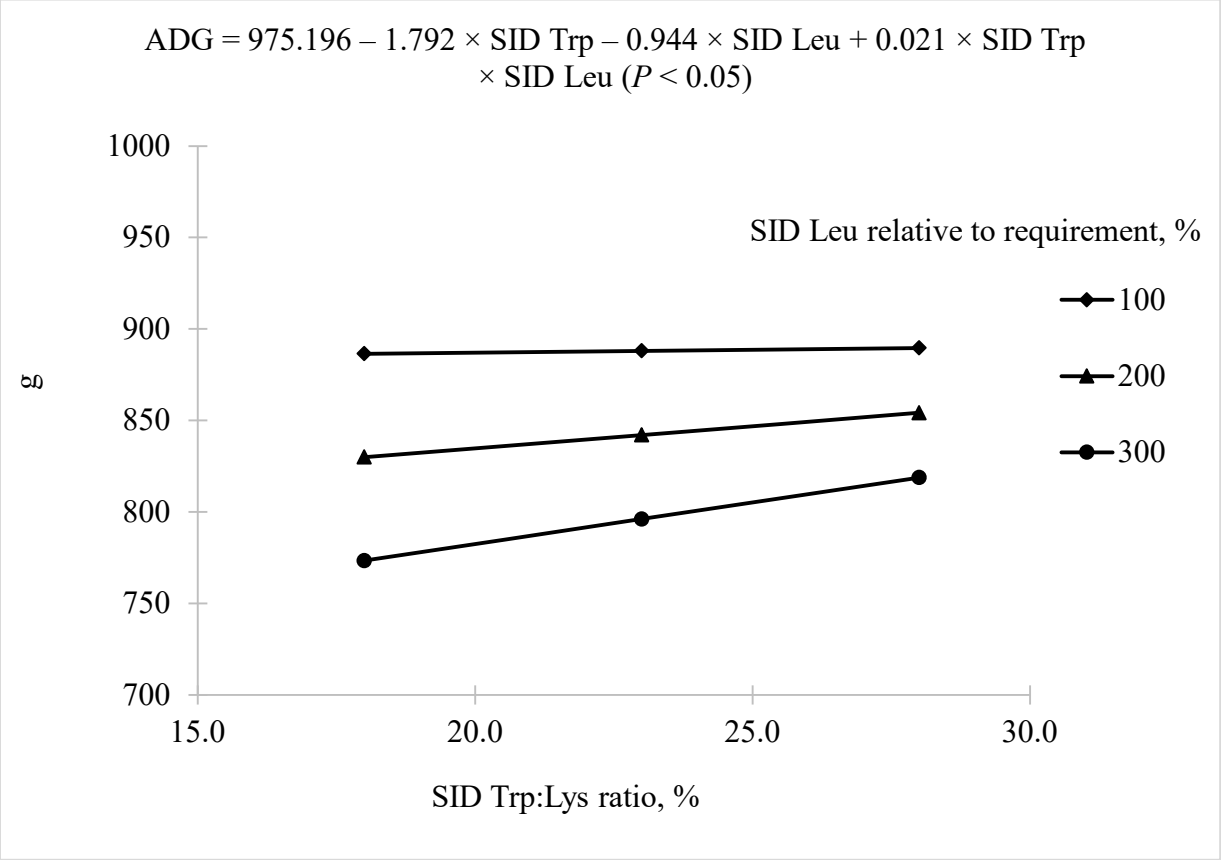

Figure 5.1. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and Leu ($P < 0.05$), for average daily gain (ADG) in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

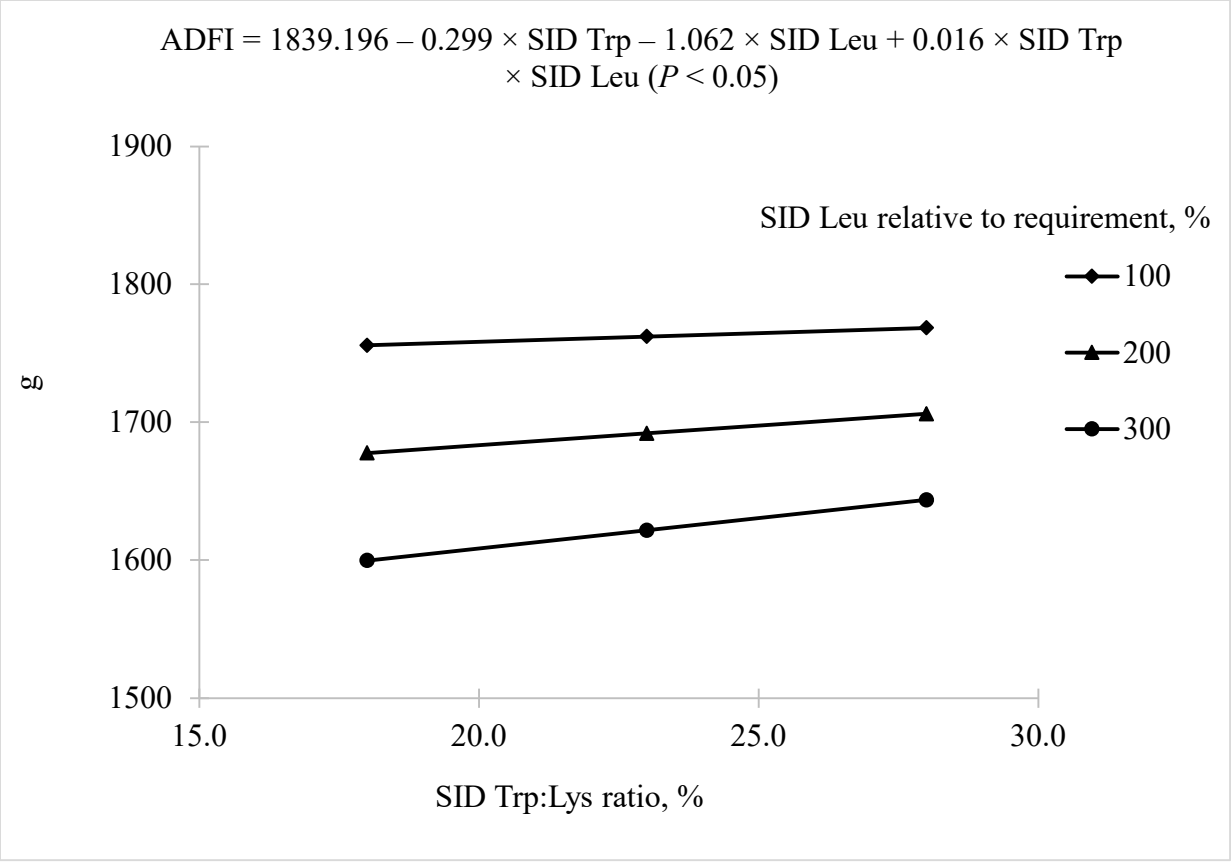

Figure 5.2. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and SID Leu ($P < 0.05$), for average daily feed intake (ADFI) in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

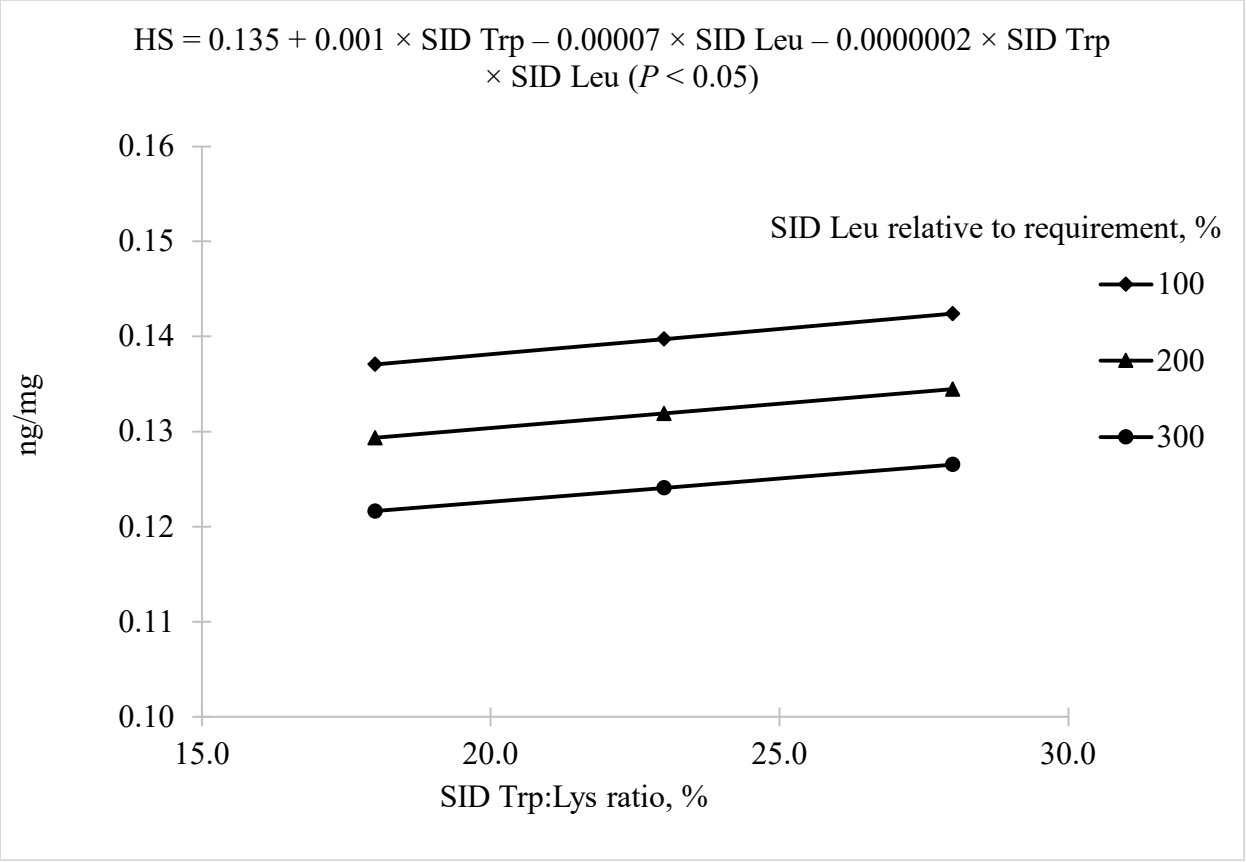

Figure 5.3. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and SID Leu ($P < 0.05$), for hypothalamic serotonin (HS) concentrations in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

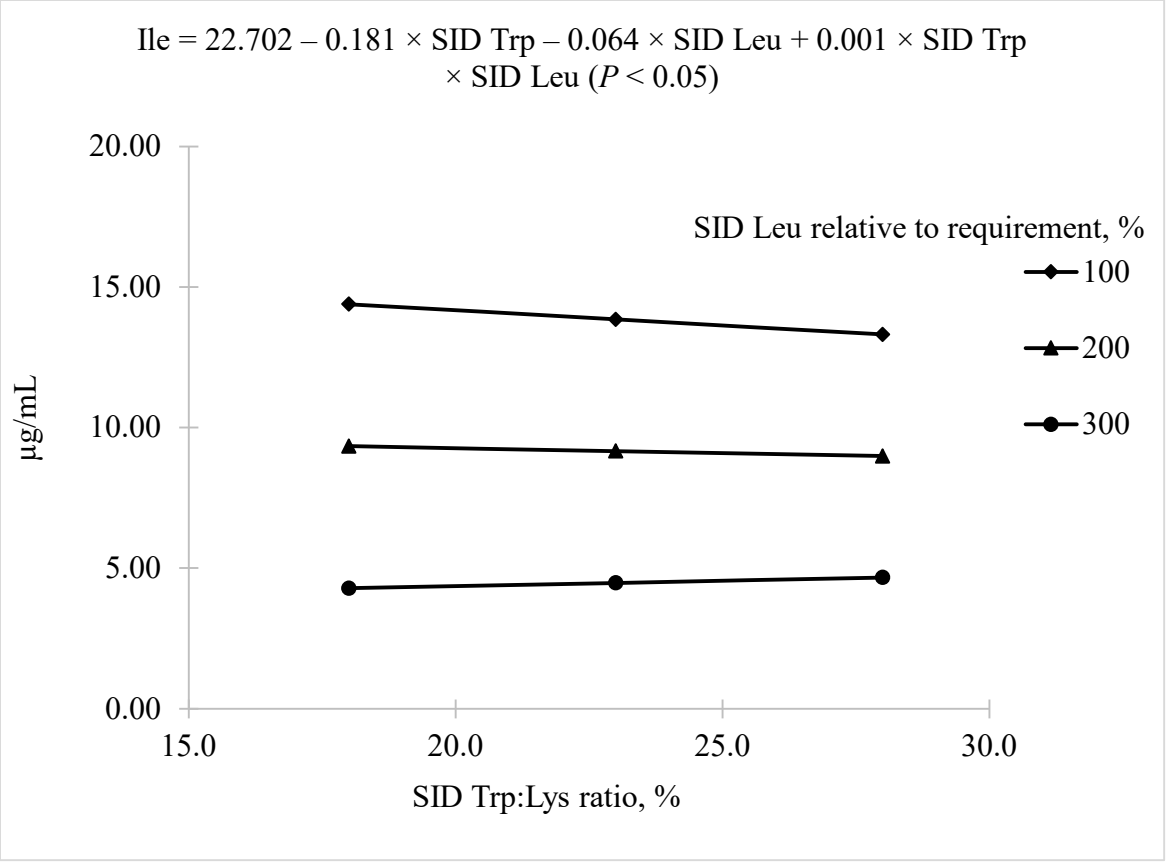

Figure 5.4. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and SID Leu ($P < 0.05$), for plasma Ile concentration in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

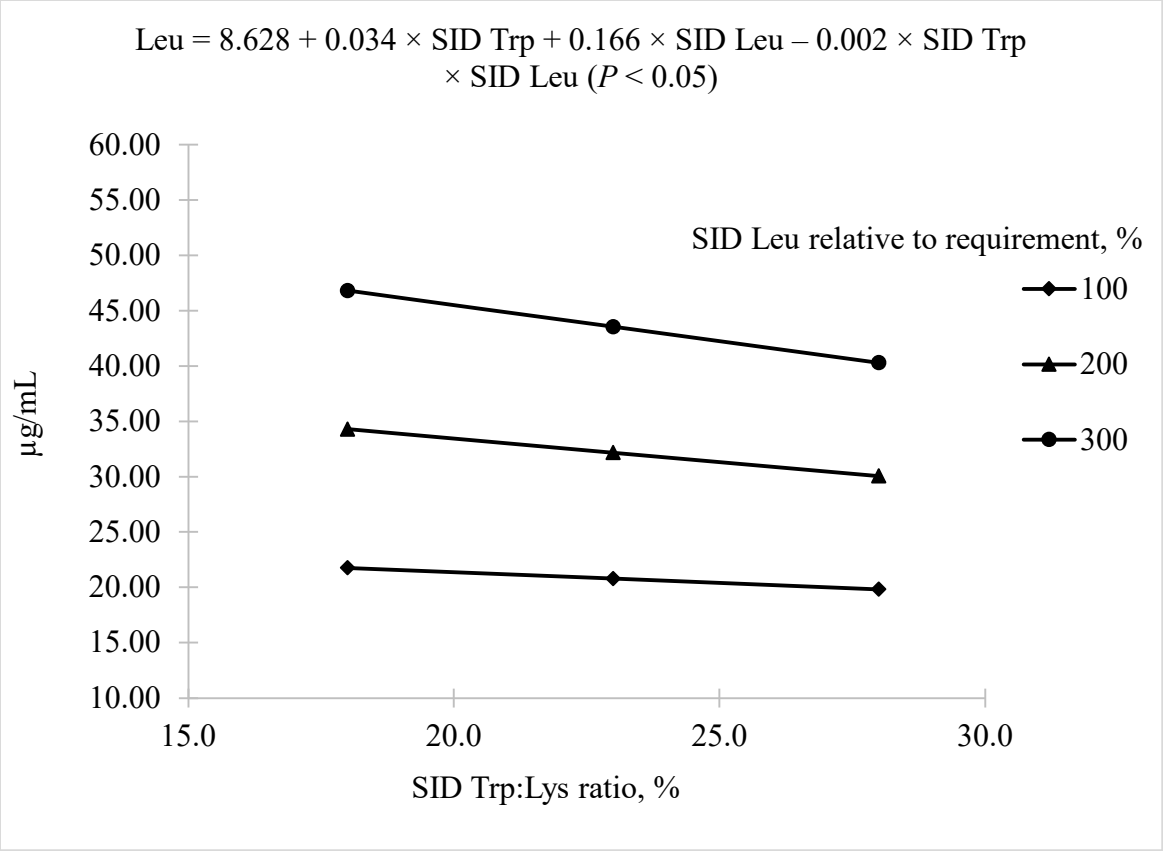

Figure 5.5. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and SID Leu ($P < 0.05$), for plasma Leu concentration in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

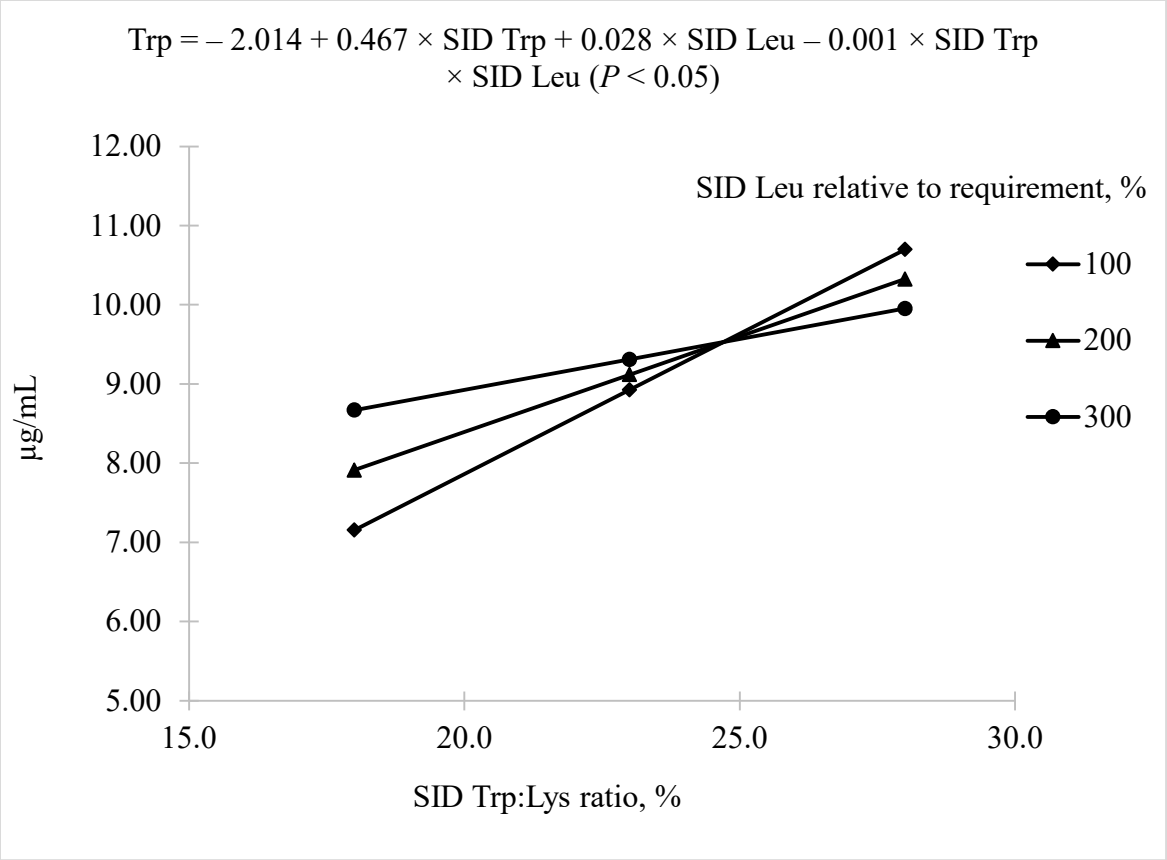

Figure 5.6. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and SID Leu ($P < 0.05$), for plasma Trp concentration in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

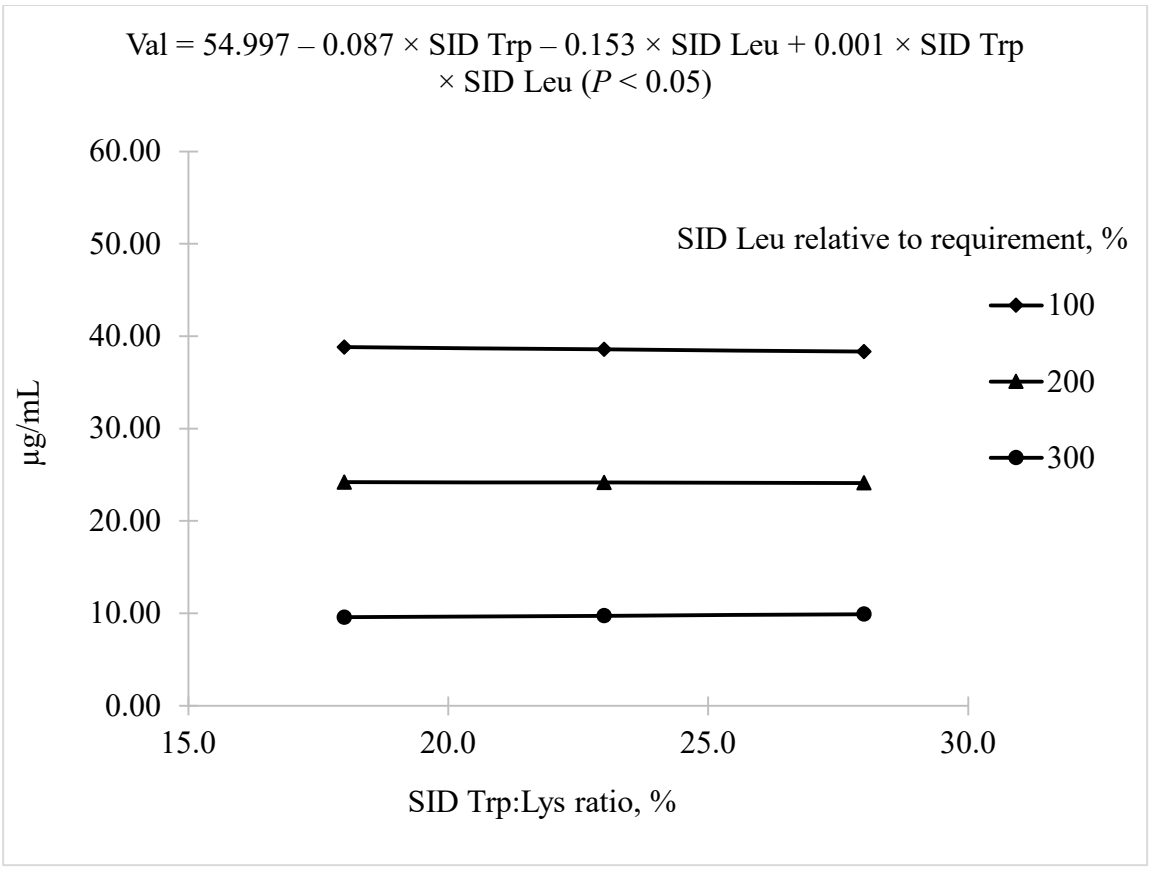

Figure 5.7. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and SID Leu ($P < 0.05$), for plasma Val concentration in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

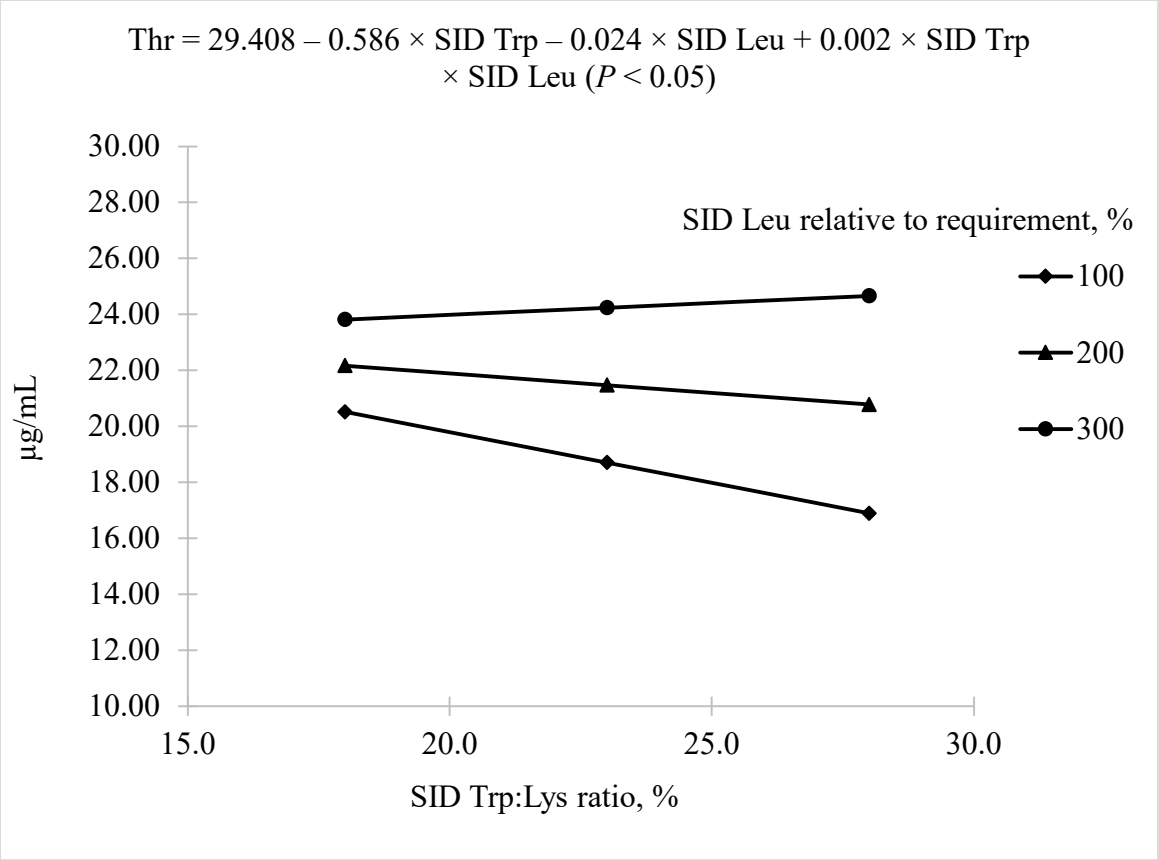

Figure 5.8. Predicted values, based on the interaction between standardized ieal digestible (SID) Trp and SID Leu ($P < 0.05$), for plasma Thr concentration in growing pigs fed diets containing from 18 to 28% SID Trp:Lys and from 100 to 300% SID Leu relative to the requirement (NRC, 2012).

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CHAPTER 6: EFFECTS OF DIETARY VALINE, ISOLEUCINE, AND TRYPTOPHAN SUPPLEMENTATIONS TO DIETS CONTAINING EXCESS LEUCINE FROM CORN PROTEIN ON NITROGEN BALANCE AND GROWTH PERFORMANCE OF GROWING PIGS

ABSTRACT

Two experiments were conducted to test the hypothesis that increasing concentrations of dietary Val, Ile, or Trp in diets containing excess Leu from corn protein will alleviate negative effects of excess dietary Leu on N balance, plasma urea N, and growth performance of growing pigs. In experiment 1, 72 barrows (initial body weight: 33.9 ± 2.6 kg) were housed in metabolism crates and randomly allotted to 8 diets and 3 blocks with 3 pigs per diet in each block. Two levels of crystalline L-Ile (0 or 0.1%), 2 levels of crystalline L-Val (0 or 0.1%), and 2 levels of crystalline L-Trp (0 or 0.05%) were added to the basal diet based on corn and high-protein corn product (**HPCP**) for a total of 8 diets that were used in a 2 × 2 × 2 factorial arrangement of treatments. Results indicated that fecal N output increased if Ile was added to diets without added Val, but that was not the case if Val was added (interaction, $P < 0.05$). Addition of Ile to diets reduced N retention, but N retention increased with Trp addition to diets without Val addition, but not if Trp was added to diets with added Val (interaction, $P < 0.05$). The biological value of protein increased if Trp was added to diets without addition of Ile, but if Ile was added, Trp addition did not increase the biological value of protein (interaction, $P < 0.05$). In Experiment 2, A total of 288 growing pigs (initial body weight: 28.6 ± 2.5 kg) were divided into 3 blocks of pigs and randomly assigned to 9 dietary treatments in a randomized complete block design. There were 2 barrows and 2 gilts per pen and 8 replicate pens per treatment. A control diet based on corn and

soybean meal and the 8 diets used in experiment 1 were fed to pigs for 28 d. Results indicated that final body weight and average daily gain of pigs fed the corn-soybean meal control diet were not different from those of pigs fed Val and Trp addition to excess-Leu basal diet, but were greater ($P < 0.001$) than those of pigs fed Val addition, Ile addition, Trp addition, Val and Ile addition, Ile and Trp addition, and Val, Ile, and Trp addition to excess-Leu basal diet. In conclusion, adding Ile alone reduced N retention, but adding Trp alone or in combination with Ile or Val increased N retention. The combination of Val and Trp supplementation may be benefical for preventing detrimental effects of excess Leu on growth performance of pigs.

Key words: branched-chain amino acids, growth performance, nitrogen balance, pigs, serotonin, tryptophan

INTRODUCTION

Leucine is a key regulator that stimulates catabolism of branched-chain amino acids (**BCAA**) in the liver (Harper et al., 1984). When diets for pigs contain excess Leu, degradation of all 3 BCAA may increase by stimulating effects of Leu or its metabolite on BCAA degrading enzymes (Wiltafsky et al., 2010). Excess dietary Leu may reduce pig feed intake and growth performance (Gatnau et al., 1995; Wiltafsky et al., 2010), which may be a consequence of the imbalanced supply of BCAA that result from increased degradation of Val and Ile. Excess dietary Leu also reduces protein synthesis, which is likely a result of reduced availability of Val and Ile (Kwon et al., 2019b).

Excess dietary Leu may also reduce synthesis of serotonin in the brain (Wessels et al., 2016) because excess Leu may prevent Trp, which is the precursor for serotonin, from being transported from blood to brain, and therefore reduce the availability of Trp for serotonin

synthesis in the brain. Serotonin is a cerebral neurotransmitter that plays an important role in feed intake regulation (Zhang et al., 2007). Therefore, if dietary Leu is in excess of the requirement, extra dietary Trp may be needed to overcome the reduction in serotonin synthesis and alleviate reduced feed intake of pigs. Indeed, increasing dietary Trp has positive effects on average daily gain and average daily feed intake, but the positive effect of increased Trp was greater if pigs were fed diets with excess Leu (Kwon et al., 2019a). Increasing concentrations of dietary Val and Ile alone or in combination also have the potential to alleviate negative effects of excess dietary Leu on growth performance of pigs (Cemin et al., 2019).

Diets based on corn and corn co-products and sorghum and sorghum co-products are rich in Leu (Sotak et al., 2015). Therefore, if diets are formulated based on high protein corn co-products, dietary Leu often exceeds the requirement of pigs (NRC, 2012). In many studies conducted to evaluate the effect of excess dietary Leu, crystalline L-Leu was the source of dietary Leu (Gatnau et al., 1995; Wiltafsky et al., 2010; Wessels et al., 2016). However, to our knowledge, no information about interactive effects between dietary BCAA and Trp on N balance and growth performance have been presented for pigs fed diets containing excess Leu that was supplied by corn protein.

Therefore, the objective of this research was to test the hypothesis that increasing concentrations of dietary Val, Ile, or Trp in diets containing excess Leu from corn protein will alleviate negative effects of excess dietary Leu supplied by corn protein on N balance, plasma urea N (**PUN**), and growth performance of growing pigs.

MATERIALS AND METHODS

The protocol for 2 experiments were reviewed and approved by the Institutional Animal

Care and Use Committee at the University of Illinois. Growing pigs that were the offspring of Line 359 boars and Camborough sows (Pig Improvement Company, Henderson, TN) were used in the experiments. A locally grown hybrid of yellow dent corn and soybean meal were obtained from the University of Illinois Feed Mill (Champaign, IL). A high-protein corn product (**HPCP**), which contained 49.6% crude protein, 1.77% Lys, and 5.88% Leu, was also obtained (NexPro; Flint Hills Resources, Wichita, KS; Table 6.1). The same batches of these ingredients were used in both experiments.

Experiment 1: Nitrogen Balance

Seventy-two growing pigs (initial body weight: 33.9 ± 2.6 kg) were assigned to 8 dietary treatments with 9 replicate pigs per treatment in a randomized complete block design. There were 3 blocks of 24 pigs with 3 pigs per diet in each block and diets were fed for 12 d. Corn and HPCP were the sources of Leu in the diets. A basal diet based on corn and 26% HPCP was formulated to contain 171% of the requirement for standardized ileal digestible (**SID**) Leu (NRC, 2012; Table 6.2). Two levels of crystalline L-Ile (0 or 0.1%), 2 levels of crystalline L-Val (0 or 0.1%), and 2 levels of crystalline L-Trp (0 or 0.05%) were added to the basal diet for a total of 8 diets that were used in a 2 × 2 × 2 factorial arrangement of treatments.

The basal diet had SID Ile:Lys, SID Val:Lys, and SID Trp:Lys ratios of 0.53:1, 0.70:1, and 0.18:1. However, addition of Ile, Val, or Trp to the basal diet resulted in diets with SID Ile:Lys, SID Val:Lys, and SID Trp:Lys ratios of 0.63:1, 0.80:1, and 0.23:1, respectively.

All diets were formulated to be isoenergetic (3,350 kcal metabolizable energy/kg) and to contain 1.00% SID Lys, which was assumed to be slightly above the SID Lys requirement for 25 to 50 kg pigs (NRC, 2012). Other indispensable amino acids (**AA**), except the 3 BCAA and Trp,

were included in all diets in excess of requirements (NRC, 2012). Glycine was included in all diets to maintain a constant concentration of dietary crude protein at 18.5%.

Pigs were individually housed in metabolism crates that were equipped with a feeder and a nipple drinker. Throughout the 12-d study, pigs were fed at 2.8 times the energy requirement for maintenance (i.e., 197 kcal/kg × body weight$^{0.60}$; NRC, 2012), which was provided each day in 2 equal meals at 0800 and 1600 h. The daily consumption of feed was recorded and water was available at all times.

The initial 5 d was considered an adaptation period to the experimental diets. Urine and fecal samples were collected from the feed provided during the following 5 d according to standard procedures for the marker to marker method (Adeola, 2001). Urine was collected in buckets with 50 mL of HCl as a preservative. Fecal samples and 10% 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.

In the morning of d 13 of each period, pigs were fed 400 g of their experimental diet and 2.5 h later, a blood sample was collected from each pig. Blood samples were collected from the jugular vein of all pigs using heparinized vacutainers (BD, Franklin Lakes, NJ). All samples were centrifuged at 1,500 × g at 4°C for 15 min and plasma was collected and stored at -80ºC until analyzed for PUN.

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 biological value of protein in the diets was calculated by expressing the retention of N as a percentage of the difference between N intake and N output in feces (Mitchell, 1924).

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) as a 2 × 2 × 2 factorial arrangement of treatments. The experimental unit was the pig and the model included SID Ile:Lys ratio, SID Val:Lys ratio, SID Trp:Lys ratio, and all possible interactions as fixed variables and block and replicate within block as random variables. Treatment means were calculated using the LSMEANS statement and if significant, means were separated using the PDIFF statement in SAS. Statistical significance and tendency were considered as $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

Experiment 2: Growth Performance

A total of 288 growing pigs with an initial body weight of 28.6 ± 2.5 kg were divided into 2 blocks of 72 pigs and 1 block of 144 pigs and randomly assigned to 9 dietary treatments in a randomized complete block design. There were 2 barrows and 2 gilts per pen and 8 replicate pens per treatment. A control diet based on corn and soybean meal was formulated and a basal diet was also formulated based on corn and the same source of HPCP as used in experiment 1. The requirement for SID Leu for 25 to 50 kg pigs is 0.99% (NRC, 2012). However, by adding 26% HPCP to the diet, the basal diet contained 171% of the requirement for SID Leu whereas the control diet only contained 139% SID Leu relative to the requirement (Table 6.2). A total of 8 diets containing the HPCP were formulated as explained for experiment 1 by adding 2 levels of crystalline L-Ile (0 or 0.1%), 2 levels of crystalline L-Val (0 or 0.1%), and 2 levels of crystalline L-Trp (0 or 0.05%) to the basal diet.

Pigs were housed in pens with floors consisting of concrete and concrete slats. Each pen was equipped with a feeder and a nipple drinker, and pigs had free access to feed and water throughout the experiment. Individual pig weights were recorded at the beginning and at the end

of the 28-d experiment. Daily feed allotments were recorded and the weight of feed left in the feeders was recorded on the last day of the experiment to calculate feed consumption.

At the beginning and on d 14 of the experiment, blood samples were collected from the jugular vein of the barrow in each pen that had a body weight that was closest to the pen average at the start of the experiment using heparinized vacutainers (BD, Franklin Lakes, NJ). At the end of the experiment, 2 blood samples were collected in heparinized vacutainers and vacutainers containing EDTA (BD, Franklin Lakes, NJ) from the same barrow in each pen that was used for bleeding at the beginning and on d 14 of the experiment. Blood samples were centrifuged at 1,500 × g at 4 °C for 15 min and plasma was collected and stored at -80 ºC until analysis. All pigs from which blood had been collected were euthanized by electrocution after blood had been collected and then exsanguinated. Brain tissue was removed, and the hypothalamus was isolated and collected into 2-mL cryogenic tubes and snap-frozen in liquid N. All hypothalamus samples were stored at -80 °C until analysis.

Data were summarized to calculate average daily gain, average daily feed intake, and gain to feed ratio for each pen of pigs and for each treatment group at the conclusion of the experiment. 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) with the experimental unit being the pen. The model included diet as fixed effect and block and replicate within block as random effects. Treatment means were calculated using the LSMEANS statement. Statistical significance and tendency were considered as $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

Sample Analyses

Samples of corn, soybean meal, and the HPCP, which were the main ingredients in the diets, and all experimental diets were analyzed for crude protein by the Kjeldahl procedure (method 984.13; AOAC Int., 2007) using a Kjeltec 8400 apparatus (FOSS Inc., Eden Prairie, MN), and 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). Gross energy was also measured using a bomb calorimeter (model 6400; Parr Instruments, Moline, IL). Ingredient samples were also analyzed for dry matter (Method 930.15; AOAC Int, 2007), ash (Method 942.05; AOAC Int, 2007), acid hydrolyzed ether extract (Method AM 5-04; AOAC Int, 2007), and total dietary fiber (Method 991.43; AOAC Int, 2007) using standard procedures as described by Navarro et al. (2018).

The frozen fecal samples were dried in a forced-air drying oven at 55°C until constant weight and ground for analysis. Fecal samples and thawed urine samples were analyzed for crude protein as explained for diets and ingredients. Plasma from blood in heparinized tubes was analyzed for PUN using a Beckman Coulter Clinical Chemistry AU analyzer (Beckman Coulter Inc., Brea, CA). Concentrations of BCAA and branched-chain α-keto acids in plasma from blood collected in EDTA tubes were measured 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 Beals et al. (2016). Concentration of serotonin in the hypothalamus was analyzed using ELISA kits developed for porcine tissues according to the manufacturer's protocol (GenWay Biotech, Inc., San Diego, CA). To obtain homogenates from the hypothalamus, frozen samples were weighed (0.5 g) and homogenized with buffer solution on ice using a handheld 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.

RESULTS

Experiment 1: Nitrogen Balance

During the adaptation period, one pig died and this pig was excluded from analysis. All other animals remained healthy and easily consumed their diets without apparent problems. Crude protein and AA concentrations in corn and the HPCP were in agreement with expected values. Analyzed values for Lys and BCAA in all diets were in agreement with formulated values (Table 6.3).

There were no 3-way interactions among main effects and feed intake and N intake were not different among treatments (Table 6.4). Fecal N output increased if Ile was added to diets without added Val, but that was not the case if Val was added (interaction, $P < 0.05$). Urine N excretion (g/5d) tended to be reduced if Trp was added to diets with no added Ile, but if Ile was added, Trp did not reduce N output in urine (interaction $P < 0.10$). Apparent total tract digestibility of N tended ($P < 0.10$) to increase with added Val in diets. Addition of Ile to diets reduced N retention, and N retention increased with Trp addition to diets without Val addition, but not in diets with added Val (interaction, $P < 0.05$). The biological value of protein increased if Trp was added to diets without addition of Ile, but if Ile was added, Trp addition did not increase the biological value of protein (interaction, $P < 0.05$).

Experiment 2: Growth Performance

All animals were healthy and readily consumed their assigned diets throughout the experimental period. Final body weight and average daily gain of pigs fed the corn-soybean meal

control diet were not different from those of pigs fed the diet with addition of Val and Trp, but greater ($P < 0.001$) than of pigs fed all other diets (Table 6.5).

There was no difference among dietary treatments for PUN on day 1 and day 14, but on d 28, PUN of pigs fed the control diet greater ($P < 0.05$) than that of pigs all other diets except the diet supplemented with Trp, but not Val or Ile (Table 6.6). Pigs fed the basal diet had lower ($P < 0.001$) concentration of plasma free Trp compared with pigs fed all other diets except the diets with added Val, Ile, or Val and Ile.

Concentration in plasma of α-keto isovalerate (**KIV**) was greater ($P < 0.001$) in pigs fed the control diet than in pigs fed the basal diet or diets with added Ile, Trp, or Ile and Trp. Concentration in plasma of α-keto β-methylvalerate (**KMV**) was greater ($P < 0.001$) in pigs fed the control diet than in pigs fed all other diets. Plasma free Val in pigs fed the control diets was not different from that of pigs fed diets with added Val, Val and Ile, Val and Trp, or Val, Ile, and Trp, but greater ($P < 0.001$) than that of pigs fed the other diets.

DISCUSSION

The objective of this research was to test the hypothesis that increasing concentrations of dietary Val, Ile, or Trp in diets containing excess Leu from HPCP may mitigate negative effects of excess dietary Leu on efficiency of AA utilization and growth performance of growing pigs. Therefore, the basal diet was formulated to contain dietary Leu well above the SID Leu requirement (NRC, 2012). In addition, a SID Val:Lys ratio of 0.70:1, a SID Ile:Lys ratio of 0.53:1, and a SID Trp:Lys ratio of 0.18:1 were used in the formulation of the basal diet which were believed to be the optimal ratios of SID Val:Lys, SID Ile:Lys, and SID Trp:Lys to maximize growth performance of pigs (NRC, 2012; van Milgen et al., 2012; 2013). The HPCP

used in the current research is a new source of corn protein from the dry-grind ethanol industry that is produced using a patented process. This new source of corn protein has greater concentrations of crude protein, Lys, and Met, but contains less fat and fiber, compared with distillers dried grains with solubles that is usually produced by the ethanol industry (NRC, 2012; Espinosa and Stein, 2018).

Results from experiment 1 indicating that there were no effects of adding dietary Val, Ile, or Trp to excess Leu diets is likely a result of the fact that all pigs were fed a similar amount of isonitrogenous diets. However, this is in contrast with results of previous research indicating that addition of Trp to a high-Leu diet increased feed intake and therefore N balance (Kwon et al., 2019a). It is possible that the reason for the difference between the 2 experiments is that the excess Leu in the previous experiment was provided by crystalline L-Leu which likely was rapidly absorbed. In contrast, the excess Leu in this experiment was provided by intact corn protein, which may have been more slowly absorbed. In addition, Leu was at 300% of the requirement in the previous experiment, but only at 171% of the requirement, which likely also contributed to the different results.

All 3 BCAA share not only the enzymes that are involved in their catabolism in skeletal muscle and liver (Harris et al., 2005), but also the AA transport system for absorption from the small intestine (Bröer, 2008). The AA transporter B^0 AT1 is Na^+-dependent transport system, which is the major transporter of BCAA, is located in the apical membrane of the enterocyte (Bröer et al., 2004). All large neutral AA such as Val, Leu, Ile, Trp, Tyr, and Phe are transported via the B^0 AT1 transporter with different affinities, but all 3 BCAA are transported with similar affinities (Bröer, 2008). This may indicate that if 1 of the BCAA is provided in excess, absorption of the other 2 BCAA may be reduced. However, excess dietary BCAA affects

expression of the B^0 AT1 transporter in jejunum and ileum of pigs (Cervantes et al., 2015). Excess Leu by itself does not affect expression of the B^0 AT1 transporter but combined excesses of all 3 BCAA appear to stimulate its expression in the jejunum and ileum (Cervantes et al., 2015). Therefore, the changes in fecal N output that were observed as an interactive effect between adding Val and adding Ile to the diet is most likely a result of increased competition for absorption in the small intestine. This may also partly explain the tendency for increased ATTD of N that was observed as dietary Val increased and the decreased retention of N that occurred as dietary Ile increased. However, reasons for the interactive effect between adding Val and adding Trp to diets on retention of N and the interactive effect between adding Ile and adding Trp on the biological value of protein are not clear.

Plasma urea N 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 dietary Leu increased 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). A deficiency in Ile and Val also may have reduced protein synthesis as indicated by the reduced N retention, which may have resulted in increased deamination of other AA and a subsequent increase in PUN (Kwon et al., 2019b). In a recent experiment conducted in our laboratory, adding dietary Val to diets with excess Leu reduced PUN, but no effect of Ile supplementation on PUN was observed regardless of dietary Leu concentration. This observation indicates that Val addition is more beneficial than Ile if diets with excess Leu are used, but current data indicated that there were no effects on PUN of adding dietary Val, Ile, or Trp to a diet containing 171% of Leu compared with the requirement.

Results of studies with pigs fed diets containing corn protein from the ethanol industry such as corn distiller dried grains with solubles indicated that growth performance was affected by the use of corn protein if a poor quality of the ingredient was used or if excess quantities of corn protein was fed to young pigs (Stein and Shurson, 2009; Woyengo et al., 2014). In general, the use of corn protein is limited, because the relatively high fiber concentration in most corn-co products contribute to reduced efficiency of utilization of nutrients by pigs (Woyengo et al., 2014). Recently, Yang et al. (2018) indicated that excess dietary Leu that was supplied by high inclusion of corn protein may contribute to a reduced ADG and ADFI of nursery pigs. Leucine is a key regulator that stimulates catabolism of BCAA in skeletal muscle and liver (Harper et al., 1984). If diets fed to pigs contain excess Leu, catabolism of all 3 BCAA may increase because of the stimulating effect of the Leu metabolite, KIC, on the branched-chain α-keto acid dehydrogenase enzyme complex (Wiltafsky et al., 2010). Comparing the control diet vs. HPCP diets in the current study, the SID Leu relative to the requirement (NRC, 2012) increased from 139% to 171%. The greater final body weight and ADG of pigs fed the control diet than in pigs fed HPCP diets that was observed is likely a result of the imbalanced supply of BCAA that result from increased catabolism of Val and Ile by excess Leu (Wiltafsky et al., 2010; Kwon et al., 2019b). Increased final body weight and ADG for pigs fed diets with Val and Trp addition to the basal diet that were observed indicates that Val and Trp supplementation may partially overcome the negative impact on growth that was caused by excess dietary Leu.

The reason for the increased PUN on day 28 in pigs fed the control diet compared with pigs fed the HPCP diets is likely a result of greater ADFI for pigs fed the control diet than for pigs fed the HPCP diets. The concentration of PUN is mostly dependent on the quantities and balance of AA that are absorbed (Nyachoti et al., 2006). No difference in hypothalamic serotonin

was observed, but adding Trp, adding Val and Trp, adding Ile and Trp, or adding Val, Ile, and Trp to the basal diet increased plasma free Trp. This observation indicates that there is a positive correlation between dieatary Trp concentration and plasma free Trp (Kwon et al., 2019a).

The first step in the catabolism of BCAA which is catalyzed by BCAA aminotransferase, produces 3 corresponding branched-chain α-keto acids, KIV, KMV, and α-keto isocaproate (**KIC**), from Val, Ile, and Leu, respectively (Harris et al., 2005). If excess Leu is included in diets for pigs, the catabolism of all 3 BCAA may increase because of increased activities of BCAA aminotransferase. However, the greater activity of the transamination enzyme may produce more KIC, which activates the branched-chain α-keto acid dehydrogenase complex and changes concentrations of branched-chain α-keto acids in blood. The changes in plasma free Val and KIV that were observed is mainly due to the increased dietary concentration of Val among dietary treatments, but the reason for the lowest plasma free Val and KIV were observed in pigs fed the basal diet may be the increased catabolism of Val that is caused by excess dietary Leu. Likewise, the reason pigs fed the control diet had the greatest plasma KMV among all dietary treatments likely is due to increase catabolism of Ile caused by excess dietary Leu in the basal diet.

The reduced concentrations of KIV and KMV that were observed in pigs fed HPCP diets are in accordance with previous data (Langer et al., 2000; Wiltafsky et al., 2010). It is likely that the increased stimulation of the branched-chain α-keto acid dehydrogenase enzyme complex 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. In the current experiment, however, no differences in plasma free Leu and KIC were observed among dietary treatments.

CONCLUSION

In conclusion, adding Ile to a diet with excess Leu reduced N retention, but if Trp was added alone or in combination with Ile or Val, N retention increased. Adding both Trp and Val to a high-Leu diet may be beneficial for preventing detrimental effects of excess Leu on growth performance of pigs. However, Trp addition did not increase feed intake of pigs.

TABLES

Table 6.1. Analyzed composition of ingredients (Experiments 1 and 2), as-fed basis[1]

Item	Corn	SBM[2]	HPCP[2]
Gross energy, kcal/kg	3,822	4,216	4,997
CP[3], %	6.50	45.94	49.60
Dry matter, %	87.22	88.64	92.84
Ash, %	1.24	6.86	3.76
Acid hydrolyzed ether extract, %	2.98	1.72	5.88
Total dietary fiber, %	11.1	17.8	37.6
Insoluble dietary fiber, %	11.0	16.5	34.9
Soluble dietary fiber, %	0.1	1.3	2.7
Indispensable amino acids, %			
Arg	0.30	3.32	2.07
His	0.20	1.22	1.27
Ile	0.26	2.26	2.12
Leu	0.83	3.59	5.88
Lys	0.25	3.05	1.77
Met	0.14	0.66	1.12
Phe	0.34	2.42	2.65
Thr	0.25	1.82	1.91
Trp	0.05	0.62	0.42
Val	0.33	2.27	2.61
Dispensable amino acids, %			

Table 6.1. (Cont.)

Ala	0.51	2.02	3.48
Asx[4]	0.46	5.28	3.43
Cys	0.15	0.68	0.95
Glx[5]	1.25	8.37	7.92
Gly	0.27	1.98	1.83
Pro	0.59	2.31	3.74
Ser	0.32	2.03	2.13
Tyr	0.19	1.65	2.12
BCAA[6]:CP ratio, %			
Ile:CP	4.00	4.92	4.27
Leu:CP	12.77	7.81	11.85
Val:CP	5.08	4.94	5.26

[1]Ingredients were analyzed in duplicate.

[2]SBM = soybean meal; HPCP = high-protein corn product (NexPro, Flint Hills Resources, Wichita, KS).

[3]CP = crude protein.

[4]Asx = Asp and Asn.

[5]Glx = Glu and Gln.

[6]BCAA = branched-chain amino acids.

Table 6.2. Ingredient composition of experimental diets (Experiments 1 and 2), as-fed basis

Item	Diet								
		High-protein corn product (Experiment. 1)							
	CON[1]	basal	+ Val	+ Ile	+ Trp	+ Val and Ile	+ Val and Trp	+ Ile and Trp	+ Val, Ile, and Trp
Ground corn	67.64	69.36	69.36	69.36	69.36	69.36	69.36	69.36	69.36
Soybean meal, 46% crude protein	27.00	-	-	-	-	-	-	-	-
High-protein corn product	-	26.00	26.00	26.00	26.00	26.00	26.00	26.00	26.00
Soybean oil	2.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
L-lysine·HCl	0.27	0.74	0.74	0.74	0.74	0.74	0.74	0.74	0.74
DL-methionine	0.06	-	-	-	-	-	-	-	-
L-threonine	0.08	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
L-tryptophan	-	0.07	0.07	0.07	0.12	0.07	0.12	0.12	0.12
L-isoleucine	-	-	-	0.10	-	0.10	-	0.10	0.10
L-valine	-	-	0.10	-	-	0.10	0.10	-	0.10
L-glycine	-	0.25	0.15	0.15	0.20	0.05	0.10	0.10	-

Table 6.2. (Cont.)

Limestone	1.00	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30
Monocalcium phosphate	0.90	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin-mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[1]CON = corn and soybean meal-based control diet (Experiment 2).

[2]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 D_3 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 B_{12}, 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 6.3. Analyzed nutrient composition of experimental diets (Experiments 1 and 2), as-fed basis[1]

Item	Diet								
		High-protein corn product (Experiment 1)							
	CON[2]	basal	+ Val	+ Ile	+ Trp	+ Val and Ile	+ Val and Trp	+ Ile and Trp	+ Val, Ile, and Trp
Gross energy, kcal/kg	3,942	4,047	4,046	4,055	4,068	4,077	4,092	4,072	4,080
Crude protein, %	17.30	18.32	18.36	18.28	18.58	18.55	18.42	18.35	18.37
Indispensable amino acids, %									
Arg	1.08	0.83	0.80	0.79	0.77	0.77	0.77	0.74	0.78
His	0.46	0.51	0.50	0.49	0.48	0.49	0.48	0.47	0.49
Ile	0.76	0.78	0.78	0.83	0.74	0.86	0.75	0.83	0.84
Leu	1.54	2.26	2.26	2.20	2.16	2.17	2.14	2.11	2.15
Lys	1.11	1.27	1.16	1.18	1.22	1.23	1.27	1.15	1.23
Met	0.30	0.41	0.41	0.40	0.40	0.39	0.40	0.39	0.40
Phe	0.87	0.99	0.98	0.95	0.94	0.94	0.93	0.91	0.93
Thr	0.70	0.83	0.80	0.80	0.78	0.80	0.78	0.77	0.79

Table 6.3. (Cont.)

Trp	0.21	0.21	0.21	0.21	0.25	0.21	0.25	0.26	0.24
Val	0.82	0.93	1.04	0.94	0.92	1.05	1.01	0.91	1.03
Dispensable amino acids, %									
Ala	0.91	1.38	1.37	1.34	1.32	1.32	1.31	1.28	1.32
Asx[3]	1.72	1.36	1.30	1.28	1.25	1.26	1.25	1.23	1.25
Cys	0.28	0.38	0.37	0.36	0.35	0.37	0.36	0.35	0.35
Glx[4]	3.10	3.25	3.24	3.16	3.10	3.10	3.09	3.01	3.05
Gly	0.71	1.08	0.83	0.87	0.96	0.77	0.78	0.75	0.70
Pro	1.04	1.50	1.52	1.48	1.48	1.46	1.44	1.40	1.43
Ser	0.75	0.84	0.83	0.81	0.81	0.77	0.78	0.76	0.78
Tyr	0.59	0.75	0.73	0.72	0.70	0.68	0.69	0.67	0.69

[1]Experimental diets were analyzed in duplicate.

[2]CON = corn and soybean meal-based control diet (Experiment 2).

[3]Asx = Asp and Asn.

[4]Glx = Glu and Gln.

Table 6.4. Effects of dietary Ile, Val, and Trp concentrations on N balance and plasma urea N of growing pigs (Experiment 1), as-fed basis[1]

Val supplementation:	0%				0.1%										
Ile supplementation:	0%		0.1%		0%		0.1%		SEM	*P-values*[1]					
Trp supplementation:	0%	0.05%	0%	0.05%	0%	0.05%	0%	0.05%		V	I	T	V × I	V × T	I × T
Feed intake, g/5 d	6,378	6,342	6,519	6,519	6,423	6,515	6,358	6,301	132	0.654	0.910	0.999	0.198	0.843	0.751
N intake, g/5 d	187	189	191	191	189	192	189	185	3.89	0.779	0.989	0.839	0.206	0.815	0.465
Fecal N output, g/5 d	40.1	40.4	47.7	43.8	38.6	43.2	39.7	40.2	2.06	0.054	0.092	0.792	0.018	0.111	0.120
Urinary N output, g/5 d	41.8	37.6	41.6	40.5	43.3	36.9	38.9	43.3	2.75	0.920	0.537	0.344	0.919	0.675	0.080
ATTD[3] of N, %	78.5	78.4	75.0	77.1	79.5	77.5	78.9	78.2	1.14	0.092	0.117	0.804	0.117	0.125	0.271
N retention, g/5 d	105	111	101	107	107	112	110	102	3.26	0.436	0.121	0.344	0.989	0.105	0.133
N retention, %	56.1	58.6	53.2	56.0	56.6	58.4	58.2	54.8	1.29	0.233	0.029	0.258	0.303	0.045	0.159
Biological value[4], %	71.5	74.9	70.9	72.8	71.2	75.3	73.7	70.1	1.48	0.940	0.195	0.173	0.990	0.261	0.032
Plasma urea N, mg/dL	8.1	8.8	7.0	8.3	8.6	7.9	7.8	8.6	0.63	0.731	0.342	0.253	0.364	0.342	0.225

[1]Interaction among Val, Ile and Trp (V × I × T) were not significant ($P > 0.05$) for all response criteria.

[2]V = Val main effect; I = Ile main effect; T = Trp main effect; V × I = interaction between Val and Ile; V × T = interaction between Val and Trp; I × T = interaction between Ile and Trp.

[3]ATTD = apparent total tract digestibility.

[4]Biological value was calculated as [N retained/(N intake – N output in feces)] × 100 (Mitchell, 1924).

Table 6.5. Effects of dietary Ile, Val, and Trp concentrations on growth performance of growing pigs (Experiment 2), as-fed basis[1]

Item	Diet									SEM	*P*-value
	CON[2]	HPCP[2]									
		basal	+ Val	+ Ile	+ Trp	+ Val and Ile	+ Val and Trp	+ Ile and Trp	+ Val, Ile, and Trp		
No. of pens	8	8	8	8	8	8	8	8	8		
Body weight, kg											
Day 1	28.7	28.5	28.7	28.7	28.4	28.5	28.5	28.7	28.5	0.83	0.969
Day 28	55.3[a]	50.7[b]	51.0[b]	50.7[b]	50.7[b]	50.2[b]	52.6[ab]	50.0[b]	51.3[b]	1.35	< 0.001
ADG, g/d[3]	950[a]	793[b]	797[b]	785[b]	797[b]	776[b]	862[ab]	760[b]	813[b]	28.8	< 0.001
ADFI, g/d[4]	1,816	1,621	1,650	1,623	1,704	1,617	1,758	1,683	1,695	57.7	0.034
G:F[5]	0.53	0.49	0.48	0.48	0.47	0.48	0.49	0.45	0.48	0.015	0.079

[1]Means within a row without a common superscript letter differ ($P < 0.05$).

[2]CON = corn and soybean meal-based control diet; HPCP = high-protein corn product-based diet.

[3]ADG = average daily gain

[4]ADFI = average daily feed intake

[5]G:F = gain to feed ratio.

Table 6.6. Effects of dietary Ile, Val, and Trp concentrations on plasma urea N, hypothalamic serotonin, and plasma free Trp of growing pigs (Experiment 2), as-fed basis[1]

	Diet										
		HPCP[2]								SEM	*P*-value
Item	CON[2]	basal	+ Val	+ Ile	+ Trp	+ Val and Ile	+ Val and Trp	+ Ile and Trp	+ Val, Ile, and Trp		
No. of pens	8	8	8	8	8	8	8	8	8		
PUN, μg/mL[3]											
Day 1	9.0	7.6	8.3	8.3	8.0	7.4	8.3	8.0	8.1	0.45	0.486
Day 14	7.9	8.0	8.9	7.6	7.5	8.5	8.8	7.8	7.4	0.71	0.773
Day 28	12.0^{a}	8.1^{b}	8.1^{b}	8.6^{b}	8.8^{ab}	7.6^{b}	8.3^{b}	8.3^{b}	7.8^{b}	0.76	0.004
HS, μg/mL[4]	0.193	0.149	0.178	0.183	0.191	0.180	0.201	0.183	0.188	0.013	0.070
Trp, μmol/L	60.5^{ab}	28.4^{c}	40.2^{bc}	43.9^{bc}	61.6^{ab}	45.9^{bc}	79.9^{a}	62.3^{ab}	59.4^{ab}	7.04	< 0.001

[1]Means within a row without a common superscript letter differ ($P < 0.05$).

[2]CON = corn and soybean meal-based control diet; HPCP = high-protein corn product-based diet.

[3]PUN = plasma urea N.

[4]HS = hypothalamic serotonin.

Table 6.7. Effects of dietary Ile, Val, and Trp concentrations on branched-chain α-keto acids (BCKA) and branched-chain amino acids (BCAA) in plasma of growing pigs (Experiment 2), as-fed basis[1]

	Diet										
		HPCP[2]								SEM	*P*-value
Item	CON[2]	basal	+ Val	+ Ile	+ Trp	+ Val and Ile	+ Val and Trp	+ Ile and Trp	+ Val, Ile, and Trp		
No. of pens	8	8	8	8	8	8	8	8	8		
BCKA, μg/mL											
KIV[3]	0.76^{a}	0.38^{c}	0.63^{abc}	0.40^{bc}	0.43^{bc}	0.57^{abc}	0.65^{ab}	0.46^{bc}	0.60^{abc}	0.078	< 0.001
KMV[3]	2.04^{a}	0.71^{b}	0.63^{b}	1.21^{b}	0.78^{b}	1.14^{b}	0.72^{b}	1.26^{b}	1.11^{b}	0.164	< 0.001
KIC[3]	10.1	12.6	12.2	13.3	11.9	11.3	12.2	11.9	12.6	0.983	0.949
BCAA, μmol/L											
Val	398^{a}	173^{d}	332^{abc}	218^{cd}	246^{bcd}	353^{ab}	368^{ab}	229^{cd}	324^{abc}	27.1	< 0.001
Ile	282	202	213	226	185	236	242	183	240	40.1	0.244
Leu	402	463	489	496	416	495	540	434	507	77.4	0.534

[1]Means within a row without a common superscript letter differ ($P < 0.05$).

[2]CON = corn and soybean meal-based control diet; HPCP = high-protein corn product-based diet.

[3]KIV = α-keto isovalerate, α-keto acid of Val; KMV = α-keto-β-methylvalerate, α-keto acid of Ile; KIC = α-keto isocaproate, α-keto acid of Leu.

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CHAPTER 7: CONCLUSION

Using co-products from the grain processing industries has become more common in swine diets as alternative sources for energy and protein because of the reduced cost of these ingredients. Many of these co-products have different protein quality when compared with traditional feed ingredients such as corn and soybean meal. Co-products from corn and sorghum usually have high Leu concentrations and if large amounts of corn or sorghum co-products are used in swine diets, pigs will have excess dietary Leu which may result in reduced feed intake and growth performance. Therefore, it is important to determine how excess dietary Leu affects feed intake and growth performance of pigs. Results of the research included in this dissertation indicate that excess dietary Leu may reduce growth performance of pigs, which is most likely due to reduced feed intake, lack of free Val and Ile as substrates for protein synthesis, and consequently reduced protein synthesis. Excess dietary Leu may also reduce serotonin synthesis in the hypothalamus, which may contribute to the reduced feed intake. A deficiency in Ile and Val caused by excess dietary Leu may have reduced protein synthesis, which may have resulted in increased deamination of other AA. However, Val supplementation may increase the efficiency of AA utilization for protein synthesis. Likewise, Trp supplementation may overcome decreased serotonin synthesis in the hypothalamus, resulting in increased feed intake of diets with excess dietary Leu. If excess dietary Leu is supplied by corn protein, the combination of Val and Trp supplementation may prevent some of the negative effects of excess Leu on growth performance of pigs. However, additional research is needed to determine how much dietary Val and Trp is required to compensate for the negative impacts of excess Leu in swine diets and to elucidate interactions among BCAA and Trp on N balance and growth performance of pigs. Understanding the relationships between BCAA and Trp utilization will enable producers to

improve efficiency of diet formulation while using feed ingredients that have high Leu concentration.