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Isoleucine and valine requirements of piglets and activity and gene expression of key enzymes of the branched-chain amino acid metabolism in response to dietary leucine excess

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Publication I

Estimates of the optimum dietary ratio of standardized ileal digestible valine to lysine for 8- to 25-kilogram pigs.

Publication II

Estimation of the optimum ratio of standardized ileal digestible isoleucine to lysine for 8- to 25-kilogram pigs in diets containing spray-dried blood cells or corn gluten feed as a protein source.

Publication III

The effects of branched-chain amino acid interactions on growth performance, blood metabolites, enzyme kinetics, and transcriptomics in weaned pigs.

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Abbreviations

%	percent
˚C	degree celcius
α-KG	α-ketoglutarate
μg	microgram
μl	microlitre
μm	micrometer
μM	micromolar
μmol	micromol
4-HPPD	4-hydroxy phenylpyruvate dioxygenase
AA	amino acid
ADFI	average daily feed intake
ADG	average daily gain
al.	alteri
Ala	alanine
AMPK	adenosine monophosphate-activated kinase
ANOVA	analysis of variance
APC	anterior piriform cortex
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
BCAA	branched-chain amino acid
BCAT	branched-chain aminotransferase
BCATc	cytosolic BCAT
BCATm	mitochondrial BCAT
BCKA	branched-chain α-keto acid
BCKDH	branched-chain α-keto acid dehydrogenase
BCKDK	branched-chain α-keto acid dehydrogenase kinase
$BCKDK^{-/-}$	BCKDK knock-out
BW	body weight
CGF	corn gluten feed
CoA	coenzyme A
CP	crude protein

Ct	cycle threshold
Cys	Cystine
d	day
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
e. g.	for example
EC	Enzyme Commission number
Exp.	experiment
g	acceleration of gravity
g	gram
G:F	gain to feed
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCN 2	general control nondepressing 2 kinase
GH	growth hormone
Gln	glutamine
Glu	glutamic acid
Gly	glycine
h	hour
His	histidine
IGF-1	insulin-like growth factor 1
IGF-ALS	insulin-like growth factor 1 acid labile subunit
Ile	isoleucine
kg	kilogram
KIC	α-keto isocaproate
KIV	α-keto isovalerate
K_m	Michaelis constant
KMV	α-keto β-methylvalerate
l	liter
Leu	leucine
Lys	lysine
M	molar
Mcal	megacalorie
ME	metabolizable energy
Met	methionine

mg	milligram
min	minute
MJ	megajoule
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimol
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
N	nitrogen
n	number
NADH	reduced form of nicotinamide adenine dinucleotide
ng	nanogram
nm	nanometre
NPY	neuropeptide Y
P	probability
Phe	phenylalanine
PLP	pyridoxal-5`-phosphate
PMP	pyridoxamine-5`-phosphate
POMC	pro-opiomelanocortin
Pro	proline
qRT-PCR	quantitative real time polymerase chain reaction
RIN	ribonucleic acid integrity number
RNA	ribonucleic acid
s	second
S	Svedberg
SD	standard deviation
SDBC	spray-dried blood cells
SE	standard error
Ser	serine
SID	standardized ileal digestible
Thr	threonine
tRNA	transfer ribonucleic acid
Trp	tryptophan

Tyr	tyrosine
V	volt
v/v	volume per volume
Val	valine
w/v	weight per volume

Abstract

Estimation of isoleucine requirement

Two growth assays and one nitrogen balance trial were conducted determining the standardized ileal digestible (SID) isoleucine to lysine ratio in 8- to 25-kg pigs using either spray-dried blood cells or corn gluten feed as a protein source. In Experiment (Exp.) 1, 48 individually penned pigs (initial body weight of 7.7 kg) were used in a six-point SID isoleucine titration (analyzed SID isoleucine levels of 0.36, 0.43, 0.50, 0.57, 0.64, and 0.72%) by addition of graded levels of crystalline isoleucine. The basal diet contained 1.00% SID lysine, 18.4% crude protein, and 13.6 MJ/kg metabolizable energy. Diets were based on wheat, barley, corn, and 7.5% spray-dried blood cells as a protein source. Dietary SID leucine and valine levels were 1.61 and 1.02%, respectively. For the 35-day period, average daily feed intake, average daily gain, and gain to feed ratio increased linearly ($P < 0.01$) and quadratically ($P < 0.04$) with increasing isoleucine supply. Estimates of optimal SID isoleucine to lysine ratios were 59% for average daily feed intake and average daily gain.

In Exp. 2, 24 nitrogen balances were conducted using the Exp. 1 diets (twelve pigs; individually penned; average body weight of 11.5 kg). Pigs were fed three times daily restrictively. Pre-collection and collection periods (seven days each) were repeated after rearranging the animals to treatments. Increasing the dietary isoleucine supply increased nitrogen retention linearly ($P < 0.01$), and nitrogen utilization linearly ($P < 0.01$) and quadratically ($P < 0.01$). An optimal SID isoleucine to lysine ratio of 54% was estimated for nitrogen retention.

In Exp. 3, 48 individually penned pigs (initial body weight of 8.0 kg) were fed grain-based diets in a six-point SID isoleucine titration (analyzed SID isoleucine levels of 0.35, 0.41, 0.49, 0.56, 0.62, and 0.69%). Dietary SID isoleucine was elevated by graded addition of crystalline isoleucine. The basal diet contained 0.97% SID lysine, 16.8% crude protein, and 13.6 MJ/kg metabolizable energy. In contrast to Exp. 1 and 2, spray-dried blood cells were excluded and corn gluten feed was used as a protein source. Dietary SID leucine and valine supplies were set to 1.05 and 0.66%. For the 42-day period, average daily feed intake, average daily gain, and gain to feed ratio increased linearly ($P < 0.01$) and quadratically ($P < 0.01$) with increasing isoleucine supply. Estimated optimal SID isoleucine to lysine ratios were 54, 54, and 49% for average daily feed intake, average daily gain, and gain to feed ratio, respectively.

These experiments suggest that the optimal SID isoleucine to lysine ratio depends on diet composition. In Exp. 1, amino acid imbalances as high leucine contents may have led to increased isoleucine nutritional needs. In 8- to 25-kg pigs fed diets without leucine excesses

an optimum SID isoleucine to lysine ratio of 54% was estimated for average daily feed intake and average daily gain.

Estimation of valine requirements

Two growth assays and one nitrogen balance trial were conducted to determine the valine requirements of weaned pigs (8- to 25-kg) as a ratio of SID valine to lysine. In Exp. 1, 48 pigs (individual housing; equal proportion of castrates and females; initial body weight of 8.0 kg) were randomly subdivided into six dietary treatment groups. Diet composition was mainly based on wheat, corn, and barley. Whey powder, field peas, and soybean meal served as a protein source. At an average SID lysine level of 0.99%, the dietary SID valine to lysine ratio was increased from 56 to 84% (planned increments of 6%) by adding crystalline valine. Other crystalline amino acids were supplied to meet optimum SID amino acid to lysine ratios based on the concept of an ideal protein. All diets were isocaloric (metabolizable energy: 13.6 MJ/kg) and isonitrogenous (crude protein: 17.8%). For the 35-day period, average daily feed intake and average daily gain increased linearly ($P < 0.01$) and quadratically ($P < 0.01$) with increasing valine supply. However, the data were not suitable for estimation of valine requirements. Thus, a second growth assay was performed.

In Exp. 2, the dietary basal level of valine was reduced to 0.49% SID valine and four increments of 0.04% and one increment of 0.08% crystalline valine were used to give six dietary treatments (48 pigs; individual housing; equal proportion of castrates and females; initial body weight of 7.9 kg). Diet composition was mainly the same as in the first growth assay but higher levels of barley and field peas were used and soybean meal was excluded. The SID lysine level averaged 0.99% and SID valine to lysine ratios ranged from 49 to 74%. Other crystalline amino acids were supplied to meet optimum SID amino acid to lysine ratios. All diets were isocaloric (metabolizable energy: 13.7 MJ/kg) and isonitrogenous (crude protein: 17.7%). For the 34-day period, average daily feed intake, average daily gain, and gain to feed ratio increased linearly ($P < 0.01$), and average daily gain and gain to feed ratio quadratically ($P < 0.01$) as the valine supply increased. Estimates of optimum SID valine to lysine ratios were 67, 66 and 61% for average daily feed intake, average daily gain, and gain to feed ratio, respectively. The estimates were confirmed by minimized levels of plasma urea and lysine, and by markedly increased levels of plasma valine.

To verify the estimates of the growth trials, a total of 24 nitrogen balances were conducted with twelve pigs using the dietary treatments of Exp. 2 (Exp. 3; equal proportion of males and females; average body weight of 14.1 kg; two balances per pig). Pigs were restrictively fed three times daily. Pre-collection and collection periods lasted seven days each and were repeated after rearranging the animals to treatments. Increasing the dietary valine supply linearly increased nitrogen retention ($P < 0.04$) and quadratically decreased plasma levels of urea ($P < 0.01$). Optimal SID valine to lysine ratios of 65 and 62% were estimated for nitrogen retention and nitrogen utilization, respectively.

We concluded that a SID valine to lysine ratio of 65 to 67% is needed for optimal performance in 8- to 25-kg pigs.

The effects of dietary leucine excesses

The impact of excess dietary leucine was studied in two growth assays with pigs (8- to 25-kg). In each trial, 48 pigs were allotted to one of six dietary groups. Diet composition was mainly based on barley, wheat, corn gluten feed, soybean meal, and corn. Crude protein content averaged 18% and SID lysine level was set at 1.12%. Crystalline amino acids were supplemented according to the concept of an ideal protein, except for the branched-chain amino acids. To guarantee that interactions between the branched-chain amino acids were not cushioned either surpluses of isoleucine (Exp. 1) or valine (Exp. 2) were avoided. The dietary leucine supply increased from treatment L100, L150, L175, to L200. The leucine supply of L200 was 200% that of L100. In the fifth treatment, the effects of a simultaneous excess of leucine and valine (Exp. 1), or of leucine and isoleucine (Exp. 2) were investigated. The sixth treatment was a positive control. In both trials, the growth performance decreased ($P < 0.05$), and plasma leucine ($P < 0.01$) and serum α-keto isocaproate levels increased ($P < 0.01$) in a linear, dose-dependent manner as the dietary leucine supply increased. Plasma levels of isoleucine and valine, and serum levels of their corresponding α-keto acids decreased, indicating increased catabolism. Linear increases in the basal activity of the branched-chain α-keto acid dehydrogenase (BCKDH) complex in liver tissue confirmed these findings. Doubling of the dietary leucine supply increased the basal BCKDH activity about threefold in both experiments ($P < 0.01$). No major alterations occurred in the messenger ribonucleic acid (mRNA) expression of branched-chain amino acid catabolism genes. In liver tissue of Exp. 2, however, the mRNA levels of growth hormone (GH) rezeptor, IGF-ALS (insulin-like growth factor 1 acid labile subunit), and IGF-1 decreased significantly with increasing dietary leucine. In conclusion, excess dietary leucine increased the catabolism of the branched-chain amino acids mainly through posttranscriptional mechanisms. The impact of excess leucine on the GH-IGF-1-axis requires further investigation.

Zusammenfassung

Ermittlung des Isoleucinbedarfs

Zur Bestimmung eines optimalen Verhältnisses von standardisiert ileal verdaulichem (standardized ileal digestible, SID) Isoleucin zu Lysin in Rationen für Absetzferkel (8 bis 25 kg) wurden zwei Wachstumsversuche und ein Stickstoffbilanzversuch durchgeführt. Über die Bestimmung des Isoleucinbedarfs hinaus sollte ein möglicher Einfluss der verwendeten Proteinträger, Hämoglobinpulver bzw. Maiskleberfutter, auf den Isoleucinbedarf untersucht werden. Für den ersten Versuch wurden 48 Ferkel mit einer durchschnittlichen Lebendmasse von 7.7 kg auf sechs Versuchsgruppen verteilt und einzeln aufgestallt. Durch Zulage von kristallinem Isoleucin wurde die Isoleucin-Versorgung schrittweise gesteigert. Folgende SID Isoleucingehalte wurden in den einzelnen Diäten ermittelt: 0.36, 0.43, 0.50, 0.57, 0.64 und 0.72%. Die Diäten bestanden hauptsächlich aus Weizen, Gerste und Mais. Als Proteinträger wurde 7.5% Hämoglobinpulver eingesetzt. Die Basaldiät enthielt 1.00% SID Lysin, 18.4% Rohprotein und 13.6 MJ/kg umsetzbare Energie. Die Verwendung des Hämoglobinpulvers führte zu erhöhten Gehalten an SID Valin (1.02%) und Leucin (1.61%). Die Versuchsdauer betrug 35 Tage. Mit steigender Isoleucinversorgung stiegen die tägliche Futteraufnahme, die täglichen Zunahmen und die Futterumsetzung linear ($P < 0.01$) und quadratisch ($P < 0.04$) an. Für die tägliche Futteraufnahme und die täglichen Zunahmen wurde ein optimales SID Isoleucin zu Lysin-Verhältnis von 59% abgeleitet.

Im Anschluss wurde mit den selben Diäten ein Stickstoffbilanzversuch durchgeführt (zwölf Ferkel; Einzeltierhaltung; 24 Stickstoffbilanzen; durchschnittliche Lebendmasse von 11.5 kg). Die Tiere wurden dreimal täglich restriktiv gefüttert. Die Vor- und die Sammelperiode dauerten jeweils sieben Tage. Nach einer Neuverteilung der Ferkel auf die Versuchsgruppen wurden eine zweite Vor- und Sammelperiode durchgeführt. Mit zunehmender Isoleucinversorgung stiegen die Stickstoffretention linear (P < 0.01) und die Stickstoffverwertung linear ($P < 0.01$) und quadratisch ($P < 0.01$) an. Für die Stickstoffretention leitete sich ein optimales SID Isoleucin zu Lysin-Verhältins von 54% ab.

Im dritten Versuch zur Ermittlung des Isoleucinbedarfs wurde eine abgewandelte Rationsgestaltung gewählt. Das Hämoglobinpulver wurde als Proteinträger durch Maiskleberfutter ersetzt. Das restliche Versuchsdesign entsprach dem des ersten Wachstumsversuches (sechs Gruppen; 48 Ferkel; 8.0 kg Lebendmasse zu Versuchsbeginn; Einzeltierhaltung). Durch die Zulage von kristallinem Isoleucin wurde eine schrittweise Steigerung der Isoleucinversorgung erreicht und folgende SID Isoleucingehalte wurden ermittelt: 0.35, 0.41, 0.49, 0.56, 0.62, 0.69%. Die Basaldiät enthielt 0.97% SID Lysin, 16.8% Rohprotein und 13.6 MJ/kg

umsetzbare Energie. Durch die Verwendung von Maiskleberfutter an Stelle von Hämoglobinpulver traten keine Überschüsse an SID Valin (0.66%) oder Leucin (1.05%) auf. Die Versuchsdauer betrug 42 Tage. Mit ansteigender Isoleucinversorgung erhöhten sich die tägliche Futteraufnahme, die täglichen Zunahmen und die Futterumsetzung linear ($P < 0.01$) und quadratisch ($P < 0.01$). Für die tägliche Futteraufnahme, die täglichen Zunahmen und die Futterumsetzung wurden optimale SID Isoleucin zu Lysin-Verhältnisse von 54, 54 und 49% abgeleitet.

Diese Versuche legen den Schluss nahe, dass das optimale SID Isoleucin zu Lysin-Verhältnis von der verwendeten Diät abhängig ist. In den Diäten des ersten Wachstumsversuches haben möglicherweise Imbalancen zwischen den Aminosäuren, wie zum Beispiel überhöhte Leucingehalte, zu einem erhöhten Bedarf an Isoleucin geführt. Enthielten die Diäten keine Überschüsse an Leucin, so wurde für die tägliche Futteraufnahme und die täglichen Zunahmen ein optimales SID Isoleucin zu Lysin-Verhältnis von 54% ermittelt.

Ermittlung des Valinbedarfs

Zur Bestimmung des Valinbedarfs des Absetzferkels (8 bis 25 kg) wurden zwei Wachstumsversuche und ein Stickstoffbilanzversuch durchgeführt. Im ersten Wachstumsversuch wurden 48 Ferkel auf sechs Versuchsgruppen aufgeteilt (Einzeltierhaltung; gleiche Anzahl von Kastraten und Weibchen; 8.0 kg durchschnittliche Lebendmasse zu Versuchsbeginn). Die Basaldiät setzte sich vornehmlich aus Weizen, Mais und Gerste zusammen, wobei Molkepulver, Ackerbohnen und Sojaextraktionsschrot als Proteinträger verwendet wurden. Die Supplementierung mit kristallinem Valin erfolgte in fünf Schritten à 0.06%. Bei einem SID Lysingehalt von 0.99% erhöhte sich dadurch das SID Valin zu Lysin-Verhältnis von 56 auf 84%. Die Supplementierung mit weiteren kristallinen Aminosäuren erfolgte gemäß den Vorgaben für ein ideales Protein. Die verwendeten Diäten waren isokalorisch (13.6 MJ/kg umsetzbare Energie) und isonitrogen (17.8% Rohprotein). Die Versuchsdauer betrug 35 Tage. Mit steigender Valinversorgung erhöhten sich die tägliche Futteraufnahme und die täglichen Zunahmen linear ($P < 0.01$) und quadratisch ($P< 0.01$). Dennoch waren die Daten nicht für eine Ableitung des Valinbedarfs geeignet, weshalb ein weiterer Wachstumsversuch nötig wurde.

Für die Durchführung des zweiten Wachstumsversuches (48 Ferkel; Einzeltierhaltung; gleiche Anzahl von Kastraten und Weibchen; 7.9 kg Lebendmasse zu Versuchsbeginn) wurde ein niedrigerer Basalgehalt an Valin und eine geringere Dosisstufung im Vergleich zum vorhergehenden Versuch gewählt. Die Zusammensetzung der Basaldiät unterschied sich von der des ersten Versuches durch höhere Gehalte an Gerste und Ackerbohnen, was den Ausschluss von Sojaextraktionsschrot ermöglichte. Die Basaldiät enthielt einen abgesenkten SID Valingehalt von 0.49%. Die Supplementierung mit kristallinem Valin erfolgte in vier Schritten à 0.04% und einem Schritt à 0.08%. Bei einem SID Lysingehalt von 0.99% ergaben sich somit SID Valin zu Lysin-Verhältnisse von 49 bis 74%. Weitere kristalline Aminosäuren wurden nach den Vorgaben für ein ideales Protein ergänzt. Die verwendeten Diäten waren isokalorisch (13.7 MJ/kg umsetzbare Energie) und isonitrogen (17.7% Rohprotein). Die Versuchsdauer betrug 34 Tage. Die ansteigende Versorgung mit Valin führte zu einer linearen Erhöhung der täglichen Futteraufnahme, der täglichen Zunahmen und der Futterumsetzung ($P < 0.01$) sowie zu einer quadratischen Erhöhung der täglichen Zunahmen und der Futterumsetzung ($P < 0.01$). Es wurden optimale SID Valin zu Lysin-Verhältnisse von 67, 66, und 61% für die tägliche Futteraufnahme, die täglichen Zunahmen und die Futterumsetzung abgeleitet. Diese Ableitungen wurden im Plasma durch Minima der Harnstoff- und Lysinspiegel und durch deutlich ansteigende Valinspiegel bestätigt.

Zur Überprüfung der Bedarfsschätzungen des zweiten Wachstumsversuches wurde anschließend unter Verwendung der selben Diäten ein Stickstoffbilanzversuch durchgeführt (zwölf Ferkel; gleiche Anzahl von Kastraten und Weibchen; 14.1 kg durchschnittliche Lebendmasse; zwei Bilanzen pro Ferkel). Die Ferkel wurden dreimal täglich restriktiv gefüttert. Die Vor- und die Sammelperiode dauerten jeweils sieben Tage und wurden nach einer Umgruppierung der Ferkel wiederholt. Mit steigender Valinversorgung erhöhte sich die Stickstoffretention linear ($P < 0.04$), während sich die Plasma-Harnstoffspiegel quadratisch erniedrigten ($P < 0.01$). Für die Stickstoffretention und die Stickstoffverwertung wurden optimale SID Valin zu Lysin-Verhältnisse von 65 und 62% abgeleitet, was die Ableitungen des Wachstumsversuches bestätigte.

Aus den vorliegenden Versuchen wurde geschlossen, dass für das Absetzferkel im Gewichtsbereich von 8 bis 25 kg zur Erzielung einer optimalen Leistung ein SID Valin zu Lysin-Verhältnis von 65 bis 67% nötig ist.

Zu den Auswirkungen einer Leucin-Überversorgung

Die Auswirkungen einer Leucin-Überversorgung auf das Absetzferkel (8 bis 25 kg) wurden in zwei Wachstumsversuchen untersucht. In jedem Versuch wurden jeweils 48 Ferkel auf sechs Versuchsgruppen aufgeteilt. Die Rationszusammensetzung bestand vornehmlich aus Gerste, Weizen, Maiskleberfutter, Sojaextraktionsschrot und Mais. Der Rohproteingehalt betrug im Durchschnitt 18% bei einem SID Lysingehalt von 1.12%. Kristalline Aminosäuren wurden gemäß den Vorgaben für ein ideales Protein supplementiert. Davon ausgenommen waren die verzweigtkettigen Aminosäuren. Um zu gewährleisten, dass sich Interaktionen zwischen den verzweigtkettigen Aminosäuren direkt auswirkten, wurden im ersten Versuch Überschüsse an Isoleucin und im zweiten Versuch Überschüsse an Valin vermieden. In den Gruppen L100, L150, L175 und L200 wurde die Leucinversorgung schrittweise erhöht. Relativ zur L100-Gruppe betrug die Leucinversorgung der L200-Gruppe 200%. In der fünften Gruppe wurden die Auswirkungen einer gleichzeitigen Überversorgung mit Leucin und Valin (Versuch I) bzw. mit Leucin und Isoleucin (Versuch II) untersucht. Die sechste Gruppe stellte eine Positivkontrolle dar. Die ansteigende Leucinversorgung führte in beiden Versuchen zu einer linearen, dosisabhängigen Depression des Wachstums ($P < 0.05$) und einer linearen, dosisabhängigen Erhöhung der Plasma-Leucin- ($P < 0.01$) und der Serum-α-Ketoisocaproatspiegel ($P < 0.01$). Gleichzeitig gingen die Plasmaspiegel von Isoleucin und Valin sowie die Serumspiegel ihrer korrespondierenden α-Ketosäuren mit steigender Leucinversorgung zurück, was auf einen erhöhten Katabolismus schließen lässt. Bestätigt wurde diese Schlussfolgerung durch eine lineare Erhöhung der basalen Aktivität des BCKDH-Komplexes (branched-chain α-keto acid dehydrogenase) im Lebergewebe. Die Verdopplung der Leucinversorgung führte in beiden Versuchen in etwa zu einer Verdreifachung der basalen BCKDH-Aktivität. Hingegen wurde die mRNA-Expression (messenger ribonucleic acid) von Enzymen, die für den Abbau der verzweigtkettigen Aminosäuren wichtig sind, nicht in größerem Umfang beeinflusst. Allerdings nahm die mRNA-Expression des Wachstumshormon (GH)-Rezeptors, der säurelabilen Untereinheit des IGF-1 (insulin-like growth factor 1) und des IGF-1 selbst mit steigender Leucinversorgung signifikant ab. Zusammenfassend lässt sich sagen, dass eine Leucinüberversorgung den Katabolismus der verzweigtkettigen Aminosäuren hauptsächlich über posttranskriptionale Mechanismen zu erhöhen scheint. Der Einfluss einer Leucinüberversorgung auf die GH-IGF-1-Achse bedarf weiterer Untersuchungen.

1 Introduction

In today's low-protein diets for pigs, isoleucine (Ile) and valine (Val) are generally discussed to be among the limiting amino acids (AA). Thus, the nutritional needs for Ile and Val must be known in order to avoid nutritional shortages and to guarantee optimal growth performance. Furthermore, a good knowledge of the animal's AA needs is essential to avoid safety margins and AA surpluses. Thus, it is the basis for optimization of diet composition which increases feed efficiency and reduces the environmental impact of animal husbandry. The use of crystalline essential AA decreases the agricultural needs for protein sources and decreases the dependency on the import of high protein feeds as soybean meal. Supplementation with essential AA enables feeding of low-protein diets and usage of protein sources of minor quality because imbalances in the AA pattern can be corrected.

In literature there is a lack of data concerning the Val and Ile requirements of weaned pigs and the present work was designed to reduce this lack of knowledge. Determining Ile and Val requirements, caution must be taken because of interactions between Ile, Val, and leucine (Leu). For the estimation of the Ile requirements of pigs, spray-dried blood cells (SDBC) have been a preferred protein source in the past because they are high in protein but extremely low in Ile. Thus, the use of SDBC enables a diet composition similar to practical diets but markedly deficient for Ile. However, the inclusion of SDBC causes surpluses of Leu. This bears the risk that the Ile requirements estimated in pigs fed SDBC-diets are overestimating the Ile requirements of pigs fed diets without surpluses of dietary Leu. This hypothesis should be elucidated in the present work.

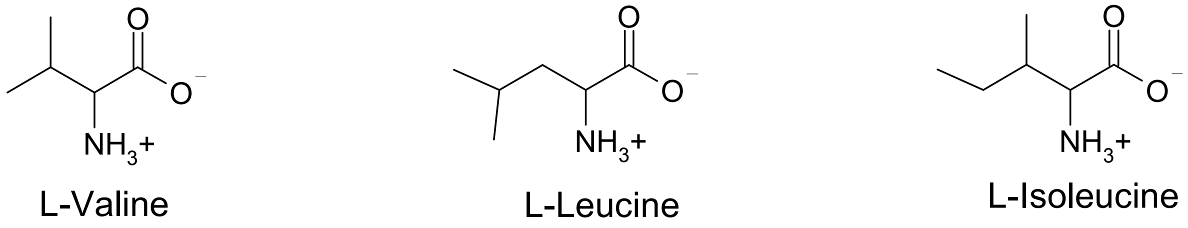
Ile, Val, and Leu have got a similar chemical structure and are summarized as branched-chain amino acids (BCAA). If diets excessive in Leu are ingested, increased plasma levels of Leu and its corresponding branched-chain α-keto acid (BCKA) α-keto isocaproate (KIC) would be expected. But the plasma BCAA and BCKA levels are highly regulated, because they are neurotoxic, especially Leu and KIC. In maple urine disease, BCAA and BCKA accumulate because of an inborn severe enzyme deficiency. This leads to poor feeding, apnoea, ketoacidosis, convulsions, coma and psychomotor delay, because BCAA and BCKA compromise brain energy metabolism by blocking the respiratory chain (Sgaravatti et al., 2003). To keep up the vital functions, it is of eminent importance to avoid accumulation of BCAA or BCKA. Thus, dietary Leu excesses might increase the catabolism of all three BCAA because the corresponding BCKA of Ile, Val, and Leu are catabolized via the same enzyme complex. In pigs, most experiments on dietary Leu excesses focused on performance parameters (Oestemer et al., 1973a; Henry et al., 1976; Taylor et al., 1984; Taylor et al., 1985). Thus, data on metabolites and BCAA catabolism are sparse or absent. This work should make a contribution to enlarge the knowledge about the effects of dietary Leu oversupply in pigs.

The introduction is divided in a general part concerning general aspects of the BCAA, as their functions in the organism and their relevance in pig nutrition. The following chapters of the introduction are dealing with the estimation of AA requirements and describe the BCAA catabolism, because these are crucial aspects of the present study.

1.1 Branched-chain amino acids and their functions

Proteins (greek: proteuo = I take the first place) are quantitatively the dominating component in all organisms – without proteins, no life is possible. They play a prominent role in every single cell. Proteins are formed out of 100 to 1.000 AA and have got a relative molecular mass of more than 10.000 to 100.000. All AA contain an amino and a carboxyl group which enables linking. For protein synthesis, about 20 different AA are needed. All mammals require the core of nine essential AA: histidine (His), Ile, Leu, lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and Val. In fast growing pigs during neonatal and immediate post-weaning phases, the endogenous synthesis of arginine (Arg) may become limiting, thus Arg is called semi-essential. Cysteine can be synthesized out of Met, and tyrosine (Tyr) out of Phe so that they are also semi-essential for pigs. The rest, alanine (Ala), asparagine (Asn), aspartate (Asp), glutamate (Glu), glutamine (Gln), glycine (Gly), proline (Pro), and serine (Ser) are non-essential AA (Boisen, 2003). The essential AA can be divided into two groups according to their degree of limitation in common diets for pigs. The firstly limiting AA are Lys, Thr, Met, and Trp. All of them are available as crystalline AA and are commonly supplemented in practical pig diets for improving protein quality and reducing crude protein (CP) oversupply. Ile, Leu, and Val belong to the secondarily limiting AA, and based on common structural features (Fig. 1) they are categorized as BCAA.

Fig. 1 Chemical structure of the branched-chain amino acids.



In the organism, the BCAA serve as a precursor for the synthesis of proteins and other AA, and as an important energy substrate during exercise and periods of stress (Ferrando et al., 1995). For example, the BCAA serve as a major source of nitrogen (N) for synthesis of Gln (Darmaun and Dechelotte, 1991). Gln is an important nutrient to many rapid dividing cells, especially in the gut and the immune system. However, Gln is not stable in solutions. Thus, conventional parenteral or enteral diets for humans are deficient in Gln and associated with gut atrophy and immune deficiency. It was found that infusion of BCAA-enriched parenteral nutrition ameliorated gut morphology and immune status. Infusion of BCAA increases the release of Gln from skeletal muscle and elevates the plasma Gln concentrations in humans and rats (McCauley et al., 1990; Abumrad et al., 1982; Platell et al., 1993).

Endogenous BCAA are a major source of nitrogen for the de-novo-synthesis of Ala. After an overnight fast, at least 20% of the nitrogen required for Ala synthesis is derived solely from Leu (Platell et al., 2000). In skeletal muscle, the BCAA serve as an oxidative substrate during periods of prolonged exercise. In athletes running a marathon the plasma levels of Gln, Ala, and BCAA were found to be decreased immediately after the marathon (Castell et al., 1997). In rats it has been shown that exercise increases the BCAA catabolism mediated by falling adenosine triphosphate (ATP) levels within the muscle (Shimomura et al., 1995). In muscle, the enhanced BCAA metabolism in conditions of a relative lack of energy as intense exercise, hypoxia, trauma, or sepsis appears to have a sparing effect on muscle glycogen degradation (Blomstrand et al., 1996). However, results reported in literature are not consistent (Madsen et al., 1996; Clarkson, 1996) and endurance exercise training blunts the acute exercise-induced activation of BCAA catabolism in human skeletal muscle (McKenzie et al., 2000).

In the brain, the BCAA serve an important role in regulating N metabolism and Gln-synthesis. They are the major external N source for the synthesis of Glu and Gln (Chuang et al., 1995). The BCAA enable the N transfer from astrocytes to neurons, provide a buffering mechanism, and prevent the excessive accumulation of the neurotransmitter Glu. Furthermore, the BCAA are an important energy source in the brain.

1.2 Isoleucine and valine in pig nutrition

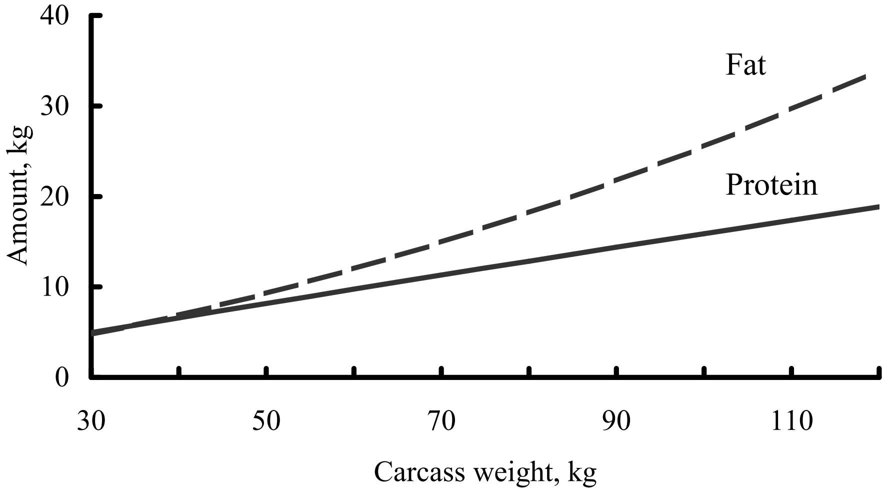
Supplementation of diets with the firstly limiting AA is the basis to reduce the dietary crude protein (CP) content without performance depression. Prevention of surface and ground water contamination with N is achievable by reductions of CP and is desirable for both economic and environmental reasons. Additionally, the use of low-protein diets has been reported to decrease the frequency and severity of digestive problems in piglets (Ball and Aherne, 1982, 1984; Lordelo et al., 2008). In average, a 10% reduction in total N excretion was reported for every 1% reduction in CP content of the diet (Kerr and Easter, 1995; Le Bellego and Noblet, 2002; Shriver et al, 2003; Lordelo et al., 2008). Reductions of CP content up to 6.6 basis points (from 18.9 to 12.3%) and simultaneous supplementation with crystalline essential AA in the diets of growing pigs has been shown to have no negative effects on N retention or animal performance, but reduced N excretion by 58% (Le Bellego et al., 2001).

Reducing the CP content and supplementing the firstly limiting AA holds the risk that the supply of the secondarily limiting AA becomes insufficient. Val and Ile are discussed to be the next-limiting AA in several low-protein diets. In corn-wheat-soybean meal-based diets (17% CP) supplemented with Lys, Met, Thr, and Trp fed to 7- to 17-kg pigs, supplementation of Val was necessary to achieve maximum performance (Lordello et al., 2008). For 10- to 20-kg pigs, His, Ile, Trp, and Val were equally limiting after Lys and Thr in sorghum-soybean meal based-diets (12% CP; Brudevold and Southern, 1994) and Thr, Met, and Val were equally limiting after Lys and Trp in corn-soybean meal-whey based-diets (13.5% CP) (Mavromichalis et al., 1998). In corn-soybean meal based-diets (11% CP) fortified with Lys, Trp, Thr, and Met fed to growing pigs, Val was fifth-limiting and either His or Ile sixth-limiting (Figueroa et al., 2003). In diets for piglets, blood products are very common in the US because of their high protein quality and AA bioavailability (DeRouchey et al., 2002; Hinson et al., 2007). Performance increasing effects of blood products were reported (DeRouchey et al., 2002). But higher inclusions (4 to 5%) of SDBC can be growth depressive because Ile becomes limiting (Hinson et al, 2007; Kerr et al., 2004b).

To avoid performance depression when low-protein diets are fed, the nutritional needs of the animal must be known. It is necessary to estimate the AA requirements for specific weight ranges, because the AA requirements change during maturation. The AA requirement of growing pigs can be divided into the requirement for maintenance and that for protein accretion. In growing animals, the AA needs for maintenance are very low compared to that for protein accretion. Maintenance contributed only for 4.7 to 6.1% of the total needs for BCAA in 50-kg pigs depositing 155 g protein per day (Heger et al., 2003). The composition of the protein accretion and its proportion of the daily gain changes while the animal is growing. In 10- to 30-kg pigs, the protein content averages 170 g per kg of body weight (BW) gain. But with increasing BW, the protein content linearly decreases to 145 g per kg of BW gain in 120-kg pigs. In contrast, the fat content linearly increases from 190 to 420 g per kg of BW gain for 30- and 120-kg pigs, respectively (Fig. 2; GfE, 2006). Because of these changes, the AA requirements have to be estimated separately for weaned, growing, and finishing pigs. Data are currently lacking for Val and are sparse for Ile requirements of 10- to 25-kg pigs. Some work on the Ile requirement of pigs has recently been published (Parr et al., 2003; Kerr et al., 2004a; Dean et al., 2005), but most experimental data for the determination of Ile requirements are dated. It is difficult to use older studies because of missing values for metabolizable energy (ME) and for the digestibility of AA, possible deficiencies in other AA and the use of CP levels different from current practice. On the other hand, most of the recent

Ile requirement trials were conducted using blood products as a protein source. SDBC were often used because of their unique AA pattern, high digestibility and high CP content (NRC, 1998). The use of SDBC enables the formulation of diets comparable to those used in practice but deficient in Ile. But a major disadvantage of SDBC are their high Leu and Val contents. Interactions among the BCAA, such as the performance depressing effects of excess dietary Leu, are known in several species (Harper et al., 1984).

Fig. 2 Carcass composition during maturation (GfE, 2006).



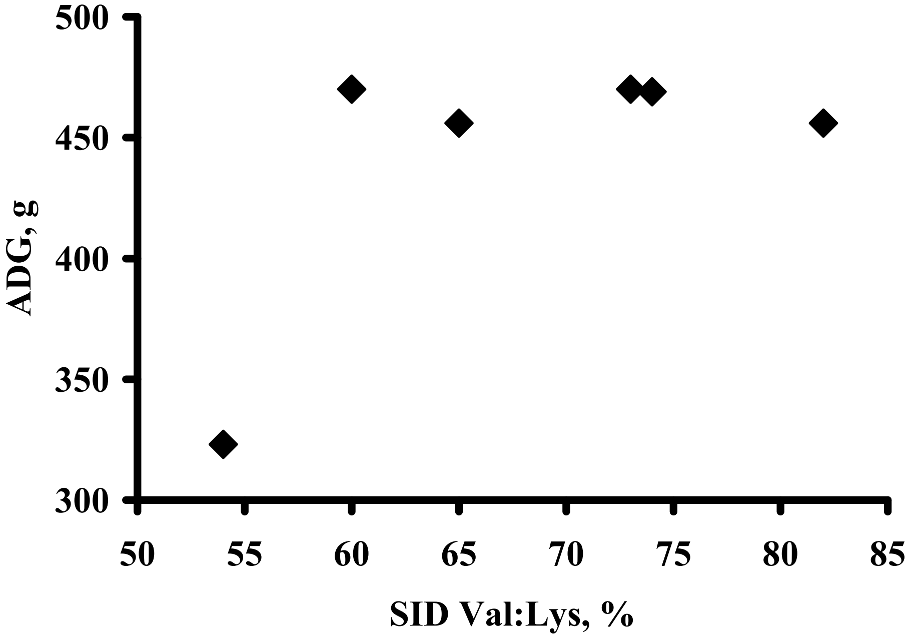
1.3 Estimation of isoleucine and valine requirements

In growing animals, AA requirements are generally estimated best using growth data as response criterion in *ad libitum* studies (Baker, 1986). The use of performance parameters for requirement estimates respects that maximal growth and feed efficiency are desired in growing pigs. *Ad libitum* feeding has the advantage that it covers both, growth responses caused by favorable effects on metabolism, and effects on feed intake. Positive effects of AA on feed intake were reported for Trp (Eder et al., 2001) and negative effects are known for dietary AA excesses or imbalances (Edmonds and Baker, 1987; Harper et al., 1984; Southern and Baker, 1982). In the present work, requirement estimates were consecutively lower for the ratio of gain to feed (G:F) and N utilization than for average daily gain (ADG) and average daily feed intake (ADFI), what illustrates the effects of AA on feed intake and confirms the importance of *ad libitum* feeding in AA requirement trials.

Another advantage of *ad libitum* feeding is that the bioavailability of the nutrients is synchronized. In AA requirement studies, crystalline AA must be supplemented to generate diets with incrementally increasing contents of the AA in question. It was reported that a one-meal-per-day-feeding regimen leads to a crystalline AA wastage because of an asynchronous availability of the AA. The protein-bound AA were not available for protein synthesis at the same time as the crystalline AA because the crystalline AA were absorbed much faster than the protein-bound AA (Batterham and Bayley, 1989; Yen et al., 2004). Thus, we used an *ad libitum*-feeding regimen for the growth assays and a three-meal-per-day-feeding regimen for the N balances.

To estimate requirements, a minimum of four dietary groups with incrementally increasing levels of the nutrient in question are needed, but six or even more levels should be preferred (Baker, 1986). The data have to be fitted to a descriptive response curve, such as broken-line, to determine the requirement. With an increasing number of dietary levels tested, the explanatory power of the model increases. In the present work, six dietary levels of Ile or Val were tested. Increments of L-Ile or L-Val were equally spaced to make statistical calculation easier (exception: the last increment in the second Val trial was double-spaced). The dietary composition of the experimental diets within one trial was kept as constant as possible to minimize side-effects. Dietary levels of the AA in question were increased by supplementation with crystalline Ile or Val. The basal Ile and Val levels, respectively, were chosen as minimal as possible using standard feed ingredients. In semipurified diets even less basal levels are possible but their use was excluded, because of transfer problems of the gained data to practical diets. A basal level low enough to generate a pronounced performance depression is crucial for the estimation of AA requirements because it is the basis for a response to the supplementation of the performance limiting AA. To confirm the slope of the response it is necessary to have a minimum of two groups within the response area. Otherwise, as seen in Fig. 3, no response curve can be fitted to the data and the experiment has to be repeated with lower basal levels of the AA in question.

Fig. 3 Average daily gain (ADG) as a function of the standardized ileal digestible valine to lysine ratio (SID Val:Lys). For details see Publication I.



The incremental supplementation of a deficient diet with the missing nutrient increases the performance parameters until the requirement is met and the animals tap their full potential. Further increments won’t increase the performance any more so that the performance will stagnate and form a plateau until the nutrient will become toxic. Again, a minimum of two groups is needed to establish the area of maximum performance. Therefore, the increments used in the present trials were planned to get three groups with an increasing and three groups with a stagnating response. Ile and Val requirement data are sparse so that the experimental design had to cope with some uncertainty. The chosen procedure covered a maximum of discrepancy to literature estimates with respect to the constrictions of the experimental facilities.

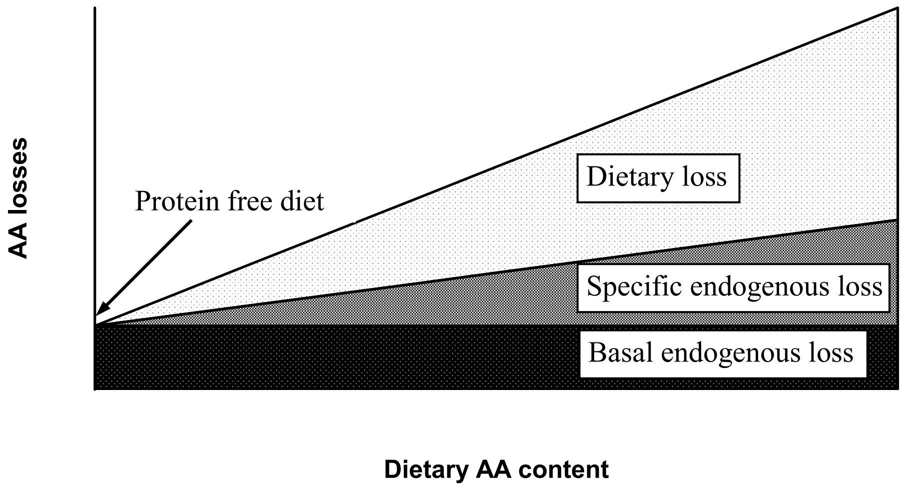
1.3.1 Ileal digestibility of amino acids

In diet formulation for pigs, estimates of ileal digestible AA are used routinely. The advantage of using ileal digestible AA is that AA undersupplies are identified which were not seen on basis of the analyzed AA. Therefore, ileal digestible AA are the basis for reducing protein oversupply and accurate crystalline AA supplementation (GfE, 2002). Ileal digestible AA expressed as standardized ileal digestible (SID) AA are at the moment the best basis for diet formulation. The standardized ileal digestibility of AA is calculated as follows:

$$\text{SID (\%)} = [(\text{AA intake} - (\text{ileal AA outflow} - \text{basal endogenous losses})) / \text{AA intake}] \times 100$$

The total ileal AA outflow contains on the one hand non-digested dietary AA and on the other hand endogenous losses (Fig. 4). The endogenous losses represent endogenously synthesized proteins and other AA containing compounds that are secreted into the intestinal lumen of the animal and are not digested and reabsorbed prior to the distal lumen (e. g.: digestive enzymes, mucoproteins, sloughed epithelial cells, serum albumin, amides). The endogenous losses can be separated into basal losses and specific losses. The basal endogenous losses are not influenced by feed ingredient composition. In contrast, the specific endogenous losses are characteristic for different feed ingredients and are influenced by their composition (anti-nutritional factors: trypsin inhibitors, lectins, tannins; amount and structure of dietary fiber).

Fig. 4 Partitioning of amino acids (AA) in ileal digesta from pigs as influenced by dietary AA content (GfE, 2005).



Correcting the ileal outflow for basal endogenous losses makes SID AA values additive in feed formulation (Stein et al., 2005), because the calculation accounts then for any components that are specific to the feed ingredient. Is a standardized amount of basal endogenous AA losses used for correction (e. g.: GfE, 2005), one yields the SID AA. The SID AA are state of the art in diet formulation for pigs (Stein et al., 2007) and the experimental diets of the present work were calculated on this basis. The SID AA were calculated for all diets by multiplication of the analyzed AA contents of the mixed diets with an AA and diet specific digestibility factor. This factor was calculated with the feed ingredient specific AA digestibility factors (INRA-AFZ-INAPG, 2004) as a weighted average taking into account the contribution of the specific feed ingredient to the total amount of the AA in the mixed diet.

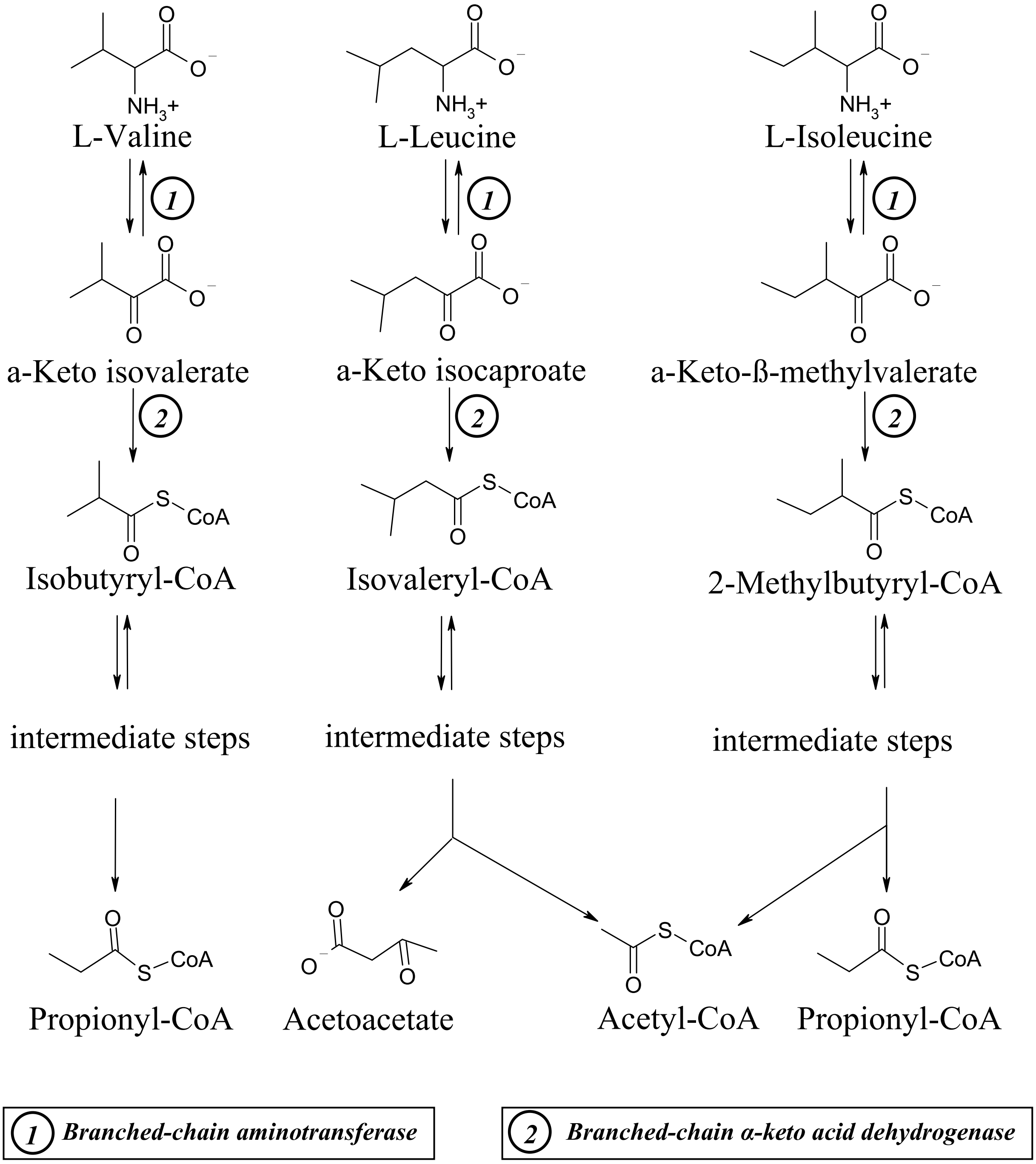
1.3.2 Ideal protein

In the present work, the requirement estimates were determined as SID Ile:Lys and SID Val:Lys ratios, respectively. The ratio to Lys is based on the ideal protein concept which was introduced by ARC (1981) and is very common in diet formulation for pigs. The principal idea is that there exists an optimal dietary pattern among essential AA that corresponds to the needs of the animal. In an ideal protein each essential AA is equally limiting, and the amount of nonessential AA N is sufficient. The ideal protein concept is an appropriate mean to reduce dietary protein oversupply. In the present work, the ideal protein concept of Chung and Baker (1992) was used for crystalline AA supplementation. In pig diets, Lys is usually the first-limiting AA. Additionally, Lys is mainly used for protein synthesis, so that the requirements of the other AA are generally expressed relative to Lys. This makes diet formulation as well as evaluation of AA patterns of feedstuffs quite simple. The impact of genotype, sex, performance level, and environment on the optimal AA:Lys ratio are much smaller than on the optimal daily AA intake, which is a great advantage and enables general conclusions. Therefore, we decided to estimate the Ile and Val requirements as SID Ile:Lys and SID Val:Lys ratios.

1.4 Branched-chain amino acid catabolism

At higher dietary contents, Leu seems to increase the BCAA catabolism and to exhibit negative effects on the nutritional status of Ile and Val, as seen in the present Ile requirement trials. Thus, two trials were conducted focusing on the BCAA catabolism and the impact of increasing dietary Leu levels. The BCAA catabolism is illustrated in Fig. 5.

Fig. 5 Schematic illustration of the branched-chain amino acid catabolism.



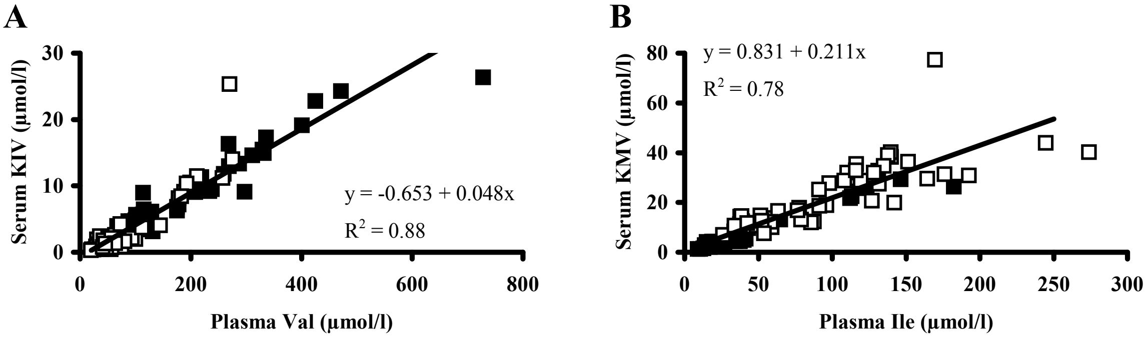
1.4.1 Transamination

The first two steps in the BCAA catabolism are catalyzed by the same enzymes for Ile, Val, and Leu which leads to a competitive situation. The first step is a transamination catalyzed by the branched-chain aminotransferase (BCAT; *EC* 2.6.1.42) isoenzymes. The BCAT isoenzymes are characterized by their location within the cell. The BCAT exists as a mitochondrial (BCATm) and a cytosolic (BCATc) form. The BCATm is widely distributed among tissues whereas the BCATc is restricted to the brain, ovary, and placenta (in rats; Hall et al., 1993). During transamination, the BCAT transfer N from the BCAA to α-ketoglutarate so that BCKA and Glu are formed. The corresponding BCKA of Val, Leu, and Ile are α-keto isovalerate (KIV), α-keto isocaproate (KIC), and α-keto β-methylvalerate (KMV), respectively (Fig. 5).

The transamination is reversible and not limiting the BCAA catabolism. It has been shown in rats that the BCATm activity in skeletal muscle and liver is 30- and 11-fold higher, respectively, than that of the branched-chain α-keto acid dehydrogenase (BCKDH) complex, which is catalyzing the following step of the BCAA catabolism (Suryawan et al., 1998). Because the BCAT is not limiting the BCAA catabolism, little impact of dietary Leu excesses on its activity is supposed. In the present work, doubling of the Leu supply was without impact on BCATm expression in skeletal muscle and liver tissue, what is in line to BCATm activities reported in literature. Leu excesses did not affect the BCATm activity in rat liver or muscle but resulted in a greater than 2-fold increase in liver BCKDH activity one hour after feeding (Block and Harper, 1984). These findings are in accordance to results of Wohlhueter and Harper (1970) for rats. They reported that a 5% surplus of L-Leu to a 9% casein diet was without effect on BCATm activity in liver, but total activity of BCKDH was doubled. However, in kidney BCATm activity was increased almost 2-fold, whereas BCKDH activity remained unchanged.

The Michaelis constant (K_m) of the BCATm varies for the individual BCAA with highest values for Val resulting in a lower rate of clearance from the plasma pool (Staten et al., 1984). In the present work, doubling of the Ile supply increased the plasma Ile levels 2.9-fold whereas doubling of the Val supply increased the plasma Val levels 4-fold. A lower rate of clearance for plasma Val than for Ile is confirmed by the regressions estimated for serum BCKA as a function of its plasma BCAA. As seen in Fig. 6, the slope was 4.4-times higher for KMV as a function of Ile than for KIV as a function of Val. Elango et al. (2002) reported that the plasma levels of Leu were consistently lower compared to the plasma levels of Ile and Val after feeding graded levels of BCAA to piglets. Thus it appears that the BCAT might regulate to some extent the plasma BCAA levels, although the BCKDH is the key enzyme regulating the BCAA catabolism.

Fig. 6 Regression estimates of serum α-keto isovalerate (KIV) as a function of plasma valine (Val; A) and of serum α-keto β-methylvalerate (KMV) as a function of plasma isoleucine (Ile; B). Diets were either limited for isoleucine (■; *n* = 48) or for valine (□; *n* = 48). For details see Publication III.



Most aminotransferases operate at substrate concentrations below the K_m, which is called a low-affinity high-capacity system (Hutson, 2006). In consequence for the BCAA catabolism, the forward reaction, the net N transfer from BCAA to Glu, occurs when the obtained BCKA are removed.

1.4.2 Decarboxylation

The BCKA are oxidatively decarboxylated by a multienzyme complex, the mitochondrial BCKDH complex (*EC* 1.2.4.4). This step commits the carbon skeleton of the BCAA irreversibly to the degradative pathway. The activity of the BCKDH is highly regulated because it is rate limiting for the catabolism of the BCAA. With respect to competitive situations, as dietary Leu excesses, the affinities of the individual BCKA are important. Wohlhueter and Harper (1970) suggested from their experiments with isolated mitochondria that the BCKDH exhibits equal affinities to all three BCKA. Their apparent K_m of BCKDH were 0.2 mM for each BCKA and it was concluded that the K_m doubtlessly exceed the liver concentrations of BCKA of rats fed 30% casein diets, which would provide large reserve of catalytic capacity (Wohlhueter and Harper, 1970). Generally, 50% inhibition is assumed if two substrates with the same affinity are present in the same concentrations, and 75% inhibition will be caused if one substrate is present at 3-times the concentration of the other. For the present work it was concluded that an increased metabolism of Ile and Val might occur, if dietary Leu excesses increase the BCKDH activity to a greater extent than the KIC levels.

1.4.3 Final degradation

The following degradative steps are different for each of the coenzyme A (CoA) derivatives but in conclusion, dehydrogenation and attachment of water follow. These reactions are consistent to the degradation of fatty acids via β-oxidation. In the end, Val catabolism gives propionyl-CoA, Ile catabolism propionyl-CoA and acetyl-CoA, and Leu catabolism acetoacetate and acetyl-CoA (Fig. 5).

Acetyl-CoA plays a key role within the metabolism. For example, it can be oxidized via citrate cycle, used for fatty acid synthesis, and cholesterol synthesis. At high levels of acetyl-CoA, two molecules of it can be metabolized to acetoacetate in the liver via the Lynen-cycle. On the one hand, acetoacetate can be catabolized to aceton and carbondioxide, which leave the organism via breathing. On the other hand, it can be transformed to β-hydroxybutyrate. This reaction is reversible. Acetoacetate and β-hydroxybutyrate are both categorized as ketone-bodies, and can be easily oxidized for energy generation in peripheric tissues and are an important source of energy in the brain during fasting.

Propionyl-CoA is further metabolized to succinyl-CoA and this can be introduced into the citrate cycle for energy generation or it is used for gluconeogenesis. Therefore, succinyl-CoA is metabolized to oxalacetate via the citrate cycle. Oxalacetate can be transformed to phosphoenol-pyruvate which is a precursor for the gluconeogenesis (Karlson, 1984).

Due to the different metabolic fates of their degradation products, Val is absolute glucogenic, and Ile is both ketogenic and glucogenic. In contrast, Leu is absolute ketogenic.

2 Aim of the present study

This work can be divided in two major fields of interest:

the estimation of Ile and Val requirements in weaned pigs and

the investigation of the impact of excess dietary Leu supply on the BCAA metabolism.

For the determination of the Ile and Val requirements, growth assays with a typical dose-response-design were conducted. The Ile and Val requirements were estimated using growth parameters as ADG, ADFI, and G:F. Plasma AA were analyzed to verify these estimates. Additionally, N balance trials were conducted with the same feed to confirm the estimates. Concerning the Ile requirements, two different diets were used to investigate the impact of different protein sources, which caused different dietary Leu levels, on the estimation of Ile requirements. The first diet contained SDBC whereas the second diet was free of SDBC and instead corn gluten feed (CGF) and whey powder were used.

After the requirement trials were conducted, the hypothesis should be elucidated whether high dietary Leu levels increase the BCAA catabolism and therewith the nutritional need for Ile and Val. Thus, two growth assays were conducted. In the first growth assay the pigs received a diet first-limiting in Ile and in the second a diet first-limiting in Val. Performance data as ADG, ADFI, and G:F were used to determine the overall impact of dietary Leu excesses. To gain more information about the impact on the BCAA metabolism, blood samples were taken for analyses of plasma AA and serum BCKA levels. Furthermore, tissue samples were taken for analyses of BCKDH activities and mRNA expression of enzymes of the BCAA metabolism.

3 Material and methods

This chapter is designed to give an overview. For details see Publication I to III.

3.1 Animals and housing conditions

In all trials, crossbreed pigs (German landrace × Pietrain) with an initial BW of 8 to 9 kg from the same commercial plant were used. The gender ratio between female and castrated male pigs was balanced. In the growth assays, 48 pigs were randomly subdivided in six treatment groups and housed individually in pens in an environmentally controlled building. Pigs had *ad libitum* access to food and water. Experimental period started after three days of adaptation to new surroundings and lasted for 35 to 42 days. In case of N balance trails, twelve pigs were subdivided to six treatment groups and housed individually in metabolism crates. Food and water were restricted. Adaptation to new surroundings lasted seven days, as well as pre-collection and collection period. Then the pigs were rearranged to the treatments and a second pre-collection and collection period were conducted.

3.2 Diet composition

In all diets, energy content was calculated to be 13.6 - 13.7 MJ ME/kg. Dietary supply with essential AA other than the one of question were adequate in accordance to the ideal protein after Chung and Baker (1992). Vitamins and minerals were added to meet or exceed recommendations (GfE, 2006; NRC, 1998). All diets contained soya oil to guarantee sufficient supply with essential fatty acids and sugar beet molasses to enhance the flavor and therewith the acceptance of the diets.

In the present work, the Ile and Val requirements were estimated as ratios to Lys. Thus, it was necessary to create experimental diets first-limiting the questioned AA and secondary-limiting Lys to get meaningful estimates. To secondarily limit Lys, average SID Lys concentrations of 1.00% were chosen. These values were decreased by 16% in comparison to German standards (GfE, 2006) and 25 to 27% in comparison to the recommendations of the NRC (1998) for 5- to 10-kg piglets. Beside this, it has been shown for the same genetics and housing conditions as used in the present work that total Lys:ME ratios of at least 0.9 g per MJ are needed for 8- to 30-kg pigs (Roth et al., 1994, 1999). In the present work, total Lys:ME ratios of 0.76 to 0.82 g per MJ were used, indicating Lys limitation. Additionally, it has been shown under commercial conditions that 1.05% SID Lys were deficient for weaned pigs (Kendall et al., 2008). The authors estimated a Lys requirement of 1.30% SID Lys for 11- to 27-kg pigs. This estimate was confirmed by Yi et al. (2006). A SID Lys requirement of 1.28 to 1.32% was estimated for optimal ADG in weaned pigs (12 to 25 kg). In experiments for the determination of Ile requirements, diets containing up to 1.10% SID Lys were proven to be secondarily limiting in Lys for weaned pigs (Fu et al., 2006b). In recent work for the determination of Val requirements, 1.00% SID Lys were limiting the ADG of 12- to 25-kg pigs fed diets based on corn, wheat, barley, and soybean meal (Barea et al., 2008). In conclusion, it is well documented in literature that the chosen level of 1.00% SID Lys is performance limiting in weaned pigs.

3.2.1 Estimation of valine requirements: Publication I

Growth assay 1: Basal diet was mainly based on wheat (30.0%), corn (24.2%), whey powder (10.0%), barley (10.0%), and field peas (7.0%). CP was analyzed at 18.0%. Basal diet contained 1.02% SID Lys. Dietary Val supply was increased by L-Val supplementation and the following SID Val:Lys ratios were estimated: 56, 62, 66, 75, 76, and 84%.

Growth assay 2: Basal diet was mainly based on wheat (30.0%), corn (24.0%), whey powder (10.0%), barley (13.9%), and field peas (10.0%). CP was analyzed at 17.7%. Basal diet contained 0.99% SID Lys. Dietary Val supply was increased by L-Val supplementation and the following SID Val:Lys ratios were estimated: 49, 55, 58, 63, 67, and 74%.

N balance trial: The same diets as in Growth assay 2 were used.

3.2.2 Estimation of isoleucine requirement: Publication II

Growth assay 1: Basal diet was mainly based on wheat (34.0%), barley (28.5%), corn (20.8%), and SDBC (7.5%). CP was analyzed at 18.4%. Basal diet contained 1.00% SID Lys and 0.36% SID Ile. Dietary Ile supply was increased in five equal increments of 0.06% by L-Ile supplementation and the following SID Ile:Lys ratios were estimated: 36, 43, 50, 56, 63, and 71%.

Growth assay 2: Basal diet was mainly based on wheat (22.8%), corn (21.8%), CGF (20.0%), barley (15%), and whey powder (5.0%). CP was analyzed at 16.8%. Basal diet contained 0.97% SID Lys and 0.35% SID Ile. Dietary Ile supply was increased in five equal steps of 0.06% by L-Ile supplementation. SID Ile:Lys ratios of 36, 44, 53, 59, 67, and 72% were calculated.

N balance trial: The same diets as in Growth assay 1 were used.

3.2.3 Interactions between the branched-chain amino acids: Publication III

Growth assay 1: The basal diet was mainly based on barley (26.8%), wheat (23.5%), CGF (15.0%), soybean meal (13.2%), and corn (10.0%). CP was analyzed at 18.1%. Basal diet was first-limiting in Ile. At a constant SID Ile level of 0.50% the Leu supply was increased and SID Leu levels were 1.15, 1.68, 1.95, and 2.21%. The SID Leu levels were 100, 150, 175, and 200% (Treatment 1 to 4) compared to Treatment 1 (basal diet). In Treatment 5, the effects of a simultaneous oversupply of Leu and Val were tested. Thus, the dietary supply of Leu and Val were doubled compared to the basal treatment (SID Leu: 2.19%; SID Val: 1.4%). Treatment 6 was a positive control (SID Leu: 1.13%; SID Ile: 6.3%; SID Val: 7.3%).

Growth assay 2: Composition of basal diet was mainly the same as in Growth assay 1. But the basal diet was first-limiting in Val (SID Val: 0.62%) instead of Ile. Again, the Leu supply was increased to give 100, 150, 175, and 200% compared to the basal diet (SID Leu: 1.11, 1.63, 1.91, and 2.17%). In Treatment 5, the effects of a simultaneous oversupply of Leu and Ile were tested by doubling their supply compared to the basal treatment (SID Leu: 2.23%; SID Ile: 1.30%). Treatment 6 was a positive control (SID Leu: 1.11%; SID Ile: 0.64%; SID Val: 0.73%).

3.3 Sample collection

In the growth assays, the animals BW and feed consumption were recorded weekly for calculation of performance parameters. In the N balances, pigs were weighed at the beginning of the pre-collection period and at the termination of the collection period. Feed consumption was recorded during the collection period.

In the N balance trials, feces and urine of individual pigs were quantitatively collected two times daily. Urine (acidified to pH ~3) was stored at 4°C. Feces were stored at –20°C until the termination of the collection period. Then, feces were freeze-dried and stored at room temperature for further analyses.

Blood samples were taken at the termination of the trials via jugular vein puncture (plasma: S-Monovette; serum: Gel-S-Monovette; Sarstedt, Nümbrecht, Germany) after a fasting period of 2.5 h. Plasma and serum were obtained by centrifugation (800 *g,* 20 min), and stored at -80°C.

In the trials dealing with the interactions between the BCAA, tissue samples of muscle (*Longissimus dorsi*), heart, liver, spleen, ileum and jejunum were taken for determination of enzyme kinetics and mRNA expressions. Thus, the pigs were killed by captive bolt pistol and exsanguinated by transsection of the carotid arteries. Pigs were at comparable postparandial conditions at the time of killing. The gained tissue samples were quick-frozen in liquid N and stored at –80°C.

3.4 Analyses

3.4.1 Feed analyses

N was analyzed using the Kjeldahl procedure (Naumann and Bassler, 1997) and CP content was calculated (N × 6.25). AA contents were analyzed by ion exchange chromatography after acid hydrolysis with HCl (Commission directive 98/64/EC, 1998). Met and Cys (Cystine) were assayed after performic acid oxidation (Commission directive 98/64/EC, 1998). Trp was determined by reverse phase high performance liquid chromatography and fluorometric detection after alkaline hydrolysis with barium hydroxide (AFNOR, 1998).

3.4.2 Nitrogen analyses of urine and feces

N analyses were performed using the Kjeldahl procedure (Naumann and Bassler, 1997).

3.4.3 Plasma free amino acids and urea

Plasma proteins were removed by precipitation with salicylsulfonic acid and centrifugation (11000 *g*, 10 min). After dilution with a lithium acetate solution, the protein-free supernatant was analysed by ion exchange chromatography on an automatic AA analyser (LC 3000, Biotronik, Hamburg, Germany; Naumann and Bassler, 1997). Plasma urea was automatically co-analyzed in the chromatograms for the determination of plasma-free AA.

3.4.4 Serum branched-chain α-keto acids

BCKA were analysed as reported by Pailla et al. (2000). BCKA were derivatised with o-phenylenediamine to give fluorescent derivates, which were separated chromatographically on a reversed-phase column (Spherisorb ODS-2, 4.6 × 250 mm, 5 µm particles, Waters, Eschborn, Germany) using a binary gradient (L-7100, Merck-Hitachi, Tokyo, Japan). Detection was performed fluorimetrically (FL Detector L-7480, Merck-Hitachi, Tokyo, Japan). α-Keto valerate was used as an internal standard.

3.4.5 Liver branched-chain α-keto acid dehydrogenase activity

BCKDH activity was assayed spectrophotometrically as described by Nakai et al. (2000). A frozen tissue sample was pulverized to a fine powder under liquid N. Thereafter, 0.25 g of the powder were homogenized (motor driven Tefflon pestle) in ice-cold extraction buffer.

Insoluble material was removed by centrifugation (20000 *g*, 5 min, 4°C) and the supernatant was made 9% (v/v) in polyethylene glycol. After 20 min on ice, a second centrifugation step (12000 *g*, 10 min, 4°C) was performed, and the pellet dissolved in suspension buffer. BCKDH activity was determined at 30°C using KIV as substrate, by measuring absorbance at 340 nm to detect NADH formation. To determine total BCKDH activity, the tissue extract was incubated (20 min, 37°C) with lambda protein phosphatase before measurement. In contrast to Nakai et al. (2000), the assay buffer was made without dihydrolipoamide dehydrogenase.

3.4.6 Total RNA extraction

Total RNA was extracted with peqGOLD TriFast (PEQLAB Biotechnologie, Erlangen, Germany). Tissue samples (50 mg) were homogenized using MatrixGreen beads in the MagNA Lyser (Roche Diagnostics, Mannheim, Germany) in the presence of peqGOLD TriFast (0.5 ml). After addition of chloroform (100 µl) and centrifugation (12000 *g*, 15 min), the homogenate was separated into three phases, with the RNA in the upper aqueous phase. The extracted RNA, free of DNA and proteins, was precipitated with isopropanol (150 µl) and centrifugation (12000 *g*, 10 min, 4˚C). Pellets were washed twice with 250 µl ethanol (75% v/v, -20˚C), followed by centrifugation (10000 *g*, 5 min, 4˚C). RNA was dissolved in 30 µl diethylpyrocarbonate (DEPC)-treated water and stored at –80˚C.

Liver samples were additionally treated with peqGOLD OptiPure (PEQLAB Biotechnologie, Erlangen, Germany) to eliminate polysaccharides. Pellets obtained by extraction with peqGOLD TriFast were mixed with 100 µl peqGOLD OptiPure. After centrifugation (3000 *g*, 10 min, 4˚C), the supernatant was removed and 100 µl sodium dodecylsufate (0.5% (w/v), pH 7) were added. After incubation for 5 min at 55˚C, 100 µl chloroform were added and the mixture was centrifuged (3000 *g*, 5 min, 4˚C). Natrium acetate (2 M, pH 5) was added to the supernatant to a final concentration of 0.2 M. RNA was precipitated with isopropanol (100 µl). Centrifugation and washing steps were as described for the extraction. Pellets were dissolved in 60 µl DEPC-treated water.

3.4.7 RNA quantity

RNA quantity was determined photometrically (Nanodrop 1000, PEQLAB Biotechnologie, Erlangen, Germany) and purity calculated from the 260/280 nm absorbance ratio. All working solutions were diluted to a RNA concentration of 10 ng/µl.

3.4.8 RNA quality

Integrity of RNA was analysed for six random samples per tissue and trial using the RNA 6000 Nano assay (Agilent Technology, Palo Alto, USA) and the 2100 Bioanalyser (Agilent Technology, Palo Alto, USA). The RNA integrity number (RIN) served as an RNA quality parameter ranging from one (the most degraded profile) to ten (the most intact profile).

3.4.9 Primer design

Primers were designed using published RNA sequences of pigs (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), if available. Otherwise they were designed as nested primers from homologous regions of cattle, human, and rat genes. Primer design accounted for primer-dimer formation, self-priming, and primer annealing temperature (60˚C) (http://fokker.wi.mit.edu/primer3/input.htm). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Primer testing included different annealing temperatures and product validation via agarose gel electrophoresis for four random samples and a negative control for each primer set.

3.4.10 PCR analysis

Quantitative real-time PCR (qRT-PCR) was conducted using a SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen Corporation, Carlsbad, USA). For each sample, 5 µl 2X SYBR Green Reaction Mix, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), and 0.2 µl SuperScript III RT/Platinum *Taq* Mix were mixed and 3.8 µl template (total RNA concentration: 10 ng/µl) were added. qRT-PCR was performed with a Rotor-Gene 6000 (Analysis Software 6.0; Corbett Life Science, Sydney, Australia) using the following protocol: hold step (55˚C, 3 min), denaturation step (95˚C, 5 min), cycling (95˚C, 15 s; 60˚C, 10 s; 68˚C, 20 s; 40 cycles), hold step (40 ˚C, 1 min), and melting curve analysis.

The products obtained by qRT-PCR were validated by 1.8% agarose gel electrophoresis at 90 V for 30 min. After gel extraction (Wizard SV Gel and PCR Clean-Up system, Promega Cooperation, Madison, USA), sequencing was performed by the Department Biology Genomics Service unit at the Ludwig-Maximilians-Universität München. Nested primers were designed from the determined sequences.

3.5 Statistical analyses

Data were analysed by ANOVA using the mixed procedure of Statistical Analysis Systems statistical software package version 9.1. (SAS Institute, Cary, NC, USA) with individual pigs as experimental units. The model included treatment and sex as fixed and litter as random effects. Initial BW was used as a covariate. Contrasts were performed to determine the linear and quadratic effects of an increasing dietary Ile, Val, or Leu supply, respectively (Lowry, 1992). The integrated matrix language procedure was used to generate orthogonal polynomial coefficients and the mixed procedure was used to compute orthogonal polynomial sums of squares. *P*-values for treatment are given in the tables.

Estimates of requirement were done by subjecting the group least square means data to least square broken-line methodology (Robbins et al., 2006).

In the trials on the BCAA interactions, the effects of doubling the Val or Ile supply at high levels of dietary Leu were tested for significance in comparison to a single Leu oversupply using the Tukey adjustment. The effects of the positive control were tested for significance in comparison with the basal group using the Tukey adjustment.

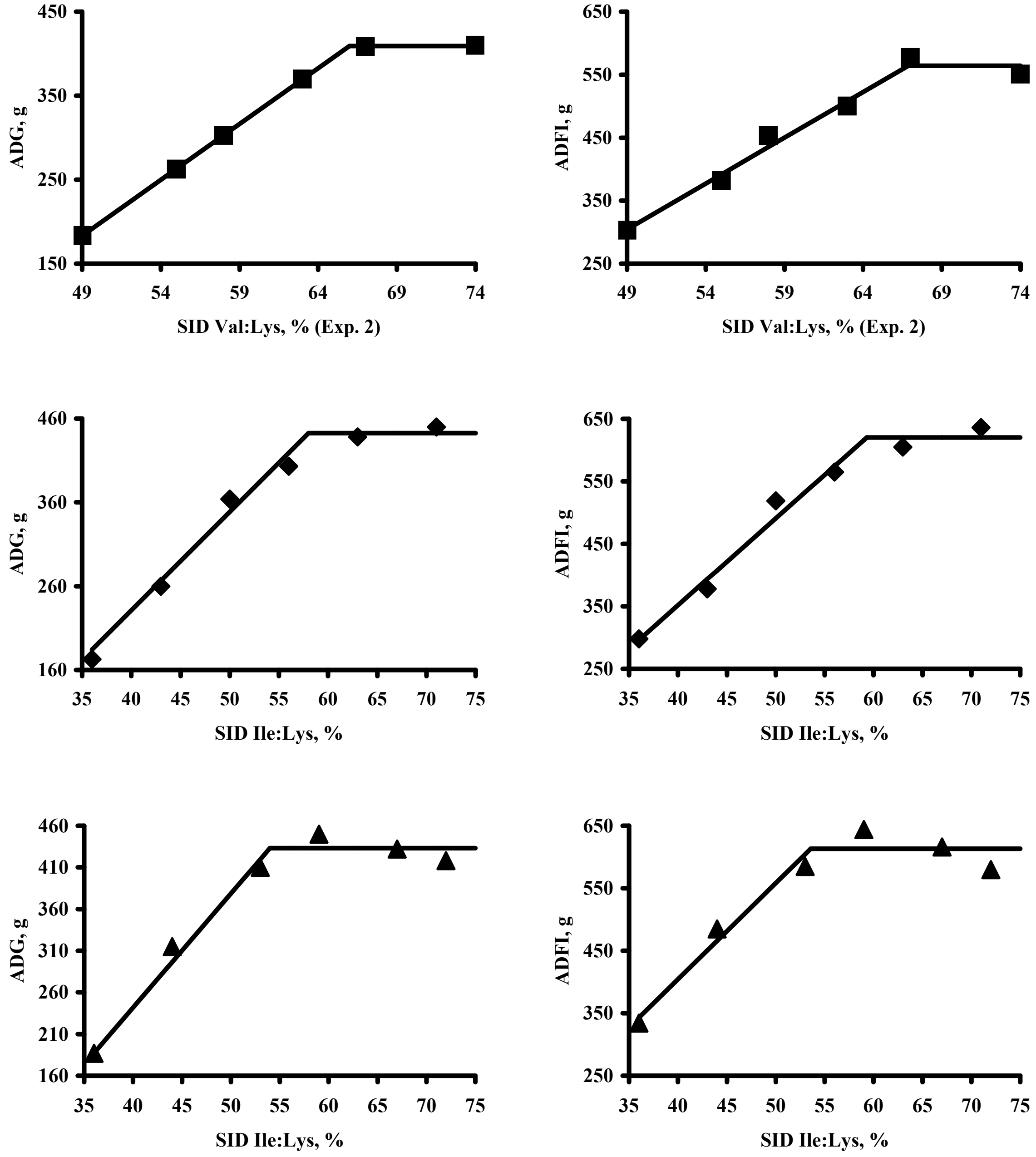
mRNA expression data were analysed using the relative quantification method, which describes the change in target gene expression relative to the control group. β-Actin, ubiquitin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were used as normalizing internal controls. For normalization, the tissue- and sample-specific arithmetic means of the three internal control genes served as a control gene index. Cycle thresholds (Ct) of the target genes were subtracted from the control gene index (ΔCt) for normalization, and treatment groups were compared to the control group (ΔΔCt; Livak and Schmittgen, 2001), using the general linear models procedure and the Tukey adjustment. To determine gene expression differences between tissues, the general linear models procedure and the Tukey adjustment were applied to Ct values.

4 Results and discussion

4.1 Estimation of isoleucine and valine requirements

To estimate requirements, the experimental data have to be fitted to a statistical model and various approaches can be used. In the present work, the broken-line methodology was used.

Fig. 7 Fitted single-slope broken-line plots of average daily gain (ADG) and average daily feed intake (ADFI) as a function of standardized ileal digestible (SID) valine (Val) to lysine (Lys) ratio (■), and as a function of SID isoleucine (Ile) to Lys ratio in diets containing spray-dried blood cells (♦) or corn gluten feed (▲) as a protein source. For details see Publication I and Publication II.



In recent work, it has been shown that the efficiency of utilization of limiting AA, including Ile, Leu, and Val, is constant at levels of intake ranging from maintenance to 85% of the requirement (Heger et al., 2003). The efficiency of utilization was almost the same for all BCAA and ranged from 81 to 82%. In the present growth assays on the estimation of Ile and Val requirements, the ADG and ADFI increased linearly as the dietary supply either of Ile or Val increased but formed a plateau at high dietary levels. The adjustment of the data to the broken-line model was very good as illustrated for the ADG and ADFI in Fig. 7. The R^2-values of the ADG ranged from 0.98 to 0.99. At the plateau phase, the deviation within one dietary group is generally highest because these animals got a sufficient nutrient supply so that performance differences due to different genetically potentials can develop. Within the plateau phase, the treatment mean values will deviate around the desired value due to naturally deviation. In the present work, pigs were allotted to treatments on the basis of weight, and sex and ancestry were equalized across treatments in a randomized block design to reduce this deviation.

4.1.1 Isoleucine requirements

The isoleucine requirements were estimated with two different diets. The first diet contained 7.5% SDBC, and optimum SID Ile:Lys ratios of 59% were estimated for ADG and ADFI. The second diet contained CGF instead of SDBC, and optimum SID Ile:Lys ratios of 54% were estimated for ADG and ADFI. The bioavailability of the supplemented crystalline L-Ile was significantly decreased when SDBC were fed. Slope-ratio was estimated with 0.67. In other words: the use of 7.5% SDBC decreased the biological efficiency of the supplemented L-Ile about 33%. It was concluded that the increased dietary Leu levels, caused by the use of SDBC, increased the BCAA catabolism, and therewith increased the nutritional need for Ile. This conclusion was supported by the plasma Ile data, as seen in Fig. 8. The basal levels of plasma Ile were the same for both diets. However, the incremental Ile supply caused a much steeper slope of the plasma Ile levels when the diets were free of SDBC. At the highest increment, the plasma Ile levels of pigs fed CGF diets were 2.7-fold increased compared to that of pigs fed SDBC diets.

Our findings are in accordance to literature. At a similar inclusion level of SDBC as in the present work, an optimal ratio of SID Ile:Lys of 62% was estimated in 5- to 9-kg pigs (6.0% SDBC; Hinson et al., 2007). This estimate is close to the present estimates of 59% SID Ile:Lys determined in diets containing 7.5% SDBC. When higher levels of SDBC were included in diet composition, increased optimal Ile:Lys ratios were reported. In 12- to 22-kg pigs fed corn-SDBC-based diets containing 10.75% SDBC, a SID Ile:Lys ratio of at least 70% has been estimated for ADG (Fu et al., 2006b). The higher nutritional need for Ile in SDBC diets has also been demonstrated in late-finishing pigs. Using corn-SDBC diets (5% SDBC) increased the nutritional need for Ile from 0.24 to 0.34% in comparison to corn-soy bean meal-based diets (Dean et al., 2005). Lower Ile estimates in SDBC-free diets were also reported for weaned pigs. In 12- to 22-kg pigs fed corn-soybean meal-based diets, increasing the SID Ile:Lys ratio from 46 to 74% had no effect indicating that the basal SID Ile:Lys ratio of 46% was already sufficient (Fu et al., 2006b). The SID Ile:Lys ratio given by the NRC (1998) is 55%, and almost the same as the estimates made for the CGF diets in the present work (54%).

4.1.2 Valine requirements

In the present work, estimates of optimum SID Val:Lys ratios were 66 and 67% for ADG and ADFI, respectively (Fig. 7). These findings were confirmed by the N balance trial. Estimates for optimum N retention were 65% SID Val:Lys. However, optimum SID Val:Lys ratios for G:F and N utilization were 61 and 62%, respectively. These findings underline the importance of *ad libitum* feeding for the estimation of AA requirements. Val seems to exhibit some positive effects on feed intake and this aspect needs further investigation. It was concluded that an optimum SID Val:Lys ratio of 65 to 67% is necessary for optimal performance in weaned pigs.

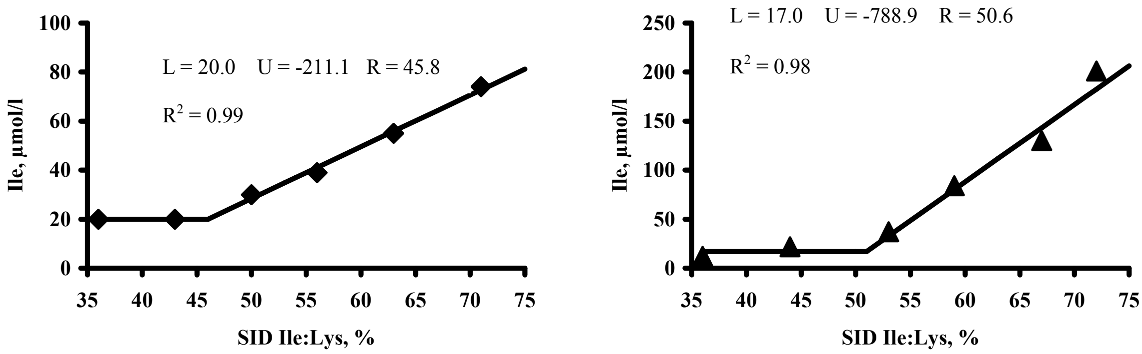
Our findings for ADG and ADFI are close to NRC (1998) recommendations for 10- to 20-kg pigs, and to the results of Chung and Baker (1992) for 10-kg pigs, with an ideal Val:Lys ratio of 68%. From recent work on the Val requirements of 12- to 25-kg pigs it was concluded that a SID Val:Lys ratio of at least 70% is needed (Barea et al., 2008) which is slightly higher than the estimates of the present study.

Other studies have estimated the Val requirements on the basis of SID Val level in the diet and not as ratio of SID Val:Lys. For 10- to 20-kg pigs, estimates for SID Val of 0.78% appear in the literature (Mavromichalis et al., 2001; Gaines et al., 2006). Comparison of these estimates with the present work is difficult because the diets used in the present work were secondly limiting Lys. Thus, in the present work only the ratio of Val to Lys is a meaningful estimate of the Val requirement and no conclusions about absolute dietary Val needs are possible. However, Mavromichalis et al. (2001) additionally expressed their estimates relative to ME and concluded that 10- to 20-kg pigs require no more than 2.22 g SID Val per Mcal of ME which is 5% higher than NRC (1998) estimates. The NRC (1998) also provides an optimal ratio of SID Val:Lys of 69% which is 3 to 5% higher than the estimates for ADG and ADFI of the present work of 66 and 67% SID Val:Lys, respectively. However, it was clearly demonstrated in the present work that SID Val:Lys ratios of 67% were sufficient to maximize performance. Higher SID Val:Lys ratios did not increase performance further on.

4.1.3 Plasma amino acids

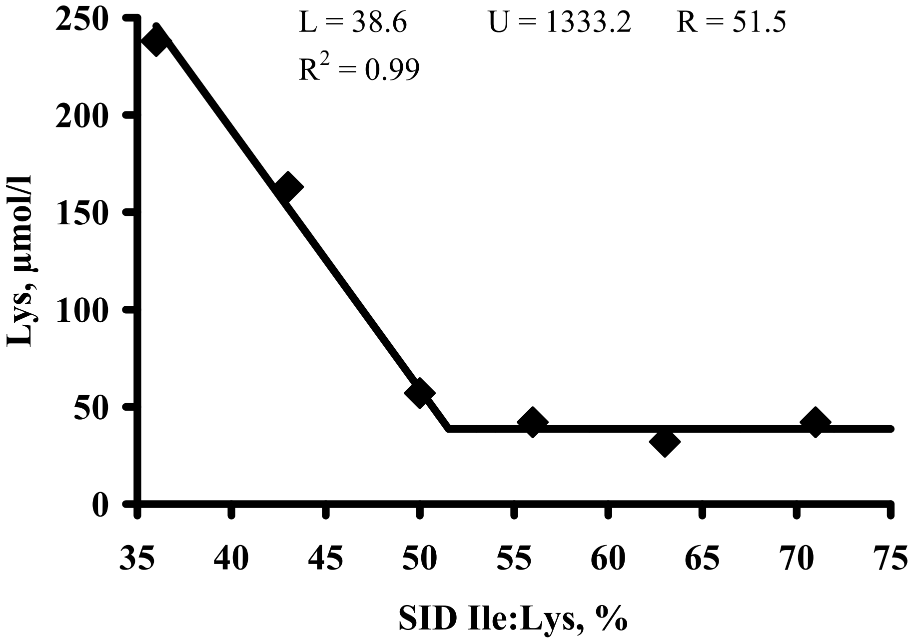
For estimation of AA requirements different parameters can be used. In the present work, performance parameters as ADG, ADFI, and G:F were used as well as plasma levels of AA and urea. N balance trails were additionally conducted to confirm the growth assay data. In general, the estimates for ADG and ADFI were consistent to each other (Fig. 7), and the plasma AA data confirmed these estimates. Increasing dietary supply of an essential AA from deficit to oversupply has little effect on its plasma levels until the requirements are met, but will markedly and linearly increase plasma levels when the diet contains adequate or excess amounts (Morrison et al., 1961; Zimmerman and Scott, 1965). Our data are in accordance to these findings (Fig. 8).

Fig. 8 Effects of increasing dietary standardized ileal digestible (SID) isoleucine (Ile) to lysine (Lys) ratio by crystalline Ile supplementation on plasma Ile levels in pigs fed diets containing spray-dried blood cells (♦) and in pigs fed diets containing corn gluten feed (▲). For details see Publication II.



Plasma concentrations of other non-limiting essential AA will tend towards minima when the requirement of the limiting AA is met, since protein synthesis should be maximal. This was also seen in the present work, and is illustrated for Lys in Fig. 9. These findings have been used to estimate AA needs in rats, chicks and pigs (Morrison et al., 1961; Zimmerman and Scott, 1965; Mitchell et al., 1968; Oestemer et al., 1973b).

Fig. 9 Effects of increasing dietary standardized ileal digestible (SID) isoleucine (Ile) to lysine (Lys) ratio by crystalline Ile supplementation on plasma Lys levels in pigs fed diets first-limiting in Ile and secondary-limiting in Lys and containing spray-dried blood cells. For details see Publication II.



The informative value of plasma AA for requirement estimates is dependent on the feeding regime. If animals are fasted overnight before blood sampling, the animals are in a postabsorptive status, body protein is mobilized and the plasma AA do not allow conclusions about the sufficiency of the fed diet. If animals were fasted for 16 h but fed 30 min before blood sampling, plasma AA data could not be used to estimate the requirement as well, because the plasma levels of the AA first-limited in the diet increased linearly (Zimmerman and Scott, 1967; Kelly and Scott, 1968). To get plasma AA data that can be used to estimate requirements, the animals have to be in the postprandial phase but close to steady state conditions. Therefore, the animals were fed *ad libitum* in the present work. Blood samples were taken in the morning after a fasting period of 2.5 h because plasma AA were found to reach maxima at one to two hours after feeding and were then constant until five hours after feeding (Braude et al., 1974; Prieto et al., 1994).

4.2 Branched-chain amino acid interactions

In the catabolism of the BCAA, the BCKDH enzyme complex plays a key role and is also important as a possible place of action for interactions between the BCAA. Thus, the BCKDH complex was a focus of the present work. The next chapter will deal with its structure because it is sophisticated but essential for the understanding of the made investigations and gained results.

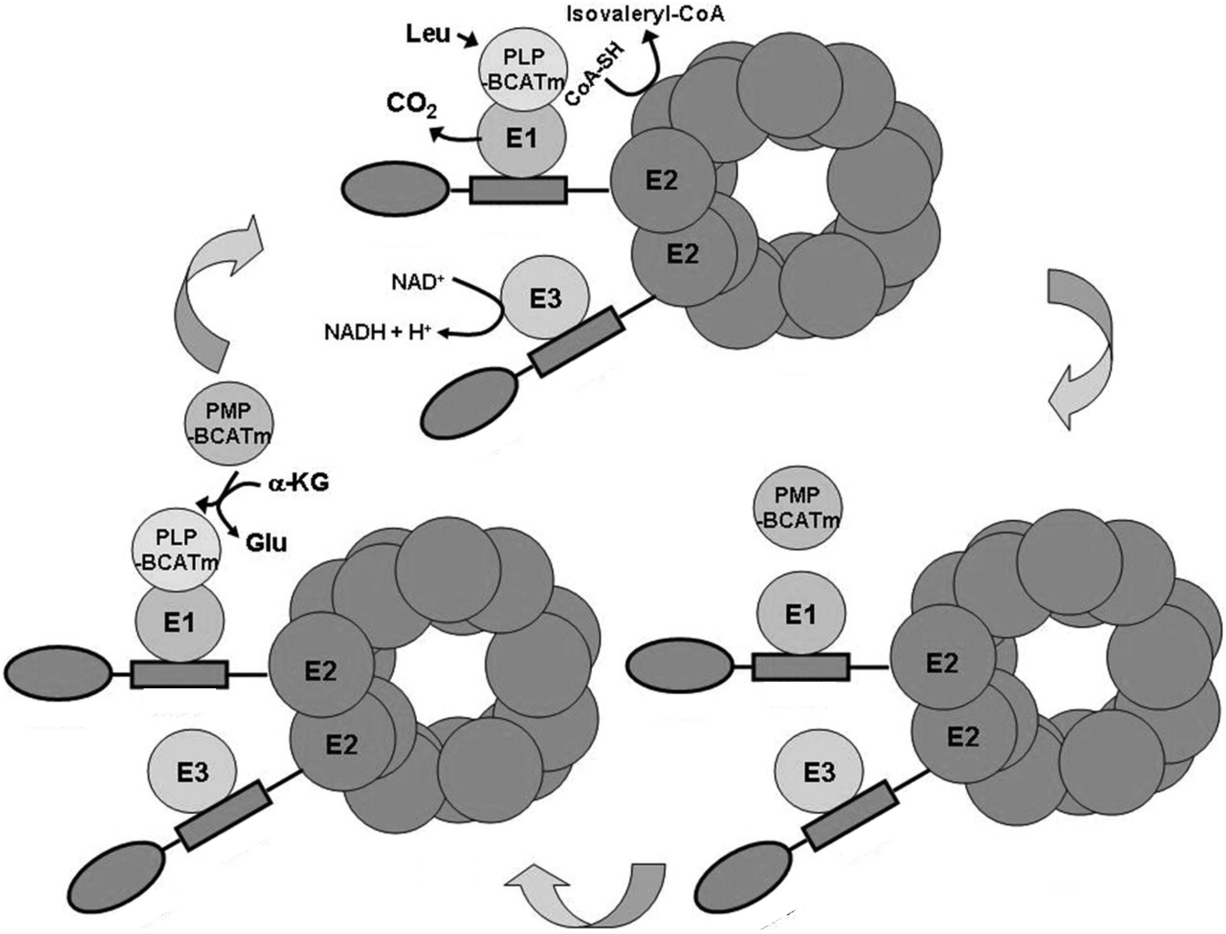
4.2.1 Structure of the branched-chain α-keto acid dehydrogenase

The BCKDH complex is the key regulatory enzyme of the BCAA catabolism and consists of three catalytic components. The E1 subunit, a heterotetramer of α and β subunits ($\alpha_2\beta_2$), is a branched-chain α-keto acid decarboxylase. The E2 subunit (homo-24-meric) is a dihydrolipoyl transacyclase and the E3 subunit (homodimeric) is a dihydrolipoyl dehydrogenase (Yeaman, 1989; Naik and Huang, 2004). The E1 and E2 components are specific for BCKDH, whereas the E3 is common to other dehydrogenases. The E2 subunits play an important role for proper structural assembly and catalytic activity of the whole BCKDH complex. The 24 E2 subunits form three independently folded domains: the first is a lipolyl bearing domain (AA 1-84), which mediates the substrate channeling (eight β-strands forming a barrel), the second is a E1/E3 binding domain (AA 104-152) and the third forms the inner-core domain (AA 168-395; Naik and Huang, 2004). The structural core of the enzyme complex binds twelve E1 tetrameres and six E3 dimeres as well as the kinase and the phosphatase through non-covalent interactions (Cook et al., 1985; Hakozaki et al, 2002). Furthermore, there is some evidence that even the BCATm is attached to the BCKDH and this metabolic unit is called BCAA-metabolon (Hakozaki et al., 2002; Fig. 10).

The activity of the BCKDH complex is highly regulated by phosphorylation and dephosphorylation (Shimomura et al., 1990, Damuni et al., 1984). The only enzyme catalyzing phosphorylation and therewith causing inactivation of the BCKDH is the branched-chain α-keto acid dehydrogenase kinase (BCKDK; Joshi et al., 2006). The BCKDK phosphorylates two Ser residues of the E1α subunit but it binds to the E2 subunit and only the bound form is active (Harris et al., 1986; Obayashi et al., 2001). Thus, there is a good correlation between the BCKDH activity and the bound form of BCKDK (Kuzuya et al., 2008). But the greatest part of the BCKDK is unbound. Therefore, there is only a slight correlation between BCKDH activity and total amount of BCKDK and no correlation was seen between BCKDK mRNA expression and BCKDK bound to the BCKDH as well as between BCKDK mRNA expression and BCKDK activity (Obayashi et al., 2001; Obayashi et al., 2004).

The present work focused on the first two steps of the BCAA catabolism because these are common to Ile, Val, and Leu so that complementary situations due to increasing Leu supply might occur here. Plasma AA, serum BCKA, and mRNA expressions of the BCATm, BCKDH subunits (E1α, E1β, E2), and BCKDK were investigated. The mRNA expression of the BCKDH E3 subunit was not measured because this enzyme is not specific to the BCKDH complex. The BCATc was not investigated because it is not expressed in the tissues of interest of the present work.

Fig. 10 Model of the branched-chain amino acid-metabolon (Islam et al., 2007). The mitochondrial brached-chain aminotransferase (BCATm) binds directly to the E1 subunit of the branched-chain α-keto acid dehydrogenase complex. α-Ketoglutarate (α-KG) binds to the pyridoxamine 5'-phosphate (PMP) form of BCATm regenerating the pyridoxal-5'-phosphate (PLP) form and initiates a new reaction cycle. Substrate is channelled from BCATm to the E1 subunit.



4.2.2 *Localization of the enzymes*

All enzymes of the catabolic pathway of the BCAA are located in the mitochondria of the cells of several tissues with the exception of the BCATc which is located in the cytosol. BCATc is restricted to brain, ovary and placenta (Hall et al., 1993; Ichihara, 1985) and was not investigated in the present work. Activity of the enzymes is tissue specific (Harper et al., 1984). In the present work, skeletal muscle, heart, liver, spleen, ileum, and colon were used for expression analyses. As shown in detail in Publication III, highest BCATm expressions were seen in heart and skeletal muscle, intermediate in spleen and jejunum, and lowest in ileum and liver. BCATm expressions in skeletal muscle and heart were increased about 20- and 130-fold, respectively, compared to that in liver tissue. Thus, muscle tissue seems to be responsible for a major part of the BCAA transamination in pigs. In literature, highest BCATm activities were reported for peripheral tissues as well. In rats, highest BCATm activities occurred in pancreas followed by stomach, heart, kidney, brain, and skeletal muscle. Lower levels were reported for the colon, small intestine, and adipose tissue, whereas BCATm was not expressed in rodent and human liver (Harper et al., 1984; Suryawan et al., 1998). In rats, the BCATm activity in heart was 10 times higher than in small intestine, whereas in humans it was just increased 1.60 times (Suryawan et al., 1998). Suryawan et al. (1998) calculated that the skeletal muscle provides for about 80 and 65% of BCAA transamination capacity in rats and humans, respectively. These findings are in line to the present work and underline the importance of muscle tissue for the transamination of the BCAA.

The relevance of muscle tissue for BCAA catabolism is quite unusual. Generally, liver is the main organ undertaking degradation of AA. In skeletal muscle, the BCAA serve as a main N donor for the formation of Gln and Ala. Gln is an important energy source for rapid dividing cells as enterocytes and immune cells. Furthermore, it is a substrate for protein synthesis and a precursor for synthesis of nucleotides. In the kidney, it is the most important precursor for ammoniagenesis. Ala is an important precursor for gluconeogenesis in liver during starvation and severe illness. After an overnight fast, at least 20% of the N required for Ala synthesis are derived from Leu, and Val and Ile are an important source of the required carbon skeletons (Platell et al., 2000). Increased needs for Gln and Ala during stages of severe illness as sepsis, trauma, or burns are covered to a major extend by BCAA deamination in skeletal muscle (Holecek, 2002). Recent literature indicated that there is a cycle between liver and muscle tissue. The BCKA which are released from muscle tissue can be used for resynthesis of BCAA in liver tissue. Then, the BCAA can be released from liver tissue and are again available to muscle tissue which completes the cycle (Holecek, 2002). This cycle is an important adaptive response that resupplies BCAA and prevents the rapid development of a negative N balance and therewith muscle-protein wastage.

The importance of liver for BCAA catabolism lays in the second catalytic step, the oxidative decarboxylation of the BCKA. In the present work, mRNA expression of BCKDH subunits (E1α, E1β, E2) was highest in liver and heart. On an average, BCKDH expressions in liver tissue were 2- to 4-fold increased compared to that in skeletal muscle. In Publication III, tissue specific mRNA expression data for the BCKDH subunits were reported the first time in pigs. It is confirmed in literature for different species that liver tissue is the main organ for BCKA decarboxylation. In rats, highest BCKDH activities were reported for liver and kidney, intermediate for heart, stomach, and pancreas, and lowest for small intestine and skeletal muscle (Suryawan et al., 1998). In humans, highest BCKDH activities were seen in kidney, brain, and liver, intermediate in heart and lowest in stomach, skeletal muscle and small intestine. The activity state of the BCKDH was also tissue specific and ranged in rats from 7% for skeletal muscle to 88% for liver whereas in humans it was 28% for liver and 26% for skeletal muscle (Suryawan et al., 1998). The liver BCKDH activity states of pigs seen in the present work were similar to that of humans and ranged from 7 to 27%.

To estimate the relative importance of a specific organ for the BCAA catabolism the catalytic capacity needs to be determined. In liver of pigs, the total BCKDH activity per g tissue was reported to be about 40-times higher than in muscle. Expressed per mg protein, the total BCKDH activity in liver was still 18-times higher than in muscle (Langer et al., 2000). With respect to the different organ weights, the BCKDH capacity of a 40-kg pig was calculated to be 25.9 and 11.5 mmol/d for liver and muscle tissue, respectively (Langer et al., 2000). In rats, the liver accounts for 83% and skeletal muscle for 3% of BCKDH capacity while in humans the liver accounts for about 13% but the skeletal muscle for about 54% of BCKDH capacity (Suryawan et al., 1998). Suryawan et al. (1998) suggested that in humans the transfer of metabolites between skeletal muscle and liver might be less extended than in rats and that in humans the skeletal muscle is the major site of BCAA catabolism.

From the present work and the data available for pigs, it can be concluded that the BCAA catabolism of pigs is somewhere between that of rats and humans. The transamination seems to take place mainly in muscle tissue whereas the decarboxylation seems to be located mainly in liver tissue. These findings confirm the suggestion made in rodents that the BCAA are not catabolized to a great extent by first-pass metabolism in the liver and are thus available to peripheral tissues.

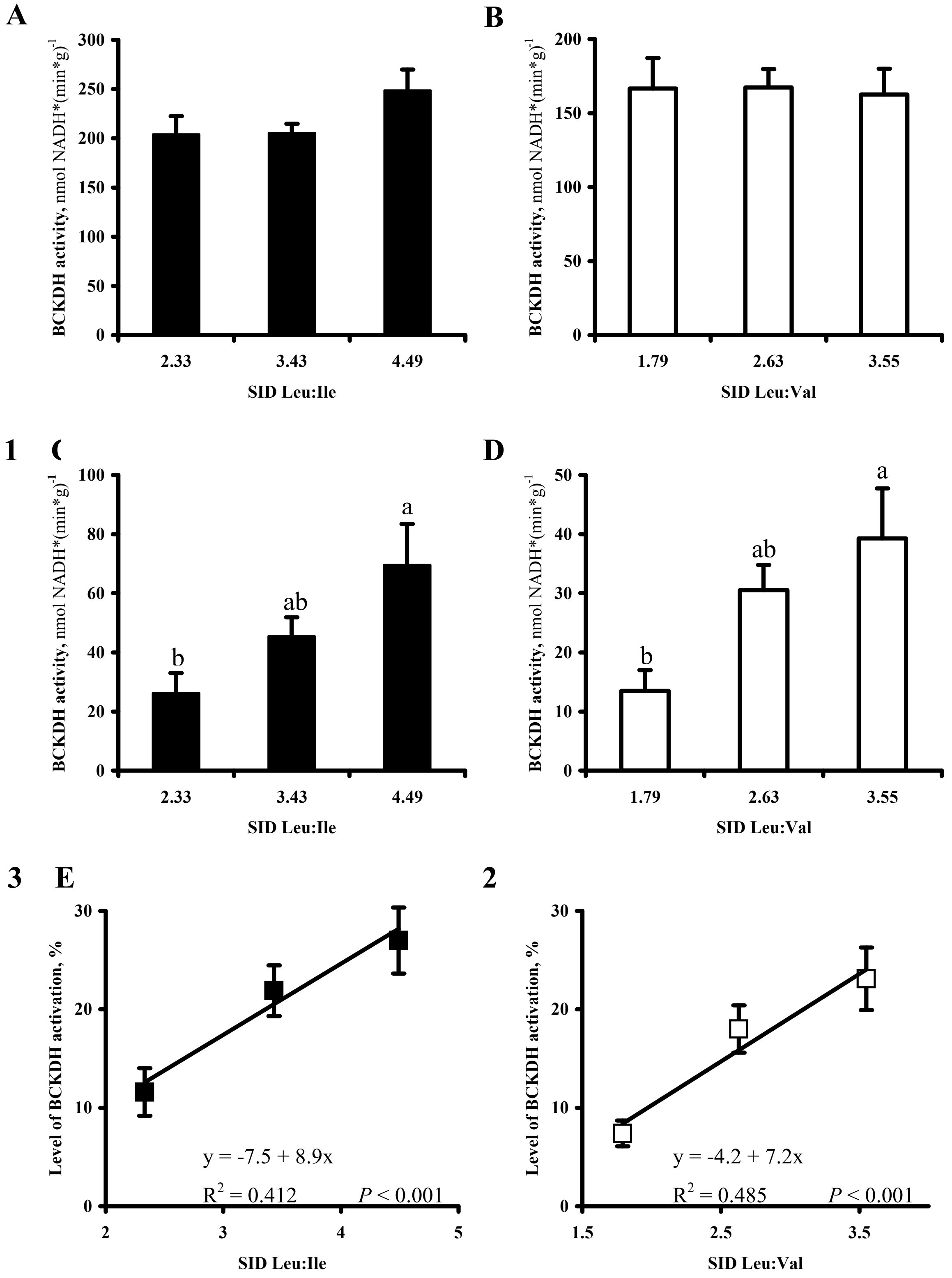
BCKDK activity is reciprocal to BCKDH activity because BCKDK phosphorylates and inactivates the BCKDH. In humans, BCKDH activity was high in liver and low in muscle and BCKDK expressions were low in liver and high in skeletal muscle (Suryawan et al., 1998). Literature lacks for mRNA expression data of BCKDK in pigs. In the present work, BCKDK expressions were highest in heart and skeletal muscle which confirms the hypothesis that skeletal muscle is not the main place of BCKA decarboxylation in pigs. In pigs, the differences in BCKDK expression between skeletal muscle and liver tissue were not as huge as seen in humans. Suryawan et al. (1998) reported that BCKDK expressions in skeletal muscle of humans were increased 11.7-fold compared to liver tissue. In the present work, BCKDK expressions in skeletal muscle of pigs were increased only 1.6-fold compared to liver. However, the BCKDK expression in human heart was 1/5 of that measured in muscle (Suryawan et al., 1998), whereas in pigs the expression in the heart was even 3.7-times that of skeletal muscle (Publication III). But BCKDK expression was at a similar level in liver and small intestine in both, humans and pigs. In conclusion, major species differences exist in the BCAA catabolism and transfer of knowledge seems problematic.

4.2.3 Activity regulation of the branched-chain α-keto acid dehydrogenase

The present work was conducted to determine whether dietary Leu excesses alter the BCAA catabolism. It was supposed that an increased Leu supply would increase the BCAA catabolism. To verify this hypothesis, the activity of the key enzyme, namely BCKDH, was measured. The activity was quantified both as total and basal activity, because its activity state is regulated. Therefore, several mechanisms are possible to increase the BCKDH activity. One is to increase its activity state, and another to increase its total amount. Especially high KIC concentrations, the corresponding BCKA of Leu, can increase the catabolism of the other BCAA by stimulation of BCKDH activity. KIC promotes the dissociation of the BCKDK and BCKDH (Murakami et al., 2005) so that the BCKDK becomes inactive. This results in a less phosphorylated and therefore more active BCKDH complex (Harris et al., 2004; Shimomura et al., 2006; Shimomura et al., 2001).

In the present work, doubling of the dietary Leu supply increased the serum KIC levels by 45 to 50% and the basal activity of the liver BCKDH 2- to 3-fold. The total activity of the BCKDH was not changed (Fig. 11). The levels of BCKDH activity observed in the present work are in accordance to levels reported for growing gilts fed semipurified diets excessive in Leu (50% excess) but marginally in Ile or Val (Langer et al., 2000). Increased BCKDH activities as a consequence of Leu excesses seen in the present work (Publication III) are in accordance to literature. Linearly increased rates of Leu oxidation were reported in rats when the dietary Leu supply exceeded the need for maximum rate of weight gain (Harper and Benjamin, 1984). Increased BCKDH activities as a consequence of dietary Leu excesses were seen in rats and pigs (Block and Harper, 1984; Kuzuya et al. 2008; Langer et al., 2000). Block et al. (1987) reported that increases in plasma Leu paralleled the changes in the activaty state of the BCKDH complex. In contrast, infusions of Val or Ile failed to activate BCKDH in the rat (Aftring et al., 1986). However, high dietary Ile or Val supply increased the BCKDH activity. Chronic feeding (for five days) of 9% casein diets (low protein for rats) containing additions of either 5% of Leu or Ile, respectively, or 4.47% of Val increased liver BCKDH activity to the same extent (Wohlhueter and Harper, 1970). However, consumption of excess Ile or Val failed to alter the plasma BCAA and BCKA pools to the extent observed after feeding high-Leu diets (Harper et al., 1970; Shinnick and Harper, 1977). In the present work, the impact of a simultaneous oversupply either with Leu and Ile or Leu and Val on performance, plasma AA, and serum BCKA was investigated. Doubling of the Ile supply increased the plasma Ile and serum KMV levels 2.9- and 2.4-fold, respectively, and doubling of the Val supply increased the plasma Val and serum KIV levels 3.9- and 5.8-fold. However, the simultaneous oversupply did not affect the performance parameter compared to a single Leu oversupply. In literature, however, it was reported that in pigs (30 to 40 kg) fed Ile-limited diets the negative effects of an Leu excess on N utilization were reversible by a simultaneous Val excess (Langer and Fuller, 2000). In contrast to Langer and Fuller (2000), there was no significant increase in performance in the present work. It could not be confirmed that a dietary excess of Val is beneficial in diets excessive in Leu but marginally in Ile.

Fig. 11 **Effects of increasing dietary standardized ileal digestible (SID) leucine (Leu) to isoleucine (Ile) ratio or SID Leu to valine (Val) ratio by crystalline Leu supplementation on branched-chain α-keto acid dehydrogenase (BCKDH) activity. Total (A + B), basal (C + D), and relative (E + F) liver BCKDH activity in pigs fed diets either limited in Ile (■) or Val (□). Means ± SE, *n* = 8. For details see Publication III.**

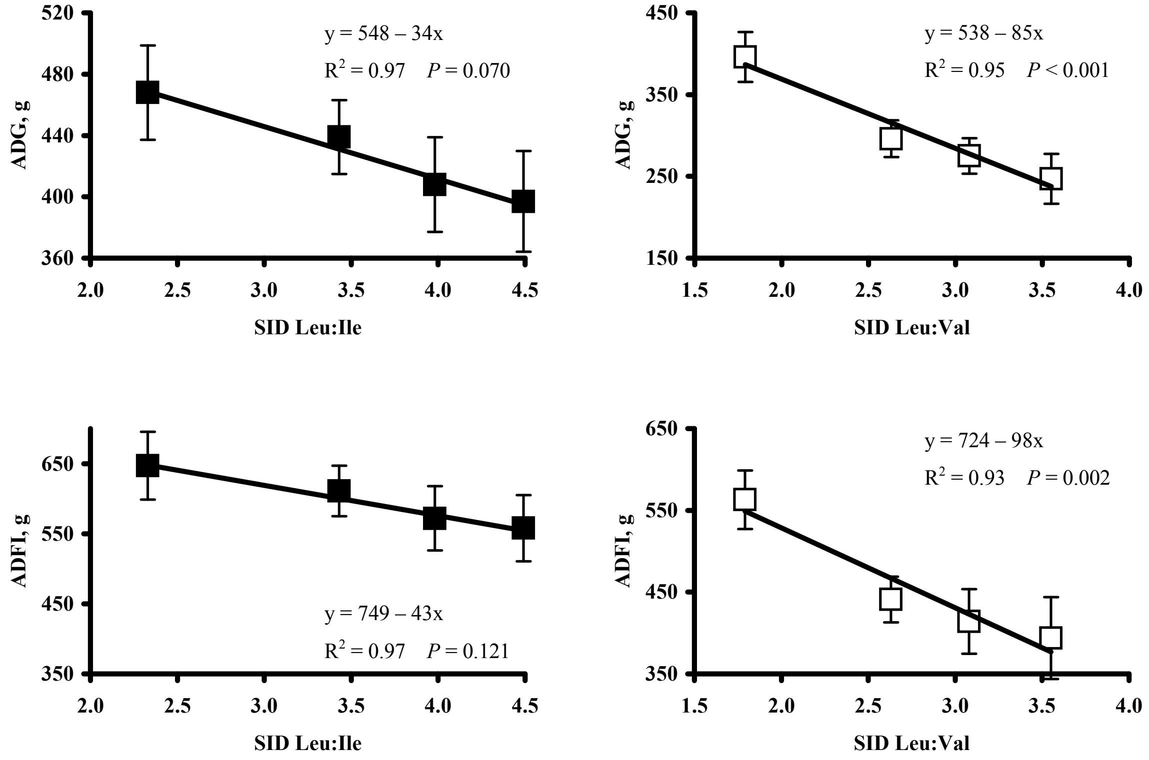


Beside the regulation of the BCKDH activity and its total amount, changes in BCKDH activity can be caused by changing its composition. Therefore, mRNA expressions of the BCKDH subunits (E1α + β, E2) were measured. For BCKDH activity regulation, the E1α subunit is essential because the activity of the whole complex is regulated by phosphorylation and dephosphorylation of the E1α subunits. It has been proposed that E1α may serve as an activator of BCKDH (Yeaman et al., 1984; Espinal et al., 1985) and variations in the BCKDH composition may contribute to alterations of the BCKDH activity state. For example, in chicks the E2 subunit binds eleven E1 subunits which is one less than in mammals and this leads to a reduced BCKDH activity in chicks (Hakozaki et al., 2002). An increased binding of E1 subunits enhances the BCKDH activity as seen in BCKDK knock-out (BCKDK$^{-/-}$) mice (Joshi et al., 2006). Metabolic disorders as diabetes also affect the BCKDH composition: increased protein expressions of E1α, E1β, and of E2 and decreased expression of BCKDK were reported for muscle and liver tissue (Lombardo et al., 1998, 1999). Beside these, changes in the BCKDH composition are part of the metabolic answer to altered nutritional supply. In starvation, increased liver BCKDH activities were accompanied by increased protein expressions of E1α in rats (Serdikoff and Adibi, 2001). In contrast, in skeletal muscle no increase of E1α protein expression occurred and no changes in BCKDK protein expression were seen (Serdikoff and Adibi, 2001). In the present work, the mRNA expression of genes encoding E1α, E1β, and E2 was not altered to a great extent, and it was suggested that the observed alterations in BCKDH activity were mainly caused by posttranscriptional mechanisms (Publication III).

4.2.4 Increased branched-chain α-keto acid dehydrogenase activity = increased branched-chain amino acid catabolism?

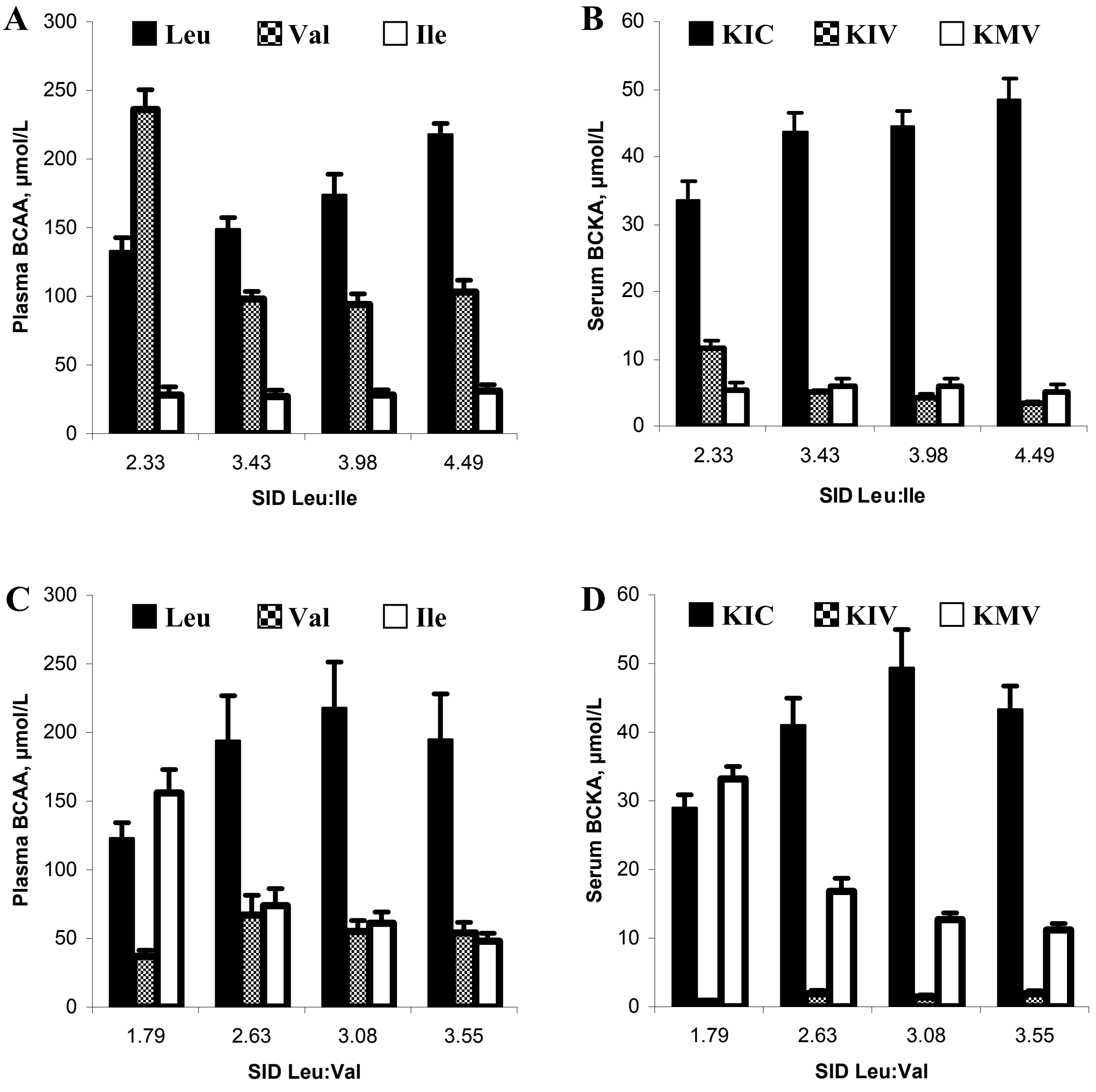
In the present work, it has been found that the basal BCKDH activities increased linearly as the dietary Leu supply increased (Fig. 11). But it remains to be discussed whether an increased catabolism of Ile and Val occurred. The diets fed in the present study were either limited in Ile or Val to guarantee that an increased BCAA catabolism would worsen the nutritional status of the animals and would not be cushioned by an oversupply of L-Ile or L-Val, respectively. The marginality of the decreased L-Ile or L-Val supply, respectively, was clearly demonstrated by a sharp decrease of their plasma AA and serum BCKA levels compared to the positive control (Publication III). As the dietary Leu supply increased, the ADFI and ADG decreased linearly (Fig. 12), and in contrast, the BCKDH activation level increased linearly (Fig. 11).

Fig. 12 Effects of increasing dietary standardized ileal digestible (SID) leucine (Leu) to isoleucine (Ile) or valine (Val) ratio by crystalline Leu supplementation on average daily gain (ADG) and average daily feed intake (ADFI) in pigs fed diets either limited in isoleucine (Ile; ■) or valine (Val; □). Means ± SE, *n* = 8. For details see Publication III.



It was concluded from the plasma AA and serum BCKA data that the increased activity of the BCKDH indeed caused an increased catabolism of the other BCAA. In pigs fed diets limited in Ile, the plasma Val and serum KIV levels decreased by 58 and 56%, respectively, as the SID Leu:Ile ratio increased from 2.33 to 3.43 (Fig. 13). In pigs fed diets limited in Val, plasma Ile and serum KMV levels decreased by 69 and 64%, respectively, as the SID Leu:Val ratio increased from 1.79 to 3.55 (Fig. 13). These findings indicate an increased catabolism of Val and Ile, when diets excessive in Leu are fed and are in accordance to literature. An increased Val and Ile catabolism was demonstrated in chicks fed diets excessive in L-Leu (Calvert et al., 1982). In growing gilts fed diets containing high levels of dietary L-Leu (50% over requirement) for seven days, reduced plasma concentrations of Val, Ile, and their corresponding BCKA occurred (Langer et al, 2000). The same effects were reported for rodents. In rats, a dietary L-Leu excess caused a depression of the plasma Val and Ile levels and their BCKA (Harper and Benjamin, 1984; Block and Harper, 1984). The negative effects of a dietary L-Leu oversupply are restorable by supplementation with L-Ile and L-Val in chicks, pigs, and kittens (Calvert et al., 1982; Fu et al., 2006a; Hargrove et al., 1988). This confirms the hypothesis of an increased nutritional need for L-Ile and L-Val in diets excessive in L-Leu.

Fig. 13 Effects of increasing leucine (Leu) supply on plasma branched-chain amino acids (BCAA) and serum branched-chain α-keto acids (BCKA) of pigs either fed diets limited in isoleucine (Ile; A + B) or valine (Val; C + D). Means ± SE, *n* = 8.



SID, standardized ileal digestible.

KIC, α-keto isocaproate; corresponding BCKA to Leu.

KIV, α-keto isovalerate; corresponding BCKA to Val.

KMV, α-keto β-methylvalerate; corresponding BCKA to Ile.

4.2.5 Alternative substrates of the branched-chain α-keto acid dehydrogenase

The BCKA are not the only substrates of the BCKDH complex. Therefore it is to question, whether an increased BCKDH activity increased the catabolism of other compounds than the BCKA.

The degradation of Thr produces 2-oxobutyrate which is a substrate to the BCKDH. This leads to the hypothesis that Thr degradation might be increased if the BCKDH activity increases. But in BCKDK$^{-/-}$ mice no changes in plasma Thr levels occurred (Joshi et al., 2006). For interpretation of these results it should be considered that liver is the main location of Thr oxidation. But in liver tissue of rodents the BCKDH is totally activated and there is no regulation by the BCKDK. Therefore, the BCKDK$^{-/-}$ mice seems not to be suitable to elucidate this hypothesis. In the present work, liver BCKDH activity states of pigs were analyzed to be between 7 to 28% and an upregulation by dephosphorylation was seen in the present work as well as in literature (Langer et al., 2000). Therefore, an impact of increased BCKDH activity on the nutritive status of Thr might exist in pigs. In the present work, however, no decreases in plasma Thr levels occurred although the BCKDH activity increased 2- to 3-fold.

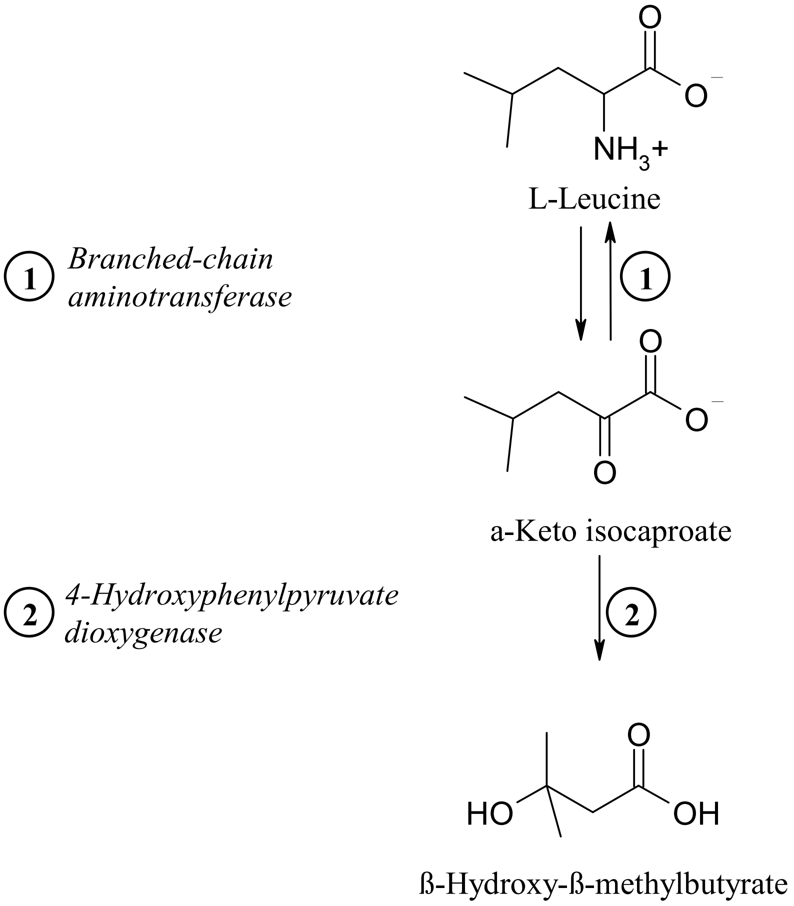
A similar situation is given for Met. In the catabolism of Met the intermediate 4-methyl-thio-2-oxobutyrate can be formed by transamination (Case and Benevenga, 1976). This oxo-acid is then decarboxylated by the BCKDH. But a major impact of increased BCKDH activity on the nutritional status of Met seems to be unlikely, because this catabolic pathway is of minor relevance. The main catabolic pathway of Met is the transsulfuration pathway. The transamination solely occurs in dietary Met excesses. But then the rate limiting reaction is the transamination and not the decarboxylation (Joshi et al., 2006). Experimental data confirm this hypothesis. In the present work, BCKDH activity increased 2- to 3-fold but no impact on plasma Met levels occurred. This is in line to results of Langer et al. (2000). They reported that plasma Met levels of pigs were not affected by either dietary Leu or BCAA excesses although the BCKDH activity was increased.

4.2.6 Alternative pathways for catabolism of leucine via 4-hydroxyphenylpyruvate dioxygenase

In phases of dietary Leu excesses, alternative degradative pathways bypassing the BCKDH complex might be of some importance for the catabolism of Leu. Additionally to its oxidation by the BCKDH, KIC can be converted to β-hydroxy-β-methylbutyrate catalyzed through 4-hydroxyphenylpyruvate dioxygenase (4-HPPD; identical to KIC dioxygenase; Crouch et al.,

2000) as illustrated in Fig. 14. β-Hydroxy-β-methylbutyrate is excreted into the urine and leaves the organism. The 4-HPPD activity was found to be 14.2% of the total BCKDH activity in human liver (Xu et al., 2000), what illustrates its importance. In the present work, no impact of an increased dietary Leu supply on the mRNA expression of liver 4-HPPD occurred in pigs fed Ile-limited diets. In Val-limited diets, however, 4-HPPD expression was significantly decreased at 50% of Leu oversupply compared to that of the basal treatment (Publication III). Feeding of BCAA rich diets to rats increased the BCKDH activity but not the 4-HPPD activity (Xu et al., 2000). In conclusion, the effects of a Leu excess on 4-HPPD require further investigation.

Fig. 14 Schematic illustration of the 4-hydroxyphenylpyruvate dioxygenase pathway



4-HPPD is an important enzyme of the Tyr catabolism (Lindstedt et al., 1992). At first, Tyr is irreversibly desaminated to 4-hydroxyphenylpyruvate. The following irreversible oxidation to homogenisate is catalyzed by the 4-HPPD. Thus, an impact of high KIC levels on the Tyr catabolism seems possible. However, no alterations in the plasma Tyr levels were seen in the present work although the dietary Leu supply increased.

4.2.7 *Leucine excess and regulation of feed intake*

It has been shown in force-fed chickens that about 70% of performance depression caused by Leu excesses were the result of a decreased feed intake (Calvert et al., 1982). It is known from preference trials that rats reject diets excessive in Leu and instead prefer an alternative diet even if it was protein-free (Rogers et al., 1967). In rats, the magnitude of ADFI reduction and weight loss caused by dietary Leu excesses were similar to the reductions caused by feeding of high-protein diets (Ropelle et al., 2008). Leu seems to function as a nutrient signal that informs the organism about the protein intake. Thus, high plasma levels of Leu signalize the organism that enough protein was ingested and that the feed intake can be stopped. That dietary Leu is indeed a nutrient signal which can cause depressions of feed intake has been proven by injection of L-Leu into the brain of rats (Cota et al., 2006; Ropelle et al. 2008). L-Leu activates the hypothalamic mTOR (mammalian target of rapamycin; a protein kinase that phosphorylates 4E-binding proteins) pathway and decreases the AMPK (adenosine monophosphate-activated kinase) activity (Ropelle et al., 2008) which leads to decreased tuberin activity and in consequence enhances the mTOR signaling additionally. Increased mTOR activity leads on the one hand to enhanced protein synthesis via increased protein synthetic efficiency, which is primarily driven by enhanced activation of translation initiation factors (eukariotic initiation factor 4E-binding protein-1 and eukariotic initiation factor G4) involved in the binding of mRNA to the 43S ribosomal complex. In consequence, protein synthesis is stimulated by upregulation of the initiation of mRNA translation (Kimball and Jefferson, 2006). Increased protein accretion due to dietary Leu excesses was reported in pigs by Escobar et al. (2006). This effect of Leu was not dependent on changes in circulating insulin levels (Escobar et al., 2006). But on the other hand, Leu excesses reduce the feed intake via mTOR signaling (Cota et al., 2006; Ropelle et al., 2008). Inhibition of mTOR with rapamycin inhibited the L-Leu induced anorexia (Cota et al., 2006). Leu excess is leading to inhibition of neuropeptide Y (NPY) and stimulation of pro-opiomelanocortin (POMC) expression (Cota et al., 2006; Ropelle et al., 2008). NPY promotes feeding, decreases energy expenditure, and silences POMC, whereas POMC promotes satiety (Gao and Horvath, 2007). Therefore, inhibition of NPY and activation of POMC cause satiety and give an explanation for the Leu induced anorexia. In conclusion, Leu serves as a signal for the protein content of the diet. Diets excessive in Leu mimic diets high in protein so that an increased protein accretion and reduced feed intake appear as a rational reaction.

Another signal to reduce the feed intake could be an essential AA deficiency provoked by dietary Leu excesses. It is known for several species (pig, golden hamster, rat, seabream), that they are not only able to select for an adequate protein content (DiBattista, 1987; Kyriazakis et al., 1990; Siegel and Collier, 1972; Vivas et al., 2006) but that they are even able to detect diets with an imbalanced AA pattern or with a single essential AA deficiency. In pigs preferences for Lys (Henry, 1987; Kirchgessner et al., 1999), Met (Roth et al., 2006), Thr (Ettle and Roth, 2005), and Trp (Ettle and Roth, 2004) were reported. The chemosensor for essential AA deficiency is housed in the anterior piriform cortex (APC) of the ventro-lateral forebrain (Gietzen et al., 2007). After ingestion of an imbalanced diet the limiting essential AA decreases in brain tissue as rapidly as in the plasma and the time to sensing essential AA deficiency is less than 30 min. In rats, depressions of plasma Ile and Val occurred between 10 and 30 min after consumption of Leu excessive diets (Block and Harper, 1984). Decreased essential AA supply in the APC leads to uncharged tRNA (transfer RNA), what induces phosphorylation of eukariotic initiation factor 2 via the general control nondepressing 2 kinase (GCN 2) in select neurons (Hao et al., 2005). Activation of these neurons transmits the signal to the lateral hypothalamus, and the learned behavior probably involves amygdaloid and hippocampal cells (Gietzen and Rogers, 2006). The general control system is a well-conserved pathway and its activation causes diet rejection and development of adaptive strategies such as foraging for complementary essential AA sources. Continued consumption of essential AA deficient or imbalanced diets causes a conditioned taste aversion to the diet as seen in the mentioned preference trials. In the present work increasing dietary L-Leu supplementation increased the catabolic activity of the BCKDH and decreasing plasma Ile and Val levels were seen. Therefore, a worsened nutritional status for Ile and Val could be involved in the regulation of the Leu induced anorexia. This hypothesis is confirmed by the observations that the performance depressing effects of high dietary Leu levels are restorable by supplementation of L-Ile and L-Val (Calvert et al., 1982; Fu et al., 2006a).

5 Conclusions

In the present work it was clearly demonstrated that the Ile and Val requirements of weaned pigs can be estimated very well using growth parameters in dose-response trials. Data fitted very well to the broken-line model, which facilitated data interpretation. The broken-line model was suitable for determining Ile and Val requirements. At dietary Ile or Val levels below the animal's requirements, growth performance data increased linearly in a dose-dependent manner as the crystalline Ile or Val supply was increased. At sufficient dietary Ile or Val levels, the growth performance data formed a plateau. Even at the highest supplementation levels of Ile or Val no decrease in performance occurred. Thus, two linear areas occurred and were well defined. According to the chosen experimental design (range of doses, number of increments) the broken-line model gained rational results.

In the growth assays, the optimal ratios of SID Ile or Val to Lys estimated for ADG and ADFI were close together with a good concordance. However, lower estimates of requirement were seen for G:F which underlines the importance of *ad libitum* feeding-regimen particularly as maximum feed intake and gain are common goals in feeding weaned pigs.
From the present data it was concluded that dietary SID Val:Lys ratios of 65 to 67% and dietary SID Ile:Lys ratios of 54% are needed for optimal growth performance in weaned pigs. These estimates hold true for diets free of dietary excesses of the other BCAA, especially free of Leu oversupply. It was demonstrated that the use of SDBC results in increased nutritional needs for Ile most likely caused by interactions between the BCAA. Thus, the Ile requirements estimated in pigs fed diets with Leu and Val excesses are overestimating the nutritional needs for Ile in diets free of these excesses. Therefore, interactions between the BCAA have to be considered in diet composition. Further investigations are needed to rework the Ile and Val requirements of pigs of different life stages because former experiments did not account for interactions between the BCAA.

Analyses of plasma AA gained valuable results confirming the requirement estimates determined for growth parameters. The chosen feeding and blood-sampling procedures were suitable to reveal the effects of an increasing Ile or Val supply on plasma AA levels and to serve as a reference to the nutritional needs. In the growth assays determining the Ile requirements using different protein sources the plasma AA were especially valuable. In these trials, the optimal SID Ile:Lys ratios for growth performance were found to be higher in diets containing SDBC compared to diets containing CGF. The plasma Ile data were used to calculate the bioefficiency of the crystalline L-Ile supplementation. The slope-ratio assay indicated a decreased bioefficiency of the supplemented L-Ile, if the diets contained SDBC instead of CGF, and confirmed the differences between these diets seen for optimal SID Ile:Lys ratios estimated for growth parameters. Furthermore, it was hypothesized that a dietary oversupply of Leu as it was caused by the use of SDBC results in increased nutritional needs for Ile because of interactions between the BCAA.

Plasma concentrations of urea were also analysed. In general, plasma levels of urea should decrease if the fed amino acid pattern becomes more balanced. This could be confirmed by the present work. Plasma concentrations of urea were at a very low level and slight decreases were seen as the AA pattern improved. However, plasma urea turned out to be an imprecise parameter and it could not be used for estimation of Ile or Val requirements.

N balance trials were conducted to confirm the growth assay data. In these trials, the pigs were fed restrictively what should equalize feed intake. However, the performance depressing effects of the Ile and Val limitation were greater than the feed restriction. Thus, the feed intake increased with increasing Ile and Val supplementation. It is to question whether feed restriction is beneficial in N balance trials when massive growth depression is anticipated. Furthermore, handling of N balance trials is very sophisticated and the daily expenditure of time is much higher than it is for growth assays. Therefore, it is to question whether the additionally gained information justifies the costs.

In the present work, it was clearly demonstrated that dietary Leu excesses should be avoided in weaned pigs fed diets either limited in Ile or Val. Growth performance linearly decreased in a dose-dependent manner as the Leu supply increased. Simultaneous oversupply of Leu and Ile or Leu and Val did not cause additional effects in comparison to a single Leu oversupply. It was concluded that Leu excesses exhibit the greatest effects in pigs and that Leu is the most important and potent BCAA according to interactions between them.

Analyses of plasma AA and serum BCKA levels were consistent to each other and indicated an increased catabolism of Ile and Val when diets excessive in Leu were ingested. These findings were confirmed by an increased activity of the BCKDH complex which is the key enzyme in the degradative pathway of the BCAA. In liver tissue, analysis of BCDKH activity was performed successfully, but in skeletal muscle BCKDH activities were below limit of detection. mRNA analyses confirmed this finding. The expression of BCKDH subunits was higher in liver than in muscle tissue. Furthermore mRNA expression analysis was in line to total BCKDH activity. Both did not change with increasing dietary Leu supply indicating mainly posttranscriptional mechanisms as a strategy of adaptation. mRNA expression analysis is a well standardized, highly sensitive, and fast method that provides deep insight into the organism. However, the present work clearly showed the limitations of mRNA analysis. To gain complex information about the effects of a nutritional treatment on the metabolism or even on the whole organism additional investigations are necessary. In the organism, there exist varied control mechanisms that work independently from mRNA expression such as phosphorylation and dephosphorylation reactions. Even if changes in mRNA expression are seen it is not proven that these changes affected the organism. Therefore, mRNA analyses are of limited informative value if they stand alone.

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7 Curriculum vitae

Name: Markus Karl Wiltafsky
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University education

2006 – 2009 **Technische Universität München, Weihenstephan**
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PhD student

PhD thesis:
Isoleucine and valine requirements of piglets and activity and gene expression of key enzymes of the branched-chain amino acid metabolism in response to dietary leucine excess

2000 – 2005 **Technische Universität München, Weihenstephan**
Agricultural Sciences

Major field of study: Animal Sciences

Diploma thesis:
Experimentelle Untersuchungen zu einer Nahrungspräferenz für Zink beim Ferkel

Alternative civilian service

1999 – 2000 **Seniorenheim Haus Stevertal (Münsterland)**

School education

1990 – 1999 **Städtisches Gymnasium Sundern (Sauerland)**
General qualification for university entrance

1986 – 1990 **Katholische Grundschule Allendorf**

8 List of publications

8.1 Peer reviewed original papers

Wiltafsky, M. K., B. Schmidtlein, F. X. Roth. 2009. Estimates of the optimum dietary ratio of standardized ileal digestible valine to lysine for 8- to 25-kilogram pigs. J. Anim. Sci. 87:2544-2564; doi. 102527/jas.2008-1221

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Wiltafsky, M. K., M. W. Pfaffl, and F. X. Roth. 2009. The effects of branched-chain amino acid interactions on growth performance, blood metabolites, enzyme kinetics, and Transcriptomics in weaned pigs. Br. J. Nutr., accepted, BJN-2009-013826

8.2 Contributions to scientific conferences

Ettle, T., M. K. Wiltafsky, and F. X. Roth. 2006. Nahrungspräferenzen für Zink beim Ferkel. In: J. Köhrle and C. Schmutzler (eds), Mineralstoffe – Molekularbiologie – Medizin, Schriftenreihe der Gesellschaft für Mineralstoffe und Spurenelemente e. V., Band 2, 09.-11.11.2006, Berlin, Herbert Utz Verlag, München, Germany

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9 Appendix

Publication I

Estimates of the optimum dietary ratio of standardized ileal digestible valine to lysine for 8- to 25-kilogram pigs.

Journal of Animal Science, volume 87, page 2544-2553, published online 09.04.09: doi. 102527/jas.2008-1221

Wiltafsky, M. K., B. Schmidtlein, and F. X. Roth
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Publication II

Estimation of the optimum ratio of standardized ileal digestible isoleucine to lysine for 8- to 25-kilogram pigs in diets containing spray-dried blood cells or corn gluten feed as a protein source.

Journal of Animal Science, volume 87, page 2554-2564, published online 27.03.09: doi.102527/jas.2008-1320

Wiltafsky, M. K., J. Bartelt, C. Relandeau, and F. X. Roth
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Publication III

The effects of branched-chain amino acid interactions on growth performance, blood metabolites, enzyme kinetics, and transcriptomics in weaned pigs.

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Wiltafsky, M. K., M. W. Pfaffl, and F. X. Roth

Publication I

Estimates of the optimum dietary ratio of standardized ileal digestible valine to lysine for 8- to 25-kilogram pigs.

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Estimates of the optimum dietary ratio of standardized ileal digestible valine to lysine for eight to twenty-five kilograms of body weight pigs

M. K. Wiltafsky, B. Schmidtlein and F. X. Roth

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Estimates of the optimum dietary ratio of standardized ileal digestible valine to lysine for eight to twenty-five kilograms of body weight pigs

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ABSTRACT: Valine is among the limiting AA in low protein diets for pigs, but data are currently lacking for empirical requirement studies. Therefore, 2 growth assays and 1 N-balance trial were conducted to determine the Val requirement of weaned pigs (8 to 25 kg of BW) expressed as the ratio of standardized ileal digestible (SID) Val to Lys. In Exp. 1, 48 pigs (individual housing; equal proportion of castrates and females; initial BW = 8.0 kg) were randomly subdivided into groups receiving 6 dietary treatments. Dietary SID Val:Lys ratio was increased by adding L-Val from 0.56 to 0.84% (planned increments of 0.06%) at an average SID Lys level of 0.99%. Other crystalline AA were supplied to meet optimum SID AA:Lys ratios based on the concept of an ideal protein. All diets were isocaloric (13.6 MJ of ME/kg) and isonitrogenous (17.8% CP). For the 35-d period, ADG and ADFI increased linearly ($P < 0.01$) and quadratically ($P < 0.01$) with increasing SID Val:Lys. However, the data were not suitable for estimation of Val requirement. Therefore, in Exp. 2 the dietary basal level of Val was reduced to 0.49% SID Val and 4 increments of 0.04% and 1 increment of 0.08% L-Val were used to give 6 dietary treatments (48 pigs; individual housing; equal proportion of castrates and females; initial BW = 7.9 kg). The SID Lys level averaged 0.99%, and SID Val:Lys ratios ranged from 0.49 to 74%. Other crystalline AA were supplied to meet optimum SID AA:Lys ratios. All diets were isocaloric (13.7 MJ of ME/kg) and isonitrogenous (17.7% CP). For the 34-d period, ADG, ADFI, and G:F increased linearly ($P < 0.01$), and ADG and G:F quadratically ($P < 0.01$) as the SID Val:Lys ratio increased. Estimates of optimum SID Val:Lys were 66, 67, and 61% for ADG, ADFI, and G:F, respectively. These estimates were confirmed by minimized concentrations of plasma urea and Lys and by markedly increased concentrations of plasma Val. To verify the estimates of the growth trials, a total of 24 N balances were conducted with 12 pigs using the dietary treatments of Exp. 2 (Exp. 3; equal proportion of males and females; average BW = 14.1 kg; 2 balances per pig). Pigs were restrictively fed 3 times daily. Precollection and collection periods lasted 7 d each and were repeated after rearranging the animals to treatments. Increasing the dietary SID Val:Lys ratio linearly increased N retention ($P < 0.04$) and quadratically decreased plasma concentrations of urea ($P < 0.01$). Optimal SID Val:Lys ratios of 65 and 62% were estimated for N retention and N utilization, respectively. We conclude that an optimum SID Val:Lys ratio of 65 to 67% is needed for optimal performance in 8- to 25-kg pigs.

Key words: growth, nitrogen balance, pig, valine requirement, valine to lysine ratio

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INTRODUCTION

The reduction of CP content in the diets of pigs is desirable for economic and environmental reasons. To maintain pig performance when low CP diets are used, supplementation with crystalline AA is necessary. Reductions of CP content up to 6.6 percentage points (from 18.9 to 12.3%) and simultaneous supplementation with crystalline essential AA in the diets of growing pigs has been shown to have no negative effect on N retention or animal performance but reduced N excretion by 58% (Le Bellego et al., 2001). Valine is a limiting AA in several low-CP diets, after Lys, Trp, Thr, and Met. For 10- to 20-kg pigs, His, Ile, Trp, and Val were equally limiting after Lys and Thr in sorghum-soybean meal based-diets (12% CP) (Brudevold and Southern, 1994), and Thr, Met, and Val were equally limiting after Lys and Trp in corn-soybean meal-whey based-diets (13.5% CP; Mavromichalis et al., 1998). In corn-soybean meal based-diets (11% CP) supplemented with Lys, Trp, Thr, and Met fed to growing pigs, Val was shown to be

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the next-limiting AA (Figueroa et al., 2003). The nutritional needs of the animal must be known to provide an optimal supply of essential AA. Data are currently lacking for Val requirements expressed as the ratio of standardized ileal digestible (**SID**) Val:Lys. Chung and Baker (1992) have estimated an optimum ratio of Val:Lys of 68% for 10-kg pigs and concluded that the ideal AA pattern of 75% reported by Wang and Fuller (1989) was excessive for Val. In recent work, a SID Val:Lys ratio of at least 70% was estimated for 12- to 25-kg pigs (Barea et al., 2009). Therefore, the present study was conducted to estimate the Val requirement for weaned pigs as the ratio of SID Val:Lys.

MATERIALS AND METHODS

Animal housing and care was conducted under supervision of the veterinary office of the Bavarian government. The handling protocol ensured proper care and treatment of all animals in conformity with the German law for animal protection.

General

To estimate SID Val requirement of piglets, 2 growth assays and 1 N balance trial were conducted with German-Landrace × Piétrain pigs (equal numbers of castrated males and females). For growth assays, pigs with an age of 31 d (1 wk postweaning) were housed in individual pens (size: 60 × 100 cm) in an environmentally controlled building with an initial room temperature of 29°C, which was progressively reduced to 25°C at the end of the trials. Artificial light was provided from 0700 to 1700 h, followed by half-light. Animals had free access to feed and drinking water provided by nipple drinkers. For the N balance trial, animals were fed 3 times daily and drinking water was offered after feeding for free intake. The pigs and their environment were monitored 3 times daily and 2 times daily in the case of the growth assays.

Diets were supplemented with vitamins and minerals to meet or exceed the nutrient requirements of 10- to 20-kg pigs (NRC, 1998). Levels of SID AA (Stein et al., 2007) were calculated for all diets by multiplying the analyzed AA content of each feed ingredients with the estimates of standardized ileal AA digestibility (Sauvant et al., 2004). It was necessary to limit the dietary SID Lys content below the requirement to enable estimation of the Val requirement as a ratio of SID Val:Lys. In all diets, a SID Lys level of 1.00% was planned. According to German standards, this Lys level is limiting for 10- to 25-kg pigs (GfE, 2008). Amino acid analyses of mixed diets and calculation of SID Lys levels averaged 0.99% SID Lys for all diets. The supply of all essential AA other than Val was calculated on the basis of SID AA to meet the definition of an ideal protein (Chung and Baker, 1992). To minimize possible antagonistic effects between the branched chain AA (**BCAA**), surpluses of Ile and Leu were avoided. Response variables used in the growth trials were ADG, ADFI, and G:F.

Exp. 1

A study using 48 crossbred piglets with an initial BW of 8.0 ± 1.0 kg was carried out over an experimental period of 35 d. Piglets were allotted to 1 of 6 dietary treatments (Table 1). For diet composition, basal SID Val level was set to 0.55%. The basal diet was based on wheat (30.0%), corn (24.2%), and barley (10.0%). Whey powder (10.0%), peas (7.0%), and soybean meal (6.1%) were used as protein sources. Dietary Lys levels were constant, and estimated SID Lys averaged 0.99%. The following average SID AA:Lys ratios were estimated for Met + Cys, Thr, Trp, Ile, Leu, Phe +Tyr, and His: 61, 65, 18, 60, 100, 94, and 31, respectively. Diets were supplemented with L-Gly and Na-L-Glu, which served as sources of nonessential AA N. Diets were planned to be isonitrogenous, and the average CP was analyzed to be 17.8%. Energy content of diets was kept constant and was calculated as 13.6 MJ of ME/kg. At constant Lys levels (estimated SID Lys of 0.99%; target: 1.00% SID Lys), diets were supplemented with crystalline L-Val (planned increments of 0.06%) to provide 6 treatments. The following SID Val:Lys ratios were estimated: 0.56, 0.62, 0.66, 0.75, 0.76, and 0.84%.

Exp. 2

In Exp. 2, 48 crossbred piglets with an initial BW of 7.9 ± 0.8 kg were allotted to 1 of 6 dietary treatments (Table 2). The experimental period was 34 d. The basal diet was similar to that of Exp. 1, but the basal Val level was reduced to 0.49% SID Val. To achieve this Val level, soybean meal was excluded, and the proportions of barley and peas were increased. At constant Lys levels (estimated SID Lys of 0.99%) the following average SID AA:Lys ratios were estimated for Met + Cys, Thr, Trp, Ile, Leu, Phe + Tyr, and His: 62, 63, 18, 63, 106, 99, and 32, respectively. Diets were supplemented with crystalline L-Val to give 6 treatments (4 increments of 0.04% and 1 increment of 0.08% L-Val). The following SID Val:Lys ratios were estimated: 0.49, 0.55, 0.58, 0.63, 0.67, and 0.74%. Diets were calculated to be isonitrogenous and isoenergenic (average CP of 17.7%; 13.7 MJ of ME/kg, calculated).

Exp. 3

Experiment 3 included 12 piglets used to conduct a total of 24 N balances (BW 14.1 ± 2.8 kg; 2 N balances per pig, 4 balances per treatment). Piglets were individually housed in metabolism cages. After 7 d of acclimation to new surroundings, 7 d of precollection and 7 d of collection followed. Allocation of piglets to treatment was rearranged, and after 7 d of precollection a second collection period (7 d) was conducted. Piglets

Table 1. Composition of experimental diets (as-fed basis; Exp. 1)

	SID[1] Val:Lys,[2] %					
Item	56	62	66	75	76	84
Ingredient, %						
Wheat	30.00	30.00	30.00	30.00	30.00	30.00
Corn	24.18	24.18	24.18	24.18	24.18	24.18
Whey powder, low lactose	10.00	10.00	10.00	10.00	10.00	10.00
Barley	9.97	9.97	9.97	9.97	9.97	9.97
Field peas	7.00	7.00	7.00	7.00	7.00	7.00
Soybean meal	6.12	6.12	6.12	6.12	6.12	6.12
Sugar beet molasses	3.00	3.00	3.00	3.00	3.00	3.00
Soy oil	1.68	1.66	1.64	1.61	1.59	1.56
Vitamin-mineral premix[3]	3.33	3.33	3.33	3.33	3.33	3.33
Biolys[4]	0.93	0.93	0.93	0.93	0.93	0.93
DL-Met	0.19	0.19	0.19	0.19	0.19	0.19
L-Thr	0.21	0.21	0.21	0.21	0.21	0.21
L-Trp	0.04	0.04	0.04	0.04	0.04	0.04
L-Val	—	0.06	0.12	0.18	0.24	0.30
L-Leu	0.04	0.04	0.04	0.04	0.04	0.04
L-Ile	0.12	0.12	0.12	0.12	0.12	0.12
L-His	0.03	0.03	0.03	0.03	0.03	0.03
L-Gly	1.71	1.67	1.63	1.60	1.56	1.53
Na-L-Glu	1.45	1.45	1.45	1.45	1.45	1.45
Analyzed composition, %						
CP	17.96	17.92	17.72	17.98	17.36	17.77
Lys	1.15	1.14	1.12	1.14	1.05	1.13
TSAA[5]	0.69	0.69	0.70	0.69	0.70	0.70
Thr	0.74	0.72	0.72	0.74	0.70	0.72
Trp	0.21	0.21	0.21	0.21	0.20	0.21
Val	0.65	0.71	0.75	0.84	0.84	0.93
Ile	0.68	0.68	0.67	0.70	0.65	0.68
Leu	1.16	1.15	1.15	1.17	1.13	1.16
Phe + Tyr	1.08	1.06	1.04	1.05	1.02	1.07
His	0.35	0.35	0.34	0.35	0.34	0.35
Calculated SID composition, %						
Lys	1.02	1.01	1.00	1.01	0.93	1.00
TSAA	0.60	0.60	0.61	0.60	0.61	0.61
Thr	0.66	0.64	0.64	0.66	0.62	0.64
Trp	0.18	0.18	0.18	0.18	0.18	0.18
Val	0.55	0.61	0.65	0.74	0.75	0.83
Ile	0.60	0.60	0.59	0.62	0.57	0.60
Leu	1.00	0.99	0.99	1.01	0.97	1.00
Phe + Tyr	0.95	0.94	0.92	0.93	0.90	0.95
His	0.31	0.31	0.30	0.31	0.30	0.31

[1] Standardized ileal digestible AA, calculated from analyzed AA concentrations and standardized ileal digestibility of the diet.

[2] Calculated with a mean dietary SID Lys content of 0.99%.

[3] Provided the following per kilogram of diet: vitamin A 5,000 IU, vitamin D_3 500 IU, vitamin E 30 mg, menadione 0.15 mg, thiamin 3 mg, riboflavin 3 mg, pyridoxine 3 mg, vitamin B_{12} 18 µg, niacin 25 mg, pantothenic acid 15 mg, biotin 50 µg, folic acid 300 µg, choline (as choline chloride) 1,000 mg; Ca 5.24 g, P 2.86 g, Zn (zinc sulfate heptahydrate) 57.48 mg, Fe (ferrous sulfate heptahydrate) 25.44 mg, Mg (magnesium oxide) 63.9 mg, I (potassium iodate) 0.12 mg, Se (sodium selenite pentahydrate) 0.10 mg.

[4] Contains the following per kilogram: Arg 6.0 g, Ile 0.4 g, Leu 7.0 g, Lys 507.0 g, Thr 4.0 g, Trp 2.0 g, TSAA 3.0 g, Val 7.0 g, P 1.6 g; Evonik Degussa GmbH, Hanau, Germany.

[5] TSAA = total sulfur AA.

were fed 3 times daily with the same diets as used in Exp. 2. Energy supply was calculated using 1.05 MJ of ME/kg of $BW^{0.75}$ for the first and 1.20 MJ of ME/kg of $BW^{0.75}$ for the second balance, respectively. For calculation of daily energy supply, piglets were weighed at the beginning of the precollection period and daily BW gains of 250 and 350 g were assumed for the first and the second balance, respectively. Feed was mixed with water (1:1) to avoid feed losses and to guarantee optimum feed intake. Room temperature was progressively decreased from 26°C at the beginning of Exp. 3 to 23°C at the experiment's end. The relative humidity was 50 to 60%.

During the collection period, feces and urine of individual piglets were collected twice daily. Whole feces and urine (acidified to pH ~3) were stored at 4°C until the end of the collection period and consolidated into animal individual samples. Feces were freeze-dried and stored for further analysis. Response variables were ADG, ADFI, G:F, plasma AA and urea, N digestibility, N utilization, and N retention.

Data Collection

Feed consumption and BW were recorded weekly in the morning for growth assays. For N balance, these data were recorded on the first day of the preparation period and the last day of the collection period.

Blood Sampling

At the termination of Exp. 2 and of each collection period within Exp. 3, 1 blood sample per pig was collected from a jugular vein in heparin-containing tubes (6 to 9 mL per sample; S-Monovetten, Sarstedt AG & Co., Nümbrecht, Germany) after a fasting period of approximately 2.5 h. Tubes were carefully shaken and cooled by ice water when they could be analyzed the same morning. Otherwise, they were frozen at −80°C.

Table 2. Composition of experimental diets (as-fed basis; Exp. 2 and 3)

	SID[1] Val:Lys,[2] %					
Item	49	55	58	63	67	74
Ingredient, %						
Wheat	30.00	30.00	30.00	30.00	30.00	30.00
Corn	24.00	24.00	24.00	24.00	24.00	24.00
Whey powder, low lactose	10.00	10.00	10.00	10.00	10.00	10.00
Barley	13.89	13.89	13.89	13.89	13.89	13.89
Field peas	10.00	10.00	10.00	10.00	10.00	10.00
Sugar beet molasses	2.00	2.00	2.00	2.00	2.00	2.00
Soy oil	1.50	1.48	1.46	1.44	1.42	1.39
Vitamin-mineral premix[3]	2.90	2.90	2.90	2.90	2.90	2.90
L-Lys·HCl	0.68	0.68	0.68	0.68	0.68	0.68
DL-Met	0.22	0.22	0.22	0.22	0.22	0.22
L-Thr	0.25	0.25	0.25	0.25	0.25	0.25
L-Trp	0.06	0.06	0.06	0.06	0.06	0.06
L-Val	—	0.04	0.08	0.12	0.16	0.24
L-Leu	0.14	0.14	0.14	0.14	0.14	0.14
L-Ile	0.22	0.22	0.22	0.22	0.22	0.22
L-His	0.08	0.08	0.08	0.08	0.08	0.08
L-Phe	0.14	0.14	0.14	0.14	0.14	0.14
L-Glu	2.08	2.08	2.08	2.08	2.08	2.08
L-Gly	1.69	1.67	1.65	1.63	1.61	1.56
L-Arg	0.15	0.15	0.15	0.15	0.15	0.15
Analyzed composition, %						
CP	17.73	17.47	17.52	17.83	17.74	17.96
Lys	1.08	1.09	1.07	1.10	1.08	1.09
TSAA[4]	0.70	0.70	0.70	0.70	0.69	0.70
Thr	0.71	0.71	0.70	0.71	0.70	0.71
Trp	0.21	0.21	0.21	0.21	0.21	0.20
Val	0.58	0.62	0.65	0.70	0.74	0.81
Ile	0.71	0.71	0.71	0.71	0.70	0.71
Leu	1.20	1.20	1.19	1.20	1.20	1.19
Phe + Tyr	1.10	1.11	1.11	1.10	1.11	1.10
His	0.36	0.36	0.36	0.36	0.36	0.37
Calculated SID composition, %						
Lys	0.99	1.00	0.98	1.01	0.99	1.00
TSAA	0.61	0.62	0.62	0.62	0.62	0.62
Thr	0.63	0.63	0.62	0.63	0.62	0.63
Trp	0.18	0.18	0.18	0.18	0.18	0.17
Val	0.49	0.54	0.57	0.62	0.66	0.73
Ile	0.63	0.63	0.63	0.63	0.63	0.63
Leu	1.05	1.05	1.04	1.05	1.05	1.04
Phe + Tyr	0.98	0.99	0.99	0.98	0.99	0.98
His	0.32	0.32	0.32	0.32	0.32	0.33

[1] Standardized ileal digestible AA, calculated from analyzed AA concentrations and standardized ileal digestibility of the diet.

[2] Calculated with a mean dietary SID Lys content of 0.99%.

[3] Provided the following per kilogram of diet: vitamin A 5,000 IU, vitamin D_3 500 IU, vitamin E 30 mg, menadione 0.15 mg, thiamin 3 mg, riboflavin 3 mg, pyridoxine 3 mg, vitamin B_{12} 18 µg, niacin 25 mg, pantothenic acid 15 mg, biotin 50 µg, folic acid 300 µg, choline (as choline chloride) 1,000 mg; Ca 4.37 g, P 2.03 g, Zn (zinc sulfate heptahydrate) 58.53 mg, Fe (ferrous sulfate heptahydrate) 57.10 mg, Cu (copper sulfate pentahydrate) 0.25 mg, I (potassium iodate) 0.11 mg, Se (sodium selenite pentahydrate) 0.09 mg.

[4] TSAA = total sulfur AA.

Table 3. Effects of increasing standardized ileal digestible (SID) Val:Lys ratio by crystalline L-Val supplementation on performance variables of weaned pigs (Exp. 1)[1]

	SID[2] Val:Lys, %							P value[3]	
Item	56	62	66	75	76	84	SEM	Linear	Quadratic
BW, kg									
d 0	8.02	7.82	7.99	8.00	7.96	7.97	0.15	0.94	0.91
d 35	19.31	24.31	23.92	24.34	24.52	23.96	0.51	<0.01	<0.01
ADG, g	324	467	456	469	472	456	13	<0.01	<0.01
ADFI, g	460	625	633	665	661	627	18	<0.01	<0.01
G:F, g/kg	700	747	721	708	714	731	4	0.38	0.71

[1] Data represent the least squares means of 8 pigs per treatment during a 35-d feeding period (31 to 65 d of age).

[2] Standardized ileal digestible AA, calculated from analyzed AA concentrations and standardized ileal digestibility of the diet.

[3] Orthogonal polynomial contrast coefficients were used to determine the linear and quadratic effect of increasing dietary SID Val:Lys ratio.

Plasma was obtained for determination of free AA and urea by centrifugation at 20°C for 20 min at 800 × *g*. The plasma proteins were removed by precipitation with salicylsulfonic acid and centrifugation at 11,000 × *g* for 10 min at 20°C.

Analysis

Diets were analyzed for CP by Kjeldahl procedure (AOAC, 2000). Analysis of dietary AA was conducted by Ajinomoto Eurolysine S.A.S (Paris, France), following Commission Directive 98/64/EC (1998) for all AA except Trp, and following AFNOR (1998) for Trp. All AA except Met, Cys, and Trp were analyzed by ion-exchange chromatography after acid hydrolysis with HCl 6 *N* (reflux for 23 h at 110°C). To avoid degradation during hydrolysis, Met and Cys were obtained by ion exchange chromatography after performic acid oxidation. Determination of Trp was conducted by reverse phase HPLC and fluorometric detection after alkaline hydrolysis with barium hydroxide for 16 h at 120°C.

Plasma free AA were analyzed by ion-exchange chromatography on an automatic AA analyzer (LC 3000; Biotronik, Hamburg, Germany). Plasma urea was coanalyzed with the plasma AA. All samples were analyzed in duplicate.

Statistical Analyses

In all trials pigs were allotted to treatments on the basis of BW, and sex and ancestry were equalized among treatments. Data were analyzed by ANOVA using the MIXED procedure (SAS Inst. Inc., Cary, NC). The experimental unit was the individual pig. The model included the effects of treatment, sex, and in the case of N balance trial, sample period, as fixed effects. Litter was used as a random effect (randomized complete block design), and initial BW was included as a covariate. Contrasts were used to determine linear and quadratic effects of SID Val:Lys (Lowry, 1992). The IML procedure of SAS was used to generate orthogonal polynomial coefficients (unequally spaced treatments), and the MIXED procedure was used to compute orthogonal polynomial contrast sums of squares. Estimates of requirement were done by subjecting the group least squares means data to least squares broken-line methodology (Robbins et al., 2006).

RESULTS

Exp. 1

Increasing the ratio of SID Val:Lys from 56 to 84% increased the ADG and the ADFI linearly ($P < 0.01$) and quadratically ($P < 0.01$; Table 3). At dietary SID Val:Lys ratios of 62 to 84% performance was constant. At these levels, ADG and ADFI were increased up to 46 and 45%, respectively, compared with a SID Val:Lys ratio of 56%. However, the data were not suitable for estimation of a Val requirement. This experiment showed that a diet with a SID Val:Lys ratio of 56% is deficient in Val for weaned pigs.

Exp. 2

Incremental supplementation with L-Val improved performance (Table 4). Increasing the SID Val:Lys ratio from 49 to 74% increased the ADG, ADFI, and G:F linearly ($P < 0.01$), and ADG and G:F quadratically ($P < 0.01$). At a SID Val:Lys ratio of 67%, ADG and ADFI were maximized. Compared with the performance at 49% SID Val:Lys, the ADG and ADFI at 67% SID Val:Lys were increased by 120 and 90%, respectively. However, G:F was maximally improved by 22% at a SID Val:Lys ratio of 63%. Broken-line analysis estimated the optimum SID Val:Lys ratios as 67, 66, and 61% for ADFI, ADG, and G:F, respectively (Table 5; Figure 1).

The dietary Val supply significantly changed plasma AA (Table 6). With increasing Val supply, plasma levels of Lys, Trp, His, and Tyr decreased linearly ($P < 0.01$). However, concentrations of Ile and Glu increased linearly ($P < 0.03$), and Cys and Val concentrations increased linearly ($P < 0.01$) and quadratically ($P < 0.01$). Raising the SID Val:Lys ratio from 49 to 74% increased plasma Val concentrations by 640%. Broken-

Table 4. Effects of increasing standardized ileal digestible (SID) Val:Lys ratio by crystalline L-Val supplementation on performance variables of weaned pigs (Exp. 2)[1]

	SID[2] Val:Lys, %							P value[3]	
Item	49	55	58	63	67	74	SEM	Linear	Quadratic
BW, kg									
d 0	7.90	7.87	7.75	7.92	7.87	7.74	0.11	0.77	0.90
d 34	14.09	16.78	18.42	20.41	21.74	21.44	0.54	<0.01	0.01
ADG, g	184	262	303	370	408	410	15	<0.01	<0.01
ADFI, g	304	382	444	501	577	568	19	<0.01	0.06
G:F, g/kg	605	682	682	739	711	725	9	<0.01	<0.01

[1] Data represent least squares means of 8 pigs per treatment; in the case of SID Val:Lys ratios of 58 and 74%, 7 pigs per treatment (1 pig each was removed for health reasons), after a 34-d feeding period (31 to 64 d of age).

[2] Standardized ileal digestible AA, calculated from analyzed AA concentrations and standardized ileal digestibility of the diet.

[3] Orthogonal polynomial contrast coefficients were used to determine the linear and quadratic effect of increasing dietary SID Val:Lys ratio.

line analysis estimated the breakpoint for plasma Val concentrations at a SID Val:Lys ratio of 60% (Table 5). Plasma urea concentrations decreased linearly ($P = 0.01$) as the SID Val:Lys ratio increased (Table 6). The urea concentration was least at a SID Val:Lys ratio of 67% and was decreased by 18% compared with a SID Val:Lys ratio of 49%.

Exp. 3

Increasing the SID Val:Lys ratio from 49 to 74% increased ADG and G:F linearly ($P < 0.02$) and quadratically ($P = 0.05$) and N retention and N utilization linearly ($P < 0.04$; Table 7). Plasma urea concentrations were affected quadratically ($P < 0.01$) as SID Val:Lys increased. Broken-line analysis estimated the optimum SID Val:Lys ratios as 65 and 62% for N retention and N utilization, respectively (Table 5; Figure 2).

DISCUSSION

The aim of the present work was to determine Val requirements of 8 to 25 kg of BW pigs as a ratio of SID Val to Lys. Thus, it was necessary to generate basal diets first limiting in Val and second limiting in Lys. To ensure the sufficient supply of all other AA and nu-

Table 5. Single-slope broken-line models estimated for performance parameter as a function of total dietary Val:Lys ratio, and for performance, N balance parameter, and plasma concentrations of Val as a function of SID[1] Val:Lys ratio

	Parameter[2]					
Item	L	U	R	RSD[3]	R[2]	Confidence 95%
	Total Val:Lys					
Exp. 2[4]						
ADG[5]	409	−1,660.2	67	8.2	0.99	65 to 69
ADFI[5]	573	−1,797.4	68	12.3	0.99	65 to 71
G:F[5]	725	−1,253.3	62	19.5	0.90	55 to 69
	SID[1] Val:Lys					
Exp. 2[4]						
ADG[5]	409	−1,330.0	66	1.0	0.99	65.7 to 66.3
ADFI[5]	569	−1,489.0	67	10.0	0.99	64 to 70
G:F[5]	725	−925.5	61	16.6	0.93	53 to 69
Plasma Val[6]	50.0	−1,490.5	60	9.7	0.99	58 to 63
Exp. 3[7]						
N retention[5,8]	9.4	−11.0	65	0.30	0.91	55 to 76
N utilization[5,9]	63.9	−36.6	62	0.39	0.98	57 to 68

[1] Standardized ileal digestible AA, calculated from analyzed AA concentrations and standardized ileal digestibility of the diet.

[2] L = plateau; U = slope; R = breakpoint.

[3] Residual SD.

[4] Estimated with treatment least squares mean values (n = 7 to 8 per treatment).

[5] Single-slope broken-line model: $y = L + U (R - x)$; $(R - x) = 0$, if $x > R$.

[6] Single-slope broken-line model: $y = L + U (R - x)$; $(R - x) = 0$, if $x < R$.

[7] Estimated with treatment least squares means values (n = 4 per treatment).

[8] N retention = N intake − (N urine + N feces).

[9] N utilization = (N retention·N intake^{-1})·100.

Table 6. Effects of increasing standardized ileal digestible (SID) Val:Lys ratio by crystalline L-Val supplementation on plasma free-AA and urea of weaned pigs (Exp. 2)[1]

	SID[2] Val:Lys, %							*P*-value[3]	
Item	49	55	58	63	67	74	SEM	Linear	Quadratic
EAA,[4] µmol/L									
Arg	73	74	77	80	82	83	3	0.19	0.95
His	49	43	39	27	34	26	2	<0.01	0.27
Ile	85	103	104	114	115	117	4	<0.01	0.20
Leu	100	110	107	102	115	107	3	0.42	0.56
Lys	88	77	41	27	38	31	6	<0.01	0.07
Met	75	76	81	84	85	85	4	0.35	0.78
Phe	59	67	62	60	58	56	2	0.31	0.41
Thr	534	582	382	425	417	494	22	0.18	0.05
Trp	28	26	24	22	21	20	1	<0.01	0.66
Val	40	47	63	90	156	255	11	<0.01	<0.01
NEAA,[5] µmol/L									
Ala	474	504	505	463	490	485	11	0.93	0.78
Asn	70	78	82	71	68	71	2	0.530	0.38
Asp	17	19	18	19	20	18	1	0.70	0.32
Cys	44	51	63	69	78	72	2	<0.01	0.01
Gln	553	584	583	503	511	532	15	0.23	0.91
Glu	98	120	114	120	137	137	5	0.02	0.67
Gly	1,951	2,078	1,964	2,095	1,972	2,023	33	0.75	0.58
Pro	289	304	306	317	290	306	12	0.82	0.71
Ser	321	352	334	315	298	296	10	0.14	0.52
Tyr	36	32	32	26	28	22	2	<0.01	0.89
Urea, mmol/L	4.14	4.06	4.07	3.74	3.38	3.52	0.12	0.01	0.91

[1] Data represent least squares means of 8 pigs per treatment and 7 pigs per treatment in the case of SID Val:Lys ratios of 58 and 74% after a 34-d feeding period (31 to 64 d of age).

[2] Standardized ileal digestible AA, calculated from analyzed AA concentrations and standardized ileal digestibility of the diet.

[3] Orthogonal polynomial contrast coefficients were used to determine the linear and quadratic effect of increasing dietary SID Val:Lys ratio.

[4] Essential AA.

[5] Nonessential AA.

trients, the composition of the experimental diets was optimized on the basis of SID AA expressed as a ratio to SID Lys in accordance with the concept of an ideal protein (Chung and Baker, 1992). The estimated ratios of SID AA:Lys were confirmed by analyses of the experimental diets and are designed to guarantee a sufficient supply relative to Lys. To secondarily limit Lys, average SID Lys concentrations of 1.00% were chosen for all experiments. Analyses of Lys and calculation with tabular digestibility averaged 0.99% SID Lys for all diets. Based on the growth performance observed in the present work, the dietary energy level, and genetic line used, the SID Lys requirement is 1.19% according to German standards (GfE, 2008), which is increased about 20% in comparison with the Lys supply of the present work. Using the same genetics and experimental conditions as in the present work, it was reported that total Lys:ME ratios of at least 0.9 g per MJ are needed for 8 to 30 kg of BW pigs (Roth et al., 1994, 1999). In the present work, total Lys:ME ratios of 0.82 and 0.79 g per MJ were used in Exp. 1 and in Exp. 2 and 3, respectively. In trials conducted on BCAA interactions, 1.12% SID Lys (1.24% total Lys) were used in cereal-based diets to enable greater performance levels as seen in the present work [M. K. Wiltafsky, M. W. Pfaffl (Lehrstuhl für Physiologie, Technische Universität München, Department für Tierwissenschaften, Freising, Germany), and F. X. Roth, unpublished data]. These trials were conducted using the same genetics, experimental conditions, and BW range as in the pres-

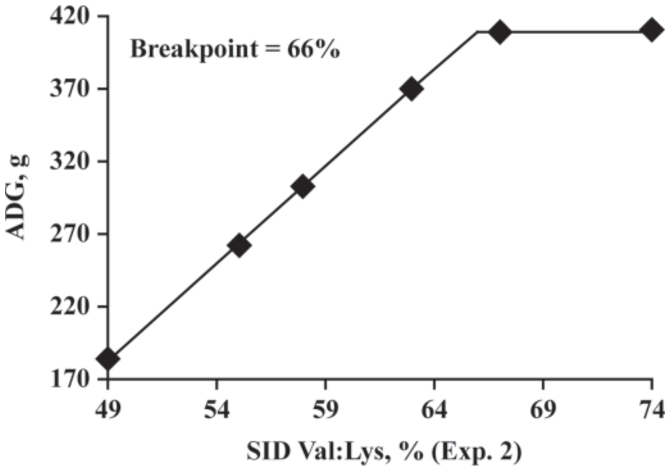

Figure 1. Fitted single-slope broken-line plot of ADG as a function of standardized ileal digestible (SID) Val:Lys ratio (Exp. 2) with treatment least squares means values (n = 7 to 8 per treatment). The optimal SID Val:Lys ratio determined by single-slope broken-line analysis was 66% (Y plateau = 409.2; slope below breakpoint = −1,330.0; R^2 = 0.99). The 95% confidence limits were 65.7 and 66.3% SID Val:Lys. The SID Val and Lys were calculated from analyzed Val and Lys concentrations and standardized ileal digestibility of the diet. For calculation of SID Val:Lys ratio, an average SID Lys of 0.99% was used.

Table 7. Effects of increasing standardized ileal digestible (SID) Val:Lys ratio by crystalline L-Val supplementation on performance and N balance parameter, and plasma urea of weaned pigs (Exp. 3)[1]

	SID[2] Val:Lys, %							*P*-value[3]	
Item	49	55	58	63	67	74	SEM	Linear	Quadratic
BW, kg	13.29	14.00	14.45	14.18	14.14	14.39	0.6	0.28	0.46
ADG, g	235	266	314	339	342	318	19	0.01	0.05
ADFI, g	466	462	509	502	524	528	27	0.18	0.85
G:F, g/kg	500	575	613	676	651	604	14	0.02	0.01
N intake, g/d	13.2	13.1	14.4	14.2	14.9	15.0	0.7	0.13	0.81
N excretion, g/d									
Feces	1.5	1.7	2.0	1.6	1.7	2.0	0.1	0.38	0.98
Urine	3.9	3.5	3.5	3.4	3.7	3.6	0.2	0.69	0.40
Total	5.4	5.2	5.5	5.1	5.4	5.6	0.3	0.84	0.59
N retention,[4] g/d	7.8	7.9	8.9	9.1	9.5	9.4	0.5	<0.04	0.43
N digestibility,[5] %	88.5	88.0	86.4	88.2	88.8	87.0	0.4	0.65	0.98
N utilization,[6] %	59.0	61.3	62.3	64.3	64.1	63.4	0.7	0.02	0.09
Urea, mmol/L	4.1	2.6	2.9	3.2	2.9	3.7	0.1	0.72	<0.01

[1] Data represent the least squares means of 4 N balances per treatment and 2 N balances per pig.

[2] Standardized ileal digestible AA, calculated from analyzed AA concentrations and standardized ileal digestibility of the diet.

[3] Orthogonal polynomial contrast coefficients were used to determine the linear and quadratic effect of increasing dietary SID Val:Lys ratio.

[4] N retention = N intake – (N urine + N feces).

[5] N digestibility = $[(\text{N intake} - \text{N feces})\cdot\text{N intake}^{-1}]\cdot 100$.

[6] N utilization = $(\text{N retention}\cdot\text{N intake}^{-1})\cdot 100$.

ent work. However, the realized ADG was 563 vs. 409 g of the present work (plateau phase of Exp. 2), indicating Lys insufficiency in the present work. In addition to these trials, recent work by Kendall et al. (2008) has shown that 1.05% SID Lys is deficient for weaned pigs raised under more commercial conditions. In 5 experiments using corn-soybean meal-based diets, the authors estimated a requirement of 1.30% SID Lys for 11 to 27 kg of BW pigs. This estimate was confirmed by Yi et al. (2006). A SID Lys requirement of 1.28 to 1.32% was estimated for optimal ADG in weaned pigs (12 to 25 kg of BW) under commercial research conditions using corn-soybean meal diets. From these data it was assumed that the SID Lys supply in the present experiments was marginal, and thus the obtained SID Val:Lys ratios are rational.

In determination of the Val requirement, dietary Ile and Leu contents must be considered. The antagonism between the BCAA has been reviewed by Harper et al. (1984). The BCAA share the first 2 steps of their catabolism and compete for the same enzymes. At first the BCAA are transaminated, and the resulting branched-chain α-keto acids are oxidatively decarboxylated by a multienzyme complex (the branched-chain α-keto acid dehydrogenase complex; **BCKDH**). The second step is irreversible and rate limiting. Especially high α-ketoisocaproate concentrations (the corresponding α-keto acid of Leu) can increase catabolism of the other BCAA by stimulation of BCKDH activity (Paxton and Harris, 1984). In rats and pigs, excess dietary Leu depresses plasma concentrations of Val, Ile, and their branched-chain α-keto acids, and increase whole-body Val oxidation by increasing BCKDH activity (Block and Harper, 1984; Langer et al., 2000). Increased dietary Leu concentrations thus result in an increased nutritional need for Ile and Val. To minimize antagonistic effects in the trials presented here, dietary excesses of Ile and Leu were avoided.

In Exp. 1, the dietary SID Val:Lys ratio incrementally increased from 56 to 84%. The ADG increased from 324 to 467 g as the SID Val:Lys ratio increased from 56 to 62%, but greater SID Val:Lys ratios did not further increase the performance. It was concluded that a SID Val:Lys ratio of 56% is deficient for weaned pigs. Estimation of the Val requirement was not possible for

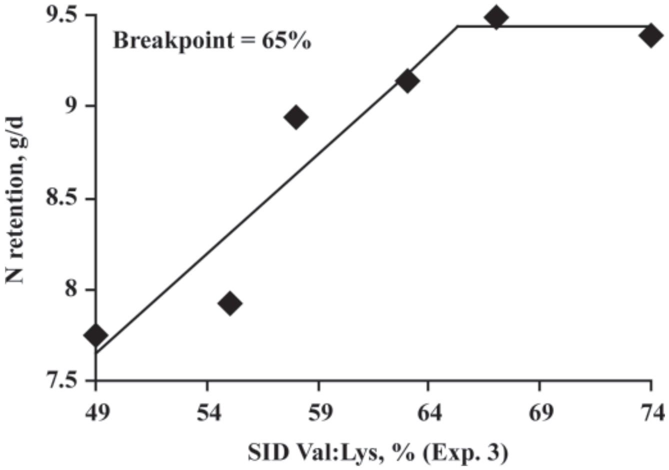

Figure 2. Fitted single-slope broken-line plot of N retention as a function of standardized ileal digestible (SID) Val:Lys ratio (Exp. 3) with treatment least squares means values (n = 4 per treatment). The optimal SID Val:Lys ratio determined by single-slope broken-line analysis was 65% (Y plateau = 9.4; slope below breakpoint = −11.0; $R^2 = 0.91$). The 95% confidence limits were 55 and 76% SID Val:Lys. The SID Val and Lys were calculated from analyzed Val and Lys concentrations and standardized ileal digestibility of the diet. For calculation of SID Val:Lys ratio, an average SID Lys concentration of 0.99% was used.

Exp. 1. This could have been caused by several factors: for example, by the basal level used, increments of l-Val supply, or number of pigs per treatment. Therefore, a second growth assay with a reduced basal Val level and smaller increments of l-Val supplementation was conducted. In Exp. 2, the SID Val:Lys ratio ranged from 49 to 74% and optimum SID Val:Lys ratios of 66 and 67% were estimated for ADG and ADFI, respectively. To verify these estimates, a N balance trial was conducted using the diets of Exp. 2. An optimal SID Val:Lys ratio of 65% was estimated for N retention and supports the ADG and ADFI results of Exp. 2. For G:F and plasma Val in Exp. 2 and N utilization in Exp. 3, breakpoints were determined at SID Val:Lys ratios of 61, 60, and 62%, respectively. These ratios were less than those estimated for ADG, ADFI, and N retention. Thus, greater SID Val:Lys ratios were necessary to maximize feed intake than required to maximize efficiency, demonstrating the importance of ad libitum feeding regimen.

Our findings regarding the responses of ADG and ADFI are close to NRC (1998) recommendations for 10 to 20 kg of BW pigs and to the results of Chung and Baker (1992) for 10 kg of BW pigs (Val:Lys ratio of 68%). From recent work investigating the Val requirements of 12 to 25 kg of BW pigs, it was concluded that a SID Val:Lys ratio of at least 70% is needed (Barea et al., 2009), which is slightly greater than the estimate from the present experiment.

Other studies have estimated the Val requirement on the basis of SID Val level in the diet. For 10 to 20 kg of BW pigs, estimates for SID Val of 0.78% appear in the literature (Mavromichalis et al., 2001a; Gaines et al., 2006); however, comparison of these estimates with the present work is difficult. Mavromichalis et al. (2001a) also expressed their estimates relative to ME and concluded that 10 to 20 kg of BW pigs require no more than 2.22 g of SID Val per Mcal of ME, which is 5% greater than NRC (1998) estimates (2.11 g of SID Val per Mcal of ME). The estimates to maximize ADG and ADFI estimated from the present work were 66 and 67% SID Val:Lys, respectively, which is 4 and 3% less than estimates of NRC (69% SID Val:Lys; 1998). However, it was clearly demonstrated in Exp. 2 and Exp. 3 that SID Val:Lys ratios of 67% were sufficient to maximize performance and greater SID Val:Lys ratios did not increase performance further.

It can be hypothesized that during evolution the AA pattern of sow milk was adjusted to the AA requirements of piglets. This assumption is supported by the fact that the AA pattern of the milk of the sow is constant and apparently not affected by dietary composition (Schneider et al., 1992). Furthermore, the AA pattern of the milk of the sow, whole-body (20 kg of BW), and deposited protein (20 to 90 kg of BW) are similar (Boisen, 2000), and calculation of daily AA requirements by daily protein deposition is described for Lys by NRC (1998). However, a large range (65 to 76%) of Val:Lys ratios has been found in the milk of the sow (Schneider et al., 1992; Boisen, 2000). Our data are in accordance with the ratio reported by Schneider et al. (1992). In recent work, Mavromichalis et al. (2001b) estimated the true ileal digestibility (**TID**) of AA in milk of sows for 17-d-old pigs. True ileal digestibility for Lys and Val was 92.1 and 87.0%, respectively. The estimated TID Val:Lys ratio was 73%.

Whole-body AA composition data can provide insight into the estimation of AA requirements, although AA requirements for maintenance and different intermediary utilization remain unconsidered. Analysis of whole-body essential AA composition of pigs from birth to 145 kg of BW revealed a Val:Lys ratio of 68% (Mahan and Shields, 1998), and a survey of literature for whole-body Val:Lys ratios suggested a range of 64 to 81% with an average of 69% (Mahan and Shields, 1998).

Performance data were confirmed by blood variables. In Exp. 2, plasma Val concentrations stayed at a reduced concentration for SID Val:Lys ratios of 49 to 58%, but rapidly increased in a linear manner for SID Val:Lys ratios of 63 to 74%. Increasing dietary supply of an AA from deficit to oversupply had little effect on plasma concentrations until the requirement is met, but will linearly increase plasma concentrations when the diet contains adequate or excess amounts (Morrison et al., 1961; Zimmerman and Scott, 1965). Plasma concentrations of other nonlimiting essential AA will be minimized once the requirement of the limiting AA is met because protein synthesis is maximal. These findings have been used to estimate AA needs in rats, chicks, and pigs (Morrison et al., 1961; Zimmerman and Scott, 1965; Mitchell et al., 1968; Oestemer et al., 1973). In Exp. 2, increased plasma Val concentrations were detected at SID Val:Lys ratios of 67 and 74%, and plasma concentrations of His, Lys, and Trp were minimized. Additionally, plasma urea concentrations were minimal at a SID Val:Lys ratio of 67% and increased again at a SID Val:Lys ratio of 74%. These results are confirmed by data from the N balance trial. Nitrogen digestibility was not affected, but N retention and utilization increased with increasing supply of dietary Val, reaching a maximum at SID Val:Lys ratios of 67 and 63%, respectively.

In conclusion, for optimal ADG and ADFI of 8 to 25 kg of BW pigs a SID Val:Lys ratio of 66 to 67% is necessary. Plasma urea and N retention data support the estimated optimal SID Val:Lys ratio established to optimize growth.

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Publication II

Estimation of the optimum ratio of standardized ileal digestible isoleucine to lysine for 8- to 25-kilogram pigs in diets containing spray-dried blood cells or corn gluten feed as a protein source.

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Estimation of the optimum ratio of standardized ileal digestible isoleucine to lysine for eight- to twenty-five-kilogram pigs in diets containing spray-dried blood cells or corn gluten feed as a protein source

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Estimation of the optimum ratio of standardized ileal digestible isoleucine to lysine for eight- to twenty-five-kilogram pigs in diets containing spray-dried blood cells or corn gluten feed as a protein source

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ABSTRACT: Two growth assays and 1 N balance trial were conducted to determine the standardized ileal digestible (SID) Ile:Lys ratio in 8- to 25-kg pigs using spray-dried blood cells or corn gluten feed as a protein source. In Exp. 1, 48 individually penned pigs (initial BW = 7.7 kg) were used in a 6-point SID Ile titration study (analyzed SID Ile of 0.36, 0.43, 0.50, 0.57, 0.64, and 0.72%) by addition of graded levels of L-Ile. The basal diet contained 1.00% SID Lys, 18.4% CP, and 13.6 MJ of ME/kg. Diets were based on wheat, barley, corn, and 7.5% spray-dried blood cells as a protein source. Dietary SID Leu and Val levels were 1.61 and 1.02%, respectively. For the 35-d period, ADG, ADFI, and G:F increased linearly ($P < 0.01$) and quadratically ($P < 0.04$) with increasing SID Ile:Lys. Estimates of optimal SID Ile:Lys ratios were 59% for ADG and ADFI. In Exp. 2, 24 N balances were conducted using the Exp. 1 diets (12 pigs; individually penned; average BW = 11.5 kg). Pigs were fed 3 times daily with an amount equal to 1.0 MJ of ME/kg of $BW^{0.75}$. Preparation and collection periods (7 d each) were repeated after rearranging the animals to treatments. Increasing the dietary SID Ile:Lys ratio increased N retention linearly ($P < 0.01$), and N utilization linearly ($P < 0.01$) and quadratically ($P < 0.01$). An optimal SID Ile:Lys ratio of 54% was estimated for N retention. In Exp. 3, 48 individually penned pigs (initial BW = 8.0 kg) were fed grain-based diets in a 6-point SID Ile titration (analyzed SID Ile of 0.35, 0.41, 0.49, 0.56, 0.62, and 0.69%). Dietary SID Ile was increased by graded addition of L-Ile. The basal diet contained 0.97% SID Lys, 16.8% CP, and 13.6 MJ of ME/kg. In contrast to Exp. 1 and 2, spray-dried blood cells were excluded and corn gluten feed was used as a protein source. Dietary SID Leu and Val were set to 1.05 and 0.66%. For the 42-d period, ADG, ADFI, and G:F increased linearly ($P < 0.01$) and quadratically ($P < 0.01$) with increasing SID Ile:Lys. Estimated optimal SID Ile:Lys ratios were 54, 54, and 49 for ADG, ADFI, and G:F, respectively. These experiments suggest that the optimal SID Ile:Lys ratio depends on diet composition. In Exp. 1, AA imbalances because of increased Leu contents may have led to increased Ile nutritional needs. For ADG and ADFI, an optimum SID Ile:Lys ratio of 54% was estimated for 8- to 25-kg pigs in diets without Leu excess.

Key words: growth, isoleucine requirement, isoleucine to lysine ratio, pig, protein source, spray-dried blood cell

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INTRODUCTION

Isoleucine is among the limiting AA in high-lean pigs fed reduced-protein diets. For 10- to 20-kg pigs His, Ile, Trp, and Val have been shown to be equally limiting after Lys and Thr in sorghum-soybean meal-based diets (12% CP; Brudevold and Southern, 1994). In corn-soybean meal-based diets (11% CP) fortified with Lys, Trp, Thr, and Met fed to growing pigs, Val was fifth-limiting, and either His or Ile sixth-limiting (Figueroa et al., 2003). Some work on the Ile requirement of pigs has recently been published (Parr et al., 2003; Kerr et al., 2004a; Dean et al., 2005), but most experimental data for the determination of Ile requirements of 10- to 25-kg pigs are dated (Oestemer et al., 1973b; Henry

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et al., 1976), and those expressed as the ratio of standardized ileal digestible (**SID**) Ile:Lys are sparse. It is difficult to use older studies because of missing values for ME and for the digestibility of AA, possible deficiencies in other AA, and the use of CP levels different from current practice. Conversely, most of the recent Ile requirement trials were conducted using blood products as a protein source. Spray-dried blood cells (**SDBC**) have often been used in diets for weaned pigs because of their unique AA pattern and increased digestibility and CP content (NRC, 1998). The use of SDBC enables the formulation of diets comparable with those used in practice but increases the dietary levels of Leu and Val. A Leu excess is known to increase the nutritional need for Ile and Val because it stimulates the catabolism of these AA (Harper et al., 1984). Hence, estimates obtained in experiments using blood products as a protein source might overestimate the Ile requirements of pigs fed diets free of these components. The present work was conducted to determine the ideal SID Ile:Lys ratio in 8- to 25-kg pigs and to investigate the impact of SDBC and corn gluten feed (**CGF**) on the determination of the Ile requirement.

MATERIALS AND METHODS

Animal housing and care were conducted under supervision of the veterinary office of the Bavarian government. The handling protocol ensured proper care and treatment of all animals in conformity with the German law for animal protection.

General

To estimate the ideal SID Ile:Lys ratio of 8- to 25-kg pigs, 2 growth trials and 1 N balance trial were conducted using SDBC or CGF as a protein source. The trials were conducted with German Landrace × Piètrain crossbred pigs weaned at 28 d of age and raised at the same commercial farm. The sex ratio within each trial was balanced. Animals were individually housed in pens (60 × 100 cm; plastic slats) in an environmentally controlled building. Feed and drinking water were provided ad libitum, unless otherwise noted. Relative humidity was 50 to 60%, and the initial room temperature of 29°C was incrementally reduced to 25°C by the end of the trials. Artificial light was provided from 0700 to 1700 h, followed by half-light.

Diet composition was optimized on the basis of SID AA. These were calculated using the analyzed AA contents of feed ingredients and estimates of SID in feed ingredients (Sauvant et al., 2004). A basal SID Ile content of 0.35% and 5 L-Ile increments of 0.075% were used. Because the ratio of SID Ile:Lys was to be estimated it was necessary to also limit Lys. Accordingly, the SID Lys level was set at 1.00% (GfE, 2008). If necessary, crystalline L-AA were supplemented to meet the conditions for an ideal protein (Chung and Baker, 1992). Diets were fortified with minerals and vitamins to meet or exceed the recommended levels for 10- to 20-kg pigs (NRC, 1998).

For calculation of the response variables ADG, ADFI, and G:F, individual pig BW and feed disappearance (as-fed basis) were recorded weekly, unless otherwise stated. Additionally, blood samples were taken at the termination of the growth trials for analysis of plasma free AA and plasma urea. Nitrogen digestibility, utilization, and retention were used in addition to the above-mentioned growth variables as response variables for the N-balance trial.

Exp. 1

A study using 48 pigs with an initial BW of 7.7 ± 0.7 kg was carried out during an experimental period of 35 d. Pigs were allotted to 1 of 6 dietary treatments (Table 1). Diet composition was mainly based on wheat, barley, and corn, with SDBC used as the protein source. The unique AA pattern of SDBC guaranteed low dietary levels of Ile, but caused some oversupply of Leu (1.61% SID Leu) and Val (1.02% SID Val). Diets were supplemented with L-Ile whereas maintaining almost constant levels of Lys (average SID Lys 1.01%). The SID Ile contents for each dietary treatment were 0.36, 0.43, 0.50, 0.57, 0.64, and 0.72%. The analyzed CP content increased from 18.4 to 18.9%. The energy content of diets maintained constant among treatments and was calculated to be 13.6 MJ of ME/kg.

Exp. 2

A total of 24 N balances (2 N balances per pig, 4 balances per treatment) were conducted with 12 pigs using the diets of Exp. 1 (Table 1). After an initial acclimation period (7 d) to new surroundings, preparation and collection periods (7 d each) followed. Allocation of pigs to treatments was then rearranged, and a second round of preparation and collection periods (7 d each) was conducted. Animals were restrictively fed 3 times daily. Daily energy supply was calculated with 1.0 MJ of ME/kg of $BW^{0.75}$. For calculation of daily energy supply, pigs were weighed at the beginning of each preparation period and BW gains of 250 and 400 g/d were assumed for the first and the second balances, respectively. Feed was mixed with water (1:1) to enhance intake and minimize feed losses. Drinking water was provided for free intake for 30 min after feeding.

During the collection period, feces and urine of individual pigs were collected twice daily. Whole feces and urine (acidified to pH ~3) were stored at 4°C until the end of the collection period and were consolidated into animal individual samples. Feces were freeze-dried and stored for further analysis.

Exp. 3

One growth assay using 48 pigs (initial BW of 8.0 ± 0.8 kg) was conducted during an experimental period

Table 1. Composition of experimental diets containing spray-dried blood cells (as-fed basis; Exp. 1 and 2)

	SID[1] Ile:Lys, %					
Item	36	43	50	56	63	71
Ingredient, %						
Wheat	34.03	34.03	34.03	34.03	34.03	34.03
Barley	28.50	28.50	28.50	28.50	28.50	28.50
Corn	20.78	20.78	20.78	20.78	20.78	20.78
Spray-dried blood cells[2]	7.50	7.50	7.50	7.50	7.50	7.50
Protex[3]	2.00	2.00	2.00	2.00	2.00	2.00
Corn starch	0.375	0.30	0.225	0.15	0.075	—
Sugar beet molasses	3.00	3.00	3.00	3.00	3.00	3.00
Soy oil	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin-mineral-premix[4]	1.49	1.49	1.49	1.49	1.49	1.49
Limestone	0.32	0.32	0.32	0.32	0.32	0.32
$CaHPO_4$	1.05	1.05	1.05	1.05	1.05	1.05
L-Lys·HCl	0.11	0.11	0.11	0.11	0.11	0.11
DL-Met	0.16	0.16	0.16	0.16	0.16	0.16
L-Thr	0.17	0.17	0.17	0.17	0.17	0.17
L-Ile	—	0.075	0.15	0.225	0.30	0.375
Analyzed composition, %						
ME,[5] MJ/kg	13.6	13.6	13.6	13.6	13.6	13.6
CP	18.4	18.4	18.5	18.6	18.9	18.9
Lys	1.07	1.06	1.08	1.08	1.09	1.09
TSAA	0.68	0.67	0.68	0.68	0.68	0.67
Thr	0.70	0.70	0.72	0.71	0.71	0.72
Trp	0.24	0.24	0.24	0.24	0.25	0.25
Val	1.09	1.10	1.13	1.12	1.12	1.14
Ile	0.42	0.49	0.56	0.63	0.70	0.78
Leu	1.73	1.74	1.75	1.74	1.75	1.76
Phe + Tyr	1.43	1.46	1.45	1.46	1.45	1.47
His	0.76	0.77	0.78	0.78	0.78	0.79
Calculated SID composition, %						
Lys	1.00	0.99	1.01	1.01	1.02	1.02
TSAA	0.60	0.59	0.60	0.60	0.60	0.59
Thr	0.62	0.62	0.64	0.63	0.63	0.64
Trp	0.21	0.21	0.21	0.21	0.21	0.21
Val	1.00	1.01	1.03	1.02	1.02	1.04
Ile	0.36	0.43	0.50	0.57	0.64	0.72
Leu	1.59	1.60	1.61	1.60	1.61	1.62
Phe + Tyr	1.28	1.31	1.30	1.31	1.30	1.32
His	0.73	0.74	0.75	0.75	0.75	0.75

[1] SID = standardized ileal digestible.

[2] Sonac, Bad Bramstedt, Germany.

[3] Consists of 97% fish meal and 3% soybean meal, 64% CP, Fritz Köster Handelsgeselschaft AG, Hamburg, Germany.

[4] Provided the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 900 IU; vitamin E (DL-α-tocopherylacetate), 30 mg; menadione, 1.88 mg; thiamin, 1.13 mg; riboflavin, 3 mg; pyridoxine, 1.88 mg; vitamin B_{12}, 15 µg; niacin, 13 mg; pantothenic acid, 9.4 mg; biotin, 150 µg; folic acid, 1.5 mg; choline (as choline chloride), 135 mg; Ca, 3.3 g; P, 0.9 g; Na, 0.75 g; Mg, 0.3 g; Zn (zinc oxide), 52.5 mg; Fe (ferrous sulfate monohydrate), 75 mg; I (calcium iodate), 0.9 mg; Se (sodium selenite pentahydrate), 0.20 mg; Cu (copper sulfate pentahydrate), 7.5 mg; Mn (mangane oxide), 30 mg.

[5] Calculated.

of 42 d. Pigs were allotted to 1 of 6 dietary treatments. To avoid Leu and Val excesses, diet composition was mainly based on wheat, corn, and barley as in Exp. 1 and 2, but protein was provided by CGF and whey powder instead of SDBC (Table 2). The basal Ile content, increments of L-Ile supplementation, and Lys levels were calculated to be the same as in Exp. 1 and 2. Dietary SID Ile levels were analyzed at 0.35, 0.41, 0.49, 0.56, 0.62 and 0.69%, with an average SID Lys level of 0.95%. Diets were calculated to be isonitrogenous and isoenergetic (16.7% CP; 13.6 MJ of ME/kg).

Analyses

Nitrogen analyses of ingredients, mixed diets, urine, and feces were performed using the Kjeldahl procedure (AOAC, 2000), and CP was calculated (N × 6.25). Analysis of total dietary AA was done by Ajinomoto Eurolysine S.A.S. (Amiens, France) after Commission Directive 98/64/EC (1998); Trp analysis followed AFNOR (1998). After acid hydrolysis with HCl (6 *N*, reflux for 23 h at 110°C) AA were analyzed by ion-exchange chromatography. To avoid degradation during hydrolysis, Met and Cys were determined by ion exchange chromatography after performic acid oxidation. Reverse-phase HPLC was used to determine Trp after alkaline hydrolysis with barium hydroxide (16 h, 120°C).

At the termination of the growth trials and after a fasting period of 2.5 h, all pigs were bled once via jugular vein puncture using heparinized tubes (6 to 9 mL, S-Monovetten, Sarstedt, Nümbrecht, Germany) for analysis of plasma free AA and urea. Samples were carefully shaken and stored on ice. Plasma was obtained by centrifugation at 800 × *g* for 20 min at 20°C and stored at −80°C until analysis. For analysis of plasma free AA, plasma proteins were removed by precipitation with salicylsulfonic acid and centrifugation at 11,000 × *g* for 10 min. The protein-free supernatant was analyzed by ion exchange chromatography using an automatic AA analyzer (LC 3000, Biotronik, Hamburg, Germany), af-

Table 2. Composition of experimental diets free of spray-dried blood cells (as-fed basis; Exp. 3)

	SID[1] Ile:Lys, %					
Item	36	44	53	59	67	72
Ingredient, %						
Wheat	22.79	22.95	23.10	23.25	23.40	23.55
Corn	21.76	21.75	21.75	21.75	21.75	21.75
Corn gluten feed	20.00	20.00	20.00	20.00	20.00	20.00
Barley	15.00	15.00	15.00	15.00	15.00	15.00
Whey powder, low lactose	5.00	5.00	5.00	5.00	5.00	5.00
Corn starch	0.38	0.30	0.23	0.15	0.08	—
Soy oil	3.01	2.97	2.94	2.91	2.88	2.85
Sugar beet molasses	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin-mineral premix[2]	1.23	1.23	1.23	1.23	1.23	1.23
Limestone	1.03	1.03	1.03	1.03	1.03	1.03
$CaHPO_4$	0.75	0.74	0.74	0.74	0.74	0.74
L-Lys·HCl	0.87	0.87	0.87	0.87	0.87	0.87
DL-Met	0.23	0.23	0.23	0.22	0.22	0.22
L-Thr	0.30	0.30	0.30	0.30	0.30	0.30
L-Trp	0.13	0.13	0.13	0.13	0.13	0.13
L-Val	0.21	0.21	0.21	0.21	0.21	0.21
L-Leu	0.14	0.14	0.14	0.13	0.13	0.13
L-Ile	—	0.075	0.15	0.225	0.30	0.375
L-His	0.07	0.07	0.07	0.07	0.07	0.07
L-Phe	0.23	0.23	0.23	0.23	0.22	0.22
L-Glu	4.88	4.77	4.66	4.55	4.44	4.33
Analyzed composition, %						
ME,[3] MJ/kg	13.6	13.6	13.6	13.6	13.6	13.6
CP	16.8	16.8	16.9	16.6	16.6	16.7
Lys	1.06	1.04	1.03	1.03	1.01	1.04
TSAA	0.69	0.70	0.71	0.70	0.70	0.70
Thr	0.70	0.68	0.68	0.68	0.68	0.68
Trp	0.24	0.23	0.24	0.23	0.24	0.24
Val	0.77	0.75	0.75	0.76	0.75	0.75
Ile	0.42	0.48	0.56	0.62	0.68	0.75
Leu	1.20	1.19	1.19	1.21	1.18	1.19
Phe + Tyr	1.12	1.10	1.11	1.11	1.09	1.11
His	0.37	0.36	0.37	0.37	0.37	0.37
Calculated SID composition, %						
Lys	0.97	0.95	0.94	0.94	0.92	0.95
TSAA	0.58	0.60	0.61	0.60	0.60	0.60
Thr	0.62	0.60	0.60	0.60	0.60	0.60
Trp	0.21	0.21	0.21	0.21	0.21	0.21
Val	0.67	0.66	0.66	0.66	0.66	0.65
Ile	0.35	0.41	0.49	0.56	0.62	0.69
Leu	1.05	1.04	1.04	1.06	1.04	1.04
Phe + Tyr	1.01	0.99	1.00	1.00	0.98	1.00
His	0.32	0.31	0.32	0.32	0.32	0.32

[1] SID = standardized ileal digestible.

[2] Provided the following per kilogram of diet: vitamin A, 5,000 IU; vitamin D_3, 500 IU; vitamin E, 30 mg; menadione, 0.15 mg; thiamin, 3 mg; riboflavin, 3 mg; pyridoxine, 3 mg; vitamin B_{12}, 18 µg; niacin, 25 mg; pantothenic acid, 15 mg; biotin, 50 µg; folic acid, 300 µg; choline (as choline chloride), 1,000 mg; Zn (zinc sulfate heptahydrate), 69.9 mg; I (potassium iodate), 0.13 mg; Se (sodium selenite pentahydrate), 0.14 mg.

[3] Calculated.

Table 3. Effects of increasing ratio of standardized ileal digestible (SID) Ile:Lys by crystalline L-Ile supplementation on performance of weaned pigs in diets containing spray-dried blood cells (Exp. 1)[1]

	SID Ile:Lys							Contrast[2] P-value	
Item	36	43	50	56	63	71	SEM	Linear	Quadratic
BW, kg									
d 0	7.71	7.70	7.69	7.70	7.58	7.71	0.11	0.89	0.91
d 14	9.24	10.04	10.81	11.15	11.53	11.46	0.21	<0.01	<0.02
d 35	13.76	16.79	20.44	21.80	23.02	23.46	0.63	<0.01	<0.01
ADG, g									
d 0 to 14	110	168	223	247	274	269	12	<0.01	<0.02
d 14 to 35	215	321	458	507	547	571	22	<0.01	<0.01
d 0 to 35	173	260	364	403	438	450	17	<0.01	<0.01
ADFI, g									
d 0 to 14	181	211	256	279	303	303	11	<0.01	0.22
d 14 to 35	377	490	694	756	809	858	31	<0.01	0.02
d 0 to 35	298	379	519	565	605	636	22	<0.01	0.03
G:F, g/kg									
d 0 to 14	600	785	856	881	921	894	22	<0.01	<0.01
d 14 to 35	569	652	663	671	677	667	8	<0.01	<0.01
d 0 to 35	574	683	702	713	723	709	9	<0.01	<0.01

[1] Data represent the least squares means of 8 pigs per treatment, in the case of SID Ile:Lys of 63% 7 pigs per treatment (1 pig was removed for health reasons), during a 35-d feeding period (31 to 65 d of age).

[2] Orthogonal polynomial contrast coefficients were used to determine linear and quadratic effects of increasing ratios of dietary standardized ileal digestible Ile:Lys.

ter dilution with lithium acetate solution. Plasma urea was automatically co-analyzed in the chromatograms for the determination of free AA in plasma.

Statistics

Pigs were allotted to treatments on the basis of BW. Sex and ancestry were equalized among treatments in a randomized block design. The experimental unit was the individual pig. Data for each response criterion were analyzed by ANOVA using the MIXED procedure (SAS Inst. Inc., Cary, NC). The model included the effects of dietary treatment and sex, and for the N balance trial, the balance number. Litter was included as random effect and initial BW was used as a covariate. Contrasts were conducted to determine the linear and quadratic effects of increasing dietary SID Ile:Lys (Lowry, 1992). The IML procedure of SAS was used to generate orthogonal polynomial coefficients, and the MIXED procedure was used to compute orthogonal polynomial contrast sums of squares. Estimates of requirement were done by subjecting the group least squares means data to least squares broken-line methodology (Robbins et al., 2006).

Further, the bioefficiency of the L-Ile supply was of interest. Thus, a slope-ratio assay was calculated for ADG of Exp. 1 and 3. A linear model was used (GLM procedure; Littell et al., 1997). Thus, treatments 5 and 6 in Exp. 1 and treatments 4 to 6 in Exp. 3 were excluded. To enable comparison across trials, alterations in ADG were expressed as percentage of improvement in performance within an experiment as a function of dietary L-Ile supplementation (basal group = 100). Approximate 95% fiducial limits were calculated (Littell et al., 1997).

RESULTS

Exp. 1

Overall ADG, ADFI, and G:F increased linearly ($P < 0.01$) and quadratically ($P < 0.04$) as the ratio of SID Ile:Lys increased from 36 to 71% (Table 3). Broken-line analysis estimated optimum SID Ile:Lys ratios of 59, 59, and 45% for overall ADFI, ADG, and G:F, respectively (Table 4; Figure 1).

Plasma concentrations of free AA are shown in Table 5. Increasing the SID Ile:Lys ratio increased plasma concentrations of Ile linearly ($P < 0.01$) and quadratically ($P < 0.01$), but decreased plasma concentrations of Lys, Trp, Tyr, and urea linearly ($P < 0.03$) and quadratically ($P < 0.03$). In addition, plasma concentrations of Leu, Asp, Gln, Glu, Gly, and Ser decreased linearly ($P < 0.04$) and plasma concentrations of Cys increased linearly ($P < 0.01$). Plasma Ile concentrations increased linearly as dietary SID Ile:Lys ratios of 46% were exceeded (Figure 2).

Exp. 2

Overall ADG and ADFI increased linearly ($P < 0.03$), and G:F increased linearly ($P < 0.01$) and quadratically ($P < 0.04$) as the ratio of SID Ile:Lys increased from 36 to 71% (Table 6). Nitrogen digestibility was not affected by increasing dietary SID Ile:Lys, but N retention increased linearly ($P < 0.01$) and N utiliza-

Table 4. Single-slope broken-line models for performance, N balance variable, and plasma concentrations of Ile as a function of standardized ileal digestible Ile:Lys

	Variable[1]				
Item	L	U	R	R^2	95% confidence interval
Exp. 1[2]					
ADG[3]	442.5	−1,173.5	59	0.98	52 to 65
ADFI[3]	621.3	−1,389.1	59	0.97	52 to 67
G:F[3]	711.6	−1,456.3	45	0.99	43 to 48
Plasma Ile[4]	20.0	−210.5	46	0.99	41 to 51
Exp. 2[5]					
N retention[3]	8.5	−18.0	54	0.88	38 to 69
Exp. 3[6]					
ADG[3]	433.3	−1,367.4	54	0.98	49 to 59
ADFI[3]	613.1	−1,537.4	54	0.96	46 to 61
G:F[3]	704.7	−1,155.7	49	0.98	43 to 55
Plasma Ile[4]	17.1	−789.8	51	0.98	44 to 57

[1] L = plateau; U = slope; R = breakpoint.

[2] Estimated with treatment least squares means values (n = 7 or 8 per treatment; 1 pig was removed for health reasons).

[3] Single-slope broken-line model: $y = L + U (R - x)$; $(R - x) = 0$, if $x > R$.

[4] Single-slope broken-line model: $y = L + U (R - x)$; $(R - x) = 0$, if $x < R$.

[5] Estimated with treatment least squares means values (n = 4 per treatment).

[6] Estimated with treatment least squares means values (n = 8 per treatment).

tion increased linearly ($P < 0.01$) and quadratically ($P < 0.01$). An optimal SID Ile:Lys ratio of 54% was estimated for N retention (Table 4).

Exp. 3

Overall ADG, ADFI, and G:F increased linearly ($P < 0.01$) and quadratically ($P < 0.01$) as SID Ile:Lys increased from 36 to 72% (Table 7). Broken-line analysis calculated for ADG, ADFI, and G:F estimated optimal SID Ile:Lys ratios of 54, 54, and 49%, respectively (Table 4; Figure 1).

Plasma Ile concentrations increased linearly ($P < 0.01$) and quadratically ($P < 0.01$) and concentrations of His, Thr, Gly, Ser, Tyr, and urea decreased linearly ($P < 0.01$) and quadratically ($P < 0.05$) as SID Ile:Lys increased (Table 8). A linear decrease ($P < 0.01$) was seen in plasma concentrations of Lys and Val, whereas concentrations of Met, Ala, Asp, Cys, Gln, and Glu increased linearly ($P < 0.02$). Plasma Ile concentrations increased linearly as dietary SID Ile:Lys ratios of 51% were exceeded (Table 4). For calculation of the bioefficiency of the L-Ile supply in Exp. 1 and 3, a slope-ratio assay was performed for ADG (Figure 3). A slope-ratio (Exp. 1/Exp. 3) of 0.775 confirmed significantly less bioefficiency of L-Ile in diets using SDBC as a protein source compared with diets containing CGF. Approximate 95% fiducial limits were calculated as 0.578 and 0.972.

DISCUSSION

The aim of the present work was to determine the ideal SID Ile:Lys ratio of 8- to 25-kg weaned pigs. It was therefore necessary to generate basal diets that were first limiting in Ile and second limiting in Lys. Hence, the composition of experimental diets was optimized on the basis of SID AA expressed as a ratio to SID Lys according to the concept of an ideal protein (Chung and Baker, 1992). To limit Lys, a dietary SID Lys concentration of 1.00% was planned. Calculation for the mixed diets with analyzed Lys contents revealed an average SID Lys content of 1.01% for Exp. 1 and 2, and 0.95% for Exp. 3. Based on the performance level realized in the present work, the formulated dietary energy level, and genetic line used, the SID Lys require-

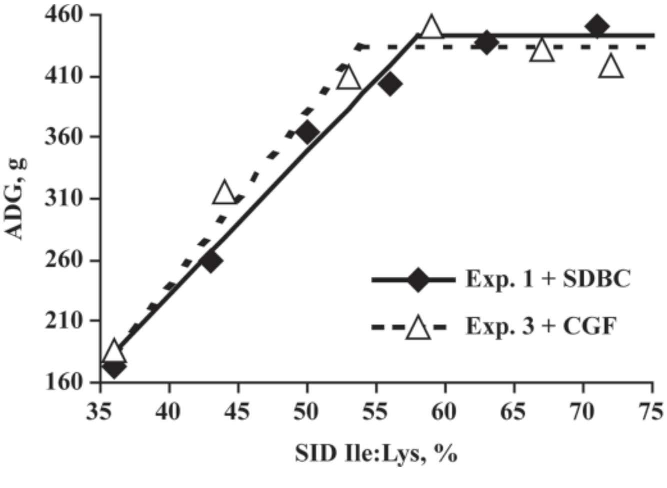

Figure 1. Fitted broken-line plot of ADG as function of standardized ileal digestible (SID) Ile:Lys with treatment least squares means values (n = 7 or 8 per treatment; in Exp. 1, 1 pig was removed for health reasons). The digestible Ile requirements determined by broken-line analysis using least squares methodology were 59% SID Ile:Lys for Exp. 1 [diets containing spray-dried blood cells (SDBC); plateau = 442.5; slope below breakpoint = −1,173.5; R^2 = 0.98; 95% confidence limits: 52 to 65] and 54% SID Ile:Lys for Exp. 3 [diets containing corn gluten feed (CGF); plateau = 433.3; slope below breakpoint = −1,367.4; R^2 = 0.98; 95% confidence limits: 49 to 59].

Table 5. Effects of increasing ratio of standardized ileal digestible (SID) Ile:Lys by crystalline L-Ile supplementation on plasma free-AA and urea of weaned pigs fed diets containing spray-dried blood cells (Exp. 1)[1]

	SID Ile:Lys							Contrast[2] *P*-value	
Item	36	43	50	56	63	71	SEM	Linear	Quadratic
EAA,[3] μmol/L									
Arg	66	52	49	62	68	72	3	0.13	<0.06
His	175	195	219	489	348	254	33	0.11	<0.10
Ile	20	20	30	39	55	73	3	<0.01	<0.01
Leu	224	220	177	170	180	187	7	<0.04	0.09
Lys	238	163	57	42	32	42	13	<0.01	<0.01
Met	60	63	60	72	61	60	3	0.94	0.36
Phe	97	92	75	84	87	84	3	0.26	0.19
Thr	490	439	353	387	365	413	21	0.21	0.12
Trp	46	38	34	34	31	37	1	0.02	<0.01
Val	366	339	305	312	325	333	9	0.32	0.10
NEAA,[4] μmol/L									
Ala	578	608	565	570	519	527	15	0.11	0.70
Asn	118	126	114	123	111	108	3	0.27	0.51
Asp	20	23	19	19	17	18	1	0.01	0.75
Cys	83	83	89	93	96	105	2	<0.01	0.55
Gln	765	910	793	758	707	686	22	<0.02	0.21
Glu	182	204	190	180	138	164	7	<0.02	0.55
Gly	1,539	1,479	1,342	1,173	1,169	1,108	37	<0.01	0.39
Pro	385	388	342	350	342	337	11	0.14	0.68
Ser	317	310	227	183	177	165	12	<0.01	0.12
Tyr	104	101	71	78	69	78	4	<0.01	<0.03
Urea, mmol/L	4.1	2.3	2.5	2.6	2.9	2.8	0.1	<0.01	<0.01

[1] Data represent the least squares means of 8 pigs per treatment, 7 pigs per treatment in the case of SID Ile:Lys = 63% (1 pig was removed for health reasons), after a 35-d feeding period (31 to 65 d of age).

[2] Orthogonal polynomial contrast coefficients were used to determine linear and quadratic effects of increasing ratios of dietary SID Ile:Lys.

[3] Essential AA.

[4] Nonessential AA.

ment is 1.18% according to German standards (GfE, 2008), which is about 20% greater in comparison with the Lys supply of the present work.

Using the same genetics as in the present work, it was reported that total Lys:ME ratios of at least 0.9 g per MJ are needed for 8- to 30-kg pigs (Roth et al., 1994, 1999). In the present work, total Lys:ME ratios of 0.79 and 0.76 g per MJ were used in Exp. 1 and 2, and in Exp. 3, respectively. Recent work by Kendall et al. (2008) has shown that 1.05% SID Lys is deficient for weaned pigs. In 5 experiments conducted under commercial conditions using corn-soybean meal-based diets, these authors estimated a Lys requirement of 1.30% SID Lys for 11- to 27-kg pigs (Triumph 4 × PIC C22). This estimate was confirmed by Yi et al. (2006) for the same genetics and BW range and conducted using comparable conditions. In experiments for the determination of Ile requirement, diets containing up to 1.10% SID Lys and up to 1.26% apparent digestible Lys were proven to be secondarily limiting in Lys for weaned pigs (Triumph 4 × PIC C 22) and for early weaned pigs (PIC C 22 × 327), respectively (James et al., 2001; Fu et al., 2006c). From these data we assumed that the SID Lys supply in the present experiments was secondarily limiting and thus the obtained SID Ile:Lys ratios are meaningful.

To generate a strong response to the increasing Ile supplementation, a basal SID Ile content of 0.35% was chosen. In Exp. 1, this low basal Ile content was achieved by the inclusion of 7.5% SDBC. The nutritional value of SDBC is characterized by high levels

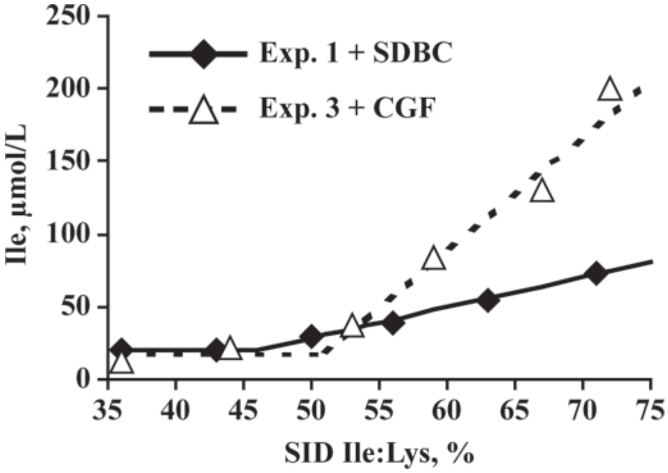

Figure 2. Fitted broken-line plot of plasma free Ile as a function of standardized ileal digestible (SID) Ile:Lys with treatment least squares means values (n = 7 or 8 per treatment; in Exp. 1, 1 pig was removed for health reasons). Breakpoints were estimated by broken-line analysis using least squares methodology at SID Ile:Lys ratios of 46 and 51% in Exp. 1 [diets containing spray-dried blood cells (SDBC); plateau = 20.0; slope below breakpoint = −210.5; R^2 = 0.99; 95% confidence limits: 41 to 51] and in Exp. 3 [diets containing corn gluten feed (CGF); plateau = 17.1; slope below breakpoint = −789.8; R^2 = 0.98; 95% confidence limits: 44 to 57], respectively.

Table 6. Effects of increasing ratio of standardized ileal digestible (SID) Ile:Lys by crystalline L-Ile supplementation on performance, N-balance, and plasma urea of weaned pigs fed diets containing spray-dried blood cells (Exp. 2)[1]

	SID Ile:Lys							Contrast[2] *P*-value	
Item	36	43	50	56	63	71	SEM	Linear	Quadratic
BW, kg	10.81	11.02	11.47	11.79	12.18	11.89	0.47	0.14	0.63
ADG, g	153	198	253	270	281	287	16	<0.01	0.19
ADFI, g	346	352	409	427	450	459	23	0.02	0.70
G:F, g/kg	441	572	611	638	647	631	22	<0.01	<0.04
N intake, g/d	10.19	10.42	12.19	12.78	13.54	13.89	0.70	<0.02	0.71
N excretion g/d									
Feces	1.65	1.77	1.81	2.07	2.24	2.00	0.11	<0.04	0.34
Urine	2.94	2.58	2.36	2.76	3.02	2.77	0.16	0.75	0.44
Total	4.68	4.35	4.18	4.83	5.25	4.77	0.26	0.32	0.74
N retention, g/d	5.51	6.07	8.00	7.95	8.29	9.12	0.47	<0.01	0.43
N digestibility, %	83.8	83.0	85.3	83.8	83.4	85.5	0.3	0.11	0.28
N utilization,[3] %	54.1	57.5	65.6	62.3	61.7	65.6	1.0	<0.01	<0.01

[1] Data represent the least squares means of 4 N-balances per treatment and 2 N-balances per pig.

[2] Orthogonal polynomial contrast coefficients were used to determine linear and quadratic effects of increasing ratios of dietary SID Ile:Lys.

[3] N utilization = $(\text{N intake} - \text{total N excretion} \cdot \text{N intake}^{-1}) \cdot 100$.

of CP (92%) and excellent AA bioavailability. For example, Lys bioavailabilities of SDBC and crystalline Lys have been shown to be similar (DeRouchey et al., 2002). Therefore, SDBC can be used in place of fish meal as a protein source in phase 1 to 3 nursery diets (Hinson et al., 2007). Inclusion of 2.5% SDBC in diets for nursery pigs has been shown to increase ADG (DeRouchey et al., 2002). However, SDBC caused depression of performance at inclusion levels of 4 to 5% in diets for nursery pigs (Kerr et al., 2004b; Hinson et al., 2007). Attention must be paid to the Ile content of diets when using SDBC. The Ile content of SDBC is extremely low (0.49%), but SDBC are rich in Leu (12.70%), Lys (8.51%), Val (8.50%), and His (6.99%; NRC, 1998). If Ile is supplemented, inclusion concentrations of up to 7.5% SDBC in diets for 8- to 25-kg pigs are possible without negative effects (Kerr et al., 2004b). This level has been used in experiments to determine Ile requirements (DeRouchey et al., 2002; Parr et al., 2003; Kerr et al., 2004a). Therefore, the level of SDBC was limited to 7.5% in the present work. The inclusion of SDBC enabled a diet composition comparable with those used in practice, but still deficient in Ile.

The use of 7.5% SDBC in Exp. 1 and 2 resulted in some oversupply of His, Leu, and Val. The average SID His, Leu and Val ratios to Lys were 75, 161, and 102%, respectively, whereas according to the ideal protein of Chung and Baker (1992), ratios of 32, 100, and 68% are

Table 7. Effects of increasing ratio of standardized ileal digestible (SID) Ile:Lys by crystalline L-Ile supplementation on performance of weaned pigs fed diets free of spray-dried blood cells (Exp. 3)[1]

	SID Ile:Lys, %							Contrast[2] *P*-value	
Item	36	44	53	59	67	72	SEM	Linear	Quadratic
BW, kg									
d 0	7.97	7.94	7.98	7.96	7.94	7.97	0.12	0.99	0.99
d 21	10.44	12.06	13.72	13.36	13.84	12.78	0.32	<0.01	<0.01
d 42	15.81	21.18	25.20	26.85	26.12	25.51	0.75	<0.01	<0.01
ADG, g									
d 0 to 21	118	195	274	257	280	229	13	<0.01	<0.01
d 21 to 42	256	434	546	642	585	606	25	<0.01	<0.01
d 0 to 42	187	315	410	450	432	418	17	<0.01	<0.01
ADFI, g									
d 0 to 21	219	283	348	334	354	301	12	<0.01	<0.01
d 21 to 42	449	687	823	953	877	858	33	<0.01	<0.01
d 0 to 42	334	485	586	644	616	580	22	<0.01	<0.01
G:F, g/kg									
d 0 to 21	529	672	770	763	766	756	18	<0.01	<0.01
d 21 to 42	570	631	666	673	669	706	9	<0.01	<0.05
d 0 to 42	557	641	702	698	698	720	10	<0.01	<0.01

[1] Data represent the least squares means of 8 pigs per treatment during a 42-d feeding period (31 to 72 d of age).

[2] Orthogonal polynomial contrast coefficients were used to determine linear and quadratic effects of increasing ratios of dietary SID Ile:Lys.

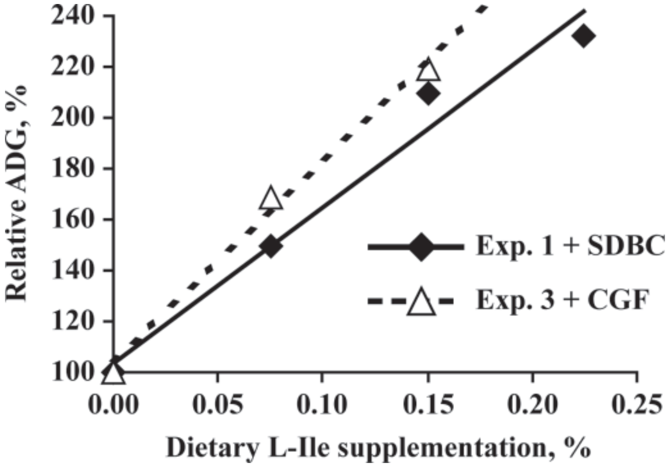

Figure 3. Slope-ratio assay of ADG as a function of dietary L-Ile supplementation (%). Regression estimates were y = 103.7 + 612x for diets containing spray dried blood cells (SDBC; Exp. 1) and y = 103.7 + 790x for diets containing corn gluten feed (CGF; Exp. 3). Slope-ratio (Exp. 1:Exp. 3) was estimated with 0.775 and approximate 95% fiducial limits were 0.578 and 0.972.

sufficient to meet the His, Leu, and Val requirements of 10-kg pigs. In this context, special attention should be paid to the Leu oversupply because of the recognized antagonistic effects of Leu excess causing decreased performance, decreased plasma concentrations of Ile and Val, and increased BCAA catabolism (Harper et al., 1984). To investigate whether the moderate oversupply of Leu in Exp. 1 and 2 increased the estimates for an optimal SID Ile:Lys ratio, a second basal diet was formulated without Leu or Val excesses (Exp. 3).

In the present work, optimal SID Ile:Lys ratios of 59% were estimated for ADG and ADFI in diets using SDBC as a protein source (Exp. 1). In contrast, optimal ratios of 54% were estimated in diets containing CGF (Exp. 3). A slope-ratio assay for ADG as a function of supplemental L-Ile confirmed that the slope in Exp. 1 was significantly decreased in comparison with Exp. 3. Slope-ratio (Exp. 1/Exp. 3) was estimated at 0.775. These greater estimates in diets containing SDBC are confirmed by the literature. At the same inclusion level of 7.5% SDBC, an optimal ratio of apparent digestible Ile:Lys of 58 to 62% was estimated in 7- to 11-kg pigs (Kerr et al., 2004a), and a SID Ile:Lys ratio of 62% was sufficient in diets for 5- to 9-kg pigs (6% SDBC; Hinson et al., 2007). These values are similar to the estimates of 59% SID Ile:Lys determined in SDBC diets (Exp. 1).

Using greater levels of SDBC increased the optimal Ile:Lys ratio. In 12- to 22-kg pigs fed corn-SDBC-based diets containing 10.75% SDBC, a SID Ile:Lys ratio of at least 70% has been estimated for ADG (Fu et al., 2006c). The greater nutritional need for Ile in SDBC diets has also been demonstrated in late-finishing pigs. Using corn-SDBC diets (5% SDBC) increased the nutritional need for Ile from 0.24 to 0.34% in compari-

Table 8. Effects of increasing ratio of standardized ileal digestible (SID) Ile:Lys by crystalline L-Ile supplementation on plasma free-AA and urea of weaned pigs fed diets free of spray-dried blood cells (Exp. 3)[1]

	SID Ile:Lys, %							Contrast[2] *P*-value	
Item	36	44	53	59	67	72	SEM	Linear	Quadratic
EAA,[3] µmol/L									
Arg	46	45	44	50	51	50	2	0.33	0.82
His	56	28	14	17	13	13	3	<0.01	<0.01
Ile	12	22	37	84	130	201	11	<0.01	<0.01
Leu	112	130	126	123	119	114	3	0.67	0.11
Lys	147	129	46	77	53	26	9	<0.01	0.15
Met	46	56	54	60	69	76	3	<0.01	0.45
Phe	55	59	50	50	47	51	2	0.06	0.61
Thr	527	451	350	332	324	404	22	<0.01	<0.01
Trp	27	26	22	27	26	24	1	0.55	0.76
Val	188	198	165	160	140	138	6	<0.01	0.72
NEAA,[4] µmol/L									
Ala	390	420	431	496	489	485	15	<0.01	0.52
Asn	75	87	66	67	66	73	2	0.09	0.32
Asp	12	12	13	17	18	14	1	<0.02	0.19
Cys	49	45	65	63	69	63	2	<0.01	0.07
Gln	639	721	763	797	859	784	23	<0.01	0.18
Glu	112	119	162	183	188	159	9	<0.01	0.13
Gly	1,189	996	707	779	737	713	36	<0.01	<0.01
Pro	250	296	274	297	287	287	9	0.242	0.30
Ser	200	198	147	144	138	151	7	<0.01	0.04
Tyr	40	33	17	19	19	16	2	<0.01	0.01
Urea, mmol/L	3.8	2.9	2.1	2.8	2.2	2.0	0.1	<0.01	<0.05

[1] Data represent the least squares means of 8 pigs per treatment after a 42-d feeding period (31 to 72 d of age).

[2] Orthogonal polynomial contrast coefficients were used to determine the linear and quadratic effect of increasing dietary SID Ile:Lys.

[3] Essential AA.

[4] Nonessential AA.

son with corn-soybean meal-based diets (Dean et al., 2005). In SDBC-free diets, Bergstrom et al. (1997) reported that the apparent digestible Ile requirement is not greater than 50% of Lys for 10- to 20-kg pigs fed corn-soybean meal diets. This is in accordance with the findings of Fu et al. (2006c) showing that in 12- to 22-kg pigs fed corn-soybean meal-based diets, increasing the SID Ile:Lys ratio from 46 to 74% had no effect. The SID Ile:Lys ratio given by the NRC (1998) is 55% and is almost the same as the estimates for the CGF diets obtained in the present work (54%; Exp. 3).

The optimal SID Ile:Lys ratios estimated for G:F were 45 and 49% in SDBC diets (Exp. 1) and in CGF diets (Exp. 3), respectively. The plateau estimated by broken-line analysis was at the same level in both trials. In contrast to the ratios estimated for ADG and ADFI, the ratio for optimal G:F was less in SDBC diets (Exp. 1) than in CGF diets (Exp. 3). In Exp. 1, G:F was maximized at lesser ratios than for ADFI and ADG. Lesser estimates for G:F than for ADG and ADFI are also reported in the literature (Oestemer et al., 1973b; Kerr et al., 2004a; Fu et al., 2006c) but not in all trials (Kerr et al., 2004a). Especially in trials with ad libitum access to feed, lesser estimates of AA requirements for G:F than for ADFI and ADG may occur. In these trials the supplemented AA exhibits positive effects on feed intake, as is the case for Trp (Eder et al., 2001). In contrast, deficiencies and imbalances of EAA are known to decrease voluntary feed intake. The detection of EAA depletion occurs in the anterior piriform cortex of the brain via uncharged tRNA (Gietzen et al., 2007) and leads to diet rejection. Therefore, marginal EAA deficiencies primarily affect feed intake, and a reduction of G:F only occurs at severe EAA deficiencies.

In the present work, plasma free AA were analyzed to obtain further information about the nutritional status of the pigs. Plasma AA concentrations are dependent on the dietary AA pattern and ADFI. Thus, comparisons among trials should be evaluated cautiously. The ADFI of Exp. 1 and 3 was similar and ranged from 298 to 636 g in Exp. 1 and from 334 to 644 g in Exp. 3. However, the plasma concentrations of Leu and Val were quite different and reflected the dietary supply. Compared with Exp. 3, the dietary Leu and Val supplies in Exp. 1 were increased by 54 and 55%, respectively, and the plasma concentrations of Leu and Val were increased by 60 and 100%, respectively. The dietary supply of Ile was the same in Exp. 1 and 3, and thus the basal plasma concentrations of Ile were also the same in both trials. However, plasma Ile concentrations in the treatment groups were increased to a different extent by the same dietary Ile supplementation. At the greatest Ile supplementation, the plasma Ile concentrations in Exp. 3 were 3-fold greater than those of Exp. 1. In conclusion, inclusion of SDBC in diet composition caused AA imbalances as dietary levels of Leu, Val, and His increased. An increased dietary supply of Ile was needed to reach the same plasma Ile concentrations as in CGF diets. These findings are in accordance with the literature. Increased dietary supply of Leu has been shown to decrease plasma concentrations of Ile and Val (Oestemer et al., 1973a; Henry et al., 1976; Taylor et al., 1984).

Plasma AA can be used to underline the estimates determined using performance data. Isoleucine requirement trials with pigs have shown the same basal concentrations of plasma Ile (17 to 38 μmol/L) as in the present work, and a linear increase in plasma Ile concentrations when dietary Ile supply exceeded requirements (Bravo et al., 1970; Oestemer et al., 1973b). Plasma urea concentrations are another indicator for the adequacy of the AA supply. In the present work, plasma urea concentrations decreased in Exp. 1 and 3 with increasing dietary L-Ile supply and underline the improved utilization of dietary AA. However, plasma urea data were not appropriate to estimate ideal Ile:Lys ratios, which is similar to the findings of Dean et al. (2005).

The effect of SDBC to increase the nutritional need for Ile is caused by an AA imbalance, mainly by increased Leu content. Supplementing corn-soybean meal diets with L-Val and L-Leu to the levels of a corn-SDBC diet decreased the performance of the pigs and resulted in similar performance compared with pigs fed corn-SDBC diets (Fu et al., 2006a). However, performance could be restored by dietary supplementation with L-Ile (Fu et al., 2006b). Most likely, the increased need for Ile in SDBC-diets is caused by increased Ile catabolism induced by excessive levels of Leu. The EAA Ile, Leu, and Val have similar chemical forms and are grouped as branched-chain AA (**BCAA**). Because of their structural similarity, they share the first 2 steps of their catabolism and compete for the same enzymes. The BCAA are first transaminated, and the resulting branched-chain α-keto acids are then oxidatively decarboxylated by a multienzyme complex, the branched-chain α-keto acid dehydrogenase complex (**BCKDH**). The second step is irreversible and rate limiting. Especially increased concentrations of the corresponding α-keto acid of Leu can increase the catabolism of the BCAA by stimulation of BCKDH activity. Dietary Leu excesses have been shown to cause a depression of plasma concentrations of Val, Ile, and their branched-chain α-keto acids by increasing BCKDH activity in rats and pigs (Block and Harper, 1984; Langer et al., 2000).

In conclusion, high dietary Leu levels increase the nutritional need for Ile. In the present work it has been shown that the use of SDBC leads to greater estimates than shown for SDBC-free diets. For optimal ADG and ADFI of 8- to 25-kg pigs optimal SID Ile:Lys ratios of 59% were estimated for the diets containing 7.5% SDBC. In contrast, optimal ratios of 54% were estimated in CGF diets. These results clearly demonstrate that estimates for the Ile requirements of pigs determined in trials using SDBC as a protein source are not applicable to SDBC-free diets and vice versa.

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Publication III

The effects of branched-chain amino acid interactions on growth performance, blood metabolites, enzyme kinetics, and transcriptomics in weaned pigs.

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The Effects of Branched-Chain Amino Acid Interactions on Growth Performance, Blood Metabolites, Enzyme Kinetics, and Transcriptomics in Weaned Pigs

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Leucine excess: Amino acid interaction: BCKDH: Pig

Abbreviations: BCAA, branched-chain amino acids; BCAT, branched-chain amino transferase; BCATc, cytosolic BCAT; BCATm, mitochondrial BCAT; BCKA, branched-chain α-keto acid; BCKDH, branched-chain α-keto acid dehydrogenase; BCKDK, branched-chain α-keto acid dehydrogenase kinase; DEPC, diethylpyrocarbonate; GAPDH, glyceraldehyde 3-phoshpate dehydrogenase; GH, growth hormone; GHR, growth hormone receptor; 4-HPPD, 4-hydroxy phenylpyruvate dioxygenase; IGF-ALS, insulin-like growth factor acid labile subunit; IGF-1, insulin-like growth factor 1; IGF-BP 3, insulin-like growth factor binding protein 3; IGFR, insulin-like growth factor receptor; Ile, isoleucine; KIC, α-keto isocaproate; KIV, α-keto isovalerate; KMV, α-keto β-methylvalerate; Leu, leucine; qRT-PCR, quantitative RT-PCR; RIN, RNA integrity number; SID, standardized ileal digestible; Val, valine.

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Running title: Amino acid interactions in pigs

The impact of excess dietary leucine was studied in two growth assays with pigs (8 to 25 kg). In each trial, forty-eight pigs were allotted to one of six dietary groups. The dietary leucine supply increased from Treatment L100 to L200 (three increments). To guarantee that interactions between the BCAA were not cushioned either surpluses of isoleucine (Expt 1) or valine (Expt 2) were avoided. In the fifth treatment, the effects of a simultaneous excess of leucine and valine (Expt 1), or of leucine and isoleucine (Expt 2) were investigated. The sixth treatment was a positive control. An increase in dietary leucine decreased growth performance, and increased plasma leucine and serum α-keto isocaproate levels in a linear, dose-dependent manner. Levels of plasma isoleucine and valine, and of serum α-keto β-methylvalerate and α-keto isovalerate, indicated increased catabolism. Linear increases in the activity of basal branched-chain α-keto acid dehydrogenase in the liver confirmed these findings. No major alterations occurred in the mRNA of branched-chain amino acid catabolism genes. In liver tissue from Expt 2, however, the mRNA levels of growth hormone receptor, insulin-like growth factor acid labile subunit, and insulin-like growth factor 1 decreased significantly with increasing dietary leucine. In conclusion, excess dietary leucine increased the catabolism of branched-chain amino acids mainly through posttranscriptional mechanisms. The impact of excess leucine on the growth hormone-insulin like growth factor-1-axis requires further investigation.

Interactions among the branched-chain amino acids (BCAA), such as the performance-depressing effects of excess dietary leucine (Leu), are known in several species(1). The impact of high dietary Leu levels needs to be elucidated in order to make correct estimates of adequate supplies and requirements for isoleucine (Ile) and valine (Val). Interactions among the BCAA include their catabolism, because all three compete for the same enzymes that catalyse the first two catabolic steps. The first step is a reversible transamination catalysed by the branched-chain amino acid transaminase isoenzymes (BCAT), yielding branched-chain α-keto acids (BCKA) that, in the second step, are oxidatively decarboxylated by a mitochondrial, multienzyme branched-chain α-keto acid dehydrogenase (BCKDH) complex. This step is irreversible, highly regulated, and rate-limiting for BCAA catabolism. The BCKDH complex consists of three catalytic components. The E1 subunit, a heterotetramer of α and β subunits, is a branched-chain α-keto acid decarboxylase. The E2 subunit is a dihydrolipoamide acyltransferase, and the E3 subunit is a dihydrolipoamide dehydrogenase(2). In contrast to the other subunits, the E3 is not BCKDH-specific and its expression is not analysed in the present work. BCKDH complex activity is regulated by covalent modification. Phosphorylation of its E1α subunits by a specific BCKDH kinase (BDKDK) causes inactivation, and dephosphorylation by a specific phosphatase causes reactivation(3,4). The abundance of the bound kinase corresponds to kinase activity(5). This regulation is unusual among amino acid-degrading enzymes, but is similar to the regulation of pyruvate dehydrogenase complex(6). Thus, high dietary Leu levels might increase the catabolism of all BCAA and the nutritional need for Ile and Val. Additionally, there is some evidence that the growth-depressive effects of amino acid deficiencies might be caused in part by impaired action of growth hormone-insulin like growth factor-1 (GH-IGF-1) axis(7). If Leu excess increases Ile and Val catabolism, these amino acids might become deficient and alter GH-IGF-1 expression.

Most experiments on excess dietary Leu in pigs have concentrated on performance parameters(8-10), so data on metabolites, BCAA catabolism and gene expression are sparse or absent. For correct diet estimates, and to guarantee sufficient supplies of Ile and Val, data on the effects of excess dietary Leu on nutritive status are needed for weaned pigs. The aim of the present work was to determine whether excess dietary Leu effects performance, plasma amino acids, serum BCKA, activity of the BCKDH complex, mRNA levels of enzymes involved in BCAA catabolism, and mRNA levels of genes related to GH-IGF-1.

Materials and methods

Animal housing and environmental conditions

Trials were conducted under supervision of the veterinary office of the Bavarian government. Animal handling and care were in accordance with the German laws for animal protection. To determine the effects of increasing dietary Leu supply to pigs fed diets without surpluses of either Ile or Val, two growth assays were conducted, each with forty-eight crossbred pigs (German Landrace x Piétrain). The pigs were raised at the same commercial plant and had an average age of 28 (SD 1) d. The initial body weight was 8·93 (SD 0·79) kg (Expt 1) and 8·40 (SD 0·91) kg (Expt 2). The ratio between castrated male and female pigs was balanced. Pigs were individually housed in pens (60 x 100 cm, plastic slats) in an environmentally controlled building. During the experimental period of 34 d, the room temperature was incrementally reduced from 29ºC at the beginning, to 25ºC by the end of the experiment. Full light was provided from 07.00 to 17.00 hours, followed by half-light. Animals had *ad libitum* access to food and water. Animals and feeders were monitored twice daily.

Diets and experimental design

Pigs were allotted to one of six dietary treatments on the basis of their weight. Gender and ancestry were equalized across treatments in a randomised block design. Basal diets were mainly based on barley and wheat (Table 1). Corn gluten feed and soybean meal were used as protein sources. Na-L-glutamate was included to enable the composition of isonitrogenous diets (analysed crude protein: 17·99 to 18·36 % in Expt 1; 17·67 to 18·31 % in Expt 2). The energy content was kept constant at 13·5 MJ metabolizable energy/kg (calculated). Diets were supplemented with essential crystalline amino acids (other than the BCAA) to meet ideal protein conditions(11). Tryptophan supplementation was increased to a standardized ileal digestible (SID) tryptophan:lysine ratio of 22 %. Analysed SID lysine levels ranged from 1·12 to 1·14 % in Expt 1, and from 1·11 to 1·12 % in Expt 2. Levels of SID amino acids in mixed diets were calculated by multiplication of the analysed amino acid contents of each feed ingredient with the estimates of standardized ileal amino acid digestibility(12). These estimates consider basal endogenous amino acid losses and are calculated as follows(13):

SID, % = {[amino acid intake – (ileal amino acid outflow) – basal endogenous ileal amino acid losses)]/ amino acid intake} × 100.

All diets were supplemented with vitamins and minerals to meet or exceed the requirements for 10- to 20-kg pigs(14).

In Expt 1, the effects of increasing the dietary Leu supply in pigs fed diets with a constant Ile supply of 5·0 g SID Ile/kg of diet were estimated. Dietary Leu level of the basal group was 11·5 g SID Leu per kg and was considered as 100 % (Treatment: L100). In Treatment L150, L175, and L200, the Leu supply was then increased to give dietary Leu levels of 150, 175, 200 % relative to Treatment L100. Ratios of SID Leu:Ile were 2·33, 3·43, 3·98, and 4·49 in Treatment L100, L150, L175, and L200, respectively. In Treatment L+V 200, the Leu and Val supply were doubled (relative to Treatment L100) to determine the effects of a simultaneous excess of Leu and Val. With the last treatment it should be tested whether the chosen Ile level of 5·0 g SID Ile/kg was first-limiting and surpluses of Ile were avoided what is a precondition to get meaningful Ile:Leu ratios. Diet composition of this positive control (PC) was equal to the basal treatment L100 but the Ile supply was increased to 6·3 g SID Ile/kg.

In Expt 2, the effects of increasing the dietary Leu supply in pigs fed diets with a constant Val level of 6·2 g SID Val/kg of diet were estimated. In the basal treatment, the Leu supply was 11·1 g SID Leu/kg and was considered as 100 % (Treatment L100). In the following treatments, the Leu supply was increased by L-Leu supplementation to give 150 (L150), 175 (L175), and 200 % (L200) of the Leu level of Treatment L100. SID Leu:Val ratios were 1·79, 2·63, 3·08, and 3·55, in Treatments L100, L150, L175, and L200, respectively. In Treatment L+I 200, the Leu and Ile supply were doubled (relative to Treatment L100) to determine the effects of a simultaneous excess of Leu and Ile. Again, the last treatment was a positive control (PC). Diet composition was equal to Treatment L100 but the Val supply was increased to 7·3 g SID Val/kg.

Sampling

Trials were terminated on d 34, when the pigs were fasted for 2·5 h. Afterwards, two blood samples per pig were taken via jugular vein puncture. The first sample was used to determine plasma free amino acids (9 ml, S-Monovette, Li-Heparin, Sarstedt, Nümbrecht, Germany). The second sample was used to determine serum BCKA (9 ml, Gel-S-Monovette, Sarstedt, Nümbrecht, Germany). The samples were cooled by ice water. Plasma and serum were obtained by centrifugation (20 min, 800 ***g***), and stored at -80ºC.

After blood sampling, the pigs remained for 3 d at the experimental piggery, where the feeding regime was switched to three meals per day. The daily amount of feed was calculated for each individual pig by multiplication of its metabolic body weight with a treatment-specific factor of average daily feed intake per kilogram metabolic body weight, estimated for the last experimental week. On d 38, all pigs of Expt 1 + 2 undergoing Treatments L100, L150, and L200 were killed by captive bolt pistol and exsanguinated by transsection of the carotid arteries, in randomised order. All pigs were fed 1/3 of their daily feed amount at 07.00 hours. The first pig was killed at 09.30 hours. For every delay, the following pigs received an extra meal (1/24 of the daily feed amount per h of delay), at 2·5 h before killing, to ensure comparable postprandial conditions. After exsanguinations, samples of skeletal muscle (musculus longissimus dorsi, seventh rib), heart, liver, spleen, jejunum, and ileum (mid parts) were removed and quick-frozen in liquid nitrogen until storage at –80°C. Response variables were daily gain, daily feed intake, feed efficiency, plasma amino acids, serum BCKA, the BCKDH activity in liver samples, mRNA of genes encoding enzymes in BCAA catabolism, and mRNA of genes related to GH-IGF-1.

Feed analyses

Crude protein content was determined using the Kjeldahl procedure(15). Analysis of dietary amino acids was carried out by Ajinomoto Eurolysine S.A.S (Paris, France). Amino acid contents were analysed by ion exchange chromatography after acid hydrolysis with HCl (6 N, reflux for 23 h at 110°C)(16). Methionine and cysteine were assayed after performic acid oxidation(16). Tryptophan was determined by reverse phase high performance liquid chromatography and fluorometric detection after alkaline hydrolysis with barium hydroxide (16 h, 120°C)(17).

Plasma free amino acids

Plasma proteins were removed by precipitation with salicylsulfonic acid and centrifugation (11000 ***g***, 10 min). After dilution with a lithium acetate solution, the protein-free supernatant was analysed by ion exchange chromatography on an automatic amino acid analyser (LC 3000, Biotronik, Hamburg, Germany)(15).

BCKA determination

BCKA were analysed as reported by Pailla *et al.*(18). BCKA were derivatised with o-phenylenediamine to give fluorescent derivates, which were separated chromatographically on a reversed-phase column (Spherisorb ODS-2, 4·6 x 250 mm, 5 μm particles, Waters, Eschborn, Germany) using a binary gradient (L-7100, Merck-Hitachi, Tokyo, Japan). Detection was performed fluorimetrically (FL Detector L-7480, Merck-Hitachi, Tokyo, Japan). α-Keto valerate was used as an internal standard.

BCKDH activity

BCKDH activity was assayed spectrophotometrically(19). A frozen tissue sample was pulverized to a fine powder under liquid nitrogen. Thereafter, 0·25 g of the powder were homogenized (motor driven Tefflon pestle) in ice-cold extraction buffer. Insoluble material was removed by centrifugation (20000 ***g***, 5 min, 4°C) and the supernatant was made 9 % (v/v) in polyethylene glycol. After 20 min on ice, a second centrifugation step (12000 ***g***, 10 min, 4°C) was performed, and the pellet dissolved in suspension buffer. BCKDH activity was determined at 30°C using α-keto isovalerate as substrate, by measuring absorbance at 340 nm to detect NADH formation. To determine total BCKDH activity, the tissue extract was incubated (20 min, 37°C) with lambda protein phosphatase before measurement. In contrast to Nakai *et al.*(19), the assay buffer was made without dihydrolipoamide dehydrogenase.

Total RNA extraction

Total RNA was extracted with peqGOLD TriFast (PEQLAB Biotechnologie, Erlangen, Germany), which uses a one-step liquid-phase separation using phenol and guanidinisothiocyanate in a single liquid phase. Tissue samples (50 mg) were homogenized using MatrixGreen beads in the MagNA Lyser (Roche Diagnostics, Mannheim, Germany) in the presence of peqGOLD TriFast (0·5 ml). After addition of chloroform (100 µl) and centrifugation (12000 ***g***, 15 min), the homogenate was separated into three phases, with the RNA in the upper aqueous phase. The extracted RNA, free of DNA and proteins, was precipitated with isopropanol (150 µl) and centrifugation (12000 ***g***, 10 min, 4˚C). Pellets were washed twice with 250 µl 75 % (v/v), -20˚C ethanol and centrifuged (10000 ***g***, 5 min, 4˚C). RNA was dissolved in 30 µl diethylpyrocarbonate (DEPC)-treated water and stored at – 80˚C.

Liver samples were additionally treated with peqGOLD OptiPure (PEQLAB Biotechnologie, Erlangen, Germany) to eliminate polysaccharides. Pellets obtained by extraction with peqGOLD TriFast were mixed with 100 µl peqGOLD OptiPure. After centrifugation (3000 ***g***, 10 min, 4˚C), the supernatant was removed and 100 µl sodium dodecylsufate (0·5 % (w/v), pH 7) were added. After incubation for 5 min at 55˚C, 100 µl chloroform were added and the mixture was centrifuged (3000 ***g***, 5 min, 4˚C). Natrium acetate (2 M, pH 5) was added to the supernatant to a final concentration of 0·2 M. RNA was precipitated with isopropanol (100 µl). Centrifugation and washing steps were as described for the extraction. Pellets were dissolved in 60 µl DEPC-treated water.

RNA quantity was determined photometrically (Nanodrop 1000, PEQLAB Biotechnologie, Erlangen, Germany) and purity calculated from the 260/280 nm absorbance ratio. All working solutions were diluted to a RNA concentration of 10 ng/µl.

RNA quality

Integrity of RNA was analysed for 6 random samples per tissue and trial using the RNA 6000 Nano assay (Agilent Technology, Palo Alto, USA) and the 2100 Bioanalyser (Agilent Technology, Palo Alto, USA). The RNA integrity number (RIN) served as an RNA quality parameter ranging from one (the most degraded profile) to ten (the most intact profile).

Primer design

Primers were designed using published RNA sequences of pigs (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), if available. Otherwise they were designed as nested primers from homologous regions of cattle, human, and rat genes. Primer design accounted for primer-dimer formation, self-priming, and primer annealing temperature (60˚C) (http://fokker.wi.mit.edu/primer3/input.htm). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table 2. Primer testing included different annealing temperatures and product validation via agarose gel electrophoresis for 4 random samples and a negative control for each primer set.

PCR analysis

Quantitative RT-PCR (qRT-PCR) was conducted using a SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen Corporation, Carlsbad, USA). For each sample, 5 µl 2X SYBR Green Reaction Mix, 0·5 µL forward primer (10 µM), 0·5 µl reverse primer (10 µM), and 0·2 µl SuperScript III RT/Platinum *Taq* Mix were mixed and 3·8 µl template (total RNA concentration: 10 ng/µl) were added. qRT-PCR was performed with a Rotor-Gene 6000 (Analysis Software 6.0; Corbett Life Science, Sydney, Australia) using the following protocol: hold step (55 ˚C, 3 min), denaturation step (95˚C, 5 min), cycling (95˚, 15 s; 60˚C, 10 s; 68˚C 20 s; 40 cycles), hold step (40 ˚C, 1 min), and melting curve analysis.

The products obtained by qRT-PCR were validated by 1·8 % agarose gel electrophoresis at 90 V for 30 min. After gel extraction (Wizard SV Gel and PCR Clean-Up system, Promega Cooperation, Madison, USA), sequencing was performed by the Department Biology Genomics Service unit at the Ludwig-Maximilans-Universität München. Nested primers were designed from the determined sequences.

Statistical analyses

Data were analysed by ANOVA using the mixed procedure of Statistical Analysis Systems statistical software package version 9.1. (SAS Institute, Cary, NC, USA), with individual pigs as experimental units. The model included treatment and sex as fixed and litter as random effects. Initial body weight was used as a covariate. Contrasts were performed to determine the linear and quadratic effects of an increasing dietary Leu supply(20). The integrated matrix language procedure was used to generate orthogonal polynomial coefficients and the mixed procedure was used to compute orthogonal polynomial sums of squares. *P*-values for treatment are given in the tables. The effects of doubling the Val or Ile supply, at high levels of dietary Leu were tested for significance using the Tukey adjustment (Expt 1: L200 vs. L+V 200; Expt 2: L200 vs. L+I 200). The effects of the PC were tested for significance using the Tukey adjustment (L100 vs. PC).

mRNA expression data were analysed using the relative quantification method, which describes the change in target gene expression relative to the control group. β-Actin, ubiquitin, and glyceraldehyde 3-phoshpate dehydrogenase (GAPDH) genes were used as normalizing internal controls for the amount of RNA added to reverse transcription reactions. Internal control gene expression was analysed for every tissue and every sample. For normalization, the tissue- and sample-specific arithmetic means of the three internal control genes served as a control gene index. Cycle thresholds of the target genes were subtracted from the control gene index (ΔCt) for normalization, and treatment groups were compared to the control group (ΔΔCt; [21]), using the SAS general linear models procedure, and the Tukey adjustment. To determine gene expression differences between tissues, the SAS general linear models procedure and the Tukey adjustment were applied to Ct values.

Results

Performance

In Expt 1, increasing the dietary Leu supply from Treatment L100 to L200 linearly decreased the daily feed intake (*P*=0·06) and gain (*P*=0·05) by 14 to 15 % (Table 3). A regression for the average daily gain (y) as a function of the SID Leu:Ile ratio (x) was estimated as y = 548·5 – 34·2 x (R^2 0.971; *P*=0·02). In contrast, no major impact was seen on feed efficiency. Doubling the Val supply at high dietary levels of Leu did not affect performance (L+V 200 vs. L200; *P*>0·05). The growth performance of PC was not different from that of L100 (*P*>0·05).

In Expt 2, incrementally increasing Leu supply from L100 to L200 linearly decreased the performance parameter in a dose-dependent manner (*P*<0·01; Table 4). The daily feed intake, daily gain, and feed efficiency decreased by 30, 38, and 11 %, respectively. A regression for the average daily gain (y) as a function of the SID Leu:Val ratio (x) was estimated as y = 538·4 – 84·7 x (R^2 0·955; *P*=0·02). In Treatment L+I 200, doubling the Ile supply did not affect performance relative to L200. In PC, increasing the Val supply significantly (*P*<0·05) increased the animals performance. Compared to L100, the daily feed intake and gain of PC were increased by 38 and 43 %, respectively.

Plasma metabolites

In Expt 1, increasing the dietary Leu supply increased plasma Leu levels linearly (*P*<0·01) and quadratically (*P*=0·03), and the serum KIC levels linearly (*P*<0·01), in a dose-dependent manner (Table 5). Doubling the Leu supply increased plasma Leu levels by 64 % and serum KIC levels by 45 %. Plasma levels of histidine, threonine, and serine increased linearly (*P*<0·01) and plasma methionine and proline were influenced quadratically (*P*=0·03). In contrast, plasma Val and serum

KIV levels decreased linearly (*P*<0·01) and quadratically (*P*<0·01), up to 60 and 72 %, respectively. However, the plasma Ile and serum KMV levels remained unaffected by increasing Leu supply (*P*>0·05).

Doubling the dietary Val supply at high dietary levels of Leu in L+V 200, increased plasma Val levels four-fold (*P*<0·05) and serum KIV levels 5·8-fold (*P*<0·05), but decreased plasma glutamic acid by 32 % (*P*<0·05) compared to L200.

Compared to L100, increasing the dietary Ile supply in PC significantly (*P*<0·05) increased the plasma Ile and serum KMV levels 4·6- and five-fold, respectively. In contrast, serum KIV levels decreased by 26 % (*P*<0·05).

In Expt 2, increasing the dietary supply of Leu increased plasma Leu linearly (*P*<0·01) and serum KIC linearly (*P*<0·01) and quadratically (*P*=0·04; Table 6). Doubling the dietary Leu supply increased the plasma Leu and serum KIC levels by 59 and 49 %, respectively. In contrast, plasma Ile and serum KMV levels decreased linearly (*P*<0·01) and quadratically (*P*=0·03) up to 69 and 66 %, respectively. Plasma levels of aspartic acid (*P*<0·01) and glutamine (*P*<0·05) also decreased linearly, whereas histidine (*P*<0·01) and phenylalanine (*P*<0·05) increased linearly (data not shown). Plasma Val levels were influenced quadratically (*P*=0·03) and serum KIV levels increased linearly (*P*<0·05).

Doubling the dietary Ile supply at high dietary Leu levels increased the plasma Ile and serum KMV levels 2·9- and 2·4-fold (*P*<0·05), respectively (L200 vs. L+I 200).

Increasing the dietary Val supply in the PC significantly (*P*<0·05) increased the plasma Val and serum KIV levels 5·6- and 13·6-fold, respectively, compared to L100. Plasma cysteine levels also increased (*P*<0·05). In contrast, plasma methionine, phenylalanine, threonine, alanine, asparagine, glycine, and serine levels decreased (*P*<0·05; data not shown).

BCKDH activities in the liver

In Expt 1, the total BCKDH activity was not altered by increasing Leu supply (Fig. 1*A*). In contrast, the basal activity (Fig. 1*C*) and the level of activation (Fig. 1*E*) increased linearly (*P*<0·01) by 165 and 135 %, respectively.

In Expt 2, increasing the dietary Leu supply did not affect the total BCKDH activity (Fig. 1*B).* In contrast, the basal activity (Fig. 1*D)* and the corresponding level of activation (Fig. 1*F)* increased linearly (*P*<0·01) in a dose-dependent manner as the Leu supply increased. Increasing the SID Leu:Val ratio from 1·79 to 3·55 increased the basal BCKDH activity and its level of activation approximately three-fold.

mRNA expression

The mean RIN value was 7·91 (SD 0·14), documenting an integer total RNA. For both Expt 1 and Expt 2, the internal control genes were unaffected by the experimental treatment ($P>0·05$). In Expt 1, increasing the dietary Leu supply did not affect the expression of the BCATm, BCKDK, or BCKDH subunit (E1α, E1β, E2) genes in skeletal muscle, heart, spleen, jejunum, and ileum (data not shown). In liver tissue, however, the mRNA levels of genes for BCKDH E1β and BCKDK were significantly altered ($P<0·05$) (Table 7). Compared to L200, mRNA of the BCKDH E1β gene was significantly lower in L150 but unchanged in L100. In contrast, the mRNA levels of the BCKDK gene increased in L200.

Tissue-specific differences were observed in the mRNA expressions of the catabolic enzymes (Table 8), and are summarized as follows:

BCATm: heart >> skeletal muscle, spleen, jejunum >> ileum, liver
BCKDH E1α: heart >> liver >> jejunum, skeletal muscle >> spleen, ileum
BCKDH E1β: heart >> liver >> jejunum >> skeletal muscle, spleen >> ileum
BCKDH E2: heart, liver >> ileum, spleen, jejunum >> skeletal muscle
BCKDK: heart >> skeletal muscle >> liver > jejunum > ileum > spleen

In Expt 2, no significant alterations were seen in mRNA expressions of the genes for BCATm, BCKDH subunits, and BCKDK neither in muscle nor in liver tissue (data not shown). The mRNA expression of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) genes in liver and muscle tissue was not influenced ($P>0·05$) in Expt 1 nor in Expt.2 (data not shown). Increasing dietary Leu supply did not affect ($P>0·05$) the mRNA expressions of the genes for GHR, IGF-BP, IGF-ALS, IGFR and IGF-1 in skeletal muscle and liver tissue of Expt 1, or in skeletal muscle in Expt 2 (data not shown). However, in liver tissue, in Expt 2, mRNA from the genes for GHR, IGF-ALS, and IGF-1 was significantly decreased ($P<0·05$; Table 9).

Discussion

In the present work, the effects of increasing dietary Leu levels in isonitrogenous diets were determined. Surpluses of Ile (Expt 1) or Val (Expt 2) were avoided to get meaningful ratios to Leu and to increase the animals' sensitivity for interactions between the BCAA. In L100, the Ile supply was set at 5·0 *g* SID Ile per kg diet (SID Leu:Ile ratio of 2·33). Incremental supplementation with Leu decreased the daily feed intake and gain in a dose-dependent manner. Increasing the SID Leu:Ile ratio from 2·33 to 4·49 (L100 to L200) decreased the daily feed intake and gain by 14 and 15 %, respectively.

In Expt 2, the Val supply was set at 6·2 g SID Val per kg diet. Incremental Leu supplementation (SID Leu:Val ratio from 1·79 to 3·55; L100 to L200) decreased the daily feed intake, daily gain, and feed efficiency linearly, in a dose-dependent manner, by 30 %, 38 %, and 11 %, respectively. In the PC, the Val supply was increased to 7·3 g SID Val per kg diet which increased the daily feed intake and gain, indicating suboptimal Val supply.

These findings are in accordance with the recent literature. Langer and Fuller(22) showed that excess dietary Leu decreases the nitrogen utilization of pigs fed diets marginal for Ile or Val by 12 to 18 %. In earlier works, however, no effect occurred after increasing the Leu:Ile ratio from 1·7 to 3·0 in 15- to 51-kg pigs(8), suggesting that increasing the Leu:Ile ratio above 3·5 may have negative effects in growing pigs(9). In contrast, we showed that excess dietary Leu linearly decreased performance, when a SID Leu:Ile ratio of 2·33 or a SID Leu:Val ratio of 1·79 was exceeded.

In 30- to 40-kg pigs fed Ile-limited diets, the negative effects of excess Leu on nitrogen utilization are reversible by a simultaneous excess of Val(22). In the present work, however, no benefits were detected for doubling the supply of Ile nor Val in Leu-excessive diets. In contrast to Langer and Fuller(22), no significant increase in performance was observed, and the benefits of excess dietary Val in diets excessive in Leu could not be confirmed.

The basal dietary Ile and Val levels in Expt 1 and 2 were chosen to be first-limiting in order to guarantee that any observed negative effects of increasing dietary Leu levels would be direct and not obscured. Compared to PC, the marginality of the Ile supply in Expt 1 was not reflected in the animals' performance, but was clearly demonstrated by a sharp decrease of plasma Ile and serum KMV levels. In Expt. 2, the marginality of the Val supply was indicated by a performance depression, and confirmed by a sharp decrease of plasma Val and serum KMV levels. The low plasma Ile and Val levels seen in the present work were in accordance with the literature(23-25).

In both trials, increasing the dietary Leu supply significantly increased the plasma Leu and serum KIC levels. Doubling the dietary Leu supply increased the plasma Leu and serum KIC levels by 64 % and 45 %, respectively, in Expt 1, and by 59 % and 49 %, respectively, in Expt 2. However, plasma Ile and Val, and serum KMV and KIV levels decreased with increasing dietary Leu levels. These findings indicate increased BCAA catabolism, in accordance with the literature. An increased catabolism of Val and Ile was seen in chicks fed diets with excess Leu(26). In growing gilts fed diets containing high levels of dietary Leu for 7 d, reduced plasma concentrations of Val, Ile, and BCKA were seen(27). The same effects were reported for rats(28,29). The negative effects of excess dietary Leu are reversed by supplementation with Ile and Val in chicks, pigs, and kittens(26,30,31), confirming the hypothesis of an increased nutritional need for Ile and Val in diets with excess Leu.

The first step of the BCAA catabolism is a transamination catalysed by the BCAT, which exists as mitochondrial (BCATm) and cytosolic (BCATc) isoenzymes. The BCAT enzymes are widely distributed among tissues, with BCATc restricted to neural tissues. Therefore, we concentrated on BCATm. In the literature, the highest BCATm activities are reported for peripheral tissues such as skeletal muscle, whereas BCATm is not expressed in rodent and human liver(1,32). To our knowledge, we are the first to publish an overview about tissue specific BCATm RNA expression in pigs. In the present work, the highest BCATm RNA expression was seen in heart and skeletal muscle, whereas the lowest expression was seen in ileum and liver. Expression of BCATm RNA in the heart increased about 137-fold compared to liver. These findings confirm the suggestion that the BCAA escape first-pass metabolism in the liver and thus are available to peripheral tissues. Increasing dietary Leu supply in the present work did not affect the mRNA levels of BCATm.

The second step in BCAA catabolism is catalysed by BCKDH. The highest BCKDH activities are reported in liver, with intermediate activity in heart, kidney and brain, and low activity in skeletal muscle(1). This is largely consistent with the mRNA levels of weaned pigs, reported for the first time in this work. The highest mRNA levels of all analysed BCKDH subunit genes (E1α, E1β, E2) were seen in heart and liver, and the lowest in skeletal muscle and ileum.

BCKDK activity is reciprocal to BCKDH activity. Therefore, decreased expression of the BCKDK gene could cause an enhanced BCKDH activity. However, a significant increase in BCKDK gene expression was seen in liver tissue at the highest Leu supply in Expt 1. Nonetheless, the alterations in BCKDK gene expression were minor changes of about 20 %, and only the bound form of the kinase regulates BCKDH activity. This should be considered in assessing the informative value of BCKDK mRNA expression with regard to changes in BCKDH activity. In this study, tissue-specific expressions of BCKDK mRNA were reported for pigs, and found to be highest in heart and skeletal muscle, and lowest in spleen and ileum. The differences between skeletal muscle and liver tissue were 1/10 of that seen in humans(32).

The KIC, the corresponding BCKA of Leu, plays an important role in the regulation of BCKDH activity. High KIC levels can increase the catabolism of the other BCAA by inhibition of the BCDKH kinase. This results in a less phosphorylated and therefore more active BCKDH complex(33). Block *et al.*(34) reported that increases in plasma Leu paralleled changes in the activity of the BCKDH complex. In contrast to Leu, Val and Ile seem to be unimportant for the regulation of BCKDH activity. Infusions of Val or Ile failed to activate BCKDH in rats(35). In the present work, doubling the dietary Leu supply increased the serum KIC levels by 45 % to 49 % and the basal activity of the liver BCKDH two- to three-fold. An increased rate of Leu oxidation was reported in rats when the dietary Leu supply exceeded the requirement for maximum rate of weight gain(28). Increased BCKDH activity was seen in rats and pigs fed diets excessive in Leu(5,27,29). The levels of BCKDH activity and its activity state in the present work were in accordance with the levels seen in growing gilts fed semipurified diets excessive in Leu, but marginal in Ile or Val(27). The liver BCKDH activity states in the present work ranged from 7 % to 27 % and are comparable to that of humans (26 %), but much smaller than that of rats (88 %)(32). The enhanced BCKDH activity in liver seen in this work resulted from an increased grade of activity. The total BCKDH activity as well as the gene expression of the BCKDH subunits (E1α, E1β, E2) remained almost unaffected, indicating a posttranscriptional regulation.

Our findings are in accordance with Matsuzaki *et al.*(36), who investigated the impact of increasing dietary Leu supply (up to 15 % of the diet) on gene expression in rats, using DNA microarrays. The data showed small alterations (less than two-fold) in the expression of BCAA catabolism enzyme genes. However, it was reported that feeding BCAA-enriched diets to rats increased the activity state of the BCKDH through a combination of decreased BCKDK activity and increased total BCKDH activity(5). The increased BCKDH catabolism reported by Kuzuya *et al.*(5) was mainly caused by an increased activity state. The total BCKDH activity increased maximally by 45 % whereas the activity state increased 8·5-fold. In conclusion, in pigs as in rats, excess dietary Leu seemed not to alter the gene expression of enzymes involved in BCAA catabolism to a great extent. Regulatory mechanisms that adapt to excess Leu appear to implicate mainly cellular posttranscriptional mechanisms.

In the context of dietary Leu oversupply, alternative pathways for Leu catabolism that bypass BCKDH might be of some importance. In addition to its oxidation by BCKDH, KIC can be converted to β-hydroxy-β-methylbutyrate by 4-HPPD (identical to KIC dioxygenase)(37). The activity of 4-HPPD was found to be 14 % of the total BCKDH activity in human liver(38), illustrating its importance. In Expt 1 and 2, increasing dietary Leu supply had no impact on expression of the liver 4-HPPD gene. Feeding a BCAA-rich diet to rats increased BCKDH, but not 4-HPPD activity(38). In conclusion, the effects of excess Leu on 4-HPPD require further investigation. 4-HPPD is an important enzyme of tyrosine catabolism(39), and an impact of high KIC levels on tyrosine catabolism seems possible. However, we found no alterations in plasma tyrosine levels as the dietary Leu supply increased.

For several species, excess dietary Leu causes a growth depression(1). The present work confirms this general effect, although the mode of action remains unexplained. The hypothesis that growth retardation is a consequence of excess dietary Leu, suggests a nutritional impact on the GH-IGF-1 axis, for which the literature contains some evidence(7). Therefore, we decided to analyse the mRNA levels of GHR, IGF-BP 3, IGF-ALS, IGFR, and IGF-1 in skeletal muscle and liver. In Expt 1, no impact on genes of the GH-IGF-1 axis in skeletal muscle or liver was observed when dietary Leu levels were increased. In contrast, increasing Leu supplementation decreased the mRNA levels of GHR, IGF-ALS, and IGF-1 genes in liver tissue in Expt 2. This is consistent with the greater extent of growth retardation in Expt 2, compared to Expt 1. Generally, fasting decreases the expression of the GH-IGF-1 axis genes(40,41). In the present work, fasting or anorexia did not occur but the daily feed intake decreased linearly with the increases in Leu supplementation. The stimulation of IGF-1 secretion by GH seems to be dependent on the availability of specific amino acids. Single deletions of arginine, proline, threonine, tryptophan, or Val caused a block of GH-stimulated IGF-1 gene expression in cultured pig hepatocytes(42). However, the present work had no essential amino acid depletion. In contrast to previous works, we showed alterations in the expression of GH-IGF-1 axis genes caused by an increasing Leu supply in a sound organism. The increased Leu supply decreased the GHR, IGFALS, and IGF-1 gene expression in Expt. 2 but not in Expt 1 indicating a more extended Leu-induced nutrient shortage for Expt 2. We conclude that the greater growth retardation seen in Expt 2 might be partially caused by decreased activity of the GH-IGF-1 axis as a result of Leu-induced Val deficiency.

In the present work, feed intake decreased as the dietary Leu content increased. In force-fed chicks, it has been found that about 70 % of performance depression caused by Leu oversupply was the result of a decreased feed intake(43). It is known from preference trials that rats reject diets excessive in Leu and instead prefer an alternative diet even if it is protein-free(44). It has been shown by injection of L-Leu into the brain of rats that dietary Leu is indeed a nutrient signal which can cause depressions of feed intake(45,46). Leucine activates the hypothalamic mTOR (mammalian target of rapamycin) pathway and decreases the AMPK (adenosine monophosphate-activated kinase) activity which results in inhibition of neuropeptide Y (NPY) and stimulation of pro-opiomelanocortin (POMC) expression(45,46). NPY promotes feeding, decreases energy expenditure, and silences POMC, whereas POMC promotes satiety(47). Therefore, inhibition of NPY and activation of POMC cause satiety and give an explanation for the Leu induced anorexia. Inhibition of mTOR with rapamycin inhibited the L-Leu induced anorexia(45).

In conclusion, growth depression as a consequence of excess dietary Leu is accompanied by decreased plasma levels of Ile and Val, and increased BCKDH activities, indicating an increase in BCAA catabolism. Regulation of BCAA catabolism mainly involved posttranscriptional mechanisms, because no major alterations were seen in gene expression of BCATm, BCKDH subunits, or BCKDK. The alterations seen in the mRNA of GHR, IGF-ALS, and IGF-1 in pigs fed Leu-excessive diets, needs further investigation.

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Table 1 Composition of experimental diets used in Expt 1 and Expt 2

	Experiment 1						Experiment 2					
	L100	L150	L175	L200	L+V 200	PC	L100	L150	L175	L200	L+I 200	PC
						Ingredient, g/kg						
Barley	267·9	273·6	276·4	279·3	287·5	269·0	267·6	273·0	275·7	278·4	284·7	268·8
Wheat	234·7	234·7	234·7	234·7	234·7	235·0	234·7	234·7	234·7	234·7	234·7	234·7
CGF	150·0	150·0	150·0	150·0	150·0	150·0	150·0	150·0	150·0	150·0	150·0	150·0
SBM	132·1	131·0	130·4	129·8	128·3	132·0	133·0	133·0	133·0	133·0	133·0	133·0
Corn	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0
SGBM	15·0	15·0	15·0	15·0	15·0	15·0	15·0	15·0	15·0	15·0	15·0	15·0
Soya oil	33·1	30·5	29·2	28·0	25·2	32·4	32·9	30·4	29·1	27·9	24·8	32·5
V+M[1]	25·3	25·3	25·3	25·2	25·1	25·3	25·3	25·3	25·2	25·1	25·0	25·3
L-Lys-HCl	7·3	7·3	7·3	7·3	7·3	7·3	7·2	7·2	7·2	7·2	7·2	7·2
DL-Met	2·2	2·2	2·2	2·2	2·2	2·2	2·2	2·1	2·1	2·1	2·1	2·2
L-Thr	2·8	2·8	2·8	2·8	2·8	2·8	2·7	2·7	2·7	2·7	2·7	2·7
L-Trp	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
L-Leu	0·9	6·4	9·2	11·9	11·9	0·9	0·7	6·1	8·9	11·6	11·6	0·7
L-Ile	-	-	-	-	-	1·4	1·6	1·6	1·6	1·5	8·1	1·6
L-Val	1·4	1·4	1·4	1·4	9·0	1·4	0·2	0·2	0·2	0·2	0·1	1·3
Na-L-Glu	26·3	18·8	15·1	11·4	-	24·3	25·9	17·7	13·6	9·6	-	24·0
						Analysed composition, g/kg						
CP	181·4	184·1	182·9	183·6	179·9	180·8	181·2	176·7	179·6	178·6	183·1	181·8
Lys	12·4	12·4	12·6	12·6	12·4	12·4	12·3	12·4	12·3	12·4	12·4	12·4
Met + Cys	7·7	7·7	7·6	7·6	7·6	7·7	7·8	7·6	7·6	7·6	7·8	7·7
Thr	7·9	8·0	8·0	8·1	8·0	8·0	8·0	7·9	7·9	8·0	8·1	8·0
Trp	2·8	2·8	2·8	2·8	2·7	2·8	2·7	2·7	2·7	2·8	2·8	2·7
Ile	5·8	5·9	5·9	5·9	5·7	7·2	7·3	7·2	7·2	7·2	13·9	7·3
Leu	13·3	18·6	21·3	23·9	23·7	13·1	12·9	18·1	20·9	23·5	24·1	12·9

Val	8·6	8·7	8·6	8·6	15·6	8·5	7·5	7·4	7·4	7·3	7·4	8·5
Phe + Tyr	13·0	12·6	12·6	12·6	12·5	12·7	12·5	12·4	12·4	12·2	12·4	12·5
His	4·0	4·0	4·0	4·0	3·9	4·0	3·9	3·8	3·9	3·8	3·9	3·9

[1] Per kilogram of diet: Retinol 1·5 mg, cholecalciferol 0·13 mg, DL-alpha-tocopherylacetate 30 mg, menadione 150 µg, thiamin 3 mg, riboflavin 3 mg, pyridoxine 3 mg, cyanocobalamin 18 µg, nicotinic acid 25 mg, pantothenic acid 15 mg, biotin 50 µg, folic acid 300 µg, choline (as choline chloride) 300 mg, Ca 4·9 g, P 1·4 g, Na 0·75 g, Mg 0·3 g, Zn (zinc oxide) 70 mg, Fe (ferrous sulfate monohydrate) 100 mg, I (calcium iodate) 1·2 mg, Se (sodium selenite pentahydrate) 0·26 mg, Cu (copper sulphate pentahydrate) 10·0 mg, Mn (mangane oxide) 40·0 mg.

CGF, corn gluten feed.

SBM, soy bean meal.

SGBM, sugar beet molasses.

V+M, vitamin and mineral mixture.

CP, crude protein.

Table 2 Sequences of primers used for qRT-PCR and product sizes

Gene	Primer	Sequence, 5'-3'	Size, base pairs
β-Actin	forward	AAC TCC ATC ATG AAG TGT GAC G	233
	reverse	GAT CCA CAT CTG CTG GAA GG	
Ubiquitin	forward	AGA TCC AGG ATA AGG AAG GCA T	198
	reverse	GCT CCA CCT CCA GGG TGA T	
GAPDH	forward	AGC AAT GCC TCC TGT ACC AC	187
	reverse	AAG CAG GGA TGA TGT TCT GG	
BCATm	forward	GCC TGA AGG CGT ACA AAG G	130
	reverse	GAT GCA CTC CAG CAA CTC G	
BCKDH E1α	forward	CCA GAT GCC CGT CCA CTA C	152
	reverse	CCC CCT CTC CGA AGT AAC AG	
BCKDH E1β	forward	GCC GAA GTC ATC CAA GAA GG	118
	reverse	TGA CCT CAC AGG ACA CTC CAA G	
BCKDH E2	forward	ACG ATA CTG CTT ATG TGG GAA AG	128
	reverse	TGT GGC CCT TTA TCT CTT GG	
BCKDK	forward	TCC GAC CAT GAT GCT CTA TTC	114
	reverse	GAA GTC CTT GAT GCG GTG AG	
4-HPPD	forward	GTC TTC TCC TCT GCC CTC AA	193
	reverse	GCA AA TTC ACC TTC CCA AAC	
GHR	forward	ATG ATG CTG CCC CTG CTA	194
	reverse	AAA GGA CCA CAC TCC CTG CT	
IGF-BP 3	forward	TCC AAG CGC GAG ACG GGA TA	113
	reverse	TCG CAG TTG GGG ATG TGG AT	
IGF-ALS	forward	CGA CGA CTA CAC GGA CGA G	241
	reverse	CGA GGT GCA GGT GGT ACA G	
IGFR	forward	GGG GAA TGG AGT GCT GTA TG	185
	reverse	CTG GTC TCA GGC TCA TCT TTG	
IGF-1	forward	CTT CAG TTC GTG TGC GGA GAC A	109
	reverse	GGA AGC AGC ACT CAT CCA CGA T	

qRT-PCR, quantitative RT-PCR.

GAPDH, glyceraldehyde 3-phoshpate dehydrogenase.

BCATm, mitochondrial branched-chain amino transferase.

BCKDH E1α, branched-chain α-keto acid dehydrogenase E1α subunit.

BCKDK, branched-chain α-ketoacid dehydrogenase kinase.

4-HPPD, 4-hydroxy phenylpyruvate dioxygenase.

GHR, growth hormone receptor.

IGFBP 3, insulin-like growth factor 1 binding protein 3.

IGFALS, insulin-like growth factor 1 acid labile subunit.

IGFR, insulin-like growth factor 1 receptor.

IGF-1, insulin-like growth factor 1.

Table 3 Effects of increasing leucine supply and of simultaneous leucine and valine excess on the performance of weaned pigs fed diets first-limiting in isoleucine (Expt 1;*n* 8)[1]

		Treatment							
		L100	L150	L175	L200	L+V 200	PC		
		SID Leu:Ile						*P* values[2]	
		2·33	3·43	3·98	4·49	-	-	linear	quadratic
IBW, kg	Mean	8·94	8·94	8·93	8·93	8·93	8·93	-	-
	SE	0·30	0·30	0·37	0·23	0·28	0·27		
FBW, kg	Mean	24·83	23·87	22·81	22·44	21·11	25·19	0·05	0·93
	SE	1·23	1·05	1·27	1·13	0·68	0·47		
FI, g/d	Mean	646	611	572	558	510	660	0·06	0·93
	SE	48	36	46	47	32	22		
Gain, g/d	Mean	468	439	408	397	358	478	0·05	0·93
	SE	31	24	31	33	20	18		
G:F, g/kg	Mean	727	722	716	715	706	724	0·31	0·99
	SE	11	12	10	13	18	12		

[1] For details of animals and procedures, see Materials and methods.

[2] Polynomial orthogonal contrasts were estimated for L100 to L200.

SID, standardized ileal digestible.

IBW, initial body weight.

FBW, final body weight.

FI, feed intake.

G:F, gain to feed.

Table 4 Effects of increasing leucine supply and of simultaneous leucine and isoleucine excess on the performance of weaned pigs fed diets first-limiting in valine (Expt 2; *n* 8)[1]

		Treatment							
		L100	L150	L175	L200	L+I 200	PC		
		SID Leu:Val						*P* values[2]	
		1·79	2·63	3·08	3·55	-	-	linear	quadratic
IBW, kg	Mean	8·67	8·66	8·65	8·66	8·71	8·65	-	-
	SE	0·34	0·52	0·27	0·20	0·42	0·32		
FBW, kg	Mean	22·29	18·88	18·19	17·25	17·36	28·08†	<0·01	0·18
	SE	0·85	0·91	1·16	1·04	1·36	1·29		
FI, g/d	Mean	568	446	420	400	388	786†	<0·01	0·14
	SE	36	28	40	50	51	48		
Gain, g/d	Mean	400	299	279	252	255	570†	<0·01	0·18
	SE	22	22	31	30	37	30		
G:F, g/kg	Mean	707	670	662	630	651	723	<0·01	0·80
	SE	14	16	25	18	19	15		

[1] For details of animals and procedures, see Materials and methods.

[2] Polynomial orthogonal contrasts were estimated for L100 to L200.

SID, standardized ileal digestible.

IBW, initial body weight.

FBW, final body weight.

FI, feed intake.

G:F, gain to feed.

Mean values were significantly different from those of the L100 group: †*P*<0·05.

Table 5 Effects of increasing leucine supply and of simultaneous leucine and isoleucine excess on plasma free amino acids and serum branched-chain α-keto acids of weaned pigs fed diets first-limiting in isoleucine (Expt 1; *n* 8)[1]

	Treatment													
	L100		L150		L175		L200		L+V 200		PC			
	SID Leu:Ile													
	2·33		3·43		3·98		4·49		-		-		*P* values[2]	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	linear	quadratic
	Amino acids, µmol/l													
Ile	28	6	27	5	28	4	31	5	27	4	130†	9	0·82	0·67
Leu	133	10	149	8	174	15	218	8	200	35	143	13	<0·01	0·03
Val	236	14	98	6	94	8	103	9	417*	49	218	14	<0·01	<0·01
∑EAS	1057	75	956	46	991	75	1171	50	1424	145	1059	64	0·24	<0·01
∑NEAS	3358	193	3294	165	3231	239	3680	141	3349	130	3384	130	0·38	0·16
	Branched-chain α-keto acids, µmol/l													
KMV	5·4	1·2	5·9	1·2	5·8	1·3	5·0	1·2	4·5	1·0	26·9†	1·3	0·92	0·61
KIC	33·4	2·9	43·7	2·7	44·4	2·5	48·5	3·6	45·0	5·4	28·6	1·7	<0·01	0·51
KIV	11·6	1·0	5·1	0·3	4·1	0·8	3·3	0·6	19·3*	1·6	8·6†	0·4	<0·01	0·01

[1] For details of animals and procedures, see Materials and methods.

[2] Polynomial orthogonal contrasts were estimated for L100 to L200.

SID, standardized ileal digestible.

∑EAS, sum of the essential amino acids.

∑NEAS, sum of the non-essential amino acids.

KMV, α-keto β-methylvalerate.

KIC, α-keto isocaproate.

KIV, α-keto isovalerate.

Mean values were significantly different from those of the L200 group: *$P<0·05$.

Mean values were significantly different from those of the L100 group: †$P<0·05$.

Table 6 Effects of increasing leucine supply and of simultaneous leucine and isoleucine excess on plasma free amino acids and serum branched-chain α-keto acids of weaned pigs fed diets first-limiting in valine (Expt 2; *n* 8)[1]

	Treatment													
	L100		L150		L175		L200		L+I 200		PC			
	SID Leu:Val													
	1·79		2·63		3·08		3·55		-		-		*P* values[2]	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	linear	quadratic
Amino acids, µmol/l														
Ile	148	17	76	12	64	8	50	6	140*	23	138	7	<0·01	0·03
Leu	127	11	198	33	221	33	198	33	208	33	127	9	0·01	0·08
Val	39	4	69	14	56	8	56	8	60	10	220†	15	0·12	0·03
∑EAS	1243	98	1155	91	1385	153	1261	137	1302	169	1066	61	0·55	0·87
∑NEAS	3823	238	3773	246	3868	328	3746	173	3417*	204	3098†	122	0·85	0·87
Branched-chain α-keto acids, µmol/l														
KMV	33·4	1·8	17·0	1·9	12·9	1·0	11·3	0·9	27·1*	2·9	40·0	5·5	<0·01	<0·01
KIC	29·2	1·9	41·2	4·0	49·6	5·6	43·4	3·4	41·9	4·6	32·1	4·0	<0·01	0·04
KIV	0·9	0·2	2·0	0·5	1·5	0·3	2·0	0·4	1·6	0·4	12·2†	2·0	<0·05	0·34

[1] For details of animals and procedures, see Materials and methods.

[2] Polynomial orthogonal contrasts were estimated for L100 to L200.

SID, standardized ileal digestible.

∑EAS, sum of the essential amino acids.

∑NEAS, sum of the non-essential amino acids.

KMV, α-keto β-methylvalerate.

KIC, α-keto isocaproate.

KIV, α-keto isovalerate.

Mean values were significantly different from those of the L200 group: *$P<0·05$.

Mean values were significantly different from those of the L100 group: †$P<0·05$.

Table 7 Effects of increasing leucine supply on mRNA of branched-chain amino acid catabolism genes in the liver of weaned pigs fed diets first-limiting in isoleucine (Expt 1)[1]

				BCKDH			
Treatment	SID Leu:Ile		BCATm	E2	E1α	E1β	BCKDK
L100	2·33	Mean	1·00	1·00	1·00	1·00^{ab}	1·00^{b}
		SE	0·23	0·14	0·10	0·08	0·06
L150	3·43	Mean	0·79	0·76	1·32	0·82^{b}	1·05^{ab}
		SE	0·15	0·17	0·13	0·04	0·04
L200	4·49	Mean	1·02	0·940	1·35	1·13^{a}	1·21^{a}
		SE	0·37	0·11	0·20	0·07	0·07

[1] $2^{-\Delta\Delta Ct}$ values; for details, see Materials and methods.

BCKDH, branched-chain α-keto acid dehydrogenase.

SID, standardized ileal digestible.

BCATm, mitochondrial branched-chain amino acid transferase.

BCKDK, branched-chain α-keto acid dehydrogenase kinase.

4-HPPD, 4-hydroxy phenylpyruvate dioxygenase.

[a,b]Mean values within a column with unlike superscript letters were significantly different (*P*<0·05).

Table 8 Tissue-specific take-off values estimated by qRT-PCR for Expt 1[1]

	LD muscle		Heart		Liver		Spleen		Jejunum		Ileum	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
BCATm	24·73^{c}	0·16	22·05^{d}	0·11	29·10^{a}	0·18	25·31^{bc}	0·17	25·70^{b}	0·17	28·31^{a}	0·36
BCKDH												
E1α	19·40^{b}	0·11	17·19^{d}	0·09	18·08^{c}	0·08	20·25^{a}	0·25	19·28^{b}	0·09	20·57^{a}	0·19
E1β	21·17^{b}	0·13	18·66^{e}	0·05	19·20^{d}	0·06	21·25^{b}	0·15	20·52^{c}	0·15	21·84^{a}	0·11
E2	20·91^{a}	0·13	18·35^{c}	0·08	18·37^{c}	0·12	19·59^{b}	0·24	19·61^{b}	0·14	19·55^{b}	0·17
BCKDK	17·43^{d}	0·09	15·45^{e}	0·06	18·13^{c}	0·05	19·23^{a}	0·27	18·28^{bc}	0·10	18·76^{ab}	0·15

[1] For details of animals and procedures, see Material and methods.

qRT-PCR, quantitative RT-PCR.

LD, longissimus dorsi.

BCATm, mitochondrial branched-chain amino acid transferase.

BCKDH, branched-chain α-keto acid dehydrogenase.

BCKDK, branched-chain α-keto acid dehydrogenase kinase.

^{a,b,c,d}Mean values within a row with unlike superscript letters were significantly different (*P*<0·05).

Table 9 Effects of increasing dietary leucine supplementation on mRNA of genes related to growth hormone-insulin-like growth factor-1 axis in the liver of pigs fed diets first-limiting in valine (Expt 2)[1]

Treatment	SID Leu:Val		GHR	IGFBP-3	IGFALS	IGFR	IGF-1
L100	1·79	Mean	$1{\cdot}00^{a}$	1·00	$1{\cdot}00^{a}$	1·00	$1{\cdot}00^{a}$
		SE	0·10	0·14	0·17	0·13	0·09
L150	2·63	Mean	$0{\cdot}62^{b}$	0·83	$0{\cdot}86^{ab}$	1·04	$0{\cdot}53^{b}$
		SE	0·09	0·10	0·19	0·09	0·09
L200	3·55	Mean	$0{\cdot}52^{b}$	0·85	$0{\cdot}58^{b}$	1·03	$0{\cdot}29^{c}$
		SE	0·10	0·13	0·08	0·09	0·07

[1] $2^{-\Delta\Delta Ct}$ values; for details, see Materials and Methods.

SID, standardized ileal digestible.

GHR, growth hormone receptor.

IGFBP-3, insulin-like growth factor binding protein 3.

IGFALS, insulin-like growth factor acid labile subunit.

IGFR, insulin-like growth factor receptor.

IGF-1, insulin-like growth factor-1.

[a,b,c] Mean values within a column with unlike superscript letters were significantly different (*P*<0·05).

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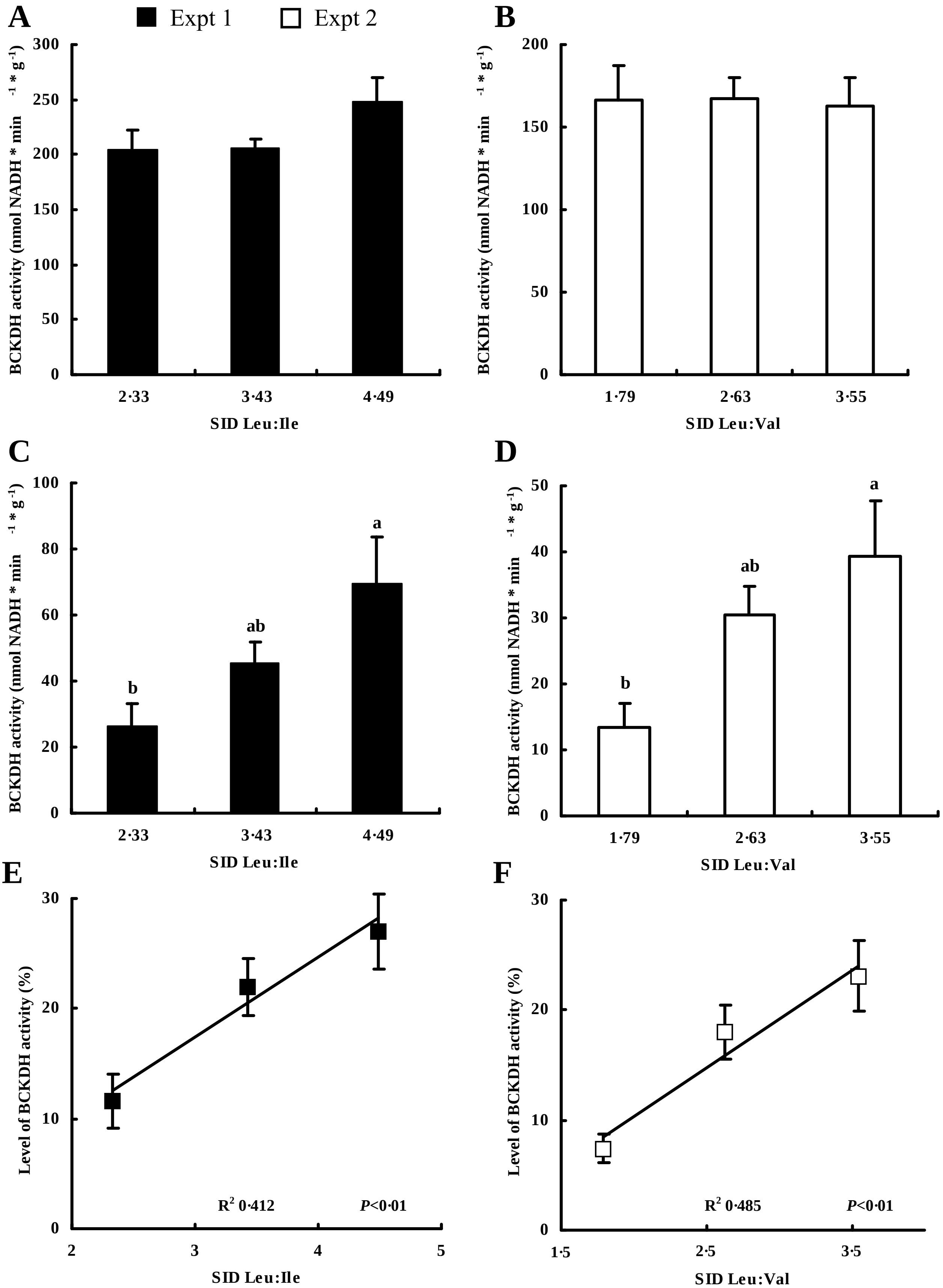
Figure Legends

Figure 1 Effects of increasing leucine supply on branched-chain α-keto acid dehydrogenase (BCKDH). Total (A and B),·basal (C and D), and relative (E and F) BCKDH activities in the liver of pigs fed diets first-limiting in isoleucine (Expt 1 ■) or valine (Expt 2 □). For E, $y = -7\cdot5 + 8\cdot9x$ and for F, $y = -4\cdot2 + 7\cdot2x$. Values are means with their standard errors depicted by vertical bars (*n* 8). ^{a,b}Mean values within a graph with unlike superscript letters were significantly different ($P<0\cdot05$).

Figure 1


A
Expt 1
Expt 2
B
BCKDH activity (nmol NADH * min-1 * g-1)
300
250
200
150
100
50
0
2·33
3·43
4·49
SID Leu:Ile
200
150
100
50
0
1·79
2·63
3·55
SID Leu:Val
C
D
100
80
60
40
20
0
b
ab
a
50
40
30
20
10
0
E
F
Level of BCKDH activity (%)
30
20
10
0
2
3
4
5
R2 0·412
P<0·01
1·5
2·5
3·5
R2 0·485
P<0·01

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