Leucine in piglets –
Nutritional requirement and consequences of excessive intakes on whole body amino acid metabolism

Dissertation

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Abstract

The concept of the “Ideal Protein” provides an approach for sustainable livestock production. The “Ideal Protein” specifies precisely the ratios of amino acids, which are required for a specific physiological performance (growth, reproduction, *etc.*). In the ideal scenario, dietary amino acids are provided neither in excess nor in deficient amounts. Consequently, all amino acids are equal limiting for the animals performance. In this case, the dietary protein could be used with highest efficiency, since nitrogen excretion and metabolic stress is minimized. Using the concept of the “Ideal Protein” the focused amino acid has to be related to a reference amino acid. The reference for the pig species is lysine, because lysine is the first-limiting amino acid for the (growth-) performance of pigs by use of commercial raw feedstuff materials for feeding.

The implementation of the “Ideal Protein” concept requires exact knowledge of the animals’ requirement for an essential amino acid. So far, literature data about the requirement of weaned piglets fed low-protein diets for leucine are sparse. Therefore, the aim of the present work was to estimate the optimum ratio among leucine and lysine for growth of piglets. To this end, the requirement for lysine was estimated in a preliminary study with crossbred [(Landrace x Large White) x Pietrain] weaned piglets. As a result of **Study I,** the requirement for standardized ileal digestible (SID) lysine was estimated to be 1.01%. To ensure a sub-limiting function of lysine in the subsequent studies, a 10% lower dietary concentration of lysine (SID 0.93%) was used in the following considerations.

Secondly, piglets requirement for leucine was estimated in a classical dose-response trial with five treatment groups (SID leucine:lysine 83%, 94%; 104%, 115%, 125%) in **Study II**. To this end, the estimates of three different statistical models were compared. A clear dependency of the requirement estimate on the model used for calculation was found. Using the linear broken-line model, the requirement of piglets in the weight range between 10 to 25 kg was estimated to be SID leucine:lysine 97,7%. By use of the quadratic broken-line model, the requirement was estimated to be 102.7%. The highest requirement was estimated with the quadratic function model (SID leucine:lysine 107,7%). The latter was the only model, which considered a decrease in growth performance of the pigs in the highest supplemented treatment group. The calculation of the expected growth performance depression caused by feeding leucine levels 10% below

the estimated requirement resulted in a low value of 2% by use of the quadratic function model.

Since available literature gives several indications on important physiological functions of leucine, which may alter the metabolism and the availability of other essential amino acids, this special branched-chain amino acid was investigated further in the present work. On the one hand, leucine stimulates anabolic processes as protein synthesis via the enzyme mechanistic target of rapamycin (mTOR). However, on the other hand, leucine regulates the catabolism of all three branched-chain amino acids leucine, valine and isoleucine and may induce a limitation of valine and isoleucine for protein synthesis.

In **Study III** the impact of a dietary leucine excess on the physiology of piglets was tested in a dose-depended manner with a control group (SID leucine:lysine 100%) and two treatment groups (SID leucine:lysine 200% and 400%). In scope of **Study III,** blood plasma was analyzed for amino acid concentrations, concentrations of branched-chain α-keto acids and free 3-hydroxybutyrate. For the first time, the concentrations of free amino acids and the basal activity of the branched-chain keto acid dehydrogenase (BCKDH) was measured in eight different porcine body tissues. Furthermore, the concentrations of serotonin were analyzed in plasma and cortical tissue. Serotonin is an orectic neurotransmitter in pigs. The brain concentrations of tryptophan define the synthesis rate of serotonin in this tissue since neurotransmitters are not capable to cross the blood-brain barrier. As a result, significant growth depressions were found in the treatment groups compared to the control group. A dietary leucine excess caused significant reduced feed intakes, in line with lower weight gains in both treatment groups. The reduced feed intake was accompanied with a reduced serotonin synthesis in brain tissue caused by the reduced uptake of tryptophan to the brain due to transport competition with leucine at the blood-brain barrier. Besides the brain concentrations of free tryptophan, the concentrations of free methionine were additionally reduced in the treatment groups. An increased basal activity of the enzyme BCKDH in six of eight analyzed tissues caused a significant reduction of valine and isoleucine (and the corresponding α-keto acids α-keto isovalerate and α-keto β-methylvalerate), combined with a significant increase of leucine and α-keto isocaprorate in plasma and tissues of the pigs from the treatment groups. The significant increase of plasma 3-hydroxybutyrate was an additional proof for the clearly accelerated amino acid metabolism, since BCKDH catalyzes the decarboxylation of the α-keto acids to acetyl-CoA. The new finding of this study

was that the pancreas seems to have higher activities of BCKDH in case of dietary leucine excess. To date, the liver is known as main tissue for amino acid metabolism.

Since it is known from literature that threonine could be degraded alternatively via BCKDH, **Study IV** was applied to test whether a dietary leucine excess could impair the availability of threonine. To this end, the growth performance of 60 weaned piglets and, simultaneously, the nitrogen balance of 20 piglets was evaluated. The piglets received two levels of leucine (SID leucine:lysine 100% *versus* 200%) and two levels of threonine (SID threonine:lysine 57% *versus* 65%) with their feeds. The growth performance remained unaffected by dietary treatment. The nitrogen retention was significantly improved with high threonine supplementation combined with adequate leucine intake. The leucine excess caused increased concentrations of plasma leucine and decreased concentrations of valine and isoleucine. The basal BCKDH activity was unaffected by feeding 200% leucine. Therefore, it could be concluded that a dietary leucine excess of 200% is not sufficient to reduce the availability of threonine via increased activity of BCKDH.

The (interaction-) **Study V** with leucine and tryptophan as nutritional factors should confirm the result of **Study III** that a dietary leucine excess reduces the availability of brain tryptophan for serotonin synthesis. To this end, each two levels of leucine (SID leucine:lysine 100% *versus* 300%) and two levels of tryptophan (SID tryptophan:lysine 18% *versus* 23%) were applied by *ad libitum* feeding to weaned piglets. As a result, the pigs in the high leucine groups consumed averagely 40 g less feedstuff per day during the entire experimental period compared to the animals with adequate leucine supply. Again, the reduced feed intake could be attributed to a reduced uptake of tryptophan to the brain, accompanied with reduction of hypothalamic serotonin synthesis. The assumption that an increased hypothalamic mTOR acitvity was additionally involved in feed intake reduction could not be confirmed based on protein expression analysis. Moreover, the imbalances (deficiencies of valine and isoleucine due to leucine excess) of the branched-chain amino acids, accompanied with the reduced hypothalamic serotonin concentrations are assumed to be the reasons for the decreased feed intake. Current literature provides various hints about the regulation of feed intake in case of consuming feedstuffs deficient in single amino acids or with imbalanced profile. The reported imbalances in plasma amino acids were evidenced in **Study V** by increased activities of BCKDH in liver and pancreas. As in **Study III,** the BCKDH activity was higher in the pancreatic tissue compared to liver.

The results shown in the present work elucidate clearly the secondary effects caused by a dietary leucine excess applied during several weeks. A leucine intake beyond the requirement firstly induces amino acid imbalances in blood plasma and body tissues, which could limit protein synthesis. This principle was clearly shown with the neurotransmitter serotonin in the brain in **Study III** and **Study V**. The described secondary effects have to be taken into account if concepts to improve the performance of athletes (e.g. bodybuilders), to use high doses of leucine for therapeutically reasons (e.g. obesity, atrophy) or the improvement of livestock performance should be developed. The use of increased amounts of dietary leucine may be useful. However, the balanced ratio to all the other large neutral amino acids has to be considered with special attention. It would be reasonable to formulate the requirement recommendations for valine and isoleucine not only in ratio to first-limiting lysine, but also in ratio to leucine in the future nutrition of pigs.

Zusammenfassung

Das „Konzept des Idealproteins“ bietet einen Ansatzpunkt zur umweltschonenden Tierproduktion. Das „Idealprotein“ definiert genau die Relationen an Aminosäuren, die für eine physiologische Leistung (Wachstum, Reproduktion, *etc*.) benötigt werden. Im Idealfall liegt keine Aminosäure im Überschuss oder im Mangel vor. In der Folge wirken alle Aminosäuren gleichermaßen limitierend. Das Protein kann unter diesen Bedingungen mit der höchsten Effizienz genutzt werden. Stickstoffexkretionen und Belastungen des Stoffwechsels werden minimiert. Bei der Umsetzung des „Idealprotein“-Konzepts wird die zu untersuchende Aminosäure in Relation zu einer Referenz-Aminosäure gesetzt. Für die Spezies Schwein ist diese Referenz-Aminosäure Lysin, weil Lysin bei Anwendung praxisüblicher Rohfutterkomponenten erstlimitierend auf die (Wachstums-) Leistung wirkt.

Die Umsetzung des „Idealprotein“-Konzepts erfordert die genaue Kenntnis des Bedarfes an essentiellen Aminosäuren. Literaturangaben zum Leucinbedarf von Absetzferkeln, die mit niedrigen Proteingehalten gefüttert werden, sind rar. Ziel dieser Arbeit war es daher das optimale Verhältnis zwischen Leucin und Lysin zu ermitteln. Hierzu wurde in einem Vorversuch mit Absetzferkeln der Dreirassenkreuzung [(deutsches Landschwein x deutsches Edelschwein) x Pietrain] der Bedarf an Lysin für optimales Wachstum ermittelt. Im Ergebnis zeigte sich, dass der Bedarf für Lysin in Getreide- und Sojabohnenbasierten Futter bei 1,01% precaecal verdaulich (pcv) liegt. Um zu gewährleisten, dass Lysin in den folgenden Versuchen als zweitlimitierende Aminosäure wirkt, wurde der in **Studie I** ermittelte Bedarf abzüglich 10% (pcv Lysin 0,93%) in allen weiteren Versuchen eingesetzt.

In **Studie II** wurde in einem klassischen Dosis-Wirkungs-Versuch mit fünf Behandlungsgruppen (pcv Leucin:Lysin 83 %, 94 %; 104 %, 115 %, 125 %) der Leucinbedarf abgleitet. Hierzu wurden die Schätzungen verschiedener statistischer Modelle verglichen. Es zeigte sich eine Abhängigkeit des errechneten Bedarfes vom Modell. Bei Anwendung des „linear broken-line” Modells wurde ein Bedarf für Schweine der Gewichtsgrößenordnung 10 bis 25 kg von pcv Leucin:Lysin 97,7 % kalkuliert. Bei Anwendung des „quadratic broken-line” Modells kann ein Bedarf von 102,7% angenommen werden. Den höchsten Wert ermittelte das „quadratic function” Modell mit pcv Leucin:Lysin 107,7%. Letzteres war das einzige der drei Modell, welches eine Verschlechterung der Leistungsparameter in der höchst-supplementierten Behandlungs-

gruppe berücksichtigte. Die Kalkulation der Höhe der Leitungseinbußen, die eine Fütterung 10 % unterhalb des Bedarfes zur Folge hätte, ergab mittels des „quadratic function" Modells einen geringen Wert von 2 %.

Die verzweigtkettige Aminosäure Leucin stand weiterhin im Fokus dieser Arbeit, weil aus der Literatur zahlreiche physiologische Funktionen bekannt sind, die den Stoffwechsel und die Verfügbarkeit anderer essentieller Aminosäuren beeinflussen. So stimuliert Leucin auf der einen Seite anabole Prozesse wie die Proteinbiosynthese über das Enzym „mechnistic target of rapamycin" (mTOR), reguliert aber auf der anderen Seite den Katabolismus der drei verzweigkettigen Aminosäuren Leucin, Valin und Isoleucin.

In **Studie III** wurde in einem Dosis-Wirkungs-Versuch mit einer Kontrollgruppe (pcv Leucin:Lysin 100%) und zwei Behandlungsgruppen (pcv Leuin:Lysin 200% und 400%) der Einfluss eines fütterungsbedingten Leucinüberschusses auf die Physiologie von Absetzferkeln getestet. Im Rahmen der **Studie III** wurden Blutproben auf Aminosäurekonzentrationen, Konzentrationen von α-Ketosäuren und freien Ketonkörpern untersucht. Erstmals wurden in acht verschiedenen porcinen Geweben die freien Aminosäurekonzentrationen und die basalen Aktivitäten der Verzweigtketten-α-Keto-Dehydrogenase (BCKDH) bestimmt. Im Cortex wurde außerdem der Gehalt an Serotonin analysiert. Serotonin ist ein Neurotransmitter der im Schwein orektisch wirkt und dessen Gehalt im Gehirn maßgeblich von der Tryptophankonzentration abhängt. Im Ergebnis zeigten sich klare Leistungseinbußen in beiden Behandlungsgruppen im Vergleich zur Kontrollgruppe. Ein nutritiver Leucinexzess führte zu einer signifikant reduzierten freiwilligen Futteraufnahme, einhergehend mit geringeren Zunahmen in den Behandlungsgruppen im Vergleich zu Tieren, die die ausbalancierte Kontrolldiät erhielten. Die Reduzierung der freiwilligen Futteraufnahme war hierbei auf eine reduzierte Serotoninsynthese im Gehirn zurückzuführen. Als Ursache konnte eine verminderte Aufnahme von Tryptophan in das Gehirngewebe angeführt werden. Neben der Konzentrationan freiem Tryptophan im Gehirngewebe, verringerten sich auch die Konzentrationen an freiem Methionin. Eine gesteigerte Aktivität des Enzymes BCKDH in sechs von acht analysierten Geweben verursachte in den beiden Behandlungsgruppen eine signifikante Reduzierung der Konzentrationen von Valin und Isoleucin (und den korrespondierenden α-Ketosäuren α-Keto-Iso-Valerat und α-Keto-Methyl-Valerat), gepaart mit einem signifikanten Konzentrationsanstieg an freiem Leucin und α-Keto-Iso-Caproat in Blutplasma und Geweben. Der signifikante Anstieg der Ketonkörperkonzentrationen im Blutplasma war ein weiterer Beweis für einen deutlich beschleunigten Aminosäurestoffwechsel, da

BCKDH die Dekarboxylierung der α-Ketosäuren zu Acetyl-CoA katalysiert. Neu war in diesem Versuch die Erkenntnis, dass die BCKDH Aktivität im Pankreas höher war als in der Leber. Bis dato gilt die Leber als das Hauptorgan für den Aminosäurestoffwechsel.

Da aus der Literatur bekannt ist, dass Threonin über alternative Stoffwechselwege verfügt, die teilweise von BCKDH katalysiert werden können, wurde in **Studie IV** getestet, ob ein nutritiver Leucinüberschuss die Verfügbarkeit von Threonin beeinflusst. Dazu wurden die Leistungsdaten von 60 Absetzferkeln erfasst, ebenso wie die Stickstoffverwertung einer kleineren Gruppe von 20 Tieren. Die Tiere erhielten mit ihrem Futter je zwei Stufen von Leucin (pcv Leucin:Lysin 100 % *versus* 200 %) und zwei Stufen von Threonin (pcv Threonin:Lysin 57 % *versus* 65 %). Es zeigten sich keine Effekte auf die Leistungsparameter der Tiere. Die Stickstoffretention konnte durch erhöhte Threoningaben bei adäquater Leucinversorgung signifikant verbessert werden. Ein Leucinüberschuss führte zu gesteigerten Konzentrationen an Leucin und reduzierten Konzentrationen an freiem Valin und Isoleucin im Blutplasma. Auch die BCKDH Aktivität veränderte sich nicht durch Gabe von 200 % Leucin. Es kann daher davon ausgegangen werden, dass ein nutritiver Leucinüberschuss von 200 % nicht ausreicht, um die Threoninverfügbarkeit zu verringern.

Da bereits in **Studie III** gezeigt werden konnte, dass ein nutritiver Leucinüberschuss die Tryptophanverfügbarkeit für die Serotoninsythese im Gehirn verringert, wurde mit **Studie V** eine Interaktionsstudie zwischen Leucin und Tryptophan durchgeführt. Dabei wurden je zwei Stufen von Leucin (pcv Leucin:Lysin 100 % *versus* 300 %) und zwei Stufen Tryptophan (pcv Tryptophan:Lysin 18 % *versus* 23 %) an Absetzferkel verfüttert. Es zeigte sich, dass die Tiere in den Leucinüberschussgruppen über einen Zeitraum von 6 Wochen durchschnittlich 40 g weniger Futter pro Tag konsumierten, als die Tiere mit adäquater Leucinversorgung. Wieder ließ sich dieses Ergebnis auf eine reduzierte Tryptophanaufnahme in das Gehirn und eine daraus resultierende reduzierte Serotoninsynthese im Hypothalamus zurückführen. Die Vermutung, dass auch eine gesteigerte mTOR Aktivität im Hypothalamus in die Reduzierung der Futteraufnahme involviert war, konnte nach Proteinexpressionsanalysen nicht bestätigt werden. Vielmehr spielen auch in diesem Versuch, neben den verringerten Serotoninkonzentrationen im Gehirn, die Imbalancen der verzweigtkettigen Aminosäuren eine Rolle. In der Literatur finden sich zahlreiche Hinweise auf eine Regulation der Futteraufnahme bei Konsum von Futtermitteln mit unausgewogenem Aminosäureprofil. Die Imbalancen im Blutplasma

wurden auch in **Studie V** durch gesteigerte BCKDH Aktivitäten in den Leucinüberschussgruppen belegt. Wieder fanden sich höhere Aktivitäten im Pankreas, als in der Leber.

Die in dieser Arbeit gezeigten Versuche veranschaulichen sehr deutlich die unerwünschten Nebeneffekte, die eine gesteigerte Leucinaufnahme über mehrere Wochen mit sich bringt. Sie führt in erster Linie zu Aminosäureimbalancen in Blutplasma und Geweben, welche die Proteinsynthese limitieren. Dieses Prinzip konnte in **Studie III** und **Studie V** sehr anschaulich am Neurotransmitter Serotonin im Gehirngewebe gezeigt werden. Diese Nebeneffekte sind zu berücksichtigen, wenn Konzepte zur Leistungssteigerung im Sport (z.B. Bodybuilding), zum Einsatz von hohen Leucinmengen zu Therapiezwecken (Fettleibigkeit, Atrophie) und zur Verbesserung der Wachstumsleistung von Nutztieren erarbeitet werden. Der Einsatz von gesteigerten Mengen an Leucin mag durchaus sinnvoll erscheinen, allerdings nur wenn auf ein ausgewogenes Verhältnis zu allen anderen langkettigen Aminosäuren geachtet wird. In der Schweinefütterung ist die Angabe Bedarfsempfehlungen insbesondere von Valin und Isoleucin nicht nur im Verhältnis zu Lysin, sondern auch im Verhältnis zu Leucin anzuraten.

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Abbreviations

Not listed below are abbreviations, which are:

- SI units and their symbols
- Mathematical characters and abbreviations
- Abbreviations listed in common

AA	Amino acids
ADFI	Average daily feed intake
ADG	Average daily gain
AEVZ	Agricultural and Nutritional Research Center
AIC	Akaike information criterion
AICC	Corrected Akaike information criterion
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
APC	Anterior piriform cortex
Aqua bd	*Aqua bisdestillata*
BCAA	Branched-chain amino acids
BCAT	Branched-chain aminotransferase
BCKA	Branched-chain α-keto acid
BCKDH	Branched-chain α-keto acid dehydrogenase
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BW	Body weight
CP	Crude protein
FAO	Food and Agriculture Organization of the United Nations
FBW	Final body weight
G:F	Gain to feed ratio
GfE	Gesellschaft für Ernährungsphysiologie
GH	Growth hormone
Gln	Glutamine
Glu	Glutamate
His	Histidine
HPLC	High performance liquid chromatography
IBW	Initial body weight
IGF-I	Insulin-like growth factor-I
Ile	Isoleucine
INRA	Institut national de la recherche agronomique
KIC	α-keto isocaproate
KIV	α-keto isovalerate

Km	Michaelis constant
KMV	α-keto β-methylvalerate
Leu	Leucine
LNAA	Large neutral amino acids
Lys	Lysine
MCP	Monocalcium phosphate
ME	Metabolizable energy
Met	Methionine
mRNA	Messenger ribonucleic acid
MSUD	Maple syrup urine disease
mTOR	Mechanistic target of rapamycin
N	Nitrogen
OPA	o-phthaldialdehyde
pcv	Precaecal vedaulich
Phe	Phenylalanine
RNA	Ribonucleic acid
SID	Standadized ileal digestible
TBST	Tris buffered saline and Tween
Thr	Threonine
tRNA	Transfer ribonucleic acid
Trp	Tryptophan
Tyr	Tyrosine
v/v	Volume per volume
Val	Valine
w/v	Weight per volume

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1 Introduction

1.1 Pork meat production, guidelines, and constraints

As the global population steadily increases and available arable land decreases, the demand for more efficient methods of animal feeding continues to rise. The Food and Agriculture Organization of the United Nations (FAO) forecasts a further reduction of 19% of available arable land (hectare per capita) by 2020 compared to 2000 (FAO, 2006). Simultaneously, the increase in population is estimated to reach its peak by 2050 with approximately 8.9 billion human beings on earth. This is an increase of 46% compared to 6.1 billion in 2000. In the past four decades, world agriculture has been growing at rates of 2.1 to 2.3% per year (FAO, 2006). In recent years, due to the growth in per capita income, the demand for animal products has increased, particularly in China, which holds a significant market share. In the developed world, the demand for pork has been constant in the last decade. In Germany, pork consumption per capita was around 52.8 kg in 2012 (German Federal Statistical Office, 2014), a high percentage when compared to world's average pork meat supply (**Figure 1**: QSEQ). In the last decade, pork production increased in Germany by 28% from 4.3 million t in 2004 to 5.5 million t in 2015 (German Federal Statistical Office, 2014). In the first half of 2015, a record high of 2.76 million t pork meat was generated in Germany, which is 67 000 t (+ 2.5%) more than in the first half of 2014 (German Federal Statistical Office, 2015).

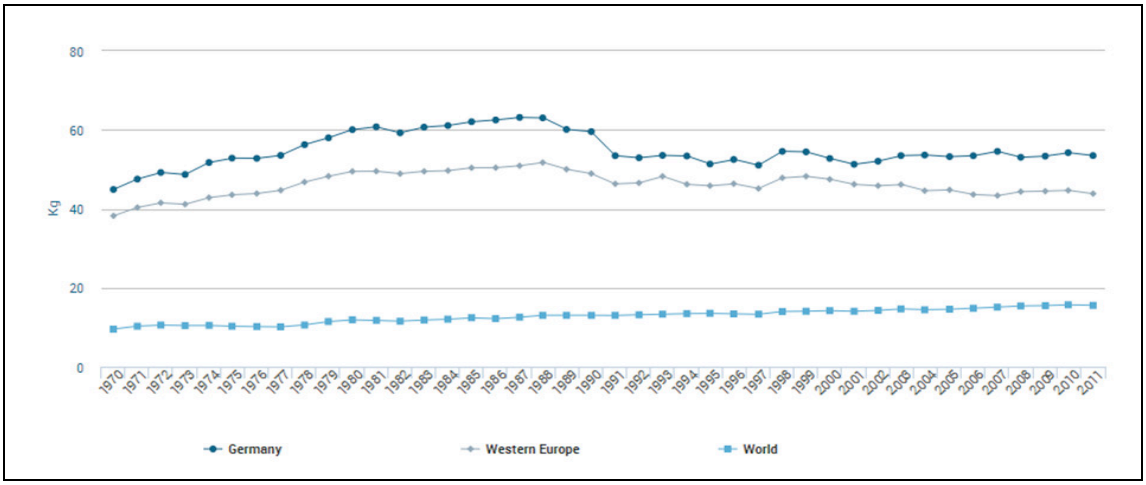

Figure 1: Quantity of pork meat supply in Germany, Western Europe and world in kg/capita/year from 1970 to 2011 (FAOSTAT, 2016)

Table 1 demonstrates that pork has the largest market share in general meat production. However, in the future there will be a shift towards higher poultry production and con-

sumption, as it is not bound by religious constraints and potentially has lower production costs. Therefore, pork production and consumption will experience a deceleration in growth as opposed to the high growth rates seen until now. Nonetheless, this does not lessen the environmental implications.

Table 1: World meat production and demand (modified to FAO, 2006)

	1999/ 2001	**1961- 2001**	**1971- 2001**	**1981- 2001**	**1991- 2001**	**1999- 2030**	**2030- 2050**
	Tsd t	Growth rates (% per year)					
Production							
Bovine meat	59,378	1.7	1.3	1.1	0.7	1.3	0.9
Ovine meat	11,337	1.7	2.0	2.1	1.6	1.7	1.2
Pig meat	**90,666**	**3.3**	**3.1**	**2.9**	**2.5**	**1.2**	**0.4**
Poultry meat	68,331	5.4	5.2	5.2	5.3	2.5	1.5
Total meat	229,713	2.9	2.9	2.9	2.7	1.7	1.0
Consumption							
Bovine meat	58,549	1.7	1.3	1.1	0.7	1.4	0.9
Ovine meat	11,187	1.7	2.0	2.1	1.6	1.7	1.2
Pig meat	**90,818**	**3.3**	**3.1**	**2.9**	**2.6**	**1.2**	**0.4**
Poultry meat	67,447	5.3	5.1	5.1	5.1	2.6	1.5
Total meat	228,000	3.0	2.9	2.8	2.7	1.7	1.0

In the past, increasing productivity has been the primary aim with respect to animal production. The environmental responsibility is becoming the primary focus. Since animal agriculture contributes to global warming, acidification, as well as land and water use, the western society has installed regulations to minimize the negative impact of animal production. Especially the nitrogen (N) input by manure into the soil could be regulated by protein contents in animal feeds. As far back as 1991, the Council of the European Union Community published the European Directive on Nitrates 91/676/EEC, which was designed to reduce water pollution due to N from farming. The Directive 96/61/EC (1996) also put into place an approach based on Integrated Pollution Prevention and Control (IPPC). According to this directive, large pig farms are authorized only if the polluting emissions in water and soil (including nitrates) and in the air (in particular ammonia) do not exceed the maximum limit of 170 kg/ha/a (European Commision, 2003). In 2012, Germany occupied a mid-table position in the ranking of European countries, with an average N output of 98.9 kg/ha/a by manure (German Federal Statistical Office, 2014). To minimize N pollution output, different strategies can be used. One of those strategies is the strong reduction of dietary protein contents in animal feeds with simultanous amino acid (AA) supplementation (European Commision,

2003). The principles are that the nutrients supplied with the feed in excess of the pigs' requirements are not absorbed by the animal or are degraded to urea. Dietary N compounds are excreted in pigs' manure, which generates ammonia (Figure 2), nitrous acid and nitrous oxide; the latter is a powerful greenhouse gas with an effect approximately 300 times that of methane. Reduced protein levels in feed are known to avoid an oversupply of non-essential AA and lead to reduced N excretion.

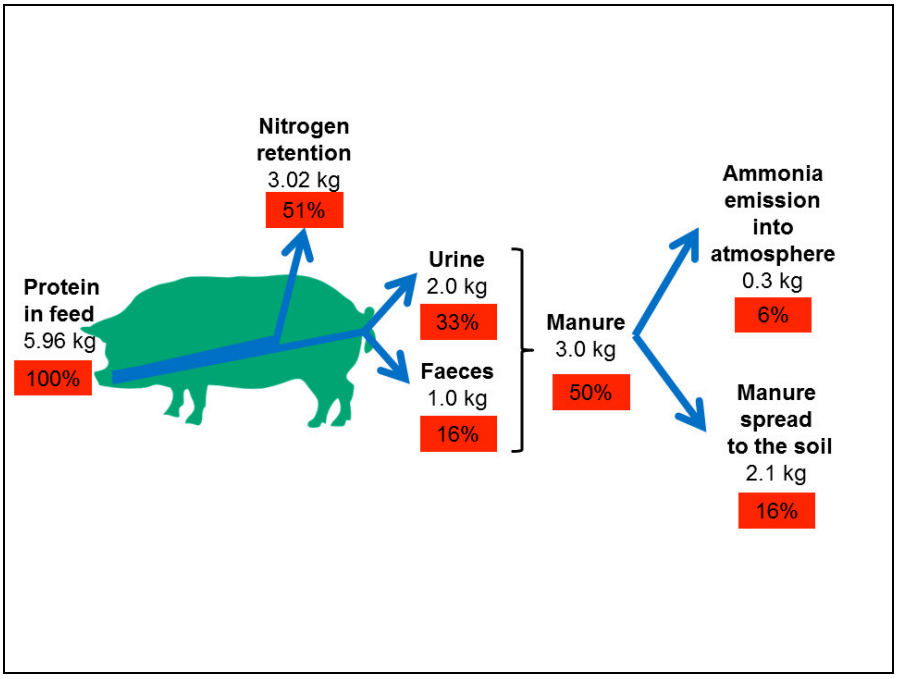

Figure 2: Consumption, utilization and losses of nitrogen in the 95-day lasting growing period of a pig fed with commercial feeds (crude protein≈17%). Data adopted from Osada *et al.* (2011)

Reduction of the concentration of noxious gases such as ammonia in livestock facilities is beneficial for both animal welfare and the health and safety of farmers and employees working in such environments.

Feed management using AA supplementation to lower dietary protein levels can potentially reduce N excretion up to 40% without detrimentally affecting animal performance (Gloaguen *et al.*, 2014). On the contrary, production performance, carcass quality as well as economic and ecological efficiency can be achieved when dietary crude protein (CP) levels are reduced (He *et al.*, 2013). Another reason for reducing dietary CP contents in pig feeds concerns the raw feedstuff materials: European cereals used as raw materials contain only minor amounts of CP (corn≈8%; barley≈11% and wheat≈14%). In contrast, soybeans contain on average 37% CP and are used to increase dietary CP.

However, soy production is associated with deforestation and generates three to four fold higher carbon dioxide emissions than regional cereals due to its transport from overseas to Europe (Garcia-Launay *et al.*, 2014). European meat consumers also reject the use of soy as it is mostly produced by genetic engineering. Reducing dietary CP is also beneficial in improving piglets' health by reducing the incidence of diarrhea since undigested proteins contribute to the proliferation of pathogenic bacteria in the gut increasing the risk of digestive disorders (Gloaguen *et al.*, 2014). In consequence, the N utilization is impaired and it is unavoidable that farmers use antibiotics against diarrhea. The use of antibiotics is also seen in a negative light by European end-consumers. Moreover, the Robert Koch Institute warns against resistances of numerous pathogens originating from the routine use of antibiotics in animal nutrition (Robert Koch Institute, 2014) Consequently, the main reasons to reduce dietary CP are for environmental protection and animal welfare.

1.2 Proteins and amino acids - Metabolism and physiological functions

Pigs require AA and not protein *per se*. The quality of protein supply is determined by its potential to cover the physical requirements in terms of AA for maintenance and performance, which is assured by a stable protein synthesis rate. The AA are derivates of carboxylic acids, containing an amino group, a carboxylic-acid group and a characteristic side-chain. AA can be grouped according to their transport affinities or essential role in nutrition or on the basis of catabolic fate of the carbon skeleton. A total of 21 AA are required for protein synthesis. Hence, they are called proteinogenic AA, occurring in L-configuration as components of peptides and proteins or free AA in both cells and body fluids. In pigs and other mammals, nine AA cannot be synthesized endogenously or in sufficient amounts. These AA, as histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), tryptophan (Trp), threonine (Thr) and valine (Val) must be delivered through the diet and are classified as nutritionally indispensable (essential) AA. For a particular feed ingredient or mixture of ingredients supplying protein, the AA, which is in shortest supply compared with the requirement of the animal, is defined as the first-limiting AA. The first-limiting AA limits the value of protein. In pigs, Lys is the first-limiting AA followed by Thr, Trp, Val and Met (Gloaguen *et al.*, 2013a). Since Lys is the first-limiting AA in pig nutrition, it was decided that Lys would be taken as a reference value and the remaining essential AA

would be referred to Lys, taking Lys as 100% (Boisen, 2003). For pigs, the “Ideal Protein“ is a concept where the AA pattern (defined as a percentage of Lys) maximizes growth, N retention or another response criterion. In this profile, all indispensable AA are equally limiting for performance, just covering the requirements for all physiological functions. The “Ideal Protein“ requirement (and hence the ratio of essential AA) differs depending on age and level of production, *i.e.* maintenance, growth or reproduction. If the dietary requirements in terms of Lys, the ideal AA profile and the stage of production are known, the requirement for the other entire essential AA can be estimated. This principle enables feeding pigs with low-CP diets, which contain an optimal AA composition – without any lack or surplus of AA. In consequence, the utilization of dietary protein would be improved and N output and emissions of greenhouse gas compounds caused by pig production is reduced. The dietary amount of CP could be reduced to ≈14% in pig feeds without reducing growth performance as long as sufficient amounts of essential AA are provided (Gloaguen *et al.*, 2014; Nørgaard *et al.*, 2014). The precondition for the use of such low-CP diets in practice is the knowledge about the exact requirement of each essential AA. In addition, protein properties and therefore AA absorption have to be taken into account because this is the first step of AA utilization. In pig feeds, the amounts of dietary AA are often expressed as standardized ileal digestible (SID) values in order to consider the availability of dietary AA for the metabolism. The SID value considers AA losses due to digestion, transport and bonding.

The AA form proteins and must be separated from the parent protein unit, before they can be absorbed from the lumen of the gut through the intestinal wall into the blood. This degradation occurs with the help of proteolytic digestive enzymes – proteases. The activity of proteases is supported by the secretion of dilute hydrochloric acid in the stomach, which results in denaturation of the protein. The process starts with denaturation of the protein and continues with the cleavage into oligopeptides, tripeptides, dipeptides or individual AA. The breakdown of the peptide chains are carried out by endopeptidases as pepsin, trypsin or chymotrypsin. The AA or peptides are absorbed by mucosal cells and finally enter the blood stream as free AA. The intestinal mucosal cells absorb AA via active transport, simple diffusion and facilitated diffusion. Absorption mainly occurs in the proximal region of the small intestine of pigs (Buraczewska, 1981) – approximately 50-75% (Leibholz, 1985). Dipeptide transport in the pig jejunum has been documented as well as the presence of a di- and tripeptide transporter 1-like transporter, which is likely to contribute to the overall AA absorption from the small intes-

tine. This transporter is able to carry a variety of di- and tripeptides but not tetrapeptides (Klang *et al.*, 2005). Moreover there are at least four Na^+-dependent AA transporters in the luminal plasma membrane of the intestinal mucosal cells responsible for transporting AA in the cytoplasm (Yao *et al.*, 2013). By measuring AA influx across the brush border membrane of the pig ileal epithelium, it has been demonstrated that bipolar AA have similar affinities for transport by system B and ASC (Munck *et al.*, 2000). A detailed overview of AA transport across the intestinal epithelium is given by Bröer (2008). However, not only the transporters determine the AA absorption. It has been determined that several free AA are more rapidly absorbed than their protein bound forms (Yen *et al.*, 2004). Once absorbed, the AA could be processed by intestinal cells as substrates for their metabolism or the AA are further transported. Enterocytes isolated from post weaning pigs also actively degrade branched-chain amino acids (BCAA). Other essential AA such as Thr and Trp are apparently less catabolized (Chen *et al.*, 2010). Since the intestinal mucins are glycoproteins, which are very rich in Thr, the Thr catabolism is marginal in the enterocytes. Even Lys is less catabolized in the enterocytes. Only 18% of what is used in the first-pass metabolism is recovered in intestinal mucosa protein (Blachier *et al.*, 2013). Non-metabolized AA are released to the portal vein and transported to the liver. There, up to 75% of the dietary AA are metabolized within the first-pass effect. The remaining 25% enter the systemic circulation and reach other peripheral tissues. Homeostasis of AA is performed by two major reactions in mammalian cells: transamination and desamination. Transamination is the centre of the entire AA metabolism. The amino group of currently not required AA is transferred on an α-keto acid, which can turn back to the corresponding AA. The α-keto acids have several metabolic functions. Desamination describes the separation of the amino group without transferring it on another molecule. Instead, cytotoxic ammonia is liberated and has to be removed by the urea cycle to become excreted.

Each AA has several functions in the metabolism and physiology of body tissues and cells. In general, all proteinogenic AA are substrates for protein synthesis and therefore essential for growth, maintenance and regulation of the body physiology. Some AA like aspartate, glutamine (Gln) and Lys are N and carbon precursors for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis. Furthermore, some AA like arginine and Gln are precursors for non-proteinogenic AA like ornithine and citrulline and therefore play important roles in detoxification pathways (*e.g.* ammonia metabolism in the urea cycle) and in intermediary metabolism. In addition, some AA like Gln, Glutamate

(Glu) and aspartate are precursors of the tricarboxylic acid cycle intermediates and represent oxidative substrates in numerous cell types, which utilize them as major energy substrates. AA can also be used indirectly as an energy source, notably in the postprandial phase, as precursors for gluconeogenesis or ketogenesis. Gluconeogenesis is a metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates such as glucogenic AA. Ketogenesis is the process by which ketone bodies (*e.g.* 3-hydroxybutyrate) are produced. In general, the production of ketone bodies serves the purpose to make available energy e.g. in case of starvation when blood glucose is unavailable.

1.3 Metabolic functions of branched-chain amino acids, threonine and tryptophan and their interactions

The BCAA Leu is the focus of the present work, since it has a central position in AA metabolism. Generally, Leu is abundant in common feed ingredients used in practical pig diets. Likely this is one reason why Leu provides hormone-like signals to tissues such as skeletal muscle, indicating overall nutrient sufficiency. In brain tissue, Leu is capable to stimulate the sensation of satiety. Due to the important roles of Leu in regulating protein synthesis and in the catabolism of all three BCAA, determination of the Leu requirement and elucidating of Leu interactions to other AA was of interest. The balance of Leu to other AA is crucial to maintain protein synthesis. Leu is known to increase protein synthesis by activation of the mechanistic target of rapamycin (mTOR) signaling pathway followed by upregulation of messenger ribonucleic acid (mRNA) translation (Escobar *et al.*, 2005; Yin *et al.*, 2010). Increased protein synthesis is dependent on the sufficient supply of substrates needed. Excess Leu could affect the requirement of the two other BCAA, Val and Ile, since a metabolite of Leu stimulates the branched-chain keto acid dehydrogenase complex (BCKDH), which catalyzes an irreversible step of BCAA catabolism (Hutson, 2006). This effect originates from α-keto isocaproate (KIC), the product of Leu transamination. The BCAA metabolism is tightly regulated to maintain levels high enough to support these important functions, but at the same time excesses are prevented via stimulation of irreversible disposal pathways. The large neutral amino acids (LNAA), including BCAA, share the same transport carriers within the body. In consequence, Leu competes with other AA for transport. There is an affinity of several transporters for Leu (Maenz and Patience, 1992; Munck *et al.*, 2000;

Smith, 2000), which causes an impaired transport of other AA and facilitates AA imbalances (synonymous with deficiencies of single AA) within the tissues. Imbalances in dietary AA supply could impair the voluntary feed intake (Ettle and Roth, 2005; Hao *et al.*, 2010). Moreover the described stimulation of mTOR by Leu causes an onset of saturation signals within the hypothalamus (Cota *et al.*, 2006). Considering these important roles of Leu and the interest to optimize low-CP diets with improved AA profiles, as well as consequences of Leu excess, it is necessary to improve the knowledge about this AA. Only a few studies report the Leu requirements for piglets after weaning (Gatnau *et al.*, 1995; Augspurger and Baker, 2004; Gloaguen *et al.*, 2013a; Gloaguen *et al.*, 2013b). Currently recommendations of the Leu requirement for growth of weaned piglets are given by the National Research Council (NRC, 2012) with SID Leu:Lys 100% or 101% recommended by Gloaguen *et al.* (2013b). The present work deals with the requirement of piglets for Leu and the mechanisms beyond, because the specific requirement is derived from the metabolic functions.

Next to Leu, the present work considers the essential AA Thr. This LNAA is usually second-limiting in European low-CP diets for pigs (Boisen, 2003). The recommendations for the Thr requirement of young pigs differs widely in current literature: the NRC (2012) recommends 59% in ratio to SID Lys, whereas the Institut national de la recherche agronomique (INRA) in France recommends 65% SID Thr:Lys according to Gloaguen *et al*. (2013b). It has been shown that increasing dietary Thr improves the N retention of piglets fed very low-CP diets (12-14%) significantly (Kerr and Easter, 1995). Thr is the most abundant AA in mucin protein produced by the intestinal mucosa (van Klinken *et al.*, 1995) and thus contributes to physical protection from microbial attachment to the mucosal surface. Therefore, Thr plays a crucial role in the maintenance of intestinal mucosal integrity and barrier function. Also, Thr is a major component of plasma gamma globulins in several animal species (Tenenhouse and Deutsch, 1966). As LNAA Thr competes with other AA for transport carriers, the availability of Thr for body tissue protein synthesis depends on the ratio to other LNAA. The relation of Thr metabolism to the metabolism of the BCAA makes Thr an interesting co-factor in the present studies: Thr has three alternative catabolic pathways (House *et al.*, 2001). Paxton *et al.* (1986) showed that the Thr metabolite 2-keto-butyrate is decarboxylated by BCKDH to propionate and carbon dioxide. However, BCKDH is the central enzyme in the catabolism of the BCAA. The common transport and the overlapping metabolisms of the BCAA and Thr encourage the assumption that availability of Thr could be

influenced by dietary Leu. This assumption was considered in several experiments, which are objects of this study.

The aromatic LNAA Trp is the third-limiting AA in European low-CP diets for pigs. This is particularly relevant with corn, which is often used as feedstuff raw material and is very poor in Trp and rich in Leu. The recommendations for the Trp requirement of young pigs differ widely according to the current literature: the NRC (2012) recommends SID Trp:Lys 16% whereas INRA in France suggests SID Trp:Lys 22% according to Gloaguen *et al.* (2013b). Trp is an indispensible AA and a dietary deficiency in Trp results in reduced feed intake (Eder *et al.*, 2001). Consequently, poor utilization of the dietary CP can cause an increased N excretion and therefore emission (Nørgaard *et al.*, 2014). Otherwise, supplementation of Trp to very low-CP diets improves N retention of growing pigs (Kerr and Easter, 1995). Apart from its role as substrate for protein synthesis, Trp has several important physiological functions. It has been reported that supplying pigs with high Trp amounts reduces aggression (Liu *et al.*, 2013). Tryptophan can also act as a modulator for controlling immune responses (Le Floc'h *et al.*, 2011). The neurotransmitter melatonin, which is produced from Trp in the gastrointestinal tract, serves as a signal for synchronizing the ingestion and digestion processes (Bubenik *et al.*, 1996). Zhang *et al.* (2007) showed the effect of Trp on feed intake by regulating ghrelin expression and secretion. Important for the present work is the role of Trp as precursor for the neurotransmitter serotonin in the brain which is thought to influence feed intake and nutrient selection behavior (Le Floc'h *et al.*, 2011). Trp is transported to the brain by a transporter located in capillaries of the blood–brain barrier. This transporter is shared with the other LNAA. Consequently, Trp entry into the brain is influenced by the ratio between Trp and LNAA, particularly, BCAA which are present in higher concentration than Trp in plasma (Hargreaves and Pardridge, 1988). Even if no common steps in the metabolisms of Trp and Leu are known, there is a presumption that there is an interaction between both LNAA. Especially the competition for transport may impair hypothalamic synthesis of serotonin and therefore the beneficial effects of the Trp metabolite within the brain. Consequently, Trp is another focus of the current work due to its interaction with LNAA and the wide range of recommendations for piglets requirement of Trp.

2 Aim of the present studies

The demand for pork meat is high in the European countries. However, at the same time the concern for environmental responsibility and animal welfare is increasing. Responsible farming and sustainable livestock feeding requires exact knowledge about the animals' requirement for essential AA and consequences of excessive intakes in order to avoid abnormal metabolism in the pig and environmental pollution. The present work focuses on the piglets' requirement for the essential AA Leu and the interactions of Leu to other AA in low protein diets. To this end, several parameters of performance and physiological response were recorded. The focus was set on:

- Growth performance (weight gain, feed intake and feed efficiency)
- N retention
- Concentrations of free AA and their metabolites in several tissues and blood plasma
- Activity of metabolic enzymes as BCKDH and mTOR

In order to estimate piglets' requirement for Leu and elucidate the consequences of excessive intakes five feeding experiments with piglets in the weight range of 8 to 28 kg were performed:

Study I	Estimation of a sub-limiting level of Lys as reference AA (to have a basis for the design of Study II to Study V)
Study II	Estimation of the Leu requirement by use of three statistical models
Study III	Characterization of tissue-specific basal activities of BCKDH in presence of adequate or excessive Leu consumption
Study IV	Considerations of interaction effects among Leu and Thr on feed efficiency, plasma AA profile and hepatic branched-chain AA catabolism
Study V	Considerations of interaction effects among Leu and Trp on appetite and appetite-regulating molecules

Following hypotheses could be derived based on the findings of the recent experiments

combined with literature review:

(I) ”*A nutritional Leu excess shifts physiological AA ratios in blood plasma and several body tissues to imbalanced levels and therefore changes metabolic processes in a negative way. This is particular the case in brain tissue where physiological AA imbalances are intensified by transport competition of the LNAA across the blood-brain barrier*.”

Recent literature showed that high dietary Leu levels increase the BCAA catabolism in line with a reduction of plasma concentrations of Val and Ile (Langer *et al*., 2000; Wiltafsky *et al*., 2010). Tissue concentrations of AA have not been analyzed in this context so far. The present work will show that a nutritional Leu excess reaches the brain, which has mutual impact on feed intake.

(II) *”A nutritional Leu excess reduces synthesis of the neurotransmitter serotonin in brain tissue due to a limitation of Trp uptake across the blood-brain barrier.”*

High levels of Leu in the brain tissue are accompanied with low concentrations of the serotonin precursor Trp since both LNAA share the same transporters at the blood-brain barrier. Consequently, serotonin synthesis is reduced by lack of its precursor AA Trp in case of excessive Leu intakes. Henry *et al*. (1992), who measured hypothalamic concentrations of 5-hydroxytryptophan subjected to dietary protein levels, made a similar assumption. However, data on brain concentrations of AA affected by dietary protein or single AA are missing in literature to date and will be completed by this point with the present work.

(III) *”Oral administration of Leu in doses up to 0.9 g/kg BW/d (SID Leu:Lys 400) is not sufficient to increase the metabolism of Thr via its’ alternative BCKDH-linked degradation pathway”*

House *et al.* (2001) could show with isolated rat hepatocytes that Thr could be alternatively degraded by the intramitochondrial oxidation of 2-oxobutyrate through either the pyruvate dehydrogenase complex or BCKDH. Since high intakes of dietary Leu could alter plasma and tissue concentrations of Thr in pigs, it was examined if the Leu-induced stimulation of

BCKDH could impair the availability of Thr for protein synthesis. The present work will show that possible changes in concentrations of plasma and tissue Thr are not induced by an increased activity of BCKDH.

The aim of the present work is to give a recommendation for the Leu requirement of weaned piglets and to give a clear overview in which ways a dietary Leu excess could modulate the voluntary feed intake. Dietary Leu excess has to be taken in into account in practical feed formulation since usual raw feedstuff materials, as corn, soy products and barley, could contain disproportional higher amounts of Leu than of other essential AA. It should be demonstrated that Leu influences not only the balance of the three BCAA by regulating their catabolism. Administered in high amounts, Leu displaces other essential AA from body tissues and fluids. As a result, other large neutral AA get lost as substrates for protein synthesis and beneficial effects of their metabolites may be interfered (herewith the dietary protein is not used efficiently). This will be shown in detail with the Trp metabolite serotonin in brain tissue, which promotes voluntary feed intake in pigs. However, the synthesis of serotonin in brain tissue is affected by the competition of Leu and Trp at the blood-brain barrier, since the brain concentration of Trp specifies the amount of possibly synthesized serotonin. So far, several studies are published considering the different topics as BCAA interactions, Trp-serotonin axis and AA requirements. To combine considerations of interaction effects among Leu and AA other than Lys or the BCAA is a new approach in estimating AA requirements and investigating the “Ideal Protein” for piglets.

3 Material and methods

3.1 General

Within the scope of the doctoral studies program, one preliminary study and four main studies were performed. The preliminary study was conducted to estimate the sub-limiting level of SID Lys for all subsequent prepared experimental diets. This approach was made to elucidate the Lys requirement of piglets under the experimental conditions, which were applied in the ongoing studies. Thereafter, the intervention studies were performed with a sub-limiting dietary Lys level. In focus of the doctoral studies program was the essential AA Leu and interactions of Leu to Thr and Trp.

Generally, crossbred piglets [Pietrain x (Large White x Landrace)] were used. The piglets were obtained from the experimental herd of the Agricultural and Nutritional Research Center (AEVZ, Merbitz, Germany). Male piglets were castrated within three days of birth. In all experiments the weaned piglets were kept in an environmentally controlled facility at a temperature of 28 °C which was reduced weekly by 1°C to a minimum of 22°C, a relative humidity between 55-60% and light from 06:00 to 18:00. The body weight (BW) and feed intake were recorded weekly. Average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) were calculated on basis of this data.

All experimental diets were based on wheat, barley, corn and soybean meal. The chemical composition of the main ingredients was determined before the experiments were conducted to formulate diets based on the actual content of nutrients. The energy content of all experimental diets was calculated to be 13.7-13.8 MJ ME/kg. With the exception of the examined AA, dietary supplies with essential AA were adequate in accordance with recommendations of NRC, 1998. In some experiments, it was necessary to add glutamic acid for isonitrogenous formulation among all diets. Vitamins and minerals were added in amounts that meet the requirements for growing pigs (GfE, 2006; NRC, 1998) by use of a premix (Mineral Premix 2588, BASU Mineralfutter GmbH, Bad Sulza, Germany). The composition of the premix is demonstrated in Table 2.

Table 2: Ingredients of 1 kg premix 2588

Ingredient	Amount	
Calcium	265	g
Sodium	60	g
Phosphorus	40	g
Magnesium	5.5	g
Iron	2.9	g
Zinc	2.2	g
Copper	750	mg
Manganese	730	mg
Cobalt	15	mg
Iodine	10	mg
Selenium	10	mg
Vitamin A	1,000,000	IU
Vitamin D	100,000	IU
Nicotinic acid	2.6	mg
Vitamin E	2	g
Pantothenic acid	1,250	g
Riboflavin	500	mg
Pyridoxine	300	mg
Thiamine	200	mg
Vitamin K	150	mg
Folic acid	30	mg
Vitamin B12	2	mg
Biotin	1	mg
Choline chloride	20	g
Aromatic substance	1.5	g

3.1.1 Study I – Estimation of a sub-limiting level of lysine as reference amino acid

This experiment was conducted to estimate the amount of sub-limiting Lys in low-CP diets in order to design further studies.

The study was performed in a one-factorial design. Three different dietary concentrations of SID Lys, which were added to a low-CP diet, were tested for their effects on growth performance of weaned piglets and compared to the performance of piglets from a control group (Control) which received higher amounts of CP with their diets (Figure 3). A total number of 56 piglets of both sexes were weaned at the age of 24 days. They were randomly subdivided into four treatment groups (n=14) and individually housed in flat deck pens. The average initial body weight (IBW) at the beginning of the experiment was 8.2±0.7 kg. The three low-CP diets contained 16.0% CP. The dietary Lys supply in these experimental diets (L1, L2, L3) was increased by supplementation of crystalline L-Lys. In the control group a high CP diet (19%) was used, which contained

1.16% SID Lys (Table 3). The experimental diets were fed for 35 days. For the preliminary study, the growth performance was recorded.

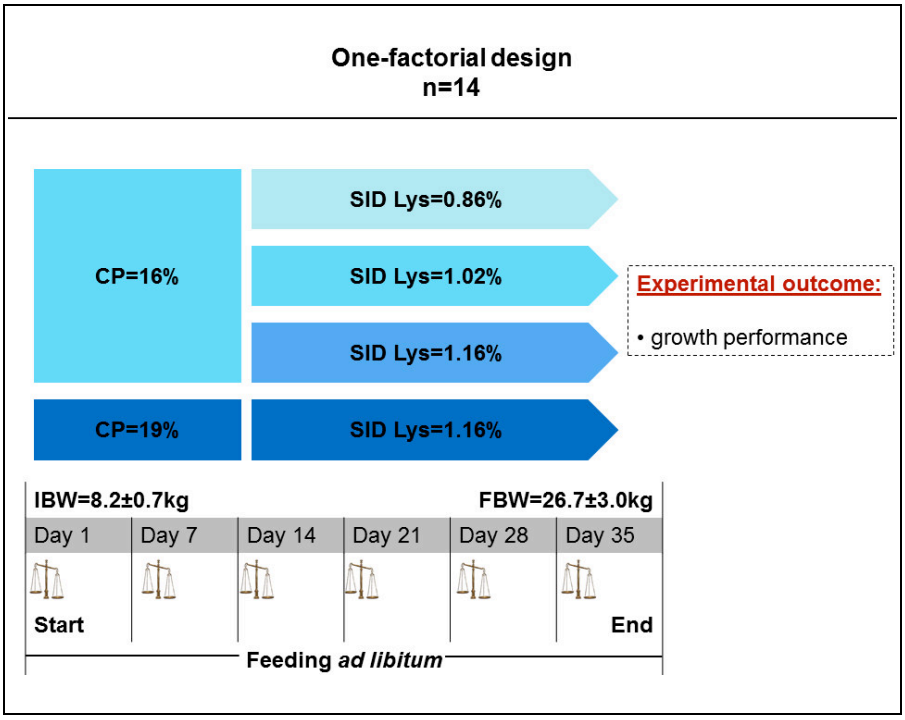

Figure 3: Study design of the preliminary study – Estimation of the limiting Lysine (Lys) amount in low crude protein (CP) diets for weaned piglets

SID: standardized ileal digestible; IBW: initial body weight; FBW: final body weight; documentation of body weight and feed intake

Table 3: Composition of the experimental diets (Study I)

Diet	**L1**	**L2**	**L3**	**Control**
Ingredients (%)				
Wheat	26.5	26.5	26.5	26.5
Barley	20	20	20	20
Corn	27.25	28.01	28.05	16.31
Soybean meal	9	9	9	22
Whey powder	6	6	6	6
Wheat bran	3	3	3	3
Soybean oil	2.4	2.0	2.0	2.5
MCP	0.5	0.5	0.5	0.5
Premix 2588	2	2	2	2
L-Lysine HCL	0.5	0.7	0.9	0.5
L-Threonine	0.19	0.29	0.39	0.23
DL-Methionine	0.15	0.24	0.34	0.23
L-Isoleucine	0.04	0.12	0.2	0.00
L-Leucine	0.00	0.13	0.28	0.00
L-Valine	0.11	0.22	0.33	0.15
L-Histidine	0.00	0.04	0.09	0.00
L-Tryptophan	0.06	0.10	0.13	0.07
L-Phenylalanine	0.00	0.15	0.15	0.00
L-Tyrosine	0.00	0.00	0.15	0.00
L-Glutamic acid	2.3	1.0	0.0	0.0
Calculated values				
ME (MJ/kg)	13.74	13.73	13.78	13.75
CP (%)	15.3	15.3	15.3	19.2
SID[1] Lys (%)	**0.86**	**1.02**	**1.16**	**1.16**
SID Thr:Lys	65	65	65	65
SID Met+Cys:Lys	60	60	60	60
SID Trp:Lys	22	22	22	22
SID Val:Lys	70	70	70	70
SID Ile:Lys	54	54	54	54
SID Leu:Lys	103	100	100	98
SID Phe+Tyr:Lys	103	102	101	110
SID His:Lys	32	32	32	32

MCP: monocalcium phosphate; CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; Thr: Threonine; Met: Methionine, Cys: Cysteine; Trp: Tryptophan; Val: Valine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine; Tyr: Tyrosine; His: Histidine

[1] Standardized ileal digestible amino acids, calculated by brutto amino acids and associated values of standardized ileal digestibility (GfE 2006)

3.1.2 Study II – Estimation of the leucine requirement by use of three statistical models

To estimate the requirement of piglets for Leu in a low-CP diet, a dose-response study was performed. The council of Saxony-Anhalt approved the experiment (Landesverwaltungsamt Sachsen-Anhalt, Germany; approval number: 42502-3-698 MLU).

The one-factorial study design is illustrated in Figure 4. The study was performed with 60 piglets of both sexes, which were weaned at the age of 28 days. During an adaptation period of one week, they were fed a commercial pre-starter diet.

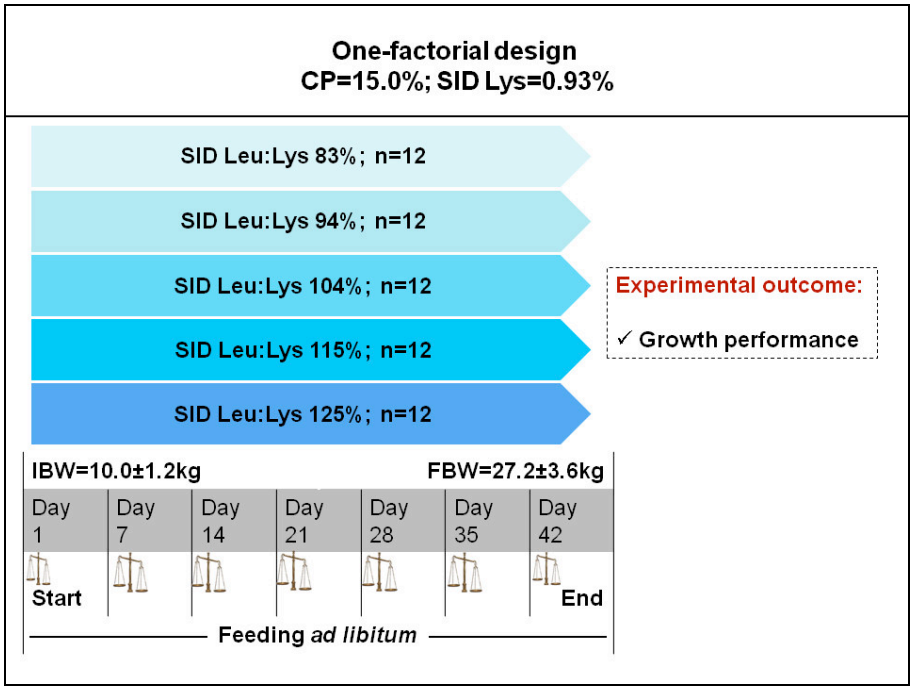

Figure 4: Experimental design of Study II – Estimation of the Leucine (Leu) requirement of weaned piglets in a weight range of 10 to 28 kg
CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; IBW: initial body weight; FBW: final body weight; documentations of body weight and feed intake

Piglets were blocked by sex, body weight and genetic (boar). Within the blocks, they were randomly assigned to five groups (n=12) and individually housed in flat deck pens. The average IBW of the piglets at the beginning of the experiment was 10.0±1.2 kg.

The analyzed CP content of the basal diet was 15.0% and the content of SID Lys at 0.93%. By supplementation of crystalline L-Leu SID Leu:Lys ratios of 83%, 94%, 104%, 115% and 125% (Table 4) were realized in the experimental diets. The diets were fed for 42 days. Growth performance was used to assess the Leu requirement.

Table 4: Composition of experimental diets (Study II)

	SID[1] Leu:Lys[2]				
Diet	83%	94%	104%	115%	125%
Ingredients (%)					
Wheat	27.3	27.3	27.3	27.3	27.3
Barley	24	24	24	24	24
Corn	26.5	26.5	26.5	26.5	26.5
Soybean meal	5	5	5	5	5
Whey powder	6	6	6	6	6
Wheat bran	3	3	3	3	3
Soybean oil	2	2	2	2	2
MCP	0.5	0.5	0.5	0.5	0.5
Premix 2588	2	2	2	2	2
L-Lysine HCL	0.67	0.67	0.67	0.67	0.67
L-Threonine	0.29	0.29	0.29	0.29	0.29
DL-Methionine	0.22	0.22	0.22	0.22	0.22
L-Isoleucine	0.18	0.18	0.18	0.18	0.18
L-Leucine	0.0	0.1	0.2	0.3	0.4
L-Valine	0.25	0.25	0.25	0.25	0.25
L-Histidine	0.05	0.05	0.05	0.05	0.05
L-Tryptophan	0.12	0.12	0.12	0.12	0.12
L-Phenylalanine	0.08	0.08	0.08	0.08	0.08
L-Tyrosine	0.15	0.15	0.15	0.15	0.15
L-Glutamic acid	1.5	1.5	1.5	1.5	1.5
Calculated values					
ME (MJ/kg)	13.66	13.65	13.63	13.62	13.61
CP (%)	14.0	14.0	13.9	13.9	13.9
SID[1] Lys (%)	0.94	0.94	0.94	0.93	0.93
SID Thr:Lys	65	65	65	65	65
SID Met+Cys:Lys	61	61	61	61	61
SID Trp:Lys	24	24	24	24	24
SID Val:Lys	72	72	72	72	72
SID Ile:Lys	59	59	59	59	59
SID Leu:Lys	**83**	**94**	**104**	**115**	**126**
SID Phe+Tyr:Lys	104	104	104	104	104
SID His:Lys	33	33	33	33	33

MCP: monocalcium phosphate; CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; Thr: Threonine; Met: Methionine, Cys: Cysteine; Trp: Tryptophan; Val: Valine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine; Tyr: Tyrosine; His: Histidine

[1] Standardized ileal digestible amino acids, calculated by brutto amino acids and associated values of standardized ileal digestibility (GfE 2006)

[2] Calculated with a mean SID Lys content of 0.93%

3.1.3 Study III – Characterization of tissue-specific basal activities of the branched-chain α-keto acid dehydrogenase (BCKDH) in presence of adequate or excessive leucine consumption

In order to elucidate the tolerance of weaned piglets to excessive amounts of dietary Leu, an excess study was performed with SID Leu levels fourfold higher than current recommendations. The council of Saxony-Anhalt approved the experiment (Landesverwaltungsamt Sachsen-Anhalt, Germany; approval number: H1-4/33A MLU).

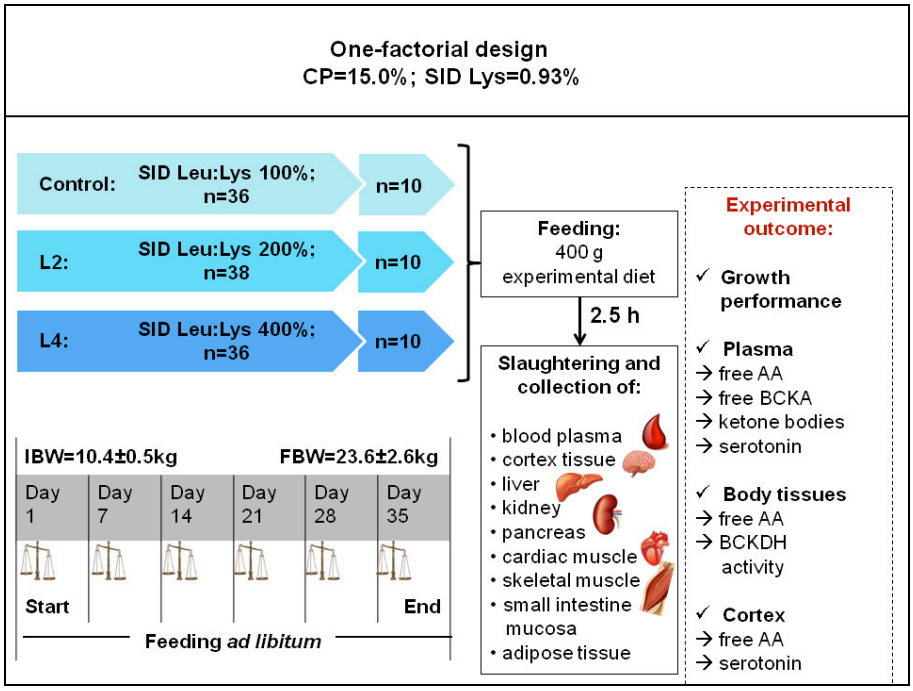

Figure 5: Experimental design of Study III – Effects of dietary Leucine (Leu) excess on physiological parameters of weaned piglets
CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; IBW: initial body weight; FBW: final body weight; AA: amino acids; BCKA: branched-chain keto acids; BCKDH: Branched-chain α-keto acid dehydrogenase; ⚖ documentation of body weight and feed intake

For the one-factorial study, 120 piglets of both sexes were weaned at the age of 28 days. During an adaption period of one week, they were fed a commercial pre-starter diet. The piglets were blocked by sex, body weight and ancestry and housed in pairs in flat deck pens. The average IBW of the piglets at the beginning of the experiment was 10.4±0.5 kg (Figure 5). Within the blocks, the piglets were randomly assigned to three treatment groups (n=36-38). Dietary Leu supply was increased by L-Leu supplementation to estimate SID Leu:Lys ratios of 100% (Control), 200% (L2) and 400% (L4; Table 5). The CP content of the basal diet was approximately 15.0% and 0.93% SID Lys. Crystalline

Leu was added to the diets at the expense of glutamicacid. The experimental diets were fed for 42 days.

At the end of the experiment, ten piglets per group were fasted overnight and received 400 g of their experimental diet 2.5 h prior to experimental slaughtering. Sampling included blood plasma and several body tissues. The blood plasma was analyzed for free AA, free branched-chain α-keto acids (BCKA), 3-hydroxybutyrate and serotonin. In brain tissue (occipital lobe and both hippocampi), liver, kidney, pancreas, skeletal muscle (*longissimus dorsi*, 7th rib), cardiac muscle, small intestine mucosa and adipose tissue, the concentrations of free AA and the basal activity of BCKDH were determined. Furthermore, the serotonin concentration was measured in brain tissue.

Table 5: Composition of experimental diets (Study III)

	SID[1] Leu:Lys[2]		
	100%	200%	400%
Diet	Control	L2	L4
Ingredients (%)			
Wheat	25.3	25.2	25.9
Barley	24	25	25
Corn	26.5	26.5	26.5
Soybean meal	7	6	5
Whey powder	6	6	6
Wheat bran	3	3	3
Soybean oil	2.2	1.8	1.0
MCP	0.5	0.5	0.5
Premix 2588	2	2	2
L-Lysine HCL	0.67	0.70	0.73
L-Threonine	0.27	0.28	0.30
DL-Methionine	0.21	0.22	0.23
L-Isoleucine	0.14	0.15	0.17
L-Leucine	0.12	1.11	3.06
L-Valine	0.25	0.26	0.28
L-Histidine	0.05	0.06	0.07
L-Tryptophan	0.12	0.12	0.13
L-Phenylalanine	0.02	0.04	0.06
L-Tyrosine	0.05	0.06	0.07
L-Glutamic acid	1.6	1.0	0.0
Calculated values			
ME (MJ/kg)	13.7	13.7	13.7
CP (%)	14.5	14.6	15.1
SID[1] Lys (%)	0.94	0.94	0.94
SID Thr:Lys	65	65	65
SID Met+Cys:Lys	61	61	61
SID Trp:Lys	25	25	25
SID Val:Lys	74	74	74
SID Ile:Lys	56	56	56
SID Leu:Lys	**100**	**200**	**400**
SID Phe+Tyr:Lys	95	95	95
SID His:Lys	35	35	35

MCP: monocalcium phosphate; CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; Thr: Threonine; Met: Methionine, Cys: Cysteine; Trp: Tryptophan; Val: Valine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine; Tyr: Tyrosine; His: Histidine

[1] Standardized ileal digestible amino acids, calculated by brutto amino acids and associated values of standardized ileal digestibility (GfE 2006)

[2] Calculated with a mean SID Lys content of 0.93%

3.1.4 Study IV – Considerations of interaction effects among leucine and threonine on feed efficiency, plasma amino acid profile and hepatic branched-chain amino acid catabolism

In order to elucidate possible interactions between Leu and Thr on piglets' growth performance, a growth study was performed with low protein diets. The same diets were used in a parallel study to measure N retention specific parameters in a balance study. The council of Saxony-Anhalt approved both experiments (Landesverwaltungsamt Sachsen-Anhalt, Germany; approval number: 42502-3-679 MLU).

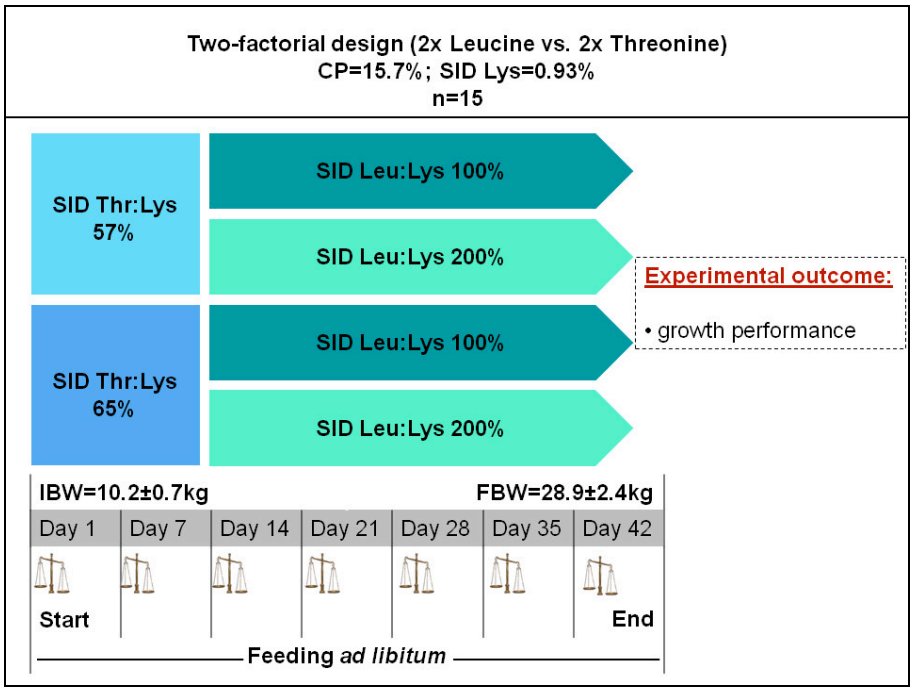

Figure 6: Experimental design of Study IV – Effects of the interactions among Leucine (Leu) and Threonine (Thr) on growth performance and physiological parameters of weaned piglets
CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; IBW: initial body weight; FBW: final body weight; documentation of body weight and feed intake

The two-factorial study design included two levels each for the two factors Leu (adequate *versus* high) and Thr (low *versus* adequate).

For the growth assay a total number of 60 female piglets were weaned at the age of 28 days. A commercial pig producer provided the piglets for this trial. During a week adaption period to the new surroundings, they were fed a commercial pre-starter diet. Thereafter, the piglets were randomly subdivided into four treatment groups (n=15) and individually housed in flat deck pens. The average IBW at the beginning of the experiment was 10.2±0.7 kg (Figure 6). The CP content of the basal diet was analyzed at 15.7% and

0.93% SID Lys in order to specify the Leu supply relative to Lys (Boisen, 2003; Barea *et al*., 2009). Dietary Leu and Thr supplies were increased by supplementation of crystalline L-Leu and L-Thr to estimate SID Thr:Leu:Lys ratios of 57:100%, 57:200%, 65:100% and 65:200%. The experimental diets were fed for 42 days.

Table 6: Composition of experimental diets (Study IV)

Diet	**57:100**	**57:200**	**65:100**	**65:200**
SID[1] Leu:Lys[2]	100%	200%	100%	200%
SID Thr:Lys	57%	57%	65%	65%
Ingredients (%)				
Wheat	26.5	26.5	26.5	26.5
Barley	20	20	20	20
Corn	26.94	27.64	26.95	27.7
Soybean meal	9	9	9	9
Whey powder	6	6	6	6
Wheat bran	3	3	3	3
Soybean oil	2	1.5	2	1.5
MCP	0.5	0.5	0.5	0.5
Premix 2588	2	2	2	2
L-Lysine HCL	0.81	0.81	0.81	0.81
L-Threonine	0.26	0.26	0.35	0.35
DL-Methionine	0.28	0.28	0.28	0.28
L-Isoleucine	0.18	0.18	0.18	0.18
L-Leucine	0.23	1.34	0.23	1.34
L-Valine	0.29	0.29	0.29	0.29
L-Histidine	0.09	0.09	0.09	0.09
L-Tryptophan	0.12	0.12	0.12	0.12
L-Phenylalanine	0.15	0.14	0.15	0.14
L-Tyrosine	0.15	0.15	0.15	0.15
L-Glutamic acid	1.50	0.20	1.40	0.05
Calculated values				
ME (MJ/kg)	13.7	13.7	13.7	13.7
CP (%)	15.0	15.0	15.0	15.0
SID[1] Lys (%)	0.93	0.93	0.93	0.93
SID Thr:Lys	**57**	**57**	**65**	**65**
SID Met+Cys:Lys	60	60	60	60
SID Trp:Lys	22	22	22	22
SID Val:Lys	74	74	74	74
SID Ile:Lys	58	58	58	58
SID Leu:Lys	**100**	**200**	**100**	**200**
SID Phe+Tyr:Lys	99	99	99	99
SID His:Lys	35	35	35	35

MCP: monocalcium phosphate; CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; Thr: Threonine; Met: Methionine, Cys: Cysteine; Trp: Tryptophan; Val: Valine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine; Tyr: Tyrosine; His: Histidine

[1] Standardized ileal digestible amino acids, calculated by brutto amino acids and associated values of standardized ileal digestibility (GfE 2006)

[2] Calculated with a mean SID Lys content of 0.93%

3.1.5 Parallel Study: Effects of the interactions among leucine and threonine on the nitrogen balance of piglets

The N-balance study was performed parallel to the growth study with the same diets. The Study aimed to find effects of the interactions among Leu and Thr on the N-utilization of piglets.

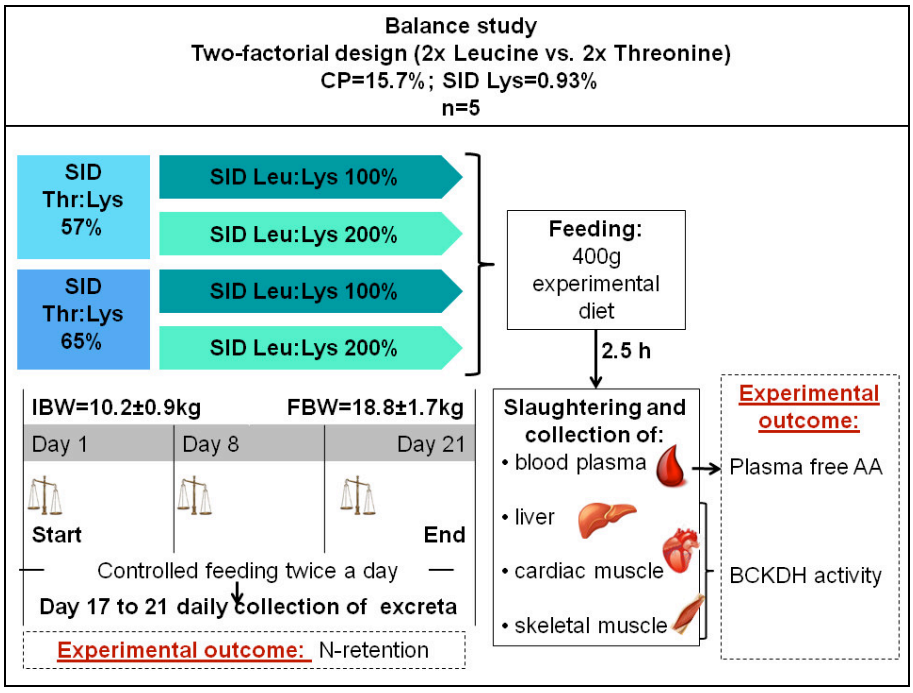

Figure 7: Experimental design - Balance study
CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; Thr: Threonine; IBW: initial body weight; FBW: final body weight; N: nitrogen; AA: amino acids; BCKDH: Branched-chain keto acid dehydrogenase; documentation of body weight and feed intake

A total number of 20 castrated male piglets at the age of 35 days were subdivided into the four treatment groups (n=5). The piglets were housed individually and were given one day of adaption to the metabolism cages. In the N-balance study, the diets described in chapter 3.2 were administered to the piglets (Table 6). Specified amounts of feed were provided twice daily and the intakes were recorded. Water was provided in the interim period. The experiment was conducted for 21 days. The piglets were weighed weekly. During a five-day collection period (day 17 to day 21), urine and feces of each piglet were quantitatively collected twice per day. The urine samples were acidified with 40% sulfuric acid to pH 3 inside the collection vessels to prevent ammonia losses. The excrements were stored at -20 °C until further analyses.

Subsequently, the piglets were slaughtered for collection of blood and tissue samples. At the end of the experiment, the piglets were fasted overnight and received 400 g of their experimental diet 2.5 h prior to experimental slaughtering. Blood plasma was collected to determine free AA concentrations. Liver and kidney tissue, cardiac and skeletal muscle (*longissimus dorsi*, 7th rib) were analyzed for the basal activity of the enzyme BCKDH.

3.1.6 Study V – Considerations of interaction effects among leucine and tryptophan on appetite and appetite-regulating molecules

To find interaction effects of Leu and Trp on the piglets' growth performance a growth assay was performed with low protein diets. The council of Saxony-Anhalt approved the experiment (Landesverwaltungsamt Sachsen-Anhalt, Germany; approval number: 42502-3-720 MLU).

The two-factorial study design included two levels for Leu (adequate *versus* excessive) and Trp (low recommendation level *versus* high recommendation level; Figure 8). 120 piglets of both sexes were weaned at the age of 21 days. During a week adaption period they were fed a commercial pre-starter diet. Piglets were blocked by sex, body weight and ancestry. Within the blocks, they were randomly assigned to four groups (n=30), that received either adequate Leu (SID Leu:Lys 100%) *versus* high Leu (SID Leu:Lys 300%) and two dietary concentrations Trp that are assumed to meet the requirement (SID Trp:Lys 18% *versus* 23%). The piglets were housed in pairs in flat deck pens. The average IBW of the piglets on day one of the experiment was 9.3±1.1 kg.

The CP content of the basal diet was analyzed at 15.7% and the content of SID Lys at 0.94%. Dietary Leu and Trp supplies were increased by supplementation of crystalline L-Leu and L-Trp to obtain SID Trp:Leu:Lys ratios of 18:100%, 18:300%, 23:100% and 23:300%. The experimental diets were fed over 42 days.

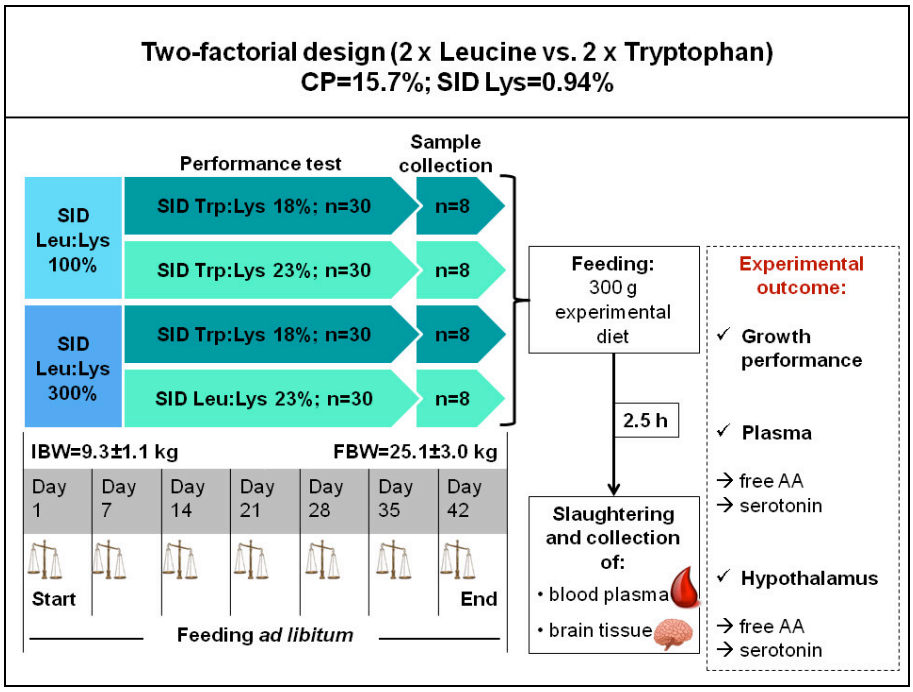

Figure 8: Experimental design of Study V - Effects of the interactions among Leucine (Leu) and Tryptophan (Trp) on growth performance and physiological parameters of weaned piglets
CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; IBW: initial body weight; FBW: final body weight; AA: amino acids; BCKDH: Branched-chain keto acid dehydrogenase; mTOR: mechanistic target of rapamycin; documentation of body weight and feed intake

At the end of the experiment, ten piglets per group were fasted overnight and received 300 g of their experimental diet 2.5 h prior to slaughtering. Sampling included blood plasma and several body tissues. In blood plasma, concentrations of free AA and concentrations of serotonin were determined. Hypothalamic tissue (tuberal area around ventromedial nucleus and arcuate nucleus) was collected to analyze mTOR protein expression and concentrations of free AA and serotonin. In the liver and pancreas, the basal activity of BCKDH was assayed spectrophotometrically.

Table 7: Composition of experimental diets (Study V)

Diet	**18:100**	**18:300**	**23:100**	**23:300**
SID[1] Leu:Lys[2]	100%	300%	100%	300%
SID Trp:Lys	18%	18%	23%	23%
Ingredients (%)				
Wheat	25.73	25.73	25.73	25.73
Barley	20	20	20	20
Corn	30	30	30	30
Soybean meal	10	10	10	10
Whey powder	6	6	6	6
Wheat bran	3	3	3	3
Soybean oil	2.2	1.5	2.2	1.5
MCP	0.5	0.5	0.5	0.5
Premix 2588	2	2	2	2
L-Lysine HCL	0.59	0.59	0.59	0.59
L-Threonine	0.24	0.24	0.24	0.24
DL-Methionine	0.2	0.2	0.2	0.2
L-Isoleucine	0.08	0.08	0.08	0.08
L-Leucine	0.02	1.90	0.02	1.90
L-Valine	0.18	0.18	0.18	0.18
L-Histidine	0.04	0.04	0.04	0.04
L-Tryptophan	0.04	0.04	0.09	0.09
L-Glutamic acid	2.10	0.05	2.10	0.05
Calculated values				
ME (MJ/kg)	13.7	13.8	13.7	13.8
CP (%)	15.6	15.7	15.7	15.7
SID[1] Lys (%)	0.93	0.94	0.93	0.94
SID Thr:Lys	65	65	65	65
SID Met+Cys:Lys	61	61	61	61
SID Trp:Lys	**18**	**18**	**23**	**23**
SID Val:Lys	72	72	72	72
SID Ile:Lys	58	58	58	58
SID Leu:Lys	**100**	**296**	**100**	**296**
SID Phe+Tyr:Lys	97	97	97	97
SID His:Lys	35	35	35	35

MCP: monocalcium phosphate; CP: crude protein; SID: standardized ileal digestible; Lys: Lysine; Thr: Threonine; Met: Methionine, Cys: Cysteine; Trp: Tryptophan; Val: Valine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine; Tyr: Tyrosine; His: Histidine

[1] Standardized ileal digestible amino acids, calculated by brutto amino acids and associated values of standardized ileal digestibility (GfE 2006)

[2] Calculated with a mean SID Lys content of 0.93%

3.2 Sample Collection

For experiment II, IV and V, samples of blood plasma and several body tissues were taken. For blood sample collection, EDTA monovettes were used (extraction of plasma: 9 ml, S-Monovette, Sarstedt, Nümbrecht, Germany). For the extraction of plasma, blood samples were centrifuged at 3000 G for 10 min. Plasma was stored at -80 °C until further analyses. The tissue samples were removed rapidly and quick-frozen in liquid N for storage at -80 °C until further analyses.

3.3 Analyses

3.3.1 Analyses of crude protein and amino acid concentrations in experimental diets

The analyses of the feedstuffs were performed by the laboratories of Ajinomoto Eurolysine SAS, Paris, France. The amount of dietary CP (N x 6.25) was obtained after N analysis according to the Dumas procedure in a Rapid N cube (Elementar France, Villeurbanne, France) using the AFNOR method NF V18-120 (AFNOR, 1997). Dietary AA content was analyzed by a JLC-500/V AminoTac Amino Acid Analyzer (Jeol, Croissy-sur-Seine, France) using the AFNOR method (standard NF EN ISO 13903). To quantify methionine and cysteine, diet samples were oxidized with performic acid prior to hydrolysation. AA were separated by ion exchange chromatography and determined by photometric detection after derivatization with ninhydrin. Total Trp was analyzed by high performance liquid chromatography (HPLC) with a fluorescence detector (RF 10AXL, Shimadzu, Bonneuil sur Marne, France) after an alkaline hydrolysis with barium hydroxide according to the method MOD0094 version G. The aminograms are shown in Appendix tables A1-A5.

3.3.2 Nitrogen content of urine and feces

Nitrogen analyses were performed according to the Kjiehldahl procedure. The 2300 Kjeltec™ (Foss, Rellingen, Germany) was used according to manufacturer's instructions. For wet fusion, 500 mg of the feces samples or 1 ml urine were added with 15 ml sulphuric acid and reduced N was liberated as ammonium sulfate. The solution was dis-

tilled at 400 °C with sodium hydroxide, which converts the ammonium salt to ammonia. The amount of ammonia and thus the amount of N present in the sample, was determined by reverse titration.

3.3.3 Plasma and tissue free amino acids and plasma branched-chain α-keto acids

The free AA in plasma were determined as isoindole derivatives by reversed phase HPLC (Hypersil ODS, 250 mm x 4 mm, 5 µm, Agilent 1100, Agilent Technologies, Waldbronn, Germany) according to Schuster (1988) with fluorescence detection after pre-column derivatisation with o-phthaldialdehyde (OPA; Sigma Aldrich, Steinheim, Germany) and mercaptopropionic acid (Teerlink, 1994; Teerlink *et al.*, 1994).
For quantification of free tissue AA, the tissue samples were prepared and chemical deproteinized as described by Aristoy and Toldra (1991): 150 mg of tissue was diluted in 0.6 ml 0.1 N hydrochloric acid, which contained 50 µM norvaline as internal standard. After homogenisation by Mixer Mill (MM 400, Retsch, Haan, Germany) at 15 Hz for 3 min, samples were centrifuged at 10.000 G and at 4 °C for 10 min. The supernatant was diluted in acetonitrile 1:2 and incubated for 30 min at 4°C. The protein-free supernatant was derivatized with 20 µl methanol-water-triethylamine-phenyl isothiocyanate (7:l:l:l) and used for further HPLC.

Concentrations of the plasma BCKA were determined by a HPLC after derivatization with o-phenylendiamine (Sigma Aldrich, Steinheim, Germany) using α-ketocaproic acid sodium salt (Sigma Aldrich, Steinheim, Germany) as internal standard (Kand'ár *et al.*, 2009). 40 µl of plasma were mixed with 4 µl 500 µM internal standard and 80 µl 1 M $HClO_4$. After an incubation period of 10 min at 4 °C and centrifugation (22,000 G, 4°C, 10 min), 50 µl of the supernatant were mixed with 50 µl 25 mM o-phenylendiamine in 2 M HCl and incubated at 50 °C for 30 min. After cooling to room temperature the samples were centrifuged again (22,000 G, 4°C, 5 min) and the supernatants were analyzed by HPLC(Hypersil ODS, 250 mm x 4 mm, 5 µm, Agilent 1100, Agilent Technologies, Waldbronn, Germany) with a at 30 °C and fluorescence detection (350 nm/410 nm). The α-keto acid derivates were eluted by a gradient of methanol and water with a flow rate of 0.8 ml/min (0 min 32.5%, 5 min 32.5%, 10 min 41.5%, 12 min 55%, 20 min 88.5%, 32 min 100% methanol). The injection volume was 10 µl.

3.3.4 Plasma 3-hydroxybutyrate

The preparation of the blood plasma for analyses of plasma 3-hydroxybutyrate concentrations was performed by use of the Autokit 3-HB (Wako Chemicals GmbH, Neuss, Germany) according to manufacturer's instructions. The wavelength used for the spectrophotometrical measurement was 405 nm.

3.3.5 Plasma and tissue concentrations of serotonin

The concentration of serotonin in plasma was analyzed by using the serotonin reagent kit for HPLC analysis (3030), the corresponding column (3130), the mobile phase (3031) and the electrochemical detector CLC 100 from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). Sample analysis was performed according to the manufacturer's protocol and the use of an Agilent 1100 HPLC (Agilent Technologies). To this end, 100 µl of plasma were mixed with 100 µl of internal standard and 100 µl precipitation reagent for 30 sec. Thereafter, the mixture was incubated for 10 min at 8°C, followed by a centrifugation step at 17,900 G for 10 min. A volume of 20 µl of the supernatant was injected into the HPLC system.

Tissue concentrations of serotonin were determined according to Barf *et al*. (1996). 10 mg of tissue samples were homogenized in 120 µl ice cold extraction solution (5 µM clorgyline containing 5 µg/ml glutathione and 20 ng/ml N-ω-methylserotonin as internal standard) using a Mixer Mill (MM 400, Retsch, Haan, Germany) at 15 Hz for 1 min and an ultrasonic bath RK 501 H (Bandelin electronic, Berlin, Germany) at 0 °C for 5 min. For protein precipitation, 10 µl 2 M $HClO_4$ were added to the homogenates followed by addition of 8 µl 2.5 M potassium acetate. After mixing and an incubation period of 15 min on ice, the homogenate was centrifuged at 15,000 G for 10 min. Subsequently, 80 µl of the supernatant was diluted with 80 µl of the mobile phase. The HPLC measurement followed (Hardie and Hirsh, 2006) using an Agilent 1100 HPLC with a Hypersil ODS column (250 mm x 4 mm, 5 µm, Agilent) at 30 °C and the electrochemical detector CLC 100 of Chromsystems. The mobile phase contained 50 mM citric acid, 50 mM acetic acid, 11 mM decanesulfonic acid and 15% acetonitrile (v/v). The pH was adjusted to 4.5 by NaOH before the addition of acetonitrile. The flow was 1 ml/min and the injection volume was 50 µl.

3.3.6 mTOR protein expression

Relative protein expression of mTOR was determined by Western Blot analysis. The contents of the used buffers and reagents is shown in Table 8. 100 mg of a frozen brain tissue sample was homogenized in 500 µl ice-cold lysis buffer using a Mixer Mill (MM 400, Retsch, Haan, Germany) at 15 Hz for 1 min. Insoluble material was removed by centrifugation (13,000 G, 4 °C, 15 min). The protein concentration of the supernatant was determined in duplicate according to Bradford (1976). Therefore, 50 µl sample or standard bovine serum albumin (BSA) was diluted with 200 µl Bradford reagent (Brilliant Blue G in phosphoric acid and methanol; Sigma Aldrich, St. Louis, Missouri, USA) and incubated for 10 min. Subsequently, the extinction measurement was performed at a wavelength of 595 nm in a Microplate Reader (Spectraflour Plus, MTX Lab Systems, Inc., McLean, Virginia, USA). The protein concentration of the sample could be calculated in the following based on the standard calibration curve.

Samples were diluted with 2 x sodium dodecyl sulfate sample buffer to a protein concentration of 2 µg/µl and incubated at 95 °C of 5 min. 20 µl lysate of each sample was loaded on an 8% SDS gel. As a standard, 5 µl molecular weight standard (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, Thermo Fisher Scientific Inc., Waltham, MA 02454, USA) and a control (mix from eight samples) was loaded. Running the gel was for 1 h at 60 mA. The gels were blotted onto nitrocellulose membranes (Protran BA 85, GE Healthcare UK limited, Buckinghamshire; UK) in a blotting chamber at 350 mA for 1 h. Thereafter, the membranes were blocked with 3% BSA (Sigma Aldrich, St. Louis, Missouri, USA) at room temperature.

Table 8: Reagents used for Western Blot analyses

Reagent	Ingredient
Lysis buffer (pH 7.8) with Halt Protease Inhibitor Mix (Thermo Fisher Scientific S.L. Barcelona, Spain)	10 mM Hepes/ NaOH 10 mM KCL 0.1% NP-40 125 µM PMSF 1 mM EDTA
2 x Sample buffer	80 µl Glycerin 26 mg SDS 250µl Stacking gel buffer 10 µl Mercaptoethanol 100 µl Bromphenol blue
Resolving gel buffer (pH 8.8)	1.5 M Tris
Resolving gel (8%)	3 ml Acryl-/ Bisacrylamid (40%) 8.5 ml *Aqua bd* 3 ml resolving gel buffer 150 µl SDS (10%) 15 µl TEMED 100 µl APS (10%)
Stacking gel buffer (pH 6.8)	0.5 M Tris
Stacking gel (4.8%)	0.6 ml Acryl-/ Bisacrylamid (40%) 3.836 ml *Aqua bd* 0.5 ml stacking gel buffer 50µl SDS (10%) 10 µl TEMED 34 µl APS (10%)
Running buffer (pH 8.3)	25 mM Tris 200 mM Glycin 1% (w/v) SDS
Transfer buffer (pH 8.3)	25 mM Tris 150 mM Glycin 10% Methanol
TBST buffer (pH 7.5)	50 mM Tris 150 mM NaCl 0.2% Tween 20

Aqua bd=aqua bidestillata; SDS= sodium dodecyl sulfate

The primary antibody (Table 9) – diluted in 3% BSA – was applied over night at 4 °C on a seesaw. Blots were washed threefold for 10 min with tris buffered saline and tween (TBS-T) and afterwards incubated with the secondary antibody (Table 9) for 1h at room temperature seesawing. Blots were washed threefold for 10 min with TBS-T. Bands were detected with Amersham ECL Prime (GE Healthcare, Munich, Germany) in a G:BOX Chemi XT4 (Syngene, Cambridge, UK). Optic density was calculated by GeneSys.Ink 1.4.0.0. (Syngene, Cambridge, UK).

Table 9: Antibodies for Western Blot analyses

Protein	**Primary antibody**	Dilution
mTOR	Cell Signaling Technology® (Danvers, MA, USA) mTOR (#7C10), Rabbit mAb	1 : 1000
Phosphorylated mTOR	Cell Signaling Technology® (Danvers, MA, USA) Phospho-mTOR (Ser2448; #2971)	1 : 1000
ß-Actin	abcam® (Cambridge, UK) beta Actin antibody [AC-15] (#ab6276)	1 : 10,000
	Secondary antibody	
m-TOR/ phosphorylated mTOR	Promega (Madison, Wisconsin, USA) Anti-rabbit, HRP (#S373B)	1:5000
ß-Actin	Promega (Madison, Wisconsin, USA) Anti-mouse, HRP (#S372B)	1:5000

mTOR: mechanistic target of rapamycin

3.3.7 Basal activity of branched-chain α-keto acid dehydrogenase (BCKDH)

The BCKDH activity was assayed by a spectrophotometrical method based on Nakai *et al.*, 2000.

Due to supply constraints of several manufacturers one essential ingredient of Buffer 1 – α-chloro isocaproate (Buffer 1; Table 10) had to be produced self-made prior to the analysis of the actual BCKDH activity. According to Harris *et al.* (1982), α-chloro isocaproate was obtained from the diazotization of (*R,S*)-Leu. 30 ml of a 0.245 M solution of $NaNO_2$ in *aqua bd* was added dropwise to 300 ml of (*R*)-Leu (Sigma Aldrich, St. Louis, Missouri, USA) diluted in 6 M HCL at 0°C. After stirring at 0 °C for 4 h, extraction with *tert*-butyl methyl ether, washing with saturated NaCl solution and drying with $MgSO_4$, the extract was dehumidified to oil, which was purified by flash chromatography. The afforded (*R,S*)- α-chloro isocaproate was added dropwise to 2 M NaOH solution at 4 °C. The resulting product mixture was filtered and evaporated to a solid which was diluted in 50 ml of warm methanol and afterwards filtered into 100 ml of 2-propanol. This solution was concentrated almost to dryness and the semisolid residue was triturated with further 100 ml of 2-propanol. After filtration and washing the solids with 2-propanol and than diethylether, drying afforded the α-chloro isocaproate, which is essential in Buffer 1 to inhibit the BCKDH kinase (an enzyme which inactivates BCKDH).

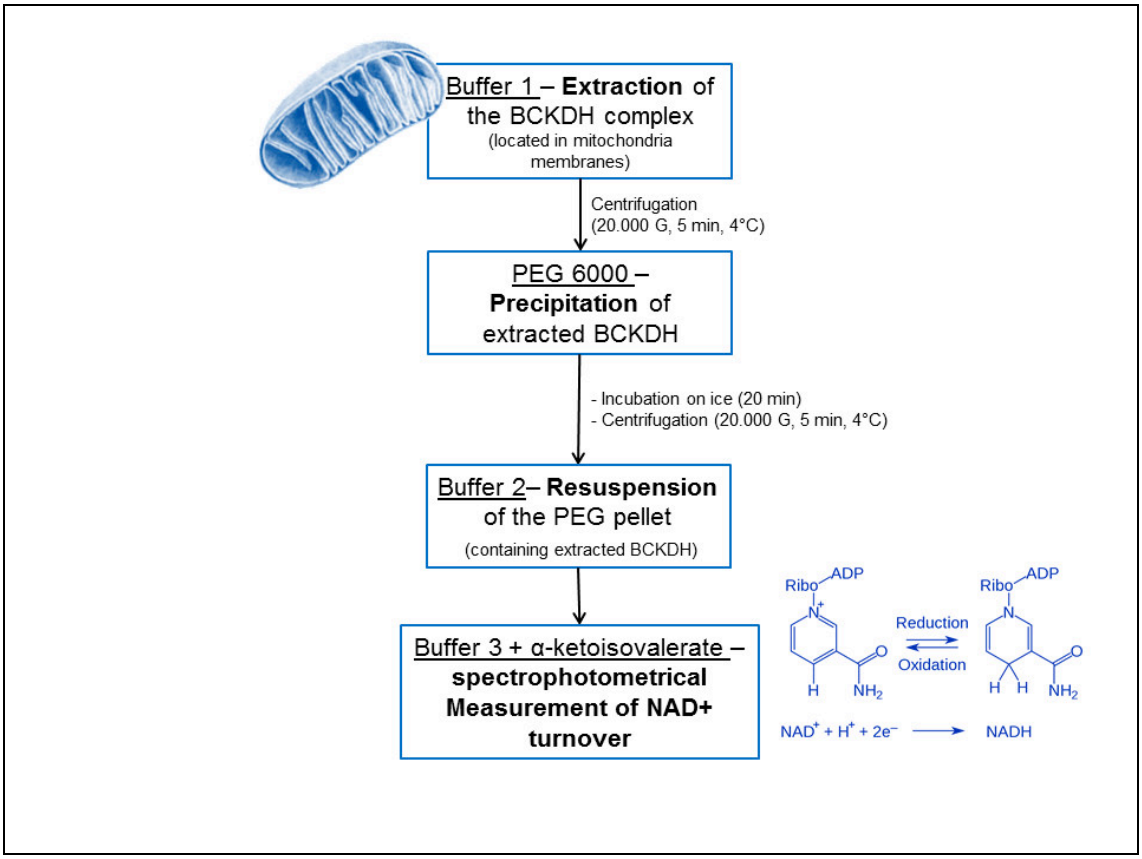

Figure 9: Spectrophotometrical measurement of the basal BCKDH activity - Methodical steps
BCKDH: Branched-chain keto acid dehydrogenase; PEG: Polyethylene glycol, NAD: Nicotinamide adenine dinucleotide

As demonstrated in Figure 9, a frozen tissue sample of 150 mg was homogenized in 1 ml ice-cold extraction buffer (Buffer 1) using a Mixer Mill (MM 400, Retsch, Haan, Germany) at 15 Hz for 3 min. Insoluble material was removed by centrifugation (20,000 G, 4°C, 5 min). The supernatant were incubated with 9% (v/v) polyethylene glycol (PEG 6000; AppliChem, Darmstadt, Germany) in *aqua bd* for 20 min on ice prior to a second centrifugation step (13,000G, 4°C, 10 min). The resulting pellet was dissolved in 250 µl suspension buffer (Buffer 2). In preparation for the spectrophotometrical measurement 40 µl of resuspended homogenate was diluted in 0.94 ml assay buffer (Buffer 3), which contained NAD^+ and CoA. The BCKDH activity was determined at 30 °C, using 20 µl of 50 mM α-keto isovalerate (Sigma Aldrich, St. Louis, Missouri, USA) as substrate, by measuring absorbance at 340 nm to detect NADH formation from NAD^+ within 6 min (except for brain samples, which were measured over 12 min).

In contrast to Nakai *et al*. (2000), the Buffer 1 was made with sodium fluoride instead of potassium fluoride and the Buffer 3 was made without dihydrolipoamide dehydrogenase.

Table 10: Reagents BCKDH activity assay according to Nakai *et al*. (2000)

Reagent	Ingredient
Buffer 1: Extraction buffer (pH 7.4 at 4°c)	50 mM Hepes 3% Triton X-100 (w/v) 2 mM EDTA 5 mM DTT 0.5 M Thiamine pyrophosphate 1 mM α-Chloro isocaproate[1] 50 mM Sodium fluoride[2] 2% Bovine serum (v/v) 0.1 mM TPCK 0.1 mg/ml Trypsin inhibitor 0.02 mg/ml Leupeptin
Buffer 2: Suspension buffer (pH 7.4 at 37°C)	25 mM HEPES 0.1% Triton X-100 (w/v) 0.2 mM EDTA 0.4 mM Thiamine pyrophosphate 1 mM DTT 50 mM KCI 0.02 mg/ml Leupeptin
Buffer 3: Assay buffer (pH 7.3 at 30°C)	60 mM Potassium phosphate 4 mM MgCl2 0.8 mM Thiamine pyrophosphate 0.8 mM CoA 2 mM NAD+ 0.2% Triton X-100 (w/v) 4 mM DTT
Substrate solution	50 mM Sodium α-ketoisovalerat
Polyethyleneglycol solution	27% PEG 6000 (w/v) in aqua bd

[1]manufactured according to Harris *et al*. (1982)
[2] modified from original

3.4 Statistical analyses

3.4.1 Statistical analysis of Study I – Estimation of a sub-limiting level of lysine as reference amino acid

The statistical analysis of all data from the preliminary study was performed using SAS (v. 9.3, SAS Inst. Inc., Cary, NC, USA). In the SAS MIXED procedure diet, sex and their interaction were considered as fixed effects. The IBW was included to the statistical model as linear covariate for final body weight (FBW), ADG and ADFI. For G:F ratio IBW had no effect and therefore was not considered for statistical analysis. Post-hoc comparisons were performed by use of Tukey test. Means were considered significantly different at $P<0.05$.

3.4.2 Statistical analysis of Study II – Estimation of the leucine requirement of weaned piglets in a weight range of 10 to 28 kg

The statistical analysis of Study II was performed using SAS (v. 9.3, SAS Inst. Inc., Cary, NC). Data were subjected to analysis of variance (ANOVA) or nonlinear regression by using the SAS procedures MIXED or NLMIXED. The statistical model in ANOVA included diet and sex as fixed effects and IBW and block as a random effect. The degrees of freedom in mixed-model, F- and t-tests were calculated using the approximation of Kenward-Roger.

The response to an increasing Leu supply was analyzed by linear broken-line, quadratic broken-line and quadratic regression models. In the linear mixed models and in the nonlinear regression models IBW was additionally included as linear covariate. If block variance was estimated to be 0, there was no consideration of block effects in the statistical model. Let yij be the response for animal j of block i with Leu supply level x and with IBW z, when the following nonlinear regression models were used for analyses. Similarly to Robbins *et al.* (2006), the (a) linear and (b) quadratic broken-line models were:

$$\text{(a)} \quad y_{ij} = L + U * (R < x) * (R - x) + V * z + b_{i+} e_{ij}$$

$$\text{(b)} \quad y_{ij} = L + U * (R < x) * (R - x)^2 + V * z + b_{i+} e_{ij}$$

The (c) quadratic function model was:

$$\text{(c)} \quad y_{ij} = L + U * x + V * x^2 + W * z + b_{i+} e_{ij}$$

L, U, V and W are regression coefficients, R is the unknown breakpoint x value and in all models is a block-specific random effect, assumed to be independently distributed from the residual-error components. The expression (R<x) is 1 for R<x and 0 otherwise. Therefore, in the broken-line models the plateau yp at values x>R is given by: where is the average of IBW. In the quadratic function model the optimal x value is given by: x_{opt}=U/(-2*V).

Selection of the statistical model to estimate the response of performance parameters to increasing Leu supply was conducted in advance using statistical information criteria Akaike information criterion (AIC) and corrected Akaike information criterion (AICC).

3.4.3 Statistical analyses of Study III – Effects of dietary leucine excess on physiological parameters of weaned piglets

Statistical analyses of Study III were performed using SPSS Statistical Software (IBM SPSS Statistics Standard 20, Armonk, NY, USA). Data were analyzed by General Linear Model ANOVA. Sex and genetics were included to the statistical model as linear covariates for all performance parameters. Since sex and genetics had no effects on performance, they were excluded from the model. Values were analyzed for homoscedasticity by Levene's test. In case of homogeneity of variance means, the three groups were compared by Tukey test or in case of unequal variances by Games-Howell test. Effects were considered to be significantly different at $P<0.05$.

3.4.4 Statistical analysis of Study IV – Effects of the interactions among leucine and threonine on growth performance and physiological parameters of weaned piglets

Statistical analyses of Study IV were performed using SPSS Statistical Software (IBM SPSS Statistics Standard 20, Armonk, NY, USA). Data were analyzed by General Linear Model two-way ANOVA. Values were analyzed for homoscedasticity by Levene's test. In case of homogeneity of variance means, the four groups were compared by Tukey test or in case of unequal variances by Games-Howell test. Effects were considered to be significantly different at $P<0.05$.

3.4.5 Statistical analysis of Study V – Effects of the interactions among leucine and tryptophan on growth performance and physiological parameters of weaned piglets

Statistical analyses of Study V were performed using SPSS Statistical Software (IBM SPSS Statistics Standard 20, Armonk, NY, USA). Data were analyzed by General Linear Model two-way ANOVA. Sex and genetics were included to the statistical model as linear covariates for all performance parameters. Since sex and genetics had no effects on performance, they were excluded from the model. Values were analyzed for homoscedasticity by Levene's test. In case of homogeneity of variance means, the four

groups were compared by Tukey test or in case of unequal variances by Games-Howell test. Effects were considered to be significantly different at $P<0.05$.

4 Results

4.1 Study I – Estimation of a sub-limiting level of lysine as reference amino acid

The preliminary study was conducted to estimate the amount of sub-limiting Lys in low-CP diets in order to design further studies. Thus, three low-CP diets were gradually added with crystalline Lys to achieve SID levels of 0.86, 1.02 and 1.16%. Those three low-CP groups were compared to a high-CP diet, which contained 1.16% SID Lys, concerning their effects on the growth performance of piglets.

The piglets used in **Study I** did not show signs of disease in response to the treatments. Table 11 shows that FBW, ADG and G:F were significantly influenced by diet, but not by sex. Furthermore, there was no interaction between diet and sex on any of the performance characteristics. Piglets that received the low-CP diets had lower FBW, lower ADG and lower G:F than piglets fed the high-protein diet (Table 11). No differences in FBW were observed between the groups that were fed the low-CP diets supplemented with different levels of SID Lys. In piglets that received the low-CP diet, increasing Lys levels were accompanied by a continuous improvement of growth performance as seen by the ADG and G:F ratio. The ADFI did not differ between the four groups of piglets. Data indicated that SID Lys levels <1.0% are limiting for growth for piglets in a weight range between 10 to 25 kg. Consequently, the amount SID Lys in the following studies was set to be 0.93%.

Table 11: Effect of dietary standardized ileal digestible (SID) Lysine (Lys) content on performance of piglets (Study I)

CP	**16**	**16**	**16**	**19**	***P*-value**	
SID Lys (%)	0.86	1.02	1.16	1.16		
	A	B	C	Con	diet	IBW
IBW (kg)	8.3±0.6	8.3±0.8	8.3±0.8	8.3±0.8	-	-
FBW (kg)	24.8±2.0^{a}	26.5±2.6^{a}	26.7±2.7^{a}	28.7±3.1^{b}	0.002	>0.001
ADG (g/day)	474±47^{a}	521±61^{ab}	531±63^{b}	587±73^{c}	>0.001	0.068
ADFI (g/day)	813±81	846±86	803±113	854±106	0.473	0.007
G:F (kg:kg)	0.60±0.02^{a}	0.63±0.03^{b}	0.68±0.03^{c}	0.71±0.03^{d}	>0.001	0.887

CP: crude protein; IBW: initial body weight; FBW: final body weight; ADG: average daily gain; ADFI: average daily feed intake; G:F: feed conversion ratio

Data are presented as means ± SD, mean values within a row not sharing a common superscript letter were significantly different by pairwise comparison (t-test, $P<0.05$); n=14 per treatment

4.2 Study II – Estimation of the leucine requirement by use of three statistical models

Study II was performed to estimate the requirement for Leu in low-CP diets for weaned piglets in a weight range of 10 to 28 kg. The study was designed as a dose-response study with five treatment groups that received experimental diets with different levels of Leu. The requirement was estimated by use of three different statistical models based on the recorded growth performance.

4.2.1 Growth performance

Table 12 demonstrates the effects of different dietary SID Leu:Lys ratios on performance characteristics. The chosen SID Leu:Lys ratios of 94%, 104% and 115%, respectively, were in the range of the supposed Leu requirement, whereas SID Leu:Lys ratios of 83% and 125% represent insufficient and excessive Leu levels, respectively.

The ADG was significantly influenced by dietary treatment (Table 12). Piglets that received 94%, 104% and 115% SID Leu:Lys ratios with their low-CP diet had higher ADG than piglets fed the diet with 83% SID Leu:Lys ratio. Piglets from the group that received the diet with 125% SID Leu:Lys ratio showed ADG that ranged between the ratios of 83% SID Leu:Lys and the groups that received diets with 94%, 104% and 115% SID Leu:Lys ratios. ADFI and G:F were not different between the 5 groups of piglets.

Table 12: Effect of dietary standardized ileal digestible (SID) Leucine (Leu) content on growth performance of weaned piglets

	SID Leu:Lys (%)					*P*-value	
	83	94	104	115	125	diet	IBW
IBW (kg)	10±1	10±1	10±1	10±1	10±1	-	-
FBW (kg)	25.3±4.0^{a}	27.3±2.8^{b}	28.4±3.7^{b}	28.3±3.2^{b}	26.8±3.8^{ab}	0.020	>0.001
ADG (g/day)	365±75^{a}	411±48^{b}	438±73^{b}	434±58^{b}	400±74^{ab}	0.020	0.075
ADFI (g/day)	615±132	654±91	707±122	686±79	651±116	0.191	0.047
G:F	0.59±0.03	0.62±0.03	0.62±0.02	0.62±0.02	0.61±0.03	0.127	0.041

Lys: Lysine; IBW: initial body weight; FBW: final body weight; ADG: average daily gain; ADFI: average daily feed intake; G:F: feed conversion ratio

Data are presented as means±SD, mean values within a row not sharing a common superscript letter were significantly different by pairwise comparison (t-test, $P<0.05$); n=11-12 per treatment

4.2.2 Assessment of the requirements by use of different statistical models

The best fitting model to estimate the response of performance parameters to increasing Leu supply was the quadratic function model according to information criteria (Table 13). Both, AIC and AICC showed lower values for the quadratic function model compared to linear broken-line and quadratic broken-line model. Table 13 shows the evaluated Leu requirements (breakpoints) for each performance parameter expressed as SID Leu:Lys ratio in response to the applied statistic model. Data reveal distinct differences in Leu requirement depending on the statistical model.

Table 13: Estimation of required dietary standardized ileal digestible Leucine:Lysine ratios to optimize performance characteristics of weaned piglets by use of linear broken-line, quadratic broken-line and quadratic function model and information criteria according to Akaike

Performance parameter	Statistical model	Breakpoint	Fit statistic -2logL	Information criteria	
				AIC	AICC
FBW (kg)	linear broken-line	97.1	266.4	278.4	280.0
	quadratic broken-line	103.8	266.4	278.4	280.0
	quadratic function	107.7	263.0	275.0	276.6
ADG (g/d)	linear broken-line	97.1	634.1	646.1	647.8
	quadratic broken-line	103.8	634.1	646.1	647.8
	quadratic function	107.7	630.7	642.7	644.4
ADFI (g/d)	linear broken-line	101.8	689.5	701.5	703.2
	quadratic broken-line	105.8	689.7	701.7	703.3
	quadratic function	107.6	687.9	699.9	701.5
G:F[1] (kg:kg)	linear broken-line	94.6	-236.4	-226.4	-225.3
	quadratic broken-line	97. 5	-236.4	-226.4	-225.3
	quadratic function	108.3	-237.2	-227.2	-226.0

AIC: Akaike information criteria; AICC: Akaike information criteria corrected; FWB: final body weight; ADG: average daily gain; ADFI: average daily feed intake; G:F: feed conversion ratio
n=11-12 per treatment
[1] block variance is estimated to be 0.

The linear-plateau model considered SID Leu:Lys ratios between 94.6% and 101.8% to be adequate. By use of the curvilinear-plateau model the optimum SID Leu:Lys ratios ranged between 97.5% and 105.8%. The quadratic-function model provided SID Leu:Lys ratios between 107.3 to 108.3% as adequate. Figure 10 illustrates that the quadratic-function model appears to describe the response of growth performance upon Leu supplementation better than the broken-line models because Leu did not reach a defined plateau. Instead, growth performance declined gradually when the piglets were fed with dietary Leu in amounts that exceeded the requirements. Instead, growth performance was smooth declining when the piglets were fed with dietary Leu in amounts that exceed the requirements. In Figure 10A the adjustment of different statistical models on

performance parameter final BW is represented. Figure 10B shows the comparison of all three models for ADG. In Figure 10C and Figure 10D the linear broken-line, curvi-linear-plateau and quadratic function are graphically represented for ADFI and G:F ratio, respectively. Based on the quadratic function model analysis, the current data show that the required SID Leu:Lys ratios to optimize final BW, ADG, ADFI and G:F ranged between 107.6% and 108.3%.

The findings based on the quadratic function model, however, show that a reduction of the SID Leu:Lys ratio from 107.7% to 97% reduced final BW, ADG, ADFI and G:F about 2.1%, 3.3%, 2.4% and 0.95%, respectively. Thereby, a reduction of 10% would have only a minor impact on performance of these animals.

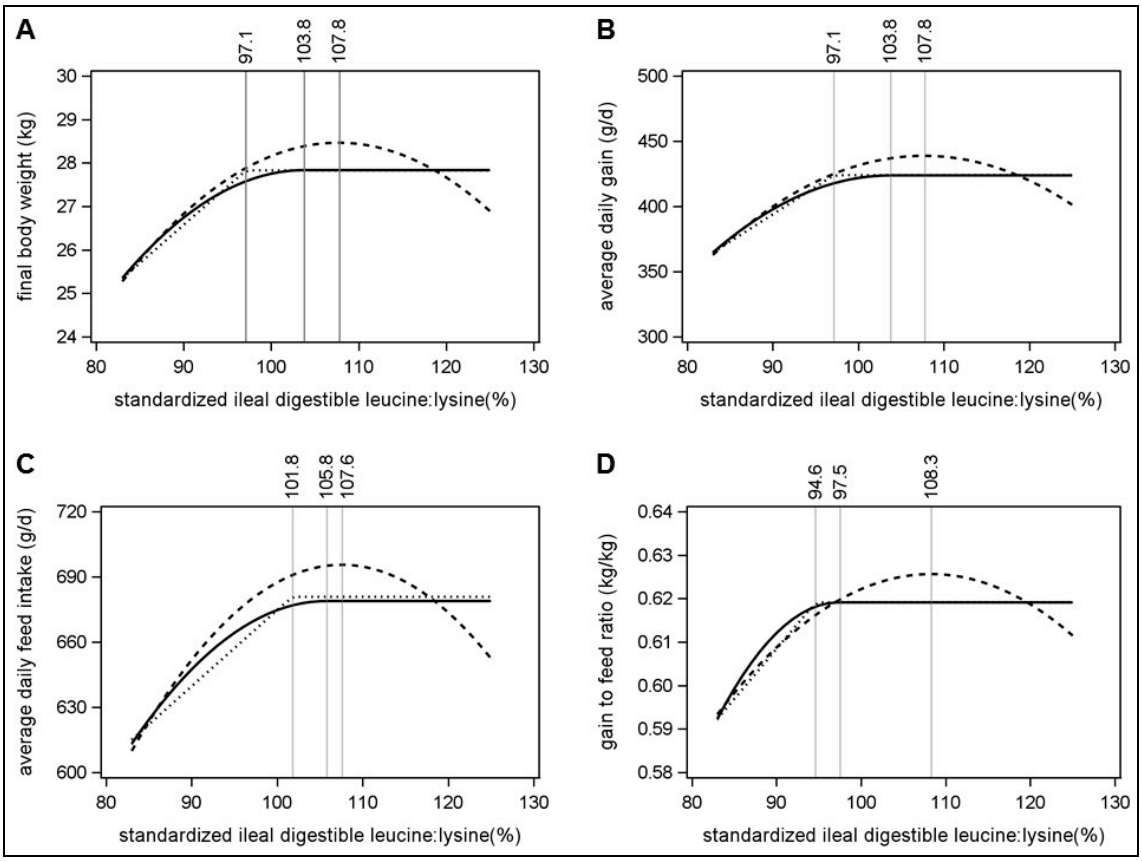

Figure 10: Comparison of different statistical models used for requirement estimation shown with response parameter (A) final body weight, (B) average daily gain, (C) average daily feed intake and (D) feed conversion ratio
Maximum response estimated by linear broken-line model (· · · ·), quadratic broken-line (——) or quadratic function (- - - -); n=11-12 per treatment

4.3 Study III – Characterization of tissue-specific basal activities of the branched-chain α-keto acid dehydrogenase (BCKDH) in presence of adequate or excessive leucine consumption

Study III was performed to elucidate the effects of excessive intake of dietary Leu on the growth performance and on the metabolism of the BCAA of piglets. The study was designed as a dose-response study with three treatment groups receiving experimental diets with different levels of Leu. Thus, the experimental parameters of a control group – which received dietary Leu according to current recommendations – were compared with two groups with non-practical diets. Those diets contained high (L2) and excessive amounts (L4) of dietary Leu.

As a result, it was found that excessive intakes of dietary Leu impaired the growth performance. Increased concentrations of dietary Leu increased the concentrations of Leu in all tissues and the concentrations of free Leu and the associated metabolites (KIC and 3-hydroxybutyrate) in blood plasma. The basal activity of pancreas, kidney, liver, cardiac muscle and brain was significantly increased due to excessive Leu intakes. Serotonin concentrations in blood plasma and brain tissue were reduced by a limited uptake of Trp in presence of high Leu concentrations.

4.3.1 Growth performance

In general, piglets of all groups appeared to be healthy. Piglets that received the L2 and L4 diets showed a significant decline in ADFI from week 1 to week 5 of the experiment compared to the control piglets (Figure 11). Taken the ADFI over the entire experimental period, the L2 group had 9% and the L4 group had 23% lower ADFI than the control group (control: 638±71g/d, L2: 583±45g/d, L4: 490±77g/d, $P<0.001$). The reduced ADFI resulted in a reduction of growth as seen by the weight gain of these pigs (control group: 411±87g/d, L2 group: 379±71g/d, L4 group: 322±80g/d; $P<0.001$). The G:F was not altered in response to the high-Leu diets (control group: 0.64±0.02 kg/kg, L2 group: 0.64±0.03 kg/kg, L4 group: 0.64±0.06 kg/kg).

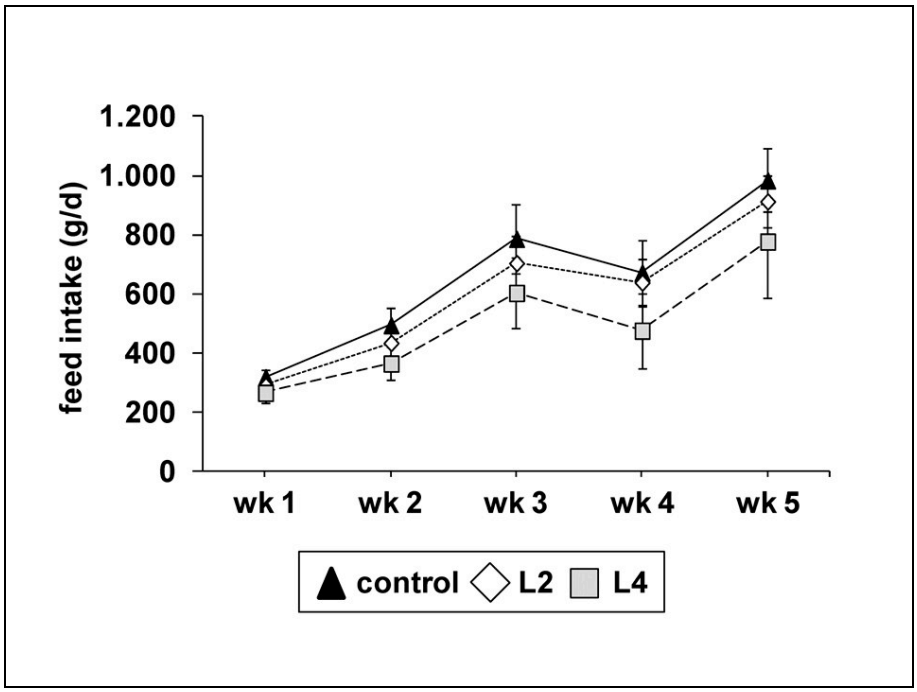

Figure 11: Daily feed intake of pigs fed diets with different Leu content
Data are presented as means ± SD; n=36-38 per treatment
Feed intake was recorded weekly (wk 1 to wk 5)

4.3.2 Basal activity of branched-chain α-keto acid dehydrogenase (BCKDH)

Data show that there were considerable tissue-specific differences in basal (control group) and stimulated BCKDH activities (Figure 12). In the control group, the highest basal BCKDH activity was found in pancreas, followed by kidney, liver, cardiac muscle, brain, skeletal muscle and adipose tissue (Figure 12). The mucosal BCKDH activity fell below the detection limits. With the exception of skeletal muscle and adipose tissue, all tissues showed an increase in BCKDH activity in response to feeding the high-Leu diets, although these stimulations of BCKDH activity were mostly significant when comparing the L4 group with the control group ($P<0.05$; Figure 12). The most marked change of BCKDH activity in response to the L4 diet was found in brain (2.5-fold), followed by liver (1.8-fold), cardiac muscle (1.7-fold) and kidney (1.2-fold). Despite the high basal activity of BCKDH in pancreas the stimulation of this enzyme complex upon feeding the high-Leu diets was marginal (Figure 12).

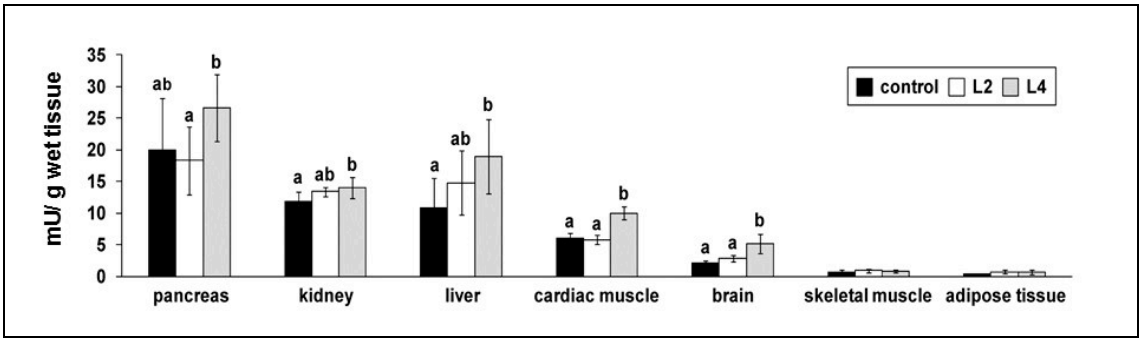

Figure 12: Branched-chain keto acid dehydrogenase (BCKDH) activity in different tissues of pigs in response to diets with different Leu contents
Data are presented as means ±SD; n=5. Superscript letters characterize significant differences by pairwise comparison (Tukey test or Games Howell test; P<0.05)
1 mU/g wet tissue = formation of 1nmol NAD^+ to NADH min^{-1}

4.3.3 Plasma amino acids, branched-chain α-keto acids, 3-hydroxybutyrate and serotonin

The plasma concentration of Leu in the groups fed the L2 and L4 diets was 2.3- and 3.3-fold higher, respectively, than that in the control group, although there were strong interindividual responses in plasma Leu upon feeding the high-Leu diets (P<0.05; Figure 13A). The increase in plasma Leu was accompanied by higher plasma levels of KIC, the transamination product of Leu and 3-hydroxybutyrate in these animals, albeit these alterations were only significant when comparing the L4 with the control group (P<0.05; Figure 13B and Figure 13C). Conversely, the plasma concentrations of Val and Ile and their corresponding branched-chain α-keto acids KIV and KMV decreased in response to the high-Leu diets (Figure 13A and Figure 13B). Plasma Leu correlated positively with plasma KIC (r=0.629; P<0.001) and negatively with KIV (r=-0.556; P<0.01) and KMV (r=-0.519; P<0.01). Remarkable is the correlation among plasma KIC and plasma Met (r=0.397; P<0.05). The concentrations of the ketone body 3-hydroxybutyrate in blood plasma increased linearly in response to dietary treatment and correlated significantly with the hepatic BCKDH activity (r=0.571; P<0.01). Figure 13D demonstrates that pigs that were placed on the high-Leu diets had lower plasma concentrations of serotonin than pigs fed the control diet (P<0.05). The ratio of BCAA:Trp in plasma showed higher values in the L4 group than in the control and L2 group (control: 11.4±0.8; L2: 11.1±2.5; L4: 13.3±2.5; P<0.05).

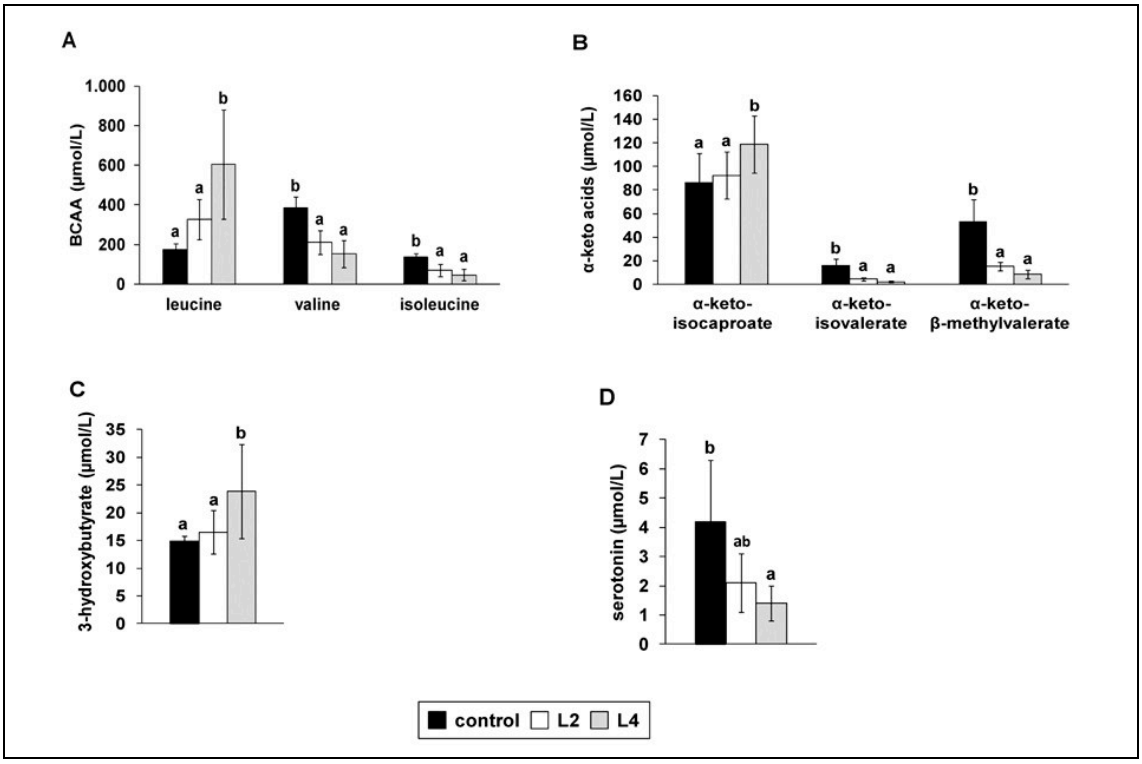

Figure 13: Final plasma concentrations of (A) branched-chain amino acids (BCAA), (B) branched-chain α-keto acids, (C) 3-hydroxybutyrate and (D) serotonin of pigs in response to diets with different Leu content
Data are presented as means ±SD; n=10. Different superscript letters characterize significant differences by pairwise comparison (Tukey test or Games Howell test; P<0.05)

4.3.4 Tissue free amino acids and cortical serotonin

The most marked changes of non-protein-bound AA in tissues in response to the high-Leu diets were observed with the individual BCAA.

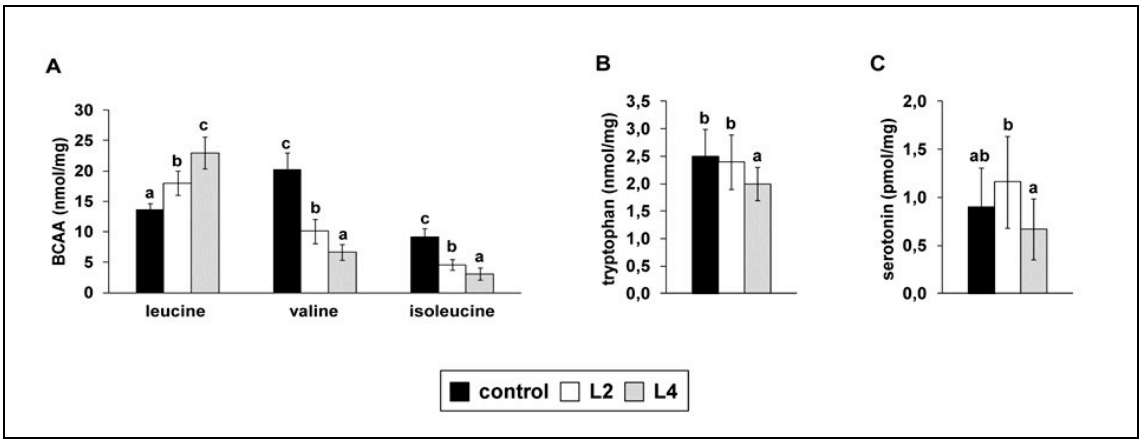

Figure 14: Concentrations of (A) branched-chain amino acids (BCAA), (B) tryptophan and (C) serotonin in brain of pigs in response to diets with different Leu content
Data are presented as means ±SD; n=10. Superscript letters characterize significant differences by pairwise comparison (Tukey test or Games Howell test; P<0.05)

In line with the plasma AA, the concentrations of Leu were markedly higher in brain (Figure 14A, $P<0.05$), pancreas (Figure 15A, $P<0.05$), liver (Figure 15B, $P<0.05$), kidney (Figure 15C, $P<0.05$), cardiac muscle (Figure 15D, $P<0.05$), skeletal muscle (Figure 15E, $P<0.05$) and duodenal mucosa (Figure 15F, $P<0.05$) of pigs fed the high-Leu diets than in the control group. In particular, the Leu concentration in the duodenal mucosa was 6.2-times higher in pigs fed the L4 diet than in those receiving the control diet. The concentrations of Val and Ile declined in all tissues, except duodenal mucosa, upon feeding the high-Leu diets (Figure 14A, Figure 5; $P<0.001$). Other plasma and tissue amino acids show marginal changes in response to the high-Leu diets (Tables A6-A12). Remarkable was the finding that cerebral tryptophan declined in response to the L4 diet (Figure 14B; $P<0.05$). The reduced Trp level in brain was accompanied by a reduction in cerebral serotonin (Figure 14C, $P<0.05$), although the decline in cerebral serotonin was only significant when comparing the L4 with the L2 group.

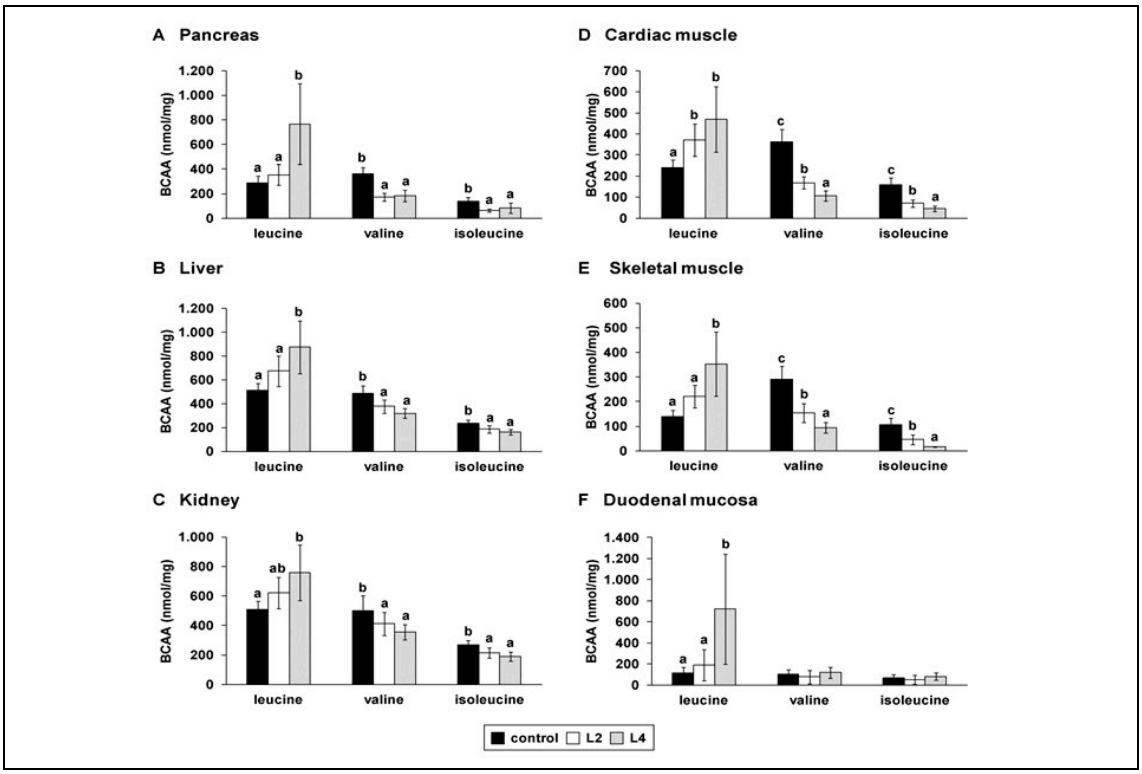

Figure 15: Concentrations of (A) branched-chain amino acids (BCAA) in different tissues of pigs in response to diets with different Leu content
Data are presented as means ±SD; n=10. Superscript letters characterize significant differences by pairwise comparison (Tukey test or Games Howell test; P<0.05)

4.4 Study IV – Considerations of interaction effects among leucine and threonine on feed efficiency, plasma amino acid profile and hepatic branched-chain amino acid catabolism

To find interaction effects of Leu and Thr on piglets' growth, physiological parameters and N-utilization, Study IV was designed with four low protein diets. The two-factorial study design included two levels each for the two factors Leu (adequate *versus* high) and Thr (low *versus* adequate). As a result, the growth performance remained unaffected by dietary treatment. Significant improvements of the N retention were observed with high supplementation of Leu and/or Thr. However, only Thr had a significant effect on the N retention. Further, it was found that high intakes of dietary Leu reduced the concentrations of plasma BCAA. Simultaneous adequate intakes of Thr lowered this effect in tendency. The dietary treatment had no effect on the basal activity of BCKDH in liver, cardiac- or skeletal muscle.

4.4.1 Growth performance

In Study IV, no significant effects on performance were observed by dietary treatment (Table 14). The statistical analysis by two-way ANOVA indicated a tendency for an effect of Thr on the ADFI (P=0.066).

Table 14: Effects of dietary Threonine (Thr) and Leucine (Leu) content on performance of piglets

Diet (Thr:Leu)	**57:100**	**57:200**	**65:100**	**65:200**	***P*-value**		
SID Leu:Lys	100%	200%	100%	200%			
SID Thr:Lys	57%	57%	65%	65%	Leu	Thr	Leu x Thr
IBW (kg)	10.2±0.6	10.1±0.7	10.3±0.7	10.1±0.7	-	-	-
FBW (kg)	28.0±2.5	28.9±2.4	29.3±2.0	29.7±2.6	0.133	0.111	0.750
ADG (g/day)	424±57	447±52	452±41	465±50	0.133	0.111	0.750
ADFI (g/day)	718±99	748±86	745±73	748±91	0.258	0.066	0.565
G:F (kg/kg)	0.60±0.04	0.61±0.02	0.61±0.04	0.61±0.03	0.635	0.710	0.537

IBW: initial body weight; FBW: final body weight; ADG: average daily gain; ADFI: average daily feed intake; G:F: feed conversion ratio
Data are presented as means ± SD; n=15 per treatment

4.4.2 Nitrogen balance

Supplementing dietary Thr improved the N retention significantly (P<0.05; Figure 16). With adequate Leu intake, the supplementation of Thr induced an increase of 31% for the ingested amount of N. With high Leu intake, the dietary amount of Thr had no effect on the N retention. Dietary Leu did not affect the N retention (P>0.05). However, supplementation of Leu enhanced the N retention in presence of low Thr intake due to interactions of Leu and Thr (P<0.05).

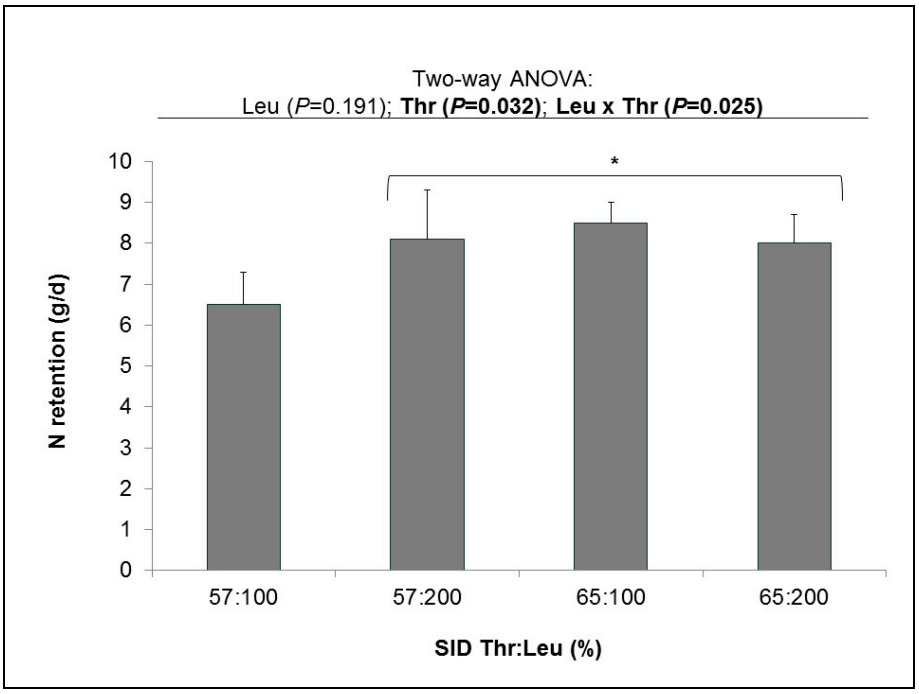

Figure 16: Effects of dietary Threonine (Thr) and Leucine (Leu) content on N retention of piglets in a weight range of 16 to 19 kg
N: nitrogen; SID: standardized ileal digestible
Data are presented as means ± SD; n=5.
Superscript letters characterize significant differences by pairwise comparison (Tukey test, P<0.05)

The N-intake was not affected by dietary treatment (P>0.05). The N-content in feces was increased with adequate Leu *versus* low Thr and high Leu *versus* high Thr, due to the interactions of both dietary AA (P<0.05). However, the N-content in feces was not affected by impact of single Leu or Thr.

4.4.3 Plasma amino acids

Blood plasma samples were analyzed for changes in the concentrations of free AA re-

lated to dietary treatment. Strong interactions among the three BCAA are demonstrated in Table 15.

Increasing the dietary amount of Leu indicated an increase of plasma Leu. In presence of high Thr intake, this effect was enhanced. However, the impact of dietary Thr on plasma Leu was only a tendency.

Table 15: Effects of dietary Threonine (Thr) and Leucine (Leu) content on plasma amino acid concentrations of piglets

Diet (Thr:Leu)	**57:100**	**57:200**	**65:100**	**65:200**	***P*-value**		
Plasma AA (µmol/l)					Leu	Thr	Leu x Thr
Lysine	401±108	326±111	429±113	412±86	0.345	0.245	0.539
Threonine	300±61^{a}	260±91^{a}	478±54^{b}	445±96^{b}	0.309	>0.001	0.926
Methionine	120±16	99±22	114±15	117±12	0.235	0.441	0.118
Tryptophan	84±8	74±28	89±17	87±16	0.512	0.292	0.646
Valine	483±34^{b}	246±41^{a}	498±35^{b}	288±25^{a}	>0.001	0.082	0.387
Isoleucine	207±14^{b}	100±14^{a}	222±22^{b}	116±16^{a}	>0.001	0.054	0.942
Leucine	227±37^{a}	391±70^{b}	230±28^{a}	478±51^{b}	>0.001	0.058	0.076
BCAA	916±81^{b}	737±119^{a}	949±80^{a}	882±90^{ab}	0.010	0.049	0.200
Histidine	115±13	84±25	105±15	103±16	0.066	0.569	0.102
Phenylalanine	131±22	125±23	146±23	146±19	0.777	0.084	0.752

AA: amino acids; BCAA: branched-chain amino acids

Data are presented as means ± SD, mean values within a row not sharing a common superscript letter were significantly different by pairwise comparison (Tukey test, $P<0.05$); n=5 per treatment, samples were taken 2.5h after feeding

Piglets that received 200% of dietary Leu showed significant lower plasma concentrations of Val and Ile than piglets fed 100% dietary Leu. High supplementation of Thr tended to dimish this effect, since plasma concentrations of Val and Ile were increased in the 65:200 group compared to group 57:200 ($P<0.1$). The plasma concentrations of Thr increased significantly in response to dietary Thr supplementation. Although, there was no significant effect of dietary Leu on plasma Thr. Other essential AA were not affected by dietary treatment ($P>0.05$).

4.4.4 Basal activity of branched-chain α-keto acid dehydrogenase (BCKDH)

The basal activity of the BCKDH enzyme complex was assayed to find possible impacts caused by dietary changes of Leu and Thr on the degradation pathway of the BCAA.

In hepatic tissue, the activity of BCKDH was 58% higher than in cardiac muscle tissue and 88% higher than in skeletal muscle tissue. Even if the BCKDH activity increased numerically in liver with increase of dietary Leu and Thr concentrations, no significant effect by dietary treatment could be demonstrated ($P>0.05$; Figure 17). Likewise, in

cardiac muscle and in skeletal muscle no significant differences of the BCKDH activity was found (P>0.05). Neither dietary Leu or Thr concentrations nor interaction of both indicated an impact on the activity of the enzyme complex (P>0.05).

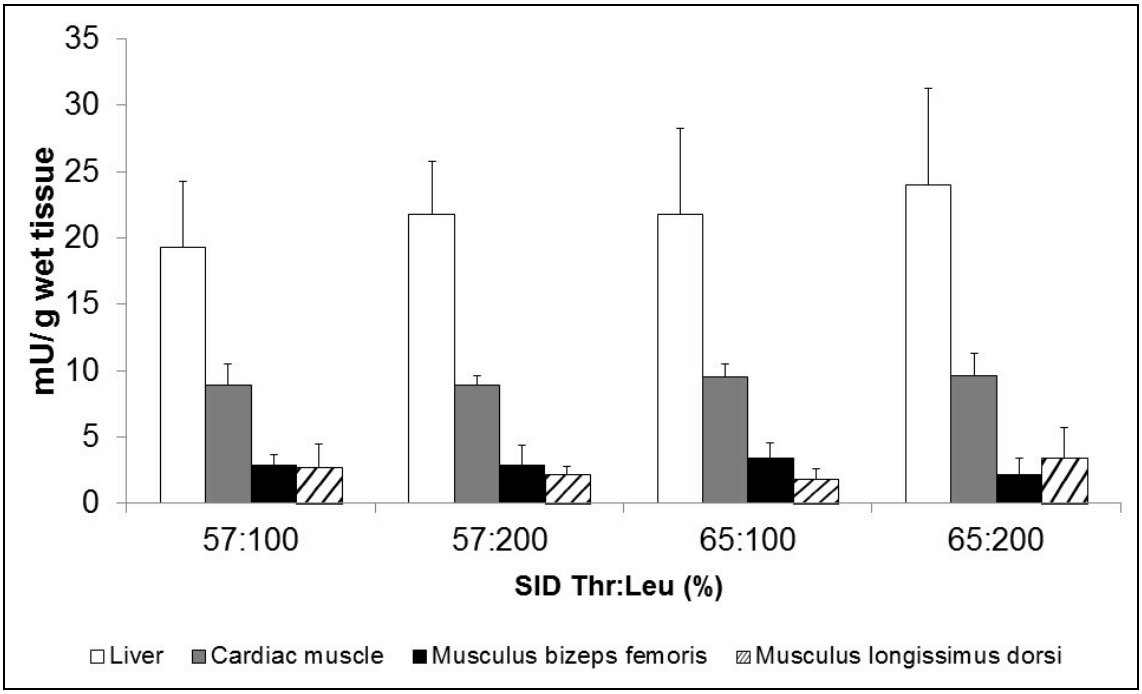

Figure 17: Effects of dietary Threonine (Thr) and Leucine (Leu) content on the basal activity of the branched-chain keto acid dehydrogenase (BCKDH) in different tissues of piglets
Data are presented as means ± SD; n=5 per treatment.
Samples were taken 2.5h after feeding
1 mU/g wet tissue = formation of 1nmol NAD^+ to NADH min^{-1}

4.5 Study V – Considerations of interaction effects among leucine and tryptophan on appetite and appetite-regulating molecules

In a two-factor study the interaction of Leu and Trp on feed intake, plasma and hypothalamic concentrations of Leu and Trp and the hypothalamic level of serotonin was elucidated. Four diets with either adequate Leu (SID Leu:Lys 100%) *versus* high Leu (SID Leu:Lys 300%) and two dietary concentrations of Trp that are assumed to meet the requirement (SID Trp:Lys 18% *versus* 23%). As a result, the growth performance was affected by dietary Leu. Further, it was found that changes in the intake of Leu affect the concentrations of plasma and hypothalamic BCAA. A reduced uptake of Trp to the hypothalamus induced a reduction of serotonin concentrations in the presence of high Leu intake. Hypothalamic mTOR expression remained unchanged by dietary treatment. The dietary treatment affected the basal activity of BCKDH in pancreas, but not in liver.

4.5.1 Growth performance

In general, piglets appeared to be healthy. Supplementing dietary Trp did not affect the growth performance. Piglets fed the high-Leu diet (Leu300 groups) consumed in average 40 g less feed per day than pigs fed the Leu100 diets. A lower final BW and a trend toward lower weight gain (Table 16) accompanied the reduced feed intake of piglets fed the Leu300 diets.

Table 16: Effects of dietary Trp and Leu content on performance of piglets

Diet	**18:100**	**18:300**	**23:100**	**23:300**	***P*-value**		
SID Leu:Lys	100%	300%	100%	300%			
SID Trp:Lys	18%	18%	23%	23%	Leu	Trp	Leu x Trp
IBW (kg)	9.4±0.9	9.1±1.1	9.5±0.9	9.1±1.1			
FBW (kg)	25.5±2.9	24.7±3.0	26.0±3.1	24.1±2.8	0.016	0.820	0.108
ADG (g/day)	384±59	372±65	393±61	358±57	0.060	0.811	0.108
ADFI (g/day)	660±59	621±67	663±68	616±66	0.046	0.748	0.931
G:F (kg/kg)	0.48±0.02^{a}	0.51±0.03^{b}	0.50±0.02^{ab}	0.49±0.03^{ab}	0.041	0.695	0.013

Data are presented as means±SD, mean values within a row not sharing a common superscript letter were significantly different by pairwise comparison (Tukey test, $P<0.05$); n=30 per treatment

4.5.2 Plasma amino acids

Analyses reveal that piglets treated with the Leu300 diets had more than two-fold higher concentrations of Leu in plasma than the Leu100 groups ($P_{Leu}<0.001$; $P_{Trp}=0.009$). In contrast, concentrations of Val ($P_{Leu}<0.001$; $P_{Trp}=0.011$) and Ile ($P_{Leu}<0.001$; $P_{Trp}=0.003$) decreased in response to the high-Leu diets. The Leu300 diets reduced the plasma concentrations of Trp, although this effect was mainly observed in the Trp23 group ($P_{Leu}=0.001$; $P_{Trp}<0.001$; $P_{Leu\ xTrp}=0.011$; Figure 18). Supplementation of 23% Trp caused less decreases in plasma BCAA and Trp in the Leu300 groups than in those groups with Leu300 and Trp18.

Additional to the BCAA and Trp, the plasma concentrations of other AA were affected by dietary treatment (Table A13): the concentration of Thr decreased due to increased intake of Leu and Trp. Concentrations of His decreased due to interactions of Leu x Trp ($P=0.009$). The plasma concentrations of Phe differed significantly due to dietary Trp ($P=0.014$) and interactions of Leu x Trp ($P=0.018$). Even semi-essential tyrosine (Tyr) decreased significantly in the high Leu groups ($P=0.006$), but increased due to Trp supplementation ($P=0.038$).

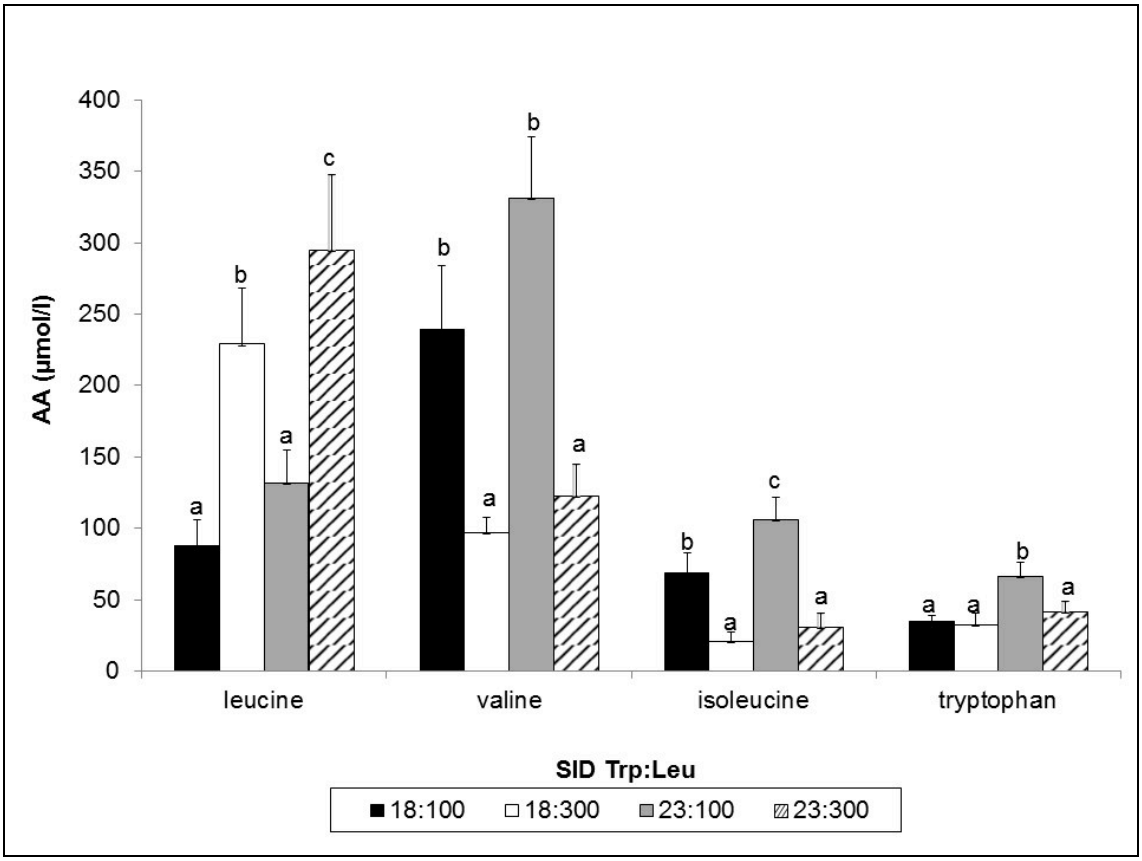

Figure 18: Final plasma concentrations of branched-chain amino acids and tryptophan of piglets in response to diets with different leucine and tryptophan content.
Data are presented as means ±SD; n=8.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test; P<0.05). Samples were taken 2.5h after feeding.

4.5.3 Free amino acids and serotonin in hypothalamic tissue

Analyses reveal that piglets treated with the Leu300 diets had 30 to 44% higher Leu concentrations in hypothalamus than the Leu100 groups (Figure 19A). In contrast, hypothalamic concentrations of Val and Ile decreased in response to the high-Leu diets (Figure 19A). Figure 19B illustrates that the Leu300 diets reduced the hypothalamic concentrations of Trp, although this effect was mainly observed in the Trp23 group. Furthermore, the changes in dietary Leu affected hypothalamic concentrations of Met (P=0.014) and Phe (P=0.013).

Piglets fed the Leu300 diets had lower hypothalamic serotonin concentrations than those fed the Leu100 diets (Figure 19C). The excess of Leu limited the uptake of Trp to the brain and therefore the serotonin concentrations in hypothalamus correlated closely with hypothalamic Trp concentrations (r=0.558, P=0.002).

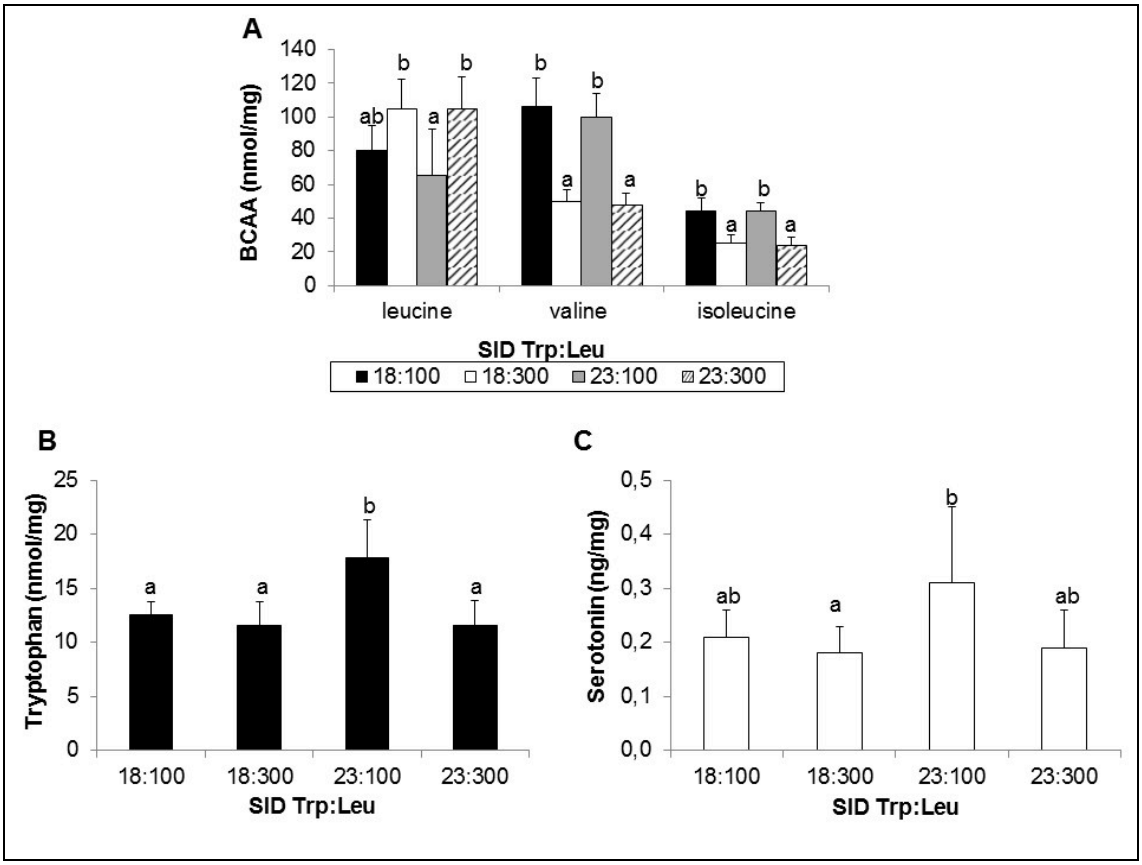

Figure 19: Concentrations of (A) branched-chain amino acids (BCAA), (B) tryptophan and (C) serotonin in brain of pigs in response to diets with different Leu and Trp contents. Data are presented as means ±SD; n=8. Superscript letters characterize significant differences by pairwise comparison (Tukey test*; P*<0.05). Samples were taken 2.5h after feeding.

4.5.4 Protein expression of mTOR

To elucidate whether changes in dietary Leu may alter the protein expression of mTOR in hypothalamus that may explain the appetite-reducing effects of excessive Leu, Western Blot analyses were performed. No significant effect of dietary treatment on protein expression of mTOR or – due to phosphorylation – activated mTOR was found (Figure 20). However, it was observed that high Leu intakes increased P-mTOR expression. Higher values were found in the 300% Leu groups compared to treatment groups with 100% Leu.

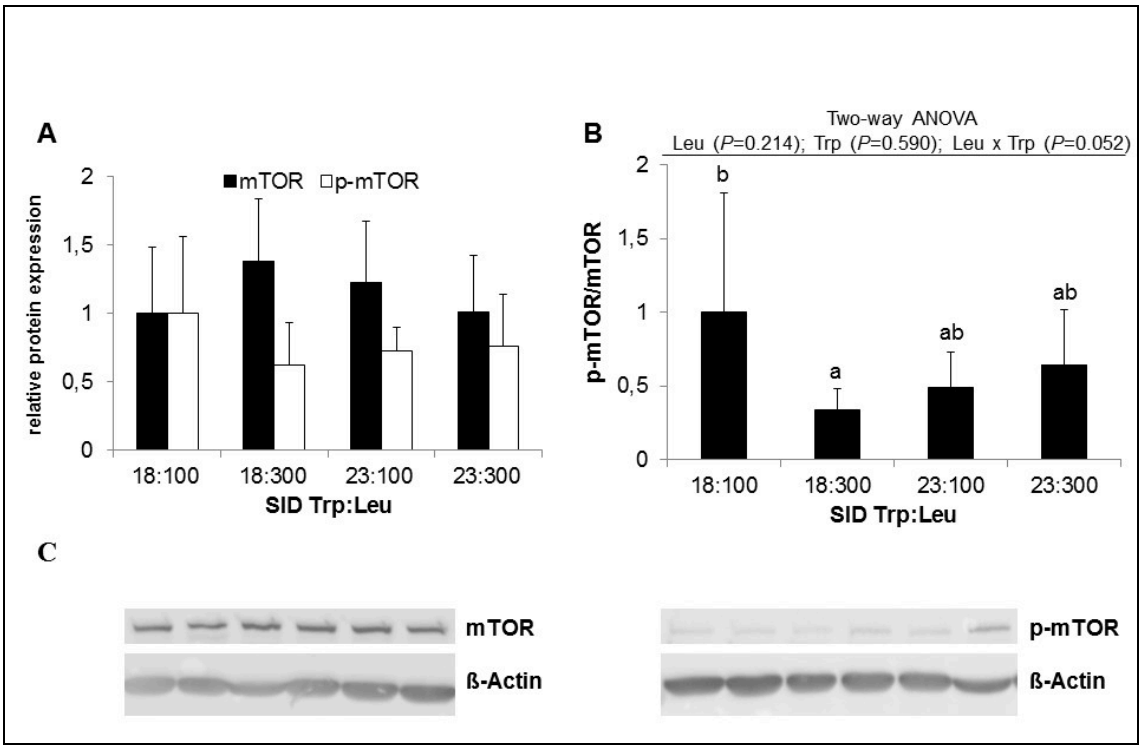

Figure 20: (A) Relative concentrations of mTOR and phosporylated mTOR and (B) Ratio of the phosphorylated form of mTOR to mTOR in hypothalamic tissue of piglets in response to dietary Leu and Trp.
Data are presented as means ±SD; n=8. Superscript letters characterize significant differences by pairwise comparison (LSD-test; $P<0.05$).
Samples were taken 2.5h after feeding. Group 18:100 was set to be 0. ß-Actin was used for normalization.

4.5.5 Basal activity of branched-chain α-keto acid dehydrogenase (BCKDH)

Data show an increased activity of hepatic BCKDH in treatment groups with high Leu supply (P=0.023). The Trp content of the experimental diets or interactions of Leu x Trp had no impact on hepatic BCKDH activity. The pancreatic activity of BCKDH was highest in the control group, lowest in the group with high Leu and low Trp and intermediate in both groups with high Trp supplementation due to interactions of Leu x Trp (Figure 21; P=0.043).

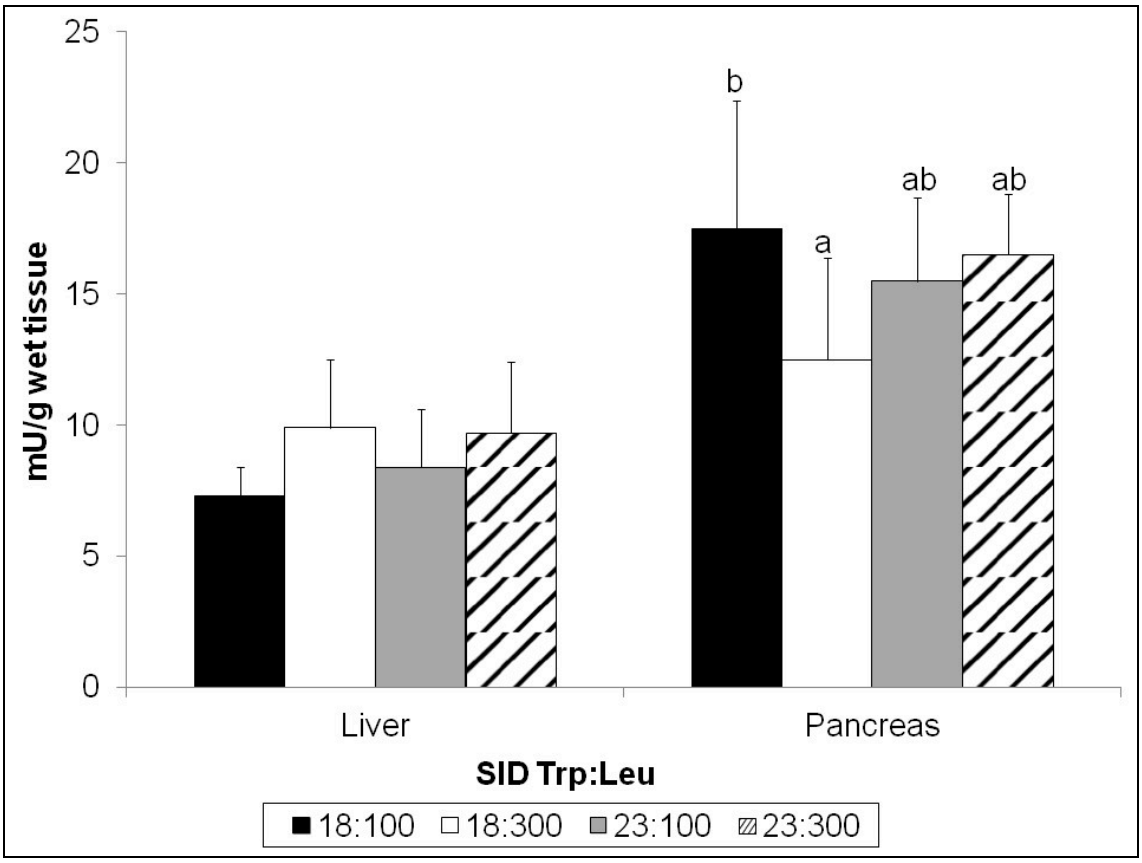

Figure 21: Branched-chain keto acid dehydrogenase (BCKDH) activity in different tissues of pigs in response to diets with different Leu contents
Data are presented as means ±SD; n=5. Superscript letters characterize significant differences by pairwisecomparison (Tukey test or Games Howell test; $P<0.05$)
1 mU/g wet tissue = formation of 1nmol NAD^+ to NADH min^{-1}

5 Discussion

5.1 Importance of leucine in pigs' physiology

Due to its central position in AA metabolism, Leu is the focus of the present work. Generally, Leu is abundant in common feed ingredients used in practical pig diets. This BCAA controls several anabolic processes. Among the AA, Leu is most effective in stimulating protein synthesis and reducing proteolysis (Escobar *et al.*, 2005; Yin *et al.*, 2010) since Leu provides hormone-like signals to tissues such as skeletal muscle, indicating overall nutrient sufficiency. Therefore, it promotes a positive N balance given that requirements for all essential AA are fulfilled, possibly by favoring the activation of mTOR. Leu has been previously shown to stimulate insulin secretion by pancreatic β-cells (Xu *et al.*, 2001). Insulin is a peptide hormone, produced by β-cells in the pancreas, regulating the metabolism of carbohydrates and fats. However, insulin regulates the protein metabolism by inducing the generation of AA transporters. Furthermore, insulin is a nutrient signal, which also stimulates mTOR activity in presence of cytosolic AA. In contrast to insulin that promotes mTOR activation only in presence of other nutrient signals as cytosolic AA, Leu is capable of stimulating mTOR activation through both insulin-dependent and -independent mechanisms (Hutson, 2006). Furthermore, Leu stimulates leptin secretion via the nutrient- and insulin-regulated mTOR pathway (Li *et al.*, 2011). In contrast to all described anabolic processes mediated by Leu, the irreversible catabolism of all three BCAA is stimulated exclusively by the Leu metabolite KIC. Therefore, high intakes of dietary Leu may reduce the availability of Val and Ile for protein synthesis.

This wealth of physiological functions indicates exact knowledge of pigs' requirements for this special AA. Data about piglets' requirements for Leu are sparse, especially for the use of low-CP diets. The few studies that exist show heterogenic results and use only one specified statistical model, respectively. For this reason, part of the present work was an estimation of the requirement of weaned piglets (fed low-CP diets) for Leu by use of three different statistical models. It was found, that Leu intakes upon the requirement decrease the voluntary feed intake. Therefore, an excess study was conducted subsequently. Data of the excess study showed that several physiological circulating AA were affected by dietary Leu excess. Consequently, two interaction studies were performed in line to generate detailed knowledge about the interactions of Leu and Thr and Leu and Trp, respectively and consequences for the AA metabolism in pigs.

5.2 Considerations of leucine effects in the physiology of pigs based on present *in vivo* studies

5.2.1 Study I and Study II – Estimation of lysine and leucine requirements with consideration of three statistical models

To estimate piglets' requirements for Lys and Leu, two dose-response studies were performed. A basal diet was added with increasing amounts of the test-AA. In **Study I,** one control group with high-CP was designed to test whether Lys-supplemented low-CP diets can compete with a high-CP diet concerning the growth performance. Data on the Leu requirement of piglets fed a low-CP diet are scarce. The few existing publications do not adequately consider the possible adverse effects of excessive BCAA intake on growth performance. The current **Study II** was conducted with five different dietary levels of SID Leu:Lys using two levels below and two levels above the presumed requirement. Besides the commonly used broken-line models, in particular the linear broken-line and the curvilinear-plateau model, the quadratic-function model was included to the considerations, because this model is the only model that considers a possible decline of growth parameters upon feeding excessive AA.

Study I was not a classical dose-response experiment, since the precondition for such a study is the comparison of more than three treatment groups. Herewith, only three treatment groups with increasing levels of Lys, supplemented to a low-CP (CP=16%) diet, were compared to a control group, which received a diet with 19% CP. However, **Study I** was imperative to create SID Lys with 0.93% as sub-limiting AA, after those AA in focus of the subsequent studies, according to Boisen (2003). This was important especially for the estimation of the Leu requirement. If the requirement for Leu (being first limiting factor for performance in the basal diet) is fulfilled, an increase of Leu level will increase the response criteria up to the point where Leu supply is not longer limiting. A further increase would result in an achievement of a plateau-value or breakpoint. At this breakpoint, Lys and Leu equally limit growth and it could be considered as the requirement. The ADG and G:F of the low-CP groups in **Study I** were lower than those parameters of the control-group, it might be assumed that another factor was limiting in the low-CP diets. The His content of all three low-CP diets in **Study I** for example was lower than recommended by NRC (2012) and could have been limiting for performance, which is a likely limitation of **Study I**. Nevertheless, the maximum

growth performance was realized in the low-CP group with 1.16% SID Lys. Consequently, 16.6 mg Lys/g gain per day was calculated as requirement of piglets for Lys. The GfE (2006) recommends 20 mg Lys/g gain per day as piglets requierement for Lys. The value of 16.6 mg Lys/g gain per day, as estimated in **Study I,** could be achieved on basis of the current data with 1.02% SID Lys. A 9% lower level of 0.93% SID Lys was used for the following studies and the risk of other limiting factors was neglected.

In **Study II,** the focus was set to the response of piglets' performance to Leu. Different statistical models were used for evaluation: the linear broken-line, the quadratic broken-line and the quadratic function model. Data revealed a dependence of the SID Leu:Lys ratio to optimize ADFI, ADG and G:F on the statistical model used for the assessment of the Leu requirement. The linear broken-line model gave a lower requirement than the quadratic broken-line model. Highest results were found by using the quadratic function model. Taking the data from the linear and quadratic broken-line models, the optimum SID Leu:Lys ratios (98% and 103%, respectively) were quite similar to those recommended by the NRC (2012) and lower than data estimated by quadratic function model. Data based on the analysis by quadratic function model demonstrated that the SID Leu:Lys ratio, which was found to optimize growth amounted 107.8%. The ratio is higher than those assessed by Gloaguen *et al.* (2013a) and also recommended, based on literature review, by the NRC (2012). Augspurger and Baker (2004), using corn-peanut meal-whey-soybean meal-based diets, determined the SID Leu requirement for 10 to 20 kg pigs to be approximately 1.05%, which corresponds to an SID Leu:Lys of 100%. However, the ratio estimated by the quadratic function model is close to the recent estimates for 28 to 43 kg pigs (García *et al*., 2015) with 104% for ADG and 109% for G:F. If the underlying statistical models are considered in the comparison of the recommendations, the present results agree with available literature. Augspurger and Baker (2004) used the broken-line model for estimation of the Leu requirement. The current linear broken-line model result is near to the recommended optimum SID Leu:Lys of ≤100%, given by Augspurger and Baker (2004) and NRC (2012). The linear broken-line model has to be assessed as risky: firstly, the linear broken-line model often forces the breakpoint to a lower value (Robbins *et al*., 2006); secondly, a nutrient supply below the requirement causes interpretation of rapid decreases in performance with this model. A higher security margin is given by the quadratic broken-line model, which is called curvilinear-plateau model within the study of Gloaguen *et al.* (2013a). The authors found a SID Leu:Lys ratio of 102% to maximize performance. This is according to the present

data, in case of estimation by quadratic broken-line model – here SID Leu:Lys 103% was found as optimal supply. As Gloaguen *et al.* (2013a) could show with the curvilinear-plateau model, a 10% lower supply of Leu caused only marginal growth depressions (2.4%). The same security assessment could be given by quadratic function model. Using this model reduction of 10% Leu supply caused also marginal growth depressions (2.0%), but a higher requirement (SID Leu:Lys 108%) than estimated by the other models. The benefit of the quadratic function is the consideration of possible growth-declining effects caused by excessive Leu consumption. This tendency was observed in the group fed SID Leu:Lys 125%. For this reason, the information criteria (Table 13) used for evaluation of the best fitting model might show lowest values for the quadratic function model. That is an indication for the best properties of quadratic function model to describe the response of piglets' growth performance to the increasing Leu supply in **Study II**, mathematically and biologically. It is remarkable that this model was used the first time for the estimation of a Leu requirement with the present study. Recent studies could show that dietary Leu intakes upon the requirement reduce the voluntary feed intake (Langer *et al.*, 2000; Wiltafsky *et al.*, 2010; Millet *et al.*, 2015, García *et al.*, 2015). The reasons will be described in detail in the following chapters. However, a limitation of the **Study II** was that only two groups, which received more Leu with their diets than required for growth optimization, were included. Thus, further studies are needed to address the safe upper range of Leu that can be used in nutrition for piglets

In conclusion, in cereal-soybean meal-based diets, the SID Leu:Lys is in average 103%, summarizing available literature and the present data of **Study II**. However, evaluation by use of different statistical models showed a dependence of the requirement on statistical model. Previous studies used solely the linear broken-line or the quadratic broken-line/curvilinear plateau model. Both models estimate lower requirements than the quadratic function model, which fitted best to the data of **Study II**. Nevertheless, three concrete recommendations for optimal SID Leu:Lys are specified based on the present data: 98% estimated by linear broken-line; 103% estimated by quadratic broken-line and 108% using quadratic function model. However, it should be noted that SID Leu:Lys ratios which are 10% below this assessed optimum would have only minor impact (-2%) on performance. Since raw feedstuff materials used in practical conditions may contain relatively high amounts of Leu, dietary Leu contents between 100 and 108% should be recommended regarding **Study II**. A dietary Leu level below 100% holds the risk for insufficient protein synthesis rates, a level beyond 108% could provoke negative

effects on growth performance, especially the feed intake, as shown with the subsequent **Study III**.

5.2.2 Study III – Characterization of tissue-specific basal activities of branched-chain α-keto acid dehydrogenase (BCKDH) in presence of adequate or excessive leucine consumption

Considerations for the use of additional Leu in animal nutrition require knowledge about the physiological functions of Leu in body protein turnover. Leu has both anabolic and catabolic effects in the metabolism of AA. This phenomenon is known as the ”Leucine Paradox” (Shimomura and Harris, 2006). On the one hand, Leu administration stimulates protein synthesis via the enzyme complex mTOR and inhibits protein degradation. mTOR is a major protein kinase that modulates translation initiation components. The phosphorylation of mTOR activates the kinase and is stimulated by both insulin and AA. A detailed review explaining regulation and structure of the mTOR complex is given by Li *et al.* (2011). Activation of the mTOR complex results in increased protein synthesis rates and cell proliferation and therefore promotes growth and regeneration.

On the other hand, Leu regulates the catabolism of all three BCAA. To balance the body’s requirement for BCAA with the supply of dietary BCAAs, the BCAA catabolic pathway is highly regulated. In consequence, the BCAA are cleared efficiently when dietary intake is in excess and conserved when dietary intake is inadequate. The first step in the BCAA catabolism is the transamination catalyzed by the branched-chain aminotransferase (BCAT) isoenzymes. In this reaction, N is transferred from a BCAA to α-ketoglutarate to form Glu and the associated BCKA. Leucine is transaminated to KIC, Ile to α-keto β-methylvalerate (KMV) and Val to α-keto isovalerate (KIV). The transamination reaction catalyzed by BCAT is reversible. In the second step, the BCKA are oxidatively decarboxylated by the mitochondrial, multienzyme BCKDH complex. This step is irreversible, highly regulated and rate limiting for BCAA catabolism. A detailed review explaining regulation and process of the BCAA catabolism and structure of the involved enzymes is given by Hutson *et al.* (2005). Tight control of the BCKDH complex activity is important for conserving as well as disposing of BCAAs, because BCAAs are required for synthesis of proteins, branched-chain fatty acids and neurotransmitters. Furthermore – it is known from diseases related to the BCAA metab-

olism that - accumulation of non-catabolized BCAA in blood and tissues may cause neurotoxic effects in human and animals (Strauss and Morton, 2003; Hutson, 2006; Scaini *et al.*, 2013).

Study III aimed to elucidate tissue-specific responses of BCKDH activity and AA derived metabolites in response to diets that contain 2- and 4-fold higher Leu amounts than required. Data showed that pigs placed on high-Leu diets consumed less feed, which may explain the decline in growth observed in these animals. An increase of the BCKDH activity in nearly all tissues except skeletal muscle and adipose tissues in response to the high-Leu diets was observed. Interestingly, the rate of increase in BCKDH activity to excessive Leu consumption was highest in brain (2.5-fold). The stimulated BCKDH activity in line with increased Leu concentration in brain of pigs fed the high-Leu diets provides evidence for an elevated transport of Leu across the blood-brain barrier. Since excessive Leu in brain is associated with neurological dysfunctions (Strauss and Morton, 2003; Hutson, 2006; Scaini *et al.*, 2013), the observed rise in cerebral BCKDH activity is presumably a protective mechanism to prevent Leu-associated neuronal disorders.

BCAA and aromatic AA such as Trp share the same brain transporters (Henry *et al.*, 1992; Nicklin *et al.*, 2009). Thus, it may be hypothesized that cerebral Trp concentration declines in response to an excessive Leu consumption. Since BCAA comprise not only of Leu but also of Ile and Val, all three BCAA were analyzed and it was found that pigs fed the high-Leu diets had increased plasma concentrations of Leu but reduced concentrations of Ile and Val. The reduction of Ile and Val upon high Leu-diets is a well-described phenomenon (Langer *et al.*, 2000; Wiltafsky *et al.*, 2010). It is partly caused by the stimulated BCKDH activity, which in turn degrades not only Leu but also Ile and Val. Calculating the total BCAA concentration, the L4 group had higher plasma BCAA levels and also a higher plasma BCAA:Trp-ratio than the control and L2 group. To investigate possible mutual interferences between BCAA and Trp in terms of cerebral uptake, a set of AA in brain was analyzed for the first time in this context and reduced concentrations of cerebral Val, Ile and Trp were found in the L4 group compared to the control and the L2 group. This confirms the assumption that an excessive Leu consumption may impair the cerebral uptake of Trp (Pérez-Cruet *et al.*, 1974) by concrete data. Trp is a precursor of serotonin. In brain, the formation of serotonin largely depends on the availability of cerebral Trp, since serotonin is not capable of crossing the blood-brain barrier (Berger *et al.*, 2009). ANOVA analysis revealed a significant impact

of dietary Leu on cerebral serotonin concentration. However, one limitation of cerebral serotonin analysis in the present study was that serotonin was quantified in a large part of the brain and not in specific cerebral regions, which comprises serotonin-synthesizing neurons. This may have caused the failing statistical difference in cerebral serotonin between the L4 group and the control group, although the L4 group had on average 26% lower cerebral serotonin concentrations than the control group. Nonetheless, this new data (showing clear changes in the composition of cerebral AA, in particular in the concentrations of BCAA and Trp, by Leu excess) are an important issue for livestock-, as well as for human science, because of following aspects: (1) therapeutic doses of Leu used to prevent muscle atrophy in hospitalized humans are within the ranges used in **Study III** (DeBandt and Cynober, 2006; Nicastro *et al.*, 2011), (2) Leu is often used in high amounts by athletes to stimulate protein synthesis in muscles and (3) Leu supplementation is discussed as strategy to improve performance and muscle protein synthesis in livestock production (Escobar *et al.*, 2005; Yin *et al.*, 2010). Although, only 1% of the body's total serotonin is detected in the brain, it has important functions in this part of the body including the control of appetite, sleep-wake rhythm, memory, temperature regulation and behavior (reviewed by Marston *et al.*, 2011). Low levels of cerebral serotonin are associated with aggressive and angry behaviors, clinical depression, Parkinson's disease, eating disorders, migraine, irritable bowel syndrome, tinnitus and bipolar diseases in humans (reviewed by Berger *et al.*, 2009; Olivier, 2015). Those possible adverse effects of high-Leu diets should be considered with the development of strategies to prevent muscle cachexia in patients and to improve growth performance in livestock.

Besides reduced cerebral serotonin, the plasma concentrations of serotonin in pigs fed the high-Leu diets were low. Plasma serotonin is normally derived from peripheral serotonin producing cells, whereby 90% of the body serotonin is suggested to be produced by enterochromaffin cells of the gut (Gershon, 2013). It could be speculated that excessive Leu may also impair the Trp uptake into enterochromaffin cells, thereby lowering serotonin synthesis in these cells. Having in mind the multiple functions of peripheral serotonin as regulator of bone mass (Karsenty and Yadav, 2011; Luiking *et al.*, 2014), platelet coagulation (Li, 1987), liver regeneration (Lesurtel *et al.*, 2006) and its function in the gastrointestinal tract (Lesurtel *et al.*, 2008), the observed reduction of peripheral serotonin can be considered as a further adverse effect of excessive Leu consumption. Not only Trp-derived serotonin regulates gastric emptying, intestinal peristalsis, intesti-

nal secretion, colonic tone and pancreatic secretion (Berger *et al.*, 2009). Melatonin is also a Trp-derived neurotransmitter involved in signaling for synchronizing the ingestion and digestion processes (Bubenik *et al.*, 1996). It is likely that the observed effects for peripheral serotonin will be relevant for melatonin in addition, whether this neurotransmitter was not in focus of **Study III** and therefore not analyzed. Serotonin is a neurotransmitter, which is also involved in appetite regulation. In pigs, high consumption of Trp significantly increased the feed intake in these animals (Henry *et al.*, 1992; Eder *et al.*, 2001; Ettle and Roth, 2003) which is presumably attributable to the role of Trp as precursor for serotonin. However, based on the present data, it could be assumed, that serotonin was not the reason for the observed feed intake reduction in the L2 and the L4 group compared to the control group. Firstly, no clear effect of the high-Leu diets on cerebral serotonin was found. Secondly, the reduction of feed intake was not only observed in the L4 group, but also in the L2 group that showed no reduction in cerebral Trp compared to the control pigs. The appetite reduction could be partly caused by the activation of hypothalamic mTOR which in turn leads to a stimulation of anorectic signals (Cota *et al.*, 2006; Li *et al.,* 2011). However, an explanation more likely is given by Hao *et al.*, 2005: An AA deficiency leads to accumulation of uncharged tRNA in the anterior piriform cortex (APC), inducing a reduction of feed intake to prevent degradation of protein in the brain. The ability of excess Leu to cause deficiencies of Val, Ile and other LNAA in blood plasma and tissues was clearly shown in **Study III**.

Leu is a ketogenic AA that could be degraded by BCKDH directly to acetyl-CoA. To test whether the high-Leu diets could modify the ketone body synthesis, plasma concentrations of 3-hydroxybutyrate were successfully analyzed as a new approach in research of piglet nutrition. It was found that pigs of the L4 group had 60% higher levels than those of the control and L2 group. Plasma ketone bodies are known to reduce appetite (Johnstone *et al.*, 2008). Since Km values of ketone body transporters are in the millimolar range (Halestrap, 2012), it could be assumed that the increase of 3-hydroxybutyrate observed in **Study III** is not high enough to pass the blood-brain barrier and to modulate appetite. However, it should be noted that any increase of circulating ketone bodies could contribute to the development of hyperuricemia and gout via competition for the same renal transporter as uric acid. The in **Study III** found increased plasma levels of the Leu-derived KIC in pigs fed the high-Leu diet is likely caused by the stimulation of BCAT, the first enzyme in BCAA catabolism. The increase of plasma KIC was accompanied by a decline of the transamination products of Val and Ile, KIV

and KMV, and confirms recent findings of a study in which pigs were fed a high-Leu diet (Wiltafsky *et al.*, 2010). Here, it was found that pigs of the L2 and the L4 group had comparably low plasma concentrations of Val and Ile and their corresponding BCKA, although a tissue-specific increase in BCKDH activity was primarily seen in the L4 but not in the L2 group. However, in liver, the BCKDH activity showed a dose-dependent increase in response to the amount of consumed Leu. Likely the liver BCKDH activity in particular, caused the reduction of plasma Val and Ile in the L2 pigs.

In contrast to the action of BCAT, the decarboxylation of the BCAA by BCKDH is an irreversible step that leads to a loss of available BCAA for protein synthesis. Thus, BCKDH activity could be used as a biomarker to assess the impact of excessive Leu supply on BCAA metabolism. Here we found marked tissue-specific differences of BCKDH activity in the growing pig with the highest basal activity in the pancreas, followed by kidney, liver, cardiac muscle, brain, skeletal muscle and adipose tissue when feeding a control diet. Since **Study III** was the first study, where several porcine tissues were analyzed for their basal BCKDH activity a comparison to previous studies could only be given for liver and muscle tissues. The range of both tissue activities is in line with previous studies of Langer *et al.* (2000) and Wiltafsky *et al.* (2010). Both studies showed similar values for the activities of BCKDH in liver and muscle in pigs. In order to elucidate whether pigs could be used as a human model to study the impact of high-Leu diet the present data should be compared with human data. In 1998, Suryawan *et al*. published data on the tissue-specific BCKDH activities of humans, African green monkey and rats and found marked differences between their BCKDH activity patterns (Suryawan *et al.*, 1998). It must be taken into account that a direct comparison between the data of Suryawan *et al.* (1998) and data of **Study III** is not possible because both studies were conducted independently from each other. Although it is striking that the tissue-specific BCKDH activities of pigs resemble human activities but not those of rats. Therefore, it is likely that pigs may be an appropriate model to study the impact of excessive Leu on human metabolism.

5.2.3 Study IV – Considerations of interaction effects among leucine and threonine on feed efficiency, plasma amino acid profile and hepatic branched-chain amino acid catabolism

Study IV aimed to elucidate interaction effects of Leu and Thr on growth performance, N retention, plasma AA and tissue specific BCKDH activity in liver and muscle tissue of weaned piglets. The rationale for this study was the finding of House *et al.* (2001), that Thr could be in part metabolized by an alternative pathway which is catalyzed by BCKDH. In **Study IV** it was found that the growth performance remained unaffected by the different dietary levels of Leu and Thr. Recent literature gives no evidence on beneficial effects of Thr on growth performance. However, 57% SID Thr:Lys is a lower level of Thr than recommended by several institutions and likely insufficient to maintain growth. Consequently, the increase of the Thr level to 65% may improve growth. An improvement of feed intake was observed in tendency with increased Thr in presence of adequate Leu intake (P=0.066). Based on the findings of **Study II** and **Study III** it was assumed that even Leu:Lys 200% would degrade the voluntary feed intake and therefore the weight gain. In **Study IV** the ratio of SID Leu:Lys 200% was not sufficient to elucidate adverse effects of Leu supplementation on growth performance, especially the feed intake. The daily feed intakes of the experimental pigs were striking high compared to other trials and compared to practical conditions. Even the BCKDH activity of liver or muscle tissue remained unaffected by dietary AA supplementation. It may be assumed that SID Leu:Lys 200% is not sufficient to increase the hepatic or muscular BCKDH activity in any case. The activity range of the three different tissue types was approximately in line with the range of the BCKDH activity of **Study III** and (Langer *et al.*, 2000; Wiltafsky *et al.*, 2010). Although it must be taken into account that a direct comparison between different studies is not possible since the analysis of BCKDH activity is a sensitive method and only samples which were treated within one batch could be compared directly. Nevertheless, the consideration of BCKDH in this content was important, since BCKDH could also be involved in the metabolism of Thr. Threonine has three independent enzymatic pathways involving (1) the Thr dehydrogenase, (2) the Thr dehydratase and, (3) the Thr aldolase (Ballevre *et al.*, 1990; Le Floc'h *et al.*, 1995; House *et al.*, 2001). In pigs, the Thr dehydrogenase is the main enzyme responsible for Thr degradation (Ballevre *et al.*, 1990). This enzyme catalyzes the oxidation of L-Thr to 2-amino-3-ketobutyric acid which is spontaneously decarboxylat-

ed to give aminoacetone or glycine and acetyl CoA when the Thr dehydrogenase is coupled with 2-amino-3-ketobutyrate CoA ligase (Le Floc'h *et al.*, 1997). The ability of the BCKDH complex to degrade also Thr metabolites indicates a connection of Thr to the BCAAs. Since Thr is metabolized by 87% trough the Thr dehydrogenase pathway and only by 10% trough the Thr dehydratase pathway, which is coupled to BCKDH, no connection was found concerning Leu and Thr in **Study IV**. However, it could be speculated that the amount of dietary Leu in the high-Leu diets was not sufficient to stimulate the BCKDH activity up to a level that provokes an increased degradation of Thr.

In **Study IV** the plasma concentrations of Thr were increased only by Thr supplementation, but not decreased by dietary Leu surplus. The high supplementation of Leu caused an increase in plasma levels of Leu and a decrease in plasma Val and Ile as seen in **Study III**. Two-way ANOVA revealed no significant effect of Thr on plasma BCAA levels. However, Thr seems to weaken BCAA imbalances induced by excessive Leu intake, since plasma concentrations of Val and Ile decreased less with high-Thr and high-Leu compared to low-Thr and high-Leu in **Study IV** ($P<0.1$; Table 15). A Thr deficiency decreases the absolute amount of intestinal mucin, resulting in impaired resistance to enteric infection (Montagne *et al.*, 2004). In turn, this affects nutrient uptake by the intestine. Consequently, the uptake of AA may be improved by Thr induced benefits for the intestinal mucin.

Imbalanced AA profiles impair N utilization and accordingly contribute to increased N output by urine and feces (Chung and Baker, 1992). However, the reduction of plasma Val and Ile of approximately 50% in the high-Leu groups of **Study IV** seems not to be sufficient to show this effect. The N output did not differ due to dietary Leu supply and the resulting plasma concentrations of Val and Ile. Unchanged activities of BCKDH in this trial could be used to interpret this result. It could be assumed that the reduction of plasma Val and Ile of the high-Leu treated pigs was caused by competition for physiological transporters in the small intestine (which was partly compensated by high-Thr) and probably by the first step of the common metabolism of the BCAA. To underline this hypothesis it would be interesting to analyze the plasma concentrations the BCKA in this trial. The lack of those data is a limitation of **Study IV.** An increased activity of BCKDH would have caused higher N-outputs in the high-Leu groups.

5.2.4 Study V – Considerations of interaction effects among leucine and tryptophan on appetite and appetite-regulating molecules

Based on the findings of **Study III**, that dietary Leu excess decreases feed intake by depletion of Trp in the brain and in consequence reduced serotonin concentrations in brain tissue, **Study V** was conducted. This study aimed to elucidate interaction effects of Leu and Trp on growth performance, appetite, appetite-regulating molecules as mTOR and serotonin in the hypothalamus, concentrations of free AA in plasma and hypothalamus and the decarboxylation of the BCAA by BCKDH in pancreas and liver. It was confirmed that piglets fed the high-Leu diet (Leu300 groups) consumed less feed than pigs fed the Leu100 diets in accordance to previous Leu excess studies (**Study III**; Wiltafsky *et al.*, 2010; Millet *et al.*, 2015). Lower final BW and a trend toward lower weight gain accompanied the reduced feed intake of piglets fed the Leu300 diets. Analyses reveal that piglets treated with the Leu300 diets had more than two-fold higher concentrations of Leu in plasma and 30 to 44% higher Leu concentrations in hypothalamus than the Leu100 groups. In contrast, plasma and hypothalamic concentrations of Val and Ile decreased in response to the high-Leu diets as already observed in **Study III.** Analyses reveal that pigs fed the Trp23 diets had markedly higher plasma concentrations of BCAA and Phe, than pigs fed Trp18 diets. However, hypothalamic concentrations of BCAA and Phe were not altered by Trp. A similar effect in blood plasma due to higher administration with Thr was observed in **Study IV**. However, **Study V** elucidated additional effects of the Leu excess on hypothalamic concentrations of Met. Concentrations of Met were markedly decreased in the Leu300 groups. This new findings show that dietary Leu excess influences not only the availability of the BCAA but also the availability of other essential AA for brain protein synthesis. Similar to Thr, Met has an alternative degradation pathway related to the BCKDH complex. The alternative transamination pathway is catalyzed by BCKDH which raises the possibility that the interactions among the BCAA may extend to the metabolism of Met (Mitchell and Benevenga, 1978). The Leu300 diets reduced the plasma and hypothalamic concentrations of Trp, although this effect was mainly observed in the Trp23 group. Beyond the effects of Trp described in **Study III**, Trp is also known to influence the voluntary feed intake firstly, via gastrointestinal hormones: Oral Trp ingestion increases plasma ghrelin level and ghrelin expression in gastric fundus (Zhang *et al.*, 2007). Furthermore it is suggested that the neurohormone melatonin, which is produced from Trp in the gastro-

intestinal tract, may serve as a signal for synchronizing the ingestion and digestion processes (Bubenik *et al.*, 1996). Secondly, Trp supplementation has been shown to induce a significant increase in feed intake which is attributable to the role of Trp as precursor for serotonin (Henry *et al.*, 1992). Since Trp serves as a precursor for serotonin, it was tested whether Trp reduction in brain is accompanied by a reduced synthesis of serotonin which is a significant stimulator of feed intake in pigs (Henry *et al.*, 1992). Here it was found that piglets fed the Leu300 diets had lower hypothalamic serotonin concentrations than those fed the Leu100 diets. Since plasma and hypothalamic Trp concentrations correlated positively with the feed intake, and hypothalamic Trp correlated positively with hypothalamic serotonin, it could be assumed that excessive intakes of Leu induces appetite suppression – at least, besides plasma imbalances of AA – via a reduction of available cerebral Trp for serotonin synthesis. It was assumed that the diminished feed intake upon excessive Leu as observed in **Study V** may also be associated the key role of Leu as mTOR stimulator in the hypothalamus. In hypothalamus activated mTOR suppresses appetite by down-regulation of orexigens and up-regulation of anorexigens (Cota *et al.*, 2006; Ropelle *et al.*, 2008; Li *et al.*, 2011). Both, mTOR and serotonin mediated appetite control is regulated by interaction with pro-opiomelanocortin (Cota *et al.*, 2006; Berger *et al.*, 2009). Since no effect of oral administration with high Leu amounts in the Leu300 groups on mTOR protein expression could be demonstrated, it may be concluded that higher amounts are required to see effects on feed intake mediated by mTOR signaling in brain tissue of pigs. Otherwise, it was observed during the analyses, that high standard deviations hampered a reliable statistical evaluation of the mTOR expression data. A reason may be the lipid-rich composition of the hypothalamus. This condition provides a limitation of **Study V**.

In **Study V** the activity of pancreatic and of hepatic BCKDH was analyzed. The increased hepatic activities in response to dietary Leu excess were in accordance to **Study III** and Wiltafsky *et al.* (2010). Herewith the findings of **Study III** were confirmed since the pancreatic tissue showed again higher activities than liver. Those activities refer to specified amounts of tissue (150 mg). However, it should be noted that the activities has to be related to total tissue weight and it would be interesting to compare the both tissues again with a calculation of the total tissue activity. It is likely that total liver activity exceeds pancreatic activity, since liver has a higher total weight.

This repeated observation in pigs' pancreatic tissue suggests that the pancreas is underestimated in its role in the BCAA metabolism, since the liver is assumed to be the main

tissue for BCAA/AA metabolism (Hutson, 1989). On the basis of a study in rats it was documented that artificially induced pancreatitis leads to a significant increase (P=0.001) of plasma BCAA, but also other AA (Zöch *et al.*, 1982). The amount of the plasma AA increase is comparable to increases associated with liver failure. In case of excessive AA consumption the pancreas shows faster uptake and higher enrichment of AA than liver (Le Floc'h *et al.,* 1997), as seen in **Study V** where AA concentrations of liver and pancreas were compared. The study of Zöch *et al.* (1982), Le Floc'h *et al.* (1997) and the high pancreatic BCKDH activities determined in **Study III** and **Study V** lead to the assumption that liver is not the only main tissue for AA metabolism and the role of the pancreas in AA metabolism should be investigated further.

To conclude, the presented data show that excessive intake of Leu was accompanied by a significant increase in hypothalamic Leu and a decrease in Trp-derived hypothalamic serotonin. Thus, it can be assumed that the Leu induced appetite suppression was partially due to a reduction of available cerebral Trp for serotonin synthesis, due to AA imbalances in plasma and brain tissue, rather than mTOR signaling in the hypothalamus.

5.3 Dietary Leucine excess decreases voluntary feed intake and overall growth performance – Considerations beyond the present results

Dietary Leu excess induces a Trp depletion in brain tissue. Consequently, the synthesis of the (in pigs) orectic neurotransmitter serotonin is reduced and the voluntary feed intake is affected negatively. However, another reason for the observed decline in feed intake upon excessive Leu in **Study III** and **Study V** is the stimulated degradation of BCAA via increased BCAT and BCKDH activities which in turn induces a deficiency of Val and Ile for protein synthesis (Wiltafsky *et al.*, 2010). Even Gloaguen *et al.* (2012) and Millet *et al.* (2015) documented decreased feed intakes of pigs fed a Val deficient diet. Generally, with a normal feeding regime the plasma concentration of Val is the highest of all BCAA, followed by Leu and Ile. This observation was made in **Study III**, **Study IV** and **Study V** as well as in literature (Langer *et al.*, 2000; Melchior *et al.*, 2004; Wiltafsky *et al.*, 2010). However, SID Leu:Lys 150% seems to be enough to shift the proportion in favor of Leu (Wiltafsky *et al.*, 2010). Val and Ile decreased inversely proportional to Leu concentrations. Feeding imbalanced AA profiles result in poor feed consumption (Chung and Baker, 1992; Gloaguen *et al.*, 2013a; Millet *et al.*, 2015). A

deficiency of dietary protein or single AAs is demonstrated to impair growth performance, immune function and increase the sensitivity of pigs to infectious challenges or stressful conditions (Le Floc'h *et al.*, 2004). Furthermore imbalanced AA profiles impair N utilization and accordingly contribute to increased N output by urine and feces (**Study IV,** Chung and Baker, 1992). Primarily, the impaired growth performance results from a reduced feed intake. However, when the dietary supply of essential AA is low, oxidation of these acids seems to be reduced in order to preserve their use for protein synthesis. This was shown in pigs which seem to be able to conserve AA such as Leu, His or Phe when these AA were deficient in the diet (Le Floc'h *et al.*, 1995). Even if several AA could be conserved, a chronically administration to imbalanced diets results in poor feed efficiency and in worst case in physiological dysfunctions. The reduced availability of essential AA as substrates for protein synthesis causes impaired muscle growth and a reduced synthesis of regulatory proteins. One clear result of the present studies is that excessive intake of Leu changes both plasma AAs and tissue AA. AA stimulate or inhibit the release of some pituitary, pancreatic and gastrointestinal hormones. Gln and Leu also increase insulin release from the pancreatic β-cells (MacDonald *et al*., 1991). AA induce the secretion of gastrointestinal hormones such as glucagon like peptide-1, cholecystokinin, melatonin and ghrelin (Xu *et al*., 2001). Growth hormone (GH) and insulin-like growth factor-I (IGF-I) control growth and the GH/IGF-I axis plays an essential role in the regulation of body growth and composition, as well as cellular proliferation and differentiation processes (Wiltafsky *et al*., 2010). The growth-inhibitory effect of AA deficiencies might be caused in part by impaired action of GH/IGF-I axis. Specific AA appear to be important in the GH/IGF-I axis. Single depletion of Thr, Trp, Val, arginine or proline caused a block of GH-stimulated IGF-I gene expression in cultured pig hepatocytes (Brameld *et al.*, 1999). The GH and IGF-I in plasma are sensitive to changes in nutritional status. Many studies have indicated that growth depression induced by undernutrition is usually followed by an increase of GH and a decrease of IGF-I in plasma of pigs (Guay and Trottier, 2006). The growth depression observed in the high Leu diets of **Study III** might be partially caused by decreased activity of the GH/IGF-I axis as a result of Leu-induced AA deficiency (especially deficiency of Val and Ile). This finding is according to (Wiltafsky *et al.*, 2010).

Besides this, pigs are capable of sensing AA imbalances which in turn leads to changes of feed selection or a reduction of feed intake (Ettle and Roth, 2003; Gloaguen *et al*., 2012). In the classical behavioural test for AA deficiency, animals detect and reject a

diet lacking an IAA; they are capable to react within 20 min of the onset of feeding (Gietzen *et al.*, 2007). This effect is autonomous from olfactory, taste, or other peripheral systems (Rudell *et al.*, 2011). The APC is the behaviourally relevant chemosensor for essential AA depletion, projecting to neural circuits that control feeding (Hao *et al.*, 2005). The APC is located in the anterior ventro-lateral forebrain. Concentrations of the limiting essential AA are decreased in the APC shortly after pigs started eating the deficient diet. In the earliest steps leading to the initiation of mRNA translation, AAs are acylated (charged) to transfer ribonucleic acid (tRNA) by their cognate amino acyl tRNA synthetases. In case of essential AA depletion, the cognate tRNA may be deacylated (uncharged). An AA deficiency leads to accumulation of uncharged tRNA in the APC. Subsequent cellular adaptation to AA deprivation is marked by decreases in global protein synthesis complemented by increased transcription of genes related to AA synthesis (Hao *et al.*, 2005; Hao *et al.*, 2010). For pigs, sensing a deficiency and neural signalling necessarily precede appropriate diet selection, which must occur before the remaining AA are metabolized and lost (Hao *et al.*, 2005). Gietzen and Rogers (2006) reported that degradation of protein in brain begins within 2 h in animals fed a diet missing a single indispensable AA. Therefore, this mechanism exists to prevent negative effects in brain caused by AA deficiency. mTOR is not involved in sensing AA deficiency (Hao *et al.*, 2010).

Due to the negative effects of high Leu intake, the suggestion to improve performance in livestock production by high Leu supplementation of young pigs (Escobar *et al.*, 2005; Yin *et al.*, 2010) has to be applied with caution: with **Study II** and **Study III** it was shown by use of growth performance parameters, that the anabolic effect of high Leu intake diminishes with advancing age. The high protein-synthesis rates of pre- and neonatal pigs, induced by Leu, are unique for this state of live. Both present studies started one week after weaning after an adaption period of one week and in both studies no higher weight gain was observed. However, the study of Escobar *et al.* (2005) was performed with neonatal pigs before weaning. Yin *et al.* (2010) used weaned piglets in the age of 21 days. In this study, the experimental period was entirely eleven days. In **Study V** the mTOR protein expression in brain was determined. However, no significant effect of high Leu supplementation on hypothalamic mTOR was found, although, activation of mTOR increased with higher AA supplementation. But nevertheless, the studies of Escobar *et al.* (2005) and Yin *et al.* (2010) cannot be applied for pigs older than those used in their studies. It could be assumed that those findings are important

for formulation of weaning feed addressed to piglets between the 21st and the 40th day of life.

The present work and literature data show that dietary AA imbalances/ deficiencies result not exclusively in an impaired growth performance; they also change the AA compositions of several tissues and have impact on numerous metabolic processes. Even if the present studies did not focus on the immune system, the current literature gives some evidence that AA play an important role in the regulation of the intestinal health and overall immune response (Montagne *et al.*, 2004). Additionally it is known that imbalanced diets cause an impaired N utilization, which has to be prevented to protect the environment against pollution from intensive pig farming. This accumulation of negative effects of imbalanced AA profiles on pigs' performance, physiology and environment elucidates the importance of feeding piglets as close as possible to their AA requirement. Since Leu is the AA with the most known physiological functions, this AA has to be considered in particular.

5.4 Further relevance for human and animal science

As obesity increases in the western civilizations, human researchers are discussing strategies to prevent or to treat this disease. Increasing intake of BCAA is assumed to assist in maintaining a healthy body weight (Qin *et al.*, 2011). Possible proposed mechanisms of BCAA on obesity including the effect of Leu supplementation on the anti-hunger hormone leptin (Lynch *et al.*, 2006), as well as the ability of BCAA to participate in the control of blood glucose. Moreover, the cross-regulation between mTOR and adenosine monophosphate-activated protein kinase (AMPK), influenced by dietary Leu contributes to the positive effects of Leu in human body weight. In clinical studies it was shown that therapeutic doses of Leu are a valid strategy to prevent muscle atrophy (DeBandt and Cynober, 2006; Nicastro *et al.*, 2011).

Some evidence is given that the BCAA metabolism is linked to human type 2 diabetes: the BCAA catabolism in type 2 diabetes is downregulated and enhanced by BCAA supplementation (Kuzuya *et al.*, 2008). Leu is known to stimulate insulin release from pancreatic β-cells (MacDonald *et al.*, 1991; Xu *et al.*, 2001). Activation of the mTOR pathway by prolonged insulin stimulation or increased AA availability induces insulin

resistance in muscle cells (Xin *et al.*, 2013). Some of the studies concerning diabetes were already performed using pigs as animal models.

Another disease related to the BCAA metabolism is the maple syrup urine disease (MSUD) – an autosomal recessive metabolic disorder which results from mutations in one or more proteins in the BCKDH complex (Harris *et al.*, 1990; Strauss and Morton, 2003). MSUD is characterized by elevated concentrations of plasma and tissue BCAA inducing oxidative stress (Scaini *et al.*, 2013). Consequences are demyelination, inhibition of brain energy metabolism and apoptosis. Chronic exposure in the brain to high concentrations (190 mmol/L Leu, 59 mmol/L Ile and 69 mmol/L Val in saline solution) of BCAAs induce an increase in concentrations of brain-derived neurotrophic factor (BDNF; Scaini *et al.*, 2013). This increase of BDNF, which causes impairment of the spartial memory and the oxidative stress are assumed to be the main reasons for brain damages related to MSUD. However, BDNF regulates the expression of cytosolic BCAT in the brain (Castellano *et al.*, 2006). It can be assumed that the relation of BDNF and cytosolic BCAT expression is a protective mechanism to prevent the brain against toxic BCAA concentrations due to increased catabolism by cytosolic BCAT. A significant accumulation of Leu caused by excessive intake in cortical and hypothalamic tissue was observed in **Study III** and **Study V**, respectively. The main symptomatology presented by MSUD patients includes ketoacidosis, failure to thrive, poor feeding, apnea, ataxia, seizures, coma, psychomotorical delay and mental retardation (Strauss and Morton, 2003). Since **Study III** demonstrated the proximity of human and pig concerning the BCAA metabolism, it could be assumed that the pig is an appropriate model to generate more knowledge about BCAA metabolism related diseases as MSUD or diabetes.

5.5 Conclusions

In scope of the present work five animal studies were conducted to elucidate interactions of Leu to other LNAA and the resulting impact on the physiology of piglets. The data collected enable a comprehensive description of these relationships by use of the previous formulated hypotheses:

(I) ”*A nutritional Leu excess shifts physiological AA ratios in blood plasma and several body tissues to unbalanced levels and therefore changes metabolic pro-*

cesses in a negative way. This is particular the case in brain tissue where physiological AA imbalances are intensified by transport competition of the LNAA across the blood-brain barrier."

Study III and **Study V** demonstrated clearly the shifts of physiological AA ratios in blood plasma and several body tissues to unbalanced levels. A dietary Leu excess causes at least a deficiency of Val and Ile for protein synthesis. However, also other LNAA could be interfered. The activity of BCKDH was analyzed as indicator for an increased BCAA metabolism and a response of BCKDH to increasing dietary Leu was demonstrated in **Study III** and **Study V**. Particularly striking here was the high activity of pancreatic BCKDH. So far it was not known that the pancreas plays a significant role in the metabolism of AA. The finding of increased BCKDH activities was confirmed by the significant increased plasma concentrations of the ketone body 3-hydroxybutyrate in the Leu excess groups of **Study III**. The findings made in brain tissue confirms **Hypothesis I**: A dietary Leu excess enters the brain! The greater affinity of the brain transporters for Leu (sevenfold higher) than other LNAA causes impaired transport of Val and Ile when Leu is in excess. A reduction of brain Val and Ile around 50% was demonstrated, while the concentrations of Leu increased and stimulated cortical BCKDH in **Study III**. The percentage stimulation of BCKDH activity was highest in the brain (2.5-fold) compared to all other tissues, since AA imbalances could cause irreversible damage to the brain.

(II) *"A nutritional Leu excess reduces synthesis of the neurotransmitter serotonin in brain tissue due to a limitation of Trp uptake across the blood-brain barrier."*

High levels of Leu in the brain tissue are accompanied with low concentrations of the serotonin precursor Trp since both LNAA share the same transporters at the blood-brain barrier and those transporters have an affinity for Leu. The results of **Study III** and **Study V** showed significant reduction in brain Trp in presence of excessive Leu intakes. As expected the serotonin synthesis was significantly reduced by lack of its precursor AA Trp which confirms **Hypothesis II**. This side effect has to be taken into account, because serotonin is a beneficial neurotransmitter. Furthermore the principle shown with serotonin in the present studies applies to other metabolites of other LNAA additionally.

(III) *"Oral administration of Leu in doses up to 0.9 g/kg BW/d (SID Leu:Lys 400) is not sufficient to increase the metabolism of Thr via its' alternative BCKDH-linked degradation pathway."*

Even if Thr concentrations in plasma and some tissues were altered in **Study III** and **Study V,** no confirmation of **Hypothesis III** could be achieved based on the present results. **Study IV** was strongly focused on possible interactions of Leu and Thr due to the common enzyme BCKDH. In **Study IV** a moderate dietary Leu excess (SID Leu:Lys 200%) could not increase the hepatic activity of BCKDH and did not result in altered plasma concentrations of Thr. Since no effect on the growth performance was observed it could be concluded that no single AA was degraded to a limiting level for protein synthesis. With the excessive levels of Leu used in in **Study III** and **Study V** it could be assumed that observed effects of Leu on Thr were based on the common and competitive transport of the LNAA.

Leucine is a central AA, which is involved in the regulation of feed intake, digestion, AA metabolism and protein synthesis. Since dietary Leu could affect the availability of other AA, with a simultaneous stimulating effect on protein synthesis, special attention has to be given to the requirement of pigs for Leu and the other BCAA. The dose-response study (**Study II**) resulted in a requirement slightly higher than the current recommendations (SID Leu:Lys 107.8%). Currently in European feedstuff large quantities of soy and corn as raw components are still used and cover or exceed the Leu requirement of growing pigs. It was shown in previous literature that addition of Val could prevent negative effects, caused by dietary Leu excess (Wiltafsky *et al*., 2010). Therefore, it is advisable, in particular, to consider pigs' requirement for Val and Ile in relation to Leu and to give the upcoming recommendations for both AA not exclusively to pigs first-limiting AA Lys.

Feeding pigs as close as possible to their AA requirement maximizes the full utilization of the genetic growth potential and simultaneously minimizes N output from pigs' production. With regard to the diverse immunoprotective effects of various AA and their interactions it seems likely that a feeding which can be precisely adapted to the needs of a defined age group, race and type of farming could be used preventively to minimize the use of antibiotics in European livestock farming. The emphasis here is primarily on the age group. Especially for Leu the requirement is strongly age-dependent. Neonates have an enormously increased mTOR capacity compared with piglets, which were held

in the present studies until the fore-fattening period. Therefore, it makes sense to further improve and apply the phase feeding which is already practiced. The distant future is consequently the Canadian concept of ”Precision Feeding”, where the daily requirement is taken into account.

Apendix

List of Tables

Tables

Table A1: Aminogram of feeds used in Study I (Estimation of a sub-limiting level of lysine as reference amino acid)[1]

	Diet			
	L1	**L2**	**L3**	**Control**
Crude protein (%)	16.2	15.8	16.1	19.1
Amino acids (%)				
Alanine	0.60	0.61	0.63	0.78
Arginine	0.72	0.73	0.70	1.03
Cystine	0.25	0.24	0.24	0.30
Glutamic acid	4.97	4.28	3.24	3.84
Glycine	0.54	0.54	0.54	0.72
Histidine	0.32	0.36	0.40	0.45
Isoleucine	0.57	0.62	0.71	0.77
Leucine	1.07	1.17	1.33	1.41
Lysine	0.97	1.11	1.29	1.29
Methionine	0.34	0.40	0.49	0.47
Phenylalanine	0.66	0.72	0.80	0.89
Serine	0.64	0.65	0.65	0.86
Threonine	0.64	0.72	0.83	0.88
Tryptophan	0.22	0.24	0.27	0.29
Tyrosine	0.44	0.50	0.56	0.61
Valine	0.74	0.82	0.93	1.01

L1: calculated 16% CP with SID Lys=0.86%; **L2**: calculated 16% CP with SID Lys=1.02%; **L3**: calculated 16% CP with SID Lys=1.16%; **Control**: calculated 19% CP with SID Lys=1.16%
CP= Crude protein; SID=standardized ileal digestible; Lys=Lysine
[1] Data are presented as means from two measurements

Table A2: Aminogram of feeds used in Study II (Estimation of the Leucine requirement by use of three statistical models)[1]

	Diet (SID Leucine:Lysine)				
	83%	**94%**	**104%**	**115%**	**125%**
Crude protein (%)	14.5	14.7	15.0	14.8	14.9
Amino acids (%)					
Alanine	0.54	0.54	0.54	0.53	0.52
Arginine	0.62	0.61	0.61	0.60	0.60
Cystine	0.23	0.24	0.23	0.23	0.232
Glutamic acid	3.96	4.08	4.05	4.03	4.01
Glycine	0.48	0.47	0.47	0.47	0.46
Histidine	0.32	0.33	0.33	0.32	0.32
Isoleucine	0.59	0.62	0.61	0.61	0.60
Leucine	0.96	1.04	1.14	1.23	1.30
Lysine	0.97	0.99	0.99	0.99	0.97
Methionine	0.36	0.37	0.38	0.38	0.37
Phenylalanine	0.64	0.65	0.64	0.65	0.64
Serine	0.57	0.56	0.56	0.56	0.56
Threonine	0.65	0.66	0.67	0.66	0.66
Tryptophan	0.24	0.25	0.25	0.25	0.25
Tyrosine	0.50	0.51	0.51	0.51	0.51
Valine	0.75	0.78	0.78	0.77	0.76

SID=standardized ileal digestible; Leu=Leucine; Lys=Lysine
[1] Data are presented as means from two measurements

Table A3: Aminogram of feeds used in Study III (Characterization of tissue-specific basal activities of the branched-chain α-keto acid dehydrogenase – BCKDH – in presence of adequate or excessive leucine consumption)[1]

	Diet		
	Control	**L2**	**L4**
Crude protein (%)	15.2	15.4	16.0
Amino acids (%)			
Alanine	0.56	0.56	0.54
Arginine	0.66	0.64	0.61
Cystine	0.24	0.24	0.24
Glutamic acid	4.26	3.73	2.94
Glycine	0.50	0.49	0.48
Histidine	0.34	0.35	0.35
Isoleucine	0.60	0.60	0.61
Leucine	1.09	1.97	3.75
Lysine	1.04	1.01	1.01
Methionine	0.37	0.37	0.38
Phenylalanine	0.63	0.63	0.62
Serine	0.58	0.58	0.57
Threonine	0.66	0.66	0.67
Tryptophan	0.25	0.25	0.26
Tyrosine	0.45	0.45	0.45
Valine	0.81	0.79	0.80

Control: calculated SID Leu:Lys=100; L2: calculated SID Leu:Lys=200; L4: calculated SID Leu:Lys=400
SID=standardized ileal digestible; Leu=Leucine; Lys=Lysine
[1] Data are presented as means from two measurements

Table A4: Aminogram of feeds used in Study IV (Considerations of interaction effects among leucine and threonine on feed efficiency, plasma amino acid profile and hepatic branched-chain amino acid catabolism)[1]

	Diet (SID Threonine:Leucine)			
	57:100	**57:200**	**65:100**	**65:200**
Crude protein (%)	15.6	15.7	15.5	15.5
Amino acids (%)				
Alanine	0.64	0.64	0.63	0.62
Arginine	0.72	0.73	0.72	0.71
Cystine	0.25	0.25	0.25	0.25
Glutamic acid	4.30	3.33	4.11	3.19
Glycine	0.54	0.54	0.54	0.53
Histidine	0.35	0.35	0.35	0.35
Isoleucine	0.64	0.66	0.64	0.63
Leucine	1.12	1.90	1.26	1.95
Lysine	1.02	1.03	0.97	1.01
Methionine	0.38	0.39	0.37	0.37
Phenylalanine	0.71	0.71	0.70	0.70
Serine	0.66	0.65	0.65	0.65
Threonine	0.63	0.64	0.69	0.70
Tryptophan	0.24	0.24	0.23	0.23
Tyrosine	0.49	0.49	0.49	0.48
Valine	0.77	0.78	0.76	0.72

SID=standardized ileal digestible
[1] Data are presented as means from two measurements

Table A5: Aminogram of feeds used in Study V (Considerations of interaction effects among leucine and tryptophan on appetite and appetite-regulating molecules)[1]

	Diet (SID Tryptophan:Leucine)			
	18:100	**18:300**	**23:100**	**23:300**
Crude protein (%)	16.0	16.6	16.5	16.4
Amino acids (%)				
Alanine	0.61	0.62	0.62	0.62
Arginine	0.71	0.73	0.73	0.73
Cystine	0.27	0.27	0.26	0.27
Glutamic acid	4.72	3.34	4.62	3.12
Glycine	0.54	0.55	0.55	0.55
Histidine	0.36	0.37	0.37	0.36
Isoleucine	0.59	0.60	0.61	0.60
Leucine	1.10	2.54	1.33	2.80
Lysine	1.00	1.04	1.04	1.00
Methionine	0.38	0.38	0.39	0.38
Phenylalanine	0.64	0.66	0.66	0.65
Serine	0.63	0.65	0.64	0.64
Threonine	0.70	0.69	0.71	0.69
Tryptophan	0.18	0.18	0.24	0.24
Tyrosine	0.44	0.46	0.45	0.45
Valine	0.77	0.79	0.80	0.79

SID=standardized ileal digestible

[1] Data are presented as means from two measurements

Table A6: Effect of dietary leucine content on amino acid concentrations in pancreas of piglets (Study III)

Tissue amino acids (nmol/mg)[1]	Diet			*P*-value
	Control	L2	L4	
Alanine	2575 ± 367	2752 ± 894	3046 ± 1450	0.635
Glutamine	1649 ± 362	1479 ± 492	1368 ± 400	0.355
Glycine	4147 ± 1140^{a}	4336 ± 354^{a}	5673 ± 1602^{b}	0.015
Histidine	56 ± 13^{a}	75 ± 19^{a}	90 ± 24^{b}	0.001
Isoleucine	138±31^{b}	65±12^{a}	84±44^{a}	<0.001
Leucine	287±58^{a}	354±83^{a}	767±330^{b}	<0.001
Lysine	256 ± 57	304 ± 118	274 ± 78	0.478
Methionine	113 ± 12	117 ± 28	130 ± 22	0.193
Threonine	1117 ± 361	1670 ± 635	1202 ± 844	0.139
Tryptophan	32 ± 6^{a}	39 ± 7^{ab}	50 ± 14^{b}	0.002
Valine	361±54^{b}	174±32^{a}	182±48^{a}	<0.001

[1] Data are presented as means ±SD; n=10.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test or Games-Howell test; $P<0.05$).

Table A7: Effect of dietary leucine content on amino acid concentrations in liver of piglets (Study III)

Tissue amino acids (nmol/mg)[1]	Diet			*P*-value
	Control	L2	L4	
Alanine	5060 ± 777	4735 ± 1122	3902 ± 1190	0.063
Glutamine	4048 ± 543	3901 ± 428	4209 ± 1322	0.747
Glycine	4090 ± 585^{ab}	4188 ± 500^{b}	3639 ± 300^{a}	0.039
Histidine	378 ± 66	452 ± 84	480 ± 122	0.062
Isoleucine	234±31^{b}	187±30^{a}	161±22^{a}	<0.001
Leucine	511±61^{a}	674±128^{b}	875±222^{c}	<0.001
Lysine	244 ± 59	241 ± 46	204 ± 57	0.212
Methionine	137 ± 21	135 ± 19	137 ± 9	0.943
Threonine	832 ± 254^{ab}	1105 ± 281^{b}	767 ± 281^{a}	0.029
Tryptophan	56 ± 7	58 ± 9	56 ± 10	0.830
Valine	488±63^{b}	377±56^{a}	319±42^{a}	<0.001

[1] Data are presented as means ±SD; n=10.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test or Games-Howell test; $P<0.05$).

Table A8: Effect of dietary leucine content on amino acid concentrations in kidney of piglets

Tissue amino acids (nmol/mg)[1]	Diet			*P*-value
	Control	L2	L4	
Alanine	2395 ± 234[b]	2053 ± 236[a]	1993 ± 161[a]	<0.001
Glutamine	1056 ± 206	848 ± 195	889 ± 165	0.049
Glycine	8003 ± 1415[a]	7919 ± 300[b]	9486 ± 2736[a]	0.044
Histidine	179 ± 28	168 ± 28	172 ± 22	0.696
Isoleucine	267±34[b]	215±34[a]	190±32[a]	<0.001
Leucine	509±58[a]	622±106[b]	758±188[b]	<0.001
Lysine	280 ± 47	255 ± 51	236 ± 57	0.171
Methionine	181 ± 23	181 ± 32	175 ± 32	0.881
Threonine	559 ± 117	628 ± 210	554 ± 205	0.605
Tryptophan	88 ± 13	90 ± 16	85 ± 18	0.779
Valine	502±102[b]	412±79[a]	356±53[a]	0.001

[1] Data are presented as means ±SD; n=10.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test or Games-Howell test; $P<0.05$).

Table A9: Effect of dietary leucine content on amino acid concentrations in cardiac muscle of piglets (Study III)

Tissue amino acids (nmol/mg)[1]	Diet			*P*-value
	Control	L2	L4	
Alanine	5169 ± 476	5249 ± 744	4901 ± 1043	0.593
Glutamine	9431 ± 1638^{b}	8628 ± 1378^{ab}	7429 ± 1764^{a}	0.033
Glycine	1042 ± 177	1136 ± 133	1142 ± 200	0.361
Histidine	107 ± 19	123 ± 30	106 ± 32	0.315
Isoleucine	160±32^{c}	71±18^{b}	45±13^{a}	<0.001
Leucine	241±35^{a}	370±77^{b}	470±156^{b}	<0.001
Lysine	133 ± 40	129 ± 70	106 ± 43	0.494
Methionine	139 ± 33	152 ± 28	143 ± 38	0.648
Threonine	423 ± 168	651 ± 234	574 ± 225	0.066
Tryptophan	46 ± 10	53 ± 10	45 ± 14	0.217
Valine	361±60^{c}	169±28^{b}	107±24^{a}	<0.001

[1] Data are presented as means ±SD; n=10.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test or Games-Howell test; $P<0.05$).

Table A10: Effect of dietary leucine content on amino acid concentrations in skeletal muscle of piglets (Study III)

Tissue amino acids (nmol/mg)[1]	Diet			*P*-value
	Control	L2	L4	
Alanine	1736 ± 320	1683 ± 310	1484 ± 188	0.148
Glutamine	2056 ± 533	1812 ± 220	1800 ± 313	0.261
Glycine	3330 ± 1096	3210 ± 682	2883 ± 824	0.467
Histidine	27 ± 20	45 ± 10	48 ± 19	0.073
Isoleucine	106±26^{c}	46±20^{b}	17±2^{a}	<0.001
Leucine	138±27^{a}	221±47^{b}	353±131^{c}	<0.001
Lysine	84 ± 31	93 ± 55	65 ± 31	0.327
Methionine	78 ± 14	85 ± 20	79 ± 9	0.538
Threonine	350 ± 103	491 ± 176	365 ± 170	0.096
Tryptophan	40 ± 10^{b}	44 ± 6^{b}	34 ± 7^{a}	0.045
Valine	290±53^{c}	154±38^{b}	95±21^{a}	<0.001

[1] Data are presented as means ±SD; n=10.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test or Games-Howell test; $P<0.05$).

Table A11: Effect of dietary leucine content on amino acid concentrations in duodenal mucosa of piglets

Tissue amino acids (nmol/mg)[1]	Diet			*P*-value
	Control	L2	L4	
Alanine	301 ± 95	262 ± 60	243 ± 58	0.261
Glutamine	130 ± 43	94 ± 46	122 ± 51	0.344
Glycine	223 ± 73	525 ± 63	271 ± 79	0.174
Histidine	18 ± 5	17 ± 7	23 ± 6	0.105
Isoleucine	67±31	49±31	82±37	0.135
Leucine	116±53[a]	190±147[a]	721±525[b]	0.001
Lysine	93 ± 56	64 ± 49	103 ± 49	0.288
Methionine	55 ± 32	41 ± 26	69 ± 33	0.180
Threonine	100 ± 38	93 ± 35	122 ± 38	0.235
Tryptophan	27 ± 16	21 ± 12	34 ± 16	0.230
Valine	102±46	77±46	118±46	0.215

[1] Data are presented as means ±SD; n=10.

Table A12: Effect of dietary leucine content on amino acid concentrations in brains of piglets (Study III)

Tissue amino acids (nmol/mg)[1]	Diet			*P*-value
	Control	L2	L4	
Alanine	88.0 ± 9.3^{b}	85.0 ± 7.9^{ab}	75.5 ± 7.9^{a}	0.036
Glutamine	603.0 ± 77.1	570.7 ± 75.1	604.7 ± 122.1	0.686
Glycine	120.5 ± 6.8^{a}	114.9 ± 12.9^{b}	134.8 ± 12.9^{b}	0.002
Histidine	6.1 ± 0.4	7.3 ± 3.0	7.2 ± 3.3	0.650
Isoleucine	9.2±1.4^{c}	4.6±0.9^{b}	3.1±1.0^{a}	<0.001
Leucine	13.6±1.1^{a}	18.0±2.0^{b}	23.0±2.6^{c}	<0.001
Lysine	9.3 ± 1.6	9.3 ± 2.2	10.1 ± 2.7	0.674
Methionine	6.7 ± 0.9^{b}	6.0 ± 1.1^{ab}	5.0 ± 1.1^{a}	0.004
Threonine	98.8 ± 22.0	122.1 ± 28.2	101.3 ± 32.7	0.163
Tryptophan	2.5 ± 0.5^{b}	2.4 ± 0.5^{b}	2.0 ± 0.0^{a}	0.029
Valine	20.2±2.8^{c}	10.1±2.0^{b}	6.6±1.3^{a}	<0.001

[1] Data are presented as means ±SD; n=10.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test or Games-Howell test*; P*<0.05).

Table A13: Effect of dietary leucine and tryptophan content on plasma amino acids of piglets (Study V)

Diet	**18:100**	**18:300**	**23:100**	**23:300**	***P*-value**		
SID Leu:Lys	100%	300%	100%	300%			
SID Trp:Lys	18%	18%	23%	23%	Leu	Trp	Leu x Trp
Plasma amino acids (µmol/l)							
BCAA	397±55^{ab}	346±45^{a}	568±80^{b}	449±81^{ab}	<0.001	0.029	0.970
Histidine	101±20^{b}	71±13^{a}	68±9^{a}	83±8^{ab}	0.756	0.190	0.009
Isoleucine	69±14^{b}	21±6^{a}	106±16^{c}	31±10^{a}	<0.001	0.003	0.089
Leucine	88±18^{a}	229±39^{b}	132±23^{a}	295±53^{c}	<0.001	0.009	0.768
Lysine	68±53	112±38	142±31	110±35	0.945	0.437	0.041
Methionine	63±17	67±5	86±15	81±13	0.548	0.098	0.978
Phenylalanine	65±8^{a}	72±5^{a}	86±2^{b}	76±10^{ab}	0.589	0.014	0.018
Threonine	639±208^{b}	479±214^{ab}	585±132^{ab}	471±190^{a}	0.058	0.506	0.114
Tryptophan	33±5^{a}	31±8^{a}	69±7^{b}	43±7^{a}	0.001	<0.001	0.011
Tyrosine	69±17^{a}	64±11^{a}	86±7^{b}	72±5^{ab}	0.006	0.038	0.802
Valine	239±45^{b}	97±11^{a}	331±43^{b}	123±22^{a}	<0.001	0.011	0.161

[1] Data are presented as means ±SD; n=8.
Different superscript letters characterize significant differences by pairwise comparison (Tukey test; $P<0.05$).

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Declaration under Oath / Eidesstattliche Erklärung

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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