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Regulation of branched-chain amino acid metabolism in major metabolic tissues of dairy cows during late pregnancy and early lactation

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English abstract

For dairy cows, the transition from late pregnancy to early lactation is characterized by dramatic changes in endocrine status, nutrient utilization as well as tissue metabolism. Specific metabolic processes, e.g., in adipose tissue (AT) thereby contribute to the physiological adaptation to the increased nutrient demands imposed by the onset of lactation. Even though AT is known to be a major site for regulating glucose and lipid metabolism, its role in systemic protein and amino acid metabolism in dairy cows is not clear. The branched-chain amino acids (BCAA) are taken up by the mammary gland in excess and are greatly used for the synthesis of (milk) protein as well as the supply of metabolic intermediates and energy. Their cellular transport and break-down are highly regulated key processes, involving the interaction of several metabolic tissues, one of them possibly AT. Yet, studies on the BCAA transporters or degrading enzymes in ruminant tissues are sparse. Thus, one aim of the present thesis was to characterize the potential capacity of bovine AT (along with liver, skeletal muscle and mammary gland) for BCAA metabolism during late gestation and early lactation by analyzing the tissue abundance (and activity) of the most relevant BCAA transporters and catabolic enzymes as well as the concentration of circulating BCAA on selected time points before and after parturition. Moreover, as high BCAA levels have been linked with obesity and certain metabolic dysfunctions such as impaired insulin sensitivity in mammals, we further aimed to investigate the effect of over-conditioning at calving on the aforementioned variables of BCAA metabolism. Overall, AT consistently had the greatest mRNA abundance of the BCAA transporters and the BCAA transaminating enzyme, branched-chain aminotransferase 2 (*BCAT2*) when compared to most other tissues, but expressed a rather low oxidative capacity for BCAA (more specifically their keto acids), indicating that AT could be an important site of BCAA uptake and initial degradation in dairy cows. Together with the marginal hepatic mRNA abundance of BCAA transporters and *BCAT2* and the high mRNA and protein abundance as well as activity of the subsequent oxidative enzyme in liver, the branched-chain α-keto acid dehydrogenase, it seems that BCAA are only degraded in the liver after being deaminated in peripheral tissues, most likely AT. Furthermore, we detected a decrease in circulating BCAA levels around parturition in our studies, which was associated with the reduced feed intake during this time. Both observations were more pronounced for cows that were over-conditioned at calving. Interestingly, despite the lower feed intake, those cows appeared to have a greater ability than normal-conditioned cows to irreversibly catabolize BCAA in AT, especially before parturition. It is likely that, due to a nutrient oversupply, the over-conditioned cows were in a more anabolic situation during late pregnancy and might have used BCAA metabolites in addition to glycolytic metabolites for synthesizing even more body fat. The present thesis thus provides information about a possible anaplerotic link between BCAA and lipid metabolism in AT of over-conditioned dairy cows and might serve as basis for further studies investigating the role of AT in systemic protein and AA metabolism in cattle during different physiological and pathophysiological conditions.

German abstract

Der Übergang von der späten Trächtigkeit zur frühen Laktation ist bei Milchkühen mit drastischen Veränderungen des endokrinen Status, der Nährstoffverwertung sowie des Gewebestoffwechsels verbunden. Spezielle Stoffwechselprozesse, beispielsweise im Fettgewebe (AT), tragen dabei zur physiologischen Anpassung an den durch die einsetzende Laktation erhöhten Nährstoffbedarf bei. Obwohl die regulativen Funktionen des AT im Glukose- und Lipidstoffwechsel bereits bekannt sind, weiß man bisher wenig über dessen Rolle im systemischen Protein- und Aminosäurestoffwechsel bei Milchkühen. Die verzweigtkettigen Aminosäuren (*branched-chain amino acids*, BCAA) werden im Übermaß von der Milchdrüse aufgenommen und dort verstärkt für die Synthese von (Milch-) Protein sowie für die Zufuhr von Stoffwechselintermediaten und Energie verwendet. Der zelluläre Transport und der Abbau der BCAA sind intensiv regulierte Kernprozesse, welche die Interaktion verschiedener metabolischer Gewebe, darunter möglicherweise auch AT, erfordern. Bislang gibt es jedoch nur wenige Studien, in welchen die BCAA-Transporter oder die BCAA-abbauenden Enzyme auf Gewebeebene bei Wiederkäuern untersucht worden sind. Demzufolge war ein Ziel dieser Arbeit die potenzielle Kapazität von bovinem AT (neben Leber, Skelettmuskulatur und Euter) für den BCAA-Stoffwechsel während der späten Trächtigkeit und frühen Laktation zu charakterisieren. Dies erfolgte durch Analyse der Gewebeexpression (und -aktivität) der relevantesten BCAA-Transporter und katabolen Enzyme sowie durch Messung der BCAA-Konzentration in der Zirkulation an ausgewählten Zeitpunkten vor sowie nach der Kalbung. Ferner, da erhöhte BCAA-Konzentrationen in Verbindung mit Übergewicht und gewissen metabolischen Dysfunktionen wie beispielsweise einer verschlechterten Insulinsensitivität bei Säugetieren stehen könnten, war es ein weiteres Ziel, den Einfluss von Überkonditionierung zum Zeitpunkt der Kalbung auf die zuvor genannten Parameter des BCAA-Stoffwechsels zu untersuchen. Fettgewebe wies generell die höchste mRNA-Expression der BCAA-Transporter und des BCAA-transaminierenden Enzyms *branched-chain aminotransferase 2* (*BCAT2*) im Vergleich zu den meisten anderen Geweben auf, jedoch zeigte es eine recht geringe oxidative Kapazität für die BCAA (genauer deren Ketosäuren). Dies deutet darauf hin, dass AT von großer Bedeutung für die Aufnahme und den initialen Abbau von BCAA bei Milchkühen sein könnte. Zusammen mit der geringen hepatischen mRNA-Expression der BCAA-Transporter und der *BCAT2* sowie der erhöhten mRNA- und Proteinexpression und Aktivität des nachfolgenden oxidativen Enzyms, der *branched-chain α-keto acid dehydrogenase*, in der Leber, weist dies darauf hin, dass BCAA nur in der Leber abgebaut werden können nachdem sie in peripheren Geweben, höchstwahrscheinlich dem AT, deaminiert wurden. Des Weiteren wurde in den dargestellten Studien eine Verringerung der BCAA-Konzentrationen zum Zeitpunkt der Kalbung festgestellt, welche mit der zeitgleich reduzierten Futteraufnahme assoziiert wurde. Diese Beobachtungen waren bei Kühen, welche zum Zeitpunkt der Kalbung überkonditioniert waren, deutlicher ausgeprägt. Interessanterweise schienen diese Tiere, trotz der geringeren Futteraufnahme, ein höheres Vermögen zu besitzen, BCAA irreversibel im AT zu katabolisieren als normalkonditionierte Kühe. Dies traf insbesondere für die Zeit vor der Kalbung zu. Es ist denkbar, dass

sich die überkonditionierten Kühe während der späten Trächtigkeit, aufgrund eines Nährstoffüberangebots, in einer mehr anabolen Stoffwechselsituation befanden und demnach zusätzlich zu glykolytischen Stoffwechselprodukten ebenfalls BCAA-Metabolite für den Aufbau von weiterem AT nutzen konnten. Die vorliegenden Ergebnisse zeigen somit eine mögliche anaplerotische Verbindung zwischen BCAA- und Lipidstoffwechsel im AT überkonditionierter Milchkühe auf. Folglich dient diese Arbeit als essentielle Grundlage weiterer Untersuchungen, um die Funktionen des AT im systemischen Protein- und Aminosäurestoffwechsel bei Kühen in verschiedenen physiologischen und pathophysiologischen Stadien zu erforschen.

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List of abbreviations

AA	amino acid(s)
ACAD8	acyl-CoA dehydrogenase family member 8
ACADSB	short/branched-chain acyl-CoA dehydrogenase
ACAT1	acetyl-CoA acetyltransferase 1
ADF_{OM}	acid detergent fiber of organic matter
AHAS	acetohydroxyacid synthase
α-KIC	α-ketoisocaproic acid
α-KIV	α-ketoisovaleric acid
α-KMV	α-ketomethylvaleric acid
Ala	alanine
ALDH6A1	aldehyde dehydrogenase 6 family member A1
$aNDF_{OM}$	ash free neutral detergent fiber of organic matter
a.p.	antepartum
ASCT2	ASC-type sodium-coupled neutral amino acid transporter type 2
AT	adipose tissue
ATP	adenosine triphosphate
AUC	area under the curve
AUH	AU RNA-binding methylglutaconyl-CoA hydratase
A-V	arteriovenous
BAIBA	ß-amino-isobutyric acid
BCAA	branched-chain amino acid(s)
BCAAT	branched-chain amino acid transporter(s)
BCAT	branched-chain aminotransferase
BCAT1 (BCATc)	branched-chain aminotransferase 1 (cytosolic isoform)
BCAT2 (BCATm)	branched-chain aminotransferase 2 (mitochondrial isoform)
BCFA	branched-chain fatty acid(s)
BCKA	branched-chain α-keto acid(s)
BCKDH	branched-chain α-keto acid dehydrogenase
BCKDHA	branched-chain α-keto acid dehydrogenase E1α subunit
BCKDHB	branched-chain α-keto acid dehydrogenase E1ß subunit
BCKDK	branched-chain α-keto acid dehydrogenase kinase

List of abbreviations

BCS	body condition score
BFT	back fat thickness
BHB	beta-hydroxybutyrate
cDNA	complementary deoxyribonucleic acid
CLA	conjugated linoleic acid(s)
CoA	coenzyme A
CV	coefficient of variation
DBT	dihydrolipoamide branched-chain transacylase
DLD	dihydrolipoyl dehydrogenase
DHAD	dihydroxyacid dehydratase
DM	dry matter
DMI	dry matter intake
E1	thiamine-dependent decarboxylase
E2	lipoate-dependent dihydrolipoamide branched-chain transacylase
E3	flavin adenine dinucleotide-dependent dihydrolipoyl dehydrogenase
EAA	essential amino acid(s)
EIF3K	eukaryotic translation initiation factor 3
EMD	emerin
FAD	flavin adenine dinucleotide
GAP	guanosine triphosphatase-activating protein
GATOR2	guanosine triphosphatase-activating protein activity towards Rags 2
Gln	glutamine
GTPase(s)	guanosine triphosphatase(s)
HADHA	hydroxyacyl-CoA dehydrogenase subunit α
HBCS	over-conditioned group
HIBADH	3-hydroxyisobutyrate dehydrogenase
HIBCH	3-hydroxyisobutyryl-CoA hydrolase
HMGCL	3-hydroxymethyl-3-methylglutaryl-CoA lyase
Hp	haptoglobin
HPCAL1	hippocalcin-like 1

List of abbreviations

HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSD17B10	hydroxysteroid 17-ß dehydrogenase 10
Ile	isoleucine
IPMDH	isopropylmalate dehydrogenase
IPMI	isopropylmalate isomerase
IPMS	isopropylmalate synthase
IR	insulin resistance
IS	insulin sensitivity
IVD	isovaleryl-CoA dehydrogenase
KARI	ketol-acid reductoisomerase
LAT1	large neutral amino acid transporter small subunit 1
LAT3	large neutral amino acid transporter small subunit 3
Leu	leucine
LRP10	lipoprotein receptor-related protein 10
M	expression stability
MCCC	methylcrotonoyl-CoA carboxylase
ME	metabolizable energy
MG	mammary gland
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MOP	4-methyl 2-oxopentanoate
mRNA	messenger ribonucleic acid
mTORC1	mechanistic target of rapamycin complex 1
MURF-1	muscle RING finger protein-1
MUT	methylmalonyl-CoA mutase
Na^+/K^+-ATPase	sodium-potassium adenosine triphosphatase
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized and reduced forms, respectively)
NBCS	normal-conditioned group
NEAA	non-essential amino acid(s)
NEFA	non-esterified fatty acid(s)
NE_L	net energy for lactation

List of abbreviations

NNB	negative nutrient balance
OCFA	odd-chain fatty acid(s)
OXCT1	3-oxoacid CoA transferase
P	phosphorylation
PCCB	propionyl-CoA carboxylase subunit ß
PDH	pyruvate dehydrogenase
POLR2A	RNA polymerase II
p.p.	postpartum
PP2Cm	protein phosphatase 2Cm
qPCR	quantitative (real-time reverse transcription) polymerase chain reaction
Rags	Ras-related guanosine triphosphatases
RNA	ribonucleic acid
S6K1	ribosomal protein S6 kinase-1
scAT	subcutaneous adipose tissue
SEM	standard error of the mean
SLC1A3	solute carrier family 1, member 3
SLC1A5	solute carrier family 1, member 5
SLC3A2	solute carrier family 3, member 2
SLC38A2	solute carrier family 38, member 2
SLC43A1	solute carrier family 43, member 1
SLC7A5	solute carrier family 7, member 5
SNAT2	sodium-dependent neutral amino acid transporter 2
TCA	tricarboxylic acid
T2DM	type 2 diabetes mellitus
V	pairwise variation
Val	valine
V_{max}	maximum reaction rate
vAT	visceral adipose tissue

1 Introduction

Sustainable safeguarding of animal performance and animal health continue to present challenges in modern agricultural systems. Through genetic selection as well as improved nutrition and management, the milk yield of German Holstein cows has increased almost threefold over the last decades (Breves, 2007). However, such high demands on the animals' metabolism are often accompanied by metabolic disorders, such as milk fever, ketosis, retained placenta, and displaced abomasum as well as reduced fertility, lameness and various infectious diseases, all of which may impact the efficiency of milk production and consequently, may lead to a shorter productive life span of high-yielding dairy cows (Goff and Horst, 1997; Sordillo et al., 2009).

The majority of these health problems occurs during the periparturient period (3 weeks before to 3 weeks after parturition; Grummer, 1995), making it one of the most critical times during a dairy cow's life. A profound knowledge of the complex physiological and pathophysiological processes emerging around this time is a prerequisite for finding any strategies which may help improve the cow's health while securing high productivity.

1.1 Dairy cow metabolism during the transition from late gestation to early lactation

During late pregnancy and early lactation, the nutrient demands imposed on the maternal organism increase substantially and rapidly as a result of (1) the growing fetus and (2) the production of colostrum and milk. Fetal development, i.e., the development of the fetus itself, fetal membranes and supplementary tissues (Bell, 1995), reaches exponential growth during the last weeks of pregnancy (Ferrell et al., 1976). Concomitantly, the alveolar system of the mammary gland (MG) is growing notably as well (Ingvartsen, 2006). At the expense of other metabolic functions, different, mostly evolutionary based, regulatory processes thereby ensure absolute metabolic priority for the support of pregnancy and lactation (Bauman and Currie, 1980). However, the sudden increase in nutrient requirements cannot be compensated by feed intake alone. This is aggravated by the fact that specific metabolic and endocrine changes around parturition usually lead to a decrease in voluntary feed consumption, which only slowly increases again after calving, and, thus, insufficient nutrient intake may often be the case (Grummer, 1995; Allen et al., 2005). With the onset of lactation, the animals enter an inevitable

state of negative nutrient balance (NNB) and systemic adaptations to NNB occur in e.g., adipose tissue (AT), liver, skeletal muscle, gut and MG, necessitating their metabolic regulation and coordination (Drackley, 1999; Herdt, 2000). In order to meet the nutrient requirements cows have to mobilize body reserves mainly from AT and muscle (Plaizier et al., 2000; Kuhla et al., 2011). Even though this situation is quite common for mammals, the severity and persistency of NNB as well as the manifestation of tissue mobilization in high-yielding dairy cows are all exceptional. Exemplary reports have shown a range of 19 to 54 kg of fat, and up to 21 kg of muscle protein being lost during early lactation (Komaragiri and Erdman, 1997; Chibisa et al., 2008). In terms of quantity, the ability to use protein is therefore restricted. Also, protein mobilization may only last until 5 weeks (wk) of lactation, whereas utilization of body fat can occur at least until 12 wk after calving (Komaragiri and Erdman, 1997). However, according to van der Drift et al. (2012), muscle protein mobilization may already occur before parturition and thus, in advance of fat mobilization.

Another notable metabolic adaptation to preserve sufficient nutrients for fetal growth and development as well as milk production is a decrease of insulin sensitivity (IS) in peripheral tissues during late pregnancy and early lactation (Bell and Bauman, 1997), oftentimes leading to an insulin-resistant state (Chagas et al., 2009). Consequently, reduced IS further shifts metabolic processes in AT and muscle (which are usually insulin-sensitive) away from maintenance or anabolism towards catabolism. Moreover, peripheral glucose utilization is reduced, facilitating a direct nutrient flow to the MG, which in lactating mammals is largely insulin-independent (Bell and Bauman, 1997). As only restricted amounts of glucose are available as energy source for peripheral tissues, alternative fuels are needed to maintain the physiological function of these tissues.

During fat mobilization (lipolysis), fatty acids from AT are released into the circulation as non-esterified fatty acids (NEFA), which can then be used as a source of energy by different tissues, such as liver and muscle, or be used as precursors for the synthesis of milk fat in the MG (Palmquist et al., 1969; Herdt, 2000). Albeit, the capacity of the liver for full NEFA oxidation is limited, resulting in either the storage of excess NEFA as triglycerides (“fatty liver syndrome”) or in incomplete NEFA oxidation, which in turn is linked to an elevated synthesis of ketone bodies, i.e., ß-hydroxybutyrate (BHB), acetone and acetoacetate (Adewuyi et al., 2005). High blood concentrations of both NEFA and BHB in dairy cows may thereby indicate an excess of NNB (McArt et al., 2013), associated with a higher incidence of metabolic disorders like fatty liver and ketosis as well as generally impaired immune functions (Drackley, 1999; Herdt, 2000).

In skeletal muscle, as the major protein reserve, increased proteolysis leads to the mobilization of amino acids (AA), which, depending on the type of AA, may be directed towards milk protein synthesis, direct oxidation, hepatic gluconeogenesis or ketogenesis (Kuhla et al., 2011; Mann et al., 2016). However, resulting from both elevated nutrient demands and concurrently reduced nutrient intake, general postpartal AA imbalances (Doepel et al., 2002; Kuhla et al., 2011) or even deficits in individual circulating AA (Meijer et al., 1995) may occur and thus, limit (milk) protein synthesis (Kuhla et al., 2011). In addition, tissue energy metabolism as well as the immune system may be affected by impaired nitrogen retention: For example, low concentrations of essential AA (EAA) in the circulation can lead to general decreases in splanchnic tissue proliferation (Gibb et al., 1992; Larsen et al., 2014) and also to reduced lymphocyte proliferation and antibody production, compromising both the innate as well as the acquired immune defense (Li et al., 2007).

1.1.1 Effect of body condition on the metabolic status of dairy cows

The body condition score (BCS) of a dairy cow is used to assess the animal's proportion of body fat [specifically subcutaneous AT (scAT)] and is an important management tool employed by animal scientists and producers, providing a gross but reasonably accurate measure of the energy status (Roche et al., 2009). Even though the scale may differ between countries, low values always signify emaciation whereas high values equate to obesity. Besides factors such as diet, milk yield and parity, BCS at calving largely affects the extent and rate of peripartum tissue mobilization (Komaragiri et al., 1998). Compared with thinner animals (mean calving BCS of 2.5; 5-point scale), cows that are over-conditioned (mean calving BCS of 4.0) and, thus, have more tissue reserves to rely on, experience greater losses in body condition as well as body weight (Treacher et al., 1986; Pires et al., 2013). At parturition, there is a negative association between BCS and dry matter intake (DMI; Hayirli et al., 2002; Matthews et al., 2012), as over-conditioned cows usually expressing a more pronounced and prolonged decline in DMI, have to face a deeper NNB (Hayirli et al., 2002), a more rapid and excessive lipolysis as well as a greater release of NEFA into the circulation than cows in moderate or lean condition (Dann et al., 2006; Pires et al., 2013). However, it has also been reported that over-conditioned cows (mean BCS of 4 at dry-off) lose more muscle fiber area after parturition than thin cows (mean BCS of 2.5 at dry-off), indicating a greater mobilization of body protein as well as fat (Reid et al., 1986). In view of a positive correlation found between muscle diameter and BCS (Kokkonen et al., 2005), BCS not only covers the assessment of fat depots, but may also assess

protein reserves in muscles located under the skin, where body condition is scored; a change in either or both factors may therefore affect BCS (Reid et al., 1986; Kokkonen et al., 2005).

It is well documented that due to the more excessive tissue mobilization, cows calving with a high BCS bear a greater risk of developing metabolic disorders and other health issues, which may compromise productivity (Gearhart et al., 1990; Bobe et al., 2004; Drackley et al., 2005; Ingvartsen, 2006). Enhanced insulin resistance (IR), a state in which physiological concentrations of insulin induce a decreased biological response in insulin-sensitive tissues (Kahn, 1978), is commonly observed during late gestation and early lactation, especially in over-conditioned cows, and seems to be related to these dysfunctions (Hayirli, 2006). Even though IR is a multi-factorial phenomenon, hyperlipidemia appears to play an important role in the development of IR, as elevated NEFA levels may compromise insulin-stimulated glucose clearance and disturb intracellular insulin-signaling pathways in liver and peripheral tissues (Hayirli, 2006; Pires et al., 2007).

Furthermore, over-conditioned cows having greater BCS losses, are more prone to oxidative stress due to an overproduction of reactive oxygen metabolites and concurrently low levels of neutralizing antioxidants (Bernabucci et al., 2005). The possible damage of lipids and other macromolecules resulting from this imbalance can lead to alterations of the plasma membrane and other cellular compartments (Toyokuni, 1999). As a consequence, vital physiological and metabolic functions in the body may be impaired (Bernabucci et al., 2005) and higher incidences of various pathologies are likely (Gröhn et al., 1989).

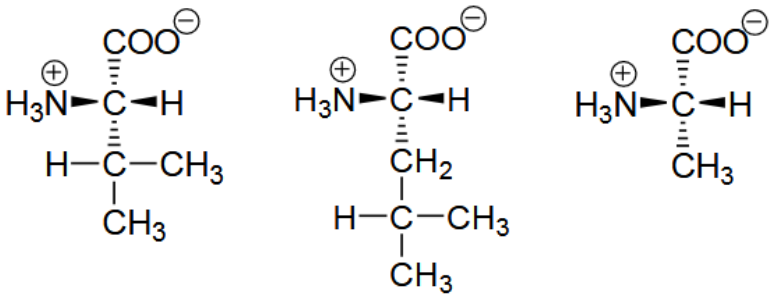
Yet, the association between some production and health variables, such as milk yield or certain diseases, and BCS is non-linear (Roche et al., 2009). Accordingly, a relatively low calving BCS (<3.0) may also have detrimental effects especially regarding fertility, e.g., delays in the resumption of estrus cycles, reduced pregnancy odds and a higher risk for uterine infections and retained placenta (Hoedemaker et al., 2009; Roche et al., 2009; Roche et al., 2013), partly due to a lower quality of oocytes (Snijders et al., 2000) and general immunosuppression (LeBlanc, 2008), respectively. An optimum calving BCS is therefore recommended to be around 3.0 to 3.25 (Roche et al., 2009).

1.2 Dietary supplementation with conjugated linoleic acids

An optimal supply in nutrients is essential for high-yielding dairy cows ensuring high milk yields and concomitantly good animal health. Research from the last decades has shown that an adequate and optimized diet (according to the respective performance level) during late gestation and early lactation can alleviate the risks for the occurrence of production diseases shortly after calving (Overton and Waldron, 2004; Ingvartsen, 2006). Using feed additives can support the metabolic adaptations of the bovine organism to the changing nutrient demands during this period. Among the different nutritional supplements, the dietary use of fatty acids, such as conjugated linoleic acids (CLA), may be one possibility to improve animal health in transition cows (Esposito et al., 2014). The CLA are octadecadienoic acids (C18), which exist as different isomers. Most studies have focused on the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 isomers. Feeding these isomers in a rumen-protected form mainly decreases milk fat secretion in dairy cows (Baumgard et al., 2000; Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2007). As the synthesis of milk fat requires over one-half of the energy needed for total milk production (Bauman et al., 2011), a reduction of milk fat synthesis can lower the caloric demand of milk production (De Veth et al., 2004) and thus, may alleviate metabolic stress. Yet, other reported effects of CLA which may possibly improve animal health and performance such as decreased NEFA and BHB, increased DMI or improved energy balance have not been consistently observed (Baumgard et al., 2001; Bernal-Santos et al., 2003; Pappritz et al., 2011; Esposito et al., 2013; 2014).

1.3 Branched-chain amino acid (BCAA) metabolism

Leucine, isoleucine and valine are collectively known as the branched-chain AA (BCAA) based on the structural similarity of their side chains (Figure 1). As they can only be synthesized by bacteria, plants and fungi, the BCAA are considered EAA for mammals. Almost always, the relative molar abundance is 2.2:1.0:1.6 leucine:isoleucine:valine, reflecting the linked nature of their synthesis and degradation (Neinast et al., 2019). Both isoleucine and valine are synthesized by the same enzymes, whereas leucine derives from α-ketoisovaleric acid, the transamination precursor of valine (Figure 2). The carbon chain of valine (and leucine) thereby originates from the readily available pyruvate, and isoleucine carbons derive from threonine, a relatively rare AA.



Leucine Isoleucine Valine

Figure 1. Chemical structure of the three branched-chain amino acids (adapted from ChemgaPedia; http://www.chemgapedia.de/).

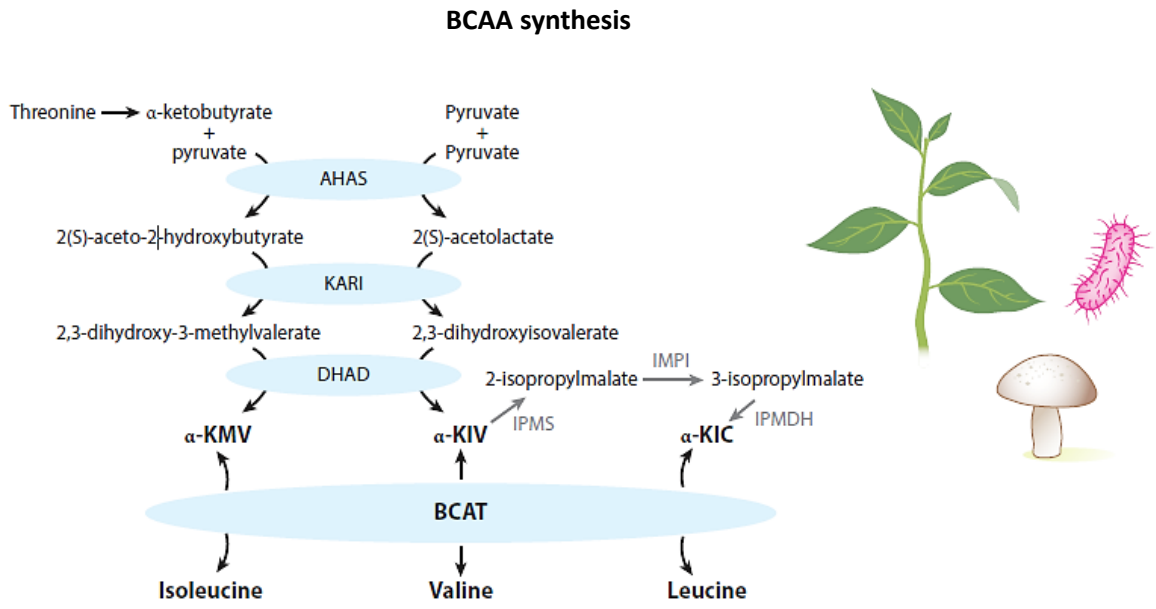


Figure 2. The synthesis of the branched-chain amino acids (BCAA) occurs in plants, bacteria and fungi. Abbreviations: AHAS, acetohydroxyacid synthase; α-KIC, α-ketoisocaproic acid; α-KIV, α-ketoisovaleric acid; α-KMV, α-ketomethylvaleric acid; BCAT, branched-chain aminotransferase; DHAD, dihydroxyacid dehydratase; IPMDH, isopropylmalate dehydrogenase; IPMI, isopropylmalate isomerase; IPMS, isopropylmalate synthase; KARI, ketol-acid reductoisomerase. Modified from Neinast et al. (2019).

The BCAA comprise around 35% of the EAA in muscle proteins (Harper et al., 1984) and 50% of the EAA in milk proteins (Mackle et al., 1999). Apart from being building blocks for proteins, BCAA are also important precursors for the synthesis of non-essential AA (NEAA), such as glutamine and alanine, as well as substrates for energy and other metabolic intermediates.

Additionally, in recent years, BCAA have emerged as signaling molecules, which may modulate the growth and metabolism of an organism. In particular leucine has been shown to independently regulate protein synthesis both *in vitro* and *in vivo,* e.g., in mammalian skeletal muscle (Lynch et al., 2002a; Suryawan et al., 2012), AT (Lynch et al., 2002a), liver (Lynch et al., 2002a; Wilson et al., 2011) and MG (Toerien et al., 2010; Appuhamy et al., 2012) via activation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway. The mTORC1 is an evolutionary conserved ubiquitous serine/threonine-specific protein kinase complex, which can also be activated by growth factors like insulin and insulin-like growth factor 1 as well as intracellular energy (Laplante and Sabatini, 2009). Under sufficient AA availability, mTORC1 stimulates both the initiation and elongation of mRNA translation, and, thus, positively controls protein synthesis (Wang and Proud, 2006). Further, mTORC1 also increases the general cellular capacity for translation by enhancing ribosomal biogenesis and, consequently, ribosome numbers (Dodd and Tee, 2012).

Upon high AA levels, mTORC1, usually located in the cytoplasm, is translocated to the lysosomes through a group of small guanosine triphosphatases (GTPases) called Ras-related GTPases or “Rags” (RagA/B, RagC/D; Efeyan et al., 2012). By changing their confirmation to an active state, the Rags, which are located at the lysosomal surface, are able to bind mTORC1 (Sancak et al., 2008; Efeyan et al., 2012). Activation of mTORC1 may then occur by leucine directly binding to sestrin2 (Saxton et al., 2016), a negative regulator of mTORC1 activity (Wolfson et al., 2016). Subsequently, sestrin2 releases a GTPase-activating protein named GAP activity towards Rags 2 (GATOR2), which positively regulates mTORC1 activity (Chantranupong et al., 2014; Wolfson et al., 2016). Once activated, mTORC1 phosphorylates several downstream targets, including the eukaryotic initiation factor 4E binding protein-1 and the ribosomal protein S6 kinase-1 (S6K1), which themselves regulate translation and ribosomal biogenesis in a more direct manner (Richter and Sonenberg, 2005; Wang and Proud, 2006). However, during conditions of restricted energy and nutrient (AA) supply, cells may rapidly reduce mTORC1 signaling in order to save substrates (Dodd and Tee, 2012).

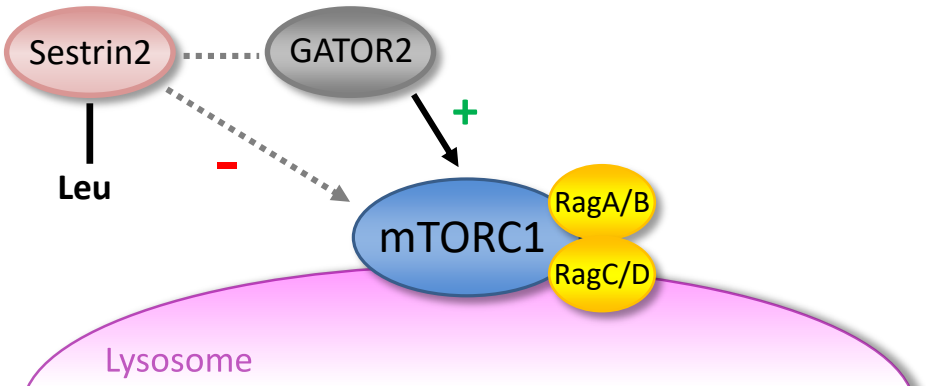


Figure 3. Activation of the mechanistic target of rapamycin complex 1 (mTORC1) via leucine (Leu). Small Ras-related GTPases (RagA/B, RagC/D) tether mTORC1 to the lysosomal membrane. By binding to sestrin2, Leu revokes the bond between sestrin2 and the GAP activity towards Rags 2 (GATOR2) protein, enabling stimulation of mTORC1 activity via GATOR2. Simplified illustration according to Saxton et al. (2016) and Wolfson et al. (2016).

Further studies showing that BCAA might also affect the release of certain hormones, like insulin (Lynch et al., 2002b), leptin (Roh et al., 2003; Lynch et al., 2006) and glucagon-like peptide-1 (Chen and Reimer, 2009), support the idea of BCAA as nutrient signals regulating metabolism. Thus, BCAA have become of particular interest in the context of many metabolic dysfunctions such as obesity, IR and type 2 diabetes mellitus (T2DM) in humans (Lynch and Adams, 2014). Albeit, during such metabolic states, BCAA metabolism seems to be profoundly altered, likely due to a dysfunction of BCAA degrading enzymes in certain tissues (see chapter 1.4.2).

1.3.1 BCAA transporters

Cellular protein synthesis and other metabolic processes are highly dependent on the continuous supply of all AA at the appropriate sites (D'Mello, 2003). As AA cannot readily pass lipid membranes, membrane-spanning transporter proteins are necessary to move AA in and out of a cell as well as between intracellular membranous compartments (Taylor, 2014). Intracellular AA homeostasis is thereby largely maintained through the external AA supply (Dodd and Tee, 2012). In most cases, AA uptake is coupled with the counter transport of certain ions, such as sodium, in an energy-dependent manner, making use of the electrochemical gradient the sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) maintains across

the plasma membrane; in other cases, one AA is transported in exchange for another (Souba and Pacitti, 1992).

Depending on their substrate specificity, transport mechanism and regulatory characteristics, AA transporters can be classified into different "systems" (Hyde et al., 2003). In case of BCAA, which are considered neutral AA, the preferred system is the sodium-independent L-type system with the large neutral amino acid transporter small subunit 1 (LAT1; Baumrucker, 1985; Hyde et al., 2003; Batistel et al., 2017) as the most relevant transport protein for BCAA uptake. The LAT1, ubiquitously expressed (Hundal and Taylor, 2009) and encoded by the solute carrier family 7 member 5 (*SLC7A5*) gene, can directly import BCAA from extracellular space in exchange for glutamine. However, other neutral AA transporters may also shuttle BCAA across the membrane or are indirectly involved in their transport, such as the A-type sodium-dependent neutral amino acid transporter 2 (SNAT2, encoded by *SLC38A2*) and the ASC-type sodium-coupled neutral amino acid transporter type 2 (ASCT2, encoded by *SLC1A5*). Both SNAT2 and ASCT2 are ubiquitously expressed as well (Fuchs and Bode, 2005; Hundal and Taylor, 2009), yet, they usually bind smaller AA like glutamine, facilitating the intracellular accumulation of this AA as efflux substrate to regulate LAT1-mediated BCAA transport (Kanai et al., 1998; Mastroberardino et al., 1998). Hence, the integration of primary active transport (export of sodium via the Na^+/K^+-ATPase) as well as secondary (sodium-dependent import of glutamine via SNAT2 and ASCT2) and tertiary active transport (import of BCAA in exchange for glutamine via LAT1) jointly modulates intracellular free AA pools (Figure 4).

While LAT1, SNAT2 and ASCT2 are all involved in the cellular import of BCAA, it seems that other transporters are responsible for the BCAA efflux back into the bloodstream. Although this area has not been studied in detail yet, there is evidence that in humans and mice, the system L transporter LAT3 (*SLC43A1*) exports BCAA mostly via facilitated diffusion, e.g., from liver, skeletal muscle and pancreas (Babu et al., 2003; Fukuhara et al., 2007; Bodoy et al., 2013).

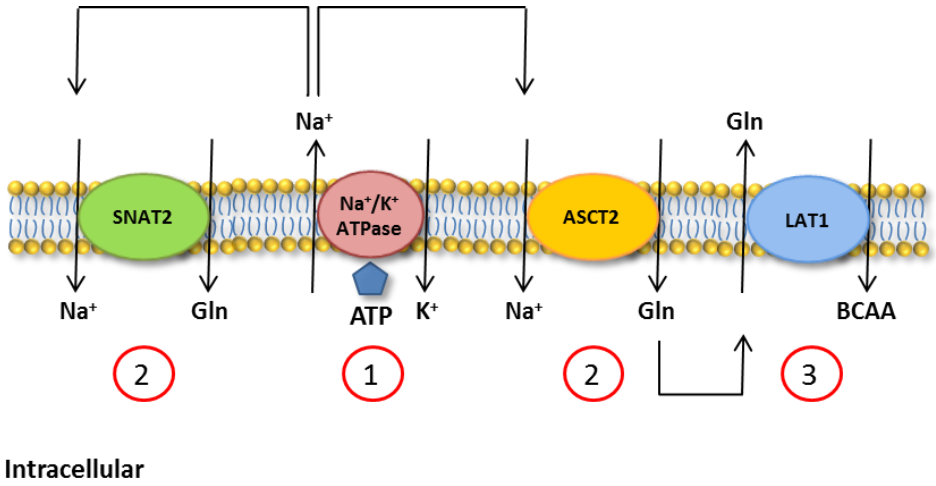


Figure 4. Mechanism of branched-chain amino acid (BCAA) uptake by the A-type sodium-dependent neutral amino acid transporter 2 (SNAT2), ASC-type sodium-coupled neutral amino acid transporter type 2 (ASCT2) and large neutral amino acid transporter small subunit 1 (LAT1). The sodium-potassium adenosine triphosphatase (Na^{+}/K^{+}-ATPase) exports intracellular sodium (primary active transport; 1), which is required by the SNAT2 and ASCT2 transporters, co-transporting extracellular sodium and glutamine (Gln) into the intracellular space (secondary active transport; 2). Via LAT1, BCAA are then imported in exchange for intracellular Gln (tertiary active transport; 3). Self-designed illustration.

During the transport process, transporters generally undergo various conformational changes, which could be used to relay signals about nutrient abundance to the inner cell (Bröer and Bröer, 2017). It has therefore been proposed that some AA transporters in mammalian cells may regulate nutrient signaling not only indirectly through their capacity to control the free intracellular AA pool but also directly through their ability to sense extracellular AA availability upstream of intracellular signaling pathways such as mTORC1 (Hyde et al., 2007; Hundal and Taylor, 2009; Pinilla et al., 2011; Taylor, 2014). This dual transporter-receptor or "transceptor" function (Thevelein and Voordeckers, 2009) thus enables cells to respond adequately and instantaneously to nutrient changes, which may be caused by different metabolic events. Especially for SNAT2 a direct (and indirect) nutrient signaling action has been observed (Hyde et al., 2007), whereas ASCT2 and LAT1 are thought to serve as conduits activating mTORC1 indirectly through the delivery of their substrates (Nicklin et al., 2009; Sinclair et al., 2013). The exact mechanism of how SNAT2 is able to transmit cellular signals is still uncertain, yet seems to involve increased transcription (Palii et al., 2004), elevated protein translation (Gaccioliy et al., 2006), increased mRNA stability as well as decreased protein degradation (Jeon et al., 2015).

1.3.2 BCAA degrading enzymes

Once an AA has entered the cell, it may either be used for protein synthesis or, if the supply exceeds the requirements, be rapidly catabolized. A unique feature of BCAA catabolism is its tissue specificity: Whereas most EAA are primarily degraded in the liver, BCAA catabolism involves the cooperation of several tissues, leading to substantial inter-organ shuttling of BCAA and their metabolites (Harper et al., 1984; Shimomura et al., 2006). Another characteristic is that the first two enzymes, the branched-chain aminotransferase (BCAT; EC 2.6.1.42) and the branched-chain α-keto acid dehydrogenase (BCKDH; EC 1.2.4.4) are common to all three BCAA (Figure 5).

The two BCAT isoforms identified in mammals, a cytosolic BCAT (BCAT1; Hall et al., 1993) and a mitochondrial BCAT (BCAT2; Hutson et al., 1992), both initiate degradation via reversible transfer of the α-amino group from the BCAA to α-ketoglutarate, resulting in the corresponding branched-chain α-keto acids (BCKA; α-ketoisocaproic acid, α-ketoisovaleric acid and α-ketomethylvaleric acid) as well as glutamate (Harris et al., 2005). In humans, expression of BCAT1 is mostly limited to the brain, whereas BCAT2 is ubiquitously expressed, but with diverging content and activity depending on the tissue. Accordingly, high levels are reported for pancreas, heart and kidney, intermediate levels for skeletal muscle, and low levels for liver (Suryawan et al., 1998). However, species-related differences may exist: Sheep muscle for instance expresses both BCAT isoforms and significant activity seems to originate from the cytosolic isoform (Faure et al., 2001). Following BCAA deamination, the resulting glutamate may act either as an amino group donor synthesizing alanine from pyruvate or as a substrate for ammonia detoxification to form glutamine (Holeček, 2018). As BCKA are potentially toxic (Harper et al., 1984; Harris et al., 2005), they may either be reaminated to BCAA (and e.g., used for protein synthesis) or further degraded.

The second catabolic step, an irreversible oxidative decarboxylation of the BCKA to branched-chain acyl-coenzyme A (CoA) derivatives (i.e., isovaleryl-CoA, α-methylbutyryl-CoA, and isobutyryl-CoA) by the BCKDH commits the BCAA to the degrading pathway (Figure 5). The BCKDH, a large multi-enzyme-complex, is located on the inner mitochondrial membrane (Harper et al., 1984). It has some structural and functional similarities to the pyruvate dehydrogenase (PDH) complex (Patel et al., 2014), an enzyme that precedes the tricarboxylic acid (TCA) cycle. Like PDH, BCKDH catalyzes the oxidative decarboxylation through the release of CO_2 and by covalently adding a CoA group to the oxidized BCKA product (Johnson et al., 1972).

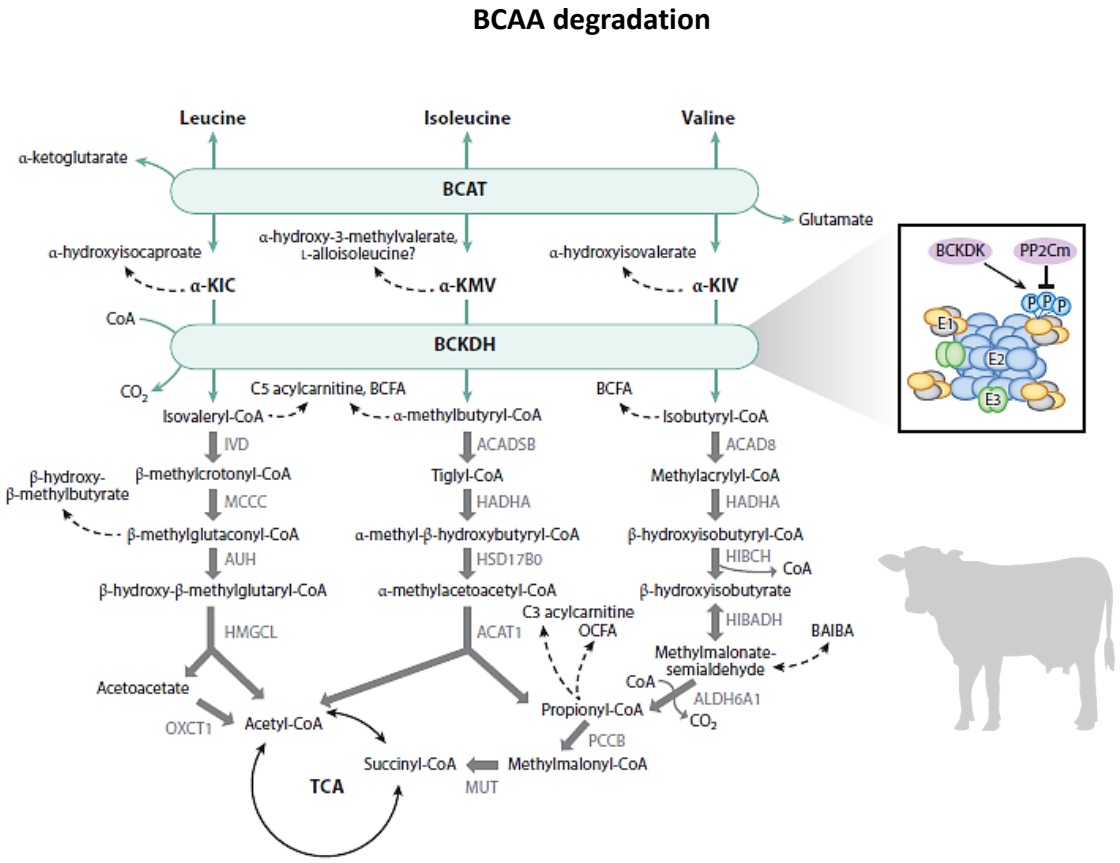


Figure 5. The degradation of branched-chain amino acids (BCAA) occurs in plants, bacteria, fungi and animals. The first two steps, a reversible transamination via the branched-chain aminotransferase (BCAT) and an irreversible oxidative decarboxylation via the branched-chain α-keto acid dehydrogenase (BCKDH), are common to all three BCAA. The BCKDH complex is composed of the different subunits E1, E2 and E3. The BCKDH kinase (BCKDK) inhibits BCKDH via phosphorylation of E1, whereas the BCKDH phosphatase (also known as protein phosphatase 2Cm; PP2Cm) activates it via dephosphorylation of E1. Further abbreviations: ACAD8, acyl-CoA dehydrogenase family member 8; ACADSB, short/branched-chain acyl-CoA dehydrogenase; ACAT1, acetyl-CoA acetyltransferase 1; α-KIC, α-ketoisocaproic acid; α-KIV, α-ketoisovaleric acid; α-KMV, α-ketomethylvaleric acid; ALDH6A1, aldehyde dehydrogenase 6 family member A1; AUH, AU RNA-binding methylglutaconyl-CoA hydratase; BAIBA, ß-amino-isobutyric acid; BCFA, branched-chain fatty acid(s); CoA, coenzyme A; HADHA, hydroxyacyl-CoA dehydrogenase subunit α; HIBADH, 3-hydroxyisobutyrate dehydrogenase; HIBCH, 3-hydroxyisobutyryl-CoA hydrolase; HMGCL, 3-hydroxymethyl-3-methylglutaryl-CoA lyase; HSD17B10, hydroxysteroid 17-ß dehydrogenase 10; IVD, isovaleryl-CoA dehydrogenase; MCCC, methylcrotonoyl-CoA carboxylase; MUT, methylmalonyl-CoA mutase; OCFA, odd-chain fatty acid(s); OXCT1, 3-oxoacid CoA transferase; P, phosphorylation; PCCB, propionyl-CoA carboxylase subunit ß; TCA, tricarboxylic acid cycle. Modified from Neinast et al. (2019).

Further, BCKDH also consists of multiple copies of three enzymes (Pettit et al., 1978; Paxton and Harris, 1982): A thiamine-dependent decarboxylase (E1) with α and ß subunits (encoded by the *BCKDHA* and *BCKDHB* genes, respectively) catalyzing the actual decarboxylation of the BCKA (Tsuruta et al., 1998); a lipoate-dependent dihydrolipoamide branched-chain transacylase (*DBT*; E2), that transfers the acyl groups from the BCKA to CoA; and a flavin adenine dinucleotide (FAD)-dependent dihydrolipoyl dehydrogenase (*DLD*; E3), that transfers the released electrons to the oxidized form of nicotinamide adenine dinucleotide (NAD^+) generating energized reduced NADH. The E1 and E2 are specific to the BCKDH, whereas E3 is also part of other α-keto acid dehydrogenases like PDH (Tsuruta et al., 1998).

Similar to BCAT(2), BCKDH is distributed ubiquitously throughout the human body with high activity in kidney, brain and liver and relatively low activity in muscle and AT (Suryawan et al., 1998). The oxidative decarboxylation is the rate-limiting step in overall BCAA catabolism, therefore activity of BCKDH is tightly regulated through phosphorylation (inactivation) and dephosphorylation (activation) of the E1α subunit by a specific kinase (branched-chain α-keto acid dehydrogenase kinase; EC 2.7.11.4) and phosphatase (branched-chain α-keto acid dehydrogenase phosphatase, also known as protein phosphatase 2Cm; EC 3.1.3.16), respectively (Shimomura et al., 1990; Lu et al., 2009).

After decarboxylation, the catabolic pathways for each of the BCAA diverge and resemble the oxidation of fatty acids, ultimately leading to ketogenic or glucogenic precursors (i.e., acetoacetate and acetyl-CoA for leucine, succinyl-CoA and acetyl-CoA for isoleucine, and succinyl-CoA for valine), some of which can enter the TCA cycle (Harper et al., 1984). Through these linkages in the TCA cycle BCAA catabolism and fatty acid metabolism are closely connected. It is assumed that e.g. in AT, where BCAA and their metabolites can also be catabolized (Herman et al., 2010), BCAA may have regulatory functions on glyceroneogenesis as well as fatty acid oxidation (Kainulainen et al., 2013). Increased BCAA degradation may thus replenish intermediates of other metabolic pathways (Shimomura et al., 2004; Green et al., 2016), a process also known as anaplerosis.

1.4 BCAA metabolism during different physiological and pathophysiological states

1.4.1 BCAA metabolism during lactation

Free AA in the blood are the most essential precursors of milk proteins (Mepham, 1982). Different adaptive mechanisms throughout the body, e.g., a decrease in AA use by extramammary tissues, an enhanced blood flow to and an elevated rate of AA extraction by the MG, thereby increase AA availability to the MG (Tesseraud et al., 1993; Tovar et al., 2001). The net mammary uptake of AA typically represents output as milk protein as well as cell growth and catabolism (Cant et al., 2018), with milk protein yield amounting to approximately 90% of uptake (Cant et al., 1993).

Due to the high demand of AA for milk protein synthesis, the capacity of the MG for AA transport is greatly increased in lactating versus non-lactating mammals, as shown by the augmented expression and activity of AA transporters (Manjarin et al., 2014) and the elevated maximum reaction rate (V_{max}) of transporters, which in turn is dependent on the number and activity of functional transporters on the cell membrane (Verma and Kansal, 1993). Some AA transport systems like system A and L also appear to be upregulated by hormones important for the establishment and maintenance of lactation, such as estradiol, prolactin and insulin (Sharma and Kansal, 1999; López et al., 2006).

Circulating BCAA, originating from a complex mixture of degraded dietary, microbial and endogenous protein, represent about 30% of total AA taken up by the bovine MG (Cant et al., 1993; Metcalf et al., 1996). Because mammary uptake of BCAA greatly exceeds their output in milk protein (Mepham, 1982; Bequette et al., 1996; Bequette and Douglass, 2010), any surplus is mostly directed towards the synthesis of NEAA (Mepham, 1982). Non-essential AA, like alanine and glutamine, are usually not extracted at levels required for milk protein synthesis (Manjarin et al., 2014), thus, BCAA serve as an essential source. Moreover, alanine and glutamine significantly contribute to gluconeogenesis (Holeček, 2002), one of the most important metabolic pathways generating energy and lactose in (lactating) ruminants (LeBlanc, 2010; De Koster and Opsomer, 2013). Alternatively, the BCAA themselves may also be (fully) oxidized to provide energy or might be incorporated into lipids (Vina and Williamson, 1981), further ensuring preservation of milk production.

During lactation, whole-body BCAA metabolism is increased, especially in the MG (Tesseraud et al., 1993; DeSantiago et al., 1998a), where BCAT activity rises by a factor of 10

and BCKDH is in a fully activated state (DeSantiago et al., 1998a). In rats, a strong positive correlation between total mammary BCAT activity and level of milk production has been observed (Tovar et al., 2001), indicating a possible metabolic connection, which could be founded by the fact that BCKA may also be readily reaminated to BCAA via BCAT and hence any conserved nitrogen might sustain the high rate of milk protein synthesis for lactation (DeSantiago et al., 2001). Due to the changes in BCAA partitioning related to lactation, the capacity for BCAA transamination and BCKA oxidation in the MG may be much higher than in other tissues, e.g., skeletal muscle and liver (DeSantiago et al., 1998b; Bequette et al., 2002), likely favoring BCAA metabolism by the MG. According to Lei et al. (2013), it may be assumed that an increased activity of BCAA catabolic enzymes in bovine mammary epithelial cells could in part be caused by lactation-related decreases in insulin and growth hormone and/or elevated cortisol and glucagon levels.

Different studies in mice, pigs and cows have shown that milk protein synthesis can be regulated by BCAA to some extent (Richert et al., 1997; Moshel et al., 2006; Rulquin and Pisulewski, 2006; Appuhamy et al., 2012; Doelman et al., 2015). By acting both as building blocks and as anabolic factors via mTORC1, BCAA, and particularly leucine, may increase protein translation (see chapter 1.3). However, despite growing evidence in this context, literature is still inconsistent and further studies are therefore warranted.

During late pregnancy and early lactation, bovine AT exhibits a distinct metabolic activity (McNamara, 1991). Besides providing lipids for energy and milk fat synthesis, AT is known to be an endocrine gland regulating, for example, glucose and lipid homeostasis through adipokine secretion. Yet, its role in BCAA metabolism is largely unknown in dairy cows.

1.4.2 BCAA metabolism during metabolic disorders

Excess lipids have long been considered as one of the main causes for IR (Morino et al., 2006; Muoio and Newgard, 2008). Recently however, numerous reports in monogastrics have found a strong connection between BCAA and metabolic disorders: Plasma concentrations of BCAA (and their metabolites) are consistently elevated in different models of obesity, IR, fatty liver and T2DM when compared to healthy subjects (She et al., 2007; Newgard et al., 2009; Nakamura et al., 2014; Lake et al., 2015; Polakof et al., 2018; David et al., 2019). As they seem to manifest already before disease onset, increased BCAA levels, at least in humans, are also considered as independent predictors of future IR (or T2DM) development (Wang et al., 2011;

McCormack et al., 2013). Albeit, the exact role of increased BCAA, meaning whether they are a causative or a resulting factor of such metabolic dysfunctions, as well as the underlying mechanisms are still under debate.

Generally, plasma BCAA levels arise from the difference between their rates of appearance and disappearance in the circulation, which in turn are affected by dietary intake and tissue proteolysis and by BCAA degradation and protein synthesis, respectively (She et al., 2013). It is suggested that alterations in BCAA tissue metabolism (i.e., reduced cellular utilization and/or incomplete degradation), especially in the AT, may not only promote elevated circulating BCAA levels (Herman et al., 2010) but may also contribute to the above mentioned dysfunctions (She et al., 2007; Polakof et al., 2018). In this context, different studies in humans, pigs and rodents have demonstrated a reduced abundance and activity of the BCAA catabolic enzymes in AT under obese and diabetic conditions (She et al., 2007; Pietiläinen et al., 2008; Lackey et al., 2013; Polakof et al., 2018); in some cases this was also associated with an upregulation of AT genes involved in inflammation (Klimcáková et al., 2011; Serralde-Zúniga et al., 2014). Consequently, the accumulation of BCAA and, more importantly, potentially toxic intermediates, such as BCKA, in blood and tissues (She et al., 2013) could trigger oxidative stress and mitochondrial dysfunction in pancreatic ß cells or elsewhere (Barschak et al., 2006; Lynch and Adams, 2014; Olson et al., 2014; Giesbertz and Daniel, 2016), thus leading to compromised cellular functions and ultimately, cell death. All of these processes, interfering with insulin signaling, are usually also involved during the manifestation of IR (Lynch and Adams, 2014). It should be noted however, that IR itself, once it is established, might also further increase BCAA levels, building some form of feed-forward loop (Mahendran et al., 2017; Wang et al., 2017).

Despite the possible role of AT in impaired BCAA catabolism, considering that systemic BCAA metabolism is substantially interorgan-dependent (Harper et al., 1984), other tissues might contribute to these perturbations as well. Accordingly, depending on the origin and severity of the metabolic disorder, alterations in BCAA metabolism in skeletal muscle (Lerin et al., 2016; David et al., 2019) and/or liver (Shin et al., 2014) might also be likely. Especially in the case of fatty liver diseases, an accumulation of BCAA, or rather their keto acids, may lead to mitochondrial overload and disruption of the BCAA degrading pathway in the liver, comprising further metabolic processes (Lake et al., 2015).

Apart from BCAA dysmetabolism, the involvement of nutrient signaling in IR has emerged as another possibility of how elevated BCAA levels are linked to impaired insulin action.

According to this theory, excessive nutrient intake associated with obesity also results in increased levels of insulin and BCAA, or more specifically leucine, which would both activate mTORC1 and downstream factors, such as S6K1 (Um et al., 2004; 2006; Tremblay et al., 2007). Chronic mTORC1/S6K1 activation may lead to an uncoupling of the insulin receptor from insulin receptor substrate-1, an important mediator of insulin signaling (Newgard et al., 2009; Lynch and Adams, 2014). Impaired insulin action would however drastically raise the demand for insulin. In relation to this, BCAA (particularly leucine) have also been reported to have a stimulatory effect on insulin secretion, e.g., in humans and rats (Lynch et al., 2002b; Newgard et al., 2009; Newsholme et al., 2014). A constant secretory pressure on the ß cells through an increased insulin demand potentiated by elevated BCAA levels may eventually lead to ß cell dysfunction and give rise to IR and other metabolic disorders (Newgard, 2012; Lynch and Adams, 2014). However, for cows this might not be the case, as a (single-dose) duodenal infusion of leucine did not induce an apparent insulin response (Sadri et al., 2017).

Even though the aforementioned studies in humans, pigs and rodents point out several possibilities of how BCAA may be connected to metabolic dysfunctions, as of yet there are no such data available in dairy cattle. And despite the fact that transition cows are often challenged with metabolic disorders showing a similar symptomology to IR or T2DM, due to the onset of lactation and all metabolic changes associated therewith, the situation might be partially different for them as compared to above mentioned species.

2 Objectives

The BCAA fulfill numerous metabolic functions throughout the body. Both their cellular transport and catabolism are tightly regulated and involve the complex interaction of several metabolic tissues. However, studies on the relevant BCAA transporters or the degrading key enzymes in different metabolic tissues of (lactating) ruminants are sparse. In high-yielding dairy cows, various tissue-specific metabolic processes, e.g., in AT, support the metabolic adaptation to the high demands for energy and nutrients in lactation. Yet, the contribution of AT to systemic protein and AA metabolism, especially during different physiological and pathophysiological states, has hardly been addressed in dairy cows so far.

With regards to these factors, the objectives of this thesis were:

(1) To characterize the potential enzymatic capacity of AT, alongside liver, muscle and MG, in BCAA metabolism in dairy cows during (late gestation and) early lactation,

(2) To analyze the concentrations of BCAA in the circulation as well as the abundance (and activity) of the BCAA transporters and catabolic enzymes in major metabolic tissues of dairy cows during late gestation and early lactation,

(3) To investigate the effect of over-conditioning at calving on the aforementioned variables of systemic and tissue BCAA metabolism in dairy cows during late gestation and early lactation.

It was hypothesized that early lactation is associated with tissue-specific changes in the BCAA transporters and catabolic key enzymes due to particular metabolic adaptations and that BCAA metabolism in AT contributes significantly to the degrading pathway. It was further assumed that over-conditioned cows, having a higher tissue mobilization than cows with normal body condition, would exhibit particular alterations in their systemic and tissue-specific BCAA metabolism.

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Changes in tissue abundance and activity of enzymes related to branched-chain amino acid catabolism in dairy cows during early lactation

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ABSTRACT

Branched-chain α-keto acid dehydrogenase (BCKDH) complex catalyzes the irreversible oxidative decarboxylation of branched-chain α-keto acids. This reaction is considered as the rate-limiting step in the overall branched-chain amino acid (BCAA) catabolic pathway in mammals. For characterizing the potential enzymatic involvement of liver, skeletal muscle, adipose tissue (AT), and mammary gland (MG) in BCAA metabolism during early lactation, tissue and blood samples were examined on d 1, 42, and 105 after parturition from 25 primiparous Holstein cows. Serum BCAA profiles were analyzed and the mRNA and protein abundance as well as the activity in the different tissues were assessed for the BCAA catabolic enzymes, partly for the branched-chain aminotransferase and completely for BCKDH. Total BCAA concentration in serum was lowest on d 1 after parturition and increased thereafter to a steady level for the duration of the experiment. Pronounced differences between the tissues were observed at all molecular levels. The mRNA abundance of

the mitochondrial isoform of branched-chain aminotransferase (*BCATm*) was greatest in AT as compared with the other tissues studied, indicating that AT might be an important contributor in the initiation of BCAA catabolism in dairy cows. From the different subunits of the BCKDH E1 component, only the mRNA for the β polypeptide (*BCKDHB*), not for the α polypeptide (*BCKDHA*), was elevated in liver. The *BCKDHA* mRNA abundance was similar across all tissues except muscle, which tended to lower values. Highest BCKDHA protein abundance was observed in both liver and MG, whereas BCKDHB protein was detectable in these tissues but could not be quantified. Adipose tissue and muscle only displayed abundance of the α subunit, with muscle having the lowest BCKDHA protein of all tissues. We found similarities in protein abundance for both BCKDH E1 subunits in liver and MG; however, the corresponding overall BCKDH enzyme activity was 7-fold greater in liver compared with MG, allowing for hepatic oxidation of BCAA transamination products. Reduced BCKDH activity in MG associated with no measurable activity in AT and muscle may favor sparing of BCAA for the synthesis of the different milk components, including nonessential AA. Deviating from previously published data on BCAA net fluxes and isotopic tracer studies in ruminants, our observed results might in part be due to complex counter-regulatory mechanisms during early lactation.

Key words: branched-chain amino acid metabolism, branched-chain amino-transferase, branched-chain α-keto acid dehydrogenase, tissue abundance/activity

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INTRODUCTION

The 3 branched-chain AA (**BCAA**), Ile, Leu, and Val, represent about 30% of total AA taken up by the bovine mammary gland (**MG**; Cant et al., 1993; Metcalf et al., 1996). The BCAA fulfill numerous metabolic functions; that is, as precursors for NEAA, as substrates for (milk) protein, as energy substrates, and as anabolic nutritional signals, regulating protein synthesis via insulin-dependent and -independent mechanisms (e.g., in muscle and mammary tissue; Bequette et al., 2002; Appuhamy et al., 2012). Homeostasis of BCAA is almost exclusively maintained at their catabolic steps.

A unique feature of BCAA catabolism in mammals is its tissue specificity; most EAA are degraded in the liver, whereas for BCAA the first catabolic step mainly occurs extrahepatically (Suryawan et al., 1998). However, subsequent catabolic steps seem to take place in liver again (Ananieva et al., 2017), necessitating substantial shuttling of BCAA and their intermediates between the periphery and liver. In this regard, degradation of BCAA (transamination or oxidative decarboxylation of the transamination products) has been studied in different tissues of ruminants, including the digestive tract (Pell et al., 1986; MacRae et al., 1997), liver (Lapierre et al., 2002), muscle (Pell et al., 1986; Bequette et al., 2002), and MG (Bequette et al., 1996a,b; Thivierge et al., 2002). However, previously published data on BCAA net fluxes and isotopic tracer studies in dairy cows have shown that, quantitatively, hepatic removal of BCAA (and their metabolites) is limited, as is their oxidation (Lapierre et al., 2002; Raggio et al., 2004; Larsen et al., 2015).

Another specific feature of BCAA catabolism is that the first 2 enzymes, the branched-chain aminotransferase (**BCAT**; EC 2.6.1.42) and the branched-chain α-keto acid dehydrogenase (**BCKDH**; EC 1.2.4.4), are common to all 3 BCAA. Two isoforms of BCAT are identified in mammals, with the mitochondrial BCAT (**BCATm**) being more abundant than the cytosolic BCAT (BCATc; Sweatt et al., 2004). Degradation begins with deamination of BCAA, catalyzed by both BCAT isoforms, resulting in the respective branched-chain α-keto acids (**BCKA**; keto-isoleucine, keto-leucine, keto-valine) and glutamate. The ensuing oxidative decarboxylation of the BCKA to branched-chain acyl-CoA derivatives occurs via the E1 component of the mitochondrial BCKDH complex. Unlike BCAT, BCKDH works irreversibly and is considered rate-limiting (Harper et al., 1984). After decarboxylation, the catabolic pathways for each of the BCAA diverge, ultimately leading to glucogenic or ketogenic precursors.

During lactation, whole-body BCAA catabolism is enhanced and BCAA are used to partially cover the increased energetic and nutritional needs of the tissues, especially the MG (Li et al., 2009). Studies on the actual BCAA catabolism enzymes in ruminants are sparse. However, flux studies in ruminants have shown that the lactating MG itself catabolizes significant amounts of BCAA (Wohlt et al., 1977; Bequette et al., 1996a). Based on BW, the relative rates of BCAA deamination and BCKA oxidation by the MG are much higher than those of the hind leg muscle (Bequette et al., 2002), likely favoring BCAA metabolism by the MG.

Besides storing lipids, adipose tissue (**AT**) is known to be an endocrine gland regulating; for example, glucose and lipid homeostasis through adipokine secretion. Nevertheless, its role in systemic protein and AA metabolism has not been widely studied, particularly in ruminants. Herman et al. (2010) reported the potential capacity for AT to regulate circulating BCAA levels in vivo. Those authors demonstrated that transplantation of wild-type AT into mice globally defective in peripheral BCAA metabolism reduced their circulating BCAA levels by 30 and 50% in the fasted and fed states, respectively. Assessing BCAA metabolism in AT via flux studies, as done for MG, liver, and muscle, is hardly possible due to the multiple locations of AT and the heterogeneity of function in different depots. Hence, in the current study, we aimed to test the potential enzymatic capacity of AT (alongside liver, muscle, and MG) in BCAA metabolism in dairy cows during early lactation. We hypothesized that (1) early lactation is associated with tissue-specific changes in the key enzymes of BCAA metabolism due to particular metabolic adaptations and that (2) BCAA enzymes in AT contribute significantly to the degrading pathway. Studying the changes in abundance and activity of BCAA catabolic enzymes in different tissues of dairy cows during early lactation in more detail would allow for estimating their importance in the adaptive processes.

MATERIALS AND METHODS

Animals and Sample Collection

Different tissues [i.e., liver, skeletal muscle, MG, subcutaneous (**scAT)** and visceral adipose tissue (**vAT**)] as well as blood samples were obtained from 25 primiparous German Holstein cows (mean ± SD age at first calving = 23 ± 0.2 mo) at 3 different slaughter time points: d 1, 42, and 105 after parturition. The animals were part of a comprehensive study conducted at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (Brunswick, Germany), on the effect of dietary CLA on performance and changes in body composition, including body fat and protein, and on energy utilization during early lactation (von Soosten et al., 2011, 2012). On the day after parturition, 5 animals were euthanized, whereas the remaining 20 cows were assigned to either a control (100 g/d of stearic acid; Silafat, BASF SE, Ludwigshafen, Germany; n = 10) or CLA-supplemented diet (100 g/d; 1.7% trans-10,cis-12, 1.6% cis-9,trans-11 CLA isomers; Lutrell Pure, BASF SE; n = 10). Five cows per group were then slaughtered at d 42 and 105. The CLA supplementation did not significantly affect any of the herein examined variables; thus,

and to increase the statistical power, treatment groups at the respective slaughter time points were pooled as d 1 (n = 5), 42 (n = 10), and 105 (n = 10).

All protocols of the study were designed and performed in strict accordance with the European Union guidelines, established by the European Community Council Directives 86/609/EEC (European Council, 1986), concerning the protection of experimental animals, with approval by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany (File Number 33.11.42502–04–071/07). Animals were kept in group pens in a freestall barn with free access to water. The partial mixed ration fed prepartum consisted of 60% corn silage and 40% grass silage (6.7 MJ of NEL/kg of DM) on a DM basis for ad libitum consumption and 2 kg of concentrate (6.7 MJ of NEL/kg of DM). Postpartum, cows received a partial mixed ration of 25% corn silage and 38% grass silage (7.5 MJ of NEL/kg of DM) and 37% concentrate (8.7 MJ of NEL/kg of DM) on a DM basis for ad libitum intake as well as an additional 3.5 kg of concentrate containing the respective rumen-protected CLA or control fat supplements. The additional 2 and 3.5 kg of concentrate were fed via a computerized concentrate feeding station (RIC; Insentec B.V., Marknesse, the Netherlands). All diets were formulated according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). A detailed description of the ingredients as well as the composition of the diets can be found in von Soosten et al. (2011). Postcalving, daily individual feed intake was recorded automatically (RIC; Insentec B.V) from wk 2 to 15 after the start of lactation. Cows were milked twice daily (Lemmer-Fullwood, Lohmar, Germany) with automatic recordings of milk yield. Milk samples were taken twice per week and stored at 4°C until analysis.

Right before slaughter, blood samples were taken via jugular venipuncture. Serum was obtained after clotting and centrifugation (3,000 × g, 10 min, 4°C) and stored at −80°C until analysis. Samples of liver, skeletal muscle (semitendinosus muscle), MG (udder parenchyma), scAT (tail head), and vAT (omental fat) were collected after slaughter, rinsed with 0.9% NaCl, and immediately snap-frozen in liquid nitrogen and stored at −80°C for the respective extractions and analyses.

Milk and Blood Analyses

Milk composition was assessed using an infrared milk analyzer (Milkoscan FT 6000 combined with a Fossomatic 5000, Foss Electric, Hillerød, Denmark). Serum BCAA concentrations were measured via HPLC in an RF-10A XL fluorescence detector (Shimadzu, Kyoto, Japan) based on o-phtaldialdehyde/3-mercaptopropionic acid derivatization as previously described (Fürst et al., 1990). Inter- and intra-assay variances were <5%.

RNA Extraction and Quantitative Real-Time Reverse Transcription-PCR

Total RNA extraction and cDNA synthesis were conducted as described by Saremi et al. (2012a). Analysis by quantitative real-time reverse transcription PCR (**qPCR**) was carried out in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) using the sample maximization method. Triplicates were run in a total volume of 10 µL, with 2 µL of cDNA (diluted 1:4) as template, 1 µL of assay-specific primer mix, 2 µL of water, 5 µL of DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific, Dreireich, Germany) for the target genes branched-chain aminotransferase, mitochondrial (*BCATm*), branched-chain α-keto acid dehydrogenase E1, α polypeptide (*BCKDHA*), and branched-chain α-keto acid dehydrogenase E1, β polypeptide (*BCKDHB*), or 5 µL of SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Steinheim, Germany) for the reference genes; analysis was carried out in Mx3000P cycler systems (1 from Stratagene, Amsterdam, the Netherlands, and 1 from Agilent, Santa Clara, CA). Each run included a negative template control, a no-reverse transcriptase control as well as 2 inter-run calibrators prepared from plate-specific pool samples. Quantification of the samples was performed against a cDNA standard curve with serial dilutions for the target genes and against a PCR product standard curve with serial dilutions for the reference genes. Target genes were normalized with the most stable reference genes, namely emerin (*EMD*), hippocalcin-like 1 (*HPCAL1*), eukaryotic translation initiation factor 3 (*EIF3K*), RNA polymerase II (*POLR2A*), and lipoprotein receptor-related protein 10 (*LRP10*), which were previously selected from a set of 9 genes according to Saremi et al. (2012b). Reference genes were evaluated based on their average expression stability (M) and pairwise variation (V) values (accepted M <1.5 and V <0.15) using the geNorm^{PLUS} algorithms of qBase^{PLUS} 2.1 software (Biogazelle, Ghent, Belgium). Primer sequences as well as qPCR conditions were

the same for a given gene in all tissues and are provided in Table 1.

Quantification of Target Proteins

Tissue samples were homogenized in ice-cold Tris/HCl lysis buffer (pH 7.4) containing protease inhibitors [cOmplete, Mini Protease Inhibitor Cocktail, 1 tablet per 10 mL; Roche Diagnostics, Mannheim, Germany; Pefabloc SC, also known as 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 1 mM, Sigma Aldrich, St. Louis, MO] for inhibiting serine, cysteine, and metalloproteases in a tissue-to-buffer ratio of 1:8 (liver), 1:4 (muscle and MG), or 1:2 (AT) using the Precellys 24 system (VWR/Peqlab Biotechnologie, Erlangen, Germany). Total protein concentration was measured via Bradford assay (Roti Nanoquant K880, Carl Roth, Karlsruhe, Germany).

Protein quantification was performed by Simple Western size-based protein assay (WES, Protein Simple, San Jose, CA), an automated capillary-based electrophoretic immunoassay, as per the manufacturer's instructions (https://www.proteinsimple.com/technical_library.html). In brief, samples were first diluted with 0.1× sample buffer to adjust the protein concentration to a dilution within the linear range (between 0.3125 and 0.9375 µg/µL depending on tissue type) and then diluted 4:1 by adding 2.5 µ L of the 5× master mix. The final samples were heated for 5 min at 95°C, placed on ice for 5 min, briefly centrifuged twice (2,500 × g, 5 s, 20°C) with short vortex spins in between, and applied to the wells in duplicates. A control sample (bovine liver) was also loaded on every plate to correct for inter-run variations (accepted inter-assay CV <10%, intra-assay CV <8%). Primary antibodies against total BCKDHA (#ab138460; Abcam, Cambridge, UK) and total BCKDHB (#ab182255; Abcam) were diluted 1:50 and 1:25, respectively. All other reagents (antibody diluent, secondary antibodies, streptavidin-HRP, luminol-S, hydrogen peroxide) loaded on the plate were obtained from ProteinSimple and used according to the recommendations. Simple Western analysis was performed with multi-image exposures and otherwise instrument default settings at room temperature (RT).

Using Compass Software (ProteinSimple), the area under the curve, which represents the signal intensity of the chemiluminescent immunoreaction, and which directly correlates with the abundance of the target protein, was assessed for each sample and normalized to the control.

Table 1. Characteristics of primers and real-time polymerase chain reaction conditions

Gene[1]	Sequences (5'-3')	NCBI accession no.	bp	Concentration (n*M*)	Mean C_q[2]	Annealing[3] (s/°C)
BCATm						
Forward	CATTTCCACATTCCCACCAT	NM_001013593.2	130	400	30.86	30/61
Reverse	AGCGTAGCCCAGAGCATTAC					
BCKDHA						
Forward	AGAACCAGCCCTTCCTCATT	NM_174506.1	108	200	26.61	30/61
Reverse	TGTCCCAGTAGTTGACCTCGT					
BCKDHB						
Forward	GGCCAAAGATCCTACGGCAGTAAT	NM_174507.2	129	400	27.09	30/61
Reverse	CCTTGTTCACACAGTGGGGT					
EMD						
Forward	GCCCTCAGCTTCACTCTCAGA	NM_203361	100	400	25.99	45/59
Reverse	GAGGCGTTCCCGATCCTT					
HPCAL1						
Forward	CCATCGACTTCAGGGAGTTC	NM_001098964	99	400	28.57	30/60
Reverse	CGTCGAGGTCATACATGCTG					
EIF3K						
Forward	CCAGGCCCACCAAGAAGAA	NM_001034489	125	400	26.68	45/59
Reverse	TTATACCTTCCAGGAGGTCCATGT					
POLR2A						
Forward	GAAGGGGGAGAGACAAACTG	X63564	86	800	24.32	60/60
Reverse	GGGAGGAAGAAGAAAAAGGG					
LRP10						
Forward	CCAGAGGATGAGGACGATGT	BC149232	139	400	25.32	30/61
Reverse	ATAGGGTTGCTGTCCCTGTG					

[1]*BCATm* = branched-chain aminotransferase, mitochondrial; *BCKDHA* = branched-chain α-keto acid dehydrogenase E1, α polypeptide; *BCKDHB* = branched-chain α-keto acid dehydrogenase E1, ß polypeptide; *EMD* = emerin; *HPCAL1* = hippocalcin like 1; *EIF3K* = eukaryotic translation initiation factor 3 subunit K; *POLR2A* = RNA polymerase II; *LRP10* = lipoprotein receptor-related protein 10.

[2]Mean quantification cycle for all tissues combined.

[3]Initial denaturation for 10 min at 95 °C; denaturation for 30 s at 95 °C; extension for 30 s at 72 °C, except for *POLR2A* (60 s at 72 °C).

Enzyme Activity

Enzyme activity of the BCKDH complex was determined spectrophotometrically in a Synergy H1 microplate reader (BioTek, Winooski, VT) according to Nakai et al. (2000). Approximately 100 mg of frozen tissue were homogenized (Precellys 24) in 1,200 µL of ice-cold extraction buffer. After removing insoluble material by centrifugation (20,000 × g, 5 min, 4°C), the supernatant was incubated with 27% (wt/vol) polyethylene glycol for 20 min on ice. Following a second centrifugation step

(12,000 × g, 10 min, 4°C), the pellet was dissolved in a suspension buffer by pipetting up and down while keeping the sample on ice. Protein concentrations of the prepared homogenates were assessed with Bradford assay (Roti Nanoquant K880, Carl Roth) and the activity assay was performed directly afterward. For this, samples were diluted 1:10 with 2× assay buffer and sterile water to a volume of 1 mL. Before adding the homogenate, the buffer-water mixture was preheated to 30°C and 200 µL of the final assay-mixture was then loaded onto 96-well polystyrene microplates. The absorbance at 340 nm and 30°C was recorded for 5 min to establish a baseline and the reaction was initiated with 20 µL of 10 mM α-ketoisovalerate (final concentration 1 mM) as substrate prewarmed to 30°C. In contrast to Nakai et al. (2000), the assay buffer was prepared without exogenous dihydrolipoamide dehydrogenase, as validated by Wessels et al. (2016). A control sample (bovine liver) with comparable BCKDH activity was included on each plate for inter-run calibration (accepted inter-assay CV <15%, intra-assay CV <5%) and sample data were normalized to the control.

Statistical Analyses

In case of the mRNA data, final results were calculated by qBase^{PLUS} (i.e., the calibrated normalized relative quantities values were used for statistical analysis of the data). Statistical analysis was carried out with SPSS 24 (IBM, Armonk, NY). Before analysis, data were tested for normal distribution and, when necessary (in case of mRNA data), log10 transformed. All analyses were conducted with the different dietary treatments in the respective model at first. Due to non-significance and an increase in statistical power, groups were combined in the final models given below.

Data for performance and serum BCAA were analyzed as repeated measures using a linear mixed model and Bonferroni post-hoc tests for correcting multiple comparisons:

$$Y_{ij} = \mu + cow_i + time_j + e_{ij},$$

where Y_{ij} = response variable, μ = overall mean, cow_i = random effect of cow (i = 1 to 25), $time_j$ = fixed effect of sampling time (j = wk 2 to 15 of lactation and d 1, 42, or 105 after parturition, respectively), and e_{ij} = residual error.

Data concerning mRNA and protein abundance as well as enzyme activity were evaluated using a linear mixed model and

Bonferroni correction according to the following equation:

$$Y_{ijk} = \mu + cow_i + tissue_j + time_k + tissue_j \times time_k + e_{ijk},$$

where Y_{ijk} = response variable, μ = overall mean, cow_i = random effect of cow (i = 1 to 25), $tissue_j$ = fixed effect of tissue type (j = liver, muscle, MG, scAT, vAT), $time_k$ = fixed effect of sampling time (k = d 1, 42, or 105 after parturition), $tissue_j \times time_k$ = fixed tissue × time interaction, and e_{ijk} = residual error.

Data are expressed as means ± standard error of the means. Level of significance was set at $P < 0.05$. Trends were declared at $0.05 < P \leq 0.10$.

RESULTS

Animal Performance

A detailed description of animal performance was reported by von Soosten et al. (2011). No significant differences between the treatment groups were observed in the performance parameters described in the current study; thus, data were merged for analysis of time effects (Figure 1). In brief, as expected, DMI changed over time ($P < 0.001$) and increased with week in milk (wk 2 versus all subsequent time points; $P < 0.05$). Milk yield reached a plateau around wk 4 after parturition. During peak lactation, cows yielded, on average, 27.5 ± 1.0 kg of milk and 0.86 ± 0.02 kg of milk protein.

Serum BCAA Concentrations

The CLA supplementation did not affect BCAA concentrations in serum. Concentrations of Leu were not affected by time ($P = 0.18$; Figure 2a), whereas concentrations of Ile and Val showed significant differences between the 3 time points ($P < 0.001$ and $P = 0.001$; Figure 2b and 2c, respectively). For Ile and Val, concentrations increased from d 1 (68 ± 14 and 138 ± 48 μ*M*) to 42 (180 ± 17 and 337 ± 21 μ*M*; both $P = 0.001$).

Serum concentrations of total BCAA (calculated as the sum of Leu, Ile, and Val) changed over time ($P = 0.002$; Figure 2d). The lowest concentrations, 298 ± 92 μ*M*, were observed on d 1 after parturition, which then increased to 652 ± 47 μ*M* on d 42 ($P = 0.002$). From d 42 to 105 after parturition, all BCAA concentrations remained unaltered.

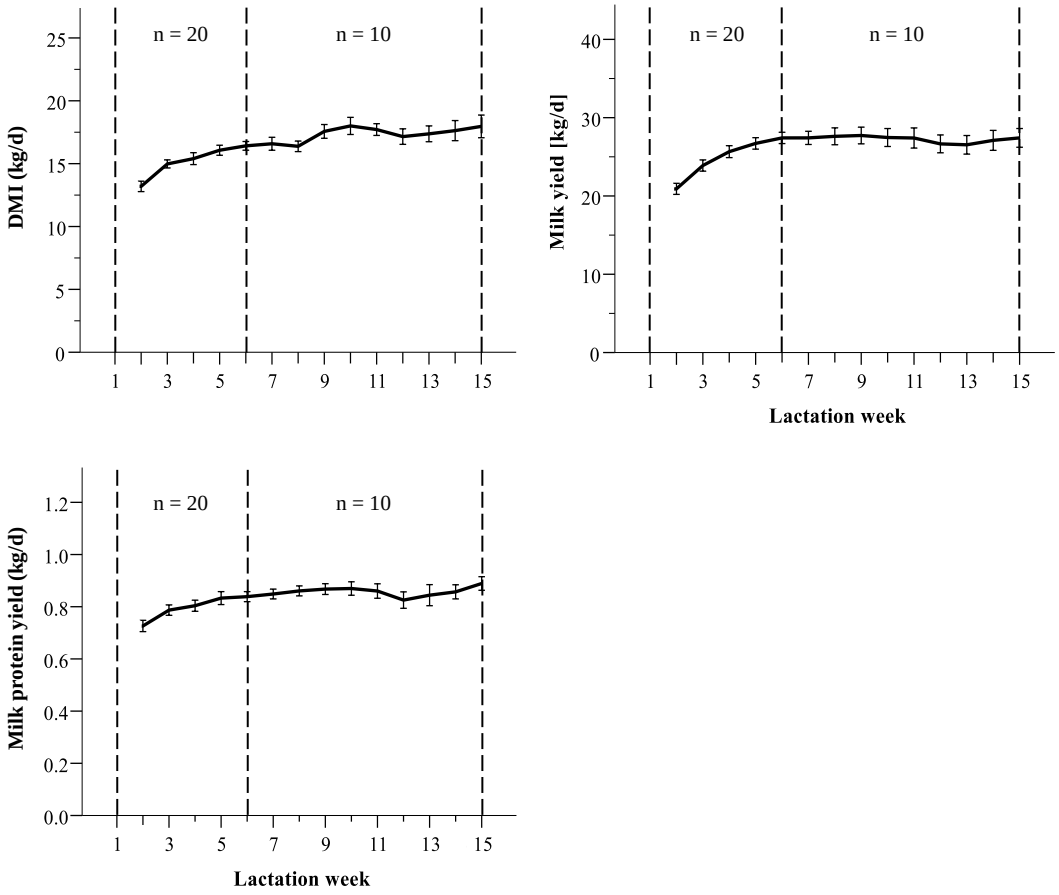

Figure 1. Weekly DMI, milk yield, and milk protein yield of cows during the experimental period. Data are given as means ± SEM. Dashed lines indicate slaughtering time points (d 1, 42, and 105 after parturition).

mRNA Abundance of BCAA Key Enzymes

The mRNA abundance of the enzymes related to BCAA catabolism did not differ between the 2 treatment groups. The *BCATm* mRNA abundance varied with tissue type and sampling time ($P < 0.001$ and $P = 0.01$, respectively; Figure 3), whereby interactions between these factors were observed ($P = 0.01$). Within tissues, we also noted time effects; that is, for scAT and vAT, *BCATm* mRNA abundance was lowest on d 1 and increased more than 3 fold until d 105 after parturition (both $P = 0.02$). For direct tissue comparisons, data from the different time points were merged, and *BCATm* mRNA was around 4-times greater in both fat depots compared with corresponding abundances in liver, muscle, and MG ($P < 0.001$). The mRNA abundance of *BCKDHA* was overall significantly influenced by tissue ($P < 0.001$) and time ($P = 0.001$), but we found no interaction of tissue × time ($P = 0.63$; Figure 4).

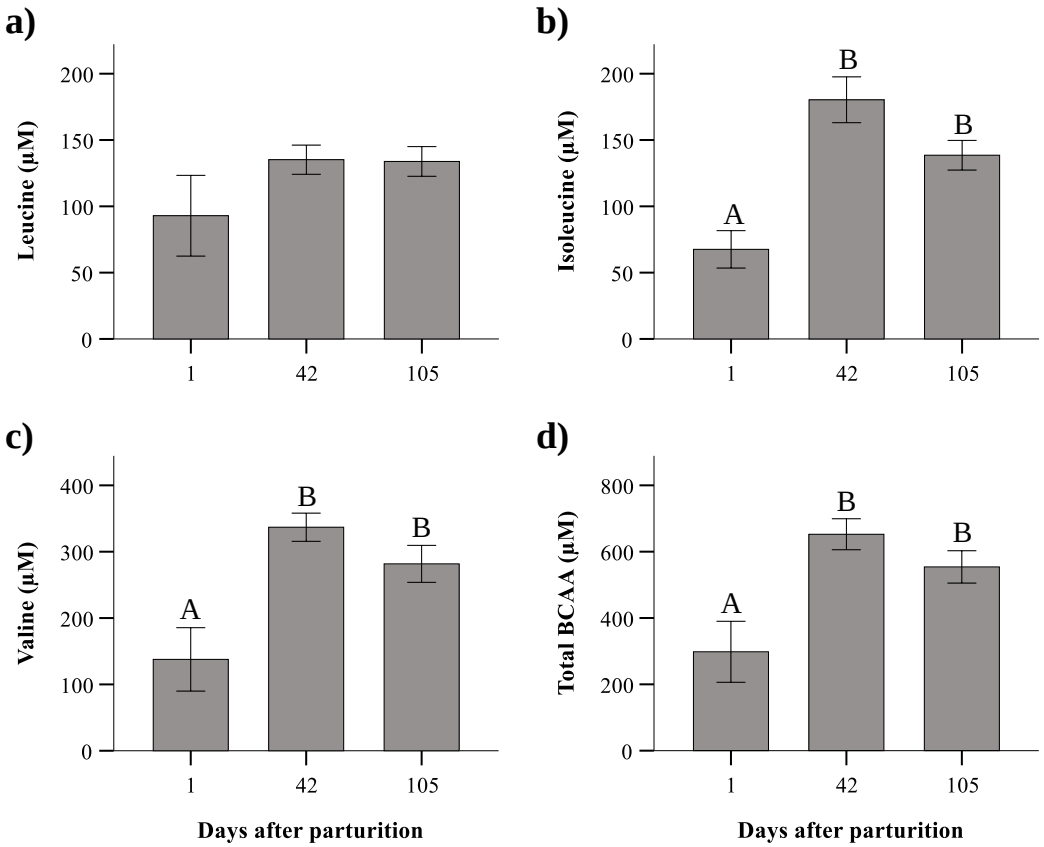

Figure 2. Serum concentrations of leucine (a), isoleucine (b), valine (c), and total branched-chain amino acids (BCAA; d) in dairy cows on d 1 (n = 5), 42 (n = 10), and 105 (n = 10) after parturition. Data are given as means ± SEM. Different letters (A,B) indicate differences ($P < 0.05$) between the 3 time points. Total BCAA are calculated as the sum of leucine, isoleucine, and valine.

Muscle tissue had the lowest *BCKDHA* mRNA, whereas liver, MG, and scAT possessed higher (all $P < 0.05$) yet similar abundance among each other. We observed an increase of *BCKDHA* mRNA over time in MG and in vAT, with the lowest values on d 1 followed by a continuous increase until d 105 ($P = 0.01$ and $P = 0.03$, respectively Tissue type and time both affected *BCKDHB* mRNA abundance ($P < 0.001$, $P = 0.01$, respectively; Figure 5), but we observed no interaction of tissue × time ($P = 0.78$). The *BCKDHB* mRNA abundance was around 5 times greater in liver than in any of the other tissues tested (each $P < 0.001$). Within MG, *BCKDHB* mRNA abundance increased steadily from d 1 to 105 ($P = 0.02$). For vAT, we noted a trend for an increase from d 1 to 105 ($P = 0.06$).

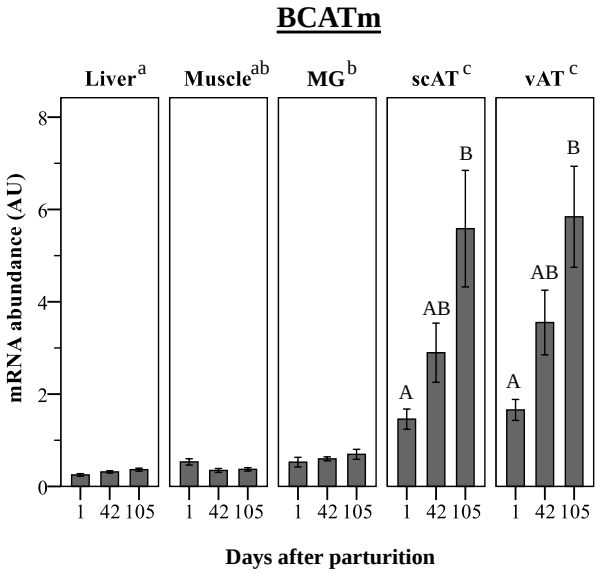

Figure 3. The mRNA abundance (arbitrary units; AU) of the mitochondrial branched-chain aminotransferase (*BCATm*) in liver, skeletal muscle, mammary gland (MG), and subcutaneous (scAT) and visceral adipose tissue (vAT) of dairy cows on d 1 (n = 5), 42 (n = 10), and 105 (n = 10) after parturition. Data are given as means ± SEM. Different lowercase letters (a–c) indicate differences ($P < 0.05$) between tissues; different uppercase letters (A,B) indicate differences ($P < 0.05$) between time points within one tissue.

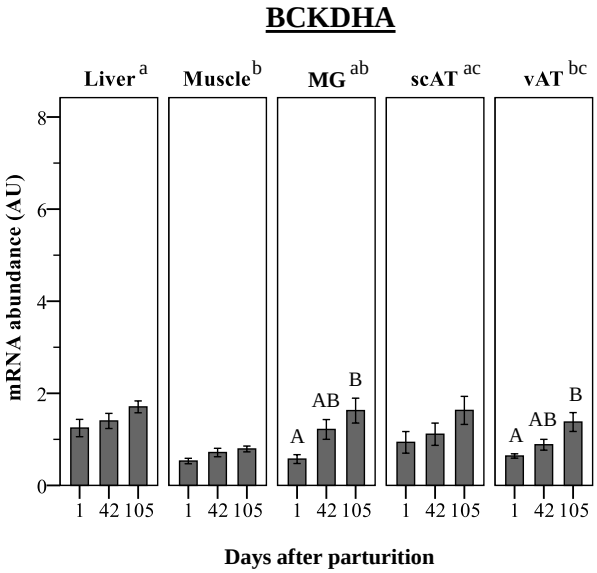

Figure 4. The mRNA abundance (arbitrary units; AU) of the branched-chain α-keto acid dehydrogenase E1, α polypeptide (BCKDHA) in liver, skeletal muscle, mammary gland (MG), and subcutaneous (scAT) and visceral adipose tissue (vAT) of dairy cows on d 1 (n = 5), 42 (n = 10), and 105 (n = 10) after parturition. Data are given as means ± SEM. Different lowercase letters (a–c) indicate differences ($P < 0.05$) between tissues; different uppercase letters (A,B) indicate differences ($P < 0.05$) between time points within one tissue.

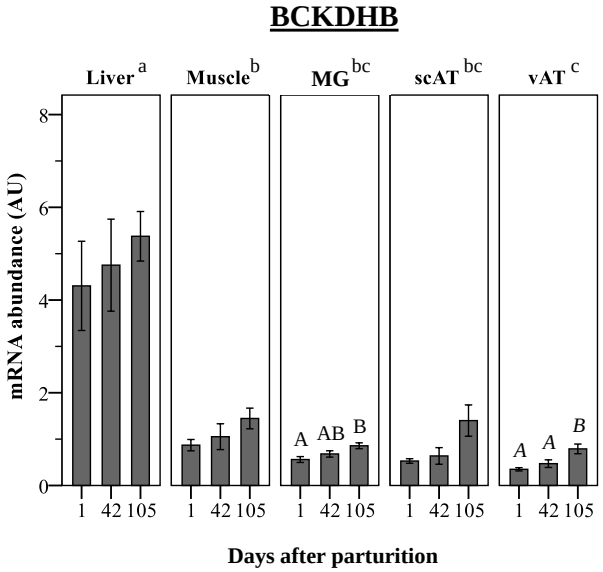

Figure 5. The mRNA abundance (arbitrary units; AU) of the branched-chain α-keto acid dehydrogenase E1, β polypeptide (*BCKDHB*) in liver, skeletal muscle, mammary gland (MG), and subcutaneous (scAT) and visceral adipose tissue (vAT) of dairy cows on d 1 (n = 5), 42 (n = 10), and 105 (n = 10) after parturition. Data are given as means ± SEM. Different lowercase letters (a–c) indicate differences ($P < 0.05$) between tissues; different uppercase letters (A,B) indicate differences ($P < 0.05$) between time points within one tissue, whereas italic letters indicate trends ($0.05 < P < 0.1$).

Protein Abundance of the BCKDH E1 Subunits

No differences related to the CLA supplementation were observed for the protein abundance of the BCKDH E1 subunits. Total BCKDHA protein abundance was affected by tissue type ($P < 0.001$), but not by time ($P = 0.07$); however, the interaction of tissue × time was significant ($P = 0.01$; Figure 6). Liver and MG expressed BCKDHA the most, followed by the 2 AT and then muscle. For MG and both AT separately, partially significant but irregular changes in BCKDHA protein over time could be detected.

Total BCKDHB protein was qualitatively detected in liver and MG, but not in muscle or AT (data not shown). However, quantification of BCKDHB protein in liver and MG was not possible due to lack of antibody saturation.

Activity of the BCKDH Complex

The activity of the whole BCKDH complex was not influenced by the CLA treatment. Even so, enzyme values for muscle and both AT were below the limit of detection.

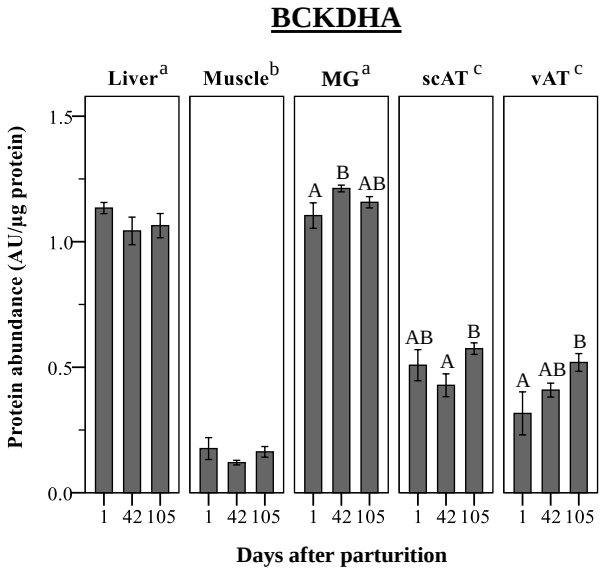

Figure 6. Protein abundance [arbitrary units (AU)/µg of protein] of the branched-chain α-keto acid dehydrogenase E1, α polypeptide (BCKDHA) in liver, skeletal muscle, mammary gland (MG), and subcutaneous (scAT) and visceral adipose tissue (vAT) of dairy cows on d 1 (n = 5), 42 (n = 10), and 105 (n = 10) after parturition. Data are given as means ± SEM. Different lowercase letters (a–c) indicate differences ($P < 0.05$) between tissues; different uppercase letters (A,B) indicate differences ($P < 0.05$) between time points within one tissue.

Activity of BCKDH was affected by tissue type ($P < 0.001$) and sampling time (P = 0.01), but we found no tissue × time interaction ($P = 0.12$; Figure 7). At all 3 time points, BCKDH activity was, on average, 7-fold greater in liver as compared with MG. Further, a slight increase of BCKDH activity in liver from d 1 to 42 after parturition was observed ($P = 0.02$). In contrast, we detected no significant time-dependent changes for MG.

DISCUSSION

In this study, we aimed to investigate the potential enzymatic capacity of different tissues in BCAA metabolism in dairy cows during early lactation by analyzing the concentrations of BCAA in the circulation as well as the mRNA and protein abundance and activity of the catabolic enzymes in liver, muscle, AT, and MG, mainly regarding the rate-limiting key enzyme BCKDH. The CLA treatment did not affect any of those variables, probably due to the timing of the supplementation, which only started on the day after parturition.

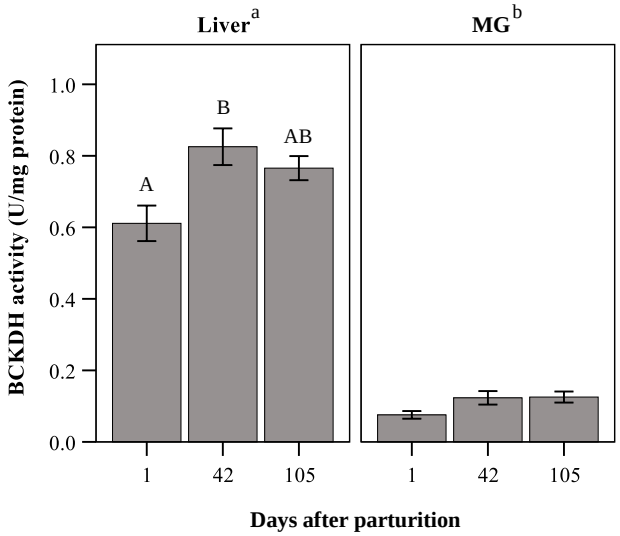

Figure 7. Enzyme activity of the branched-chain α-keto acid dehydrogenase (BCKDH) complex in liver and mammary gland (MG) of dairy cows on d 1 (n = 5), 42 (n = 10), and 105 (n = 10) after parturition. Data are given as means ± SEM. Different lowercase letters (a,b) indicate differences ($P < 0.05$) between tissues; different uppercase letters (A,B) indicate differences ($P < 0.05$) between time points within one tissue.

Further, the applied CLA dosage, although in the common range for the primarily targeted effect in dairy cows (i.e., milk fat reduction), might not have been sufficient for the supplements to accumulate in the body. Using the same study population, von Soosten et al. (2013) reported only marginal CLA concentrations in the tissues [e.g., 0.01% trans-10,cis-12 CLA in scAT, vAT, MG, and offal (containing liver) of CLA-supplemented cows versus no detectable CLA in the control group; contents in muscle were below the limit of detection for both groups] and, in case of the cis-9,trans-11 CLA isomer, no change in the amount of CLA because of supplementation. In summary, it can be assumed that because of the overall low transfer of the supplemented isomers into the body, the CLA treatment was not effective enough to elicit a response at tissue level and, thus, affect BCAA metabolism.

Concerning the tissue distribution of BCAA catabolic enzymes, the current literature is quite ambiguous and is mainly based on studies in nonruminants. To our knowledge, this is the first report on tissue-specific differences in abundance as well as activity of BCAA key enzymes in dairy cows, allowing for estimating the importance of different tissues in the adaptive processes occurring during early lactation.

It is likely that the observed lower BCAA concentrations on d 1 after parturition were due to reduced feed intake before calving, which is described in several publications (e.g., Grummer, 1995). In our study, DMI could only be recorded from the second week of lactation onwards; nevertheless, DMI was still lower at wk 2 compared with all subsequent time points. Moreover, metabolic stress, related to the negative energy balance during the transition from late pregnancy to lactation, is associated with an increase in positive acute phase proteins such as serum amyloid A (Meglia et al., 2005) and haptoglobin (Saremi et al., 2012a). Both proteins, containing BCAA and in the milligrams per milliliter range close to parturition, require substantial AA supply. In addition, it is known that human immune cells oxidize BCAA in vitro to release energy and to use them for the synthesis of new cells or effector molecules (Calder, 2006).

After absorption, most EAA are degraded by the liver, whereas catabolism of BCAA seems to be under the joint control of several metabolically active tissues (Goodwin et al., 1987; Suryawan et al., 1998). Based on data from AA net fluxes and isotopic tracer studies in dairy cows, hepatic removal of BCAA (and their metabolites) has been described as greatly reduced even at high AA supply (Wray-Cahen et al., 1997; Lapierre et al., 2002; Raggio et al., 2004) both in dry and lactating animals. However, some variations in the extent of hepatic removal relative to portal absorption have been reported (Raggio et al., 2004), with variation likely due to differences in the metabolic need of AA in various physiological stages. Pell et al. (1986), using both radiolabeled Leu and keto-leucine, the deamination product of Leu, showed that around 75% of the keto-leucine released from the hindquarter, portal drained viscera, and remaining tissues of infused sheep is oxidized in the liver, compared with only 17% in the hindquarter of fed sheep. Goodwin et al. (1987) and Papet et al. (1988) observed highest BCKDH activity in ovine liver (and kidney) compared with muscle, AT, and jejunum, demonstrating hepatic involvement in BCAA metabolism to some extent. Even if full hepatic BCKA oxidation may be relatively low, high BCKDH activity would ensure the removal of excess BCKA, which are toxic at high blood concentrations.

In general, it has been acknowledged that the initial step in BCAA catabolism, the reversible deamination via BCAT, may occur extrahepatically [e.g., in muscle (Bequette et al., 2002), AT (Bergen et al., 1988), or MG (Bequette et al., 1996a)]. Depending on the tissue, this is followed by either the export of the resulting BCKA into the blood stream or direct oxidative

decarboxylation of BCKA to acyl-CoA derivatives via BCKDH and, hence, not adding to the plasma flux (Lapierre et al., 2002).

In the current study, *BCATm* mRNA in liver was marginal, which may point to low BCAA transamination in this tissue and, consequently, a greater availability of absorbed BCAA to peripheral tissues. However, measuring mRNA cannot be used as surrogate for the corresponding protein; thus, further studies including protein abundance and activity of BCAT(m) are warranted to support our assumption.

Surprisingly, low *BCATm* mRNA abundance was also detected in MG and skeletal muscle. According to Mepham (1982), and confirmed in other bovine studies (e.g., Raggio et al., 2006; Larsen et al., 2014), BCAA (and Lys), arising primarily from muscle mobilization, are usually taken up in excess to their output as milk protein, even during early lactation, supporting the synthesis of NEAA as mammary uptake of NEAA is generally insufficient for milk protein synthesis. For this, at least some transamination is necessary; however, it may be possible that the decreased *BCATm* abundance in MG in our study was a result of a counter-effective downregulation to avoid further promotion of postpartum AA deficiencies.

Moreover, it has been reported that sheep muscle (*M. longissimus dorsi*) expresses both BCAT isoforms, *BCATm* and *BCATc*, and that significant activity may be attributed to the cytosolic isoform (Faure et al., 2001). It remains to be clarified whether BCAA transamination in muscle and MG of dairy cows may also in part or even largely be mediated by *BCATc*.

The mRNA abundance of *BCATm* was greatest in both scAT and vAT compared with all other tissues, which indicates that, on a transcriptional level, AT might be an important contributor in the initiation of BCAA catabolism in dairy cattle. This notion is in line with a study by Bergen et al. (1988), who showed that Leu deamination activity as well as the predicted deamination capacity based on body and tissue weights was highest in scAT compared with muscle, liver, and kidney of mature sheep. More recently, Green et al. (2016) demonstrated the importance of BCKA oxidation in adipogenic differentiation and that inhibition of BCAA catabolism compromised adipogenesis. Moreover, it was assumed that, through linkages in the citric acid cycle, BCAA catabolism might regulate glyceroneogenesis as well as fatty acid oxidation in AT for the maintenance of lipid homeostasis (Kainulainen et al., 2013). The extent of a possible contribution

of BCAA catabolism to milk fat synthesis is yet unclear.

During early lactation, bovine AT exhibits a pronounced metabolic activity (McNamara, 1991). Glutamate, resulting from BCAA deamination in AT, may be used for the synthesis of NEAA, such as Ala and Gln (Tischler and Goldberg, 1980), providing important "N shuttles" between different tissues and therefore supporting lactogenic processes in the MG.

Initially avoiding hepatic degradation, BCAA or their metabolites may still be catabolized in the liver to some degree, most likely to maintain BCAA/BCKA homeostasis (Ananieva et al., 2017). Therefore, it could be assumed that liver tissue generally has a high *BCKDH* mRNA expression. This expectation was only met by the findings for the β subunit, but not for the α subunit of the BCKDH E1 component. Although *BCKDHA* mRNA in liver was equally low in MG and AT, *BCKDHB* mRNA abundance was more than 5-fold greater in liver compared with all other tissues. Most studies in mammals focused on assessing the α subunit of the BCKDH E1 component (Harris et al., 1997; Herman et al., 2010; Nichols et al., 2016), assuming it to be one of the main subunits responsive to nutritional and hormonal influences and therefore regulating enzymatic activity. Until now, it has not been clear whether this also applies for the ruminant organism. The sole upregulation of *BCKDHB* mRNA could imply that the β subunit might also partake in the hepatic disposal of BCAA (or more specifically, BCKA) in dairy cows. Yet, in the current study, this could not be fully supported at the level of protein, as only a qualitative detection of BCKDHB protein was possible. Both *BCKDHA* as well as *BCKDHB* mRNA increased in MG and vAT during the course of the experiment, indicating a possible enhancement of BCKA oxidation in these tissues to meet the rising energetic and nutritional demands with progressing lactation. In partial support of this, DeSantiago et al. (1998) described a 10-fold increase of Leu oxidation in MG of rats in peak lactation in comparison to nonlactating rats. No data have been published yet regarding BCAA metabolism in AT of lactating individuals.

Highest BCKDHA protein abundance was observed in liver and MG, followed by AT, and lowest in muscle, suggesting a relatively low oxidative capacity of the latter tissue in dairy cows during early lactation. The actual changes of BCKDHA protein abundance with time in MG and AT were rather negligible and did not follow any regular patterns; therefore, interpretation is not reasonable. Based on calculations of isotopic transfers in Lapierre et al. (2002), liver contributes only 3 to 19% to whole-body Leu oxidation compared

with 31 to 37% for the portal drained viscera in lactating dairy cows. However, the high capacity for hepatic oxidation of BCKA enabled through increased BCKDH abundance (and activity), as shown in our study, could be a counter-regulatory response to the quantitatively low liver net flux of BCAA and their metabolites, ensuring all possible substrates or energy sources be used for milk production. Moreover, the possibility of hepatic BCKA degradation could be seen as a form of preventive measure, only truly affecting hepatic fluxes in case of perilously high BCKA concentrations in the circulation, which under regular feeding regimens would naturally be avoided.

Likewise, the observed elevated BCKDH protein abundance in MG may facilitate irreversible degradation of BCAA deamination products and, therefore, provide additional energy for lactation; however, oxidation might vary with AA supply (Bequette et al., 1996a). In a different study, Bequette et al. (2002) described a higher oxidation rate of 4-methyl 2-oxopentanoate (**MOP**; keto-leucine) in the hind leg compared with MG of lactating goats. For the hind leg, however, the uptake of MOP was negative throughout the whole experiment, indicating endogenous MOP production for which those authors did not correct. Further, it has already been hypothesized that, during early lactation, ovine skeletal muscle is less responsive to both AA supplies (Tauveron et al., 1994) and insulin (Debras et al., 1989) compared with mid lactation, ensuring optimal availability of nutrients for lactation. A downregulation of BCAA degrading enzymes in muscle may therefore support the flux of BCAA (and their intermediates) toward the MG.

Activity of the BCKDH complex is regulated through phosphorylation (inactivation) and dephosphorylation (activation) of the E1α subunit of the enzyme complex by a specific kinase (branched-chain α-keto acid dehydrogenase kinase; EC 2.7.11.4) and phosphatase (branched-chain α-keto acid dehydrogenase phosphatase; EC 3.1.3.16), respectively (Shimomura et al., 1990; Lu et al., 2009). In mature sheep, BCKDH is almost fully activated in the liver (Goodwin et al., 1987); hence, the ability of liver to respond acutely to metabolic events is limited and probably requires the synthesis of additional enzyme protein (Lapierre et al., 2002). On the contrary, in tissues such as skeletal muscle or AT, most of the BCKDH enzyme is inactive (Goodwin et al., 1987; Papet et al., 1988) and thus acutely sensitive to metabolic regulators such as insulin, energy status, and BCAA supply (Aftring et al., 1986; Hutson, 1986). The presence of BCKDH in its inactive form might therefore explain the unquantifiable

activities of the enzyme in muscle and AT in the present study. Moreover, a high ratio of BCAT to BCKDH activity in muscle, favoring the release of BCKA into the circulation instead of their oxidation, as well as a high BCKDH-to-BCAT activity ratio in liver, allowing for BCKA oxidation (Hutson et al., 2005), would both support a continuous exchange of metabolites between these tissues.

During lactation, the MG is prioritized in nutrient use over other peripheral tissues (Bauman and Currie, 1980), and BCAA uptake by the MG was shown to be enhanced in lactating ruminants (Davis et al., 1978; Guinard and Rulquin, 1994). Consequently, protein synthesis in other tissues, such as muscle, declines (Baracos et al., 1991), resulting in increased relative contributions from the splanchnic tissues and MG to whole-body protein metabolism (Lapierre et al., 2002). Our results have shown that, despite the similarities in protein abundance in liver and MG, the corresponding BCKDH enzyme activity was only increased in liver, probably due to physiological stimuli regulating BCAA homeostasis or distinct tissue-specific responses to insulin. This form of decoupling might lead to a greater amount of interorgan shuttling of BCAA and their metabolites, which could allow for a more differential regulation and support BCAA conservation. Accordingly, the lower enzyme activity observed in the MG possibly directs the flux of BCAA (or rather their keto acids) to de novo protein synthesis as EAA and NEAA.

However, having measured specific enzyme activity (i.e., enzyme activity related to protein mass) under saturated substrate conditions ex vivo may not necessarily reflect the real activity within the cell. Therefore, the applicability of results obtained to in vivo systems might have some limitations, particularly because data on BCAA/BCKA tissue transfers are missing in our study. However, for the investigated tissues, BCKDH activity was measured under the same conditions; thus, a tissue-wise comparison of the enzymatic capacity for BCKA oxidation is still possible.

Nevertheless, given that BCKDH catalyzes the rate-limiting step in the BCAA catabolic pathway, alterations in enzyme activity may play an important role in determining the metabolic fate of BCAA; that is, whether these AA should be used as energy sources or, eventually, for the synthesis of (milk) protein during lactation.

CONCLUSIONS

The presented data reveal that the key enzymes of BCAA catabolism vary in their contribution to BCAA degradation in major metabolic tissues of dairy cows. The greatest abundance of *BCATm* mRNA in both scAT and vAT as compared with other tissues may indicate that AT is an important contributor in the reversible degradation of BCAA to BCKA during early lactation. Reduced BCKDH enzyme activity in MG along with no measurable activity in AT and muscle may favor sparing of BCAA for the synthesis of the different milk components, including NEAA. Among the tissues studied, liver had high BCKDH abundance and the greatest enzyme activity. Based on previous flux studies in ruminants and our observations, these data might suggest a counter-regulatory mechanism employed by liver during early lactation. It is also likely that BCKDH is fully activated in the liver and, hence, the ability of liver to respond acutely to metabolic events might be limited. Given that protein abundance may not proportionally correspond to mRNA, future research that includes protein abundance and activity of BCAT(m) may provide additional clues to the involvement of the studied tissues in BCAA metabolism in dairy cows.

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Branched-chain amino acids: Abundance of their transporters and metabolizing enzymes in adipose tissue, skeletal muscle and liver of dairy cows at high or normal body condition

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ABSTRACT

Branched-chain amino acids (BCAA) are major components of milk protein and important precursors for non-essential AA. Thus, the BCAA transport and break-down play a key role in the metabolic adaptation to the high nutrient demands in lactation. However, in monogastrics, increased BCAA levels have been linked with obesity and certain metabolic disorders such as impaired insulin sensitivity. Our objective was to study the effect of over-conditioning at calving on plasma BCAA levels as well as the tissue abundance of the most relevant BCAA transporters and degrading enzymes in dairy cows during late pregnancy and early lactation. Thirty-eight Holstein cows were allocated 15 wk antepartum to either a

normal- (NBCS) or over-conditioned (HBCS) group, receiving 6.8 or 7.2 MJ of NE_L/kg of DM, respectively, during late lactation to reach the targeted differences in body condition score (BCS) and back fat thickness (BFT; NBCS: BCS < 3.5, BFT < 1.2 cm; HBCS: BCS > 3.75, BFT > 1.4 cm) until dry-off. During the dry period and next lactation, cows were fed the same diets, whereby differences in BCS and BFT were maintained: prepartum means were 3.16 ± 0.06 and 1.03 ± 0.07 cm (NBCS) vs. 3.77 ± 0.08 and 1.89 ± 0.11 cm (HBCS), postpartum means were 2.89 ± 0.06 and 0.81 ± 0.05 cm (NBCS) vs. 3.30 ± 0.06 and 1.38 ± 0.08 cm (HBCS). Blood and biopsies from liver, *M. semitendinosus*, and subcutaneous adipose tissue (scAT) were sampled at d 49 antepartum, 3, 21 and 84 postpartum. Free BCAA were analyzed and the mRNA abundance of solute carrier family 1 member 5 (*SLC1A5*), *SLC7A5*, and *SLC38A2* as well as branched-chain aminotransferase 2 (*BCAT2*), branched-chain α-keto acid dehydrogenase E1α (*BCKDHA*), and branched-chain α-keto acid dehydrogenase E1ß (*BCKDHB*) as well as the protein abundance of BCKDHA were assessed. Concentrations of all BCAA changed with time, most markedly in HBCS cows, with a nadir around calving. Apart from Ile, neither individual nor total BCAA differed between groups. The HBCS group had greater *BCKDHA* mRNA as well as higher prepartum BCKDHA protein abundance in scAT than NBCS cows pointing to a greater oxidative capacity for the irreversible degradation of BCAA transamination products in scAT of over-conditioned cows. Prepartum hepatic BCKDHA protein abundance was lower in HBCS than in NBCS cows. In both groups, *SLC1A5*, *SLC7A5* and *BCAT2* mRNA were most abundant in scAT, whereas *SLC38A2* was higher in scAT and muscle compared with liver, and *BCKDHA* and *BCKDHB* mRNA were greatest in liver and muscle, respectively. Our results indicate that scAT may be a major site of BCAA uptake and initial catabolism, with the former, however, being independent of BCS and time relative to calving in dairy cows.

Keywords: Branched-chain amino acid transporter, branched-chain amino acid enzyme, body condition

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INTRODUCTION

During the transition from late pregnancy to lactation, dairy cows have to mobilize body reserves from adipose tissue (**AT**) and skeletal muscle to cover the increasing nutrient demands imposed by the onset of milk production (Plaizier et al., 2000; Kuhla et al., 2011). The extent of peripartal tissue mobilization is thereby largely influenced by body condition. After calving, over-conditioned cows (BCS ≥ 3.75; 5-point scale) mobilize relatively more body reserves than cows in moderate (2.75 ≤ BCS ≤ 3.5) or lean condition (BCS ≤ 2.5; Reid et al., 1986; Drackley et al., 2005; Pires et al., 2013). A greater reduction before parturition as well as a slower increase of feed intake after parturition are often observed in cows with high BCS (Garnsworthy and Topps, 1982; Hayirli et al., 2002; Roche et al., 2009) and are considered as the main reasons for such increased tissue mobilization (Roche et al., 2009). Consequently, over-conditioned cows undergo a more pronounced and prolonged negative energy and protein balance and bear a greater risk for developing metabolic disorders such as ketosis, mastitis or milk fever (Holtenius et al., 2003; Roche et al., 2009). Enhanced peripheral insulin resistance (**IR**), a frequent issue in modern high-yielding dairy cows during early lactation (Chagas et al., 2009), is often associated with the development of these dysfunctions. Further, in obese humans, rodents and pigs, elevated levels of branched-chain amino acids (**BCAA**) have been linked with IR (Newgard et al., 2009; Polakof et al., 2018).

Circulating BCAA, generally originated from a complex mixture of degraded dietary, microbial and endogenous protein, may be used by tissues as precursors for NEAA and as substrates for milk protein synthesis as well as for generating energy (DeSantiago et al., 1998; Thivierge et al., 2002). Additionally, BCAA, in particular Leu, have emerged as signaling molecules, directly activating the mechanistic target of rapamycin complex 1 (**mTORC1**) pathway and thus regulating tissue protein synthesis in both ruminants (Toerien et al., 2010; Appuhamy et al., 2012) and nonruminants (Lynch et al., 2002; Suryawan et al., 2012).

For the cellular uptake of AA, specific transporters are required. In case of BCAA, the preferred transporter is the sodium-independent L-type large neutral amino acid transporter small subunit 1 (**LAT1**; Baumrucker, 1985; Hyde et al., 2003; Batistel et al., 2017), which is encoded by the solute carrier family 7 member 5 (*SLC7A5*) gene and can directly import BCAA from extracellular space in exchange for Gln. Nonetheless, other neutral transporters, likewise ubiquitously expressed (Fuchs and Bode, 2005; Hundal and Taylor, 2009), may also directly

transport BCAA or are indirectly involved in their transport, such as the A-type sodium-coupled neutral amino acid transporter 2 (**SNAT2**, encoded by *SLC38A2*) and the ASC-type sodium-dependent neutral amino acid transporter type 2 (**ASCT2**, encoded by *SLC1A5*). Both SNAT2 and ASCT2 prefer the cellular import of smaller AA such as Gln, enabling the cytoplasmic accumulation of this AA as "exchange currency" for the BCAA transport via LAT1 (Kanai et al., 1998; Mastroberardino et al., 1998).

In contrast to most other EAA, only a small proportion of absorbed BCAA is directly removed by the ruminant liver (Lapierre et al., 2002; Raggio et al., 2004) and at least the initial catabolic step, the reversible deamination via the branched-chain aminotransferase (**BCAT**; EC 2.6.1.42), yielding the respective branched-chain α-keto acids (**BCKA**; ketoisoleucine, ketoleucine, ketovaline) and glutamate, may occur extrahepatically [e.g., in muscle (Bequette et al., 2002) and AT (Bergen et al., 1988)]. However, the subsequent oxidative decarboxylation of the BCKA to branched-chain acyl CoA derivatives, catalyzed by the rate-limiting branched-chain α-keto acid dehydrogenase (**BCKDH**; EC 1.2.4.4) complex, may take place in liver again in order to maintain physiological BCAA/BCKA levels (Ananieva et al., 2017; Webb et al., 2019). Whole-body BCAA homeostasis is therefore highly dependent on the cross-talk between various tissues.

So far, the tissue abundance of BCAA transporters (**BCAAT**) has been assessed in bovine mammary gland (Bionaz and Loor, 2011), placenta (Batistel et al., 2017) and AT (Liang et al., 2019). However, no comparative study has been conducted yet comparing different tissues of transition dairy cows.

Different studies have shown that AT may be an important site for BCAA metabolism (Bergen et al., 1988; Herman et al., 2010; Liang et al., 2019; Webb et al., 2019), in some cases even during periods of negative energy balance. Regardless of this however, a possible link between BCAA degradation and fatty acid metabolism, in particular glyceroneogenesis and fatty acid oxidation might exist via the tricarboxylic acid (**TCA**) cycle (Kainulainen et al., 2013). As body condition may influence both lipid as well as protein mobilization in transition dairy cows (Kokkonen et al., 2005), we hypothesized that over-conditioned cows, having a more intense mobilization of body reserves than normal-conditioned cows due to the greater metabolic challenge during early lactation, would also show particular alterations within their systemic and tissue-specific BCAA metabolism.

Thus, we aimed (1) to investigate the changes in plasma BCAA concentrations in cows that were over- or normal-conditioned at calving and (2) to identify the differences in the mRNA abundance of the most relevant BCAAT as well as the mRNA and protein abundance of the BCAA catabolizing enzymes in subcutaneous AT (**scAT**), muscle and liver of these animals during the transition from late pregnancy to early lactation.

MATERIALS AND METHODS

Animals and sample collection

The trial was conducted at the experimental station of the Educational and Research Center for Animal Husbandry, Hofgut Neumühle, Münchweiler a.d. Alsenz, Germany. All protocols of the study were designed and performed in strict accordance with the European Union Guidelines concerning the protection of experimental animals, with approval by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany [G 14-20-071]). The animals were part of a trial aiming to establish an experimental model of high versus normal body tissue mobilization during the transition from pregnancy to lactation. A detailed description of the experimental design together with performance data has already been reported (Schuh et al., 2019). Briefly, 15 wk before calving, 38 multiparous German Holstein cows (average parity 2.9 ± 0.3) were allocated to either a normal-conditioned (**NBCS**; n = 19) or high-conditioned group (**HBCS**; n = 19). To reach the targeted differences in body condition score (BCS) and back fat thickness (BFT) in the experimental groups (NBCS: BCS < 3.5 and BFT < 1.2 cm; HBCS: BCS > 3.75 and BFT > 1.4 cm) until dry-off (wk 7 antepartum, **a.p.**), NBCS cows received a low-energy ration (6.8 MJ of NE_L/kg of DM) and HBCS cows were fed a ration with greater energy density (7.2 MJ of NE_L/kg of DM) during late lactation. During the dry-period and subsequent lactation both groups received the same diets. The animals obtained all diets for ad libitum intake as total mixed rations consisting of 74% roughage and 26% concentrate in the low-energy ration and 63% roughage and 37% concentrate in the high-energy ration. All diets were formulated according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). A detailed description of the ingredients as well as the composition of the diets is given in Supplemental Table S1 (https://doi.org/10.3168/jds.2019-17147).

During the whole trial (15 wk a.p. until 15 wk postpartum, **p.p.**), BCS and BFT were assessed every 2 wk by the same person. Animals were kept in a freestall

barn with free access to water and feed. Daily individual feed intake was recorded automatically (RIC; Insentec, B.V; Marknesse, The Netherlands) from wk 3 a.p. until wk 14 p.p. Cows were milked twice daily (GEA Farm Technologies GmbH, Boenen, Germany) with automatic recordings of milk yield. Milk samples were taken weekly and stored at 4°C until analysis. In addition, blood from the *V. caudalis mediana* was collected weekly, and samples from d 49 a.p. and 3, 21 and 84 p.p. were used herein. Plasma was obtained after centrifugation (10 min at 2000 × *g*, 4°C) and stored at -20°C until analysis.

Further, tissue biopsies from liver, skeletal muscle and scAT were collected at d 49 (±5) a.p. and 3 (±2), 21 (±2) and 84 (±2) p.p. under local anesthesia (procaine hydrochloride, 20 mg/mL, 8 mL per biopsy in case of liver and muscle, 9 mL per biopsy for scAT; Richter Pharma AG, Wels, Austria) and while the animals were sedated (xylazine i.v., 20 mg/mL, 0.1 mL/100 kg BW; CP-Pharma Handels GmbH, Burgdorf, Germany) and fixed in a headlock. In total, 28, 31 and 24 animals had the full sets of liver, muscle and scAT biopsies, respectively. Liver tissue was obtained by performing a small incision through the skin at the 11th and 12th intercostal space on a line between the olecranon and the tuber coxae with a 14G biopsy needle (Dispomed Witt oHG, Gelnhausen, Germany). Samples of muscle (*M. semitendinosus*) were collected through a 1-cm incision with a Bard Magnum biopsy instrument and Bard Magnum core tissue biopsy needles (12 gauge × 20 cm, C.R. Bard Inc., Tempe, AZ). Adipose tissue was excised from the tail head region with a scalpel through an incision of 1 cm width. Immediately after sampling, incisions were closed with a sterile needle and sterile absorbable suture (Spool suture PGA, USP 1, EP 4, LOT 15B27, Henry Schein U.K. Holdings Ltd, Gillingham, U.K.). To prevent infection and for analgesia, respectively, oxytetracycline hydrochloride was applied to the skin (25 mg/mL, EngemycinTM, MSD Animal Health Innovation GmbH, Schwabenheim an der Selz, Germany) and a ketoprofen injection (100 mg/mL, 3 mL/100 kg BW; Streuli Pharma AG, Uznach, Switzerland) was given. All tissue samples were rinsed with 0.9% NaCl to remove any blood contamination, immediately snap-frozen in liquid nitrogen and stored at -80°C for the respective extractions and analyses.

Milk and Blood Analyses

Milk composition was assessed using an infrared milk analyzer (Bentley FTS, Bentley Instruments, Inc., Chaska, MN, USA). Plasma BCAA concentrations were measured via high performance liquid

chromatography (HPLC) in a RF-10A XL fluorescence detector (Shimadzu, Kyoto, Japan) based on o-phtaldialdehyde/3-mercaptopropionic acid derivatization as previously described (Fürst et al., 1990). Inter- and intra-assay variances were <5%.

RNA Extraction and Quantitative Real-Time Reverse Transcription-PCR

Tissues (20, 100, 200 mg of liver, muscle and scAT, respectively) were homogenized in 1 mL of QIAzol (Qiagen N.V., Hilden, Germany) using the Precellys 24 system (VWR/Peqlab Biotechnologie, Erlangen, Germany). The extracted total RNA was purified with spin columns according to the Qiagen mini kit protocol (RNeasy Mini Kit, Qiagen). The concentration and the purity of the RNA obtained were assessed with the Nanodrop 1000 (VWR/Peqlab Biotechnologie) by measuring the absorbance at 260 and 280 nm. Using ethidium bromide denaturing RNA gel electrophoresis, the integrity of the RNA was checked. For cDNA synthesis, 250 ng total RNA per 20 µL reaction were reverse transcribed in duplicate for each sample with RevertAid reverse transcriptase (Thermo Fisher Scientific, Dreieich, Germany) in a thermocycler Alpha-SC (Analytik Jena, Jena, Germany).

Analysis by quantitative real-time reverse transcription PCR (**qPCR**) was carried out in Mx3000P qPCR systems (Stratagene, Amsterdam, The Netherlands, and Agilent, Santa Clara, CA) in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Samples were run as triplicates in a total volume of 10 µL, with 2 µL cDNA (diluted 1:4) as template, 1 µL assay-specific primer mix, 2 µL water, and 5 µL DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific). Relative quantification of the target genes, i.e., solute carrier family 1, member 5 (*SLC1A5*), solute carrier family 38, member 2 (*SLC38A2*), solute carrier family 7, member 5 (*SLC7A5*), branched-chain aminotransferase 2 (*BCAT2*), branched-chain α-keto acid dehydrogenase E1α (*BCKDHA*), and branched-chain α-keto acid dehydrogenase E1ß (*BCKDHB*), was performed with standard curves using cDNA serial dilutions to calculate the abundance based on run-specific PCR efficiency. Each run included a no-template control and a no-reverse-transcriptase control.

To remove any systematic bias caused by random and technical variations between the plates, a factor correction, taking into account all overlapping technical and biological replicates, was conducted using Factor-qPCR software (Ruijter et al., 2015). Target genes were normalized with the 4

most stable reference genes, namely hippocalcin-like 1 (*HPCAL1*), emerin (*EMD*), RNA polymerase II (*POLR2A*), and eucariotic translation initiation factor 3 (*EIF3K*), which were evaluated based on their average expression stability (M) and pairwise variation (V) values (M < 1.0 and V = 0.18) using the geNorm^{PLUS} algorithms of qBase^{PLUS} 3.2 software (Biogazelle, Ghent, Belgium). Primer sequences as well as qPCR conditions were the same for a given gene in all tissues and are provided in Table 1.

Protein quantification

Protein extraction and quantification via Simple Western size-based protein assay (WES, ProteinSimple, San Jose, CA) were described earlier (Webb et al., 2019). In brief, after tissue homogenization, samples were diluted with 0.1 × sample buffer to a protein concentration of 0.325 mg/mL for liver and 0.5 mg/mL for muscle and scAT, respectively and then mixed with 5 × master mix containing 40 mM dithiothreitol. Following denaturation (5 min, 95°C), samples were pipetted in duplicate onto the plate. A control sample (bovine liver) was also loaded on every plate to correct for inter-run variations. The primary antibody against total BCKDHA (#ab138460; Abcam, Cambridge, UK) was diluted 1:50. All other reagents (antibody diluent, secondary antibody, streptavidin-HRP, luminol-S, and hydrogen peroxide) were obtained from ProteinSimple and used according to the recommendations. Simple Western analysis was performed with instrument default settings at room temperature. Using Compass Software (ProteinSimple), the area under the curve was assessed for each sample and normalized to the control of the applicable plate of the sample and over all plates, respectively.

Statistical Analyses

In case of the mRNA data, final results (i.e., calibrated normalized relative quantities) were calculated by qBase^{PLUS}. Statistical analysis for all data was carried out with SPSS 25 (IBM, Armonk, NY). Before analysis, data were tested for normal distribution and when necessary (in case of mRNA data) were $\log_{10}$ transformed. Using a Linear Mixed Model and Bonferroni correction for multiple comparisons, data were analyzed as repeated measures with “group” (treatment), “time” (week or day relative to calving), “tissue type” (in case of mRNA and protein data) and the interaction thereof as fixed effects, and “cow” as a random factor. For all graphs, nontransformed data (means ± SEM) were used. The level of significance was set at *P*

< 0.05. Trends were declared at $0.05 < P \leq 0.10$.

Table 1. Characteristics of primers and real-time polymerase chain reaction conditions

Gene[1]	Sequences (5'-3')	NCBI[2] accession no.	bp	Concentration (n*M*)	Mean C_q[3]	Annealing[4] (s/°C)
BCAT2						
Forward	CATTTCCACATTCCCACCAT	NM_001013593.2	130	400	28.69	30/61
Reverse	AGCGTAGCCCAGAGCATTAC					
BCKDHA						
Forward	AGAACCAGCCCTTCCTCATT	NM_174506.1	108	200	25.99	30/61
Reverse	TGTCCCAGTAGTTGACCTCGT					
BCKDHB						
Forward	GGCCAAAGATCCTACGGCAGTAAT	NM_174507.2	129	400	25.42	30/61
Reverse	CCTTGTTCACACAGTGGGGT					
EIF3K						
Forward	CCAGGCCCACCAAGAAGAA	NM_001034489	125	400	26.35	45/59
Reverse	TTATACCTTCCAGGAGGTCCATGT					
EMD						
Forward	GCCCTCAGCTTCACTCTCAGA	NM_203361	100	400	25.59	45/59
Reverse	GAGGCGTTCCCGATCCTT					
HPCAL1						
Forward	CCATCGACTTCAGGGAGTTC	NM_001098964	99	400	27.05	30/60
Reverse	CGTCGAGGTCATACATGCTG					
POLR2A						
Forward	GAAGGGGGAGAGACAAACTG	X63564	86	800	24.97	60/60
Reverse	GGGAGGAAGAAGAAAAAGGG					
SLC1A5						
Forward	GGCTAGCAGCTGTTTACTCCT	NM_174601.2	129	200/400	27.31	30/60
Reverse	AGTCTGGGGGCTAGAAGACG					
SLC38A2						
Forward	TGAAAAGCCATTATGCCGATGT	NM_001082424.1	148	400	24.06	30/60
Reverse	CCCACAATCGCATTGCTCAG					
SLC7A5						
Forward	GGGTGACGTAGCCAATCTGG	NM_174613.2	107	200	29.59	30/60
Reverse	ATCCCCCATAGGCAAAGAGG					

[1]*BCAT2* = branched-chain aminotransferase 2; *BCKDHA* = branched chain α-keto acid dehydrogenase E1α; *BCKDHB* = branched chain α-keto acid dehydrogenase E1ß; *EMD* = emerin; *HPCAL1* = hippocalcin like 1; *EIF3K* = eukaryotic translation initiation factor 3 subunit K; *POLR2A* = RNA polymerase II; *SLC1A5* = solute carrier family 1, member 5; *SLC38A2* = solute carrier family 38, member 2; *SLC7A5* = solute carrier family 7, member 5.
[2]NCBI = National Center for Biotechnology Information.
[3]Mean quantification cycle for all tissues combined.
[4]Initial denaturation for 10 min at 95°C; denaturation for 30 s at 95°C; extension for 30 s at 72°C, except for *POLR2A* and *SLC1A5* (60 s at 72°C).

RESULTS

Body condition and animal performance

A summary of disease incidence in the cows studied herein is provided in Supplemental Table S2 (https://doi.org/10.3168/jds.2019-17147). In the present study, the number of animals was too small to allow for valid comparisons of disease incidence. All cows enrolled in the study (n = 40) were free of disease, including mastitis. Results from 2 cows (one cow from each group), that failed to complete the sampling schedule, were excluded, thus data of 38 cows were used. No clinical health events occurred before calving. Mastitis was the most common clinical ailment postpartum. Numerical differences existed between HBCS and NBCS cows in the number of cows affected by clinical events (i.e., total clinical events were observed for 27 HBCS vs. 15 NBCS cows, including 4 HBCS vs. 2 NBCS cows with ketosis and 4 HBCS vs. 2 NBCS cows with milk fever).

A more detailed description of variables characterizing body condition as well as animal performance is given in Schuh et al. (2019). In brief, both BCS and BFT differed between the two treatment groups during the whole study, with HBCS cows having greater values than NBCS cows (both $P < 0.001$; Figure 1). Prepartum BCS and BFT values (as means ± SEM) were 3.77 ± 0.08 and 1.89 ± 0.11 cm for HBCS vs. 3.16 ± 0.06 and 1.03 ± 0.07 cm for NBCS, whereas postpartum values were 3.30 ± 0.06 and 1.38 ± 0.08 cm for HBCS vs. 2.89 ± 0.06 and 0.81 ± 0.05 cm for NBCS. During the dry period, when both groups received the same diet, body condition increased independent of treatment. Previously established differences between NBCS and HBCS cows remained until wk 1 a.p. (Δ = 0.7 BCS points, 1.1 cm BFT). Average calving BCS and BFT were 4.04 ± 0.07 and 2.24 ± 0.09 cm for the HBCS group and 3.38 ± 0.09 and 1.28 ± 0.08 cm for the NBCS group, respectively. With the onset of lactation, body condition decreased in both groups, whereby the decline until wk 15 p.p. was greater for HBCS (Δ = 0.9 BCS points, 1.2 cm BFT) compared to NBCS cows (Δ = 0.6 BCS points, 0.7 cm BFT).

Due to technical conditions, daily individual feed intake a.p. could only be recorded from wk 3 a.p. onwards (Figure 2A). Group ($P = 0.04$), time ($P < 0.001$) and the interaction of both ($P = 0.001$) all influenced DMI: Before calving, NBCS cows had a higher DMI than HBCS cows. At wk 1 p.p. both groups reached the same nadir. Thereafter DMI increased faster for NBCS compared to HBCS until levels became equal again at wk 11 p.p. When DMI was adjusted for BW, the pattern was similar, except that the differences between

the two groups became clearer (P = 0.001; Figure 2B) and included more time points.

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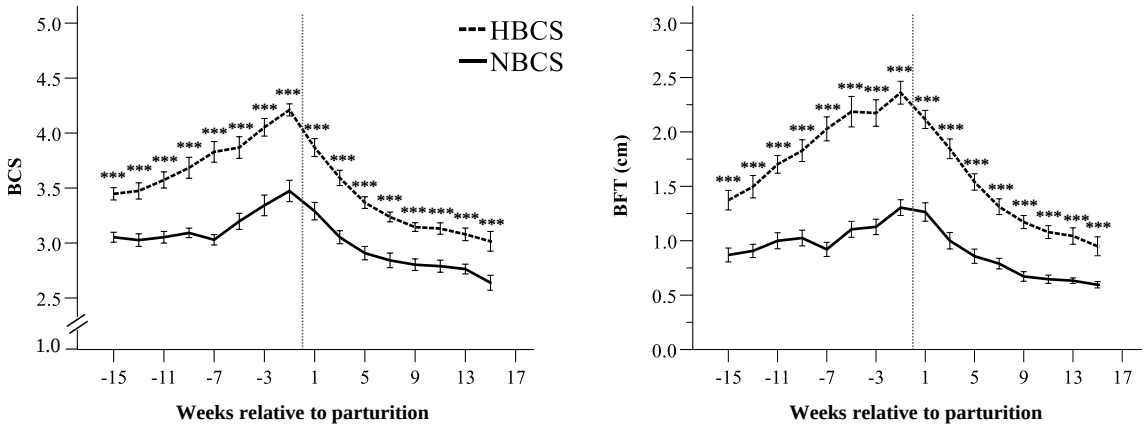

Figure 1. Changes in BCS and back fat thickness (BFT) from wk 15 antepartum to wk 15 postpartum of high BCS (HBCS; n = 19) and normal BCS (NBCS; n = 19) cows [modified from Schuh et al. (2019)]. Data are given as means ± SEM. Asterisks (***) indicate differences ($P < 0.001$) between HBCS and NBCS within one time point. The vertical dotted lines indicate calving.

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No group difference was observed for overall milk yield (Figure 2C). However, the interaction of group × time was significant ($P < 0.001$). During the first 4 wk of lactation, cows in the NBCS group yielded on average 2.3 kg milk more than cows in the HBCS group. Milk protein yield did not differ between the two treatment groups (Figure 2D).

Plasma BCAA concentrations

Plasma concentrations of Leu, Ile and Val as well as total BCAA, calculated as the sum of the three, were not different between the two groups (Figure 3). Albeit for Ile, a significant group × time interaction was observed ($P = 0.03$): At d 3 p.p. HBCS cows tended to have lower Ile concentrations than NBCS cows ($P = 0.08$) and vice versa at d 21 ($P = 0.02$). Concentrations of all BCAA changed with time ($P \leq 0.01$). However, this mainly applied to the HBCS group: From pre-calving to calving, their concentrations mostly decreased and increased again thereafter to initial values. Changes in total AA concentration were also more prominent for HBCS cows, with highest values observed on d 21 p.p. (Supplemental Table S3; https://doi.org/10.3168/jds.2019-17147).

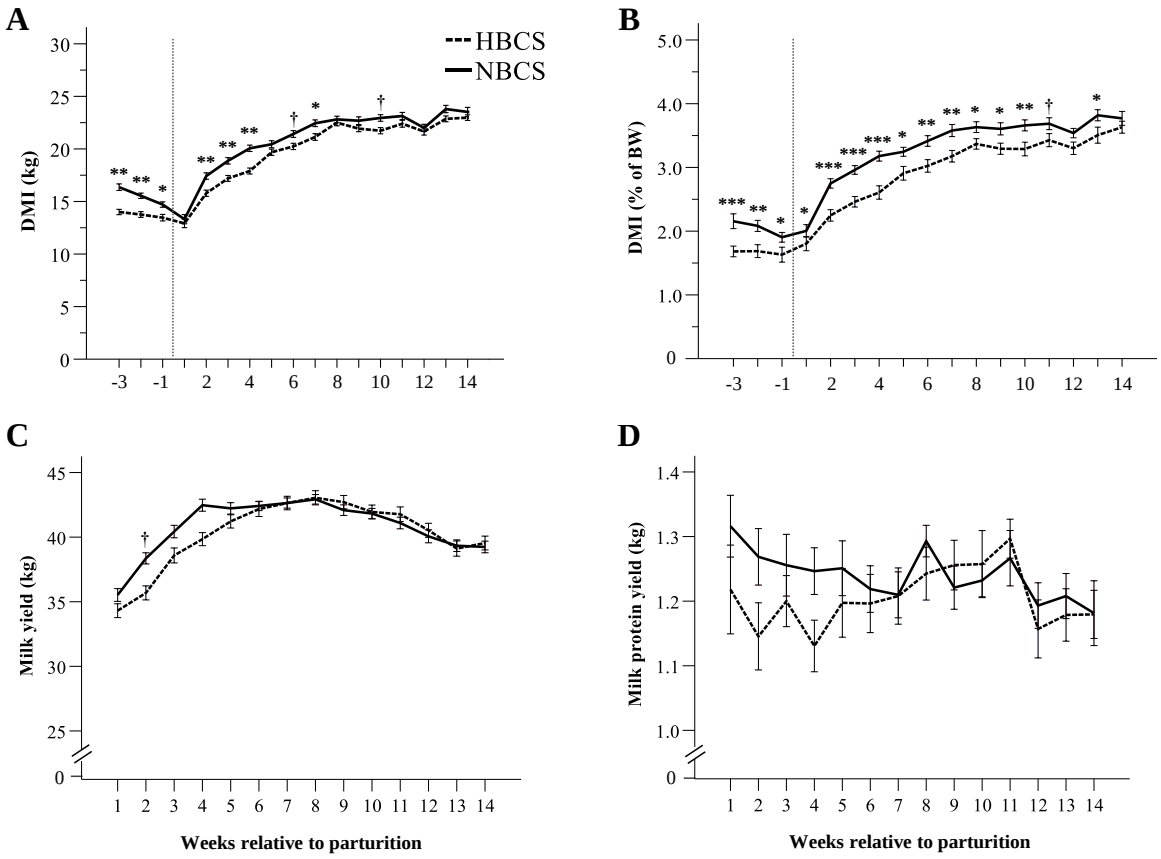

Figure 2. Changes in DMI (A) and DMI as % of BW (B) from wk 3 antepartum to wk 14 postpartum (p.p.), milk yield (C) and milk protein yield (D) from wk 1 to wk 14 p.p. of high BCS (HBCS; n = 19) and normal BCS (NBCS; n = 19) cows [in part modified from Schuh et al. (2019)]. Data are given as means ± SEM. Asterisks (*, **, ***) indicate differences ($0.01 < P < 0.05$, $0.001 < P < 0.01$ and $P < 0.001$, respectively) between HBCS and NBCS within one time point. Trends ($0.05 < P \leq 0.1$) are marked with daggers (†). The vertical dotted lines indicate calving.

mRNA abundance of the BCAA transporters and enzymes

The mRNA abundance of the three BCAA transporters assessed in this study was not affected by over-conditioning at calving (Table 2). For *SLC1A5*, the abundance varied with tissue type and time (both $P < 0.001$), whereby interactions between these factors were observed ($P < 0.001$; Figure 4A). Over all time points and for both groups, *SLC1A5* mRNA in scAT was on average 30-fold higher than in liver and 9-fold higher compared to muscle (both $P < 0.001$). Time effects were most prominent for muscle in which the abundance increased continuously from d 49 a.p. to d 21 p.p. and decreased again at the last sampling time point, both for the HBCS and NBCS groups.

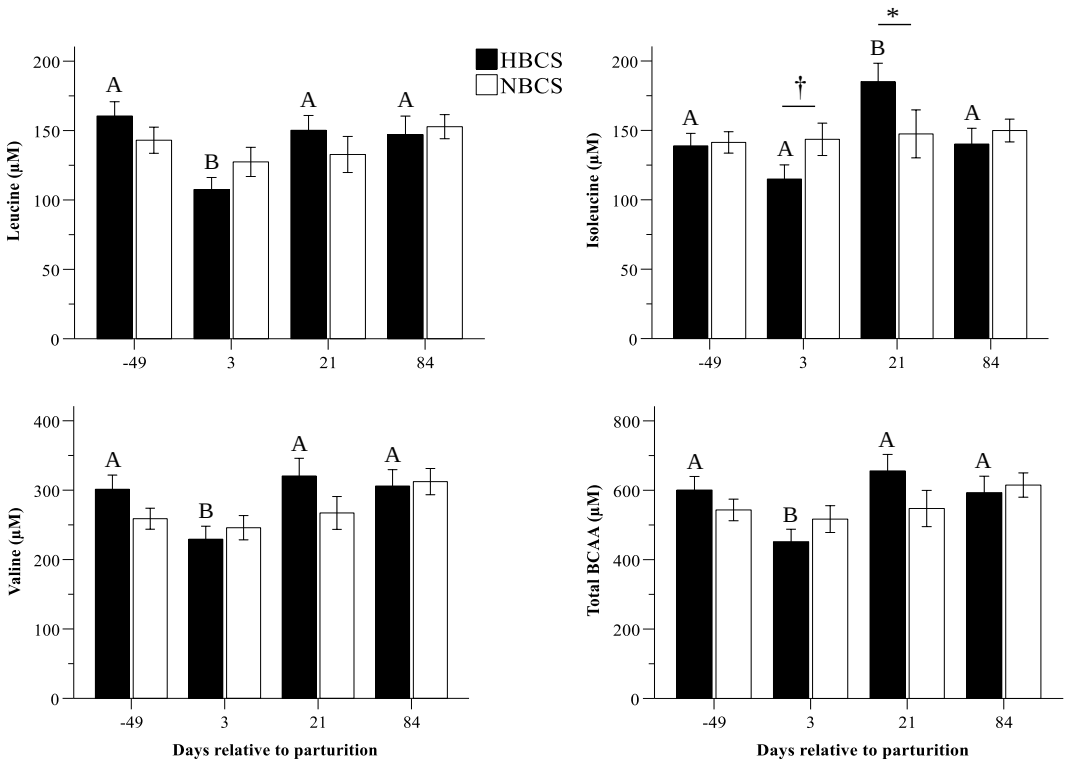

Figure 3. Plasma concentrations of free leucine, isoleucine, valine, and total branched-chain amino acids (BCAA) of high BCS (HBCS; n = 19) and normal BCS (NBCS; n = 19) cows on d -49, 3, 21 and 84 relative to parturition. Data are given as means ± SEM. Different letters (A,B) indicate differences ($P < 0.05$) between time points within the HBCS group. Asterisks (*) indicate differences ($P < 0.05$) between HBCS and NBCS within one time point. Trends ($0.05 < P \leq 0.1$) are marked with daggers (†). Total BCAA were calculated as the sum of leucine, isoleucine and valine.

Table 2. Level of significance (*P*-values) for the effects of body condition (group), type of tissue, time relative to calving, as well as the respective interactions on the abundance of transporters and enzymes related to branched-chain AA metabolism in dairy cows during late pregnancy and early lactation

Variable[1]	Group	Tissue	Time	Group × Tissue	Group × Time	Tissue × Time	Group × Tissue × Time
SLC1A5	0.149	**<0.001**	**<0.001**	0.668	0.845	**<0.001**	0.399
SLC7A5	0.618	**<0.001**	**<0.001**	0.97	0.919	**<0.001**	0.521
SLC38A2	0.107	**<0.001**	**0.002**	0.856	0.273	**<0.001**	0.823
BCAT2	0.345	**<0.001**	**<0.001**	0.522	**0.011**	**<0.001**	0.519
BCKDHA	**0.002**	**<0.001**	**0.028**	**0.002**	0.067	**<0.001**	**0.033**
BCKDHB	0.902	**<0.001**	**0.037**	0.93	0.153	**<0.001**	0.772
BCKDHA[2]	0.119	**<0.001**	0.062	**0.008**	0.795	**<0.001**	**0.03**

[1]*SLC1A5* = solute carrier family 1 member 5; *SLC7A5* = solute carrier family 7 member 5; *SLC38A2* = solute carrier family 38 member 2; *BCAT2* = branched-chain aminotransferase 2; *BCKDHA* = branched-chain α-keto acid dehydrogenase E1α; *BCKDHB* = branched-chain α-keto acid dehydrogenase E1ß.

[2]Protein abundance.

The mRNA abundance of *SLC7A5* was altogether influenced by tissue and time as well as tissue × time (all $P < 0.001$; Figure 4B). Again, over all time points and independent of group, highest abundance was detected in scAT compared with the other two tissues. However, changes with time were only observed for liver and muscle. Hepatic *SLC7A5* mRNA was expressed at fairly constant levels until d 84 p.p. when values decreased by factor 2 compared to the previous time points p.p. in the HBCS group. For NBCS cows, hepatic *SLC7A5* mRNA abundance was lower at d 84 p.p. than at d 49 a.p. and 3 p.p. In muscle, *SLC7A5 mRNA* abundance increased from d 49 a.p. to d 21 p.p. and, in case of NBCS cows, levels decreased again thereafter.

Abundance of *SLC38A2* changed with tissue, time and the interaction thereof (all $P < 0.01$; Figure 4C). On average, levels in both scAT and muscle were about 4-times higher than in liver. Time dependent changes were only detected for the latter tissue and were more notable in the NBCS group, where *SLC38A2* mRNA decreased at d 84 p.p. compared with previous time points (bar d 49 a.p.).

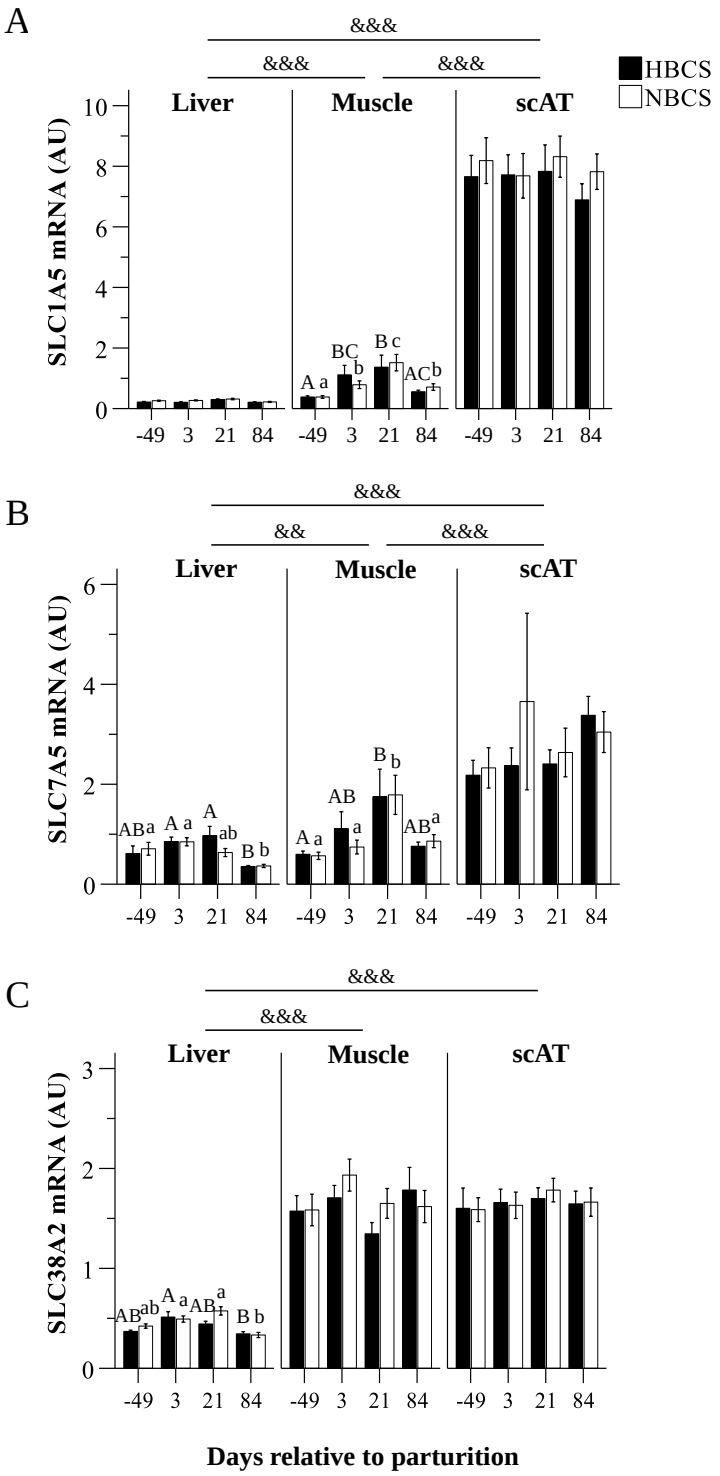

Figure 4. mRNA abundance of the main branched-chain AA transporters, solute carrier family 1 member 5 (*SLC1A5*, A), *SLC7A5* (B), and *SLC38A2* (C), in liver, skeletal muscle, and subcutaneous adipose tissue (scAT) of high BCS (HBCS; n = 19) and normal BCS (NBCS; n = 19) cows on d -49, 3, 21 and 84 relative to parturition. Data are given as means ± SEM. Differences between tissues are marked with && ($0.001 < P < 0.01$) or &&& ($P < 0.001$). Different uppercase letters (A-C) indicate differences ($P < 0.05$) between time points within HBCS. Different lowercase letters (a-c) designate differences ($P < 0.05$) between time points within NBCS. AU = arbitrary units.

The mRNA abundance of the enzymes related to BCAA catabolism was primarily influenced by type of tissue and time of sampling (Table 2).

For *BCAT2*, no difference between the two groups was observed (Figure 5A). Abundance only changed with tissue and time (both $P < 0.001$). Interactions of group × time as well as tissue × time were significant (both $P \leq 0.01$). Regardless of time points and for both groups, *BCAT2* abundance was on average 5.5- and 7-times greater in scAT compared to muscle and liver (all $P < 0.001$), respectively. In case of muscle, *BCAT2* mRNA abundance in the NBCS group increased from d 49 a.p. to all subsequent time points (all $P < 0.05$, except for d 49 a.p. vs. 21 p.p.; $P = 0.07$). For scAT, *BCAT2* abundance in both groups was highest at d 49 a.p. compared to all other time points (all $P < 0.001$) and decreased until d 21 p.p. At d 84 p.p., levels seemed to slightly increase again, albeit for HBCS cows, initial values were not reached again.

The mRNA abundance of *BCKDHA* was overall influenced by group, tissue and time (all $P < 0.05$) with significant interactions of group × tissue ($P = 0.002$), tissue × time ($P < 0.001$) and group × tissue × time ($P = 0.03$; Figure 5B). Considering the 3 tissues in this study, scAT showed the strongest group effect: At all time points except for d 84 p.p., *BCKDHA* mRNA in scAT was between 1.4- to 1.9-times higher in HBCS than in NBCS cows (all $P < 0.05$). Abundance in muscle and liver did not differ between the two groups. Independent of group, the mRNA abundance of *BCKDHA* was mostly greatest in liver compared to the other tissues ($P < 0.001$, bar d 3 p.p. in muscle). Changes with time were observed for all tissues but liver. In muscle, *BCKDHA* abundance in the HBCS group increased more than 3-fold from d 49 a.p. to d 3 p.p. and declined to previous levels thereafter. In scAT of HBCS cows, *BCKDHA* mRNA was higher at d 49 a.p. compared to subsequent time points (except d 3 p.p.). For NBCS cows, *BCKDHA* mRNA in muscle and scAT only changed numerically.

Tissue type and time (both $P < 0.05$) but not group affected *BCKDHB* mRNA abundance (Figure 5C). In addition, a significant tissue × time interaction was observed ($P < 0.001$). Overall, the *BCKDHB* abundance was higher in muscle, and partly liver, compared to scAT (muscle vs. scAT: $P < 0.001$, except for d 49 a.p.; liver vs. scAT: $P < 0.001$, except for d 49 a.p.). Time dependent changes were only detected for scAT, where for both groups, *BCKDHB* mRNA decreased by a factor of 2 from d 49 a.p. to subsequent time points.

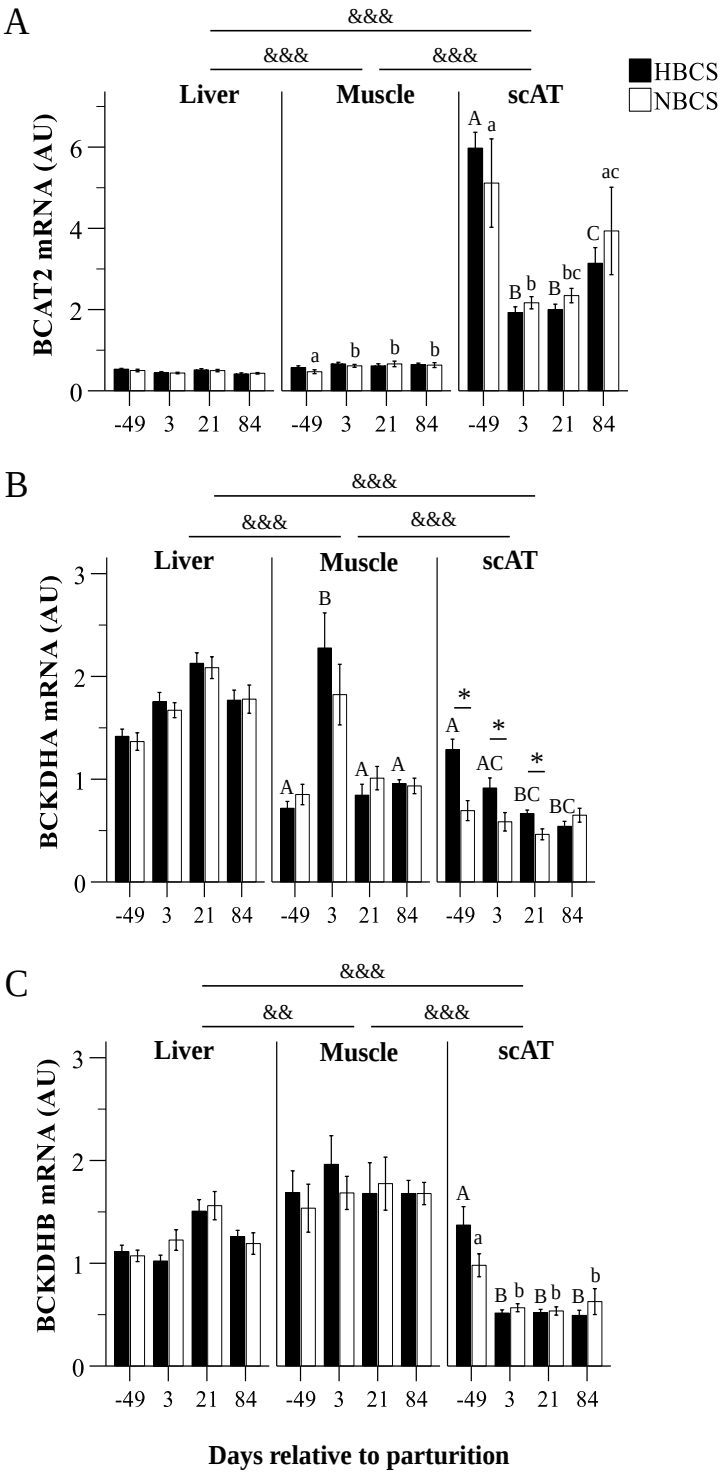

Figure 5. mRNA abundance of the branched-chain AA enzymes, branched-chain aminotransferase 2 (*BCAT2*, A), branched-chain α-keto acid dehydrogenase E1α (*BCKDHA*, B) and branched-chain α-keto acid dehydrogenase E1ß (*BCKDHB*, C), in liver, skeletal muscle, and subcutaneous adipose tissue (scAT) of high BCS (HBCS; n = 19) and normal BCS (NBCS; n = 19) cows on d -49, 3, 21 and 84 relative to parturition. Data are given as means ± SEM. Differences between tissues are marked with && ($0.001 < P < 0.01$) or &&& ($P < 0.001$). Asterisks (*) indicate differences ($P < 0.05$) between HBCS and NBCS within one time point. Different uppercase letters (A-C) indicate differences ($P < 0.05$) between time points within HBCS. Different lowercase letters (a-c) designate differences ($P < 0.05$) between time points within NBCS. AU = arbitrary units.

Protein abundance of the BCKDHA

Due to limitations in the amount of tissue available, 10 animals per group with the most complete sets of biopsies were selected for the measurement of BCKDHA protein abundance (Figure 6). Overall, cows with high or normal body condition at calving were not different in their BCKDHA protein. However, significant group × tissue (P = 0.008) and group × tissue × time interactions (P = 0.03) were observed. At d 49 a.p., BCKDHA protein abundance in liver of HBCS cows was lower compared with NBCS cows (P < 0.001), and vice versa in scAT (P = 0.04). In general, liver had approximately 13-times greater BCKDHA abundance than muscle and 9-times higher values than scAT. Because of significant interactions of tissue and time (P < 0.001) and, as mentioned above, group, tissue and time, time effects were different within tissues and treatment groups: For liver, BCKDHA abundance of both groups increased from d 49 a.p. to d 3 p.p. and decreased again subsequently (P < 0.001). But, unlike the NBCS group, BCKDHA protein in liver of HBCS cows did not reach initial values again. In scAT of HBCS cows, highest abundance was observed at d 49 a.p. which then decreased around the factor of 4.6 at the following time points (P < 0.001). No time effects were detected for scAT in the NBCS group or for muscle in either group.

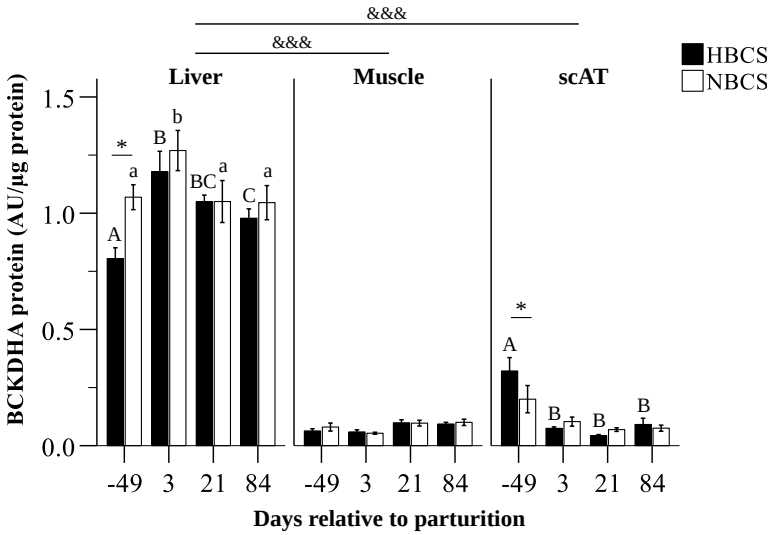

Figure 6. Protein abundance of the branched-chain α-keto acid dehydrogenase E1α (BCKDHA) in liver, skeletal muscle, and subcutaneous adipose tissue (scAT) of high BCS (HBCS; n = 19) and normal BCS (NBCS; n = 19) cows on d -49, 3, 21 and 84 relative to parturition. Data are given as means ± SEM. Differences (P < 0.001) between tissues are marked with &&&. Asterisks (*) indicate differences (P < 0.05) between HBCS and NBCS within one time point. Different uppercase letters (A-C) illustrate differences (P < 0.05) between time points within HBCS. Different lowercase letters (a-b) designate differences (P < 0.05) between time points within NBCS. AU = arbitrary units.

DISCUSSION

An experimental model characterizing dairy cows with normal versus high tissue mobilization was successfully established (Schuh et al., 2019). The over-conditioned cows in our trial were more metabolically challenged during early lactation than the cows with normal body condition due to a more severe and persistent negative energy balance, a higher lipomobilization, and probably a lower insulin sensitivity (**IS**; Holtenius et al., 2003; Roche et al., 2009; Pires et al., 2013; Schuh et al., 2019). Most likely, these differences were related to the slower postpartal increase of feed intake and the lower feed intake in general that was observed in HBCS cows, both of which were especially obvious, when DMI was expressed as a percentage of BW.

The BCAA are particularly responsive to insulin, and during metabolic states with reduced IS, such as obesity, their metabolism seems to be profoundly altered in monogastrics (She et al., 2007; Newgard et al., 2009; Polakof et al., 2018). Even though the underlying mechanisms in this relationship are not fully understood yet, (i.e., the cause and effect of such metabolic dysfunctions), it is assumed that a reduced BCAA oxidative capacity in scAT may be a contributing factor (Polakof et al., 2018). Nevertheless, studies in humans have shown that, depending on the degrees of obesity and decreased IS, an impaired BCAA catabolism in other tissues, such as skeletal muscle (Lerin et al., 2016) and liver (Lee et al., 2018), may also affect or be caused by alterations in IS.

In this study we wanted to explore whether the plasma concentrations of BCAA as well as the abundance of BCAA transporters and enzymes in liver, skeletal muscle and scAT differed between normal- and over-conditioned dairy cows during late pregnancy and early lactation. Apart from the increased nutrient and energy demands of the mammary gland for lactogenesis, systemic AA concentrations around calving are greatly affected by DMI (Meijer et al., 1995; Doepel et al., 2002; Zhou et al., 2016). The herein observed lower feed intake of HBCS cows therefore most probably also accounted for the more pronounced drop of plasma BCAA concentrations in this group around the time of parturition. Even though total AA did not decrease around calving for HBCS cows, animals in this group overall had greater reductions in circulating AA from prepartum to postpartum as compared to NBCS cows. Yet, as over-conditioned cows are metabolically more challenged and likely closer to a pro-inflammatory status than animals in normal body condition (Akbar et al., 2015; Roche et al., 2015; Vailati-Riboni et al., 2016), HBCS cows might have had to generate more immune

cells and positive acute phase proteins, such as haptoglobin (Hp), all of which containing substantial portions of BCAA (Morimatsu et al., 1991; Calder, 2006). However, in our study, for the assessed serum Hp concentrations, differences between the two groups were not detectable, likely due to the high interindividual variation (Schuh et al., 2019). To fully understand the relationship between circulating BCAA and inflammatory processes in cows differing in body condition, further indicators of immune responses should therefore be taken into account.

In ruminants and nonruminants, whole-body BCAA metabolism is increased during lactation (Tesseraud et al., 1993; DeSantiago et al., 1998; Li et al., 2009) and BCAA as well as their catabolized products (succinyl-CoA, acetyl-CoA, acetoacetate), some of which may eventually enter the TCA cycle, are used to partially cover the elevated energy and nutrient demands for milk production (Li et al., 2009). Further, BCAA can also fulfill different tissue-specific regulatory functions: the regulation of glyceroneogenesis and fatty acid oxidation in AT through linkages in the TCA cycle (Kainulainen et al., 2013), the regulation of protein synthesis in muscle via activation of the mTORC1 pathway (Dodd and Tee, 2012), and the modulation of cell growth and proliferation in liver also via mTORC1 signaling (Kimura and Ogihara, 2005). Clearly, in order to exert these effects, transporters are needed to bring BCAA into the cell.

As insulin also partly mediates cellular AA uptake and increases mRNA encoding AA transporters, such as *SLC7A5* in mammalian cells (Malmberg and Adams, 2008), we hypothesized that cows differing in body condition and likely expressing different degrees of IS (Holtenius et al., 2003; Dann et al., 2006; Jaakson et al., 2018; Schuh et al., 2019), would also show variations in their BCAAT mRNA abundance. However, within the present study model this was not the case. During early lactation, nutrient availability is, regardless of body condition, thought to be physiologically limited for all tissues except the mammary gland (Bauman and Currie, 1980). Yet, the consistently high abundance of *SLC1A5*, *SLC7A5* and *SLC38A2* mRNA in scAT of both normal- and over-conditioned cows indicates that AT, or rather scAT, may still be a major site of BCAA metabolism, or more specifically BCAA uptake, that seems however independent of BCS and time relative to calving at transcription level in dairy cows. As adipose metabolism is crucial for contributing to the efficiency of milk production during the transition period (Khan et al., 2013), an upregulation of BCAA uptake would clearly increase the intracellular availability of BCAA. Partially

supporting this is data by Liang et al. (2019), showing that transporters for BCAA, i.e., *SLC7A5*, *SLC1A5* and *SLC3A2*, were constantly expressed at mRNA level from d -10 to 30 relative to parturition in bovine scAT. In addition, the protein abundance of SLC1A5 and SLC1A3/SLC3A1 was gradually increasing from late pregnancy to early lactation pointing to an actually growing capability of AT for BCAA uptake even during periods of negative energy balance. However, it should be noted that the SLC1A3/SLC3A1 and SLC3A2 transporters are not clearly specified in the aforementioned paper.

The changes over time detected for muscle *SLC1A5* and *SLC7A5* mRNA of both HBCS and NBCS cows, with peaks at d 21 p.p., could however imply a more acute form of adaptive response in this tissue, likely to the slowly increasing DMI. Consistent with this notion, EAA ingestion led to an upregulation of mRNA and protein abundance of AA transporters in human muscle within 3 h post meal (Drummond et al., 2010). For liver, time effects herein were only limited to a downregulation toward the latest sampling time point, probably mirroring the attenuated metabolic pressure on the tissue caused by the slowly instating decrease in milk production at that stage.

According to our results, the transport of BCAA into liver and partly muscle appears to be lower than into scAT, assuming not a primary demand of BCAA in these tissues during late pregnancy and early lactation. Nonetheless, as mRNA measurements are not fully representative of the actual protein, future studies comparing different metabolic tissues for their ability to use AA, should include the assessment of protein abundance and transporter activity in order to support this idea. Yet, based on the possibility that, at translation level, AA may be readily taken up by scAT especially toward the end of the transition period (Liang et al., 2019), it is certainly conceivable that BCAA (and other AA) are utilized within cellular adipose metabolism, i.e., to sustain metabolic turnover.

Following cellular uptake, BCAA may be used either for anabolic processes or, if the supply exceeds the requirement, be rapidly catabolized in the different tissues (Blouin et al., 2002). Generally, the liver is considered as the major organ of AA disposal; however, its capacity for direct BCAA degradation is limited in ruminants (Lapierre et al., 2002; Raggio et al., 2004). It has been acknowledged though, that the initial step in BCAA catabolism, the reversible deamination via BCAT mostly occurs in extrahepatic tissues, such as muscle and AT (Bergen et al., 1988; Lapierre et al., 2002; Zhang et al., 2017).

Consequently, and in support of previously published data (Suryawan et al., 1998; Webb et al., 2019), we detected lowest *BCAT2* mRNA abundance in liver. This may be indicative of a reduced hepatic BCAA transamination capacity, allowing for a greater availability of BCAA to peripheral tissues regardless of differences in DMI. In fact, greatest *BCAT2* abundance was observed in scAT: Together with the observed elevated abundance of BCAAT in scAT, it seems that scAT not only may be important for BCAA uptake but also for their initial degradation.

Human studies have shown that prolonged periods of negative energy balance may lead to decreases in body mass due to losses of both body fat and skeletal muscle, with a ratio of approximately 75% AT and 25% fat-free mass (Carbone et al., 2012). And because body condition might affect lipid as well as protein mobilization in transition dairy cows (Kokkonen et al., 2005), we presumed that the over-conditioned cows in our study, being metabolically more challenged because of a more severe negative energy balance associated with a more reduced early lactation DMI (Schuh et al., 2019), could have had a higher tissue mobilization in general. The observed differences in pre- and postpartum BCS and BW between the two groups (HBCS: prepartum 3.77 ± 0.08 and 810 ± 28 kg, postpartum 3.30 ± 0.06 and 674 ± 11 kg; NBCS: prepartum 3.16 ± 0.06 and 715 ± 25 kg, postpartum 2.89 ± 0.06 and 631 ± 12 kg) would clearly be in favor of this. Further data collected from the same study population as used herewith, indicate that high BCS and BFT were associated with a greater mRNA abundance of the two muscle-specific ligases muscle RING finger protein-1 (*MuRF-1*) and *atrogin-1* (Ghaffari et al., 2019), and consequently, a more stimulated proteolysis in these cows seems likely. Over-conditioned cows might then also have a greater reservoir of BCAA and counteractively a higher tissue abundance of BCAA degrading enzymes. However, only the latter could be confirmed in parts. The decreased feed and thus protein intake of HBCS cows, leading to at least greater reductions in plasma BCAA around the time of parturition, might have masked any such effects concerning circulating BCAA.

As the rate-limiting enzyme of BCAA catabolism and due to the tight regulation of its activity (Harris et al., 1994), the BCKDH complex is considered probably the most important key enzyme in overall BCAA metabolism. For the mRNA abundance of *BCKDHA*, the E1α subunit of BCKDH, we were able to observe group differences in scAT, with HBCS cows having almost continuously higher *BCKDHA* mRNA abundances than NBCS cows. Thus, despite the lesser feed intake and probably because

of the higher BW, HBCS cows could have had a greater capacity for the irreversible degradation of BCAA, or rather their keto acids, in scAT than NBCS animals. This would be in contrast to most studies in nonruminants, where a greater body condition (i.e., obesity) has often been associated with a downregulation of adipose BCAA catabolism (She et al., 2007; Pietiläinen et al., 2008; Connor et al., 2010; Polakof et al., 2018). However, there is also some evidence of increased BCAA oxidation in obese subjects likely due to increased protein turnover (Welle et al., 1992; She et al., 2013). Nonetheless, the differences in *BCKDHA* mRNA between HBCS and NBCS slightly decreased over time possibly reflecting the greater metabolic change of HBCS cows starting off in a more anabolic situation prepartum and then, in consequence of the possibly greater tissue mobilization, approaching a more catabolic situation during early lactation as compared to NBCS cows.

We did not see any group differences concerning the mRNA abundance of the E1ß subunit of BCKDH (*BCKDHB*). Based on studies in nonruminants by Shimomura et al. (1990) and Lu et al. (2009), it has been concluded that the E1α subunit is the enzyme complex's main site of phosphorylation (deactivation) and dephosphorylation (activation) by a specific kinase and phosphatase, respectively. Further, the E1α subunit seems more responsive to nutritional and hormonal influences than other subunits (Harris et al., 1997; Shimomura et al., 2001; Kadota et al., 2013). Any metabolic differences caused by variations in body condition of the cows may therefore rather be expected for *BCKDHA* than *BCKDHB*. However, previously published results by our group showing greater *BCKDHB* mRNA abundance in liver as compared to muscle and scAT in early-lactating dairy cows (Webb et al., 2019) could not be confirmed. Herein we observed highest *BCKDHB* mRNA in muscle, followed by liver and then scAT. In contrast to the present study, the animals used in the prior trial were all primiparous cows, not yet fully mature. The existence of age-dependent changes in BCAA catabolism has been well documented for different tissues in sheep: Both BCAT and BCKDH activities seem to be affected by the ever-changing nutrient demand and cellular composition of tissues such as liver, muscle and AT during various developmental stages (Goodwin et al., 1987; Bergen et al., 1988; Faure et al., 2001). It is possible that this is also valid for dairy cows of different ages.

At all time points and for both groups, greatest BCKDHA protein abundance was observed in liver, followed by scAT and muscle, which had almost similar values. Even though flux studies have shown that

hepatic degradation of BCAA/BCKA is limited in lactating dairy cows (Lapierre et al., 2002; Raggio et al., 2004), upregulation of the main enzyme responsible for irreversible liver BCKA oxidation could be seen as a negative feedback mechanism compensating for the possibly low hepatic BCAA flux and thus, ensuring overall BCAA/BCKA homeostasis. Different human studies have shown that in the context of metabolic disorders such as IR and non-alcoholic fatty liver, concentrations of BCAA and their downstream catabolites, like short-chain acylcarnitines, can be elevated in plasma (Newgard et al., 2009) and liver (Lake et al., 2015), respectively, due to a dysregulation of BCAA degrading enzymes. In transition dairy cows, propionyl-carnitine (i.a. a by-product of Ile and Val catabolism) has been reported to be increased in plasma during various disease states (i.e., mastitis, metritis, laminitis, and retained placenta; Hailemariam et al., 2014). Yet, to our knowledge, BCAA metabolism in relation to the acylcarnitine status of metabolically challenged cows has not been described yet.

At d 49 a.p. the protein abundance of BCKDHA was lower in liver and higher in scAT of HBCS compared to NBCS cows. At this time cows were dried off and previous differential feeding (starting 15 wk a.p.) was ceased. An energetic surplus appears likely and could explain the more reduced need of HBCS cows to oxidize BCAA transamination products. Due to over-conditioning and despite lower DMI, animals in the HBCS group had greater fat reserves and probably an increased metabolic turnover in AT allowing for more BCKA to be degraded in this tissue as well as for the respective end products to be used for anabolic processes like glyceroneogenesis. By testing different tracer substrates for their contribution to TCA metabolism as well as adipocyte differentiation, Green et al. (2016) found that catabolism of BCAA accounts for one-third of lipogenic acetyl-CoA generation and that functional knockdown of BCAA degradation may impair lipid accumulation and adipocyte differentiation. Through increased BCKA oxidation and the donation of additional carbon sources, BCAA catabolism thus fuels anaplerosis as well as de novo lipogenesis. It is therefore possible that, before parturition, HBCS cows also used BCAA, or rather their metabolites, to build up more body fat. Normal-conditioned cows, being in a less anabolic state, were on the other hand probably sufficiently supplied by glycolytic processes generating acetyl-CoA and further fatty acids and glycerol for prepartum lipogenesis. The metabolic shift to lactation, however, seemed to level off previously established differences in BCKDHA protein abundance between the

two groups, leading to decreased postpartal values in scAT of both HBCS and NBCS cows. Contrary to previously described results in obese rats, humans and pigs (She et al., 2007; Newgard et al., 2009; Polakof et al., 2018), we did not detect any general downregulation of BCAA catabolic enzymes in the AT of HBCS cows compared to NBCS cows. However, the event of lactation is a very specific situation and may lead to changes of many regulatory processes as well as shifts in metabolic priorities of nutrients. It might also be that in our case over-conditioning was not severe enough to elicit such a strong tissue response as observed in the aforementioned studies.

Regardless of body condition, hepatic BCKDHA abundance peaked at d 3 p.p., which could be related to the fact that during the first weeks after parturition energy balance is usually the most negative in dairy cows (e.g., Tienken et al., 2015). Herein, despite differences in DMI, energy balance of both groups reached the same nadir in the first week after calving. To antagonize this energy deficit, protein abundance of BCKDHA may be increased around calving to enable the generation of additional energy via irreversible oxidation of BCKA. As the energy balance slowly increases again during lactation, the need for additional energy diminishes and BCKDHA protein may decrease again.

CONCLUSIONS

In this study, cows calving with high BCS were likely oversupplied with nutrients during late pregnancy and were therefore in a more anabolic situation than the normal-conditioned animals. Consequently, as indicated by the lower hepatic BCKDHA protein abundance prepartum, the over-conditioned cows may have relied less on the oxidation of BCAA transamination products for the generation of energy. However, given the greater prepartal BCKDHA protein abundance in scAT, high BCS cows may have instead catabolized BCKA in AT supporting metabolic turnover therein. Steadily increased mRNA abundance of the most relevant BCAAT as well as BCAT2 in scAT during late pregnancy and early lactation indicates that AT, or more precisely scAT, may be an important site of BCAA uptake and initial degradation in dairy cows that is by contrast not influenced by body condition.

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Supplemental Table S1. Ingredients and chemical composition of the diets [from Schuh et al (2019)]

	Late lactation		Dry period	Early lactation
	Wk 15 to 7 a.p.		Wk 7 a.p. to parturition	Wk 1 to 14 p.p.
Item	HBCS	NBCS	HBCS / NBCS	HBCS / NBCS
Ingredient				
Grass silage	22.4	32.0	32.0	22.4
Corn silage	20.7	32.0	32.0	20.7
Pressed beet pulp silage	12.5	-	-	12.5
Hay	5.5	5.4	5.4	5.5
Straw	2.3	4.1	4.1	2.3
Vitamin and mineral mix[1]	0.4	0.7	0.7	0.4
Concentrate[2]	36.2	25.8	25.8	36.2
Chemical composition				
ME (MJ/kg DM)	10.8	10.6	10.6	10.8
NE_L (MJ/kg DM)	7.2	6.8	6.8	7.2
Crude protein (g/kg DM)	170	157	157	170
Utilizable crude protein (g/kg DM)	156	149	149	156
$aNDF_{OM}$ † (g/kg DM)	359	382	382	359
ADF_{OM} ‡ (g/kg DM)	204	223	223	204
Ruminal N balance (g/d)	3.4	2.3	2.3	3.4

[1]Provided per kg total mixed ration (on DM basis): Ca, 0.36 g; P, 0.36 g; sodium, 0.36 g; Mg, 0.40 g; Zn, 28 mg; Mn, 17 mg; Cu, 6.0 mg; Co, 0.24 mg; I, 0.80 mg; Se, 0.21 mg; vitamin A, 4.000 IU, vitamin D, 600 IU, vitamin E, 20 mg (RINDAMIN K11 ATG, Schaumann, Pinneberg, Germany).

[2]Concentrate portion consisting of barley (6.5% of DM), corn grain (8.8% of DM), soybean meal (5.9% of DM), and canola meal (6.5% of DM).

† $aNDF_{OM}$, ash free Neutral Detergent Fiber of organic matter.

‡ ADF_{OM}, Acid Detergent Fiber of organic matter.

Supplemental Table S2. Number of cows affected by clinical conditions occurring from calving to the first 6 weeks after calving in HBCS and NBCS cows

Clinical condition	HBCS (n = 19)		NBCS (n = 19)	
	No. cows	No. cases	No. cows	No. cases
Mastitis	8	16	6	11
Ketosis	4	5	2	2
Milk fever	4	5	2	4
Locomotion	7	8	3	4
Retained fetal membranes/endometritis	1	1	2	2
Other conditions[1]	3	4	3	3
Total clinical conditions	27	39	15	26

[1] dystocia, inflammation of the conjunctiva of the eye

Supplemental Table S3. Plasma amino acid (AA) concentrations (µmol/L) of high (HBCS) and normal body condition score cows (NBCS) on d -49, 3, 21 and 84 relative to parturition

		Time				*P*-value[1]		
	Group	-49	3	21	84	G	T	G × T
Ala	HBCS	283.2 ± 26.6^{ab}	209 ± 16.4^{c}	241.8 ± 25.6^{bc}	329.5 ± 25.8^{a}	0.214	**<0.001**	0.729
	NBCS	294.6 ± 22.5^{ab}	257.7 ± 19.9^{b}	253.6 ± 17.4^{b}	329.4 ± 37.4^{a}			
Arg	HBCS	78.4 ± 5.5^{a}	48.7 ± 3.2^{b}	66.8 ± 6.9^{a}	84.1 ± 7.2^{a}	0.471	**<0.001**	0.238
	NBCS	75.7 ± 4.7^{ab}	59.3 ± 4.8^{b}	60.6 ± 4.4^{b}	96.1 ± 8.3^{a}			
Asn	HBCS	37.4 ± 2.7^{b}	35 ± 3^{b}	49.1 ± 4.1^{a}	56.7 ± 5.2^{a}	0.421	**<0.001**	0.309
	NBCS	42.2 ± 3.5^{b}	39.9 ± 3.5^{b}	43.8 ± 2.9^{b}	62.6 ± 6.3^{a}			
Glu	HBCS	78.5 ± 6.2^{a,*}	52.0 ± 3.9^{b,*}	62.7 ± 5.4^{ab}	65.7 ± 4.6^{ab}	0.240	**<0.001**	**0.020**
	NBCS	89.3 ± 7.0^{a}	66.3 ± 5.3^{b}	58.7 ± 3.2^{b}	58.8 ± 2.6^{b}			
Gln	HBCS	352.8 ± 28.8	320.1 ± 28.3	326.8 ± 36.8	294.7 ± 23	0.911	**0.017**	0.414
	NBCS	347.6 ± 24.8	338.7 ± 24.2	279.4 ± 22.3	288.3 ± 19.0			
Gly	HBCS	244.1 ± 30.3^{c}	554.8 ± 46.1^{b}	854.6 ± 57.9^{a,*}	433.1 ± 42.2^{b}	**0.019**	**<0.001**	**<0.001**
	NBCS	304.5 ± 35.7^{c}	552.1 ± 52.2^{ab}	576.4 ± 48.8^{a}	416.1 ± 51.4^{bc}			
His	HBCS	39.6 ± 3.9	30.3 ± 1.9	32.5 ± 3.7	35.8 ± 3.9	0.485	0.093	0.483
	NBCS	33.6 ± 3.5	33.1 ± 3.9	26.7 ± 2.3	34.3 ± 3.5			
Ile	HBCS	139.4 ± 10.1^{b}	115.7 ± 9.9^{b}	179.1 ± 13.2^{a,*}	140.8 ± 12.6^{b}	0.930	**0.010**	**0.027**
	NBCS	142.2 ± 8.6	142.2 ± 12.3	158.9 ± 26.3	150.9 ± 9.7			
Leu	HBCS	160.2 ± 11.5^{a}	108.7 ± 8.0^{b}	145.6 ± 11.3^{a}	149.0 ± 14.8^{a}	0.792	**0.003**	0.181
	NBCS	141.4 ± 10.9	128.3 ± 11.1	136.2 ± 18.0	162.7 ± 12.2			
Lys	HBCS	110.0 ± 9.7^{a}	63.5 ± 4.9^{c}	81.8 ± 6.2^{bc}	94.5 ± 8.9^{ab}	0.168	**<0.001**	0.065
	NBCS	81.2 ± 7.1^{ab}	66.2 ± 5.6^{b}	64.4 ± 4.4^{b}	95.6 ± 10.7^{a}			
Met	HBCS	28.9 ± 2.4^{a}	22.3 ± 2.1^{b}	20.3 ± 2.2^{b}	23.7 ± 2.7^{ab}	0.851	**0.001**	0.237
	NBCS	25.6 ± 2.1	25.9 ± 2.3	18.0 ± 1.7	22.1 ± 1.9			
Phe	HBCS	50.0 ± 3.3	45.3 ± 3.1	43.3 ± 3.2	47.5 ± 3.7	0.366	0.164	0.384
	NBCS	51.3 ± 3.1	53.5 ± 4.8	42.2 ± 2.9	48.9 ± 2.8			
Ser	HBCS	84.8 ± 5.4^{b}	102.6 ± 8.6^{ab}	114.5 ± 9.0^{a}	111.0 ± 9.6^{ab}	0.405	**<0.001**	0.259
	NBCS	86.3 ± 6.8^{b}	125.5 ± 10.9^{a}	112.1 ± 7.5^{a}	110.0 ± 11.9^{ab}			
Thr	HBCS	104.3 ± 8.3^{ab}	78.9 ± 8.3^{c}	92.4 ± 10.8^{bc}	136.1 ± 14.8^{a}	0.806	**<0.001**	0.847
	NBCS	107.2 ± 9.2^{ab}	87.2 ± 8.1^{b}	88.8 ± 7.7^{b}	152.1 ± 15.9^{a}			
Trp	HBCS	32.0 ± 2.5^{a}	23.2 ± 2.1^{b}	29.3 ± 2.2^{ab}	37.9 ± 3.6^{a}	0.307	**<0.001**	0.536
	NBCS	35.0 ± 4.5^{ab}	28.6 ± 2.6^{b}	27.7 ± 2.2^{b}	39.2 ± 3.2^{a}			
Tyr	HBCS	59 ± 5^{a}	32.2 ± 3.0^{c}	42.8 ± 3.6^{bc}	56.3 ± 6.8^{ab}	0.685	**<0.001**	0.424
	NBCS	55.7 ± 3.9^{a}	40.4 ± 4.1^{b}	39.3 ± 2.4^{b}	57.4 ± 5.2^{a}			
Val	HBCS	298.8 ± 22.9^{a}	228.0 ± 18.6^{b}	306.2 ± 25.6^{a}	307.5 ± 25.6^{a}	0.324	**0.002**	0.167
	NBCS	254.4 ± 16.7	247.7 ± 18.4	275.7 ± 30.3	318.3 ± 20.7			

Total AA	HBCS	2181.6 ± 151.1[b]	2070.3 ± 130.2[b]	2689.6 ± 184.1[a]	2404.1 ± 178.3[ab]	0.615	**0.028**	0.052
	NBCS	2167.7 ± 139.8	2292.5 ± 152.4	2262.6 ± 135.8	2442.7 ± 186.5			
Total BCAA	HBCS	600.7 ± 39.1[a]	451.8 ± 36.0[b]	655.8 ± 47.6[a]	593.2 ± 47.4[a]	0.568	**0.008**	0.117
	NBCS	538.0 ± 35.4	518.2 ± 40.8	570.9 ± 73.9	631.9 ± 41.3			
3-MH[2]	HBCS	4.3 ± 1.4[b]	16.0 ± 2.9[a]	7.3 ± 2.2[b]	3.0 ± 1.3[b]	0.587	**<0.001**	0.233
	NBCS	7.4 ± 2.8[b]	12.1 ± 2.2[a]	4.1 ± 0.9[b]	3.3 ± 0.5[b]			

[1] Statistical comparisons: G = group effect; T = time effect; G × T = group by time interaction.
Means in a row with superscripts without a common letter differ (P < 0.05).
* Different from NBCS (P < 0.05) within the same time point.
[2] 3-methylhistidine.

5 General discussion and conclusions

The overall aim of the present thesis was to investigate the regulation of BCAA metabolism in major metabolic tissues of dairy cows during late pregnancy and early lactation. For this, two studies were carried out (manuscript I and II). The first one (manuscript I) was conducted to characterize the potential enzymatic capacity of AT, alongside liver, muscle and MG, in BCAA metabolism in dairy cows during early lactation by assessing the serum BCAA profile, the tissue abundance as well as the activity of the main BCAA catabolic enzymes (in part for BCAT and completely for BCKDH). The second study (manuscript II) aimed at examining the changes in BCAA metabolism, i.e., the plasma BCAA concentrations and the abundance of the BCAA transporters and degrading enzymes in AT, muscle and liver of transition cows that were either normal- or over-conditioned at calving. It was hypothesized that early lactation is associated with tissue-specific changes in the BCAA transporters and catabolic enzymes and that BCAA metabolism in AT contributes significantly to the degrading pathway. Moreover, it was assumed that over-conditioned cows, having a higher tissue mobilization than cows with normal body condition, would exhibit specific alterations in their systemic and tissue-specific BCAA metabolism.

Potential capacity of different bovine tissues for BCAA metabolism

After absorption, most EAA are degraded in the liver, whereas catabolism of BCAA may occur in a wide range of metabolic tissues, e.g., liver, AT, muscle and kidney, both in ruminants and nonruminants (Goodwin et al., 1987; Suryawan et al., 1998), requiring significant interorgan transport of BCAA and their metabolites. Generally, the uptake and output of AA across an organ may provide an indication of the tissue's metabolic capacity to degrade or use them for protein synthesis. This can be assessed indirectly by measuring the arteriovenous (A-V) difference via indwelling catheters, i.e., the AA concentration in the artery leading into the organ (afferent vessel) and in the vein leading out of the organ (efferent vessel), respectively (Baumrucker, 1985). The A-V difference may then be multiplied by the rate of blood (or plasma) flow across the respective organ to obtain a more quantitative entity: the organ-specific AA net flux (Brosnan, 2003). A negative flux thereby indicates substrate uptake (or removal) and a positive flux substrate release. It should be noted though, that measuring nutrient fluxes may also have its boundaries, e.g., underestimation of actual transport rates as cellular transport

can occur bidirectionally (Baumrucker, 1985) or inaccurate assumption of constant intracellular substrate pools (Nielsen, 2003). Assessing the tissue abundance and activity of specific AA transporters and enzymes may however be an alternative estimate of AA flux and might give additional information about possible contributions of and regulatory targets within metabolically important tissues.

For decades it has been known that AT is an important storage of lipids as well as an endocrine gland regulating lipid and glucose metabolism. Albeit, its role in systemic AA and protein metabolism has been less appreciated, especially in ruminants. It has already been described that, *in vivo*, human AT can take up significant amounts of glutamate and may in turn release glutamine and alanine (Frayn et al., 1991), all of which are important by-products of BCAA metabolism. In a later study, Herman et al. (2010) demonstrated the possible capacity of murine AT to catabolize circulating BCAA and that BCAA degrading enzymes in AT may contribute to the coordinated regulation to modulate circulating BCAA levels. Very recently, it was also shown for dairy cows that through upregulation (mRNA and protein) of different transporters (e.g., SLC1A5, SLC1A3 and SLC38A1), bovine AT may take up significant amounts of AA from plasma depending on nutrient availability (Liang et al., 2019). According to the results of the present studies, where up to 5 different tissues (liver, skeletal muscle, MG, one or two types of AT) were compared for their potential capacity to metabolize BCAA, AT consistently had the greatest mRNA abundance of the BCAA transporters, i.e., *SLC1A5*, *SLC7A5* and *SLC38A2*, and the BCAA transaminating enzyme *BCAT2*, underscoring that AT may be an important site of BCAA uptake and initial degradation in dairy cows. In support of this, earlier studies by Bergen et al. (1988) and Papet et al. (1988) have revealed that, *in vitro*, ovine AT may exhibit highest leucine deamination and intermediate (keto-)leucine decarboxylation activity when compared to other tissues like muscle, liver and kidney.

As some end products of BCAA catabolism (e.g., succinyl-CoA and acetyl-CoA) may enter the TCA cycle, there is a close connection between BCAA and fatty acid metabolism (Kainulainen et al., 2013). But also, at virtually every other step of BCAA catabolism, the resulting metabolites may be diverted for fatty acid metabolism or other anaplerotic pathways (see Figure 5; chapter 1.3.2). Correspondingly, it has been reported that AT readily converts BCAA carbon skeletons into *de novo* fatty acids and that insulin may increase the conversion rate of leucine to lipids in murine AT but not in liver or muscle (Rosenthal et al., 1974). Furthermore, Green et al. (2016) observed that inhibition of BCAA catabolism (via knockdown of *Bckdha* in 3T3-L1 cells) can compromise adipocyte differentiation, demonstrating the importance of BCKA oxidation in adipogenesis. It should be noted however that with the onset

of lactation, dairy cows are usually already in a catabolic situation (with low circulating insulin levels and decreased peripheral insulin sensitivity), thus, any excess BCAA not directed towards the MG likely contribute more towards the generation of local cellular energy via the TCA cycle rather than towards anabolic processes such as lipogenesis or adipogenesis. In fact, unpublished data from one of our trials showed that close to parturition, as the cows enter a NNB, mRNA abundance of the key enzyme for fatty acid synthesis (fatty acid synthase) in scAT is down-regulated as compared to later time points. In contrast, around mid-lactation, when the cows achieve a state of positive energy balance again and mobilization of lipid storages subsides (Schuh et al., 2019), and peripheral IS is likely improved again, BCAA, or rather their metabolites, might be used for the restoration of previously lost body reserves. This is in agreement with our observation that in some cases BCKDHA mRNA and protein abundance in scAT may decrease over time during early lactation, illustrating some form of adjustability of AT for irreversible BCAA degradation depending on the metabolic status of the animal. However, according to Goodwin et al. (1987) and also based on our own findings for BCKDH, it is apparent that, despite high mRNA and protein abundances, the actual enzyme itself is acutely sensitive to metabolic regulators such as insulin, energy status and BCAA supply (Aftring et al., 1986; Hutson, 1986), and thus, under particular metabolic circumstances, might not be fully active in certain tissues (e.g., AT or muscle).

Concerning the potential capacity of other tissues for BCAA metabolism, studies have shown that, due to a considerably high expression and activity of BCAA enzymes (based on tissue weight) and the elevated respective net fluxes, skeletal muscle may also metabolize significant amounts of BCAA (Wijayasinghe et al., 1983; Pell et al., 1986; Suryawan et al., 1998; Faure et al., 2001). Yet, our results could not quite confirm this: Except for *SLC38A2*, which is only indirectly involved in BCAA transport, and *BCKDHB*, mRNA of BCAA transporters and degrading enzymes was less abundant in skeletal muscle than in AT or most other tissues by comparison. Further, muscle displayed the lowest BCKDHA protein abundance and, as for AT, the activity of the whole BCKDH complex was below the limit of detection. Thus, the overall capacity of semitendinosus muscle for BCAA metabolism seems to be restricted to BCAA supply through muscle mobilization, but otherwise appears to be relatively minor in dairy cows during the herein studied periods. As stated above, with the onset of lactation, anabolic processes, i.e., protein synthesis in this case, requiring BCAA, have a subordinate role in peripheral tissues. However, a generally low abundance of muscle BCAA metabolism related parameters was already observable before parturition [day (d) 49 *prepartum*]. In cattle, the last trimester of gestation is marked by intense fetal growth (Ferrell

et al., 1976), and self-evidently, there is a great demand for AA. Due to the high uteroplacental uptake and catabolism of BCAA (Lemons and Schreiner, 1984; Goodwin et al., 1987), it is suggested that BCAA are particularly important for fetal metabolism. In sheep, during late pregnancy, maternal tissues (particularly muscle) exhibit low BCAT activity, while fetal muscle and placenta both display high corresponding activities (Goodwin et al., 1987). This adaptation, also conceivable for dairy cows, may limit maternal BCAA (or rather BCKA) oxidation while BCAA utilization for protein synthesis in the growing fetus and placenta is likely promoted via increased reamination (Goodwin et al., 1987).

Surprisingly, our results also revealed comparably low *BCAT2*, *BCKDHA* and *BCKDHB* mRNA abundance in MG. Even though the present work is lacking protein data for BCAT2, this observation is in contrast to previous studies, where, during lactation, MG displays greatly elevated abundance and activity of both BCAA catabolic key enzymes as well as increased respective fluxes in relation to other metabolic tissues (DeSantiago et al., 1998a; Bequette et al., 2002; Li et al., 2009). Usually, BCAA are taken up by the MG in excess to their output as milk protein, supporting the synthesis of NEAA (Mepham, 1982). For this, at least some transamination would be necessary; however, in our study, counter-regulatory mechanisms might have caused a downregulation of BCAA enzyme mRNA to avoid a further promotion of AA deficiency during early lactation. Albeit, the low BCKDH enzyme activity we observed in MG (despite a high corresponding protein abundance), was associated with BCAA being spared from oxidation and possibly directed to *de novo* protein synthesis. This would be in support of the idea that via mTORC1 activation BCAA, particularly leucine, may stimulate milk protein synthesis under certain conditions (Moshel et al., 2006; Appuhamy et al., 2012; Doelman et al., 2015). If less (keto-) leucine is irreversibly oxidized, more protein could theoretically be synthesized, thus ensuring milk production.

Previously published data on BCAA net fluxes and isotopic tracer studies in dairy cows have illustrated that hepatic degradation of BCAA (and their keto acids) may be greatly reduced as compared to other splanchnic tissues or whole body degradation (Lapierre et al., 2002; Raggio et al., 2004; Larsen et al., 2015). Our results confirm that possibly due to the marginal hepatic abundance of BCAA transporters and BCAT2, BCAA cannot be directly degraded in the liver, but are first available to peripheral tissues, most likely AT. The elevated hepatic mRNA and protein abundance as well as the activity of BCKDH detected herein suggest that bovine liver could still be a significant site of BCAA metabolism and that only after BCAA are converted to their respective keto acids, the liver may oxidize them (Harper et al., 1984; Pell et al., 1986; Zhang et al., 2017). Even if full hepatic BCAA/BCKA oxidation may be comparably low in

dairy cows, high BCKDH abundance and activity, and thus, high oxidative capacity in the liver, would ensure clearance of toxic BCAA metabolites from portal blood and avoid plasma hyperaminoacidemia. As AA cannot be stored in tissues (unlike lipids and carbohydrates), regulation of circulating AA via catabolic enzymes plays an important role. However, depending on the physiological situation of the animal, the metabolic capacity of tissues for degradation may change.

BCAA metabolism during late gestation and early lactation in dairy cows

In both studies, a decrease of individual and total serum/plasma BCAA concentrations was observed around parturition. Generally, this can be associated with the lower feed intake the animals expressed during that time (Von Soosten et al., 2011; Schuh et al., 2019), as apart from microbial and endogenous protein degradation, circulating AA are largely affected by dietary intake. During late pregnancy, close to parturition, feed intake in dairy cows is physiologically reduced (Grummer, 1995; Allen et al., 2005), most likely due to endocrine changes, the initiation of lipomobilization and hence, the increase in anorexiant metabolites, as well as the limited rumen capacity because of the growing fetus (Ingvartsen, 2006). However, metabolic stress, related to the NNB during the transition period, is linked to an enhanced immune response, e.g., an increase in immune cells and positive acute phase proteins (Esposito et al., 2014), some of which containing substantial amounts of BCAA (Morimatsu et al., 1991; Calder, 2006), and, thus, may further reduce their availability in the circulation. This is particularly applicable for our second trial, where cows, continuously differing in body condition, expressed differences in their tissue mobilization (Schuh et al., 2019) and therefore might have had dissimilar manifestations of metabolic stress. In fact, it was shown that, compared with the normal-conditioned cows [NBCS; BCS < 3.5; back fat thickness (BFT) < 1.2 cm], the cows calving with high body condition (HBCS; BCS > 3.75; BFT > 1.4 cm) were metabolically more challenged because of a more severe and persistent NNB, a higher lipomobilization and possibly a lower IS (Holtenius et al., 2003; Roche et al., 2009; Pires et al., 2013; Schuh et al., 2019). Further, over-conditioned cows displayed a lower feed intake than NBCS cows throughout the study period (Schuh et al., 2019) and could have been closer to a pro-inflammatory status (Akbar et al., 2015; Roche et al., 2015; Vailati-Riboni et al., 2016) as the nadir of their plasma BCAA concentrations around calving was more pronounced.

Prolonged periods of NNB may lead to losses of both body fat and skeletal muscle, with a ratio of approximately 75% AT and 25% lean mass (Carbone et al., 2012). And because body

condition might affect lipid as well as protein mobilization in transition dairy cows (Kokkonen et al., 2005), it was assumed that HBCS cows, could have had a higher tissue mobilization in general. In support of this, different data from our trial indicate that high BCS and BFT were associated with a greater mRNA abundance of the two muscle-specific ligases muscle RING finger protein-1 (*MuRF-1*) and *atrogin-1* (Ghaffari et al., 2019), thus, an increased proteolysis in these cows seems likely. Consequently, the HBCS cows would then also have had a greater pool of BCAA and, counteractively, an increased tissue abundance of BCAA transporters and catabolic enzymes. However, this could only be confirmed in parts. The complex metabolic changes and adaptations occurring in the bovine organism during the shift from pregnancy to lactation might in some cases have had a greater effect on BCAA metabolism than the differences in body condition of the animals.

During lactation, when energy and nutrient demands rise substantially, whole-body BCAA transport and degradation mostly appear elevated as compared to non-lactating states (Tesseraud et al., 1993; DeSantiago et al., 1998a). Accordingly, except for scAT, where we observed constant expression levels of the BCAA transporters, we mainly detected increases in tissue transporter mRNA from *prepartum* (d 49 *prepartum*) to *postpartum* for both groups, with respective peaks at d 21 after calving (despite overall low abundance in muscle and liver). The downregulation at the last sampling time point (d 84 after parturition) thereby likely reflected the attenuated metabolic pressure on the tissues and the reduced demand for BCAA as milk production slowly started to decrease at that time. In partial support of this, data by Liang et al. (2019) revealed that transporters for BCAA, i.e., *SLC7A5*, *SLC1A5* and *SLC3A2*, were steadily expressed at mRNA level from d 10 *prepartum* to 30 *postpartum* in bovine scAT. Additionally, the protein abundance of SLC1A5 and SLC1A3 was gradually increasing during this time period pointing to an actually growing capability of AT for BCAA uptake even during periods of NNB.

Concerning the BCAA enzymes, the changes over time were less consistent in tissues other than AT. It seemed that overall, especially for HBCS cows, mRNA levels of BCAA degrading enzymes in scAT were constantly higher before than after parturition, suggesting an elevated capacity for adipose BCAA catabolism in these cows before the onset of lactation. Even though a greater *prepartum* BCAA catabolism is in contrast to what is reported in the literature until now (Tesseraud et al., 1993; DeSantiago et al., 1998a), one possible explanation could be that over-conditioned animals having a greater fat mass, or more specifically a larger size and number of adipocytes (Jo et al., 2009), generally possess a higher abundance of mitochondrial BCAA enzymes in this tissue, but that during early lactation, when tissue mobilization reaches

its maximum, the greater lipolysis in HBCS cows might diminish these circumstances. In addition, over-conditioned cows are more likely to experience a nutrient oversupply during late pregnancy (Ingvartsen, 2006); an upregulation of catabolizing enzymes in peripheral tissues would therefore be an efficient countermeasure to regulate elevated nutrient levels.

As the rate-limiting enzyme in BCAA catabolism and due to the sensitive regulation of its activity (Harris et al., 1994), the mitochondrial BCKDH complex is considered probably the most important key enzyme in overall BCAA metabolism. The E1α subunit (BCKDHA) is thereby especially responsive to nutritional and hormonal influences (Harris et al., 1997; Shimomura et al., 2001; Kadota et al., 2013) and is most likely the enzyme's main site of regulation (Shimomura et al., 1990; Lu et al., 2009). For both the mRNA and protein abundance of BCKDHA, we observed group differences in scAT, with HBCS cows having higher expression values than NBCS cows. Therefore, despite the lesser feed intake, HBCS cows could have had a greater ability to irreversibly catabolize BCAA, or rather their keto acids, in scAT compared to NBCS animals. Moreover, the differences in scAT *BCKDHA* mRNA between the two groups, which were almost permanently observed, slightly decreased over time possibly reflecting the bigger metabolic change of HBCS cows: The HBCS cows, having greater tissue reserves, started off in a more anabolic situation but due to the higher mobilization rate, reached a more catabolic condition during early lactation, thus, leveling up to the NBCS group. Further, it is likely that due to the nutrient surplus, animals in the HBCS group had an increased metabolic turnover in AT during late pregnancy allowing for more BCAA metabolites to also be used for synthesizing even more body fat. Normal-conditioned cows, being in a less anabolic state *prepartum* could have been sufficiently supplied by glycolytic processes providing acetyl-CoA and fatty acids and might have been less in need of BCAA to additionally support lipogenesis. This possible anaplerotic link between BCAA catabolism and lipid metabolism in HBCS cows is further substantiated by a study of Schäff et al. (2012), where a more intensive mobilization of body fat after calving was associated with a lower feed intake and increased anaplerosis, TCA cycling and mitochondrial oxidation in dairy cows.

Interestingly, in liver we observed a different picture: *Prepartum*, HBCS cows had a lower hepatic BCKDHA protein abundance than NBCS cows. Yet, for both groups abundance peaked around parturition and particularly for the HBCS group values were lower before than after calving. It appears as if *prepartum* over-conditioned cows were less in need of hepatic BCKA oxidation than normal-conditioned cows. However, at calving, when energy balance is usually the most negative (Tienken et al., 2015), protein abundance of BCKDHA in the liver could be upregulated for the generation of additional energy via irreversible BCKA oxidation. As the

energy balance slowly increases again during early lactation, the need for additional energy may decrease. The subsequent decline of BCKDHA protein could thus reflect the lower metabolic pressure on the liver.

Within our first trial only the time from d 1 to 105 after parturition was covered. Both the mRNA as well as the protein abundance of the assessed BCAA enzymes mostly increased during the course of the experiment, especially in AT and MG, allowing in this case for enhanced BCAA degradation in these tissues with progressing lactation. However, as for the assessed BCKDH activity, only liver showed time dependent changes which could point to more metabolic flexibility in this tissue.

Conclusions

The herein presented studies show that the metabolism of BCAA is a tightly regulated system, which involves the complex interactions of several metabolic tissues to fulfill the ever-changing nutrient demands of high-yielding dairy cows in different physiological and pathophysiological conditions. Studying the changes of BCAA transporters and catabolic enzymes in different tissues of dairy cows during the transition from late gestation to early lactation has revealed that AT may be an important site of BCAA uptake and (initial) degradation at that time, thus, confirming our hypothesis. Enhanced capacity for BCAA catabolism in AT, particularly observed for the cows with high body condition, might be linked with lipid metabolism through increased anaplerosis, especially during late gestation, when animals are in a more anabolic situation and may use additional substrates such as BCAA metabolites to build up body fat. However, further research involving e.g., radio-labelled isotopes should address more quantitatively the exact contributions of BCAA catabolism to lipogenic and adipogenic processes in dairy cows.

According to the mostly low abundance (and activity) of BCAA transporters and enzymes, semitendinosus muscle appears to play a subsidiary role in BCAA metabolism of transition dairy cows, that is except for the supply of BCAA through muscle mobilization. Whether other types of muscle could have a greater capacity for BCAA transport and degradation is not clear yet and should be investigated in the future. Intriguingly, we also observed mainly low abundance and activity of BCAA enzymes in MG. In contrast to studies in other mammalian species, where the NNB might not be as deep and persistent, actual BCAA/BCKA degradation in the MG could be downregulated in high-yielding dairy cows to avoid further deficiencies and spare BCAA for milk protein synthesis.

Despite probably reduced hepatic BCAA/BCKA fluxes, a counter-regulatory mechanism seems to allow for activation of the BCKA oxidative pathway in bovine liver, ensuring the degradation of possibly toxic BCAA metabolites. And depending on the manifestation of the energy deficit dairy cows experience during the transition period, hepatic BCKA oxidation may contribute more or less to the generation of energy in support of lactation. Here it seems that *prepartum*, especially cows with high body condition and likely oversupplied with nutrients during late gestation, might rely less on additional energy stemming from hepatic BCKA oxidation and instead may have catabolized BCAA transamination products in AT supporting the metabolic turnover therein.

6 Summary

The transition from late pregnancy to early lactation is one of the most crucial times in a dairy cow's life. It is characterized by drastic changes in nutrient balance as well as metabolic and endocrine shifts throughout the whole body. To cover the increased nutrient demands of lactation, dairy cows have to mobilize body reserves from adipose tissue (AT) and skeletal muscle. The extent of tissue mobilization is thereby more pronounced in cows that are over-conditioned at calving. Consequently, those animals are more susceptible to develop metabolic disorders and a more enhanced peripheral insulin resistance (IR) than cows in normal body condition. While body fat is generally repartitioned to provide fatty acids for the synthesis of energy and milk fat, body protein is mobilized to release amino acids (AA) for protein synthesis, direct oxidation and hepatic gluconeogenesis. However, specific regulatory processes within the tissues are also involved and may be linked with each other. Although it is well established that AT can regulate glucose and lipid metabolism through adipokine secretion, its role in systemic protein and AA metabolism is less well-appreciated, especially in ruminants. The three branched-chain amino acids (BCAA), leucine, isoleucine and valine, are taken up by the bovine mammary gland (MG) in excess as they are major components of milk protein and important precursors for the synthesis of non-essential AA, energy and other metabolic intermediates. Further, high BCAA levels have been associated with obesity and certain metabolic dysfunctions, such as increased IR in mammals. Both the cellular transport and break-down of BCAA are tightly regulated processes, requiring the cooperation of different organs, one of them possibly AT. In terms of the many tissue-specific changes occurring during the periparturient period, assessing the actual BCAA transporters and degrading enzymes in major metabolic tissues may provide useful information about possibly regulated targets within BCAA metabolism and elucidate the general contributions of these tissues to BCAA metabolism in transition dairy cows.

Thus, the aims of the present thesis were (1) to evaluate the potential enzymatic capacity of AT as well as liver, skeletal muscle and MG in BCAA metabolism in dairy cows during (late gestation and) early lactation, (2) to analyze the concentrations of circulating BCAA as well as the tissue abundance (and activity) of the most important BCAA transporters and enzymes in major metabolic tissues of dairy cows during late gestation and early lactation and (3) to investigate the effect of over-conditioning at calving on the aforementioned parameters of systemic and tissue BCAA metabolism in dairy cows during late gestation and early lactation.

In the first study (manuscript I), we aimed to test the potential enzymatic capacity of AT (alongside liver, muscle and MG) in BCAA metabolism in dairy cows during early lactation. We hypothesized that early lactation is associated with tissue-specific changes in the key enzymes of BCAA metabolism due to particular metabolic adaptations and that BCAA enzymes in AT contribute significantly to the degrading pathway. For this, tissue samples (i.e., subcutaneous and visceral AT, liver, *M. semitendinosus* and udder parenchyma) and blood were collected on days (d) 1, 42 and 105 *postpartum* (*p.p.*) from 25 primiparous Holstein cows during slaughter. Serum BCAA profiles were analyzed and the mRNA and protein abundance as well as the activity in the different tissues were assessed for the BCAA catabolic enzymes, partly for the branched-chain aminotransferase (BCAT) and completely for the branched-chain α-keto acid dehydrogenase (BCKDH). Total BCAA as well as individual isoleucine and valine concentrations in serum were lowest on d 1 *p.p.* and increased thereafter to a steady level for the duration of the experiment. This was associated with the physiologically reduced feed and thus protein intake around calving as well as a possible increase in immune parameters (often observed in cows shortly after parturition), the latter containing and using significant amounts of BCAA. Pronounced differences between the tissues were detected at all molecular levels. The mRNA abundance of the mitochondrial isoform of BCAT (*BCAT2*) was greater in both types of AT than in the other tissues studied, indicating that AT could be an important site for initial BCAA degradation during early lactation in dairy cows. Surprisingly, muscle mostly tended to overall low mRNA, protein and activity levels of BCAA enzymes which in this case could imply a rather subsidiary involvement in BCAA catabolism. High BCKDH abundance and activity in liver, possibly a countermeasure towards the therein usually reduced BCAA flux in cows, was linked to a greater hepatic oxidative capacity for BCAA or rather their keto acids, supposably to prevent hyperaminoacidemia and remove toxic BCAA intermediates. Despite elevated protein abundance, the corresponding BCKDH activity was reduced in MG. Therefore, it was assumed that, during early lactation, BCAA and their metabolites might be spared from mammary oxidation and instead conserved for milk protein synthesis.

Within the second study (manuscript II) the objectives were to determine the changes in circulating BCAA levels in cows that were either over- or normal-conditioned at calving and to identify the differences in the mRNA abundance of the most relevant BCAA transporters as well as the mRNA and protein abundance of the BCAA key enzymes in AT, muscle and liver of these animals during the transition from late pregnancy to early lactation. It was hypothesized that over-conditioned cows, having a higher tissue mobilization and likely showing a more decreased insulin sensitivity than cows with normal body condition, would exhibit specific

alterations in BCAA metabolism. The experimental set-up included 38 multiparous Holstein cows that were allocated 15 weeks *antepartum* (*a.p.*) to a normal-conditioned (NBCS; n = 19) or high-conditioned group (HBCS; n = 19), receiving differential feeding during late lactation to reach the targeted differences in body condition score (BCS) and back fat thickness (BFT) (NBCS: BCS < 3.5 and BFT < 1.2 cm; HBCS: BCS > 3.75 and BFT > 1.4 cm) until dry-off. During the dry period and the next lactation, cows were fed the same diets, whereby differences in BCS and BFT were retained. Blood and biopsies from subcutaneous AT (scAT), *M. semitendinosus* and liver were sampled at days 49 *a.p.*, 3, 21 and 84 *p.p.* Overall, HBCS cows were more metabolically challenged during early lactation than NBCS cows due to a more severe and persistent negative energy balance, a greater lipolysis and probably a lower insulin sensitivity after calving. For HBCS cows, we detected a nadir of total BCAA (and individual leucine and valine) plasma concentrations around parturition, which could be explained by the generally more decreased feed intake of this group. But it is also possible that because of the greater metabolic stress, HBCS cows were closer to a pro-inflammatory status around calving and thus had to use more AA for the generation of immune parameters further reducing BCAA levels. The greatest differences in BCAA transporter abundance were related to the type of tissue: Compared to the other tissues, we found steadily increased mRNA abundance of the BCAA transporters in scAT without any changes due to the body condition of the animals or the time relative to calving. As for the first study, we also detected highest *BCAT2* mRNA in (sc)AT in study II (with no effects of BCS and BFT). Taken together, it therefore seems that, independent of body condition, (sc)AT could be important for both BCAA uptake and initial degradation in dairy cows. In contrast, liver (and partly muscle) expressed only low mRNA levels of the BCAA transporters and *BCAT2* pointing to a decreased BCAA uptake and deamination capacity therein. We further observed that, in comparison to the NBCS group, the HBCS cows had higher BCKDH E1α (*BCKDHA*) mRNA and, *prepartum*, also higher BCKDHA protein levels in scAT suggesting a greater ability to irreversibly catabolize BCAA in AT, especially before calving. However, it was assumed that over-conditioned animals, having larger tissue reserves, were likely oversupplied with nutrients during late pregnancy and thus in a more anabolic situation as compared to normal-conditioned cows. As the lower hepatic BCKDHA protein abundance of HBCS cows before calving implies, over-conditioned animals may have relied less on the oxidation of BCAA transamination products for energy generation, but instead, through linkages in fatty acid and adipose BCAA metabolism, could have also used BCAA metabolites for synthesizing even more body fat before parturition.

In summary, the present thesis shows that the metabolism of BCAA in dairy cows is a complex and highly regulated system. Studying the tissue-specific changes of BCAA transporters and enzymes during the transition from late pregnancy to early lactation has demonstrated amongst others that AT may be a significant site of BCAA uptake and (initial) degradation in dairy cows at that time. For over-conditioned cows, increased BCAA catabolism in AT might be connected with lipid metabolism, especially during late gestation, when animals are still building up tissue reserves to support the impending lactation. However, further studies are needed to assess more quantitatively the exact contributions of BCAA metabolism to lipogenic and adipogenic processes in dairy cows.

7 Zusammenfassung

Der Übergang von der späten Trächtigkeit zur frühen Laktation ist eine der kritischsten Phasen im Leben einer Milchkuh, welche sich vor allem durch systemische Veränderungen der Nährstoffbilanz, des Stoffwechsels und des Hormonhaushalts auszeichnet. Um den erhöhten Nährstoffbedarf der Laktation zu decken, müssen Kühe Körperreserven in Form von Fettgewebe (AT) und Skelettmuskulatur mobilisieren. Dabei ist das Ausmaß dieser Gewebemobilisierung um einiges ausgeprägter bei Tieren, welche zum Zeitpunkt der Kalbung überkonditioniert sind. Infolgedessen unterliegen diese Kühe einem höheren Risiko für die Entwicklung metabolischer Erkrankungen und einer erhöhten Insulinresistenz (IR) als normalkonditionierte Tiere. Während die Umverteilung des Körperfettes generell der Bereitstellung von Fettsäuren zur Synthese von Energie und Milchfett dient, werden die aus dem Körperprotein mobilisierten Aminosäuren vor allem für die Proteinsynthese, die direkte Oxidation und die hepatische Glukoneogenese genutzt. Es existieren jedoch auch spezifische regulatorische Prozesse innerhalb der Gewebe, die sich zudem gegenseitig beeinflussen können. Obwohl allgemein bekannt ist, dass AT sowohl den Glukose- als auch den Lipidstoffwechsel durch die Sekretion von Adipokinen regulieren kann, ist dessen Funktion im systemischen Protein- und Aminosäurestoffwechsel weniger geläufig, insbesondere bei Wiederkäuern. Die drei verzweigtkettigen Aminosäuren (*branched-chain amino acids*; BCAA), Leucin, Isoleucin und Valin, werden im Übermaß von der bovinen Milchdrüse aufgenommen, da sie Hauptbestandteile des Milchproteins und wichtige Vorläufer für die Synthese von nicht-essentiellen Aminosäuren, Energie und anderen metabolischen Intermediaten sind. Des Weiteren werden erhöhte BCAA-Konzentrationen mit Übergewicht und gewissen metabolischen Erkrankungen, wie z.B. einer erhöhten IR bei Säugetieren, assoziiert. Sowohl der zelluläre Transport als auch der Abbau der BCAA sind hochregulierte Prozesse, welche die Interaktion mehrerer Gewebe erfordern, u.a. vermutlich AT. Bezogen auf die vielen gewebespezifischen Veränderungen während der Transitphase, kann eine Erfassung der eigentlichen BCAA-Transporter und abbauenden Enzyme in den verschiedenen metabolischen Geweben peripartaler Milchkühe über potentiell regulierte Zielparameter innerhalb des BCAA-Stoffwechsels Auskunft geben und helfen den allgemeinen Beitrag der einzelnen Gewebe zum Stoffwechsel der BCAA abzuschätzen.

Demnach waren die Ziele der vorliegenden Arbeit (1) die Beurteilung der potentiellen enzymatischen Kapazität von AT sowie Leber, Skelettmuskel und Euter im BCAA-

Stoffwechsel von Kühen während der (späten Trächtigkeit und) frühen Laktation, (2) die Erfassung der zirkulierenden BCAA-Konzentration sowie der Gewebeexpression (und -aktivität) der relevantesten BCAA-Transporter und -Enzyme in wichtigen metabolischen Geweben von Kühen während der späten Trächtigkeit und frühen Laktation und (3) die Untersuchung des Einflusses einer Überkonditionierung zum Zeitpunkt der Kalbung auf die zuvor genannten Parameter des systemischen und gewebebezogenen BCAA-Stoffwechsels bei Kühen währen der späten Trächtigkeit und frühen Laktation.

In der ersten Studie (Manuskript I) wurde die potentielle enzymatische Kapazität von AT (neben Leber, Muskel und Euter) im BCAA-Stoffwechsel von Milchkühen während der frühen Laktation erfasst. Die Hypothese war, dass die Frühlaktation, aufgrund von speziellen metabolischen Anpassungen, mit gewebespezifischen Veränderungen der Schlüsselenzyme des BCAA-Stoffwechsels verbunden ist und dass die BCAA-Enzyme im AT entscheidend zum Abbau beitragen. Hierfür wurden an den Tagen 1, 42 und 105 *postpartum* (*p.p.*) Gewebeproben (subkutanes und viszerales AT, Leber, *M. semitendinosus* und Euterparenchym) und Blut von 25 geschlachteten primiparen Holsteinkühen entnommen. Die Serum-BCAA-Profile wurden analysiert und die mRNA- und Proteinexpression sowie die Aktivität der BCAA-Enzyme wurden in den verschiedenen Geweben erfasst, teilweise im Falle der *branched-chain aminotransferase* (BCAT) und vollständig für die *branched-chain α-keto acid dehydrogenase* (BCKDH). Die Gesamt-BCAA-Konzentration (und individuelle Isoleucin- und Valinkonzentration) war an Tag 1 *p.p.* am geringsten und stieg in Folge für die verbleibende Dauer des Versuches auf ein stetiges Level an. Die geringe BCAA-Konzentration um die Kalbung herum wurde dabei einerseits mit der zu dieser Zeit physiologisch reduzierten Futter- bzw. Proteinaufnahme und andererseits mit einem möglichen Anstieg von BCAA-enthaltenden und BCAA-verwendenden Immunparametern assoziiert. Demzufolge wird bei Kühen insbesondere eine Erhöhung von positiven Akute-Phase-Proteinen und Immunzellen kurz nach der Kalbung beobachtet. Zwischen den verschiedenen Geweben wurden ausgeprägte Unterschiede auf jeglichen molekularen Ebenen festgestellt. Die mRNA-Expression der mitochondrialen BCAT (*BCAT2*) war in beiden AT höher als in den anderen untersuchten Geweben, was zu der Annahme führt, dass AT von großer Bedeutung für den initialen Abbau der BCAA während der Frühlaktation bei Milchkühen sein könnte. Erstaunlicherweise tendierte Muskel meistens zu allgemein geringen mRNA-, Protein- und Aktivitätswerten der BCAA-Enzyme. In diesem Fall könnte das eine eher nebensächliche Rolle des bovinen Muskelgewebes im BCAA-Katabolismus vermuten lassen. Eine hohe BCKDH-Expression und -Aktivität in der Leber, möglicherweise eine Gegenmaßnahme zu den dort meist reduzierten BCAA-Flüssen,

wurde mit einer erhöhten hepatischen oxidativen Kapazität für BCAA verbunden. Dies könnte u.a. dazu dienen, eine Hyperaminoazidämie und die Akkumulierung potentiell toxischer BCAA-Metabolite zu vermeiden. Trotz der erhöhten Proteinexpression, war die korrespondierende BCKDH-Aktivität im Euter reduziert. Es wurde daher angenommen, dass BCAA und deren Metabolite währen der frühen Laktation dort weniger oxidiert werden und stattdessen eher für die Milchproteinsynthese aufgespart werden.

Innerhalb der zweiten Studie (Manuskript II) sollten die Veränderungen der zirkulierenden BCAA-Konzentrationen bei zum Zeitpunkt der Kalbung über- oder normalkonditionierten Milchkühen bestimmt werden und die Unterschiede der mRNA-Expression der relevantesten BCAA-Transporter sowie die mRNA- und Proteinexpression der BCAA-Schlüsselenzyme in AT, Muskel und Leber dieser Tiere während der späten Trächtigkeit und frühen Laktation identifiziert werden. Die Hypothese war, dass überkonditionierte Kühe, die eine erhöhte Gewebemobilisierung und vermutlich eine stärker verringerte Insulinsensitivität als Kühe mit normaler Körperkondition aufweisen, spezifische Veränderungen im BCAA-Stoffwechsel zeigen. Für den Versuch wurden 38 multipare Holsteinkühe eingesetzt, welche 15 Wochen *antepartum* (*a.p.*) entweder einer normalkonditionierten (NBCS; n = 19) oder einer überkonditionierten Gruppe (HBCS; n = 19) zugeteilt wurden. Die Gruppen erhielten jeweils unterschiedlich angepasste Fütterungen während der späten Laktation, um die angestrebten Ziele hinsichtlich des *Body Condition Scores* (BCS) und der Rückenfettdicke (RFD) (NBCS: BCS < 3.5 und RFD < 1.2 cm; HBCS: BCS > 3.75 und RFD > 1.4 cm) bis zum Zeitpunkt des Trockenstellens zu erreichen. An den Tagen 49 *a.p.*, 3, 21 und 84 *p.p.* wurden Blut und Gewebebiopsien von subkutanem AT (scAT), *M. semitendinosus* und der Leber entnommen. Insgesamt waren die HBCS-Kühe während der Frühlaktation aufgrund einer stärker ausgeprägten und länger anhaltenden negativen Energiebilanz, einer erhöhten Lipolyse und vermutlich einer geringeren Insulinsensitivität stärker metabolisch belastet als die NBCS-Kühe. Bei der HBCS-Gruppe wurden ein Tiefstand der Gesamt-BCAA-Konzentration (und individueller Leucin- und Valinkonzentrationen) im Plasma an Tag 3 *p.p.* verzeichnet. Dies könnte zum einen an der generell geringeren Futteraufnahme dieser Gruppe liegen, zum anderen aber auch der Tatsache geschuldet sein, dass die HBCS-Kühe sich aufgrund des erhöhten metabolischen Stresses möglichweise eher in einem proinflammatorischen Zustand um die Kalbung herum befanden und somit mehr Aminosäuren für die Generierung von Immunparametern verwenden mussten. Die größten Unterschiede bei der Expression der BCAA-Transporter standen in Zusammenhang mit den verschiedenen Gewebearten. Verglichen mit den anderen Geweben konnten wir in scAT eine stetig erhöhte mRNA-

Expression der BCAA-Transporter vorfinden, ungeachtet der unterschiedlichen Körperkondition oder der Zeit relativ zur Kalbung. Auch in dieser Studie wurde die höchste *BCAT2* mRNA-Expression in AT detektiert (ohne Einfluss von BCS oder RFD). Zusammengefasst deutet dies darauf hin, dass bei Milchkühen (sc)AT, unabhängig von der Körperkondition, sowohl für die zelluläre BCAA-Aufnahme als auch deren Abbau von großer Bedeutung sein kann. Im Gegensatz hierzu exprimierte Leber (und teilweise Muskel) nur begrenzte Mengen an BCAA-Transportern und *BCAT2*, was auf eine dort geringere BCAA-Aufnahme und Deaminierungskapazität schließen lässt. Ferner wurde beobachtet, dass die HBCS-Kühe eine höhere BCKDH E1α (*BCKDHA*) mRNA- und *antepartum* auch eine höhere BCKDHA-Proteinexpression in scAT aufwiesen als die NBCS-Kühe. Dies könnte insbesondere vor der Kalbung ein höheres Vermögen der HBCS-Tiere für den irreversiblen BCAA-Abbau im AT bedeuten. Allerdings wurde angenommen, dass die überkonditionierten Kühe während der späten Trächtigkeit mit Nährstoffen überversorgt waren und sich daher im Vergleich zu den normalkonditionierten Tieren eher in einer anabolen Situation befanden. Wie die geringere hepatische BCKDHA-Proteinexpression der HBCS-Tiere vor der Kalbung suggeriert, waren die überkonditionierten Kühe womöglich weniger auf die Oxidation der BCAA-Deaminierungsprodukte zur Energiegewinnung angewiesen und könnten die BCAA-Metabolite stattdessen, über Verknüpfungen des Lipid- und BCAA-Stoffwechsels im AT, vermehrt zum Aufbau von weiterem Körperfett genutzt haben.

Zusammenfassend zeigt die vorliegende Dissertation, dass der Stoffwechsel der BCAA ein komplexes und stark reguliertes System bei Milchkühen ist. Die Untersuchungen der gewebespezifischen Veränderungen der BCAA-Transporter und -Enzyme während des Übergangs von der späten Trächtigkeit zur frühen Laktation haben u.a. belegt, dass AT von signifikanter Bedeutung für die Aufnahme und den (initialen) Abbau der BCAA bei Milchkühen zu dieser Zeit sein kann. Bei überkonditionierten Kühen könnte ein erhöhter BCAA-Katabolismus im AT mit dem Lipidstoffwechsel verbunden sein. Dies gilt besonders für die späte Trächtigkeit, einem Zeitraum in dem die Tiere hauptsächlich noch Körperreserven aufbauen um die anstehende Laktation zu unterstützen. Jedoch sind weitere Studien erforderlich, um die genaue Mitwirkung des BCAA-Stoffwechsels an lipogenen und adipogenen Prozessen in Milchkühen quantitativ zu erfassen.

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10 Publications derived from this doctorate thesis and from related work

1) **Webb, L. A.**, M. H. Ghaffari, H. Sadri, K. Schuh, V. Zamarian, C. Koch, N. Trakooljul, K. Wimmers, C. Lecchi, F. Ceciliani, and H. Sauerwein. 2020. Profiling of circulating microRNA (miRNA) and pathway analysis in normal- versus over-conditioned dairy cows during the dry period and early lactation. Submitted to J. Dairy Sci.
2) **Webb, L. A.**, H. Sadri, K. Schuh, S. Egert, P. Stehle, I. Meyer, C. Koch, G. Dusel, and H. Sauerwein. 2020. Branched-chain amino acids: Abundance of their transporters and metabolizing enzymes in adipose tissue, skeletal muscle and liver of dairy cows at high or normal body condition. J. Dairy Sci. 103: 2847-2863. https://doi.org/10.3168/jds.2019-17147
3) **Webb, L. A.**, H. Sadri, D. von Soosten, S. Dänicke, S. Egert, P. Stehle, and H. Sauerwein. 2019. Changes in tissue abundance and activity of enzymes related to branched-chain amino acid catabolism in dairy cows during early lactation. J. Dairy Sci. 102: 3556-3568. https://doi.org/10.3168/jds.2018-14463
4) Schuh, K., H. Sadri, S. Häussler, **L. A. Webb**, C. Urh, M. Wagner, C. Koch, J. Frahm, S. Dänicke, G. Dusel, and H. Sauerwein. 2019. Comparison of performance and metabolism from late pregnancy to early lactation in dairy cows with elevated versus normal body condition at dry-off. Animal 13: 1478-1488. https://doi.org/10.1017/S1751731118003385
5) Ghaffari, M. H., H. Sadri, K. Schuh, **L. Webb**, G. Dusel, C. Koch, and H. Sauerwein. 2018. Effect of over-conditioning on mTOR and ubiquitin proteasome gene expression in muscle of dairy cows. p. 181. *In* Proceedings of the 69th Annual Meeting of the European Federation of Animal Science, Dubrovnik, Croatia, August 27-31.
6) **Webb, L. A.**, H. Sadri, K. Schuh, S. Egert, P. Stehle, C. Koch, G. Dusel, and H. Sauerwein. 2019. Plasma branched-chain amino acids (BCAA) and mRNA abundance of 3 different BCAA transporters in adipose tissue, muscle and liver of dairy cows with high or normal body condition score. J. Dairy Sci. 102, Supplement 1, 271.
7) **Webb, L. A.**, H. Sauerwein, D. von Soosten, S. Dänicke, and H. Sadri. 2018. Expression and activity of the branched-chain a-keto acid dehydrogenase (BCKDH) in different tissues of early-lactating dairy cows. J. Dairy Sci. 101, Supplement 2, 148.

8) Urh, C, K. Schuh, V. Zamarian, **L. Webb**, C. Lecchi, M. T. A. Alaedin, H. Sadri, M. H. Ghaffari, G. Dusel, C. Koch, N. Trakooljul, K. Wimmers, F. Ceciliani, and H. Sauerwein. 2018. Profiling peripheral microRNA in normal-versus over-conditioned dairy cows during dry-off and early lactation. J. Anim. Sci. 96, Supplement 3, 357. https://doi.org/10.1093/jas/sky404.785

9) **Webb, L. A.**, K. Schuh, G. Dusel, M. Wagner, C. Koch, H. Sauerwein, and H. Sadri. 2017. Plasma thyroid hormone concentrations in dairy cows of high versus normal body condition. p. 176. *In* Proceedings of the Meeting of the German Society of Nutrition Physiology 26, Göttingen, Germany, March 14-16.