Institut für Tierwissenschaften

Metabolic profiling in serum and muscle of dairy cows during the periparturient period and the subsequent lactation

Dissertation

zur Erlangung des Grades

Doktor der Agrarwissenschaft (Dr. agr.)

der
Landwirtschaftlichen Fakultät
der
Rheinischen Friedrich–Wilhelms–Universität Bonn

von

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Guiyang, P. R. China

Bonn 2018

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Tag der mündlichen Prüfung:	27.11.2018

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

To my family

English Abstract

The transition period in dairy cows, spanning three weeks before and after parturition, is characterized by complex metabolic processes required for the homeorhetic adaptation to the needs of late pregnancy and the onset of lactation. Adaptive failure during the periparturient period may impair animal health and performance. Besides liver and systemic investigations performed in blood, the role of adipose tissue that is mostly affected by the mobilization of body reserves at that time, formed a research focus. Skeletal muscle is the largest organ in the body and is also involved in the adaptive reactions during the transition period, but alterations of metabolic pathways around parturition were scarcely characterized. Therefore, the aim of this dissertation was to characterize the metabolome in skeletal muscle and in serum of dairy cows during the transition from late pregnancy to early lactation. The oxidative capacity for fatty acids (FA) in skeletal muscle may also contribute to reducing the metabolic load of FA, and thus we took the profiles of muscle and blood serum FA esters of carnitine, i.e. the acylcarnitines (**ACC**) in focus. In addition, a dietary supplementation with conjugated linoleic acids (**CLA**) was tested for potential effects on the target variables. To achieve the objectives, a targeted metabolomics approach was used in which up to 188 endogenous metabolites from six different compound classes can be quantified. The ACC metabolism was further investigated by assessing the mRNA abundance of carnitine acyltransferases in muscle. Results of this study indicate that FA oxidation, as well as the metabolism of arginine, tryptophan, phosphatidylcholines, and sphingomyelines were the metabolic pathways that were mainly influenced by the transition from late gestation to early lactation. These altered metabolic pathways may reflect dysregulated lipid metabolism, impaired insulin action, and increased inflammatory status in dairy cows around parturition. Furthermore, when combining the results with the data from the mRNA abundance of carnitine acyltransferases, the results also indicate that β-oxidation in muscle mitochondria increased around parturition, but was likely exceeding the capacity of acetyl-CoA utilization in the tricarboxylic acid cycle. The present dissertation adds on to understanding the multifaceted metabolic adaptation of dairy cows during the transition from late pregnancy to early lactation.

German Abstract

Die sogenannte Transitphase bei Milchkühen umfasst einen Zeitraum von rund drei Wochen vor und drei Wochen nach der Geburt. Sie ist durch komplexe Stoffwechselprozesse charakterisiert, die für die homörhetische Anpassung an die Bedürfnisse der Spätträchtigkeit und des Laktationsbeginns notwendig sind. Störungen in dieser Anpassung während der Transitperiode können die Gesundheit und Leistungsfähigkeit der Tiere beeinträchtigen. Neben in Leber und Blut durchgeführten Untersuchungen steht auch das Fettgewebe, das in dieser Zeit am stärksten für die Mobilisierung von Körperreserven zuständig ist, im Fokus der Forschung. Der Skelettmuskel ist das größte Organ des Körpers und ist ebenso an den Anpassungsreaktionen während der Übergangszeit beteiligt. Änderungen innerhalb der Stoffwechselwege rund um die Geburt, wurden hier aber bisher kaum beschrieben. Deshalb war es das Ziel dieser Dissertation, die Veränderungen im Metabolom des Skelettmuskels und des Serums von Milchkühen während der Transitphase zu beschreiben. Die Oxidation von Fettsäuren (FA) im Skelettmuskel kann auch dazu beitragen, die metabolische Belastung durch FA zu reduzieren, weswegen die Profile der FA-Cartinitin-Ester (ACC) in Muskulatur und Blutserum von besonderem Interesse waren. Der weiteren wurde eine Supplementierung der Futterration mit konjugierten Linolsäuren (CLA) auf mögliche Auswirkungen auf die Zielvariablen hin untersucht. Um diese Ziele zu erreichen, nutzten wir einen metabolomischen Ansatz („*targeted*“), in dem bis zu 188 endogene Metaboliten aus sechs verschiedenen Stoffgruppen quantifiziert werden können. Der ACC-Metabolismus wurde zusätzlich durch die Bewertung der mRNA-Konzentration von Carnitin-Acyltransferasen in Muskelgewebe untersucht. Die Ergebnisse dieser Studie zeigen, dass die FA-Oxidation, sowie der Stoffwechsel von Arginin, Tryptophan, der Phosphatidylcholine und der Sphingomyeline hauptsächlich während der Übergangszeit von der späten Schwangerschaft bis zur frühen Laktation beeinflusst wurden. Diese veränderten Stoffwechselwege können an Dysregulationen im Fettstoffwechsel, der beeinträchtigte Insulinwirkung und dem erhöhten Entzündungsstatus bei Milchkühen um die Geburt beteiligt sein. In Kombination mit den RNA-Daten zeigt sich zudem, dass die β-Oxidation in den Muskelmitochondrien um die Geburt erhöht ist, aber die Kapazität der Acetyl-CoA-Nutzung im Tricarbonsäure -Zyklus wahrscheinlich überschritten ist. Die vorliegende Dissertation soll zum besseren Verständnis der vielschichtigen Stoffwechselanpassung von Milchkühen beim Übergang von der späten Trächtigkeit in die frühe Laktation beitragen.

List of abbreviations

3-MH	3-methylhistidine
4EBP1	EIF4E Binding Protein 1
AA	Amino acid
ACBP	Acyl-CoA-binding protein
ACC	Acylcarnitine
ACC2	Acetyl-CoA carboxylase 2
ACSL	Acyl-CoA synthetase
ADMA	Asymmetric dimethylarginine
AIC	Akaike's information criterion
AMPK	AMP-activated protein kinase
AT	Adipose tissue
BIC	Bayesian information criterion
*c*9,*t*11	*cis*-9,*trans*-11
CI	Chemical ionization
CLA	Conjugated linoleic acids
CPT	Carnitine palmitoyltransferase
DM	Dry matte
DMI	Dry matter intake
EB	Energy balance
EI	Electron ionization
EIF3K	Eukaryotic translation initiation factor 3
EMD	Emerin
ESI	Electrospray ionization
FA	Fatty acid
FABPc	Cytoplasmic FA-binding protein
FABPpm	Membrane-bound FA binding protein
FAO	Fatty acid oxidation
FAT/CD36	FA translocase CD36
FATP	FA transport proteins
FDR	False discovery rate
GC	Gas chromatography
GH	Growth hormone
glog	Generalized log
GLUT4	Glucose transporter 4
H1	Sum of hexoses – about 90-95% glucose
IGF-1	Insulin-like growth factor 1
JAK2	Janus kinase 2
KNN	*k*-nearest neighbors
LA	Linoleic acid
LC	Liquid chromatography
LOD	Limit of detection
LRP10	Low-density lipoprotein receptor–related protein 10
lysoPC	Lysophosphatidylcholines

m/z	Mass-to-charge
Met-SO	Methionine sulfoxide
MFD	Milk fat depression
MP	Metabolizable protein
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
MUFA	Monosaturated FA
NAD	Nicotinamide adenosine dinucleotide
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NPB	Negative protein balance
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
Orn	Ornithine
PC	Phosphatidylcholine
PC aa	diacyl-phosphatidylcholine
PC ae	acyl-alkyl- phosphatidylcholine
PCA	Principle component analysis
PE	Phosphatidylethanolamine
PLS-DA	Partial least squares discriminant analysis
PMR	Partial mixed ration
POLR2A	RNA polymerase II
PUFA	Polyunsaturated FA
RQUICKI	Revised Quantitative Insulin Sensitivity Check Index
S6K1	p70S6 Kinase 1
SCM	Significantly changed metabolites
SDMA	Symmetric dimethylarginine
SM	Sphingomyelin
SM OH	Hydroxysphingomyelins
STAT5	Signal transducer and activator of transcription 5
T2DM	Type 2 diabetes mellitus
*t*4-OH-Pro	*trans*-4-hydroxyproline
TAG	triacylglycerol
TCA	Tricarboxylic acid
UCP	Uncoupling protein
UPS	Ubiquitin proteasome system
VIP	Variable importance of projection
VLDL	Very low-density lipoproteins

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1. Introduction

1.1. Negative protein balance during the transition period

The growing global demand for milk and dairy products drives the selection of cows for high milk yields. A significant portion of the nutrients to support milk production comes from the mobilization of body reserves, i. e., adipose tissue (**AT**) and skeletal muscle, as dry matter intake (**DMI**) is suppressed in early lactation. Pursuit of high production levels is pushing cows to mobilize more body reserves and thus widens the gap between energy intake and requirements. Modern dairy cows are thus faced with more drastic and abrupt metabolic fluctuations (Drackley et al., 2005) that impose a risk for metabolic disorders during the transition from late pregnancy to lactation which in turn will impair production profitability.

The transition period, encompassing 3 weeks (**wk**) before and after calving, is characterized by extensive physiological and metabolic changes (Drackley, 1999). A successful and smooth transition period is crucial for health and performance, and thus profitability. The nutritional demands of the fetus increase exponentially during late gestation whereas feed intake declines during the last wk of gestation (NRC, 2001; Hayirli and Grummer, 2004), resulting in reduced nutrient intake. As a consequence, cows enter a negative nutrient balance which triggers a coordinated change in metabolism among multiple organs necessary to support the increased demands for nutrients and energy, also designated as homeorhetic adaptation (Bauman and Currie, 1980; Casey et al., 2009). Most research dealing with dairy science was focused on the negative energy balance (**NEB**), however, the negative protein balance (**NPB**) was hardly investigated.

Formulating diets to meet the requirements of dairy cows for metabolizable protein (**MP**) during early lactation is very challenging, because dairy cows are unable to consume enough dry matter (**DM**) and protein to meet the mammary and non-mammary amino acid (**AA**) requirements. The protein requirements of dairy cows during the transition period are classically considered to comprise the needs for maintenance, body growth, and lactation (NRC, 2001), but the potential loss of body protein to support fetal requirements has not been taken into consideration. The body`s protein pool is usually supported by intestinal absorption of MP, and may also be repartitioned between different organs, whereby skeletal muscle forms the largest depot. The calculated protein balance of high-yielding dairy cows was demonstrated to decrease to a nadir of -600 g MP/d at 7 d post partum, thereafter it increased to reach a zero balance around 23 d

post partum. The authors (Bell et al., 2000) further estimated that the cows may need to mobilize around 1000 g tissue protein per day to meet the needs for lactation during the first 7 – 10 d post partum.

When cows are in NPB, protein mobilization from endogenous tissues (e.g. skeletal muscle and visceral tissue) is necessary to provide AA for milk protein synthesis and protein synthesis in other organs, for direct oxidation as well as for hepatic gluconeogenesis. However, excessive mobilization can be associated with increased incidence of metabolic disorders, immune dysfunction, and poor lactation and reproductive performance (Drackley, 1999). The breakdown of muscle protein is associated with the release of 3-methylhistidine (**3-MH**; formed by the posttranslational methylation of peptide-bound histidine) which can be used as an indicator to characterize mobilization of muscle protein. Earlier studies have shown that protein mobilization rate from muscle increased from 3 wk ante partum to parturition, and then decreased until 4 wk post partum (Doepel et al., 2002). Further studies including assessment of 3-MH in and thickness of *M. longissimus* evidenced that protein mobilization starts before parturition, and interestingly even before lipid mobilization (van der Drift et al., 2012). Moreover, the latter authors observed a large variation in protein mobilization of cows kept under similar conditions, likely due to the individual differences in milk production, feed intake, metabolic adaptation to NEB, or ante partum fat and muscle thickness (van der Drift et al., 2012). The observation that protein mobilization occurred before parturition and in advance of fat mobilization indicates that protein mobilization might result from a pre-partum AA deficiency rather than just resulting from NEB (van der Drift et al., 2012). Besides the needs of lactation, AA released from muscle protein breakdown are also used for hepatic gluconeogenesis and thus limit the generation of ketone bodies (Bell et al., 2000; Schäff et al., 2013). Larsen and Kristensen (2013) estimated that glucogenic AA derived from muscle proteolysis are primarily utilized for milk protein synthesis. They concluded that only alanine is likely to contribute to the liver`s release of glucose through its role in the inter-organ transfer of nitrogen from catabolized AA (Larsen and Kristensen, 2013). They challenged the dogma that AA are significant contributors to hepatic gluconeogenesis in early lactation. Using a proteomics approach, Kuhla et al. (2011) reported that muscle breakdown products in early lactation support hepatic gluconeogenesis and milk production, and also provide signals regulating feed intake.

1.2. Skeletal muscle

1.2.1. Structure of skeletal muscle

Skeletal muscle, the largest internal organ in mammals, is the main labile source of AA in the body and metabolically very active in regulating systemic metabolism (Baskin et al., 2015; Lindstedt, 2016). For instance, skeletal muscle is accounting for more than 80% of insulin-stimulated glucose disposal and thus is quantitatively one of the most important sites of insulin action (Patti and Corvera, 2010).

Skeletal muscle comprises several components including muscle fibers, connective tissues, motor nerves, and blood vessels. The cytoplasm of muscle fiber is called sarcoplasm, containing cellular organelles such as nuclei, mitochondria, endoplasmic reticulum (sarcoplasmic reticulum), proteasome and other cytosolic proteins. Muscle fibers (myofibrils), known as muscle cells, are the basic units of skeletal muscle. Within each muscle fiber, the sarcomere is a contractile unit, consisting of repeated units of interlocking thick myosin and thin actin myofilaments. The myosin is at the center of the sarcomere, whereas the actin attaches to a Z disc at the end of the sarcomere (Frontera and Ochala, 2015). As the myosin and actin compose most of the muscle proteins, any loss or modification of these proteins would affect muscle size, and probably leading to muscle atrophy and impaired muscle function. The degradation products from myosin and actin (e. g., 3-MH and 14-kDa actin fragment) (Du et al., 2004) could thus in turn reflect changes in skeletal muscle homeostasis.

1.2.2. Muscle homeostasis: turnover of muscle protein

Homeostasis in muscle is maintained through the dynamic turnover between protein synthesis and degradation. Briefly, protein synthesis is mainly controlled via several pathways whereby the mammalian target of rapamycin (**mTOR**) and the ubiquitin proteasome system (**UPS**) are considered as the major regulators of protein synthesis and protein degradation, respectively (McCarthy and Esser, 2010). The mTOR complex 1(**mTORC1**) is a crucial signaling node that integrates environmental cues (e. g., growth factors, stress, AA, and energy) and promotes protein synthesis largely through the phosphorylation of two key effectors, p70S6 Kinase 1 (**S6K1**) and eIF4E Binding Protein (**4EBP1**) (Saxton and Sabatini, 2017). The UPS accounts for the main proteolytic pathway in muscle, through which proteins are selectively targeted for

degradation by the 20S proteasome following covalent modification with ubiquitin (Bilodeau et al., 2016). Both of these pathways are associated with and could be influenced by insulin. The mTOR pathway can not only be regulated by insulin, insulin-like growth factor 1 (**IGF-1**), and glucose, but also plays a key role in mediating insulin resistance (Liu et al., 2012; Yoon, 2017). Studies on muscle wasting indicate that insulin resistance accelerates the UPS thereby causing muscle protein degradation (Wang et al., 2006). Thus, it is conceivable that muscle homeostasis could influence whole-body insulin action.

1.2.3. The role of muscle in systemic insulin resistance

The aforementioned homeorhetic adaptations ensure energy and nutrient partitioning towards the mammary gland in the early lactation period. Decreased plasma insulin, IGF-1, leptin, and thyroid hormone, and increased plasma growth hormone (**GH**), cortisol, glucagon and catecholamine post partum support elevated endogenous glucose production and delivery of glucose and non-esterified fatty acid (**NEFA**) to the mammary gland (Knegsel et al., 2014). For instance, increased GH concentrations post partum exert lipolytic effects and thereby increase plasma NEFA concentrations (Houseknecht et al., 1995; Contreras et al., 2017). Meanwhile, GH also promotes hepatic gluconeogenesis for providing glucose needed for synthesizing milk lactose (Knapp et al., 1992). Growth hormone also modulates hepatic IGF-1 production via the Janus kinase 2 (**JAK2**)/signal transducer and activator of transcription 5 (**STAT5**) signaling pathway, and promotes the utilization of NEFA in the mammary gland and in muscle by increasing blood flow (Renaville et al., 2002). Furthermore, glucose uptake by the muscle is reduced (Bell and Bauman, 1997; Spachmann et al., 2013) and skeletal muscle turns into the main site of oxidation of fat-derived fuels (Schäff et al., 2013).

Skeletal muscle is a main contributor to whole-body glucose metabolism and its insulin sensitivity is reduced around parturition (De Koster and Opsomer, 2013). Dysregulation of muscle metabolism has been shown to be related with changes in insulin sensitivity (Turcotte and Fisher, 2008; DeFronzo and Tripathy, 2009; Collins-Hooper et al., 2015). Reduced insulin sensitivity is a metabolic state in which peripheral tissues, such as skeletal muscle, are less responsive to the anabolic effects of insulin (De Koster and Opsomer, 2013). In humans, the role of insulin resistance in muscle has been under investigation in subjects with type 2 diabetes (**T2DM**) (Collins-Hooper et al., 2015; Perry et al., 2016). The importance of FA and FA-derived

metabolites in the development of insulin resistance was addressed (Blaak, 2003, 2004, 2005). The severity of insulin resistance is linked with accumulation of intramuscular lipids (Goodpaster et al., 2001), including ceramides, diacylglycerols, and FA-CoAs (Chavez and Summers, 2003; Adams et al., 2004; Bell et al., 2006; Pickersgill et al., 2007; Galadari et al., 2013). The underlying mechanisms are not entirely clear yet (Koves et al., 2008), but it has been proposed that lipid accumulation might be due to decreased rates of FA oxidation (**FAO**), increased rates of FA uptake, or both in insulin-resistant muscle (Turcotte and Fisher, 2008).

1.2.4. Fatty acid oxidation in muscle

The NEFA enter skeletal muscle cells through passive diffusion over the plasma membrane or via transport proteins such as the membrane-bound FA binding protein (**FABPpm**), the FA transport proteins (**FATP**), and the FA translocase CD36 (**FAT/CD36**) in the plasma membrane (Bonen et al., 1998; Turcotte and Fisher, 2008; Holloway et al., 2010; Jeppesen et al., 2011). Once inside the cell, FA are reversibly bound to the abundantly expressed cytoplasmic FA-binding protein (**FABPc**), which protects against the lipotoxic accumulation of free FA and traffics them throughout cellular compartments (Watt and Hoy, 2012). Thereafter, FA are activated as acyl-CoA via acyl-CoA synthetase (**ACSL**), bound to the acyl-CoA-binding protein (**ACBP**) to transporting in the cytosol for the final generation of energy via ß-oxidation.

The ß-oxidation occurs in both mitochondria and peroxisomes. The mitochondria catalyze the ß-oxidation of short, medium, and long-chain FA, while the peroxisomes are responsible for special FA including very long-chain FA (> C22), and branched FA (Reddy and Hashimoto, 2001). In mitochondria, acyl-CoA is fully degraded to acetyl-CoA. However, the final products in the peroxisomal ß-oxidation are short or medium-chain acyl-CoA which are transferred to the mitochondria for complete ß-oxidation (Reddy and Hashimoto, 2001; Schrader et al., 2015).

In dairy cows, plasma FA are mainly composed of saturated long-chain FA including palmitate (C16:0) and stearate (C18:0), and oleic acid (C18:1n9c) as a monounsaturated FA (Leroy et al., 2005; Tyburczy et al., 2008). For the mitochondrial generation of energy from long-chain FA, they should be transported from the cytoplasm into the mitochondrial matrix across the mitochondrial membranes as acylcarnitines (**ACC**) through a carnitine-dependent transport shuttle (McGarry et al., 1978). This system (Figure 1) is made up of two separate proteins

located in the outer (carnitine palmitoyltransferase 1; **CPT1**) and inner (carnitine palmitoyltransferase 2; **CPT2**) mitochondrial membranes. While CPT2 is a ubiquitous protein, the CPT1b is the major isoform that exists in muscle (Flanagan et al., 2010). The carnitine shuttle is closely associated with FAO efficiency (Bruce et al., 2009; Morash and McClelland, 2011; Qu et al., 2016). However, most studies performed in this regard were focusing on liver (Mizutani et al., 1999; Dann and Drackley, 2005; Li et al., 2017). Once inside the mitochondria, carnitine and long-chain acyl-CoA are regenerated by CPT2, and can then be further oxidized for ATP production through mitochondrial β-oxidation and the tricarboxylic acid (**TCA**) cycle (Flanagan et al., 2010). Taken together, the carnitine shuttle is a relevant step to regulate mitochondrial FAO in skeletal muscle.

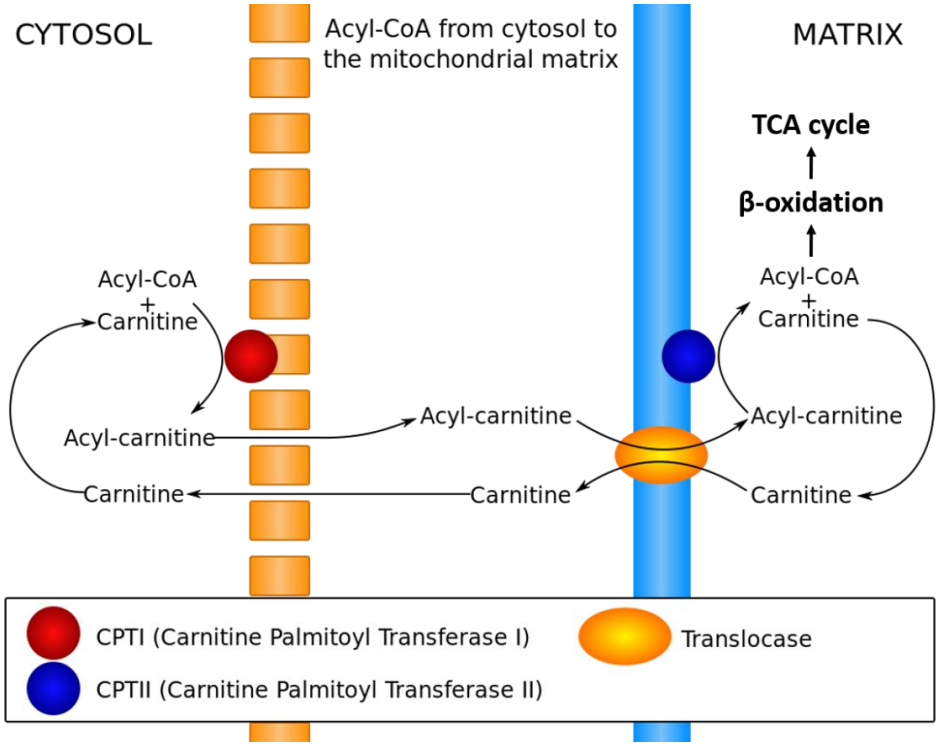

Figure. 1. A schematic pathway illustrating the carnitine shuttle (Amended from *https://en.wikipedia.org/wiki/Carnitine-acylcarnitine_translocase#/media/File:Acyl-CoA_from_cytosol_to_the_mitochondrial_matrix.svg*).

1.3. Conjugated linoleic acids

Conjugated linoleic acids (**CLA**) are a family of stereo and positional isomers of linoleic acid (**LA**, C18:2) with conjugated double bonds (C18:2n-6). For CLA there are several health claims including anticarcinogenic and antiatherogenic effects, immune modulation, and changes in body composition (Chouinard et al., 1999; Pariza, 2004). Ruminant fats, particularly dairy products, are the major dietary source of CLA for humans. The CLA are synthesized as an intermediate through the microbial biohydrogenation of dietary LA in the rumen or are synthesized endogenously from *trans*-11 C18.1 by Δ^9-desaturase (Kelly et al., 1998; Griinari et al., 2000). The CLA include 28 known isomers, the most abundant natural isomer (70-90%) is the *cis*-9,*trans*-11 (***c*9,*t*11**), whereas another predominant isomer, *trans*-10,*cis*-12 (***t*10,*c*12**) is known of its dose-dependent milk fat depression (**MFD**) effect (Baumgard et al., 2001; Bauman et al., 2008; Bauman et al., 2011). The CLA can also be industrially produced by alkaline isomerization from LA rich vegetable oils (Koba and Yanagita, 2014). Commercially available CLA supplements are usually provided as the equal mix of aforementioned 2 isomers.

1.3.1. Use of CLA in early-lactation dairy cows

The use of CLA supplements (mainly *t*10,*c*12 CLA) for dairy cows is aiming at a suppression of milk fat synthesis during early lactation (de Veth et al., 2004). The dosage-dependent MFD effect of *t*10,*c*12 is well described by Baumgard et al. (2001), who reported a curvilinear reduction of milk fat yield (25, 33 and 50%, respectively) with increasing quantities of abomasal infusion *t*10,*c*12 CLA (3.5, 7.0, and 14.0 g/d, respectively). Later feeding studies with varying supplementation lengths (i. e. from 7 to 140 d) evidenced that CLA could be used as an MFD agent in mid-lactation cows (Perfield et al., 2002; Peterson et al., 2003; de Veth et al., 2005; Moore et al., 2005; de Veth et al., 2006; Kay et al., 2007). Feeding CLA starting ante or post partum with different dosages (i. e. from 2.4 to 15 g/d *t*10,*c*12 CLA) resulted in decreased milk fat content and yield (Moore et al., 2004; Castaneda-Gutierrez et al., 2007; Odens et al., 2007; Pappritz et al., 2011; von Soosten et al., 2011). Two other isomers, *t*9,*c*11 and *c*10,*t*12 CLA, were also reported to suppress milk fat content (Saebo et al., 2005; Perfield et al., 2007). The molecular mechanisms of CLA-induced MFD include down-regulation of the mRNA abundance and (or) enzyme activity of lipid synthesis-related enzymes in the mammary gland (Bauman et al., 2011; Han et al., 2012). The portion of FA synthesized *de novo* (< C16) in milk fat is

decreased with feeding *t*10,*c*12 CLA (Peterson et al., 2003; Pappritz et al., 2011; Hotger et al., 2013). Besides, the *t*10,*c*12 isomer inhibits *de novo* FA synthesis related genes in an *in vitro* model (Zhang et al., 2018). Therefore, the t10,c12 CLA-induced MFD is likely targeting *de novo* FA synthesis in mammary gland.

The energy requirements for milk fat synthesis account for one-half of the energy requirement for milk production, therefore supplementation with CLA could be used to save energy and reduce the degree of negative energy balance. Supplementing 178 g/d CLA (29% *t*10,*c*12) in mid-lactating cows resulted in greater net energy balance (**EB**) when compared with cows receiving a CLA-free and 22 g/d (29% *t*10,*c*12) CLA supplement (Sippel et al., 2009). An improved EB status was observed in CLA fed lactating goats (10 g/d, 29.9% *t*10,*c*12; Baldin et al., 2013). However, the effects of CLA supplementation on EB in early lactation are inconsistent: A large dose of CLA (600 g/d, 5.6% *t*10,*c*12) resulted in an improvement of EB (Odens et al., 2007), but in many other studies either no or negative effects on the EB post partum were demonstrated (Castaneda-Gutierrez et al., 2007; Hötger et al., 2013; Schäfers et al., 2017). Cows receiving CLA showed a more severe EB during early lactation resulting from decreased DMI (Pappritz et al., 2011). Besides, the evidence for CLA-induced improvement of EB status, and related parameters is limited (Oliveira et al., 2018).

An explanation for insignificant CLA effects on energy metabolism in early lactation is that CLA induced MFD increases the available energy that can be repartitioned towards milk or milk protein synthesis when NEB occurs (Bauman et al., 2008). Supplementing cows with 50 g/d CLA (9% *t*10,*c*12) from 2 wk before to 9 wk after parturition was associated lower endogenous glucose production, pointing to a CLA-related improvement of whole-body energy utilization efficiency that enables less glucose utilization for milk fat synthesis (Hötger et al., 2013). Feeding 7.6 g/d each of the *t*10,*c*12 and the *c*9,*t*11 CLA isomer resulted in an attenuated postpartal increase of serum adiponectin thus acting towards prolongatig the peripheral insulin resistance and drain of nutrients towards the mammary gland (Singh et al., 2014). Likewise, a repartitioning of energy to AT was also observed (Harvatine et al., 2009; von Soosten et al., 2012), suggesting a more efficient utilization of metabolizable energy. Indeed, the CLA-induced nutrient repartitioning effect is affected by the dosage, the formulation (e. g., rumen-protection) as well as the time and duration of treatment.

1.3.2. Effects of CLA in skeletal muscle

Only few studies addressed the effects of CLA treatment on skeletal muscle in dairy cows (von Soosten et al., 2012; Kramer et al., 2013). One of the well-known effects of CLA is to modulate of body composition by reducing body fat and/or increasing lean body mass (Steck et al., 2007; Halade et al., 2009; Lehnen et al., 2015), suggesting that CLA could targets skeletal muscle metabolism. CLA supplementation promoted muscle FAO by upregulating expression of uncoupling protein (**UCP**), a group of mitochondrial inner membrane proteins involved in the combustion of stored energy into heat (Busiello et al., 2015), and CPT1 mRNA in mice (Ryder et al., 2001; Zabala et al., 2006; Ribot et al., 2007). In addition, studies on murine skeletal muscle cells showed that both the *c*9,*t*11 and the *t*10,*c*12 CLA isomers could activate AMP-activated protein kinase (**AMPK**) signaling, the principle initial fuel and energy status sensing regulator (Qin et al., 2009; Mohankumar et al., 2012, 2013). The activated AMPK phosphorylates acetyl-CoA carboxylase and inhibits its activation by citrate, thereby suppressing the synthesis of malonyl-CoA, which inhibits CPT1 (Ruderman et al., 2003). As consequence of activating the carnitine shuttle, mitochondrial β-oxidation increases. Besides, AMPK could improve muscle insulin sensitivity by inducing glucose transporter 4 (**GLUT4**) (Habegger et al., 2012; Zachariah Tom et al., 2014). The *c*9,*t*11 CLA activated AMPK at lower concentration (around 50 μM), while the *t*10,*c*12 isomer activated AMPK in a dosage-dependent manner up to 120 μM, and then plateaued (Mohankumar et al., 2012). Thus, it is likely that the CLA profile in muscle is important in affecting AMPK signaling and downstream metabolic pathways.

1.4. Metabolomics, a system approach for research on dairy cows` physiology

Metabolomics is the large-scale study of small molecules (molecular weight less than 1500 Da), commonly known as intermediates and products of cellular metabolism, within cells, biofluids, tissues or organisms in order to achieve a systemic view of metabolic status (Cambiaghi et al., 2017). Collectively, these small molecules within a biological system are known as the metabolome. Since the last decades, metabolomics has widely been used for clarifying disease etiologies, characterizing metabolic signatures, and discovering novel biomarkers (Nordstrom and Lewensohn, 2010; Wishart, 2016; Trivedi et al., 2017). While transcriptomics, genomics, and proteomics indicate what might happen, metabolomics is aiming to identify what is happening in a system (Cambiaghi et al., 2017; Ceciliani et al., 2018). Thus metabolomics

provides a bridge in understanding the relations starting from DNA over mRNA and proteins to the functions of enzymes, their production of metabolites and the signaling exerted by phosphorylation and hormones — all in all to eventually understand the complexity of biology. To conduct a metabolomics study, the workflow consists of sample preparation, instrumental analysis, data acquisition and analysis, and biological interpretation (Brown et al., 2005; Cambiaghi et al., 2017). Different approaches and analytic platforms can be selected depending on the aim of the study, experimental design, and most importantly, the physical and chemical properties of the metabolome of interest.

1.4.1. Metabolomics platforms and approaches

In general, the metabolomics approaches can be divided into non-targeted and targeted metabolomics (Dunn, 2008; Cambiaghi et al., 2017). The non-targeted metabolomics is mainly discovery-based and is an unbiased method since it is attempting to capture all possible metabolites. It focuses on previously unknown information about how a system may respond to (patho-)physiological or environmental stimuli (i.e., healthy vs. diseased, supplemented vs. control, knockout vs. wild-type). The harvested spectra usually contain segments of 1000 compounds, often including unknow/unidentified ones. However, the resulting spectra are often hard to match with corresponding metabolites due to the following reasons: 1) the available libraries (e.g., Livestock Metabolome Database, LMDB, http://lmdb.ca/) that contain lesser metabolites (1070, as per 11. April 2018) than the estimated number of metabolites in the body (over 100,000). 2) Peak overlap and shift are common and thus make it hard to identify separate peaks (Alonso et al., 2015; Riekeberg and Powers, 2017).

In contrast, targeted metabolomics focuses on exact quantification and identification of a group of defined known compounds (usually in a specifically metabolic pathway) in the system driven by *a priori* hypothesis (Roberts et al., 2012). Accordingly, it is possible to optimize sample preparation and instrument analytical conditions based on the metabolome's features (e.g., polarity, molecular weight, functional groups), which in turn reduces the number of detected metabolites. A typical targeted metabolomics study may cover 200 compounds (e.g., the targeted metabolomic kits from Biocrates Life Sciences AG) with developed protocols for each group of compounds. Collectively, reproducibility, sensitivity and quantification performance are better in

the targeted metabolomics by selecting suitable analytical methods. A targeted metabolomics approach was performed for the current thesis.

The nuclear magnetic resonance spectroscopy (**NMR**) and mass spectrometry (**MS**) are the major analytical methods to characterize the metabolome. The principle of them has been reviewed in detail (Darbeau, 2006; Aksenov et al., 2017). Briefly, the signal of NMR is based on the spin of an atomic nucleus. An NMR spectrum can provide a great deal of information about the chemical structure of organic compounds. ^{1}H-NMR spectroscopy is most commonly applied in metabolomics research, since hydrogen is naturally abundant and is present in almost all organic molecules. NMR is a rapid, quantitative, highly reproducible, and non-destructive method that enables direct measurement on the collected samples or even *in vivo* (Pfeuffer et al., 1999). In fact, samples for the NMR assay can be stored for a long time, and they are usually analyzed with minimum or even without sample preparation (Le Gall, 2015). These features have made NMR a promising platform for large-scale, high-throughput non-targeted metabolomics studies. However, the lower sensitivity (e.g., heavily overlapping peaks) of traditional one dimensional (**1D**)^{1}H-NMR compared with other methods (e.g., MS, two dimensional ^{1}H-NMR), limits its use in analyzing low-abundance metabolites (Pan and Raftery, 2007; Emwas, 2015; Giskeødegård et al., 2015).

Unlike NMR, the principle behind MS is to measure the mass-to-charge (***m/z***) ratio of ions at vacuum from ionized compounds through a magnetic field (Aksenov et al., 2017). In practice, the MS is usually integrated with other separation methods in order to achieve high selectivities, such as gas chromatography (**GC**) and liquid chromatography (**LC**) (Emwas et al., 2015; Zhao et al., 2015). In this manner, prepared samples are first separated in gas or liquid phase and subsequently are ionized via ion sources such as electron ionization (**EI**), chemical ionization (**CI**), and electrospray ionization (**ESI**) (Yin et al., 2015). In biology studies, ESI is a routine approach to analyze large molecules at low *m/z* values such as peptides. The yielded molecular ions, and smaller fragment ions can be detected by a mass analyzer, and then matching the resulting *m/z* with corresponding compounds in the database. To achieve accurate molecular identification, the selected products (also known as precursor ions) from the first MS can be further fragmented in a second MS to produce another group of ions (namely product ions) (tandem MS, also known as MS/MS or MS^{2}) (Finehout and Lee, 2004).

An advantage of GC-MS is the well-established MS spectra database for multiple biological species (e.g., National Institute of Standards Technology, NIST, https://chemdata.nist.gov/; (Riekeberg and Powers, 2017). This is particularly suitable for the analysis of volatile, thermally-stable, lower molecular weight metabolites (< 500 Da) (Emwas et al., 2015). Compared to GC-MS, LC-MS is a better choice for investigating higher molecular weight (100 - 2000 Da), non-volatile, and polar compounds (Zhao et al., 2015). The disadvantages of LC-MS compared with GC-MS are longer analysis times and lower separation efficiency (Yin et al., 2015). Currently, to capture a metabolome in a given condition, there is a growing trend of combining several of the aforementioned technologies in one study (Chen et al., 2014; Deng et al., 2016; Goldansaz et al., 2017).

1.4.2. Metabolomics data analysis: from processing to interpretation

Each metabolomics platform yields large and complex data sets compared with traditional lab assays, making data analysis procedures non-trivial, sophisticated, and time-consuming. A simplified workflow (Figure 2) on the acquired raw data (i. e., spectra or NMR data) in order to reveal alterations in specific metabolic pathways consists of preprocessing, data preparation and analysis, and functional interpretation (Brown et al., 2005).

Preprocessing of the data is generally defined as those steps to convert the instrument outputs into metabolite concentrations (Brown et al., 2005; Enot et al., 2011; Euceda et al., 2015). Briefly, this procedure includes noise filtering, retention time correction, peak detection and integration, and chromatogram alignment. Finally, spectra are matched with the respective compounds in public or private databases (Tautenhahn et al., 2012; Vettukattil, 2015).

After preprocessing the data, many approaches may be used to extract useful information from the data. However, statistical inference cannot be directly drawn from this set of data, because of the following reasons: 1) Missing values (are represented by the symbol NA, i.e., not available) are usually those compounds with concentrations below the limit of detection (Di Guida et al., 2016). 2) Variation from samples that are not related to experimental design, e.g., sample injection that needs to be corrected, and 3) Large differences in level and ranges of metabolites that may influence weight of the metabolites in the subsequent analysis model (van den Berg et

al., 2006). Thus, metabolomics data sets need to be "cleaned" or processed before performing any statistical analysis as reviewed in detail by van den Berg et al. (2006) and Yang et al. (2015).

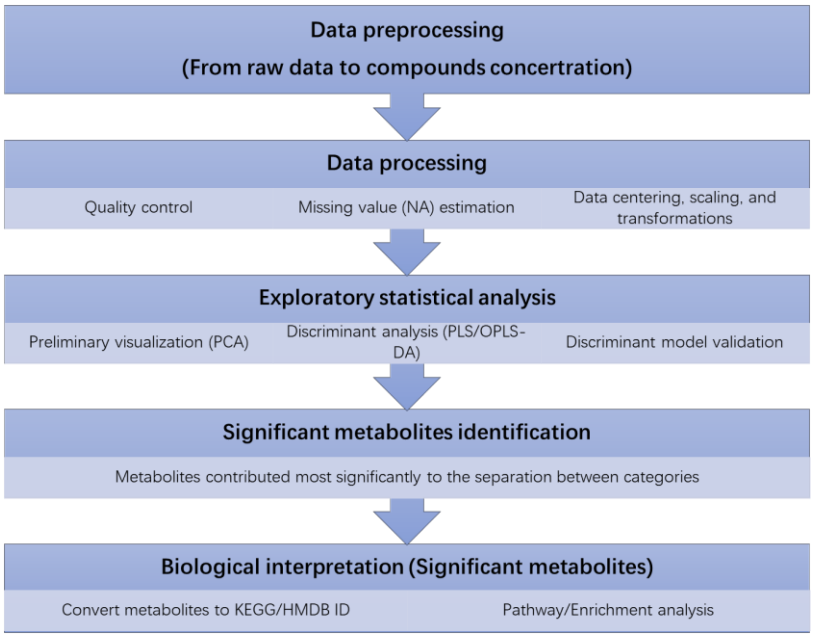

Figure. 2. Overall view of a simplified workflow of metabolomics analysis (mainly suitable for targeted metabolomics) from acquired raw data (i. e., spectra or NMR data) to interpretation.

The key challenge of analyzing -omics data set is the high-dimensionality, that is, the number of variables (***k***, compounds) is much larger than observations (***n***, experimental units) (Xia et al., 2013). In a typical metabolomics study, the *k* is usually several folds more than the *n*. This "large *k*, small *n*" problem, also known as "curse of dimensionality," increases the amount of data needed to support the results. It often grows exponentially with the dimensionality and consequently, increases the probability of type 1 errors (Saccenti et al., 2014). Another challenge is that the aim of a metabolomic study is usually describing the pattern of metabolites, i. e. a cluster of metabolites in the same pathway or a group of metabolites has similar functions, but not a single metabolite (Cambiaghi et al., 2017). Recently, many algorithms, even machine learning approaches have been introduced into metabolomics data analysis (Cuperlovic-Culf, 2018).

Currently, there is also interest in taken both univariate and multivariate approaches into consideration for analysis of metabolomics data. In the univariate analysis, such as t-tests,

ANOVA, and their correspondent non-parametrical forms like Kruskal-Wallis tests, each variable is considered separately in each time-point. However, the limitation of univariate approaches is that they do not take variable correlations or interactions into consideration (Saccenti et al., 2014). Thus, univariate methods might fail to discriminate between groups since uninformative variables might mask the difference when it is too small. Moreover, multiple testing corrections to compensate the "large *k* small *n*" problem might, in turn, increase the risk of false negatives.

Multivariate approaches, by definition, consider all variables simultaneously (Worley and Powers, 2013). The principle component analysis (**PCA**), partial least squares discriminant analysis (**PLS-DA**), and orthogonal projections to latent structures discriminant analysis (**OPLS-DA**) are commonly used approaches and are available as packages/toolboxes in open-source or commercial software (e.g., R, MATLAB, and the golden standard SIMCA) (Enot et al., 2011; Chokkathukalam et al., 2013; Wheelock and Wheelock, 2013; Xia et al., 2015; Spicer et al., 2017; Rodriguez-Martinez et al., 2018). In short, PCA extracts the dominant sources of variation without prior knowledge of data classification, also known as an unsupervised method. It is usually the first step, particularly for checking outliers and group distributions (Worley and Powers, 2013). In contrast, the PLS-DA and OPLS-DA are supervised methods which aim to describe the inherent patterns between the data (***X*** matrix, e.g., metabolites concentrations) with the response matrix containing response variables (***Y*** matrix, e.g., category, group), thereby finding a small set of metabolites that contributes most significantly to the separation between groups (Worley and Powers, 2013). The discriminate models need to be validated to ensure that the separation performance does not result from noise in the dataset, that is, by chance only (Triba et al., 2015). Once the discriminate models are validated, important metabolites could be selected based on their weights in contributing to the model separation (Xia and Wishart, 2016, Cambiaghi et al., 2017).

The final step in most of the metabolomics studies is interpretation of the data. Pathway analysis is performed using the selected most important metabolites to interprete biological function; that is, "translating" the changes in the concentrations of the metabolites into regulation of metabolic pathway(s). This procedure aims at describing the inter-relationship and interactions of these metabolites in context of the available knowledge frameworks. Relevant research questions are for instance: 1) How important are these metabolites in influencing a known pathway? 2) How

many metabolites are regulated in the given pathway? The selected metabolites from the last step are mapped and visualized in the pathway network, the commonly used public databases are KEGG, BioCyc, and Reactome (Xia et al., 2013; Xia et al., 2015; Xia and Wishart, 2016).

1.4.3. Application of metabolomics in research on dairy cows

Metabolomics approaches have been employed to reveal changes that occur in a biology system, and thereby facilitate and improve our understanding about rumen health, dietary changes, etiology of metabolic diseases, etc. They enable comprehensive identification and quantification of metabolites in biofluids and tissues. Most studies performed during the past years, have focused on blood, milk, urine and tissues like liver. However, there are only few studies in dairy cows that have employed metabolomics for the studying skeletal muscle (Kamila and Beata, 2016; Goldansaz et al., 2017).

The classical use of metabolomics is biomarker discovery for diagnosis and monitoring metabolic or disease status. Kenéz et al. (2016) demonstrated a notable shift of the serum metabolic phenotype from 42 d ante partum to 100 d post partum in dairy cows, pointing to disrupted lipid metabolism. The serum phosphatidylcholines (**PC**) have been reported as the most significant metabolites contributing to discriminate between healthy controls and cows with different stages of fatty liver (Imhasly et al., 2014). When comparing cows with different levels of lipolysis, PC, mainly those with diacyl-residues, sphingomyelins (**SM**) and ACC were found to be different between the groups (Humer et al., 2016). By using GC/MS, carbohydrates, FA, AA, even sitosterol and vitamin E isomers, etc. 2-piperidinecarboxylic acid and *cis*-9-hexadecenoic acid were identified to be closely associated with metabolic perturbations in ketosis (Zhang et al., 2013). By using ^{1}H-NMR, 25 plasma metabolites, including acetoacetate, acetone, lactate, glucose, choline, glutamic acid, and glutamine were identified to be different among healthy and ketotic cows (Sun et al., 2014a). These authors further reported potential biomarkers in the plasma for diagnosis of milk fever and fatty liver in dairy cows (Sun et al., 2014b; Xu et al., 2016). In addition, several metabolic pathways including Lys degradation, biotin metabolism, Tyr metabolism, urea cycle, Arg-Pro metabolism, protein biosynthesis, Met metabolism, phospholipid biosynthesis, Val-Leu-Ile degradation, betaine metabolism, Asp metabolism, His metabolism, and β-Ala metabolism were found to be perturbed in cows with ketosis during the onset and progression of the disease (Ametaj et al., 2016).

Metabolomics is also applied to study changes in the metabolic status of animals in response to different treatments. Increasing the portion of barley grain in the diet (from 30% to 45% of DM) in dairy cows resulted in decreased rumen pH (i. e. below 5.8 during 6 – 12 h after feeding), accompanied by increases in the ruminal concentrations of potentially harmful metabolites such as methylamine and endotoxin; in addition, enhanced concentrations of glucose, alanine, maltose, propionate, uracil, valerate, xanthine, and phenylacetate were reported (Ametaj et al., 2010). Replacing corn stover with alfalfa hay in lactating cows was associated with elevation of 55, 8, 28, and 31 metabolites in rumen fluid, milk, serum and urine, respectively, pointing to the changes that occurred in glycine, serine, and threonine metabolism as well as tyrosine and phenylalanine metabolism (Sun et al., 2015). In the study conducted by Sundekilde et al. (2013), the increases in milk lactate, butyrate, isoleucine, acetate, and β-hydroxybutyrate, and the decrease in hippurate and fumarate were associated with the somatic cell count in milk. Moreover, the authors found several metabolites in milk that were associated with milk protein content and rennet-induced coagulation properties, which may be considered as quality markers for cheese milk (Sundekilde et al., 2014). In a targeted metabolomics study conducted in our group, a single-dose duodenal infusion of Leu in dairy cows affected multiple intermediary metabolic pathways including AA and energy metabolism (Sadri et al., 2017a). In another study conducted in our group, non-targeted metabolomics was performed for testing the effects of cinnamon supplement aiming to ameliorate metabolic stress in transition dairy cows. The data pointed lipolytic and ketogenic effects of cinnamon supplementation during the transition from late gestation to early lactation in dairy cows (Sadri et al., 2017).

2. Objectives

As mentioned in the introduction, it is clear that the adaptation to the onset of lactation puts high yielding dairy cows in metabolic stress. The skeletal muscle is not only the primary labile source of AA, but also plays important roles in regulating the systemic metabolic homeostasis. The muscle metabolome is considered as a promising object to understand the shifted networks; however, related studies were lacking. Additionally, a rate-limiting step of FA oxidation has been previously attributed to the transport of FA across the mitochondrial inner membranes via CPT1, which converts the long-chain acyl-CoA to long-chain ACC. Furthermore, supplementation with CLA in early lactation cows has been associated with attenuated NEB degree, and were reported to affect lipid metabolism. The effect of CLA on skeletal muscle is unclear. Therefore, the present dissertation was aimed to determine:

1). to characterize the serum and the skeletal muscle metabolome in context of metabolic changes occurring during the transition from late pregnancy to early lactation in dairy cows,

2). to determine changes in serum and muscle concentrations of ACC and mRNA abundance of muscle carnitine acyltransferases from late pregnancy to lactation, and

3). to test whether dietary supplementation with CLA altered these compared with control-fat supplemented cows.

3. Manuscript 1 (To be submitted)

Targeted assessment of serum and skeletal muscle metabolome of dairy cows during the transition from late pregnancy to early lactation

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Contributions

H. Sauerwein, H. Sadri, S.D. and J.R. conceptualized and designed the experiments. Y. Y. performed experiments, analyzed data and wrote the original draft. C.P. and J.A. performed metabolomics analyses. H. Sadri and H. Sauerwein supervised the analysis and edited the manuscript. All authors discussed the results and commented on the manuscript at all stages.

Competing interests

The authors declare no competing interests.

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ABSTRACT

The periparturient period in dairy cows is characterized by a complexity of metabolic processes required to maintain homeostasis. Skeletal muscle, the largest internal organ in mammals, serves a variety of important roles in maintaining body homeostasis. The objective of this study was to characterize serum and skeletal muscle metabolome of dairy cows during the transition from late pregnancy to early lactation. The metabolome was characterized in serum and *M. semitendinosus* samples collected from 21 Holstein cows at days -21, 1, 21, and 70 relative to calving using a targeted quantitative metabolomics approach. Out of 188 metabolites, 80 and 52, respectively, in serum and muscle contributed most significantly to the separation among the 4 time-points. Furthermore, fatty acid oxidation, arginine metabolism, tryptophan metabolism, phosphatidylcholine and lysophosphatidylcholines metabolism, and sphingomyeline metabolism were found to be the most important metabolic pathways influenced by the transition from late gestation to early lactation. The altered metabolic pathways may reflect changed lipid metabolism, impaired insulin action, and increased inflammatory status in dairy cows around parturition. These data contribute towards an in-depth understanding of the multifaceted metabolic adaptation of dairy cows during the transition from late pregnancy to early lactation.

INTRODUCTION

The periparturient period in dairy cows, spanning 3 weeks before and after parturition, is characterized by extensive changes in metabolic, endocrine, and immune functions[1]. Reduced feed intake during the last 3 weeks of gestation and increased nutrient demand for milk synthesis at the onset of lactation commonly result in a negative nutrient balance[2]. The selection of dairy cows for milk yield has imposed even greater metabolic challenges and albeit the ability to cope with such challenges is increasingly considered by considering fitness traits in breeding strategies, the incidence of so called production diseases related to the metabolic stress has not substantially decreased[3]. Adaptive failure during the periparturient period may thus impair animal health and performance[3,4] whereby dysregulated lipid metabolism, impaired insulin action, and increased inflammation status are the main contributors[5-8]. Skeletal muscle, the primary labile source of amino acids (**AA**), plays important roles in regulating the systemic metabolic homeostasis[9]. Muscle protein mobilization seems to start even before parturation[10], in order to overcome negative nutrient balance through providing AA for milk protein synthesis, hepatic gluconeogenesis, and for the immune system[11,12]. Moreover, the oxidative capacity for fatty acids (**FA**) in skeletal muscle contributes to the reduction of the metabolic load imposed by lipolysis and the resulting increase of FA in the circulation and hepatic accumulation of FA[12,13] . The quantitative contribution of skeletal muscle to FA metabolism in dairy cows during the periparturient period is not known. We have recently reported elevated concentrations of muscle long-chain acylcarnitines around parturition pointing to increased FA β-oxidation which seems not to be entirely met by upregulation of the downstream metabolic pathways, such as the tricarboxylic acid (**TCA**) cycle and respiratory chain[14].

Metabolomics is a powerful tool for acquiring information from whole sets of low-molecular weight metabolites in a sample. Therefore, the metabolome, representing the terminal downstream product of the genome, transcriptome, and the proteome can provide a direct measure of physiological changes in dairy cows[15], e.g. in particular condition such as ketosis[16-18], heat stress[19], rumen health[20,21] and footrot[22]. A targeted metabolomics performed to characterize phenotypes of metabolic transition from late pregnancy to early lactation in dairy cows revealed that the highest ranked metabolites were related to the whole-body changes in FA oxidation, associated with altered patterns of glycerophospholipids, and sphingolipids[23]. In human studies, the muscle metabolome is considered as a promising object to understand the metabolic

networks such as bioenergetic status, glucose and FA metabolism[24-26]; however, there are only few studies performed in dairy cows to explore the metabolome in skeletal muscle[14,19].

As stated above, in the dairy cow, late gestation and early lactation are characterized by a complexity of metabolic processes required to maintain homeostasis whereby the role of skeletal muscle is less well investigated than is adipose tissue and liver. Thus, the objective of this study was to characterize the serum and the skeletal muscle metabolome in context of metabolic changes occurring during the transition from late pregnancy to early lactation in dairy cows. An overview of the experimental design and work flow of the data processing is shown in Fig.1.

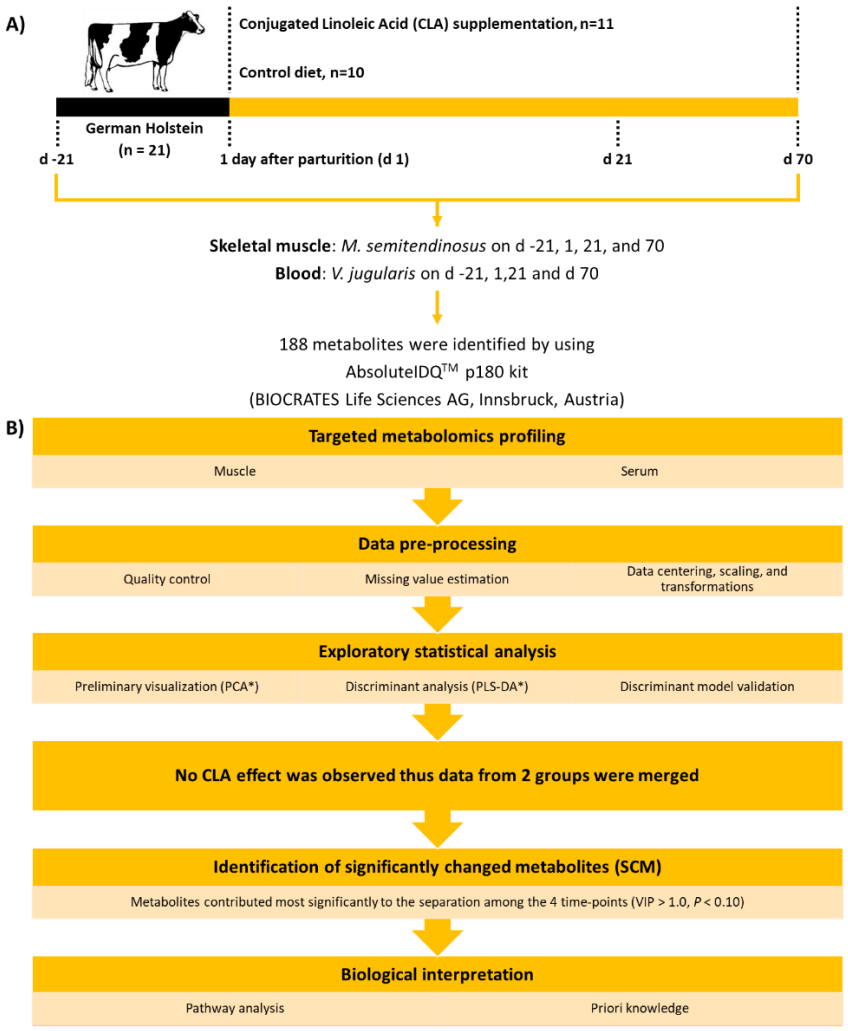

Figure 1. A) experimental design and B) flowchart of significantly changed metabolites (SCM) identification and data interpretation. PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in projection.

METHODS

Animal management and sample collection

All animal experiments were in accordance with the European Community regulations concerning the protection of experimental animals and the guidelines of the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany). The experimental design and zootechnical data were reported previously[27]. Briefly, twenty one pluriparous German Holstein cows, housed in a free stall barn, were fed ad libitum with a partial mixed ration (**PMR**) according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). The PMR (6.8 MJ of NE_L/kg of DM) consisted of 37.8% corn silage, 25.2% grass silage, and 37% concentrate (DM basis). From days in milk 1 to 182, the cows received either a dietary supplement, i.e., conjugated linoleic acids group (group **CLA**; n = 11) or a control fat supplement (group **CTR**; n = 10). The animals in the CLA group received 100 g/d encapsulated rumen-protected CLA (Lutrell Pure, BASF, Ludwigshafen, Germany) supplying 7.6 g of *cis*-9, *trans*-11 CLA and 7.6 g of *trans*-10, *cis*-12 CLA per day. The animals in the CTR group received 100 g/d of rumen-protected control fat supplement (Silafat, BASF) in which CLA was substituted by stearic acid to form an isoenergetic control diet using a FA with the same number of carbon atoms as in CLA. The supplements were provided with 4 kg of additional concentrate (8.8 MJ of NE_L/kg DM).

Muscle biopsies from *M. semitendinosus* were taken on d -21, 1, 21, and 70 relative to parturition. The biopsies were cut to pieces of about 25 mg each and weighted freshly. Afterwards the samples were snap-frozen in liquid nitrogen and stored at -80 °C until analysis. Blood samples were collected from a jugular vein on d -21, 1, 21, and 70 relative to parturition. Blood serum was prepared (1,500 × *g* at 4 °C for 20 min) and stored at -80 °C until analysis.

Estimation of insulin sensitivity

Data of serum non-esterified FA (**NEFA**), glucose, and insulin needed for the estimation of insulin sensitivity through calculating the "Revised Quantitative Insulin Sensitivity Check Index" (**RQUICKI**)[28] were reported elsewhere[27].

Targeted metabolomics measurements

The metabolome profiles in muscle and serum were determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) and flow injection-electrospray ionization-tandem mass spectrometry (FIA-ESI-MS/MS) analyses through a targeted metabolomics approach using the Absolute*IDQ*™ p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). The assay allows for the simultaneous quantification of 188 metabolites including free carnitine (**C0**), 40 acylcarnitines (**ACC**), 21 AA (19 proteinogenic + citrulline + ornithine [**Orn**]), 19 biogenic amines, hexoses (sum of hexoses – about 90-95% glucose; **H1**), 90 glycerophospholipids (76 phosphatidylcholines [**PC**] and 14 lysophosphatidylcholines [**lysoPC**]), and 15 sphingolipids. The abbreviations Cx:y are used to describe the total number of carbons and double bonds in lipid fatty acid chains, respectively. All analyses were performed in the Helmholtz Zentrum München, German Research Center for Environmental Health, Genome Analysis Center. In case of serum, 10 µL of the thawed sample have been applied directly to the assay. In case of muscle, frozen samples were homogenized and extracted using homogenization tubes with ceramic beads (1.4 mm) and a Precellys 24 homogenizer with an integrated cooling unit (PEQLAB Biotechnology GmbH, Germany). Using the measured weight of the fresh muscle samples, the appropriate volume of extraction solvent was calculated for each individual piece of tissue. To each mg of frozen muscle tissue were added 3 µL of a dry ice cooled mixture of ethanol/phosphate buffer (85/15 v/v). After centrifugation, 10 µL of the homogenate supernatant were applied to the well plate of the p180 kit. The assay procedures of the Absolute*IDQ*™ p180 Kit, the detailed description of the tissue preparation and the metabolite nomenclature have been described in detail previously[29,30]. Sample handling was performed by a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK), beside standard laboratory equipment. Mass spectrometric analyses were done on an API 4000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the Met*IDQ*™ software package, which is an integral part of the Absolute*IDQ*™ Kit. Internal standards were used as reference for the calculation of metabolite concentrations.

The concentrations of the serum samples were given in µM, the concentrations of the tissue samples in pmol/mg wet tissue and the concentrations of tissue homogenate in µM. The LOD was set to three times the values of zero samples (PBS for serum, ethanol/phosphate buffer for tissue homogenate).

Data processing and identification of significantly changed metabolites

Statistical analyses of the metabolomics data were performed according to previously published protocols using MetaboAnalyst 3.0[31,32]. As quality control, variables containing more than 50% missing values (i. e., values lower than LOD) were not considered for the statistical analysis. The *k*-nearest neighbors (**KNN**) algorithm was used to estimate the values of missing data in remaining variables. In the preliminary data mining, no treatment effects or its interactions with time were observed; thus, the data of 2 groups were merged (Supplemental Fig. 1). Data were generalized log (**glog**) transformed and then Pareto-scaled to correct for heteroskedasticity, to reduce the skewness of the data, and to reduce mask effects[33]. Multivariate approaches including principal component analysis (**PCA**), and partial least squares discriminant analysis (**PLS-DA**) were performed. The PCA, an un-supervised method, was used to visualize clusters and trends of the datasets. The PLS-DA, a supervised method, was conducted to perform classification and to identify those metabolites showing significant differences among the 4-time points. To validate class discrimination and to avoid overfitting of the PLS-DA model, 10-fold cross-validation and 2000 times permutation tests were performed. Variable importance in projection (**VIP**), representing the weighted sum of squares of the PLS loading, which takes the amount of orthogonal variance explained by each component into account, were used to rank the metabolites based on their importance (VIP score $\geq$ 1) in discriminating different time-points. Additionally, a one-way ANOVA with Tukey's HSD test was performed on the data to further confirm the significance of important metabolites identified using PCA and PLS-DA models. The threshold of significance was set at false discovery rate (**FDR**) $\leq$ 0.10. Those metabolites that were identified as significantly changed metabolites (**SCM**) in serum and muscle were manually classified into different metabolic pathway based on prior knowledge, PubChem (https://pubchem.ncbi.nlm.nih.gov/), Human Metabolome Database (http://www.hmdb.ca/), and KEGG (http://www.genome.jp/kegg/).

Correlation analyses

The Spearman's rank-order correlation was used to reveal correlations between muscle and serum metabolome, blood NEFA, glucose, insulin, as well as RQUICKI using PROC CORR of SAS, release 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

RQUICKI

As shown in Fig. 2, RQUICKI tended to decrease on d 1 as compared to d 21 (P = 0.106).

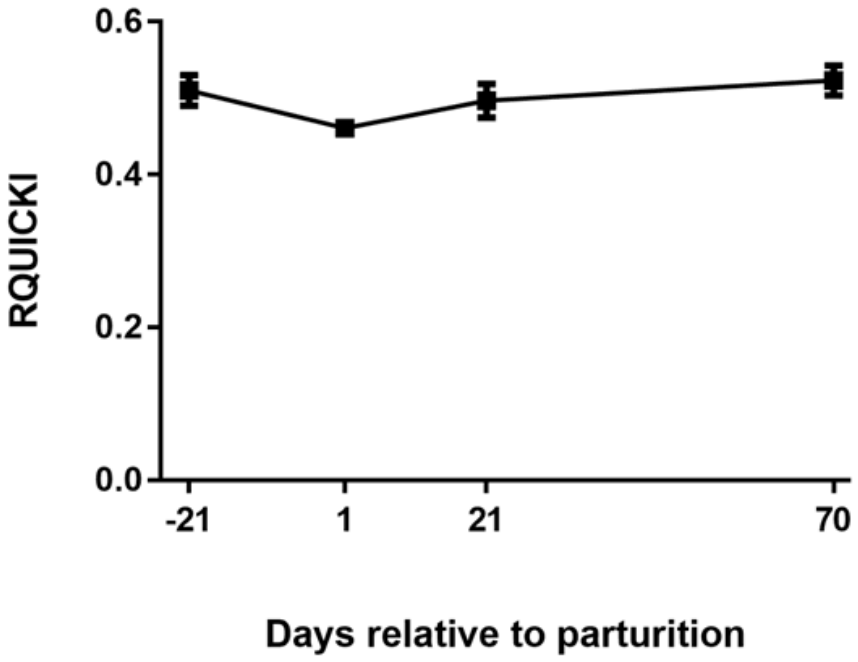

Figure 2. Time course of the estimated insulin sensitivity as measured by the revised quantitative insulin sensitivity check index (RQUICKI) in dairy cows during late gestation and early lactation (P = 0.106). Data are means ± SEM.

Muscle and serum metabolome

In our experimental setup we wanted to compare metabolomics signatures from intrinsic muscle metabolism and milk. In total, 184 and 176 metabolites in muscle and serum, respectively, passed the data quality check. The PCA score plots show clear separations between pre- and post partum periods in both muscle and serum metabolites (Supplemental Fig. 2A and B). The PLS-DA score plots of muscle and serum data again represented a clear separation among the 4 time-points. The 2 PLS-DA models identified 5 components with satisfactory modeling and predictive abilities around 80% (in case of both R^2 and Q^2; Supplemental Fig. 3A and C). To avoid model overfitting, 2000 times random permutation tests were performed (P = 0/2000; Supplemental Fig. 2B and D), indicating that the models were valid. Using the validated PLS-DA models (Supplemental Fig. 4A and B), a total of 52 metabolites in muscle and of 80 in serum that contributed most significantly (VIP $\geq$ 1.0; ANOVA $P \leq 0.10$) to the separation among the 4 time-points were identified and are presented in the Supplemental Table 1. Out of the SCM in muscle and serum, 31 metabolites were common between muscle and serum (Fig. 3A). Heatmap of the SCM in muscle and serum also depicted time-dependent alterations in muscle and serum metabolome (Fig. 3B and C).

Characterization of the key metabolic pathways

Five potential metabolic pathways were selected based on the SCM in muscle and serum. The metabolites involved in these potential metabolic pathways are presented as heat maps in Figs. 4-8. FA oxidation, arginine (**Arg**) metabolism, tryptophan (**Trp**) metabolism, PC and lysoPC metabolism, and sphingomyeline (**SM**) metabolism were found to be the most important metabolic pathways influenced by the transition from late gestation to early lactation.

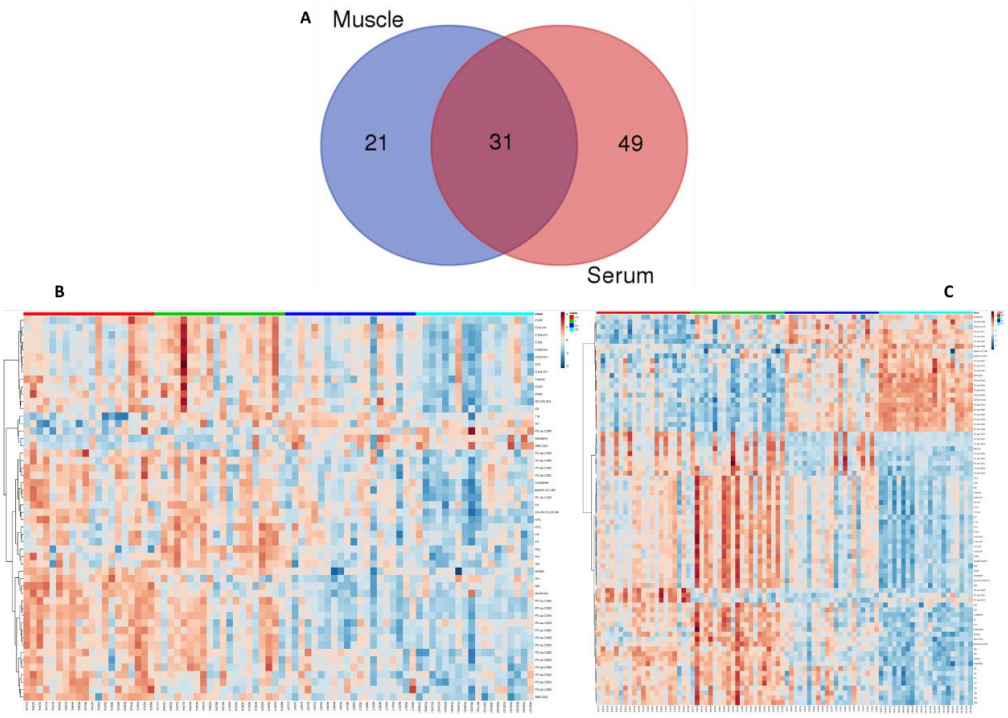

Figure 3. A) Venn diagram of significantly changed metabolites in muscle and serum. Patterns of muscle (B) and serum (C) significantly changed metabolites on d -21, 1, 21 to 70 days relative to parturition are shown in heatmap.

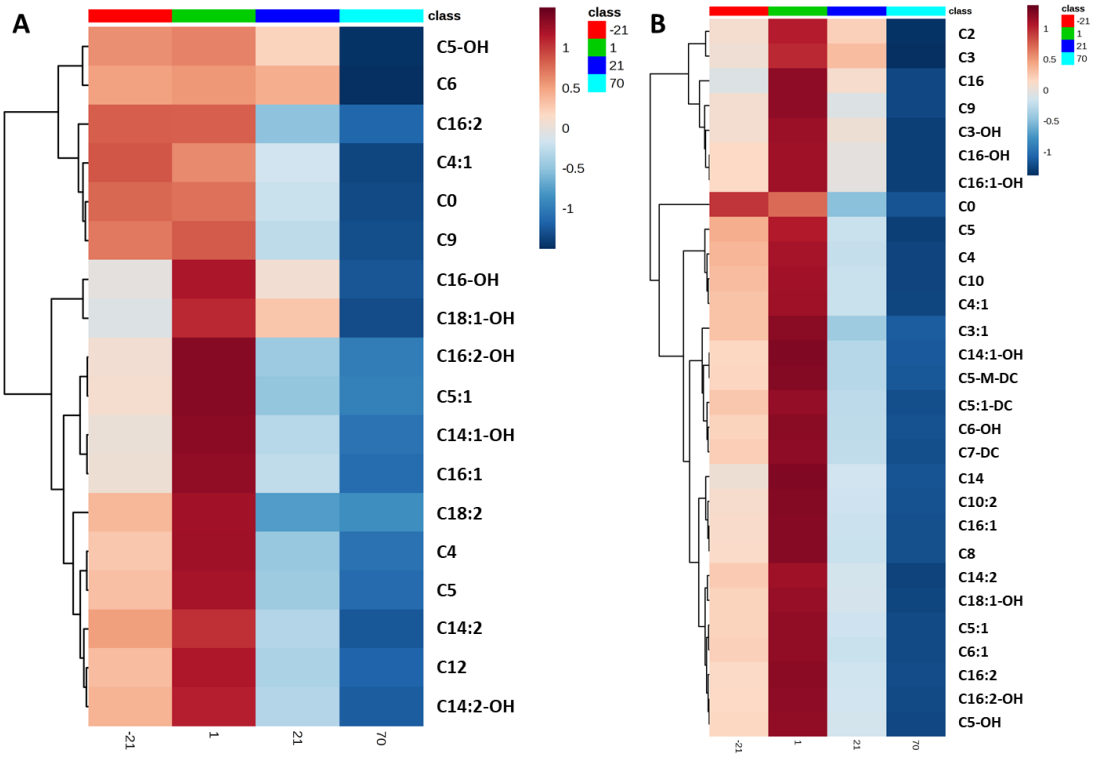

Figure 4. Heatmap of significantly changed metabolites related to carnitine shuttle in A) muscle and B) serum of dairy cows on d -21, 1, 21 to 70 days relative to parturition.

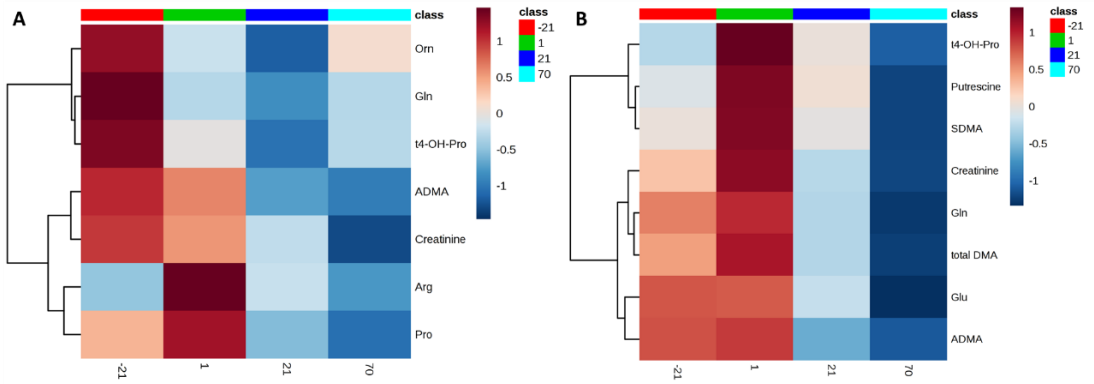

Figure 5. Heatmap of significantly changed metabolites related to arginine metabolism in A) muscle and B) serum of dairy cows on d -21, 1, 21 to 70 days relative to parturition.

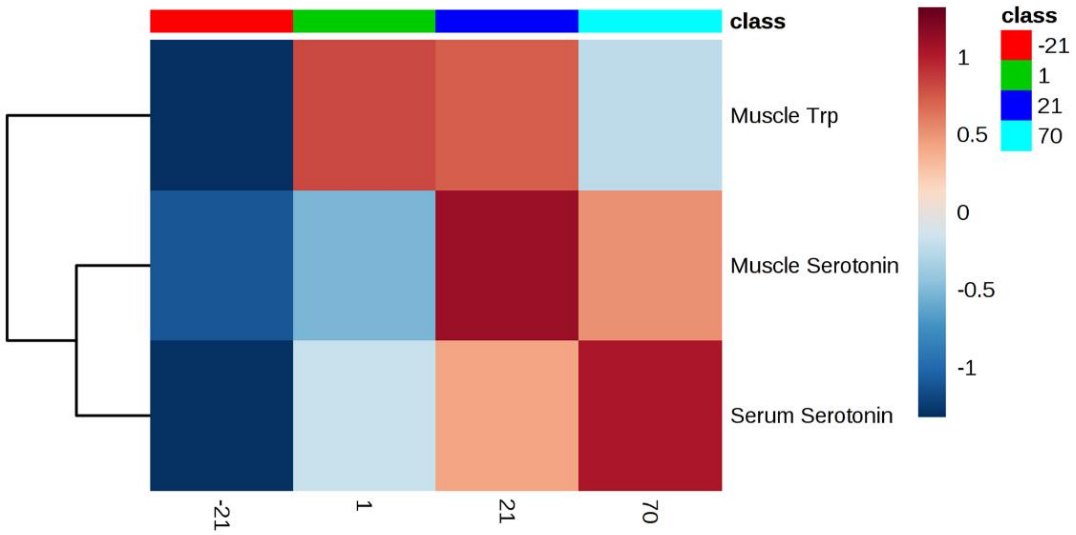

Figure 6. Heatmap of significantly changed metabolites related to tryptophan metabolism in muscle and serum of dairy cows on d -21, 1, 21 to 70 days relative to parturition.

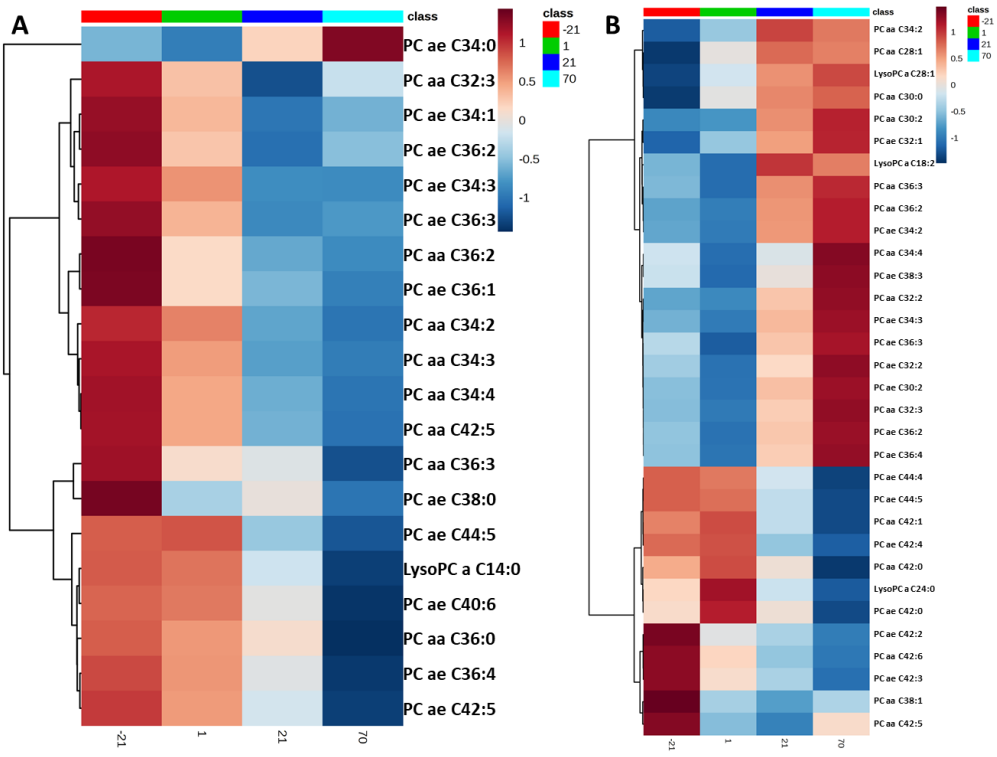

Figure 7. Heatmap of significantly changed metabolites related to phosphatidylcholine (PC) and lysophosphatidylcholines (LysoPC) metabolism in A) muscle and B) serum of dairy cows on d -21, 1, 21 to 70 days relative to parturition

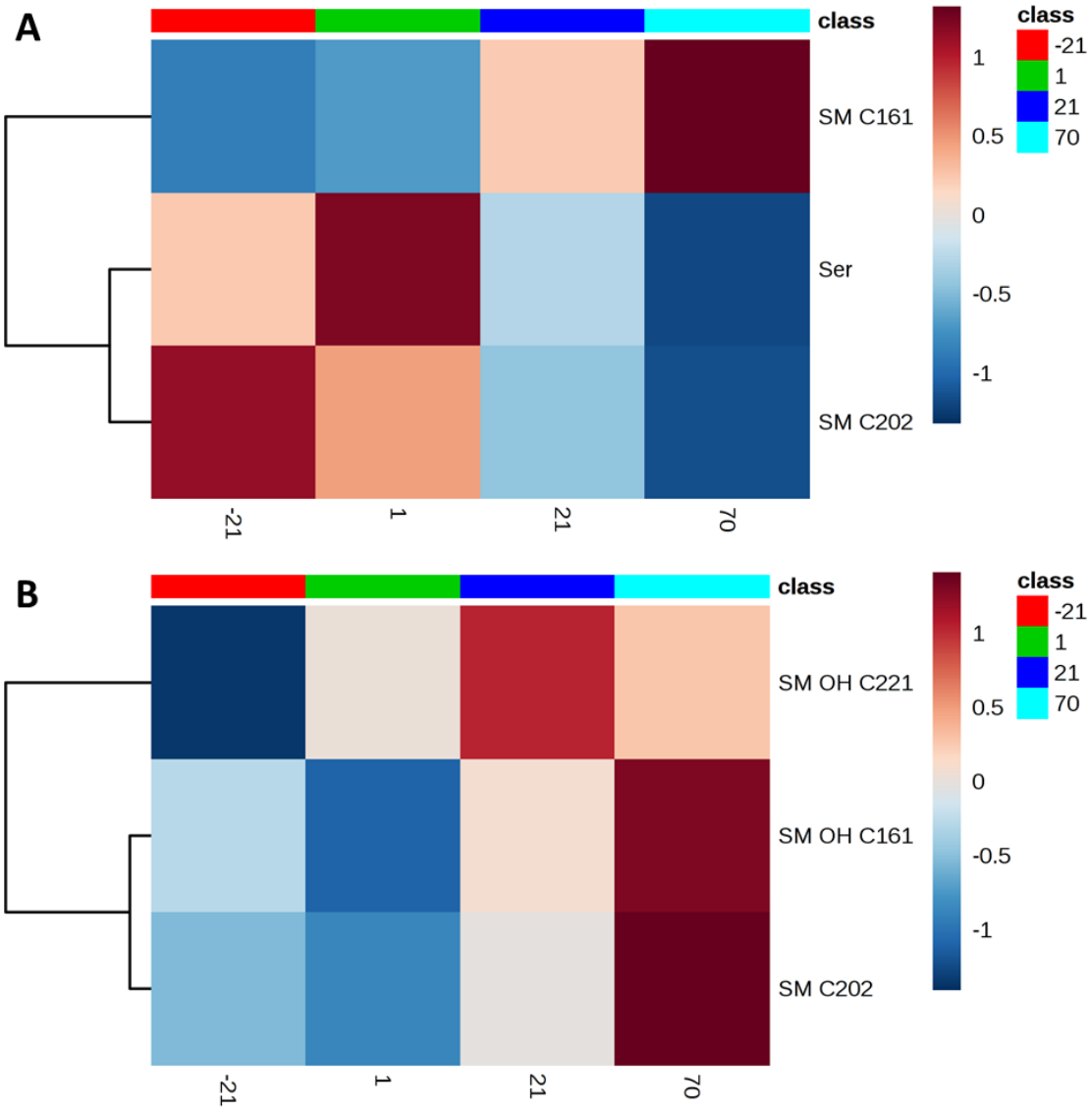

Figure 8. Heatmap of significantly changed metabolites related to sphingomyelins (SM) metabolism in A) muscle and B) serum of dairy cows on d -21, 1, 21 to 70 days relative to parturition.

Association of muscle and serum SCM with conventional metabolic parameters

In Fig. 9 the significant ($P \leq 0.10$, $|r| \geq 0.2$) spearman's rank correlation coefficients between blood serum parameters (NEFA, glucose, insulin, and RQUICKI) and identified SCM in muscle and serum are presented. Most of the muscle long-chain ACC (C14-C18) were positively correlated with NEFA and negatively with RQUICKI. Serum long-chain ACC (C14 and C16)

were positively correlated with NEFA and negatively with glucose, whereas serum short-chain ACC (C5) and C0 were correlated negatively with NEFA and positively with insulin. Serum acetylcarnitine (**C2**) was negatively correlated with RQUICKI.

Muscle long-chain (> C32) diacyl-phosphatidylcholines (**PC aa**) and acyl-alkyl-phosphatidylcholines ae (**PC ae**) were negatively correlated with NEFA and positively with glucose and insulin. Additionally, muscle PC ae C36:4 was negatively correlated with RQUICKI. Serum shorter-chain PC aa (C30 and C28:1) were found to be negatively correlated with serum glucose and insulin. Serum lysoPC a C18:2 along with lysoPC C24:0 were negatively correlated with NEFA and positively with RQUICKI. Muscle SM C20:2, was found to be negatively correlated with NEFA and positively with insulin.

Most of the metabolites related to Arg metabolism including muscle and serum asymmetric dimethylarginine (**ADMA**), glutamine (**Gln**), and muscle Orn, *trans*-4-hydroxyproline (***t*4-OH-Pro**) were negatively correlated with NEFA, and positively with glucose and insulin. In contrast, positive correlations were observed between muscle Arg, and serum creatinine and symmetric dimethylarginine (**SDMA**) with NEFA. Moreover, muscle Arg was negatively correlated with insulin and RQUICKI. There was a negative correlation observed between serum SDMA and glucose. Serum *t*4-OH-Pro was negatively correlated with insulin and glucose.

Muscle Trp and serotonin were positively correlated with NEFA and negatively with glucose and insulin.

Serum histidine (**His**), and tyrosine (**Tyr**) were negatively correlated with NEFA and positively with glucose. Serum Tyr was positively correlated with insulin. Serum Tyr and methionine sulfoxide (**Met-SO**) were positively correlated with RQUICKI. Muscle H1 was positively correlated with NEFA, and negatively with insulin. Muscle taurine was positively correlated with glucose and insulin but negatively with RQUICKI.

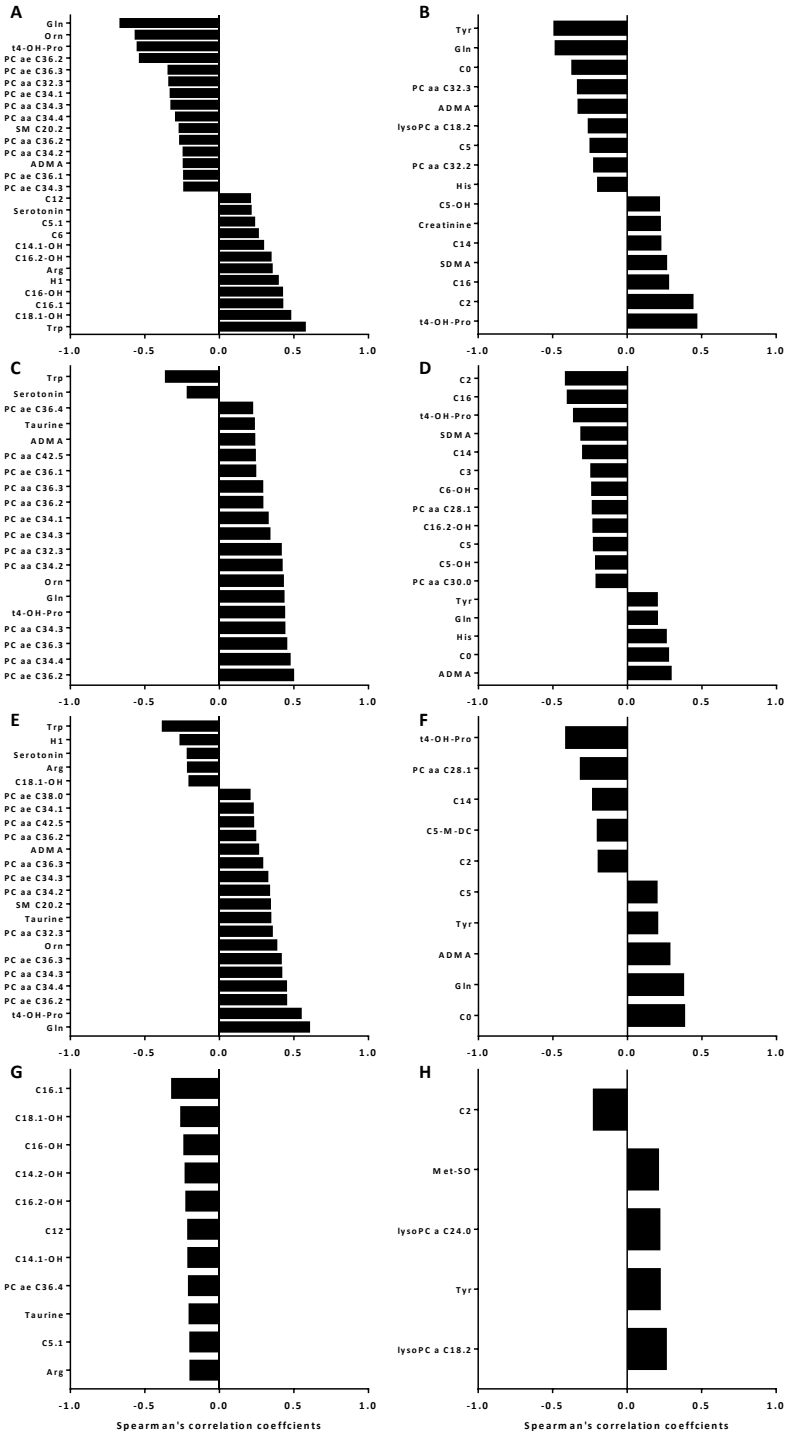

Figure 9. Correlations between muscle (A, C, E, G) and serum (B, D, F, H) metabolites with conventionally used parameters ($P < 0.10$). (NEFA: A and B; Glucose: C and D; Insulin: E and F; and RQUICKI: G and H). The bars shows spearman's rank correlation coefficients.

DISCUSSION

Carnitine and the majority of the ACC were identified as SCM in both muscle and serum. Accumulated concentrations of ACC in both muscle and serum, varying in carbon chain length, were observed around parturition and were associated with greater NEFA concentrations and a trend for reduced RQUICKI. In dairy cows, plasma FA is mainly comprised of saturated FA, including palmitate (C16:0) and stearate (C18:0), and oleic acid (C18:1n9c) as a monounsaturated FA[34,35]. For generating energy, long-chain FA need to be transported from the cytoplasm into the mitochondrial matrix across the mitochondrial membranes through a carnitine-dependent transport shuttle. Dysregulation of carnitine metabolism may lead to incomplete mitochondrial FA oxidation, resulting in accumulation of ACC, which may be associated with the development of insulin resistance[36-38]. Most lipid-derived ACC were elevated around parturition, which is consistent with the lactation-induced rise in circulating FA. Most long-chain ACC species in muscle were decreased from d 1 to d 21, with little or no changes afterwards, suggesting insufficient adaptation of their metabolism in response to the metabolic load of FA around calving (Fig. 4A and B). Taken together, in metabolic situations when FA availability is high, β-oxidation is elevated and/or glucose metabolism is impaired, depletion of several TCA cycle intermediates is likely, so that the mitochondrial status around parturition is compromised.

Arginine, metabolically interconvertible with the AA proline (**Pro**) and glutamate (**Glu**), serves as a precursor for synthesis of protein, nitric oxide (**NO**), creatinine, polyamines, agmatine, and urea. Metabolism of Arg through nitric oxide synthase (**NOS**) or arginase[39] regulates the biosynthesis of these physiologically important metabolites, pointing also to important pathophysiological events, such as insulin resistance and tissue regeneration. Dimethylarginine (**DMA**) consists of ADMA and SDMA. The ADMA is an endogenous inhibitor of NO synthase through displacing Arg from the substrate binding site. Increased circulating ADMA levels impair the endothelium-derived NO-mediated vasodilation[40]. In muscle, elevated ADMA inhibited glucose uptake, pointing to its association with insulin resistance[41]. Indeed, the circulating ADMA concentrations are related with insulin sensitivity[42]. This could be explained by the NO pathway as this pathway is inhibited in case of diabetic condition[43,44]. In the current study, the greater muscle and serum concentrations of ADMA observed on d -21 and d 1

compared to other 2 time-points (Fig. 5A and B), might reflect reduced insulin sensitivity in dairy cows around calving.

Creatinine is produced non-enzymatically from muscle creatine and phosphocreatinine and is excreted in blood proportional to muscle mass[45]. In the present study, the concentrations of creatinine in muscle and serum were higher on d -21 and 1 compared with the other time-points (Fig. 5A and B), suggesting that probably increased skeletal muscle degradation may have induced a decrease in muscle mass on d 21 and 70.

Polyamines (mainly putrescine, spermidine, spermine), mainly synthesized from L-Arg, are small aliphatic polycations. They are involved in many functions including oxidation, and autophagy, due to their natural affinity with negatively charged molecules such as DNA, RNA and proteins[46]. Elevated concentrations of polyamines were observed in muscle hypertrophy and regeneration, probably reflecting the stimulation of DNA synthesis and cell proliferation[47,48]. As the precursor of spermidine and spermine, putrescine was found to be associated with tumor necrosis factor, and consequently with inflammation[46]. In the current study, the concentrations of putrescine in serum increased from d -21 to d 1 (Fig. 5B), probably reflecting an inflammatory status in dairy cows during this period[6].

In addition to its indispensable role in protein synthesis, Trp is the precursor in two important metabolic pathways during the course of its degradation. In mammals, Trp is metabolized via 2 metabolic pathways including biosynthesis of serotonin and kynurenine. The major non-protein route of Trp metabolism that accounts for 95% of total body Trp metabolism is the kynurenine pathway, leading to the formation of nicotinamide adenosine dinucleotide (**NAD**). Kynurenine is the first stable intermediate that is formed in the kynurenine pathway[49,50]. The key enzymes of the kynurenine pathway are activated by the stress hormones or inflammatory factors[49,51,52], and consequently the metabolites derived from this pathway were reported to contribute to the development of insulin resistance[53]. The remaining portions of Trp are hydroxylated for the synthesis of serotonin. Serotonin is not only a key neurotransmitter but also a modulator of cell proliferation, insulin action, and cytokine production[54,55]. An imbalance of these 2 metabolic pathways have been shown to be involved in the development of insulin resistance such as type 2 diabetes mellitus (**T2DM**)[53,56]. In the current study, the increase in the Trp concentrations in muscle from d -21 to d 21 (Fig. 6) was accompanied by increased kynurenine/Trp ratios in serum. The kynurenine/Trp ratio is used for evaluation of the enzyme that catalyzes the conversion of

Trp into kynurenine[57]. The rising kynurenine/Trp ratio from d -21 to d 21 may thus suggest that less Trp was entering the serotonin pathway in favor of the kynurenine pathway around parturition. In turn, this is likely linked with the development of insulin resistance[53].

Phosphatidylcholines form the largest portion (around 50%) of the total cellular membrane phospholipids, and are important for membrane properties, cell integrity, and cellular signaling[58]. Manipulation of cellular membrane composition in muscle, especially the PC: phosphatidylethanolamine (**PE**) ratio was reversely associated with insulin sensitivity[59,60]. In the current study, muscle PC were predominantly decreased from d -21 to d 70 (Fig. 7A). In addition, muscle PC ae 36.4 was negatively correlated with RQUICKI (Fig. 9G); thus, likely reflecting a status of insulin resistance. Disruption of PC content might induce triacylglyceride (**TAG**) accumulation in liver and greater oxidative challenges[61,62]. The PC aa are essential for hepatic secretion of very low-density lipoprotein (**VLDL**), which is responsible for the TAG package and export from the liver, whereas PC ae also act as antioxidants in the circulation[61,63]. Previous studies have shown a perturbation in the serum PC and lysoPC patterns in dairy cows in response to excessive lipolysis[64,65] and lactation challenges[23,66]. Imhasly et al. (2014) observed a reduction in the longer PC ($\geq$ 36) in cows diagnosed with hepatic lipidosis. Likewise, Humer et al. (2016) reported a similar PC perturbation, i.e., PC with more than 40 carbons were reduced in cows undergoing extensive lipolysis cows in the early post partum period. The authors of the aforementioned study speculated that the longer PC might protect from high lipid mobilization via a currently unknow mechanism[64]. Interestingly, in outr study, serum PC aa and PC ae showed different pattern (Fig. 7B). The serum PC aa seems to be more influenced by degree of saturation than chain length. From the 14 serum PC aa identified as SCM, only polyunsaturated FA (**PUFA**) PC aa carrying 30 to 36 carbon were decreased from d -21 to d 1 and increased thereafter. This might indicate a shift from PUFA towards saturated and/or monosaturated FA (**MUFA**) PC in the membrane during the peripartum period. These changes might result in an increase of membrane stiffness which may be associated with abnormalities in glucose transporters (**GLUT**) translocation in muscle[67]. This is supported, at least in part, by increased serum glucose from d -21 to d 1.

In the present study, serum PC ae presented a chain length related pattern: serum PC ae containing shorter FA moieties ($\leq$ 38) were predominantly decreased from d -21 to d 1, whereas those containing longer FA ($\geq$ 42) followed a reverse pattern (Fig. 7B). This might suggest that

the shorter PC ae are more important in acting as serum antioxidants[61,63], as oxidative stress is increased around calving. Based on previous studies[64,67,68] and on our observations, we thus speculate that not only the carbon chain length, but also the position of the double bonds and conformation of the PC may determine the membrane-related cell functions during the transition from late pregnancy to early lactation. LysoPC, a PC breakdown product, is a major lysophospholipid in plasma and tissues[69]. It is not only involved in the transportation of the glycerophospholipid components such as FA, phosphatidylglycerol and choline between tissues, but also is a major phospholipid of the oxidized low-density lipoproteins[70]. As an effector of FA-induced insulin resistance[71], plasma lysoPC levels were changed in subjects with T2DM[72]. Greater levels of lysoPC a C18:0, but not unsaturated ones, were reported in obese subjects, suggesting that shorter and saturated lysoPC may play a role in impaired insulin signaling observed in these subjects[64,73]. In the present study, muscle short and saturated lysoPC (lysoPC a C14:0) concentrations were greater on d -21 and d 1, than thereafter (Fig. 7A). However, serum lysoPC a C18:2 concentrations showed almost a reverse pattern as compared with that of lysoPC a C14:0 (Fig. 7B), and were positively correlated with RQUICKI (Fig. 9H). In humans, unsaturated lysoPC were associated with improved insulin sensitivity[74]. Whether lysoPC acts in the same manner in dairy cows is currently unknown; however, the different patterns observed in their profiles during the transition from late pregnancy to early lactation in the present study may reflect a contribution of these metabolites in the adaptive processes likely including insulin signaling.

In the present study, the concentrations of most SM and hydroxysphingomyelins (**SM OH**) in muscle and serum reached a nadir at parturition and increased post partum. The SM, making up about 10-15% of lipids within the plasma membrane, are a molecularly diverse group of phospholipids. They are composed of a backbone of ceramide base and a phosphocholine head group[75,76]. SM is knowingly regulating the physical properties of membranes, while ceramide has been suggested as a therapeutic target for T2DM[77,78]. Remodeling of SM and ceramide metabolism is recently noted in the development of insulin resistance. In human and dairy cows, circulating ceramide levels were observed inversely correlated with insulin sensitivity[5,64,79,80]. The *de novo* synthesis of ceramide, that is, condensation of palmitate and serine (**Ser**), may attributed to insulin resistance[79,81]. The activation of sphingomyelinase and subsequent hydrolysis of SM to produce ceramide in response to pro-inflammatory signals is also well

characterized in dairy cows[79]. In the present study, muscle and serum SM that identified as SCM (Fig. 8) and serum total SM were principally decreased from d -21 to d 1 and then increased thereafter, likely reflecting elevated circulation profiles.

CONCLUSIONS

Out of 188 metabolites, 52 metabolites in muscle and 80 metabolites in serum contributed most significantly to the separation among the 4 time-points. Based on the SCM in muscle and serum, five potential metabolic pathways including FA oxidation, Arg metabolism, Trp metabolism, PC and lysoPC metabolism, and SM metabolism were found to be the most important metabolic pathways impacted by the transition from late gestation to early lactation. The altered metabolic pathways are intriguing and warrant further study. These data contribute towards an in-depth understanding of the metabolic and physiological changes occurring in dairy cows during the transition from late pregnancy to early lactation and identifies metabolic pathways for future targeted analysis.

ACKNOWLEDGMENTS

Y. Yang is recipient of a scholarship from the China Scholarship Council (CSC).

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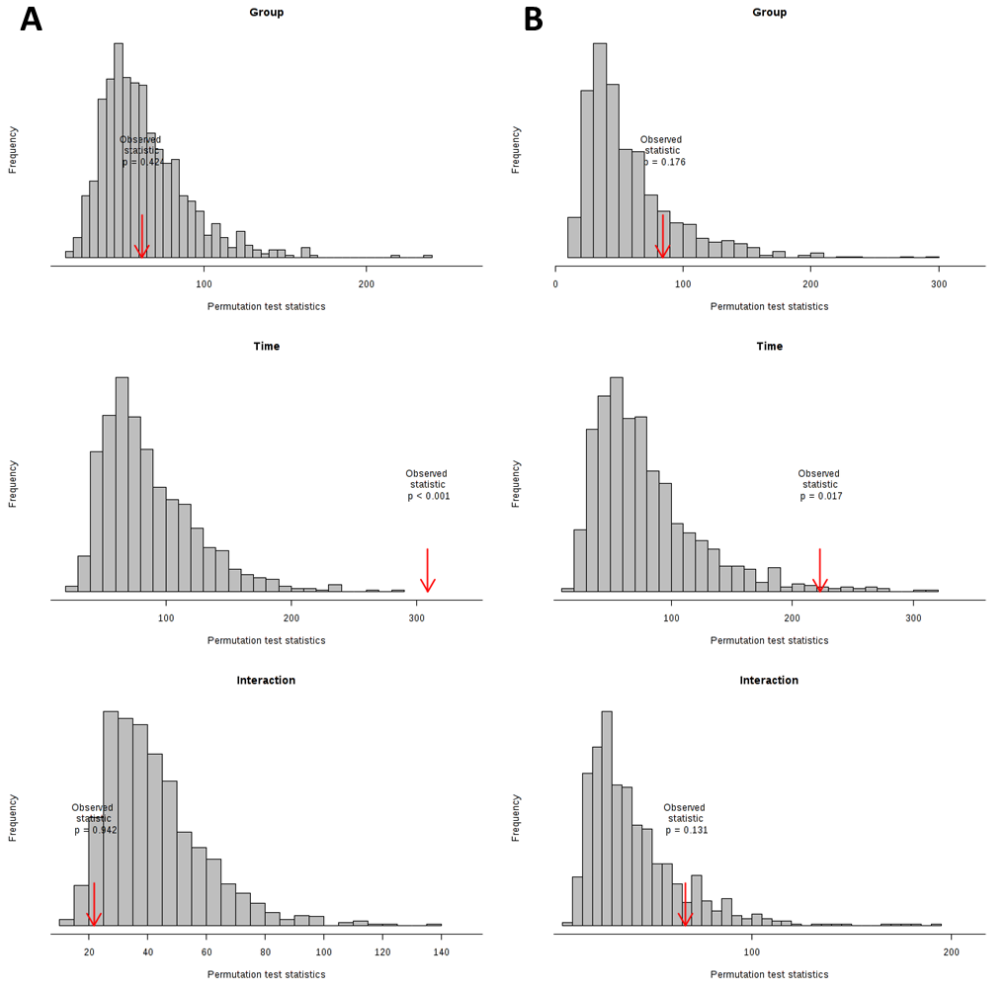
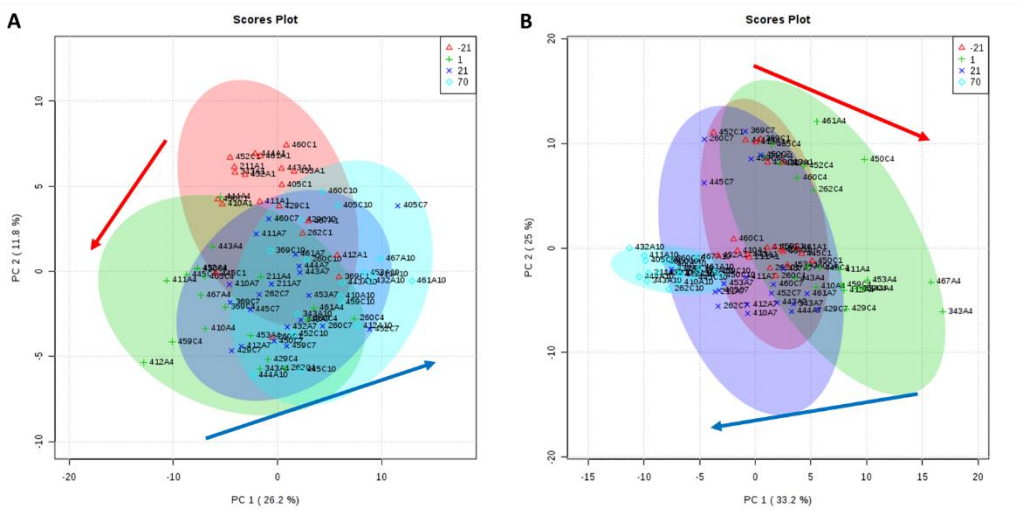
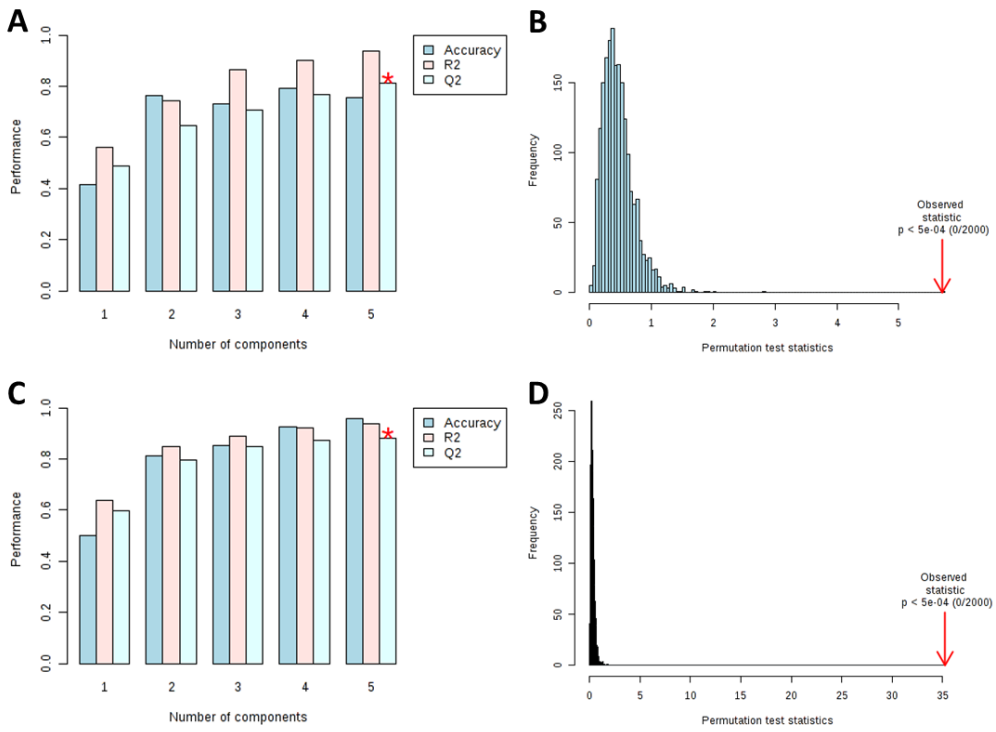


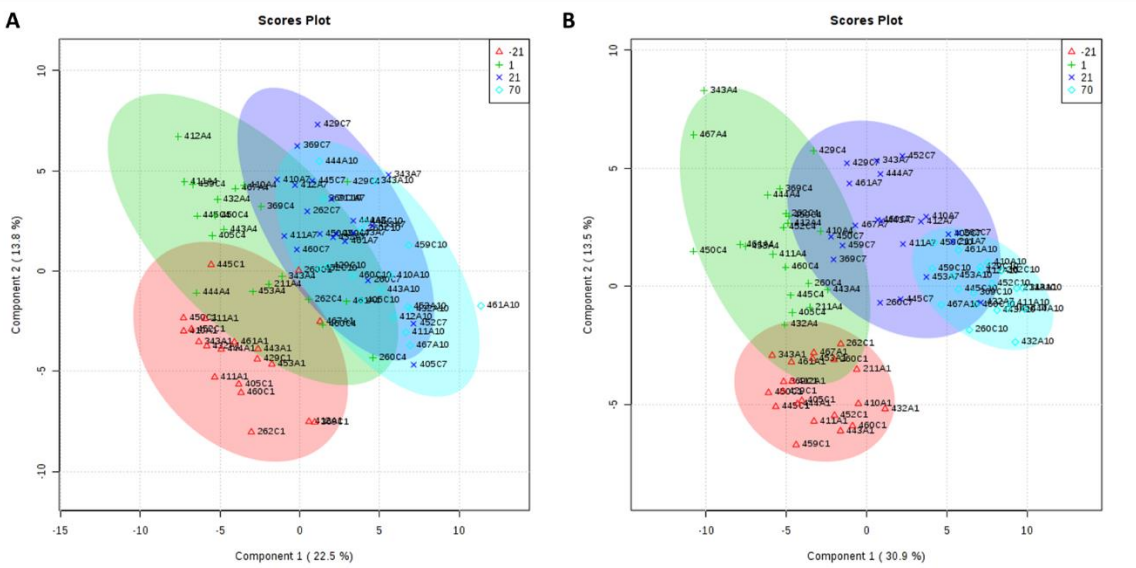
S. Figure 1. ANOVA-simultaneous component analysis (ASCA) of group (CLA supplementation and Control), time (d -21, 1, 21 and 70) and group × time interaction dairy cow's A) muscle and B) serum metabolites. The effects were tested by 1,000 times of permutation tests based on separation distance. The histogram shows the group separation distance formed by these datasets randomly reassigned class labels. The red arrow represents the group separation distance of the original classifier. The further away to the right of the distribution formed by randomly permuted data, the more significant the discrimination. Only time effects reached significant value.



S. Figure 2. PCA score plots of dairy cows A) muscle; and B) serum metabolites on d -21, 1, 21 and 70 days relative to parturition of the first two principal components. Each symbol represents the principal components of one cow at one sampling day. Arrows with different colors indicate directions of pattern shift from d -21 to d 1 (Red) and from d 1 to d 70 (Blue).



S. Figure 3. Validation of partial least squares-discriminant (PLS-DA) model discriminating dairy cow's muscle and serum metabolites from late gestation to early lactation. Prediction accuracy, R^2 (Goodness of fit) and Q^2 (Predictive ability) of PLS-DA models of A) muscle and C) serum using different number of components were calculated by 10-fold cross-validation. The red asterisk (*) indicates the best classifier which used in the current model. The PLS-DA model of B) muscle and D) serum were further validated by 2,000 times of permutation tests based on separation distance. The histogram shows the group separation distance formed by these datasets randomly reassigned class labels. The red arrow represents the group separation distance of the original classifier. The further away to the right of the distribution formed by randomly permuted data, the more significant the discrimination. The p-value is calculated as the proportion of the times that class separation based on randomly labeled sample is at least as good as the one based on the original data.



S. Figure 1. PLS-DA scores plots of dairy cows A) muscle; and B) serum metabolites showing a significant separation among d -21, 1, 21 and 70 days relative to parturition.

S. Table. 1. Complete list of Muscle and serum significantly changed metabolites (SCM) selected based on VIP $\geq$ 1.0 obtained from PLS-DA model and false discovery rate (FDR) $\leq$ 0.10 from ANOVA, respectively.

Tissue	Metabolites	VIP	FDR
	ADMA	2.615	< 0.0001
	Arg	1.061	< 0.0001
	C0	1.050	< 0.0001
	C12	1.065	< 0.0001
	C14:1-OH	1.027	< 0.0001
	C14:2	1.287	< 0.0001
	C14:2-OH	1.160	< 0.0001
	C16:1	1.202	< 0.0001
	C16:2	2.035	< 0.0001
	C16:2-OH	1.152	< 0.0001
	C16-OH	1.009	< 0.0001
	C18:1-OH	1.008	< 0.0001
	C18:2	1.617	< 0.0001
	C4	1.079	< 0.0001
	C4:1	2.115	< 0.0001
	C5	1.229	< 0.0001
	C5:1	1.180	< 0.0001
	C5-OH	1.688	< 0.0001
	C6	1.305	< 0.0001
	C9	1.411	< 0.0001
	Creatinine	1.784	< 0.0001
	Gln	2.345	< 0.0001
Muscle	H1	1.218	< 0.0001
	lysoPC a C14:0	1.097	0.0009
	Orn	1.848	< 0.0001
	PC aa C32:3	1.040	< 0.0001
	PC aa C34:2	1.976	< 0.0001
	PC aa C34:3	2.965	0.0009
	PC aa C34:4	2.282	< 0.0001
	PC aa C36:0	1.516	< 0.0001
	PC aa C36:2	1.123	< 0.0001
	PC aa C36:3	1.419	< 0.0001
	PC aa C42:5	1.345	< 0.0001
	PC ae C34:0	1.087	< 0.0001
	PC ae C34:1	1.182	< 0.0001
	PC ae C34:3	1.919	< 0.0001
	PC ae C36:1	1.016	0.0006
	PC ae C36:2	1.702	< 0.0001
	PC ae C36:3	1.823	< 0.0001
	PC ae C36:4	1.357	< 0.0001
	PC ae C38:0	1.128	0.0083
	PC ae C40:6	1.121	< 0.0001
	PC ae C42:5	1.140	< 0.0001
	PC ae C44:5	1.014	< 0.0001
	Pro	1.413	< 0.0001

	Ser	1.107	< 0.0001
	Serotonin	2.113	0.0002
	SM C16:1	1.014	0.0007
	SM C20:2	2.958	< 0.0001
	t4-OH-Pro	1.884	< 0.0001
	Taurine	1.258	< 0.0001
	Trp	1.096	0.0002
Serum	ADMA	2.238	< 0.0001
	C0	2.057	< 0.0001
	C10	1.329	< 0.0001
	C10:2	1.028	< 0.0001
	C14	1.050	< 0.0001
	C14:1-OH	1.134	< 0.0001
	C14:2	1.201	< 0.0001
	C16	1.010	< 0.0001
	C16:1	1.108	< 0.0001
	C16:1-OH	1.084	< 0.0001
	C16:2	1.075	< 0.0001
	C16:2-OH	1.097	< 0.0001
	C16-OH	1.214	< 0.0001
	C18:1-OH	1.234	< 0.0001
	C2	1.292	< 0.0001
	C3	1.090	< 0.0001
	C3:1	1.076	< 0.0001
	C3-OH	1.052	< 0.0001
	C4	1.659	< 0.0001
	C4:1	1.248	< 0.0001
	C5	1.361	< 0.0001
	C5:1	1.163	< 0.0001
	C5:1-DC	1.191	< 0.0001
	C5-M-DC	1.127	< 0.0001
	C5-OH	1.297	< 0.0001
	C6:1	1.163	< 0.0001
	C6-OH	1.252	< 0.0001
	C7-DC	1.177	< 0.0001
	C8	1.103	< 0.0001
	C9	1.013	< 0.0001
	Creatinine	1.570	< 0.0001
	Gln	1.432	< 0.0001
	Glu	1.772	< 0.0001
	H1	1.373	< 0.0001
	His	1.376	< 0.0001
	Leu	1.346	< 0.0001
	Lys	1.032	< 0.0001
	lysoPC a C18:2	1.066	0.0002
	lysoPC a C24:0	1.170	< 0.0001
	lysoPC a C28:1	1.157	< 0.0001
	Met-SO	1.534	0.0001
	PC aa C28:1	1.533	< 0.0001
	PC aa C30:0	1.035	< 0.0001

PC aa C30:2	1.017	< 0.0001
PC aa C32:2	1.200	< 0.0001
PC aa C32:3	1.981	< 0.0001
PC aa C34:2	1.643	< 0.0001
PC aa C34:4	1.442	< 0.0001
PC aa C36:2	1.572	< 0.0001
PC aa C36:3	1.290	< 0.0001
PC aa C38:1	1.293	< 0.0001
PC aa C42:0	1.062	< 0.0001
PC aa C42:1	1.012	0.0013
PC aa C42:5	1.089	< 0.0001
PC aa C42:6	1.674	< 0.0001
PC ae C30:2	1.090	< 0.0001
PC ae C32:1	1.075	< 0.0001
PC ae C32:2	1.025	< 0.0001
PC ae C34:2	1.559	< 0.0001
PC ae C34:3	2.025	< 0.0001
PC ae C36:2	1.830	< 0.0001
PC ae C36:3	1.381	< 0.0001
PC ae C36:4	1.631	< 0.0001
PC ae C38:3	1.098	< 0.0001
PC ae C42:0	1.018	< 0.0001
PC ae C42:2	1.412	< 0.0001
PC ae C42:3	1.319	0.0024
PC ae C42:4	1.237	0.0249
PC ae C44:4	1.363	< 0.0001
PC ae C44:5	1.184	< 0.0001
Phe	1.416	< 0.0001
Putrescine	1.029	< 0.0001
SDMA	1.286	< 0.0001
Serotonin	1.783	0.0002
SM C20:2	1.553	< 0.0001
SM OH C16:1	1.112	< 0.0001
SM OH C22:1	1.078	< 0.0001
*t*4-OH-Pro	1.027	< 0.0001
total DMA	2.005	< 0.0001
Tyr	1.137	< 0.0001

4. Manuscript 2 (Accepted: J. Dairy Sci., 2018)

Acylcarnitine profiles in serum and muscle of dairy cows receiving conjugated linoleic acids or a control fat supplement during early lactation

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ABSTRACT

Acylcarnitines (**ACC**) are formed when fatty acid (**FA**)-CoA enters the mitochondria for β-oxidation and the tricarboxylic acid (**TCA**) cycle through the carnitine shuttle. Concentrations of ACC may vary depending on the metabolic conditions, but can accumulate when rates of β-oxidation exceed those of TCA. This study aimed to characterize muscle and blood serum acylcarnitine profiles, to determine the mRNA abundance of muscle carnitine acyltransferases, and to test whether dietary supplementation (from d 1 in milk) with conjugated linoleic acids (CLA; 100 g/d; each 12% of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA; n = 11) altered these compared with control-fat supplemented cows (CTR; n = 10). Blood samples and biopsies from *M. semitendinosus* were collected on d -21, 1, 21, and 70 relative to parturition. Serum and muscle ACC profiles were quantified using a targeted metabolomics approach. The CLA supplement did not affect the variables examined. The serum concentration of free carnitine decreased with the onset of lactation. The concentrations of acetylcarnitine (C2), hydroxybutyrylcarnitine (C4-OH), and the sum of short-chain ACC (C2-C5) in serum were greater from d -21 to 21 than thereafter. The serum concentrations of long-chain ACC tetradecenoylcarnitine (C14:1), and octadecenoylcarnitine (C18:1) concentrations were greater on d 1 and 21 compared with d -21. Muscle carnitine remained unchanged, whereas short and medium- chain ACC, including propenoylcarnitine (C3:1), C4-OH, hydroxyhexanoylcarnitine (C6-OH), hexenoylcarnitine (C6:1), and pimelylcarnitine (C7-DC) were increased on d 21 compared with d -21, and decreased thereafter. In muscle, the concentrations of long-chain ACC (from C14 to C18) were elevated on d 1. The mRNA abundance of carnitine palmitoyltransferase 1, muscle isoform (***CPT1B***) increased 2.8-fold from d -21 to d 1 (P = 0.02), followed by a decline to nearly prepartum values by d 70, whereas that of *CPT2* did not change over time. The majority of serum and muscle short-chain and long-chain ACC were positively correlated with the FA concentrations in serum, whereas serum carnitine and C5 were negatively correlated

with FA. Time-related changes in the serum and muscle ACC profiles were demonstrated, that were not affected by the CLA supplement at the dosage used in the present study. The elevated concentrations of long-chain ACC species in muscle and of serum C2 around parturition point to incomplete FA oxidation, likely due to insufficient metabolic adaptation in response to the load of FA around parturition.

Key Words: acylcarnitine, carnitine shuttle, skeletal muscle, conjugated linoleic acid, early lactation

INTRODUCTION

The transition from late gestation through early lactation in dairy cow is associated with a substantial mobilization of body reserves, in particular fat, leading to a marked increase in circulating concentrations of fatty acids (**FA**) which are oxidized by hepatic and extrahepatic tissues as an energy source (Grummer, 1993). However, in the liver, the oxidative capacity for FA and also for exporting FA via very low-density lipoproteins (**VLDL**) is limited and thus fatty liver may result from increased lipolysis (Grummer, 2008). Peroxisomal β-oxidation, as an auxiliary pathway for oxidizing FA during extensive NEFA mobilization, helps to dampen accumulation of fat in the liver (Grum et al., 1994, 1996, 2002). In addition, the oxidative capacity for FA in other tissues such as skeletal muscle may also contribute in reducing the metabolic load of FA on the liver (Kuhla et al., 2011; Schäff et al., 2013), though the quantitative contribution of skeletal muscle to FA metabolism in dairy cows during the periparturient is not known.

In dairy cows, plasma FA mainly comprise saturated FA, including palmitic acid (C16:0) and stearic acid (C18:0), and oleic acid (C18:1n9c) as a monounsaturated FA (Leroy et al., 2005; Tyburczy et al., 2008). For generating energy from long-chain FA, they need to be transported from the cytoplasm into the mitochondrial matrix across the mitochondrial membranes through a carnitine-dependent transport shuttle. This transport system is regulated by carnitine acyltransferases, i.e. carnitine

palmitoyltransferase 1 (CPT1; present in the mitochondrial outer membrane), and CPT2 (located on the matrix side of the inner membrane) (Flanagan et al., 2010; Schooneman et al., 2014). Once inside the mitochondria, carnitine and long-chain acyl-CoA are regenerated by CPT2, which can then be further oxidized via the tricarboxylic acid (**TCA**) cycle and respiratory chain to provide ATP (Schooneman et al., 2013). Deficiencies in these enzymes or impaired functions, or depletion of TCA cycle intermediates may lead to incomplete mitochondrial FA oxidation, resulting in accumulation of acylcarnitines (**ACC**), which may be associated with development of insulin resistance as documented in human studies (Adams et al., 2009; Mihalik et al., 2010; Sun et al., 2016).

Serum ACC undergo time-related changes in dairy cows during the transition from late pregnancy to early lactation (Kenéz et al., 2016) and differ between cows experiencing excessive versus low lipolysis as classified via the serum FA concentrations post partum (Humer et al., 2016). The plasma ACC profile may reflect the intra-mitochondrial acyl-CoA pattern; however, it is not clear to what extent circulating levels of ACC reflect tissue ACC metabolism, as plasma ACC represent the sum from different tissues, mainly skeletal muscle and liver (Schooneman et al., 2014; Xu et al., 2016).

Supplementation with *trans*-10, *cis*-12 CLA is used to reduce milk fat content in early-lactation dairy cows as a dietary strategy in order to improve energy status to counteract the physiological negative energy balance (Sippel et al., 2009; Schlegel et al., 2012). The *trans*-10, *cis*-12 isomer is referred as the most effective isomer lowering milk fat content mainly through inhibition of de novo FA synthesis in the mammary gland, accompanied by a reduction of FA uptake from triacylglycerol rich lipoproteins due to inhibition of lipoprotein lipase activity (Bauman et al., 2011). A number studies have examined the effects of CLA on hepatic lipid metabolism in growing beef cattle (Shibani et al., 2011) or dairy cows (Schlegel et al., 2012) through assessing expression of genes involved in lipid metabolism; but to our knowledge there is no evidence evaluating the effects of CLA on lipid metabolism in ruminant

skeletal muscle. Considering skeletal muscle as principal contributor to the serum ACC pool (Koves et al., 2008), we tested the hypothesis that dietary supplementation with CLA may alter the expression of muscle carnitine acyltransferases in conjunct with free carnitine and ACC profiles in both serum and muscle of dairy cows as compared with control-fat supplemented cows to address potential changes in the capacity for mitochondrial β-oxidation of FA in skeletal muscle in context with the negative energy balance typical for early lactation. Using serum samples and biopsies from semitendinosus muscle, we aimed to determine changes in serum and muscle concentrations of ACC and muscle expression of *CPT1B* and *CPT2* mRNA related to treatment and time from late pregnancy to lactation.

MATERIAL AND METHODS

Animals, Treatment, and Experimental Design

All animal experiments were in accordance with the European Community regulations concerning the protection of experimental animals and the guidelines of the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany, File Number 33.14.42502-04-071/07). The experimental design and zootechnical data were reported previously (Pappritz et al., 2011a). A subset of animals and samples from this study, i.e., only multiparous cows, was considered for the current study. Briefly, 21 Holstein cows, housed in a free stall barn were fed *ad libitum* with a partial mixed ration (**PMR**) according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). The PMR (6.8 MJ of NE_L/kg of DM) consisted of 37.8% corn silage, 25.2% grass silage, and 37% concentrate (DM basis). At 1 DIM, cows were allotted to either the CLA group (n = 11) or the control group (CTR; n = 10). The animals in the CLA group received 100 g/d encapsulated rumen-protected CLA (Lutrell Pure, BASF SE, Ludwigshafen, Germany) supplying 7.6 g of *cis*-9, *trans*-11 CLA and 7.6 g of *trans*-10, *cis*-12 CLA per day. The animals in the CTR group received 100 g/d of rumen-protected control fat supplement (Silafat, BASF) in which CLA was substituted by stearic acid to form an isoenergetic control

diet using a fatty acid with the same number of carbon atoms as in CLA. The supplements were provided with 4 kg of additional concentrate (8.8 MJ of NE_L/kg DM) from DIM 1 throughout the observation period.

Blood and Muscle Tissue Sampling

Blood samples were taken from the jugular vein on d -21, 1, 21, and 70 relative to parturition using evacuated tubes. Cows were sampled after the morning milking before they had access to the new fresh ration. Blood samples were allowed to clot and centrifuged at 1,500 × *g* at 4 °C for 20 min. Serum was recovered and frozen (-80 °C) until analysis. Muscle biopsies (M. semitendinosus) were collected on d -21, 1, 21, and 70 relative to parturition and were snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

Estimation of insulin sensitivity

Data needed for the estimation of insulin sensitivity were reported elsewhere (Pappritz et al., 2011a). Insulin sensitivity was estimated by calculating the "Revised Quantitative Insulin Sensitivity Check Index" (**RQUICKI**) from the data of blood glucose, insulin, and FA (Holtenius and Holtenius, 2007).

Acylcarnitine Profiling

The acylcarnitine profiles in muscle and serum were determined by FIA-ESI-MS/MS profiling through targeted metabolomics using the AbsoluteIDQ^{TM} p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). Free carnitine and 40 acylcarnitines (Cx:y) were simultaneously quantified. The abbreviations Cx:y are used to describe the total number of carbons and double bonds of all chains, respectively. All analyses were performed in the Helmholtz Zentrum München (GmbH), German Research Center for Environmental Health, Genome Analysis Center. In case of serum, 10 µL of the thawed sample have been applied directly to the assay. In case of muscle, 25 mg of frozen samples were homogenized and

extracted using homogenization tubes with ceramic beads (1.4 mm) and a Precellys 24 homogenizer with an integrated cooling unit (PEQLAB Biotechnology GmbH, Germany). To each mg of frozen muscle tissue were added 3 µL of a dry ice cooled mixture of ethanol/phosphate buffer (85/15 v/v). After centrifugation, 10 µL of the homogenate supernatant were applied to the well plate of the p180 kit. The assay procedures of the AbsoluteIDQ™ p180 Kit, the detailed description of the tissue preparation and the metabolite nomenclature have been described in detail previously (Zukunft et al., 2013 and 2018). Sample handling was performed by a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK), beside standard laboratory equipment. Mass spectrometric analyses were done on an API 4000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the Met*IDQ*™ software package, which is an integral part of the Absolute*IDQ*™ kit. Internal standards were used as reference for the calculation of metabolite concentrations. The concentrations of the plasma samples were given in µM, the concentrations of the tissue samples in pmol/mg tissue and the concentrations of tissue homogenate in µM.

RNA Extraction and Quantitative real-time Reverse Transcription-PCR

The preparation of the samples including RNA extraction and cDNA synthesis was described in detail previously (Saremi et al., 2012a,b; Sadri et al., 2015). Quantification of the mRNA of the targeted genes was performed in an Mx3000P cycler (Agilent, Santa Clara, CA) and in accordance with MIQE guidelines (Bustin et al., 2009). Primers sequences and the real-time PCR conditions are shown in Table 1.

The reaction was performed in triplicate in a total volume of 10 µL consisting of 2 µL of cDNA (diluted 1:4) as template, 1 µL of primer mix, 2 µL of water, and 5 µL of the DyNAmo ColorFlash SYBR Green qPCR Kit master mix (Thermo Scientific,

Germany). For each PCR run, a negative-template control for quantitative PCR, as well as a negative-template control and no-reverse transcriptase control of cDNA were included. A standard curve was generated using serial dilutions of cDNA to calculate efficiency-corrected relative quantities of the targets (run-specific target amplification efficiency). A set of 2 inter-run calibrators was used for each PCR plate to correct for run to run variation. The mRNA abundance of the target genes was normalized using the 4 most stable reference genes (Saremi et al., 2012a), namely Emerin (**EMD**), RNA polymerase II (**POLR2A**), eukaryotic translation initiation factor 3 (**EIF3K**), and low-density lipoprotein receptor–related protein 10 (**LRP10**) using qBase^{PLUS} 2.0 (Biogazelle, Ghent, Belgium).

Table 1. Characteristics of the primers and the real-time PCR conditions

Gene[1]	Sequence (5' -3')	NCBI accession no.	Length (bp)	Annealing conditions (s/°C)	Mean efficiency
CPT1B					
Forward	GCAGATGATGGCTATGGA	NM_001034349.2	78	20/61	90.2
Reverse	GGAGAACTTGCTGGAGAC				
CPT2					
Forward	GTAGCCAGTAAGCACTATTC	NM_001045889.2	180	60/59	97.0
Reverse	CCAAGTCTTACCTCCTGATA				
EMD					
Forward	GCCCTCAGCTTCACTCTCAGA	NM_203361	100	45/59	95.5
Reverse	GAGGCGTTCCCGATCCTT				
POLR2A					
Forward	GAAGGGGGAGAGACAAACTG	X63564	86	60/60	100.4
Reverse	GGGAGGAAGAAGAAAAAGGG				
EIF3K					
Forward	CCAGGCCCACCAAGAAGAA	NM_001034489	180	60/59	89.8
Reverse	TTATACCTTCCAGGAGGTCCATGT				
LRP10					
Forward	CCAGAGGATGAGGACGATGT	BC149232	125	45/59	98.4
Reverse	ATAGGGTTGCTGTCCCTGTG				

[1]*CPT1B* = carnitine palmitoyltransferase 1, muscle isoform; *CPT2* = carnitine palmitoyltransferase 2; *EMD* = emerin; *POLR2A* = RNA polymerase II; *EIF3K* = eukaryotic translation initiation factor 3; *LRP10* = low-density lipoprotein receptor–related protein 10.

Statistical Analysis

Statistical analysis of the data was carried out using SAS software (version 9.2; SAS Institute Inc., Cary, NC). The data were tested for normality before analysis using the UNIVARIATE procedure. When the data were not normally distributed (BW, BCS, DMI, and blood glucose, insulin, NEFA, and RQUICKI), they were transformed using a $\log_{10}$ transformation before analysis. Body weight, BCS, DMI, blood metabolites and mRNA data were analyzed using repeated measures in the MIXED procedure of SAS. The model included treatment, time, and interaction of treatment × time as the fixed effects and cow as the random effect. No significant effect of treatment or interaction of treatment × time were observed on the tested variables. Therefore, data from the 2 feeding groups were merged for the final statistical analysis of the data. The appropriate covariance structure for all repeated statements was determined according to the Akaike's information criterion (**AIC**) and Bayesian information criterion (**BIC**). The Tukey-Kramer adjustment was applied to account for multiple comparisons. The threshold of significance was set at $P \leq 0.05$; trends were declared at $0.05 < P \leq 0.10$.

Serum and muscle ACC data was analyzed with MetaboAnalyst 3.0 (Xia et al., 2015). A preliminary statistical analysis of the data showed no significant effect of treatment or interaction of treatment × time. Thus, data from the 2 feeding groups were merged for the final statistical analysis. The *k*-nearest neighbors (**KNN**) algorithm was used to estimate the values of missing data. Metabolites with more than 50% of missing values (i. e., values lower then LOD) were omitted. Data were generalized log (**glog**) transformed and Pareto-scaled to correct for heteroskedasticity, to reduce the skewness of the data, and to reduce mask effects (van den Berg et al., 2006). Principle component analysis (**PCA**), partial least squares discriminant analysis (**PLS-DA**), and variable importance of projection (VIP) were conducted to identify those metabolites showing significant differences among the 4 time-points. The PLS-DA models were validated by 10-fold cross validation and 2000 times permutation tests (Figures 3 and 4; Szymanska et al., 2012). The VIP score was used to rank the metabolites based on

their importance in discriminating different time-points. In addition, a one-way ANOVA followed by Tukey's HSD test was performed on the data, in order to further confirm the significance of important metabolites identified from PCA and PLS-DA. The threshold of significance was set at false discovery rate (FDR) $\leq$ 0.10.

The Spearman's rank-order correlation was used to reveal correlations between AC profiles, mRNA data, and RQUICKI using PROC CORR. The *P* values were adjusted for multiple comparisons by calculating FDR using PROC MULTTEST. The cut-off condition of correlation analyses was set as $|\rho| \geq 0.20$ and FDR $\leq$ 0.10.

RESULTS

BW, BCS, and DMI

Neither BW nor BCS and DMI were different between groups. As shown in Figure 1 for the merged groups, both BW and BCS decreased with time ($P < 0.0001$), whereas DMI increased with time ($P = 0.0002$).

RQUICKI

For RQUICKI, no treatment effects were observed, but and it tended to decrease by about 4% from d 1 to d 21 (Figure 2; $P = 0.106$).

Acylcarnitine Profiles in Serum

The serum ACC concentrations were not influenced by CLA supplementation, but changes related to time were observed. With unsupervised and supervised pattern recognition analyses, clear separations between d -21 and post partum time-points in serum were observed (Figures 3A and 3B). Moreover, the top 15 ACC that contributed most significantly to the separation between the 4 time-points were identified by the respective validated PLS-DA model and VIP (Figures 3C and 3D). In order to further confirm the specificity and significance of important metabolites identified from PCA and PLS-DA, we performed univariate analysis using one-way ANOVA and Tukey's HSD test on each metabolite. In total, 7 serum ACC and 3

related indices changed over time (VIP $\geq$ 1.0, FDR < 0.10, Figure 4). The serum concentrations of free carnitine decreased with the onset of lactation. The serum concentrations of acetylcarnitine (C2), butyrylcarnitine (C4), and sum of the short-chain ACC concentrations (C2-C5) were elevated around parturition compared to d 70. The serum concentrations of hydroxybutyrylcarnitine (C4-OH) and octadecenoylcarnitine (C18:1) increased from d -21 to d 21 and then remained unchanged. The CPT1 ratio, the ratio of free carnitine to the sum of palmitoylcarnitine and stearoylcarnitine [carnitine/(C16:1+C18:0)] decreased with the onset of lactation. The CPT2 ratio [(C16:0+C18:1)/C2] was higher after parturition compared to prepartum values.

Acylcarnitine Profiles in Skeletal Muscle

The muscle ACC concentrations did not differ between the groups, but changes related to time were observed. Score plots of PCA and PLS-DA of dairy cow's muscle ACC are presented in Figure 5A and 5B. The top 15 metabolites that contributed most significantly to the observed separation are shown in Figure 5C. The top 15 metabolites contributing most significantly (VIP score > 1) to the observed separation are shown in Figure 5C. Time course of the selected metabolites identified to have a VIP score > 1.0, and FDR < 0.10 are shown in Figure 6. The muscle concentrations of carnitine remained unchanged (data not shown). Muscle short and medium-chain ACC, including propenoylcarnitine (C3:1), C4-OH, hydroxyhexanoylcarnitine (C6-OH), hexenoylcarnitine (C6:1), were elevated from d -21 to d 21 and decreased thereafter. Muscle long-chain ACC including tetradecanoylcarnitine (C14), C14:1, hexadecanoylcarnitine (C16), hydroxyhexadecenoylcarnitine (C16:1-OH), C18:1, hydroxyoctadecenoylcarnitine (C18:1-OH), and sum of the long-chain ACC (C12-C18) changed over time ($P \leq$ 0.002) and followed a similar pattern; that is, they increased from d -21 to d 1, declined to nearly pre-partum values by d 21 and then remained unchanged. The CPT1 ratio in muscle was decreased from d -21 to a nadir on d 1, and then increased thereafter. In contrast, the muscle CPT2 ratio increased towards parturition, and then decreased thereafter.

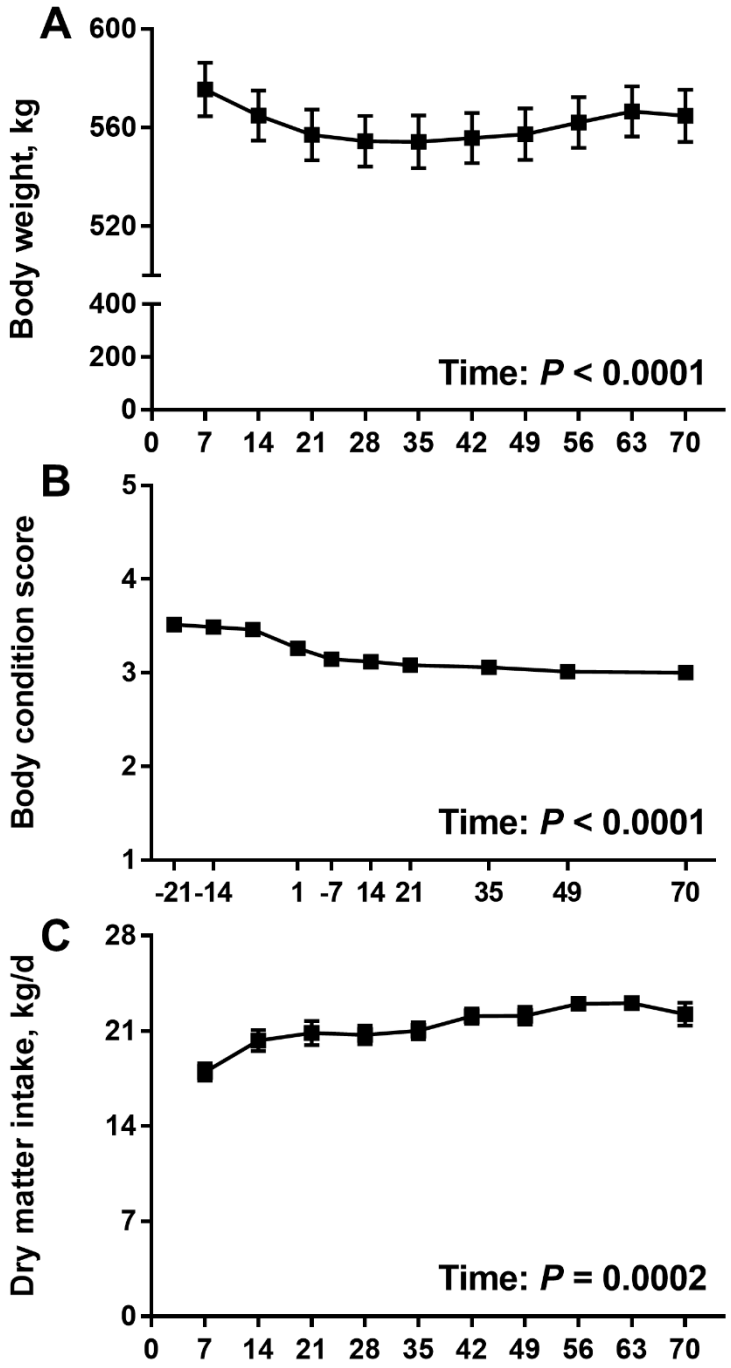

Figure 1. Time course of body weight (**A**), body condition score (**B**), and dry matter intake (**C**) in dairy cows during late gestation and early lactation. Data are means ± standard error of the mean (SEM). Time effect: $P < 0.0001$ (body weight and body condition score) and 0.0002 (dry matter intake).

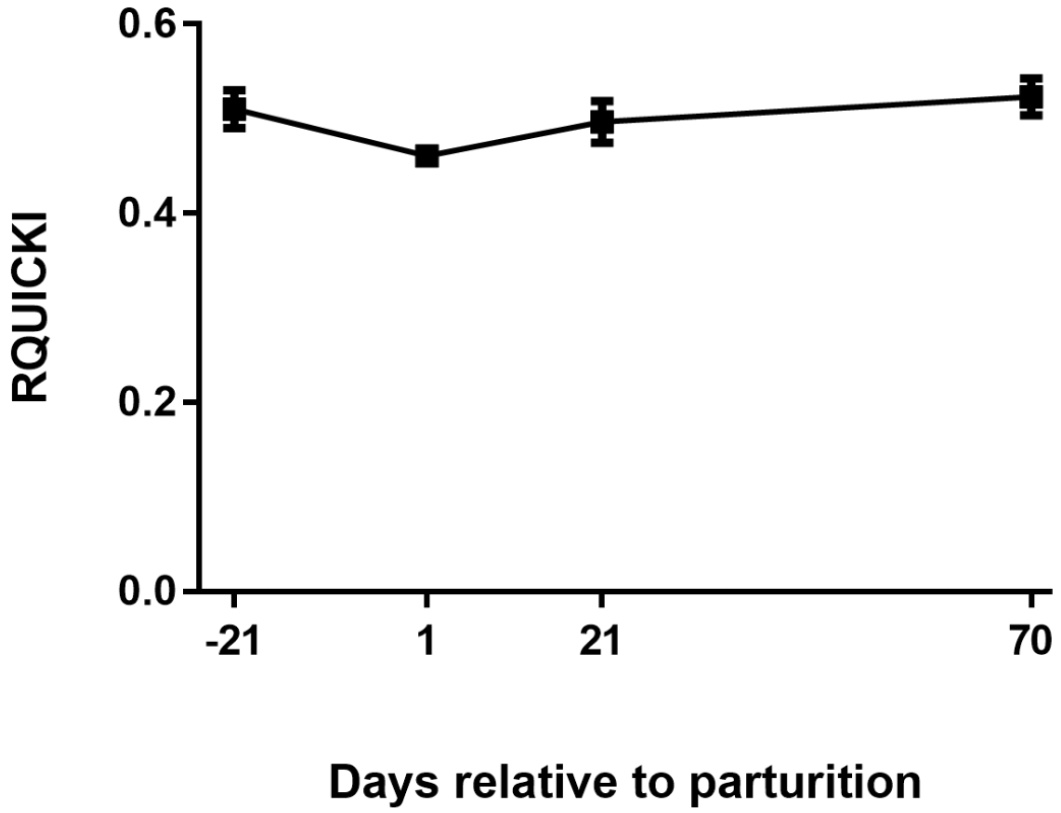

Figure 2. Time course of the estimated insulin sensitivity as measured by the revised quantitative insulin sensitivity check index (RQUICKI) in dairy cows during late gestation and early lactation (P = 0.106). Data are means ± SEM.

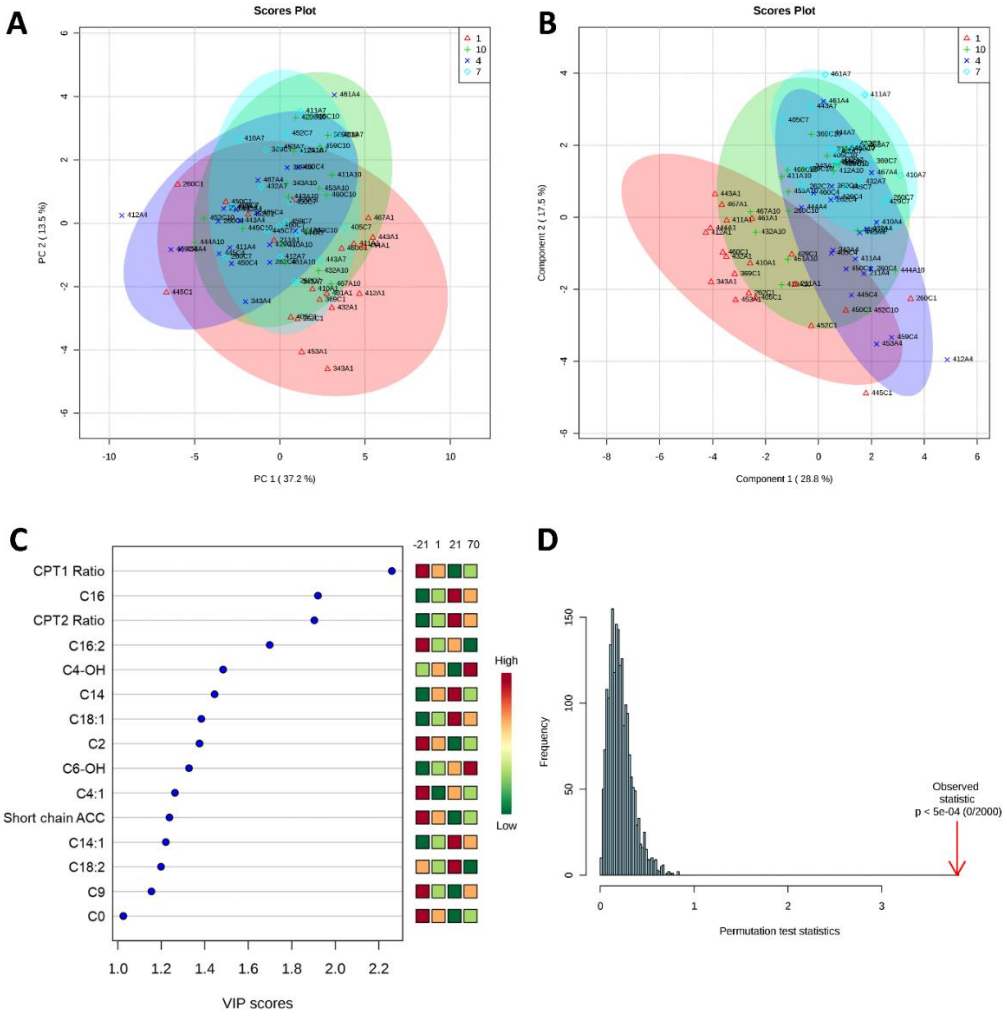

Figure 3. Score plots of principal component analysis (PCA, **A**) and Partial least squares-discriminant (PLS-DA, **B**) of dairy cow's serum acylcarnitine on d -21 (Red), 1 (Green), 21 (Blue), and 70 (Cyan) relative to calving. Top 15 metabolites that contributed most significantly to the separation between the 4 time-points were identified according to weights in PLS-DA model by using variable importance in projection (VIP, **C**). The PLS-DA model was further validated by 2000 times of permutation tests based on separation distance (**D**). The histogram shows the group separation distance formed by these datasets randomly reassigned class labels. The red arrow represents the group separation distance of the original classifier. The further away to the right of the distribution formed by randomly permuted data, the more significant the discrimination. The p-value is calculated as the proportion of the times that class separation based on randomly labeled sample is at least as good as the one based on the original data.

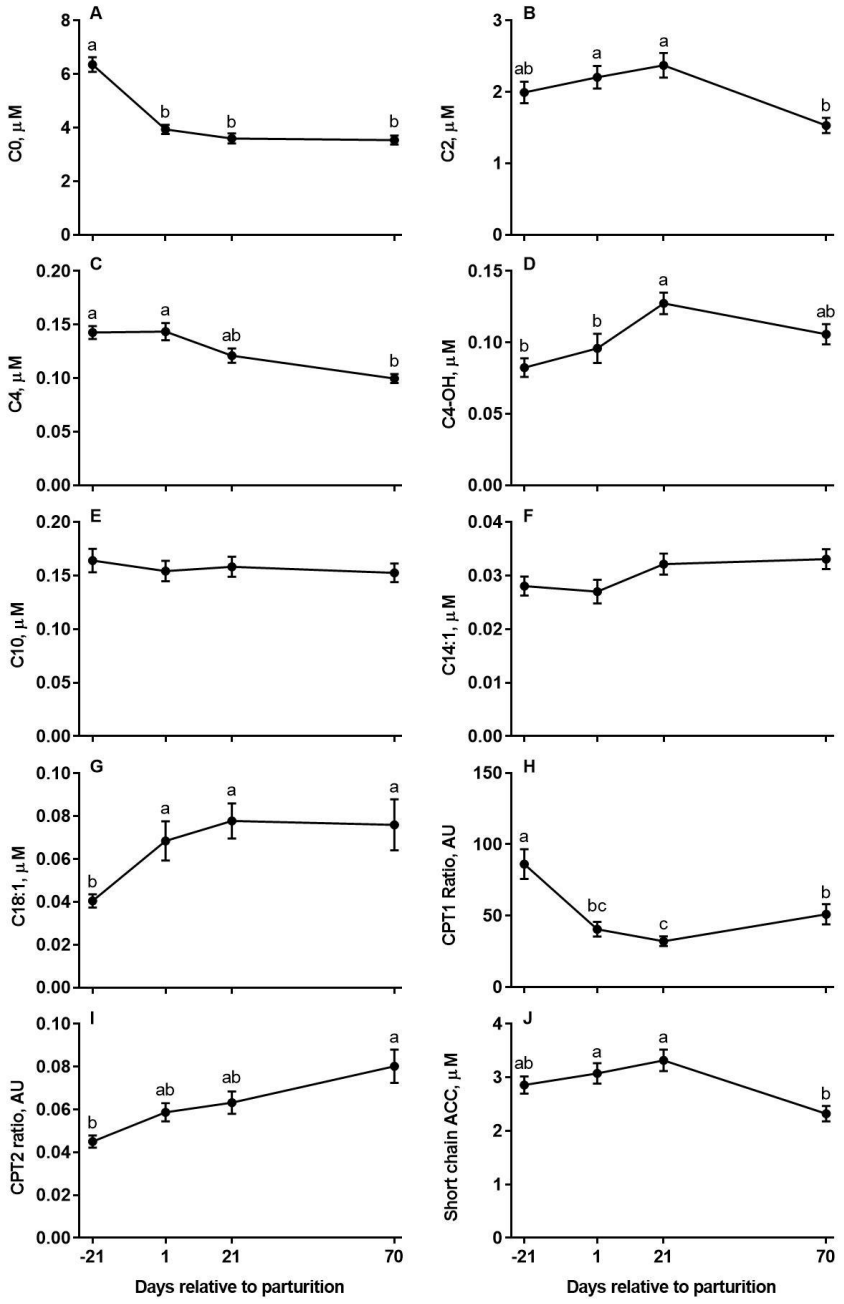

Figure 4. Time course of selected metabolites in serum of dairy cows identified to have a VIP (variable importance in projection) score > 1.0, and FDR < 0.1: free carnitine (C0, **A**), acetylcarnitine (C2, **B**), butyrylcarnitine (C4, **C**), (**D**) hydroxybutyrylcarnitine (C4-OH, **D**), decanoylcarnitine (C10, **E**), tetradecenoylcarnitine (C14:1, **F**), octadecenoylcarnitine (C18:1, **G**), carnitine palmitoyltransferase (CPT)1 ratio (**H**), CPT2 ratio (**I**), and sum of short-chain acylcarnitine (Short-chain ACC; C2-C5, **J**). Data are means ± SEM. Differences between different time points identified by post-hoc testing (Tukey's HSD) after ANOVA are indicated by different letters.

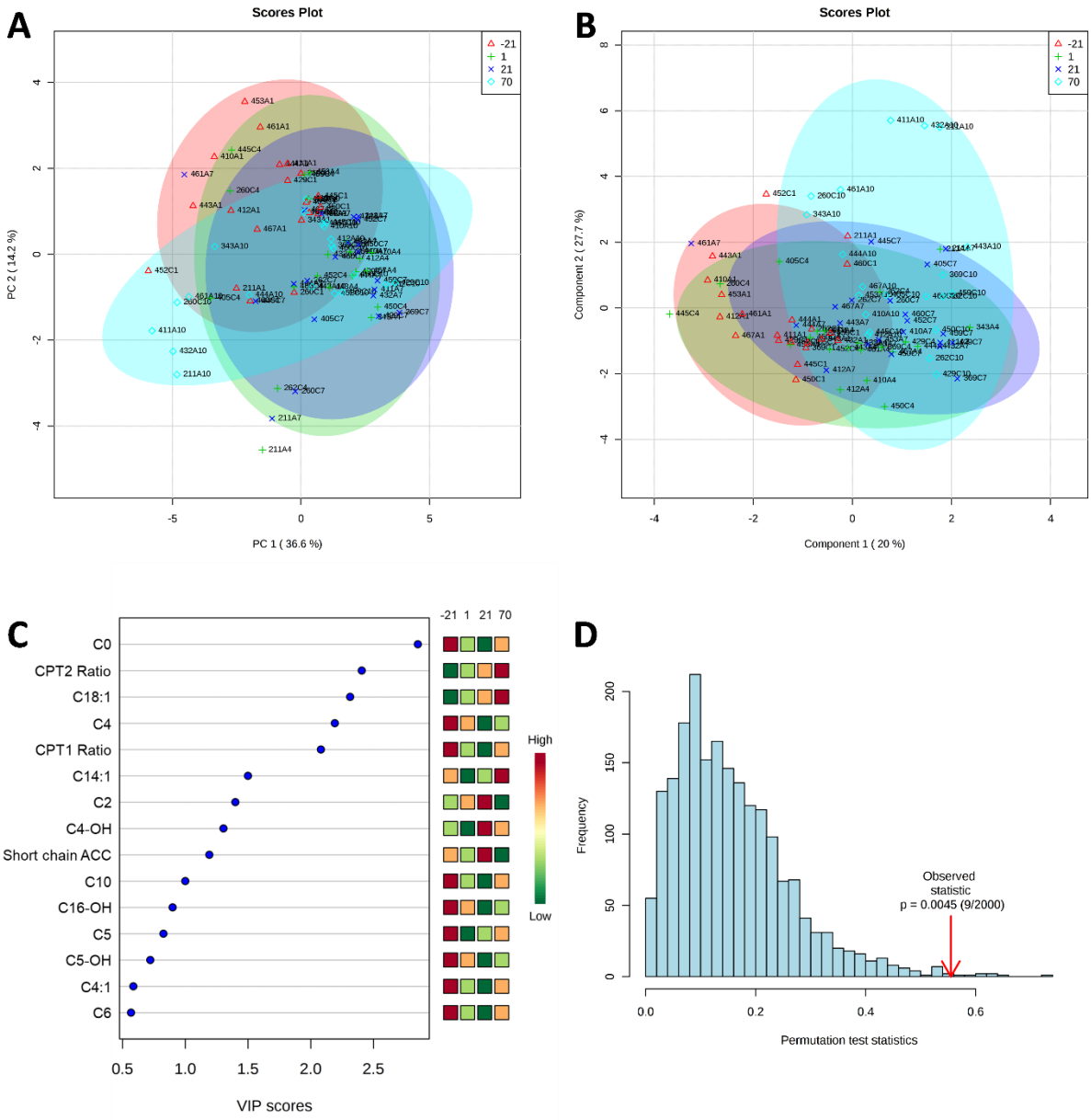

Figure 5. Score plots of principal component analysis (PCA, **A**) and Partial least squares-discriminant (PLS-DA, **B**) of dairy cow's muscle acylcarnitine on d -21 (Red), 1 (Green), 21 (Blue), and 70 (Cyan) relative to calving. Top 15 metabolites that contributed most significantly to the separation between the 4 time-points were identified according to weights in PLS-DA model by using variable importance in projection (VIP**, C**). The PLS-DA model were further validated by 2000 times of permutation tests based on separation distance (**D**). The histogram shows the group separation distance formed by these datasets randomly reassigned class labels. The red arrow represents the group separation distance of the original classifier. The further away to the right of the distribution formed by randomly permuted data, the more significant the discrimination. The p-value is calculated as the proportion of the times that class separation based on randomly labeled sample is at least as good as the one based on the original data.

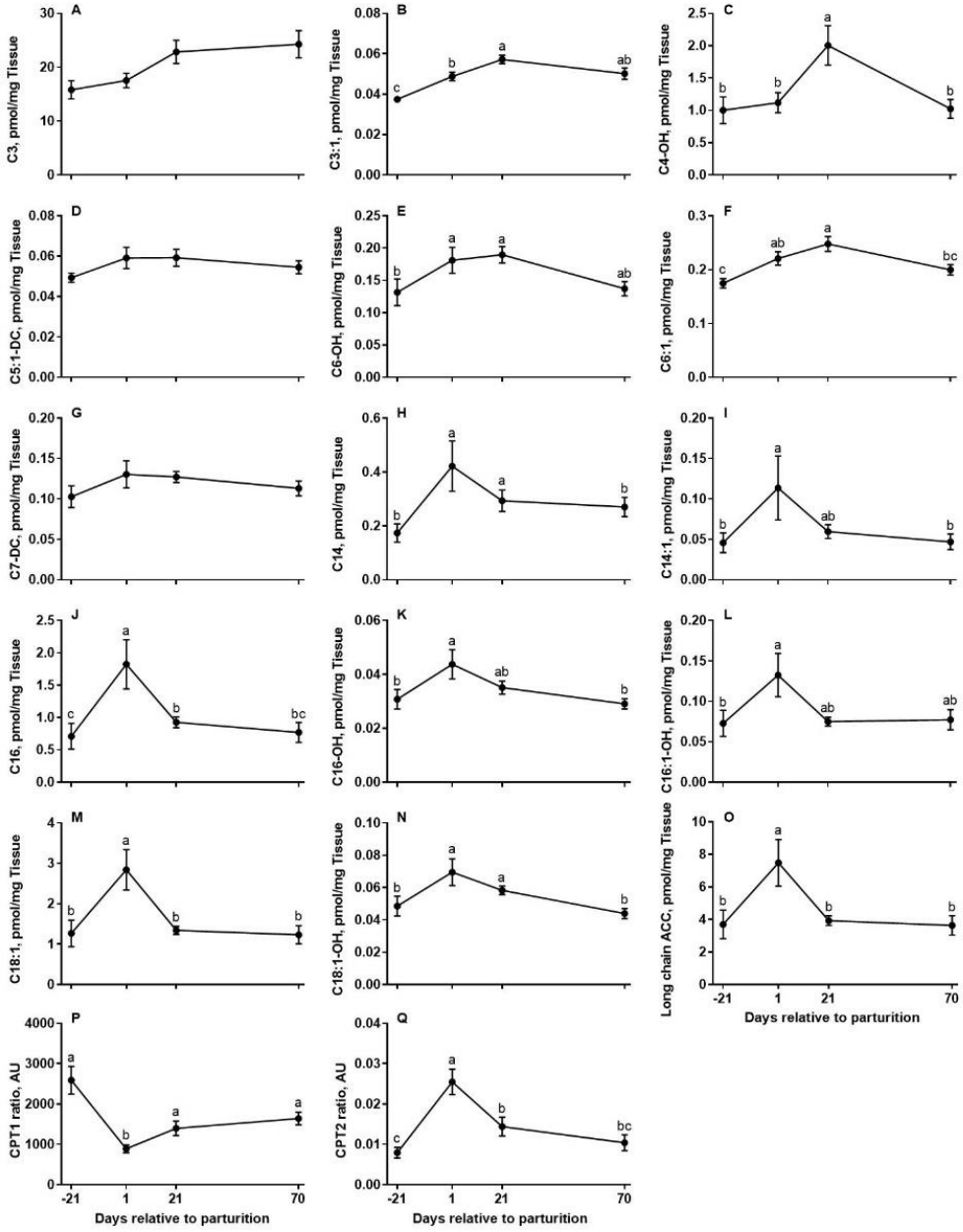

Figure 6. Time course of selected metabolites in muscle of dairy cows identified to have a VIP (variable importance in projection) score > 1.0, and FDR < 0.1: propionylcarnitine (C3, **A**), propenoylcarnitine (C3:1, **B**), hydroxybutyrylcarnitine (C4-OH, **C**), glutaconylcarnitine (C5:1-DC, **D**), Hydroxyhexanoylcarnitine (C6-OH, **E**), hexenoylcarnitine (C6:1, **F**), pimelylcarnitine (C7-DC, **G**), tetradecanoylcarnitine (C14, **H**), tetradecenoylcarnitine (C14:1, **I**), hexadecanoylcarnitine (C16, **J**), hydroxyhexadecanoylcarnitine (C16-OH, **K**), hydroxyhexadecenoylcarnitine (C16:1-OH, **L**), octadecenoylcarnitine (C18:1, **M**), hydroxyoctadecenoylcarnitine (C18:1-OH, **N**), sum of the long-chain acylcarnitine (Long-chain ACC; C12+, **O**), carnitine palmitoyltransferase (CPT)1 ratio (**P**), and CPT2 ratio (**Q**). Data are means ± SEM. Differences between different time points identified by post-hoc testing (Tukey's HSD) after ANOVA are indicated by different letters.

mRNA Abundance of Carnitine Acyl Transferases in Skeletal Muscle

No differences were observed between the treatment groups, and thus merged data were analyzed for time-dependent effects. The mRNA abundance of *CPT1B* increased 2.8-fold from d -21 to d 1 (P = 0.02), followed by a decline to nearly prepartum values by d 70 (Figure 7). The mRNA abundance of *CPT2* remained unchanged over time.

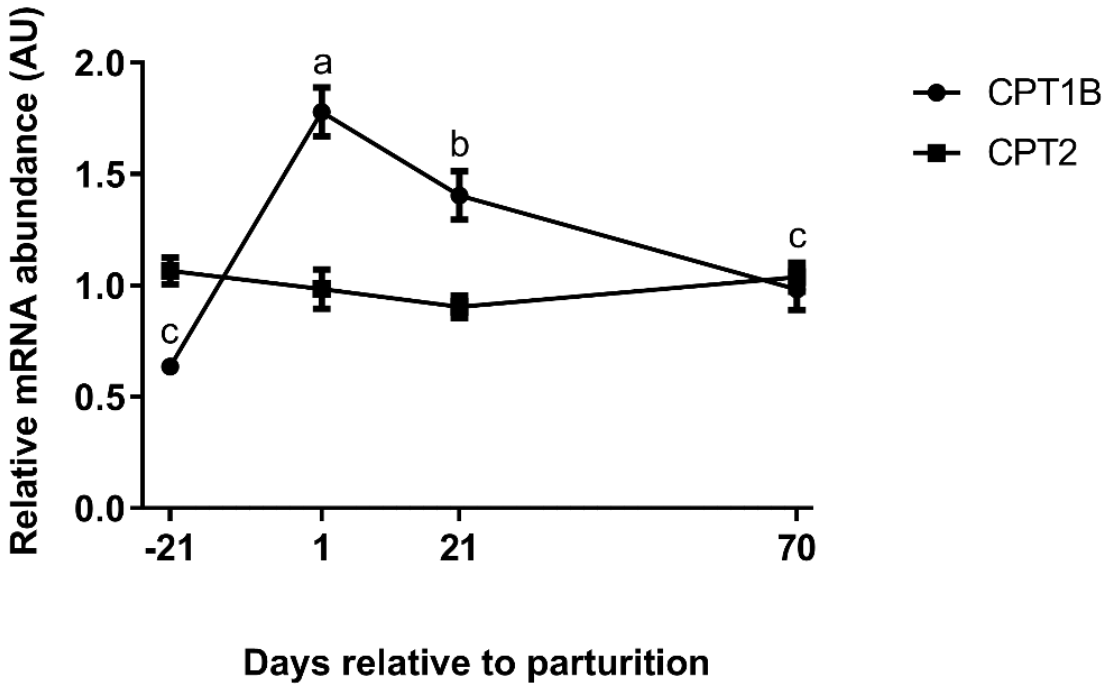

Figure 7. Time course of muscle mRNA abundance (means ± SEM) of carnitine acyltransferases (CPT1B and CPT2) in dairy cows during late gestation and early lactation. Differences between different time points identified by post-hoc testing (Tukey's HSD) after ANOVA are indicated by different letters.

Associations of Acylcarnitine Profiles with Conventional Parameters and mRNA Abundance

As shown in Figure 8, across all time-points, correlation analysis revealed a negative correlation between serum FA and CPT1 ratio in muscle and serum ($P < .0001$; ρ = -0.46 and -0.66, respectively). Positive correlations were observed between serum FA with most of the muscle and serum long-chain ACC (C14-C18. $P < 0.03$; $\rho > 0.25$), several muscle short- and medium-chain ACC (C3, C4, C6, C7, C10 derived ACC, and C12:1. $P < 0.03$; $\rho > 0.246$), serum C2 ($P < .0001$; $\rho = 0.44$), and C4-OH ($P < 0.01$; $\rho = 0.30$) as well as muscle CPT2 ratio ($P < .0001$; $\rho = 0.46$). Moreover, FA concentrations were positively related with the *CPT1B* mRNA abundance ($P < .0001$; $\rho = 0.64$). RQUICKI were neither associated with the aforementioned ACC nor mRNA abundance.

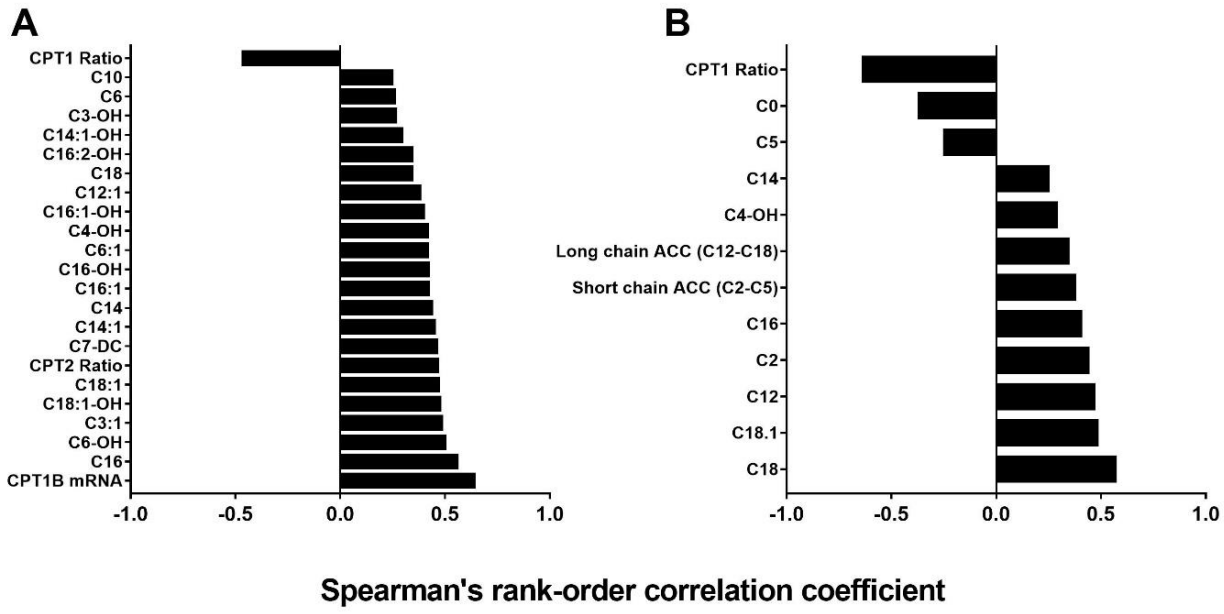

Figure 8. Significant Spearman's rank-order correlation coefficient (ρ) between serum FA concentration with muscle (**A**) and serum (**B**) ACC profiles as well as mRNA abundance. The P values were adjusted for multiple comparisons by calculating the false discovery ratio (FDR). The cut-off condition of correlation analyses was set as $|\rho| > 0.20$ and FDR < 0.10.

DISCUSSION

Skeletal muscle is important for coping with the increasing concentrations of FA at the end of pregnancy and the onset of lactation. The capacity of oxidizing FA is likely changing depending on the supply with FA and the physiological status of the animal. We herein characterized the longitudinal changes in ACC both in the circulation and in skeletal muscle in dairy cows during the transition from pregnancy into lactation by means of targeted metabolomics and investigated whether dietary supplementation (from d 1 in milk) with CLA altered these compared with control-fat supplemented cows. In laboratory animals, CLA supplementation has been associated with metabolic changes favoring the increase of lipolysis, the reduction of lipoprotein lipase activity, accompanied by the oxidation of FA in the adipose and muscle tissues, due to increased CPT-1 activity and action, or possibly as a result of inhibiting adipocyte differentiation (Botelho et al., 2005; Churruca et al., 2009; Lehnen et al., 2015). In contrast to our hypothesis, the treatment with CLA tested in the animal feeding trial from which the samples were obtained did not affect the variables targeted herein and also other "classical" variables used to characterize the metabolic effects like e.g. FA. In consequence, the groups could be pooled thus increasing sample size. The reasons for the lack of a CLA response in the examined variables are not known, but are likely related to the dosage used and the availability of the CLA isomers in the intermediary metabolism as well as the timing of the supplementation which started only with the first day in milk. The CLA dosages commonly used in dairy cows are far below those tested in laboratory animals and in humans. However, for the main targeted effect in dairy cows, i.e., milk fat reduction, the relatively low dosages are, as manifold proven, effective. Also the CLA-treated animals in our study had 12% less milk fat than the control cows; this effect was evident after 28 d of lactation and CLA supplementation (Pappritz et al., 2011a). However, the transfer of CLA into milk was low and largely limited to the *trans*-10, *cis*-12 isoform (0.03% versus 0.004% in the control group; Pappritz et al., 2011a). In a more detailed approach using cows fitted with ruminal and duodenal cannulas, the actual duodenal availability of *trans*-10, *cis*-12 CLA was determined to be low, i.e., between 5 and 16% (Pappritz et

al., 2011b), though this is in accordance with the protection rate of 9-34% reported for the calcium salts of the CLA (de Veth et al., 2005). Pappritz et al. (2011b) have shown that major portions of the CLA reaching the duodenum are excreted via milk (36-48%) or faeces (~ 50%), and thus only a small proportion of the CLA, i.e. 2-14%, may reach different tissues and cells. Using the same CLA treatment in primiparous cows that were sequentially slaughtered during lactation, Von Soosten et al. (2013) reported only low tissue concentrations. For example, in adipose tissues maximally 0.02% of total FA were *trans*-10, *cis*-12 CLA in supplemented cows; the CLA content in control cows remained below the limit of detection (<0.01% of total FA). In skeletal muscle tissue, CLA were not detectable (Von Soosten et al., 2013). We thus speculate that a substantially higher dose of CLA would be required to affect FA oxidation in muscle and/or the studied muscle was less sensitive and responsive to CLA. In the current study, muscle samples were biopsied only from the M. semitendinosus, and we also did not determine muscle fiber type composition in the samples. Skeletal muscle, a heterogeneous and highly structured tissue, is composed of a set of fiber types differing in their functional and metabolic profiles (Gunawan et al., 2007) and it is probable that the response of skeletal muscle to the CLA supplement is fiber type dependent and thus warrants further investigation.

As expected, we observed greater FA serum concentrations around calving and in the first weeks of lactation pointing to increased lipolysis around parturition as a response to the massively augmented need for energy to accomplish fetal growth and milk synthesis. Carnitine and its acyl esters, i.e. ACC, are indispensable for the mitochondrial β-oxidation of FA through facilitating the transfer of long-chain FA from the cytoplasm to the mitochondrial matrix across the mitochondrial membranes. Once inside mitochondria, the enzyme *CPT2* reconverts the ACC back into free carnitine and the respective long-chain acyl-CoA, which can then be oxidized for ATP production through β-oxidation and the TCA cycle (Schooneman et al., 2013). Carnitine is mainly synthesized in the liver from the essential AA lysine and methionine (Krajcovicová-Kudlácková et al., 2000). Skeletal muscle, harboring the highest concentrations, is unable to synthesize carnitine and thus needs to take

carnitine from blood. In accordance with a previous study (Schooneman et al., 2014), we observed carnitine levels being unchanged in muscle but decreasing in serum, likely due to increased carnitine excretion in milk (Shennan et al., 1998) and its uptake by the muscle to maintain the intracellular concentrations. In addition, carnitine maintains the balance between free and esterified CoA, and is required for the mitochondrial efflux of excess acyl groups (Sharma and Black, 2009). Thus, changes in individual serum and tissue ACC may imply changes in specific metabolic pathways, and is therefore commonly used in neonatal screening for metabolic disturbances (Meyburg et al., 2002). The C2, the shortest ACC, derives from acetyl-CoA via the action of carnitine acetyltransferase for transport out of the mitochondria (Flanagan et al., 2010). Acetylcarnitine is the universal degradation product of all metabolic substrates, and is thus the most abundant ACC in the tissues and circulation. In the current study, serum C2 concentrations were elevated around parturition, pointing to an increased FA β-oxidation in mitochondria relative to the TCA cycle flux. In addition, C4-OH which can be derived from the CoA ester of the ketone body 3-hydroxybutyrate (Soeters et al., 2012; Schooneman et al., 2013) had higher concentrations around parturition in both serum and muscle. The higher C4-OH concentration which is thought to reflect ketogenesis (Xu et al., 2016) is also consistent with an excess pool of acetyl-CoA around parturition. Amino acid catabolism is a source of odd-chain species such as C3 and C5 (Flanagan et al., 2010). In the current study, muscle concentrations of C3 and C3:1 were slightly higher in after than before calving, whereas those in serum did not show time-dependent changes. These data imply that most lipid-derived ACC increased around parturition, which is consistent with the lactation-induced rise in circulating FA.

We hypothesized that lactation-induced alterations in the ACC profiles are caused by incomplete FA oxidation, as long-chain ACC species were elevated around parturition in both serum and muscle (more notable in the latter). Most long-chain ACC species in muscle decreased from d 1 to d 21, with little or no changes afterwards, suggesting insufficient adaptation of their metabolism in response to the metabolic load of FA around parturition. However, it should be noted that due to the study design, we were

unable to assess potential changes during the first days after calving, a period of rapid and substantial metabolic changes in dairy cows. It is likely that FA oxidation should be in relative excess to oxidation in TCA and respiratory chain in order to guarantee continuous supply of energy. Peroxisomal β-oxidation, independent of carnitine-mediated transport, is the second pathway through which long-chain FA can be oxidized, and unlike mitochondrial β-oxidation, is not regulated by energy demands of the cell (Osmundsen et al., 1991; Drackley, 1999). For bovine liver, the relative contribution of peroxisomal β-oxidation to total oxidative capacity has been shown to be >50%, suggesting that this pathway may be a component of the adaptations of FA metabolism in liver during the periparturient period, and thus helping the liver to cope with the large influx of NEFA from body fat mobilization (Grum et al., 1994, 1996, 2002). The main function of peroxisomal β-oxidation is the shortening of the NEFA chains, preparing them to be completely oxidized in the mitochondria (Drackley, 1999). The role of peroxisomes in metabolism of FA in the skeletal muscle of ruminants has not been determined. It is also probable that peroxisomal oxidation plays a role as an "overflow" pathway to oxidize FA in muscle of dairy cows during extensive NEFA mobilization that warrants future investigations.

The CPT1 ratio, as a potential marker for CPT1 deficiency (Fingerhut et al., 2001), which is a rate limiting enzyme for long-chain FA entry into the mitochondria for β-oxidation, was also evaluated. An elevation of this ratio has been described in CPT1 deficiency (Fingerhut et al., 2001). In this study, the ratio significantly decreased with the onset of lactation in both serum and muscle, reflecting increased mitochondrial entrance of long-chain FA. Incomplete FA β-oxidation downstream of CPT1 is associated with elevated levels of plasma ACC (Koves et al., 2008), as acyl-CoA in the mitochondrial matrix can be converted into ACC for transport out of the mitochondria (Koves et al., 2008; Millington and Stevens 2011; Violante et al., 2013). The CPT2 ratio, calculated as the ratio of C16:0+C18:1 to C2, is a potential marker to describe CPT2 deficiency (Gempel et al., 2002). Deficiency of CPT2 is associated with a pronounced elevation of C16:0 and C18:1 ACC, while C2 is low, pointing to a

significant reduction in long-chain FA oxidation. In the present study, the ratio was increased around parturition, likely indicating deficiency and/or impaired CPT2 functions. In this situation, long-chain ACC cannot be converted to their corresponding acyl-CoA esters, resulting in accumulation of long-chain ACC in the mitochondrial matrix which are subsequently transported out of the mitochondria to the blood stream (Flanagan et al., 2010; Schooneman et al., 2013). It has been shown that high-fat overfeeding and an increased lipid exposure to skeletal muscle was associated with an increased expression of genes involved in the FA β-oxidation pathway, including *CPT1* that regulates the entry of acyl-CoA into the mitochondrial matrix (Muoio and Newgard, 2006; Noland et al., 2007; Turner et al., 2007). Interestingly, in the current study, the mRNA abundance of *CPT1B* (muscle isoform) increased 2.8-fold from d -21 to d 1, followed by a decline thereafter, whereas that of *CPT2* remained unchanged over time. These data may suggest a physiological increase in the capacity of long-chain fatty acyl-CoA entry into muscle mitochondria around parturition, but does not seem to coincide with upregulation of downstream metabolic pathways, such as the TCA cycle and respiratory chain. Thus, it is likely that post-CPT1 events including deficiency, or impaired in CPT2 function and depletion of several TCA cycle intermediates cause an accumulation of ACC in skeletal muscle around parturition.

CONCLUSIONS

The serum and muscle concentrations of the ACC as well as the mRNA expression of the carnitine acyltransferases – *CPT1B* and *CPT2* changed with time, but they were not affected by the CLA supplement at the dosage used. Muscle carnitine remained unchanged despite a decline in the serum concentrations, likely due to increased carnitine excretion with milk and its uptake by the muscle to maintain the intracellular concentrations. The elevated concentrations of muscle long-chain ACC species and serum C2 around parturition point to increased FA β-oxidation which does not seem to coincide with an upregulation of downstream metabolic pathways, such as the TCA cycle and respiratory chain.

ACKNOWLEDGEMENTS

The authors express their appreciation to Inga Hofs and Isabella Israel (Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, Germany) for their excellent laboratory assistance as well as to Evonik Nutrition & Care GmbH (Rodenbacher Chaussee 4, 63457 Hanau, Germany) for partial financial support of this experiment. Y. Yang is recipient of a scholarship from the China Scholarship Council (CSC).

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5. General discussion and future research prospective

Experimental design and treatment effect

The treatment with CLA did not affect the variables targeted in this project. Thus, the groups were merged to increase sample size. The reasons for the lack of a CLA response in the examined variables are not known, but are likely related to the dosage used and the availability of the CLA isomers in the intermediary metabolism as well as the timing of the supplementation which started only with the first day in milk. The applied CLA dosage, although being in the common range for the primarily targeted effect in dairy cows, i.e. milk fat reduction (Pappritz et al., 2011a), is far below those tested in laboratory animals and in humans. Using the same CLA treatment in primiparous cows that were sequentially slaughtered during lactation, Von Soosten et al. (2013) reported only low tissue concentrations of *trans*-10, *cis*-12 CLA and those of skeletal muscle tissue were not detectable. Thus, it is likely that the CLA treatment was not effective enough to elicit a response at tissue level, and thus affect the variables tested herein.

Altered metabolic pathways point to insulin resistance around parturition

In dairy cows, late gestation and early lactation periods are associated with a moderate degree of reduced peripheral tissue insulin sensitivity (De Koster and Opsomer, 2013). This promotes mobilization of body reserves and facilitates the adequate supply of nutrients to the foetus and mammary tissue (Bell, 1995; Bell and Bauman, 1997). Maternal insulin resistance is considered as a part of the homeorhetic adaptations in the periparturient cow, which may manifest as decreased insulin sensitivity or decreased insulin responsiveness. A state of insulin resistance has been ascribed to multiple factors, including growth hormone (Smith et al., 1997) and several potential mediators such as tumor necrosis factor-alpha, NEFA, adiponectin, resistin, and other adipokines and/or adipomyokines (Havel, 2002; Kirwan et al., 2002; Pires et al., 2007; Choi, 2016). Skeletal muscle, the largest internal organ in mammals, is a highly metabolic active tissue and is crucial for maintaining metabolic homeostasis. Thus,

development of insulin resistance across skeletal muscle tissue is certainly a significant factor in the peripheral insulin resistance in the periparturient cow. In the current project, FA oxidation, Arg metabolism, Trp metabolism, PC and lysoPC metabolism, and SM metabolism were found to be the most important metabolic pathways influenced by the transition from late gestation to early lactation (Manuscript 2). The altered metabolic pathways point to dysregulated lipid metabolism (Manuscript 2 and 3), and insulin resistance in dairy cows around parturition (Manuscript 3). Dysregulated lipid metabolism in the skeletal muscle has been linked to lipid-induced insulin resistance, that is characterized by elevated plasma FA in human studies (Savage et al., 2007; Samuel et al., 2010; Zhang et al., 2013a). The underlying molecular mechanisms responsible for lipid-induced insulin resistance are not yet well understood, but likely comprise the following aspects: 1) a mismatch between FA oxidation and downstream metabolic pathway such as the TCA, 2) inflammatory cytokines that cause endoplasmic reticulum stress and mitochondrial dysfunction, and 3) production of bioactive lipids (Goodpaster et al., 2001; Hotamisligil and Erbay, 2008; Muoio, 2010; Coen and Goodpaster, 2012; Jornayvaz and Shulman, 2012; Newgard, 2012; Schooneman et al., 2013). However, insulin sensitivity, estimated by calculating the RQUICKI (Holtenius and Holtenius, 2007), only tended to decrease by about 4% from d 1 to d 21 and does not indeed illustrate the occurrence of insulin resistance (Manuscript 2). The RQUICKI is calculated based on the logarithmic transformation of the fasting glucose, NEFA, and insulin and its use has been well proven in human medicine (Muniyappa et al., 2008). During the past decade, this surrogate index has also been extensively used for predicting insulin resistance in dairy cows. However, the applicability of this index needs further investigations and validations (De Koster and Opsomer, 2013). The fasting state in humans is critical in order to gets reliable estimation of insulin resistance using the surrogate indexes (Muniyappa et al., 2008). In contrast to nonruminants, because of the continuous flow of digesta to the small intestine, the secretion of bile, enzymes, and digestive juices is continuous in ruminants and thus they are not subjected to large

diurnal changes. Therefore, in dairy cows, it is not possible to reach a fasting state whereby insulin and glucose levels are in a balanced state (De Koster and Opsomer, 2013). In addition, late gestation and early lactation periods in dairy cows are associated with substantial changes in the concentrations of glucose, insulin, NEFA, and BHBA, and thus probably reduce the suitability of this surrogate index to be used in periparturient cows (De Koster and Opsomer, 2013).

Taken together, this project contributes to an in-depth understanding of the changes in the different metabolic pathways in insulin-sensitive muscle tissue, and may help to identify metabolic pathways for future targeted analyses, and thereby may help unraveling the underlying pathogenesis of insulin resistance in the periparturient cows.

Post-CPT1 events may play important roles in regulating FA oxidation

Alterations in muscle FA oxidation are considered as major contributors in the development of insulin resistance, though the exact underlying molecular mechanisms linking mitochondrial FA flux, capability of FA oxidation and downstream utilization, and insulin resistance are not yet fully understood. Our results point to increased FA β-oxidation which does not seem to coincide with an upregulation of downstream metabolic pathways (Manuscript 2). After entry into the mitochondrial matrix, FA-CoA is metabolized to acetyl-CoA through β-oxidation. Thereafter, acetyl-CoA generated from both β-oxidation and glycolysis enters the TCA cycle. A rate-limiting step of FA oxidation has been previously attributed to the transport of FA across the mitochondrial inner membranes via CPT1, which converts the long-chain acyl-CoA to long-chain acylcarnitine. Accumulation of malonyl-CoA, generated from acetyl-CoA via acetyl-CoA carboxylase 2 (**ACC2**) (Aguer et al., 2015), is a potent allosteric inhibitor of CPT1 and thereby resulting in accumulation of cytosolic long-chain FA and decreasing FA oxidation rates.

However, recently several post-CPT1 events have also been suggested as potential factors influencing FA oxidation and utilization. Kim et al. (2000) have suggested that

defects at both CPT1 and mitochondrial content levels (reflected by citrate synthase activity) could contribute to the reduced lipid oxidation in human skeletal muscle. Additionally, lipid-induced insulin resistance in skeletal muscle was associated with decreased mitochondrial number, lower levels of TCA intermediates, decrement in electron transport chain activity, and defect in mitochondrial oxidative phosphorylation (Petersen et al., 2004, Ritov et al., 2005, Befroy et al., 2007, Koves et al., 2008). More in-depth analysis is required to unravel molecular processes that underlie mechanisms of post-CPT1 events including TCA cycle enzymes and/or intermediates causing the accumulation of long-chain ACC in muscle of dairy cows around parturition.

6. Summary

The transition from late gestation to early lactation in dairy cows is characterized by extensive changes in metabolic, endocrine, and immune functions. The adaptation to these physiological changes is highly variable and compromised adaptation may result in production diseases. The characterization of the skeletal muscle metabolome is of special interest, since this tissue is not only the primary labile source of amino acids, but also plays important roles in regulating the systemic metabolic homeostasis. In human studies, the muscle metabolome is considered as a promising object to understand the metabolic networks such as bioenergetic status, glucose and fatty acid (**FA**) metabolism; however, there are only few studies performed in dairy cows to explore the metabolome in skeletal muscle. Therefore, the experiment conducted herein aimed 1) to characterize the serum and the skeletal muscle metabolome in context of the metabolic changes occurring during the transition from late pregnancy to early lactation in dairy cows, 2) to determine changes in serum and muscle concentrations of acylcarnitines (**ACC**) and mRNA abundance of muscle carnitine acyltransferases from late pregnancy to lactation, and 3) to test whether dietary supplementation with conjugated linoleic acids (**CLA**) may alter these when compared with control-fat supplemented cows.

For the present experiment 21 pluriparous German Holstein cows were studied from d 21 prepartum until d 70 post partum. The animals were allocated to two different feeding groups, receiving either a CLA (100 g/d per cow; each 12% of trans-10, cis-12 and cis-9, trans-11 CLA) or a control-fat dietary supplementation from d 1 post partum throughout the observation period. Samples from skeletal muscle, and blood were collected on d -21, 1, 21, and 70 relative to calving.

Within **manuscript 1**, we aimed to unravel metabolic pathway shifts in skeletal muscle and serum from late gestation to early lactation. A target metabolomics approach was applied to discover the longitudinal patterns of metabolites from skeletal muscle and serum from 21 d before to 70 d after parturition. Out of 188 metabolites, 80 in serum and 52 in muscle, respectively, contributed most significantly to the separation among the 4 time-points. Furthermore, FA oxidation,

arginine metabolism, tryptophan metabolism, phosphatidylcholine (**PC**) and lysophosphatidylcholines (**LysoPC**) metabolism, and sphingomyeline (**SM**) metabolism were found to be the most important metabolic pathways influenced by the transition from late gestation to early lactation. The altered metabolic pathways may reflect dysregulated lipid metabolism, impaired insulin action, and increased inflammatory status in dairy cows around parturition.

Based on the aforementioned findings, the aim of **manuscript 2** was to characterize muscle and blood serum ACC profiles, to determine the mRNA abundance of muscle carnitine acyltransferases, and to test whether dietary supplementation with CLA altered these compared with control-fat supplemented cows. The CLA supplement did not affect the variables examined. The serum concentration of free carnitine decreased with the onset of lactation. The majority of serum and muscle short-chain and long-chain ACC was positively correlated with the FA concentrations in serum, whereas serum carnitine and C5 were negatively correlated with FA. Time-related changes in the serum and muscle ACC profiles as well as the mRNA expression of the carnitine acyltransferases – CPT1B and CPT2 were demonstrated, that were not affected by the CLA supplement at the dosage used in the present study. Muscle carnitine remained unchanged despite a decline in the serum concentrations, likely due to increased carnitine excretion with milk and its uptake by the muscle to maintain the intracellular concentrations. The elevated concentrations of long-chain ACC species in muscle (from C14 to C18) and of serum acetylcarnitine (C2) around parturition point to incomplete FA oxidation, likely due to insufficient metabolic adaptation in response to the load of FA around parturition.

In summary, these results show that targeted metabolomics provides a powerful approach to expand the understanding of the changes in metabolic pathways during the transition from late gestation to early lactation. More research, i.e., untargeted metabolomics, proteomics and transcriptomics is warranted to elucidate intermediates causing the accumulation of long-chain ACC in muscle of dairy cows around parturition.

7. Zusammenfassung

Die Übergangszeit von der späten Trächtigkeit zur frühen Laktation ist bei Milchkühen durch umfangreiche Veränderungen der Stoffwechsel-, Hormon- und Immunfunktionen charakterisiert. Die Anpassung an diese physiologischen Veränderungen ist sehr variabel und eine beeinträchtigte Anpassung kann zu Produktionskrankheiten führen. Die Besonderheiten des Skelettmuskel-Stoffwechsels sind von besonderem Interesse, weil dieses Gewebe nicht nur die Hauptquelle für Aminosäuren ist, sondern auch eine wichtige Rolle bei der Regulierung der systemischen Stoffwechsel-Homöostase spielt. In humanen Studien gilt das Muskelmetabolom zum Verständnis der Stoffwechselnetzwerke wie Bioenergiestatus, Glukose- und Fettsäure(FA)-Metabolismus als vielversprechend; es gibt jedoch nur wenige Studien über den Stoffwechsel im Skelettmuskel von Milchkühen. Daher waren die hier gezeigten Untersuchungen auf folgende Aspekte konzentriert: 1) auf die Charakterisierung des Serum- und des Skelettmuskel-Metaboloms im Kontext der Veränderungen, die während der Übergangszeit von der späten Trächtigkeit zur frühen Laktation bei Milchkühen auftreten, 2) auf die Bestimmung von Konzentrationsänderungen von Acylcarnitinen (ACC) in Serum und Muskelgewebe und der Menge der mRNA von Carnitin-Acyltransferasen im Muskel, und 3) auf den möglichen Einfluss von mit dem Futter supplementierten konjugierten Linolsäuren (CLA).

Dafür wurden 21 pluripare Kühe der Rasse Deutsche Holstein im Zeitraum von 21 Tagen vor bis 70 Tage nach der Geburt untersucht. Die Tiere wurden in zwei Fütterungsgruppen eingeteilt und erhielten während des Beobachtungszeitraums entweder eine CLA-Zulage (100 g/Tag pro Kuh; jeweils 12% *trans*-10,*cis*-12 und *cis*-9,*trans*-*11* CLA) oder eine Kontroll-Fett Nahrungsergänzung ab Tag 1 nach der Geburt. Skelettmuskel- und Blutproben wurden an Tag -21, 1, 21 und 70, bezogen auf die Kalbung, gewonnen.

Im ersten Manuskript wurden die Verschiebungen in den Stoffwechselwegen in Skelettmuskel und Serum von der späten Trächtigkeit bis zur frühen Laktation

charakterisiert. Dazu wurde ein gezielter („*targeted*“) Metabolomik-Ansatz verwendet, um die longitudinalen Veränderungen der Metaboliten zu erfassen. Von 188 Metaboliten trugen 80 im Serum bzw. 52 im Muskel am stärksten zur Trennung zwischen den vier Zeitpunkten bei. Außerdem wurden die FA-Oxidation, der Arginin-, Tryptophan-, Phosphatidylcholin- (PC), Lysophosphatidylcholin- (LysoPC), und Sphingomyelin-(SM) Metabolismus als die hauptsächlich während der Transitphase veränderten Stoffwechelwege identifiziert. Diese veränderten Stoffwechselwege könnten den fehlregulierten Fettstoffwechsel, die beeinträchtigte Insulinwirkung und den erhöhten Entzündungsstatus bei Kühen um die Geburt wiederspiegeln.

Basierend auf den oben genannten Ergebnissen, wurden, wie im zweiten Manuskript beschreiben, die ACC-Profile im Muskel und Blutserum charakterisiert und die Menge an Carnitin-Acyltransferasen-mRNA im Muskel bestimmt. Zudem wurde gestestet, ob die Fütterung mit CLA diese Variablen im Vergleich zu mit Kontroll-Fett gefütterten Kühen, verändert. Die CLA-Gabe hatte keinen Einfluss auf die untersuchten Variablen. Die Serumkonzentration an freiem Carnitin sank mit dem Beginn der Laktation. Die Mehrheit der kurz- und langkettigen- ACC in Serum und Muskel korrelierte positiv mit den FA-Konzentrationen im Serum, während Serum-Carnitin und C5 negativ mit FA korrelierten. Zeitbedingte Veränderungen im Serum- und Muskel-ACC-Profil, sowie die mRNA-Expression der Carnitin-Acyltransferasen - CPT1B und CPT2 konnten nachgewiesen werden, Muskel-Carnitin blieb unverändert, hingegen waren abnehmende Serumkonzentrationen zu beobachten. Möglicherweise war dies durch eine erhöhte Carnitinausscheidung mit der Milch und die Aufnahme in die Muskulatur zur Aufrechterhaltung der intrazellulären Konzentrationen begründet. Die erhöhten Konzentrationen von langkettigen ACC-Formen im Muskel (von C14 bis C18) und von Serumacetylcarnitin (C2) um den Zeitpunkt der Geburt herum, deuten auf eine unvollständige FA-Oxidation; möglicherweise wegen einer unzureichenden metabolischen Anpassung als Reaktion auf die Belastung durch FA um die Geburt.

Zusammenfassend, zeigen diese Ergebnisse, dass der verwendete, gezielte Metabolomics-Analytik eine geeignete Methode darstellt, um das Verständnis für die Veränderungen der Stoffwechselwege während der Transitphase der Milchkuh zu erweitern. Mit Nutzung von „OMICS-Methoden“ wie ungezielte („non-targeted“) Metabolomics, Proteomics und Transcriptomics, könnten die Zwischenprodukte, die die Anhäufung von langkettigen ACC im Muskel von Milchkühen im geburtsnahem Zeitraum verursachen, klären helfen.

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9. Acknowledgements

Finally, my 4 years PhD journey is about to come to an end. I would first like to thank my supervisor, Prof. Dr. Dr. Helga Sauerwein for not only providing with the opportunity to do my doctorate in her group but also for supporting me throughout my time here at the University of Bonn. I am really appreciating all the efforts, time, and especially patience to boost me to finish my dissertation. I also would like to thank my daily supervisor, Dr. Hassan Sadri. I am your first PhD student and I know you always ready to sharing your knowledge and experience on almost every aspect in the PhD study, even when I was lost with my project and writing. Words are not enough to express my gratitude. I also would like to thank my second supervisor, Prof. Dr. Karl-Heinz Südekum for his endless support.

My great appreciation also goes to my colleagues. Dr. Morteza Ghaffari, thank you for your valuable suggestion on my dissertation and scientific discussion about omics. I am looking forward to learn more things from you. Barbara and Thomas, thanks for all your help on those WB experiments and for the latest information about remaining coffee amounts. Frau. Rzesnik, Susanne, Stephanie, Ute, Hannelore, Karin, Katharina, Christiane, Laura, I had many good times with you all. Many thanks to the “Haus 5” people, I got a lot of help from you all. Inga, we always discussed about funny parts in cooking (Ants climbing the tree!) and family issues. Isabella, thank you for teaching me from cutting those frozen sample to preparing box to shipment. I really love your fancy sports car. Katharina, I will surely remember our nice talks during coffee and ice cream break. Taher, it was great to see all these nice pictures from your hometown.

I would like to thank Yang Liu, Kaihong Huang and Xiaoming Song. We both arrived in Bonn 4 years ago, and I will always remember these nice, pleasant and relaxing time together. Special thanks give to Dr. Fan Yang and Zongkai Zhang. I am lucky to have you guys around and thank you for all the nice lunch and dinners, and of course our nice talks about study, jobs, and future. I will miss all the funny moments in

playing games on weekend nights! Dr. Qin Yang. Thank you for all these help and suggestion about life and works in Germany.

I would like to express my gratitude to my family members, especially my girlfriend, Dr. Ting Gong. I know keep a long-distance relationship is hard, but we made it. I got a lot of support from you all in the past 4 years, especially in those dark times. I also would like to thank my uncle and my grandmother, who departed from me. I know you were always love me and I believe you all will keep on lead me.

Finally, I would like to thank the Chinese Scholarship Council for financing my stay in Germany to do science. I will keep going.

10. Publications and abstracts derived from this doctorate thesis

Publications

1). **Yang, Y.,** Sadri, H., Prehn, C., Adamski, J., Rehage, J., Dänicke, S., Saremi, B. and Sauerwein, H. 2018. Acylcarnitine profiles in serum and muscle of dairy cows receiving conjugated linoleic acids or a control fat supplement during early lactation. J. Dairy Sci. (Accepted)

Abstracts in conferences

1). **Yang, Y.,** Sauerwein, H., Prehn, C., Adamski, J., Rehage, J., Dänicke, S., Saremi, B. and Sadri, H. 2017. Proteasome activity and expression of E3 ubiquitin ligases in muscle of periparturient dairy cows. 68th Annual Meeting of the European Federation of Animal Science, 28 August - 1 September, Tallinn, Estonia, Page 148. ISBN: 978-90-8686-312-9.

2). **Yang, Y.,** Prehn, C., Adamski, J., Rehage, J., Dänicke, S., Sauerwein, H. and Sadri, H. 2017. Muscle-targeted metabolomics in dairy cows during the transition from late pregnancy to early lactation. J. Dairy Sci., 100, Suppl. 2, Page 152.

3). **Yang, Y.,** Sadri, H., Prehn, C., Adamski, J., Rehage, J., Dänicke, S., Saremi, B. and Sauerwein, H. 2017. Acylcarnitine profiles in serum and skeletal muscle and mRNA expression of the carnitine acyltransferases - CPT1B and CPT2 in muscle of dairy cows around parturition. Proceedings of the Society of Nutrition Physiology. Band 26, Page 151. ISBN: 978-3-7690-4110-1.

4). **Yang, Y.,** Sauerwein, H., Prehn, C., Adamski, J., Rehage, J., Dänicke, S. and Sadri, H. 2016. Branched-chain amino acids (BCAA) in serum and skeletal muscle and mRNA expression of BCAA catabolizing enzymes in muscle of dairy cows around parturition. J. Anim. Sci., 94, Suppl. 5/J. Dairy Sci., 99, Suppl. 1, Page 505.

5). **Yang, Y.,** Prehn, C., Adamski, J., Rehage, J., Dänicke, S., Sauerwein, H. and Sadri, H. 2016. Muscle and serum acylcarnitine profiles in dairy cows during the periparturient period. 67th Annual Meeting of the European Federation of Animal Science, 29 August - 1 September, Belfast, United Kingdom, Page 241. ISBN: 978-90-8686-284-9.

6). **Yang, Y.,** Rehage, J., Dänicke, S., Sauerwein, H. and Sadri, H. 2016. Supplementation of dairy cows with conjugated linoleic acid: effects on the

mRNA abundance of genes related to protein synthesis and proteolysis in the skeletal muscle. Proceedings of the Society of Nutrition Physiology. Band 25, Page 97. ISBN: 978-3-7690-4109-5.

7). **Yang, Y.,** Sauerwein, H., Dänicke, S., Rehage, J. and Sadri, H. 2015. Longitudinal characterization of the gene expression of key components of the mTOR signaling and ubiquitin proteasome system in skeletal muscle of dairy cows during the periparturient period and subsequent lactation. J. Anim. Sci., 93, Suppl. s3/J. Dairy Sci., 98, Suppl. 2, Page 708.